

Methods in ENZYMOLOGY

Volume 434

Lipodomies and Bioactive Lipids:
Lipids and Cell Signaling

Edited by

H. Alex Brown





VOLUME FOUR HUNDRED AND THIRTY-FOUR

**METHODS IN
ENZYMOLOGY**

**Lipodomics and
Bioactive Lipids: Lipids
and Cell Signaling**

METHODS IN ENZYMOLOGY

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METHODS IN ENZYMOMOLOGY

Lipodomics and Bioactive Lipids: Lipids and Cell Signaling

EDITED BY

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CONTENTS

<i>Contributors</i>	<i>xi</i>
<i>Preface</i>	<i>xvii</i>
<i>Volumes in Series</i>	<i>xix</i>

1. Phospholipase A₁ Assays Using a Radiolabeled Substrate and Mass Spectrometry **1**

Rei Morikawa, Masafumi Tsujimoto, Hiroyuki Arai, and Junken Aoki

1. Introduction	2
2. Types of PLA ₁	2
3. Conventional PLA ₁ Assay Using Radiolabeled Substrates	3
4. Novel PLA ₁ Assay Using ESI-MS	6
5. Perspective	11
Acknowledgments	11
References	11

2. Real-Time Cell Assays of Phospholipase A₂s Using Fluorogenic Phospholipids **15**

Debasis Manna and Wonhwa Cho

1. Introduction	16
2. Fluorogenic PLA ₂ Substrates	18
3. Measuring Cellular sPLA ₂ Activity Using PED6 and Red-PED6	23
4. Measuring Cellular cPLA ₂ α Activity Using DAPC	24
References	26

3. Analysis and Pharmacological Targeting of Phospholipase C β Interactions with G Proteins **29**

David M. Lehmann, Chujun Yuan, and Alan V. Smrcka

1. Introduction	30
2. Methods	31
3. Concluding Remarks	46
Acknowledgment	47
References	47

4. Biochemical Analysis of Phospholipase D 49

H. Alex Brown, Lee G. Henage, Anita M. Preininger,
Yun Xiang, and John H. Exton

1. Introduction	50
2. Assay of Recombinant PLD <i>In Vitro</i>	52
3. Regulated PLD1 Activity	59
4. Preparation of Activators of PLD1	60
5. Effects of Activators on PLD1 Activity	61
6. Synergy Between PLD1 Activators	62
7. Binding of PLD1 to Phospholipid Vesicles	63
8. Kinetic Parameters of PLD1 Catalytic Activity	65
9. Kinetic Analyses of Synergistic Responses	69
10. Phosphatidylinositol 4,5-Bisphosphate is an Essential PLD1 Activator	69
11. <i>In Vivo</i> PLD Assay Using Radioisotopes	74
12. <i>In Vivo</i> PLD Assay Using Deuterated 1-Butanol	74
13. Fluorescent <i>In Vitro</i> PLD Assay	77
14. Real-Time Diacylglycerol Lipase Assay	80
Acknowledgments	85
References	85

5. Measurement of Autotaxin/Lysophospholipase D Activity 89

Andrew J. Morris and Susan S. Smyth

1. Introduction	90
2. Overview of Methods for Determination of Autotaxin/LysoPLD Activity	93
3. Expression of V5-Tagged Autotaxin/LysoPLD in HEK293 Cells	94
4. Measurement of Autotaxin/LysoPLD Activity Using Radiolabeled Substrates	95
5. Measurement of Autotaxin/LysoPLD Activity Using Fluorogenic Substrates	98
6. Concluding Comments	100
Acknowledgment	102
References	102

6. Platelet-Activating Factor 105

John S. Owen, Michael J. Thomas, and Robert L. Wykle

1. Introduction	105
2. Procedure	107
Acknowledgments	115
References	115

7. Quantitative Measurement of Phosphatidylinositol 3,4,5-trisphosphate	117
Hervé Guillou, Len R. Stephens, and Phillip T. Hawkins	
1. Introduction	118
2. Measuring Levels of Radioactively Labeled Phosphoinositides in Isolated Cells	120
3. Measuring PtdIns(3,4,5)P ₃ by Protein–Lipid Overlay	122
4. Conclusions	126
Acknowledgments	128
References	128
8. Measuring Phosphorylated Akt and Other Phosphoinositide 3-kinase-Regulated Phosphoproteins in Primary Lymphocytes	131
Amber C. Donahue, Michael G. Kharas, and David A. Fruman	
1. Overview	132
2. Choosing a Downstream Readout: General Considerations	134
3. Protocols for Detection of PI3K-Regulated Phosphoproteins by Immunoblot	137
4. Protocols for Detection of Phosphoproteins by Flow Cytometry	142
5. Discussion	147
Acknowledgments	150
References	150
9. Regulation of Phosphatidylinositol 4-Phosphate 5-kinase Activity by Partner Proteins	155
Yasunori Kanaho, Kazuhisa Nakayama, Michael A. Frohman, and Takeaki Yokozeki	
1. Introduction	156
2. Protocols	158
Acknowledgments	166
References	166
10. Biochemical Analysis of Inositol Phosphate Kinases	171
James C. Otto, Sashidhar Mulugu, Peter C. Fridy, Shean-Tai Chiou, Blaine N. Armbruster, Anthony A. Ribeiro, and John D. York	
1. Introduction	172
2. Experimental Methods	174
3. Conclusions	182
Acknowledgments	183
References	183

11. Analysis of Phosphoinositides and Their Aqueous Metabolites	187
Christopher P. Berrie, Cristiano Iurisci, Enza Piccolo, Renzo Bagnati, and Daniela Corda	
1. Introduction	188
2. Cell Sample Extraction	191
3. Lipid Phase: TLC, HPLC Separation, and Desalting	197
4. Aqueous Phase: HPLC Separation, Desalting, and Ascintillant Extraction	203
5. Chemical Identification	212
6. ESI-MS/MS Identification	214
7. Standards	219
8. Final Considerations	226
Acknowledgments	227
References	227
12. Combination of C₁₇ Sphingoid Base Homologues and Mass Spectrometry Analysis as a New Approach to Study Sphingolipid Metabolism	233
Stefka Spassieva, Jacek Bielawski, Viviana Anelli, and Lina M. Obeid	
1. Introduction	234
2. Mass Spectrometry Analysis	235
3. Ceramide Synthase	236
4. <i>In Vitro</i> Ceramide Synthase Method	236
5. Sphingosine Kinase	237
6. <i>In Vitro</i> Sphingosine Kinase Method	238
7. In Cells Labeling with C ₁₇ Sphingoid Base	239
Acknowledgments	240
References	240
13. Measurement of Mammalian Sphingosine-1-Phosphate Phosphohydrolase Activity <i>In Vitro</i> and <i>In Vivo</i>	243
Michael Maceyka, Sheldon Milstien, and Sarah Spiegel	
1. Introduction	244
2. Principle	249
3. Measurement of SPP Activity in Cell Lysates	249
4. Measurement of SPP Activity in Live Cells	252
Acknowledgments	253
References	253
14. A Rapid and Sensitive Method to Measure Secretion of Sphingosine-1-Phosphate	257
Poulami Mitra, Shawn G. Payne, Sheldon Milstien, and Sarah Spiegel	
1. Introduction	258
2. Measurement of S1P	259

3. Conclusions and Perspectives	262
Acknowledgments	263
References	263
15. Ceramide Kinase and Ceramide-1-Phosphate	265
Dayanjan S. Wijesinghe, Nadia F. Lamour, Antonio Gomez-Munoz, and Charles E. Chalfant	
1. Introduction	266
2. Recombinant Expression and Kinetic Analysis of CERK	269
3. <i>In Vitro</i> Kinetic Analysis of CERK Activity Using Mixed Micellar Assays	272
4. Effective Delivery of C1P to Cells in Tissue Culture to Study Biological Effects	278
5. Analysis of Levels of Kinase-Derived C1P in Cells	281
6. Analysis of CERK Localization in Cells	284
7. Analysis of CERK Function by siRNA-Mediated Manipulation of CERK Expression	286
8. Analysis of CERK mRNA Levels by Q-PCR	288
Acknowledgments	289
References	290
16. Measurement of Mammalian Diacylglycerol Kinase Activity <i>In Vitro</i> and in Cells	293
Richard M. Epand and Matthew K. Topham	
1. Introduction	294
2. <i>In Vitro</i> Assay of DGK	295
3. Measuring DGK Activity in Subcellular Compartments	300
4. Measuring DGK Activity in Cultured Cells	301
5. Summary	303
References	303
17. Lipid Phosphate Phosphatases from <i>Saccharomyces cerevisiae</i>	305
George M. Carman and Wen-I Wu	
1. Introduction	306
2. Preparation of Radiolabeled Substrates	307
3. Assay Methods	307
4. Growth of Yeast	308
5. Purification Procedure	308
6. Properties of <i>DPP1</i> - and <i>LPP1</i> -Encoded Lipid Phosphate Phosphatases	311
Acknowledgment	313
References	313
<i>Author Index</i>	317
<i>Subject Index</i>	335

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PREFACE

Lipid metabolism and cellular signaling are highly integrated processes that regulate cell growth, proliferation, and survival. Lipids have essential roles in cellular functions, including determinants of membrane structure, serving as docking sites for cytosolic proteins, and allosteric modulators. Abnormalities in lipid composition have established roles in human diseases, including diabetes, coronary disease, obesity, neurodegenerative diseases, and cancer. In the post-genomic era, we look at epigenetic factors and metabolomic biomarkers to better understand the molecular mechanisms of complex cellular processes and realize the benefits of personalized medicine.

Recent advances in lipid profiling and quantitative analysis provide an opportunity to define new roles of lipids in complex biological functions. Lipidomics was developed to be a systems biology approach to better understand contextual changes in lipid composition within an organelle, cell, or tissue as a result of challenge, stress, or metabolism. It provides an approach for determining precursor–product relationships as well as ordering the temporal and spatial events that constitute vital processes. This volume of *Methods in Enzymology* is one of a three-volume set on *Lipidomics and Bioactive Lipids* designed to provide state-of-the-art techniques in profiling and quantification of lipids using mass spectrometry and other analytical techniques used to determine the roles of lipids in cell function and disease. The first volume (432), *Mass-Spectrometry-Based Lipid Analysis*, provides current techniques to profile lipids using qualitative and quantitative approaches. The cell liposome is composed of thousands of molecular species of lipids; thus, generating a detailed description of the membrane composition presents both analytical and bioinformatic challenges. This volume includes the methodologies developed by the National Institute of General Medicine large-scale collaborative initiative, LIPID MAPS (www.lipidmaps.org), as well as an overview of international lipidomics projects. The second volume (433), *Specialized Analytical Methods and Lipids in Disease*, presents applications of lipid analysis to understanding disease processes, in addition to describing more specialized analytical approaches. The third volume (434), *Lipids and Cell Signaling*, is a series of chapters focused on lipid-signaling molecules and enzymes.

The goal of these volumes is to provide a guide to techniques used in profiling and quantification of cellular lipids with an emphasis on lipid signaling pathways. Many of the leaders in the emerging field of lipidomics have contributed to these volumes, and I am grateful for their comments in

shaping the content. I hope that this guide will satisfy the needs of students who are interested in lipid structure and function as well as experienced researchers. It must be noted that many of the solvents, reagents, and instrumentation described in these chapters have the potential to be harmful to health. Readers should consult material safety data sheets, follow instrument instructions, and be properly trained in laboratory procedures before attempting any of the methods described.

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PHOSPHOLIPASE A₁ ASSAYS USING A RADIOLABELED SUBSTRATE AND MASS SPECTROMETRY

Rei Morikawa,^{*,†} Masafumi Tsujimoto,[†] Hiroyuki Arai,^{*,§} and Junken Aoki^{*,‡}

Contents

1. Introduction	2
2. Types of PLA ₁	2
3. Conventional PLA ₁ Assay Using Radiolabeled Substrates	3
3.1. Materials	3
3.2. Preparation of radiolabeled phospholipid substrates	3
3.3. Assays	5
4. Novel PLA ₁ Assay Using ESI-MS	6
4.1. Materials	6
4.2. Preparation of recombinant PLA ₁	6
4.3. Phospholipase A ₁ assay	8
4.4. PLA ₁ activity of intracellular PLA ₁ s	10
5. Perspective	11
Acknowledgments	11
References	11

Abstract

Although a number of phospholipase A₁s (PLA₁s) have been identified in the recent decade, the physiological functions of PLA₁s remain almost elusive. The major reason for this is the poor availability of assay methods. In many studies, radiolabeled phospholipid substrates have been used to measure PLA₁ activity. This chapter describes the conventional PLA₁ assay using a radiolabeled substrate and a novel PLA₁ assay using electrospray ionization mass spectrometry.

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1. INTRODUCTION

Phospholipase A₁s (PLA₁s) are enzymes that hydrolyze the acyl group of phospholipids at the *sn*-1 position, liberate fatty acid, and produce 2-acyl-1-lysophospholipid. PLA₁ activity has been detected in various cells and tissues (Higgs and Glomset, 1994; Inoue and Okuyama, 1984; Pete *et al.*, 1994; Sato *et al.*, 1997); currently, six extracellular and three intracellular PLA₁s are known in mammals (see later). The physiological roles of these PLA₁ isozymes are largely unknown (Aoki *et al.*, 2002, 2007; Inoue and Aoki, 2006; Inoue *et al.*, 2004).

To measure PLA₁ activity, phosphatidylcholine (PC) has been used as the substrate in many studies. However, some PLA₁s, such as phosphatidylserine-specific PLA₁ (PS-PLA₁) and membrane-associated phosphatidic acid-selective PLA₁ (mPA-PLA₁), were reported to show PLA₁ activity toward certain phospholipids other than PC (Hiramatsu *et al.*, 2003; Sato *et al.*, 1997; Sonoda *et al.*, 2002). Thus, it is important to test various phospholipids as substrates. PLA₁ activities are normally assayed with a fluorescent- or radio-labeled acyl chain at the *sn*-1 position of the glycerol backbone. This assay is very sensitive, but because the substrates must be synthesized individually, only a few substrates have been examined so far.

A recently developed method that utilizes electrospray ionization mass spectrometry (ESI-MS) has made it possible to assay PLA₁s using a wide range of phospholipids as substrates. ESI-MS has been shown to be a powerful tool for the analysis of lipids with high sensitivity and simplicity (Han and Gross, 2005; Milne *et al.*, 2006; Pulfer and Murphy, 2003; Taguchi *et al.*, 2000). This method quantifies the liberated fatty acid using ESI-MS and does not require fluorescent- or radiolabeled substrates. It also makes it possible to test various phospholipids with regard to head groups and fatty acid species. In addition, by using phospholipids with different fatty acids at the *sn*-1 and *sn*-2 positions, PLA₁ and PLA₂ activities can be easily distinguished by analyzing the fatty acid liberated. At the same time, the by-products of the reaction, lysophospholipids, can be detected, which helps understand the biochemical properties of the enzyme. This chapter describes the conventional PLA₁ assay using a radiolabeled substrate and the novel PLA₁ assay using ESI-MS.

2. TYPES OF PLA₁

Phospholipase A₁ isozymes are classified into two groups based on their primary structure and cellular distribution: extracellular PLA₁ family and intracellular PLA₁ family. Extracellular PLA₁s consist of phosphatidylserine

(PS)-specific PLA₁ (PS-PLA₁), membrane-associated phosphatidic acid-selective PLA₁s (mPA-PLA₁ α and mPA-PLA₁ β), hepatic lipase (HL), endothelial lipase (EL), and pancreatic lipase-related protein-2 (PLRP-2), all of which belong to the lipase gene family (Carriere *et al.*, 1998; Hide *et al.*, 1992; Hiramatsu *et al.*, 2003; Sato *et al.*, 1997; Sonoda *et al.*, 2002; Wong and Schotz, 2002) (Table 1.1). Intracellular PLA₁s consist of three members, PA-preferring PLA₁ (PA-PLA₁), KIAA0725, and p125 in mammals (Table 1.1), which do not show any sequence homology to classical phospholipases and are conserved in a wide range of eukaryotes (Higgs *et al.*, 1998; Nakajima *et al.*, 2002; Tani *et al.*, 1999).

3. CONVENTIONAL PLA₁ ASSAY USING RADIOLABELED SUBSTRATES

3.1. Materials

1,2-di[¹⁴C]oleoyl-*sn*-glycero-3-phosphocholine is from Dupont NEN. *Rhyzopus delemer* lipase (pure grade, 6000 units/mg) and *Naja naja* PLA₂ are from Seikagaku Kogyo (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. Phospholipase D from *Actinomadura* was kindly donated by Dr. S. Kato of Meito Sangyo Co. (Tokyo, Japan). Other chemicals are from Wako (Osaka, Japan). Silica thin-layer chromatography (TLC) plates, Silica Gel 60, are from Merck.

3.2. Preparation of radiolabeled phospholipid substrates

To produce 1-[¹⁴C]oleoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1-[¹⁴C]oleoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, and 1-[¹⁴C]oleoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA), 1,2-di[¹⁴C]oleoyl-*sn*-glycero-3-phosphocholine (PC) is first converted to 1-[¹⁴C]oleoyl-*sn*-glycero-3-phosphocholine (lysoPC) by the PLA₂ reaction catalyzed by PLA₂ from *N. naja* (from *Naja mossambica*). For the PLA₂ reaction, phospholipid films are first prepared. Diethyl ether/ethanol (95/5; v/v), 5 mM Tris-HCl (pH 8.9), 2.5 mM CaCl₂, and 50 units of PLA₂ are added to lipids films, and the mixture is incubated at 25° for 120 min. Every 30 min, PLA₂ (50 units) is added to the reaction mixture. The reaction is stopped by adding EDTA (40 mM), and the reaction products are extracted by the method of Bligh and Dyer (1959). 1-[¹⁴C]oleoyl-*sn*-glycero-3-phosphocholine is then reacylated (oleic acid) using rat liver microsome as described elsewhere (Arai *et al.*, 1986). The resulting PC is converted to PE, PS, and PA by transphosphatidylation catalyzed by bacterial phospholipase D (from *Actinomadura*). For preparation of PS, diethyl ether and an equal volume of buffer containing L-serine (5 M), 100 mM CaCl₂, 100 mM NaOAc (pH 5.5), and 25 units

Table 1.1 Mammalian PLA₁s^a

Family	Substrate	Possible functions	References
Extracellular PLA₁s			
PS-PLA ₁	PS specific	LPS production, PS elimination	Nagai <i>et al.</i> , 1999; Sato <i>et al.</i> , 1997
mPA-PLA ₁ α	PA specific	LPA production	Jin <i>et al.</i> , 2002; Sonoda <i>et al.</i> , 2002
mPA-PLA ₁ β	PA specific	LPA production and TG metabolism	Hiramatsu <i>et al.</i> , 2003
EL	PC	HDL metabolism	Hirata <i>et al.</i> , 1999; Ishida <i>et al.</i> , 2003; Jaye <i>et al.</i> , 1999; Jin <i>et al.</i> , 2003; Ma <i>et al.</i> , 2003; McCoy <i>et al.</i> , 2002
HL	PC	HDL metabolism	Grosser <i>et al.</i> , 1981; Homanics <i>et al.</i> , 1995; Jensen <i>et al.</i> , 1982; Laboda <i>et al.</i> , 1986; McCoy <i>et al.</i> , 2002
PLRP-2	PC	Digestion of dietary lipids	Roussel <i>et al.</i> , 1998; Thirstrup <i>et al.</i> , 1994
Intracellular PLA₁s			
PA-PLA ₁	PA	?	Han <i>et al.</i> , 2001; Higgs and Glomset, 1994, 1996; Higgs <i>et al.</i> , 1998; Pete <i>et al.</i> , 1994; Uchiyama <i>et al.</i> , 1999
KIAA0725	PA, PS, PE	?	Nakajima <i>et al.</i> , 2002
p125	—	Vesicular transport	Shimoi <i>et al.</i> , 2005; Tani <i>et al.</i> , 1999

^a PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS-PLA₁, PS-specific PLA₁; mPA-PLA₁, membrane-associated PA-selective PLA₁; HL, hepatic lipase; EL, endothelial lipase; PLRP-2, pancreatic lipase-related protein-2; PA-PLA₁, PA-preferring phospholipase A₁; LPS, lysophosphatidylserine; LPA, lysophosphatidic acid; TG, triacylglycerol.

PLD are added to the lipid film prepared from PC and incubated at 45°. Every 15 min, PLD (25 units) is added. At 120 min, the reaction is stopped by adding EDTA (80 mM), and the reaction products are extracted by the method of Bligh and Dyer (1959). For preparation of PE, ethanolamine (5 M) is added to the reaction mixture instead. To produce lysoPC, PC is subjected to the PLA₂ reaction catalyzed by PLA₂ from *N. naja*. To produce 1-[¹⁴C]oleoyl-*sn*-glycero-3-phosphoethanolamine (lysoPE), 1-[¹⁴C]oleoyl-*sn*-glycero-3-phosphoserine (lysoPS), and 1-[¹⁴C]oleoyl-*sn*-glycero-3-phosphate (lysoPA), PC is first converted to PE, PS, and PA by transphosphatidylolation catalyzed by bacterial phospholipase D (from *Actinonema*), and the resulting phospholipids are converted to lyso derivatives by the PLA₂ reaction catalyzed by PLA₂ from *N. naja*. All labeled substrates thus prepared are isolated as single components using preparative TLC. They all show a single spot on silica TLC. Hydrolysis of the phospholipid substrates by lipase from *R. delemere* confirms that 95% of the radioactivity in 1-[¹⁴C]oleoyl-2-oleoyl-glycerophospholipids is in position 1.

3.3. Assays

Phospholipase A₁ activity is measured using phospholipids whose *sn*-1 position is selectively radiolabeled as substrates (see the previous section). The specific activities of labeled phospholipids are adjusted to 2000 dpm/nmol by dilution with nonlabeled phospholipids. Multilamellar liposomes are prepared by sonicating the phospholipid emulsion. The reaction mixture contains enzyme and 40 μM phospholipid substrates in a total volume of 200 μl. For extracellular PLA₁s such as PS-PLA₁ and mPA-PLA₁α, Tris-HCl (100 mM, pH 7.5) and CaCl₂ (4 mM) are added to the reaction mixture. For intracellular PLA₁s such as PA-PLA₁ and KIAA0725, Tris-HCl (100 mM, pH 7.4) and EDTA (1 mM) are added. As is the case for other lipophilic enzymes, the substrate specificity of PLA₁ is affected by the presence of detergent such as Triton X-100 (0.1%). Thus, we routinely perform the assays in both the presence and the absence of the detergent. Some PLA₁s exhibit lysophospholipase L₁ activity in addition to PLA₁ activity. Lysophospholipase L₁ assays are performed essentially the same as PLA₁ assays, except that 1-[¹⁴C]oleoyl-*sn*-glycerophospholipids are used as substrates. After incubating the reaction mixture for an appropriate time, the reaction is stopped by adding 1.25 ml of Dole's reagent (78:20:2 [v/v/v] 2-propanol, *n*-heptane, and 1 N H₂SO₄). To extract fatty acid specifically, water (0.5 ml) and *n*-heptane (0.25 ml) are added and mixed by vortexing for 5 min. After centrifugation, 0.8 ml of the upper layer is mixed with 0.75 ml of heptane and silica gel (Wako gel C-200) and centrifuged to absorb the aqueous phase completely. The radioactivity of the resulting solution is quantified using a liquid scintillation counter. Enzyme activities are expressed as the percentage of phospholipids hydrolyzed over the total phospholipids.

4. NOVEL PLA₁ ASSAY USING ESI-MS

We have developed a novel assay to detect PLA₁ activity by measuring fatty acids liberated from nonlabeled phospholipid substrates using ESI-MS. Although PLA₁ activity can be evaluated by the formation of either fatty acids or lysophospholipids, we usually evaluate PLA₁ activity exclusively by the formation of fatty acids liberated because it enables us to compare the activities toward different phospholipids by analyzing the same fatty acid. This method can also be used to detect PLA₂ activity. In addition, it is possible to detect multiple phospholipase activities using two or more phospholipids with different fatty acid compositions as substrates. For example, by utilizing PC and PE, simultaneous detection of PLA₁ and PLA₂ activity toward both PC and PE is possible.

4.1. Materials

In this method, it is important to utilize asymmetric phospholipids with different fatty acids at the *sn*-1 and *sn*-2 positions of the glycerol backbone to distinguish the PLA₁ and PLA₂ reactions. PC, PE, PS, PA, and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol (PI) are from Avanti Polar Lipids (Birmingham, AL); 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol-3-monophosphate [PI(3)P], 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol-4-monophosphate [PI(4)P], 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol-3-monophosphate [PI(5)P], 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol-3, 5-bisphosphate [PI(3,5)P₂], and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol-4,5-bisphosphate [PI(4,5)P₂] are from Cell Signals (Columbus, OH); and fatty acids (15:0, 16:0, 18:0) are from Nu-Chek-Prep (Elysian, MN). *R. delemer* lipase (pure grade, 6000 units/mg) is from Seikagaku Kogyo, and anti-FLAG M2 affinity gel and FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) are from Sigma. Expression plasmids, pFLAG-CMV2 carrying PA-PLA₁, KIAA0725, and p125 cDNAs, were kindly donated by Dr. M. Tagaya and K. Tani (Tokyo University of Pharmacy and Life Science).

4.2. Preparation of recombinant PLA₁

To prepare recombinant intracellular PLA₁s (PA-PLA₁, KIAA0725, and p125), HeLa cells are transfected with expression plasmids using Lipofectamine 2000 reagent. Twenty-four hours after transfection, cells are suspended in lysis buffer (25 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5 mg/ml leupeptin, 2 mM pepstatin, and 2 mg/ml aprotinin) and stirred gently for 15 min at 4°C. The resulting

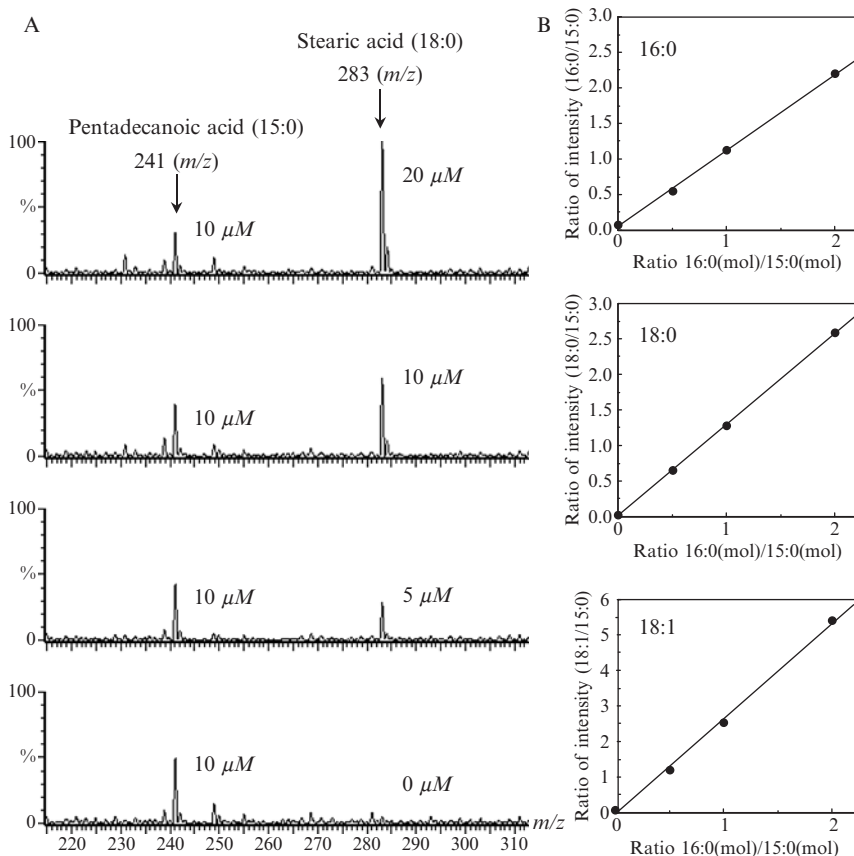


Figure 1.1 Standard curves of fatty acids determined by ESI-MS. Mixtures of pentadecanoic acid (15:0, internal standard, 10 μM) and various amount of palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1) were injected into ESI-MS. The intensities of each fatty acid were normalized by the intensity of the internal standard (15:0, pentadecanoic acid) and plotted. (A) Mass spectra of pentadecanoic acid and stearic acid. (B) Standard curves of palmitic acid, stearic acid, and oleic acid. Spectra were obtained by negative ion mode.

lysate is centrifuged at 20,000g for 20 min, and the supernatant is mixed with anti-FLAG M2 affinity gel. The tube containing the suspension is rotated for 1 h at 4°, and the gel is washed with the lysis buffer three times. FLAG proteins are eluted with 100 μl of elution buffer (25 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 2 mM EDTA, 0.1% of Triton X-100, and 100 ng/ml of FLAG peptide) three times. The elute solutions are pooled and used for the PLA₁ assay.

Extracellular PLA₁s (PS-PLA₁ and mPA-PLA₁ α and β) are expressed using a baculovirus system and are purified as described previously (Hiramatsu *et al.*,

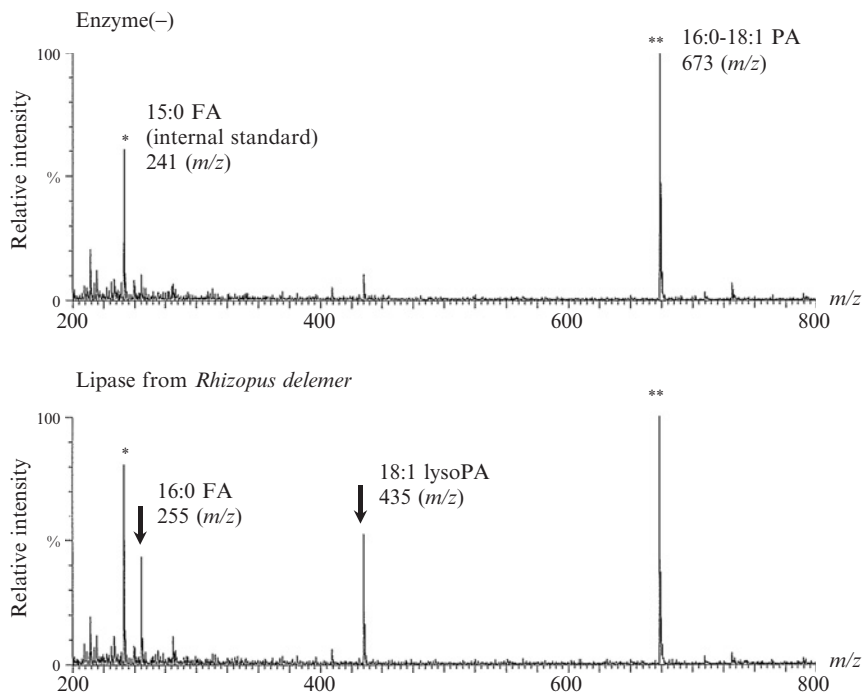
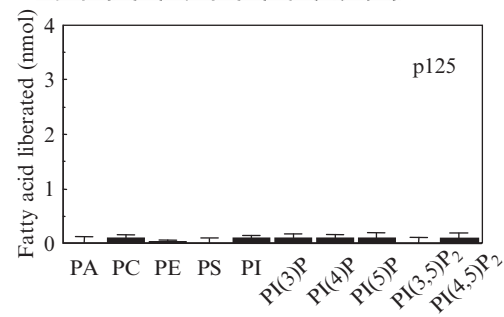
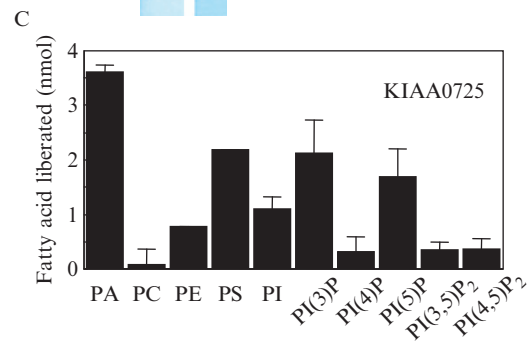
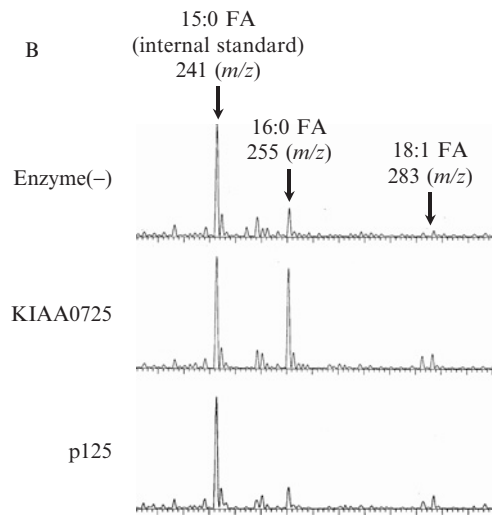
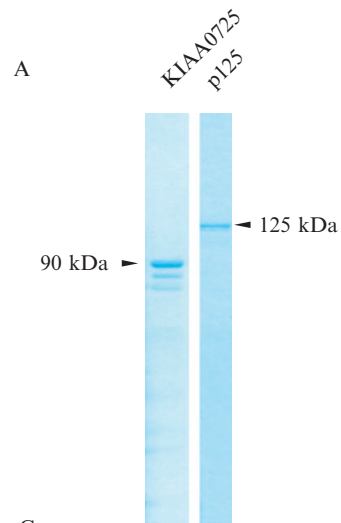


Figure 1.2 Mass spectra of 1-palmitoyl-2-oleoyl-PA after the PLA₁ reaction. 1-Palmitoyl-2-oleoyl-PA (16:0–18:1) was incubated in the presence or absence of lipase from *Rhizopus delemere*, which has PLA₁ activity toward phospholipids. The resulting lipids were subjected to ESI-MS and analyzed by negative ion mode. In the presence of lipase, both palmitic acid (255 m/z) and lysophosphatidic acid (lysoPA, 435 m/z) were detected.

2003; Nagai *et al.*, 1999; Sato *et al.*, 1997; Sonoda *et al.*, 2002). Briefly, the culture supernatant of Sf9 insect cells infected with baculoviruses expressing each extracellular PLA₁ is subjected to heparin column chromatography, and PLA₁s are eluted by a linear gradient of 0.1 to 1.5 M NaCl. The active fractions are pooled and used for the PLA₁ assay.

4.3. Phospholipase A₁ assay

The phospholipid is dried and suspended in water at a concentration of 100 μ M. Fifty milliliters of the phospholipid solution is added to 75 μ l of assay buffer containing purified enzyme in a total volume of 125 μ l and incubated at 37° for 1 h. The final concentrations of the reaction mixture are 40 μ M phospholipid, 100 mM Tris-HCl (pH 7.5), and 1 mM EDTA (or 4 mM CaCl₂ for extracellular PLA₁ and lipase from *R. delemere*). After terminating the reaction by the addition of 1 ml of methanol, 2.5 nmol of



pentadecanoic acid (15:0) is added as an internal standard. The total lipids are extracted by a modified method of Bligh and Dyer (1959). Briefly, water (775 μ l) and chloroform (1000 μ l) are added to the reaction mixture and mixed by vortexing for 5 min. After centrifugation, the organic phase is dried and resolved in chloroform/methanol (2:1; v:v).

Mass spectra of fatty acids and lysophospholipids are obtained by introducing the samples into a Quattro Micro tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source (ESI) at a flow rate of 30 μ l/min. Acetonitrile/methanol/water (2:3:1; v:v:v) containing 0.1% ammonium formate (pH 6.4) is used as the eluting solvent. For detection of fatty acids, the mass spectrometer is operated in the negative ion scan mode. The nitrogen drying gas flow rate is 12 liters/min, and its temperature is 80°. The capillary voltage is set at 3.7 kV, and the cone voltage is set at 50 V.

Figure 1.1 shows standard curves for quantification of each fatty acid. Phospholipids or lysophospholipids injected together with the standard fatty acids did not affect the standard curves (data not shown). When 1-palmitoyl-2-oleoyl-PA was incubated with lipase from *R. delemer*, which was used as a standard PLA₁ control, palmitic acid, but not oleic acid, was detected in the mass spectra (Fig. 1.2), showing that the enzyme preferentially releases palmitic acid from 1-palmitoyl-2-oleoyl-PA by its PLA₁ activity. The substrate, 1-palmitoyl-2-oleoyl-PA, and the by-product, 2-oleoyl-lysoPA, were also detected (see Fig. 1.2).

4.4. PLA₁ activity of intracellular PLA₁s

The method was applied to detect the PLA₁ activity of intracellular PLA₁s, KIAA0725, and p125. Using 1-palmitoyl-2-oleoyl-PA, PC, PE, PS, and PI as substrates, palmitic acid was liberated exclusively by incubating with KIAA0725 (Fig. 1.3B and data not shown), confirming that KIAA0725 shows PLA₁ activity as reported previously using radiolabeled substrates (Nakajima *et al.*, 2002). Figure 1.3C shows the substrate specificity of KIAA0725 and p125. KIAA0725 hydrolyzed not only PA, PE, and PS, but

Figure 1.3 Application of the novel PLA₁ assay to substrate determination of KIAA0725 and p125. (A) Purity of the recombinant enzymes used was confirmed by CBB staining. (B) Mass spectra of fatty acids obtained after incubating 1-palmitoyl-2-oleoyl-PA in the presence or absence of PLA₁s. When KIAA0725 was used as an enzyme source, a remarkable increase in the peak height of palmitic acid, but not oleic acid, was observed, showing that KIAA0725 specifically hydrolyzes the fatty acid at the *sn*-1 position of 1-palmitoyl-2-oleoyl-PA. (C) Substrate specificity of KIAA0725 and p125. KIAA0725 showed significant preference for acidic phospholipids such as PA, PS, PI, PI (3)P, and PI(5)P. In contrast, p125 did not show any detectable PLA₁ activity toward the various phospholipids tested.

also PI, PI(3)P, and PI(5)P, indicating that acidic phospholipids are preferable substrates for KIAA0725. In contrast, as reported previously (Tani *et al.*, 1999), another member of the intracellular PLA₁ family, p125, did not show any detectable PLA activity toward any phospholipids examined.

5. PERSPECTIVE

This chapter described two assays for measurement of PLA₁ activity. The study of PLA₁ has not advanced as much as other phospholipases predominantly because of the poor availability of assays. We believe that these assays will definitely help identify and characterize the PLA₁ isozymes. Although the study of PLA₁ has just started, a certain number of PLA₁ isozymes have been identified in the past decade and we will be able to know more about the physiological roles of them in the near future.

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REFERENCES

- Aoki, J., Inoue, A., Makide, K., Saiki, N., and Arai, H. (2007). Structure and function of extracellular phospholipase A1 belonging to the pancreatic lipase gene family. *Biochimie* **89**, 197–204.
- Aoki, J., Nagai, Y., Hosono, H., Inoue, K., and Arai, H. (2002). Structure and function of phosphatidylserine-specific phospholipase A1. *Biochim. Biophys. Acta* **1582**, 26–32.
- Arai, H., Inoue, K., Nishikawa, K., Banno, Y., Nozawa, Y., and Nojima, S. (1986). Properties of acid phospholipases in lysosome and extracellular medium of *Tetrahymena pyriformis*. *J. Biochem. (Tokyo)* **99**, 125–133.
- Bligh, E. C., and Dyer, W. F. (1959). A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Carriere, F., Withers-Martinez, C., van Tilbeurgh, H., Roussel, A., Cambillau, C., and Verger, R. (1998). Structural basis for the substrate selectivity of pancreatic lipases and some related proteins. *Biochim. Biophys. Acta* **1376**, 417–432.
- Grosser, J., Schrecker, O., and Greten, H. (1981). Function of hepatic triglyceride lipase in lipoprotein metabolism. *J. Lipid Res.* **22**, 437–442.
- Han, M. H., Han, D. K., Aebersold, R. H., and Glomset, J. A. (2001). Effects of protein kinase CK2, extracellular signal-regulated kinase 2, and protein phosphatase 2A on a phosphatidic acid-preferring phospholipase A1. *J. Biol. Chem.* **276**, 27698–27708.

- Han, X., and Gross, R. W. (2005). Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom. Rev.* **24**, 367–412.
- Hide, W. A., Chan, L., and Li, W. H. (1992). Structure and evolution of the lipase superfamily. *J. Lipid Res.* **33**, 167–178.
- Higgs, H. N., and Glomset, J. A. (1994). Identification of a phosphatidic acid-preferring phospholipase A1 from bovine brain and testis. *Proc. Natl. Acad. Sci. USA* **91**, 9574–9578.
- Higgs, H. N., and Glomset, J. A. (1996). Purification and properties of a phosphatidic acid-preferring phospholipase A1 from bovine testis: Examination of the molecular basis of its activation. *J. Biol. Chem.* **271**, 10874–10883.
- Higgs, H. N., Han, M. H., Johnson, G. E., and Glomset, J. A. (1998). Cloning of a phosphatidic acid-preferring phospholipase A1 from bovine testis. *J. Biol. Chem.* **273**, 5468–5477.
- Hiramatsu, T., Sonoda, H., Takanezawa, Y., Morikawa, R., Ishida, M., Kasahara, K., Sanai, Y., Taguchi, R., Aoki, J., and Arai, H. (2003). Biochemical and molecular characterization of two phosphatidic acid-selective phospholipase A1s, mPA-PLA1alpha and mPA-PLA1beta. *J. Biol. Chem.* **278**, 49438–49447.
- Hirata, K., Dichek, H. L., Cioffi, J. A., Choi, S. Y., Leeper, N. J., Quintana, L., Kronmal, G. S., Cooper, A. D., and Quertermous, T. (1999). Cloning of a unique lipase from endothelial cells extends the lipase gene family. *J. Biol. Chem.* **274**, 14170–14175.
- Homanics, G. E., de Silva, H. V., Osada, J., Zhang, S. H., Wong, H., Borensztajn, J., and Maeda, N. (1995). Mild dyslipidemia in mice following targeted inactivation of the hepatic lipase gene. *J. Biol. Chem.* **270**, 2974–2980.
- Inoue, A., and Aoki, J. (2006). Phospholipase A1s: Structure, distribution and function. *Future Lipidol.* **1**, 687–700.
- Inoue, K., Arai, H., and Aoki, J. (2004). Phospholipase A1 structures, physiological and patho-physiological roles in mammals. Wiley-VCH Verlag GmbH, Wein Heim.
- Inoue, M., and Okuyama, H. (1984). Phospholipase A1 acting on phosphatidic acid in porcine platelet membranes. *J. Biol. Chem.* **259**, 5083–5086.
- Ishida, T., Choi, S., Kundu, R. K., Hirata, K., Rubin, E. M., Cooper, A. D., and Quertermous, T. (2003). Endothelial lipase is a major determinant of HDL level. *J. Clin. Invest.* **111**, 347–355.
- Jaye, M., Lynch, K. J., Krawiec, J., Marchadier, D., Maugeais, C., Doan, K., South, V., Amin, D., Perrone, M., and Rader, D. J. (1999). A novel endothelial-derived lipase that modulates HDL metabolism. *Nat. Genet.* **21**, 424–428.
- Jensen, G. L., Daggy, B., and Bensadoun, A. (1982). Triacylglycerol lipase, monoacylglycerol lipase and phospholipase activities of highly purified rat hepatic lipase. *Biochim. Biophys. Acta* **710**, 464–470.
- Jin, W., Broedl, U. C., Monajemi, H., Glick, J. M., and Rader, D. J. (2002). Lipase H, a new member of the triglyceride lipase family synthesized by the intestine. *Genomics* **80**, 268–273.
- Jin, W., Millar, J. S., Broedl, U., Glick, J. M., and Rader, D. J. (2003). Inhibition of endothelial lipase causes increased HDL cholesterol levels *in vivo*. *J. Clin. Invest.* **111**, 357–362.
- Laboda, H. M., Glick, J. M., and Phillips, M. C. (1986). Hydrolysis of lipid monolayers and the substrate specificity of hepatic lipase. *Biochim. Biophys. Acta* **876**, 233–242.
- Ma, K., Cilingiroglu, M., Otvos, J. D., Ballantyne, C. M., Marian, A. J., and Chan, L. (2003). Endothelial lipase is a major genetic determinant for high-density lipoprotein concentration, structure, and metabolism. *Proc. Natl. Acad. Sci. USA* **100**, 2748–2753.
- McCoy, M. G., Sun, G. S., Marchadier, D., Maugeais, C., Glick, J. M., and Rader, D. J. (2002). Characterization of the lipolytic activity of endothelial lipase. *J. Lipid Res.* **43**, 921–929.
- Milne, S., Ivanova, P., Forrester, J., and Alex Brown, H. (2006). Lipidomics: An analysis of cellular lipids by ESI-MS. *Methods* **39**, 92–103.

- Nagai, Y., Aoki, J., Sato, T., Amano, K., Matsuda, Y., Arai, H., and Inoue, K. (1999). An alternative splicing form of phosphatidylserine-specific phospholipase A1 that exhibits lysophosphatidylserine-specific lysophospholipase activity in humans. *J. Biol. Chem.* **274**, 11053–11059.
- Nakajima, K., Sonoda, H., Mizoguchi, T., Aoki, J., Arai, H., Nagahama, M., Tagaya, M., and Tani, K. (2002). A novel phospholipase A1 with sequence homology to a mammalian Sec23p-interacting protein, p125. *J. Biol. Chem.* **277**, 11329–11335.
- Pete, M. J., Ross, A. H., and Exton, J. H. (1994). Purification and properties of phospholipase A1 from bovine brain. *J. Biol. Chem.* **269**, 19494–19500.
- Pulfer, M., and Murphy, R. C. (2003). Electrospray mass spectrometry of phospholipids. *Mass Spectrom. Rev.* **22**, 332–364.
- Roussel, A., Yang, Y., Ferrato, F., Verger, R., Cambillau, C., and Lowe, M. (1998). Structure and activity of rat pancreatic lipase-related protein 2. *J. Biol. Chem.* **273**, 32121–32128.
- Sato, T., Aoki, J., Nagai, Y., Dohmae, N., Takio, K., Doi, T., Arai, H., and Inoue, K. (1997). Serine phospholipid-specific phospholipase A that is secreted from activated platelets. A new member of the lipase family. *J. Biol. Chem.* **272**, 2192–2198.
- Shimoi, W., Ezawa, I., Nakamoto, K., Uesaki, S., Gabreski, G., Aridor, M., Yamamoto, A., Nagahama, M., Tagaya, M., and Tani, K. (2005). p125 is localized in endoplasmic reticulum exit sites and involved in their organization. *J. Biol. Chem.* **280**, 10141–10148.
- Sonoda, H., Aoki, J., Hiramatsu, T., Ishida, M., Bandoh, K., Nagai, Y., Taguchi, R., Inoue, K., and Arai, H. (2002). A novel phosphatidic acid-selective phospholipase A1 that produces lysophosphatidic acid. *J. Biol. Chem.* **277**, 34254–34263.
- Taguchi, R., Hayakawa, J., Takeuchi, Y., and Ishida, M. (2000). Two-dimensional analysis of phospholipids by capillary liquid chromatography/electrospray ionization mass spectrometry. *J. Mass Spectrom.* **35**, 953–966.
- Tani, K., Mizoguchi, T., Iwamatsu, A., Hatsuzawa, K., and Tagaya, M. (1999). p125 is a novel mammalian Sec23p-interacting protein with structural similarity to phospholipid-modifying proteins. *J. Biol. Chem.* **274**, 20505–20512.
- Thirstrup, K., Verger, R., and Carriere, F. (1994). Evidence for a pancreatic lipase subfamily with new kinetic properties. *Biochemistry* **33**, 2748–2756.
- Uchiyama, S., Miyazaki, Y., Amakasu, Y., Kuwata, H., Nakatani, Y., Atsumi, G., Murakami, M., and Kudo, I. (1999). Characterization of heparin low-affinity phospholipase A1 present in brain and testicular tissue. *J. Biochem.* **125**, 1001–1010.
- Wong, H., and Schotz, M. C. (2002). The lipase gene family. *J. Lipid Res.* **43**, 993–999.

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REAL-TIME CELL ASSAYS OF PHOSPHOLIPASE A₂S USING FLUOROGENIC PHOSPHOLIPIDS

Debasis Manna *and* Wonhwa Cho

Contents

1. Introduction	16
2. Fluorogenic PLA ₂ Substrates	18
2.1. Substrate specificity of PLA ₂ s	18
2.2. Selection of fluorophores and design of fluorogenic PLA ₂ substrates	18
2.3. Real-Time cellular PLA ₂ assay using fluorogenic phospholipids	22
3. Measuring Cellular sPLA ₂ Activity Using PED6 and Red-PED6	23
3.1. Materials	23
3.2. Labeling of cell membranes with PED6 and monitoring cellular sPLA ₂ activities	23
4. Measuring Cellular cPLA ₂ α Activity Using DAPC	24
4.1. Synthesis of DAPC	24
4.2. Labeling of cell membranes with DAPC and monitoring cellular cPLA ₂ α activity	25
References	26

Abstract

Phospholipase A₂s (PLA₂s) are a superfamily of enzymes involved in production of a wide variety of lipid mediators, including arachidonic acid, lysophospholipids, platelet activation factor, and eicosanoids. Fluorescence-based, real-time cellular activity assays for PLA₂s have been developed as a tool for studying the function and spatiotemporal regulation of PLA₂s. Recent progress in fluorogenic phospholipid design, genetic methods, and multiphoton multichannel microscopy allows simultaneous and continuous measurement of cellular localization and activity of PLA₂ in an isoform-selective manner. These assays should aid in elucidating the physiological roles and regulatory mechanisms of diverse PLA₂

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isoforms, developing isoform-specific PLA₂ inhibitors, and analyzing lipidomics data of PLA₂ products.

1. INTRODUCTION

Phospholipase A₂s (PLA₂s) are a superfamily of intracellular and secreted enzymes that catalyze the hydrolysis of the *sn*-2 ester of phospholipids (Fig. 2.1) (Schaloske and Dennis, 2006). PLA₂ hydrolysis produces fatty acids and lysophospholipids with different acyl chains and head groups, which can either act directly as bioactive signals or serve as precursors for other bioactive lipids (e.g., eicosanoids from arachidonic acid [AA]). Thus, identification of PLA₂ isoforms involved in the production of specific lipid mediators under different physiological conditions and determination of their regulatory mechanisms and sites of cellular actions are important for understanding the basis of diverse cellular processes regulated by PLA₂-derived lipid mediators and diseases caused by dysfunctional PLA₂ signaling pathways. Furthermore, because of the profound effect of PLA₂s on cellular lipid turnover and dynamics, elucidation of their spatiotemporal dynamics and activation should aid greatly in the analysis and interpretation of lipidomics data.

Phospholipase A₂s are, in general, subdivided into three subfamilies: secretory PLA₂s (sPLA₂s: groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII) (Murakami and Kudo, 2001; Valentin and Lambeau, 2000), Ca²⁺-dependent cytosolic PLA₂s (cPLA₂α, β, γ, δ, ε, and ζ; cPLA₂γ is Ca²⁺ independent) (Kita *et al.*, 2006; Leslie, 1997), and Ca²⁺-independent intracellular PLA₂s (iPLA₂β and γ) (Balsinde *et al.*, 2006). Among these PLA₂s, cPLA₂α (group IVA) has been shown to be involved in inflammation by gene knockout studies (Bonventre *et al.*, 1997; Uozumi *et al.*, 1997). Similar studies have also implicated iPLA₂β in spermatogenesis and insulin signaling (Bao *et al.*, 2004, 2006) and group V PLA₂ (gVPLA₂) in inflammation (Satake *et al.*, 2004). However, physiological roles of other PLA₂s have not been fully defined. To date, a majority of mechanistic studies on PLA₂s have relied on the separate use of confocal imaging and activity assays to monitor the cellular location and activity of PLA₂s, respectively. Confocal imaging of various PLA₂ isoforms, either endogenous or exogenous, has revealed that these enzymes can be localized at different membranes upon stimulation (Balestrieri *et al.*, 2006; Bingham *et al.*, 1999; Evans *et al.*, 2001; Kim *et al.*, 2002). However, establishing a direct correlation between PLA₂ localization and activation has been elusive because the radiometric assay (i.e., the measuring the release of radioactive fatty acids from the cells whose membranes have been radiolabeled) (Leslie and Gelb, 2004) used routinely for cellular PLA₂ activity measurements does not allow unequivocal determination of the site of lipolytic action and the identity of a PLA₂ isoform responsible for the hydrolysis. Recent development

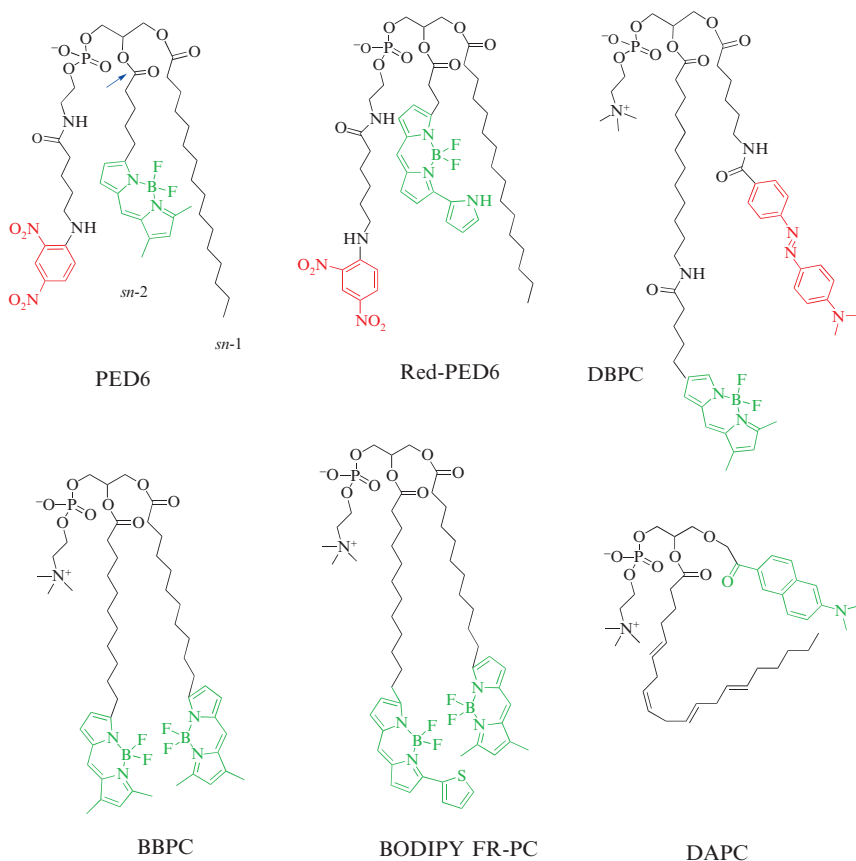


Figure 2.1 Structures of fluorogenic substrates used for the real-time cell activity assay of PLA₂. Fluorophores and quenchers are shown in green and red, respectively. PED6 has the BODIPY fluorophore in the *sn*-2 acyl group whose fluorescence is quenched by the 2,4-dinitrophenyl moiety in the head group. PLA₂-catalyzed hydrolysis of *sn*-2 ester (blue arrow) relieves the fluorescence quenching and thus enhances the emission intensity of BODIPY. Red-PED6 has the BODIPY 576/589 fluorophore in place of BODIPY. DBPC also contains a BODIPY group appended to the end of *sn*-2 acyl chain, but in this molecule the BODIPY fluorescence is quenched by the DABCYL group in the *sn*-1 acyl chain. This design allows variation in the head group structure. In BBPC, two BODIPY groups are attached to the *sn*-1 and *sn*-2 positions, respectively, and are self-quenched. BODIPY FR-PC is a FRET-based substrate with the *sn*-1 BODIPY 505/512 group as a FRET donor and the *sn*-2 BODIPY 558/568 group as an acceptor. DAPC contains the environmentally sensitive DAN probe in the *sn*-1 position and the arachidonoyl group in the *sn*-2 position. PLA₂ hydrolysis releases the DAN-containing lysophosphatidylcholine to the medium, causing a large change in fluorescence.

of real-time cellular PLA₂ activity assays, which allow simultaneous measurements of PLA₂ localization and activity if a fluorescently labeled PLA₂ is used for the analysis, has provided a powerful new tool for studying the

spatiotemporal regulation of PLA₂s (Farber *et al.*, 1999, 2001; Feng *et al.*, 2002; Hendrickson *et al.*, 1999; Kim *et al.*, 2002; Rose and Prestwich, 2006; Wijewickrama *et al.*, 2006). Real-time cell assays pose greater technical challenges than *in vitro* assays and radiometric cell assays because the activity of a particular enzyme must be measured continuously in a highly heterogeneous and dynamic environment, which entails a sensitive, rapid, specific, and high-resolution detection and quantification of signals (Baruch *et al.*, 2004; Cho, 2006). In general, fluorescence measurements meet these requirements and thus microscope-based assays using fluorogenic phospholipids have been developed for the real-time cellular activity measurement of PLA₂s (Farber *et al.*, 1999, 2001; Hendrickson *et al.*, 1999; Kim *et al.*, 2002; Wijewickrama *et al.*, 2006). This chapter summarizes the utility and limitations of the currently available real-time fluorescence cell assays for PLA₂s and describes the protocols for cellular sPLA₂ and cPLA₂ α assays.

2. FLUOROGENIC PLA₂ SUBSTRATES

2.1. Substrate specificity of PLA₂s

A main challenge in developing a fluorescence-based cellular PLA₂ activity assay is the preparation of fluorogenic substrates that can be specifically recognized by individual PLA₂ isoforms in the cell. PLA₂s can have two types of substrate selectivity: head group and acyl group selectivity. Most PLA₂s, including all sPLA₂s, have definite head group selectivity, although the degree of head group selectivity varies among species (Lichtenbergova *et al.*, 1998; Singer *et al.*, 2002). However, they exhibit a much smaller degree of acyl group selectivity. To date, only cPLA₂ α has been shown to have pronounced selectivity for the *sn*-2 arachidonoyl group (Clark *et al.*, 1991; Sharp *et al.*, 1991), hence its critical involvement in AA production and eicosanoid-mediated inflammation. Also, many PLA₂s cannot effectively bind and hydrolyze synthetic phospholipids with a bulky head group. Therefore, a fluorophore and other substituents should be preferably introduced to the *sn*-1 or *sn*-2 acyl chain to avoid perturbation of the head group structure of phospholipid. The design of cPLA₂ α -specific substrates should also take into account the requirement that the *sn*-2 arachidonoyl group be preserved.

2.2. Selection of fluorophores and design of fluorogenic PLA₂ substrates

For real-time cell assays that require continuous and prolonged monitoring of fluorescence, it is essential to use fluorophores that are excited at a longer wavelength (i.e., >500 nm; see Table 2.1) and less prone to photobleaching

Table 2.1 Fluorogenic phospholipids used for cellular PLA₂ activity assay

Compounds	λ_{ex} (nm) ^a	λ_{em} (nm) ^a	PLA ₂ isoform selectivity	References
PED6	500	512	sPLA ₂ and zebrafish PLA ₂	Farber <i>et al.</i> , 1999, 2001; Hendrickson <i>et al.</i> , 1999; Kim <i>et al.</i> , 2002; Wijewickrama <i>et al.</i> , 2006
Red-PED6	579	589	sPLA ₂	Wijewickrama <i>et al.</i> , 2006
DBPC	500	512	sPLA ₂	Feng <i>et al.</i> , 2002; Rose and Prestwich, 2006
BBPC	500	512	sPLA ₂	Feng <i>et al.</i> , 2002
DAPC	378 (720 ^b)	460 (in CHCl ₃) 560 (in H ₂ O)	cPLA ₂ α	

^a λ_{ex} and λ_{em} indicate wavelength values for maximal fluorescence excitation and emission, respectively.

^b Excitation wavelength for two-photon microscopy.

to avoid cell death and nonspecific signal reduction, respectively. This entails incorporation of a relatively bulky fluorophore into the acyl chain of the phospholipid, which would inevitably perturb the phospholipid structure and may lower efficacy for some PLA₂ isoforms. Phospholipids with a bulky fluorophore may also disrupt the local membrane structure, affecting normal cellular functions and the subcellular localization of fluorogenic phospholipids in an unpredictable manner. Two-photon microscopy (Sanchez and Gratton, 2005), which employs longer wavelength excitation (theoretically twice of that used for single-photon excitation), alleviates this constraint greatly and thus allows for more flexible design of PLA₂ substrates with a wider selection of fluorophores.

To date, a majority of fluorogenic PLA₂ substrates have been designed based on the so-called “dequenching by hydrolysis” principle. In this approach, both a fluorophore and a quencher are incorporated into the same phospholipid molecule, resulting in internal quenching. The PLA₂ hydrolysis liberates a fluorophore or a quencher and thereby relieves internal quenching, resulting in enhanced fluorescence emission intensity. A representative fluorogenic phospholipid designed by this principle is *N*-([6-(2,4-dinitrophenyl)amino]hexanoyl)-1-hexadecanoyl-2-(BODIPY-pentanoyl)-*sn*-glycero-3-phosphoethanolamine (PED6) (Hendrickson *et al.*, 1999), which contains a BODIPY fluorophore in the *sn*-2 acyl chain, the fluorescence of which is quenched by the 2,4-dinitrophenyl moiety appended to the head group (see Fig. 2.1). Release of the BODIPY fluorophore by PLA₂ catalysis would relieve this quenching, hence the increase in fluorescence intensity. For this compound, attaching the 2,4-dinitrophenyl group to the head group does not significantly interfere with its interaction with PLA₂s, presumably because of the insertion of a relatively long flexible linker (i.e., 4-CH₂-) that minimizes unfavorable interactions between the PLA₂ active site and the quencher. A structural analog of PED6 that contains the BODIPY 576/589 fluorophore in place of BODIPY has been developed (Wijewickrama *et al.*, 2006). This so-called Red-PED6 can be used orthogonally with PED6 for simultaneously monitoring the PLA₂ activity in two different types of cells. Red-PED is also useful for activity assay for the PLA₂ labeled with a green or yellow fluorescence protein. Both PED6 and Red-PED6 are good substrates for most sPLA₂s (specific activity up to 20 μmol/min/mg), and cell studies using PED6 and Red-PED6 have provided valuable information about the function and regulation of different sPLA₂s (Farber *et al.*, 1999, 2001; Hendrickson *et al.*, 1999; Kim *et al.*, 2002; Wijewickrama *et al.*, 2006). PED6 was initially reported to be a good substrate for cPLA₂α (Hendrickson *et al.*, 1999) and was used to monitor the intracellular PLA₂ activity of zebrafish (Farber *et al.*, 2001); however, it has been shown that cPLA₂α has less than 1% of specific activity of major types of sPLA₂ (i.e., groups IIA, V, and X) toward both PED6 and Red-PED6 (Wijewickrama *et al.*, 2006). Therefore, these compounds may not be suitable

for the cellular cPLA₂ α assay under normal physiological conditions. Another drawback of PED6-like compounds is that they do not allow variation of the head group structure. To overcome this, a series of fluorogenic phospholipids that contain the BODIPY fluorophore at the *sn*-2 acyl chain and the Dabcyl group as quencher at the *sn*-1 acyl chain (see Fig. 2.1) have been prepared (Feng *et al.*, 2002; Rose and Prestwich, 2006). These compounds, with the head group varying from phosphatidylcholine (1-[6-*p*-methyl red]aminohexanoyl)-2-O-(12-[5-BODIPY-pentanoyl]aminododecanoyl)-*sn*-3-glycerophosphocholine; DBPC), -ethanolamine, -glycerol, to phosphatic acid, have been used for the cellular real-time PLA₂ assay. It has not been demonstrated, however, that they can be used specifically for the cPLA₂ α assay. Other fluorogenic phospholipids with a fluorophore and a quencher at different locations and ones with two identical fluorophores appended to the *sn*-1 and *sn*-2 acyl group (see Fig. 2.1; BBPC), respectively, have been also reported (Hendrickson *et al.*, 1999), but their use has been largely limited to the *in vitro* PLA₂ activity assay.

The second group of fluorogenic PLA₂ substrates contains two fluorophores that act as a fluorescence resonance energy transfer (FRET) donor and acceptor, respectively. For example, BODIPY FR-PC has the BODIPY 505/512 group as a FRET donor at the *sn*-1 position and the BODIPY 558/568 group as an acceptor at the *sn*-2 position (see Fig. 2.1) (Farber *et al.*, 2001). The PLA₂ assay using this compound follows the decrease in FRET signal as the PLA₂-catalyzed release of the *sn*-2 acyl group abolishes FRET. BODIPY FR-PC has been used for monitoring the endogenous PLA₂ activity of zebrafish (Farber *et al.*, 2001). An advantage of the FRET-based assay over the dequenching-based assay is that the fluorescence signal can be detected both before (as FRET) and after the hydrolysis (as emission intensity of the donor). However, a major drawback of the FRET assay is low sensitivity because the FRET efficiency is typically low and because the assay monitors the decrease in signal as a function of time.

Most recently, phospholipids containing an environmentally sensitive fluorophore have been developed in our laboratory for the cPLA₂ α assay. cPLA₂ α has strong preference for the *sn*-2 arachidonoyl group, but the aforementioned substrate designs make it difficult to incorporate the *sn*-2 arachidonoyl group in a fluorogenic substrate. If a fluorophore whose fluorescence emission is sensitive to the solvent polarity, such as 2-dimethylamino-6-acyl-naphthalene (DAN) (Weber and Farris, 1979), is appended to the *sn*-1 group of the phospholipid, the *sn*-2 arachidonoyl group can be preserved, yet the PLA₂ hydrolysis would cause a fluorescence change because release of the water-soluble DAN-containing lysophosphatidylcholine from the membrane results in a large drop in fluorescence intensity at 460 to 500 nm. For instance, 1-O-(1-[6-dimethylamino]naphthoylacyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine (DAPC; see Fig. 2.1), which contains the DAN group at the *sn*-1 position via an ether

linkage, has been prepared as a first-generation, environment-sensitive cPLA₂ α substrate. (The ether linkage is used to prevent hydrolysis of the *sn*-1 group by the lysophospholipase activity of cPLA₂ α or by phospholipases A₁.) A full description of this new group of fluorogenic substrates will be published elsewhere. In this chapter, general properties of DAPC and the assay protocol using this substrate are described briefly.

In vitro measurements show that DAPC is a good substrate for cPLA₂ α (the specific activity can reach 100 nmol/min/mg depending on the assay condition) and is a much better substrate for cPLA₂ α than for major types of sPLA₂s at low to submicromolar Ca²⁺, which is a physiologically relevant range of Ca²⁺ for cPLA₂ α . When rat basophilic leukemia cells are labeled with DAPC, a rapid decrease in fluorescence intensity is seen primarily at the perinuclear region in response to cell stimulation with ionomycin, which is abrogated by the treatment of cells with a cPLA₂ α inhibitor, methyl arachidonoyl fluorophosphonate, but not by an iPLA₂ inhibitor, bromoenol lactone (BEL). An obvious advantage of this assay over other methods is that it allows a specific cPLA₂ α assay. sPLA₂s do not show detectable activity toward DAPC at the cellular Ca²⁺ concentration, and the potential contribution from iPLA₂ can be assessed readily by treating the cells with an iPLA₂ inhibitor, such as BEL. Although this assay monitors the decrease in fluorescence signal, it still offers good sensitivity because of the high molecular brightness of the membrane-incorporated DAN group (Weber and Farris, 1979). However, the DAN group may undergo significant photobleaching and cells may be damaged by continuous irradiation with an ultraviolet (UV) laser, both of which can be avoided to a large extent by two-photon excitation.

2.3. Real-Time cellular PLA₂ assay using fluorogenic phospholipids

Real-time cellular PLA₂ assays can serve as a powerful tool for studying the spatiotemporal dynamics and activation of various PLA₂ isoforms. To achieve this goal, however, one must first establish an effective and consistent labeling method that allows uniform distribution of fluorogenic lipids over cell membranes while causing minimal cell damage. Among several membrane-labeling techniques available, a method using mixed vesicles has been used most frequently (see later) (Farber *et al.*, 2001). Because the membrane-labeling efficiency and the subcellular distribution of fluorogenic phospholipid may vary significantly depending on the cell type and the nature of fluorogenic lipid, careful controls must be performed on a trial-and-error basis to achieve optimal cell labeling. Typically, cells can be used for the activity assay when a majority (i.e., >50%) of cells display uniform fluorophore distribution and healthy morphology.

An isoform-specific PLA₂ assay can be achieved by selecting an appropriate fluorogenic substrate (e.g., DAPC for cPLA₂ α and Red-PED6 for sPLA₂) and an isoform-specific agonist and can be confirmed using isoform-selective inhibitors and/or genetic techniques, such as gene knockdown by RNA interference. Most PLA₂ cell assays can be performed using a conventional one-photon confocal microscope with a proper set of filters (see Table 2.1), but a two-photon microscope should serve better for a prolonged continuous assay, especially for those phospholipids with the excitation maximum in the UV range (e.g., DAPC). Finally, current fluorogenic phospholipids allow only qualitative to semiquantitative activity measurements because neither ratio-metric measurement nor substrate concentration determination through calibration is feasible. Improvement in fluorogenic substrate design, as well as in fluorescence microscopy detection, would lead to the development of a true quantitative real-time *in situ* assay for PLA₂ that can be used for functional and mechanistic studies of PLA₂s and PLA₂-specific drug development in various cells, tissues, and whole organisms.

3. MEASURING CELLULAR SPLA₂ ACTIVITY USING PED6 AND RED-PED6

3.1. Materials

PED6 is available commercially from Invitrogen, and Red-PED is synthesized from 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine *N*-(6-[(2,4-dinitrophenyl)amino]hexanoyl) and succinimidyl 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a,diaza-*s*-indacene-3-propionate (Invitrogen) (Wijewickrama *et al.*, 2006). The purity of the fluorogenic phospholipids is critical for the low background fluorescence and the high sensitivity of the assay. Therefore, extreme care must be taken to remove fluorescent impurities, including fluorescent fatty acids and lysophospholipids, which increase the background fluorescence greatly throughout the cell interior and membranes. It is recommended that the purity of fluorescent phospholipids be checked immediately before use by thin-layer chromatography and the compounds be further purified if necessary. Also, any precipitate formed in the lipid sample and during vesicle preparation should be removed by centrifugation or filtration.

3.2. Labeling of cell membranes with PED6 and monitoring cellular SPLA₂ activities

1. Mix 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS)/cholesterol/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG)/PED6 (or Red-PED6) (107:31:20:1 in mole ratio, 300 nmol total) each dissolved

in chloroform and dry the mixture under N_2 . Resuspend the residue in 10 μ l ethanol and add 10 μ l of Dulbecco's modified Eagles medium (DMEM). Dry the mixture again under N_2 until the volume is reduced to about 7 μ l to ensure that most of the ethanol is evaporated. Add an additional 10 μ l of DMEM to the mixture and sonicate the mixture on ice for 20 min to prepare vesicles.

2. Twenty-four hours prior to labeling, seed the cells into eight-well plates and keep them in DMEM supplemented with 10% fetal bovine serum at 37° with 5% CO_2 . Remove media and add 10 μ l of the vesicle solution and 90 μ l of DMEM, both prewarmed to 37°, to cells in each well and incubate the cells for 25 to 50 min at 37°. Adjust the amount of vesicles added and the incubation period depending on the cell type. The degree of membrane labeling can be assessed by monitoring the low but definite fluorescence emission of PED6 with the high detector gain. Rinse vesicle-treated cells five times with phosphate-buffered saline or DMEM without phenol red (37°) to remove unincorporated dye.
3. Add sPLA₂ exogenously or add an agonist to stimulate the secretion and activation of sPLA₂. Start imaging with a confocal microscope using a 488-nm krypton/argon laser and a 530-nm band-pass filter. Adjust the detector gain to eliminate background autofluorescence. A 40× (or higher magnification) water immersion objective with a 1.2 numerical aperture can be used for experiments. PED6 and Red-PED start to show a significant degree of photobleaching after 10 min with single-photon excitation under normal conditions. If the assay must be performed for a longer period, it is necessary to lower the laser intensity and enhance the laser-scanning rate to minimize photobleaching. It is also recommended that the net fluorescence change be calculated after the calibration of photobleaching.

4. MEASURING CELLULAR cPLA₂α ACTIVITY USING DAPC

4.1. Synthesis of DAPC

1. 6-Bromoacetyl-2-(dimethylamino)naphthalene is synthesized from commercially available 6-acetyl-2-methoxynaphthalene according to the reported procedure (Nitz *et al.*, 2002). ^1H NMR (300 MHz, $CDCl_3$) δ = 8.31 (m, 1H), 7.88 (m, 1H), 7.78 (m, 1H), 7.61 (m, 1H), 7.12 (m, 1H), 6.83 (m, 1H), 4.51 (s, 2H), and 3.10 (m, 6H).
2. Add anhydrous triethylamine (0.32 ml, 2.27 mmol) to a stirred solution of *sn*-glycero-3-phosphocholine (1:1 cadmium chloride adduct, 0.1 g,

- 0.23 mmol) in dry chloroform (3 ml) that is kept under N₂ atmosphere and at 0°. After 15 min of stirring, add a solution of 6-bromoacetyl-2-(dimethylamino)naphthalene (0.09 g, 0.23 mmol) in chloroform and stir the mixture for another 48 h at 23°. Concentrate the reaction mixture *in vacuo* and purify 1-O-(1-[6-dimethylamino]naphthoylacetyl)-2-hydroxyl-*sn*-glycero-3-phosphocholine (about 59% yield) by flash silica gel chromatography using 10 to 50% methanol/chloroform as eluent. ¹H NMR (300 MHz, CDCl₃): δ = 8.83 (m, 1H), 8.03 (m, 1H), 7.86 (m, 1H), 7.56 (m, 1H), 6.97 (m, 1H), 6.72 (m, 1H), 5.25 (s, 2H), 3.76 (m, 5H), 3.02 (m, 6H), and 1.28 (m, 10H).
3. To a stirred solution of *N,N'*-dicyclohexylcarbodiimide (0.05 g, 0.25 mmol) in dry carbon tetrachloride (3 ml) kept under N₂ atmosphere and at 23°, add a solution of AA (0.19 g, 0.51 mmol) in carbon tetrachloride (3 ml) and a catalytic amount of 4-(dimethylamino)pyridine. Stir the mixture at 23° for 6 h and remove the precipitate by filtration. Concentrate the filtrate *in vacuo* and dissolve the residue in 2 ml anhydrous pyridine. Add a solution of 1-O-(1-[6-dimethylamino]naphthoylacetyl)-2-hydroxyl-*sn*-glycero-3-phosphocholine (0.15 g, 0.23 mmol) in anhydrous pyridine (2 ml) and a catalytic amount of 4-(dimethylamino)pyridine to the reaction mixture. After stirring at 50° for 18 h, concentrate the reaction mixture *in vacuo* and purify DAPC twice by flash silica gel chromatography using 10 to 30% methanol/chloroform and 0 to 2% methanol/chloroform as eluents, respectively. ¹H NMR (300 MHz, CDCl₃): δ = 9.10 (m, 1H), 8.25 (m, 2H), 8.05 (m, 1H), 7.35 (m, 1H), 6.95 (m, 1H), 5.34 (m, 10H), 3.84 (m, 5H), 3.09 (m, 6H), 2.87 (m, 4H), 2.79 (m, 6H), 2.38 (m, 2H), 2.05 (m, 4H), 1.75 (m, 5H), 1.42 (m, 6H), 1.28 (m, 6H), and 0.88 (m, 3H).

4.2. Labeling of cell membranes with DAPC and monitoring cellular cPLA₂ α activity

1. Label cell membranes using POPS/cholesterol/POPG/DAPC (107:31:20:1) mixed vesicles as described for PED6 and Red-PED6.
2. Add Ca²⁺ ionophore or an agonist to activate cPLA₂ α and start imaging, preferably with a two-photon microscope with the detector gain adjusted to eliminate background autofluorescence. For two-photon excitation of DAPC, a 720-nm ultrafast pulsed beam from a tunable Tsunami laser and a 515-nm band-pass filter are used. One-photon excitation is possible but not recommended because DAPC starts to photobleach within 3 min of excitation. With two-photon excitation, photobleaching is negligible in the first 10 min and can be further suppressed by faster scanning.

REFERENCES

- Balestrieri, B., Hsu, V. W., Gilbert, H., Leslie, C. C., Han, W. K., Bonventre, J. V., and Arm, J. P. (2006). Group V secretory phospholipase A2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. *J. Biol. Chem.* **281**, 6691–6698.
- Balsinde, J., Perez, R., and Balboa, M. A. (2006). Calcium-independent phospholipase A2 and apoptosis. *Biochim. Biophys. Acta* **1761**, 1344–1350.
- Bao, S., Miller, D. J., Ma, Z., Wohltmann, M., Eng, G., Ramanadham, S., Moley, K., and Turk, J. (2004). Male mice that do not express group VIA phospholipase A2 produce spermatozoa with impaired motility and have greatly reduced fertility. *J. Biol. Chem.* **279**, 38194–38200.
- Bao, S., Song, H., Wohltmann, M., Ramanadham, S., Jin, W., Bohrer, A., and Turk, J. (2006). Insulin secretory responses and phospholipid composition of pancreatic islets from mice that do not express group VIA phospholipase A2 and effects of metabolic stress on glucose homeostasis. *J. Biol. Chem.* **281**, 20958–20973.
- Baruch, A., Jeffery, D. A., and Bogoy, M. (2004). Enzyme activity: It's all about image. *Trends Cell Biol.* **14**, 29–35.
- Bingham, C. O., 3rd, Fijneman, R. J., Friend, D. S., Goddeau, R. P., Rogers, R. A., Austen, K. F., and Arm, J. P. (1999). Low molecular weight group IIA and group V phospholipase A(2) enzymes have different intracellular locations in mouse bone marrow-derived mast cells. *J. Biol. Chem.* **274**, 31476–31484.
- Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997). Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* **390**, 622–625.
- Cho, W. (2006). Seeing is believing: Real-time cellular activity assay for phospholipase A2. *ACS Chem. Biol.* **1**, 65–66.
- Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991). A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043–1051.
- Evans, J. H., Spencer, D. M., Zweifach, A., and Leslie, C. C. (2001). Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. *J. Biol. Chem.* **276**, 30150–30160.
- Farber, S. A., Olson, E. S., Clark, J. D., and Halpern, M. E. (1999). Characterization of Ca2+-dependent phospholipase A2 activity during zebrafish embryogenesis. *J. Biol. Chem.* **274**, 19338–19346.
- Farber, S. A., Pack, M., Ho, S. Y., Johnson, I. D., Wagner, D. S., Dosch, R., Mullins, M. C., Hendrickson, H. S., Hendrickson, E. K., and Halpern, M. E. (2001). Genetic analysis of digestive physiology using fluorescent phospholipid reporters. *Science* **292**, 1385–1388.
- Feng, L., Manabe, K., Shope, J. C., Widmer, S., DeWald, D. B., and Prestwich, G. D. (2002). A real-time fluorogenic phospholipase A(2) assay for biochemical and cellular activity measurements. *Chem. Biol.* **9**, 795–803.
- Hendrickson, H. S., Hendrickson, E. K., Johnson, I. D., and Farber, S. A. (1999). Intramolecularly quenched BODIPY-labeled phospholipid analogs in phospholipase A(2) and platelet-activating factor acetylhydrolase assays and *in vivo* fluorescence imaging. *Anal. Biochem.* **276**, 27–35.
- Kim, Y. J., Kim, K. P., Rhee, H. J., Das, S., Rafter, J. D., Oh, Y. S., and Cho, W. (2002). Internalized group v secretory phospholipase A2 acts on the perinuclear membranes. *J. Biol. Chem.* **277**, 9358–13174.

- Kita, Y., Ohto, T., Uozumi, N., and Shimizu, T. (2006). Biochemical properties and pathophysiological roles of cytosolic phospholipase A2s. *Biochim. Biophys. Acta* **1761**, 1317–1322.
- Leslie, C. C. (1997). Properties and regulation of cytosolic phospholipase A2. *J. Biol. Chem.* **272**, 16709–16712.
- Leslie, C. C., and Gelb, M. H. (2004). Assaying phospholipase A2 activity. *Methods Mol. Biol.* **284**, 229–242.
- Lichtenbergova, L., Yoon, E. T., and Cho, W. (1998). Membrane penetration of cytosolic phospholipase A₂ is necessary for its interfacial catalysis and arachidonate specificity. *Biochemistry* **37**, 14128–14136.
- Murakami, M., and Kudo, I. (2001). Diversity and regulatory functions of mammalian secretory phospholipase A2s. *Adv. Immunol.* **77**, 163–194.
- Nitz, M., Mezo, A. R., Ali, M. H., and Imperiali, B. (2002). Enantioselective synthesis and application of the highly fluorescent and environment-sensitive amino acid 6-(2-dimethylaminonaphthoyl) alanine (DANA). *Chem. Commun. (Camb.)* 1912–1913.
- Rose, T. M., and Prestwich, G. D. (2006). Fluorogenic phospholipids as head group-selective reporters of phospholipase A activity. *ACS Chem. Biol.* **1**, 83–91.
- Sanchez, S. A., and Gratton, E. (2005). Lipid-protein interactions revealed by two-photon microscopy and fluorescence correlation spectroscopy. *Acc. Chem. Res.* **38**, 469–477.
- Satake, Y., Diaz, B. L., Balestrieri, B., Lam, B. K., Kanaoka, Y., Grusby, M. J., and Arm, J. P. (2004). Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. *J. Biol. Chem.* **279**, 16488–16494.
- Schaloske, R. H., and Dennis, E. A. (2006). The phospholipase A2 superfamily and its group numbering system. *Biochim. Biophys. Acta* **1761**, 1246–1259.
- Sharp, J. D., White, D. L., Chiou, X. G., Gooden, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskin, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., et al. (1991). Molecular cloning and expression of human Ca²⁺-sensitive cytosolic phospholipase A₂. *J. Biol. Chem.* **266**, 14850–14853.
- Singer, A. G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., and Gelb, M. H. (2002). Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. *J. Biol. Chem.* **277**, 48535–48549.
- Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997). Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature* **390**, 618–622.
- Valentin, E., and Lambeau, G. (2000). Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. *Biochim. Biophys. Acta* **1488**, 59–70.
- Weber, G., and Farris, F. J. (1979). Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)naphthalene. *Biochemistry* **18**, 3075–3078.
- Wijewickrama, G. T., Kim, J. H., Kim, Y. J., Abraham, A., Oh, Y., Ananthanarayanan, B., Kwatia, M., Ackerman, S. J., and Cho, W. (2006). Systematic evaluation of transcellular activities of secretory phospholipases A2: High activity of group V phospholipases A2 to induce eicosanoid biosynthesis in neighboring inflammatory cells. *J. Biol. Chem.* **281**, 10935–10944.

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ANALYSIS AND PHARMACOLOGICAL TARGETING OF PHOSPHOLIPASE C β INTERACTIONS WITH G PROTEINS

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Contents

1. Introduction	30
2. Methods	31
2.1. Protein expression and purification	31
2.2. PLC β assay	37
2.3. PLC assay optimization	41
2.4. Application of the PLC assay to evaluate peptide/small molecule modulation of G $\beta\gamma$ -dependent PLC activity	42
2.5. Evaluation of G $\beta\gamma$ -PLC binding	43
3. Concluding Remarks	46
Acknowledgment	47
References	47

Abstract

Phosphatidylinositol-specific phospholipase C enzymes (PLC) catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate generating the second messengers diacylglycerol and inositol 1,4,5-triphosphate. Mammalian phosphoinositide-specific phospholipase C β (PLC β) activity is regulated by the α_q family of G-protein α subunits and by G $\beta\gamma$ subunits. Regulation of PLC β enzymatic activity can be assayed by reconstituting purified G-protein subunits with purified PLC β in the presence of phospholipid vesicles containing the substrate phosphatidylinositol 4,5-bisphosphate. This chapter describes methods for expression and purification of PLC β and G $\beta\gamma$ from insect cells, assay of G-protein-dependent regulation of PLC activity, and assessment of G-protein-PLC direct binding interactions. This combination of functional and direct binding analysis provides a powerful approach to characterizing PLC and G-protein interfaces, identifying

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inhibitors of this interaction, and potentially uncovering new modes of PLC regulation.

1. INTRODUCTION

Agonist binding to specific G-protein-coupled receptors liberates the α and $\beta\gamma$ subunits to interact directly with and activate phosphoinositide-specific phospholipase C β isoforms (PLC β) (Rhee, 2001; Singer *et al.*, 1997). PLC β is a key enzyme in hydrolysis of the minor membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) (Exton, 1996; Rhee *et al.*, 1991; Singer *et al.*, 1997). This reaction produces two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol, which in turn stimulate intracellular calcium release and activation of protein kinase C, respectively.

To date, 13 different PLC isozymes have been identified and grouped into β , γ , δ , ϵ , ζ , and η subfamilies, each composed of multiple isoforms (Harden and Sondek, 2006; Rhee, 2001). PLC β isozymes are activated through interactions with the α subunits of the pertussis toxin-insensitive G α_q family and the $\beta\gamma$ subunits of heterotrimeric G proteins (Rhee, 2001; Singer *et al.*, 1997). Examples of G-protein-coupled receptors that utilize this G α_q /PLC β pathway include bradykinin, bombesin, angiotensin, histamine, vasopressin, muscarinic acetylcholine, α_1 -adrenergic, thyroid-stimulating hormone, and endothelin1 receptors. The GTP γ S/AlF₄⁻ activated G α_q or G α_{11} subunits stimulate PLC β isoforms with the rank order of potency: PLC β 4 > PLC β 1 > PLC β 3 > PLC β 2 (Smrcka and Sternweis, 1993).

The sensitivity of PLC β isozymes to G $\beta\gamma$ subunits differs from that of G α_q and decreases in the order PLC β 3 > PLC β 2 > PLC β 1, whereas PLC β 4 is not activated to any extent (Lee and Rhee, 1996; Smrcka and Sternweis, 1993). The m2 and m4 muscarinics, acetylcholine, interleukin-8, V2 vasopressin receptor, and luteinizing hormone receptors can activate the pathway mediated by G $\beta\gamma$ subunits. PLC β 2 and β 3 are also activated by the small GTP-binding protein Rac (Harden and Sondek, 2006).

Much of the original evidence for direct regulation of PLC isoforms by G proteins comes from reconstitution of purified PLC isoforms with purified G-protein subunits in assays of phospholipase C activity (Camps *et al.*, 1992; Smrcka *et al.*, 1991; Taylor and Exton, 1991; Waldo *et al.*, 1991). These studies have been followed by extensive experimentation to determine the mechanisms for PLC regulation. The three-dimensional structure of Rac1 bound to PLC has been solved, giving some insight into the nature of G-protein-PLC interactions (Jezyk *et al.*, 2006), but in the absence of other structural information, analyses of interactions between heterotrimeric G-protein subunits and PLC β have relied on biochemical approaches. In particular, assessment of effects of PLC β and G-protein mutations (Bonacci *et al.*, 2005; Panchenko *et al.*, 1998; Wang *et al.*, 2000) and inhibitory peptides on G-protein-

dependent regulation in PLC enzymatic assays (Sankaran *et al.*, 1998), in combination with analysis of binding interactions (Dowal *et al.*, 2001), have been used to identify regions on both G proteins and phospholipases critical for G protein–PLC interactions. Results of these analyses from several laboratories are summarized briefly here. The COOH terminus of PLC β is essential for binding and activation of PLC β by the G α_q subunit (Lee *et al.*, 1993). G $\beta\gamma$ subunits interact with PLC β 2 at the catalytic domain (Bonacci *et al.*, 2005; Kuang *et al.*, 1996; Sankaran *et al.*, 1998), the pleckstrin homology (PH) domain (Dowal *et al.*, 2001; Wang *et al.*, 2000), or both. Recent experiments have exploited information about G $\beta\gamma$ /PLC β interfaces to identify inhibitors of G-protein-dependent regulation of PLC (Bonacci *et al.*, 2006; Scott *et al.*, 2001).

A full understanding of the nature of these interactions and the mechanism of action of inhibitors relies on a combination of both functional reconstitution assays and binding assays. This chapter describes complementary methods for assessment of functional interactions using enzymatic PIP₂ hydrolysis assays (Smrcka and Sternweis, 1993; Smrcka *et al.*, 1991) and two PLC–G protein-binding assays we have developed. Methods are also described for purification of PLC β isoforms (1, 2, and 3) and G $\beta\gamma$ from a baculovirus insect cell expression system. Applications of these methods to evaluation of small molecule inhibitors of G $\beta\gamma$ -dependent regulation of PLC β enzymatic activity and in analyzing the effects of PLC mutations on regulation by G $\beta\gamma$ are also discussed.

2. METHODS

2.1. Protein expression and purification

2.1.1. Expression of recombinant 6His PLC β in insect cells

The procedure described here is equally applicable to purification of N-terminally 6His-tagged PLC β 1, 2, and 3. Infect 800 ml of Sf9 cells (ATCC) at a density of 1.5 to 2×10^6 cells/ml with 10 ml of 6His PLC β baculovirus (multiplicity of infection [MOI] = 1) and incubate with continuous shaking (125 rpm) for 48 h at 27°. PLC β 1, β 2, and β 3 baculoviruses can be obtained from our laboratory. Maximal expression of PLC β occurs at 48 h. Once the cells are harvested, all processing from cell lysis until the purified protein is obtained is conducted at 4° to minimize proteolytic degradation. All buffers should be made just prior to use (minimize inactivation of some protease inhibitors and oxidation of the dithiothreitol [DTT]) and chilled to 4°.

Harvest cells by centrifugation at 2500 rpm for 20 min (Beckman centrifuge, JA10 rotor). Resuspend cells in 40 ml of phosphate-buffered saline (PBS; 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl) containing a basic protease inhibitor cocktail (PTT: 133 μ M

phenylmethylsulfonyl fluoride; 21 $\mu\text{g/ml}$ N^a -*p*-tosyl-L-lysine chloromethyl ketone; 21 $\mu\text{g/ml}$ tosylphenylalanyl chloromethyl ketone), 0.1 mM EDTA, and 0.1 mM EGTA, transfer to a 50-ml conical tube, and centrifuge again at 1000g for 20 min at 4°. Discard the supernatant and continue or freeze the pellet rapidly by submerging the tube in liquid N₂ and store at -70° until further processing is done.

2.1.1.1. Cell lysis Suspend Sf9 cells expressing 6HisPLC β in 25 ml Sf9 cell lysis buffer [50 mM Na-HEPES, pH 7.4, 0.1 M ethylenedis(oxyethylene)triacetic acid (Na-EGTA), 0.1 M ethylenediamine tetracetic acid (Na-EDTA), 0.1 mM DTT, 100 mM NaCl, and full protease inhibitor (PI) cocktail containing PTT supplemented with other protease inhibitors, 1 $\mu\text{g/ml}$ aprotinin, 2 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin A, 21 $\mu\text{g/ml}$ tosyl-larginine methyl ester, 10 mg/ml soybean trypsin inhibitor (SBTI)]. If the cells have been stored at -70°, use room temperature lysis buffer, otherwise use buffer that has been chilled to 4°. Freeze the suspended cells in liquid N₂ and thaw in a 37° water bath until only a small amount of ice remains. Repeat three more times. During the process of thawing, the temperature of the lysate should not exceed 4° to prevent any degradation of the protein either because of proteolytic cleavage or because of changes in the temperature. Adjust the volume of the thawed lysate to 45 ml with ice-cold lysis buffer (50 mM Na-HEPES, pH 7.4, 0.1 M Na-EGTA, 0.1 M Na-EDTA, 0.1 mM DTT, 100 mM NaCl, and PI) and add 15 ml of ice-cold 4 M NaCl to obtain a final concentration of 1 M NaCl. Centrifuge the cell extract at 40,000 rpm in a Beckman Ti60 rotor for 45 min at 4° and collect the supernatant. Dilute the supernatant by adding 240 ml of dilution buffer [10 mM Na-Hepes, pH 8.0, 10 mM β -mercaptoethanol (β ME), 0.1 mM EGTA, 0.1 mM EDTA, 0.5% polyoxyethylene 10 lauryl ether (C₁₂E₁₀) and PI] to obtain a fivefold dilution. Centrifuge the diluted extract at 40,000 rpm in a Ti45 rotor (100,000g) for 45 min at 4° and collect the supernatant. Discard the pellets (save 100 μl of supernatant in a microfuge tube as “load” for later analysis).

2.1.1.2. Affinity chromatography using Ni-NTA agarose All the steps for affinity chromatography are done at 4°. Pack a 2 $\times$ 8-cm glass column with 4 ml of Ni-NTA agarose (Qiagen) and equilibrate with 10 bed volumes of dilution buffer. Load the supernatant onto the column such that the flow rate does not exceed 2 to 2.5 ml/min and collect the flow through for later analysis. Wash the column with 80 ml of wash buffer 1 (10 mM Na-HEPES, pH 8.0, 0.1 mM EGTA, 0.1 mM EDTA, 800 mM NaCl, 0.5% C₁₂E₁₀, 15 mM imidazole, plus PI). Wash the column with 12 ml of wash buffer 2 (10 mM Na-HEPES, pH 8.0, 0.1 mM EGTA, 0.1 mM EDTA, 100 mM NaCl, 15 mM imidazole, plus PI without SBTI). Elute the protein with six successive 4-ml applications of the elution buffer (10 mM Na-HEPES, pH 8.0, 0.1 mM EGTA, 0.1 mM EDTA, 50 mM

NaCl, 125 mM imidazole, plus PI without SBTI), collecting each elution in a separate tube. Analyze the load, flow through, wash, and elution fractions (10 μ l each) on a 9% SDS-PAGE gel followed by staining with Coomassie blue. Pool the fractions containing PLC β from the Ni-NTA column based on the extent of purity as seen on the SDS-PAGE gel. PLC β can be identified as a strongly staining band running at between 130 and 150 kDa. At this point the intact PLC β is usually the most intensely staining band but a number of contaminants, some of which may be proteolytic products, will be visible. Store at 4° until ready to be loaded onto a heparin-Sepharose column. Preferably this column is run the same day but the sample can be saved on ice overnight.

2.1.1.3. Heparin-sepharose chromatography Equilibrate a 5-ml prepacked Hi-Trap heparin HP column (GE Healthcare) with 20 ml of heparin-Sepharose buffer A (low salt gradient buffer; 20 mM Na-HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1 mM DTT, plus PI without SBTI) using a 10-ml syringe. Be sure not to push air into the column during this procedure. Using a syringe, load the pooled fractions on the column, collect the flow through, and store at 4° for later analysis. Wash the column with 25 ml of heparin buffer A using the 10-ml syringe. Elute with a 150-ml linear gradient from heparin buffer A to heparin buffer B (20 mM Na-HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 800 mM NaCl, 1 mM DTT, plus PI without SBTI), collecting 6-ml fractions. A fast protein liquid chromatography (FPLC) or other chromatography system may be used to accurately control the gradient or a gradient maker and a peristaltic pump can also be used. The Hi-Trap heparin column may be reused for several purifications if regenerated with 5 column volumes of 1 M NaCl followed by a low salt wash. Analyze each of the fractions for PLC β by SDS-PAGE on a 9% gel staining with Coomassie blue. Pool all the fractions containing pure PLC β enzyme running at between 130 and 150 kDa (eluting at $\approx$ 300 mM NaCl). Concentrate the pooled fractions to 1 to 2 ml using a 10-ml Millipore diafiltration device with a PM 30 membrane filter at 4° or with a Centriprep centrifuge-based concentrator (50-kDa cutoff) following the manufacturer's instructions. Estimate the yield of the protein with an Amido black protein assay (Schaffner and Weissmann, 1973) or Bradford protein assay. In general, we obtain 1 to 5 mg of PLC β protein from 1 liter of Sf9 cells. Make small aliquots of the concentrated sample in microfuge tubes and freeze rapidly in liquid N₂ before storing at -70°. Usually the aliquots of PLC are thawed immediately before use and then discarded, as freeze/thaw results in loss of PLC enzymatic activity, and very small amounts of protein (1–500 ng) are needed for a full set of PLC assays. Thus the more small aliquots that are made, the more use will be obtained from a single preparation.

2.1.2. Expression and purification of $G\beta\gamma$ in insect cells

Depending on the experiment, either wild-type or biotinylated β ($bG\beta$) subunits can be used to assess interactions with $PLC\beta$. This section describes preparation of wild-type and $bG\beta\gamma$ subunits for PLC interaction assays.

2.1.2.1. Preparation of baculovirus for $bG\beta\gamma$ expression As discussed, to complement functional assays for PLC regulation we have developed direct binding assays to evaluate the effects of mutations or inhibitors on protein binding. In these assays we use $G\beta\gamma$ subunits that have been biotinylated *in vivo* at the amino terminus with a biotin acceptor tag. $bG\beta\gamma$ subunits are mixed with PLC, and binding is assessed by pull-down approaches. The major advantage of the biotinylation approach is that background binding of $PLC\beta$ to the beads is negligible. Site-specific tagging of $G\beta$ allows multiple $G\beta$ subunit isoforms and/or mutants to be compared for binding without having to consider the possible effects of the mutations on a chemical biotinylation reaction. To create the site-specific $b\beta$ subunits, the cDNA for the rat $G\beta 1$ subunit is subcloned into a baculovirus transfer vector for expression of amino-terminal fusions of a biotin acceptor peptide (Duffy *et al.*, 1998). The biotin acceptor peptide is a sequence of 20 amino acids that is the substrate for the enzyme biotin holoenzyme synthetase (BirA). When a protein fused to the biotin acceptor peptide is coexpressed with BirA, the protein becomes biotinylated *in vivo* at a specific lysine residue in the acceptor peptide sequence. The $\beta 1$ subunit is amplified by a polymerase chain reaction with pfu polymerase and cloned into the baculovirus transfer vector PDW464 in frame with the biotin acceptor sequence at *Ascl* and *EcoRI* restriction sites to yield MAGGLNDIFEAQKIEWHEDTGGA... $\beta 1$ sequence with the lysine residue being the site of biotinylation. The baculovirus is generated using recombination in bacteria as described in the Bac to Bac system (Invitrogen), amplified, and used as discussed later for infection of insect cells.

Expression and purification of $G\beta\gamma$ have been reported previously from Sf9 insect cell cultures (Kozasa and Gilman, 1995). We, and others, have found that the yield of $G\beta\gamma$ is nearly two times greater with High Five cells (when compared to Sf9 cells) (Davis *et al.*, 2005; unpublished observations). Given these findings, we describe the methods necessary to culture High Five cells, expression of $G\beta\gamma$, and pertinent caveats of the system.

High Five cells (Invitrogen) are grown in suspension, shaking at 125 rpm in Bellco flasks at 27 to 28°, and have a doubling time of 24 h. High Five cells have a tendency to aggregate and clump at high cell density. Large aggregates are difficult to count accurately and reduce viral infection efficiency. It is very difficult to revert to monodisperse cells once large aggregates appear. Antiaggregating agents will prevent large clumps from forming, extending the life span of the cultures. However, some antiaggregating agents (e.g., heparin and dextran) prevent viral infection and

consequently must be removed prior to infection with baculovirus. Although the manufacturer recommends heparin sulfate (25 mg/ml) to prevent cell aggregation, in our hands it was very difficult (if not impossible) to remove the heparin from the culture prior to viral infection. We use dextran (sodium salt from *Leuconostu* ssp., M_r 5000; Fluka) because it effectively prevents cell aggregation and, more importantly, dextran can be washed away easily prior to viral infection. A 1000 $\times$ concentrated stock of dextran in water can be used to supplement Sf-900 II media to a final concentration of 25 μ g/ml dextran. Passage High Five cells in Sf-900 II medium (Invitrogen/Life Technologies) supplemented with dextran at an optimal density of 0.75 to 2×10^6 cells/ml. Do not routinely culture High Five cells at greater than 3×10^6 cells/ml for extended periods. Cultures that exceed 4×10^6 cells/ml become unusable. Typically, High Five cell cultures can be passaged up to 22 to 25 times. Thereafter, cultures stop doubling and need to be discarded.

Because the maximal cell density is relatively low, cells should be successively scaled from a 200-ml culture to a density of 4×10^6 cells/ml to 800-ml culture to obtain a sufficient number of cells for expression of $G\beta\gamma$. As noted earlier, prior to infection with viruses the dextran must be washed away. To remove dextran prior to scaling up to an 800-ml culture, centrifuge the cells from the 200-ml culture at 1800g (RT6000B Sorvall) in sterile, 200-ml conical polycarbonate disposable centrifuge tubes (Falcon) for 5 min and discard the supernatant. Resuspend the cell pellet in 200 ml Sf-900 II medium, pellet the cells by centrifugation, and again discard the supernatant. Resuspend the cell pellet in 800 ml dextran-free Sf-900 II medium in a 2-liter Belco flask (final cell density 1×10^6 /ml). Incubate the culture as described earlier for 12 h and make a note of the cell density prior to infection. The cell density should be approximately 1.5 to 1.8×10^6 cells/ml. Triply infect the 800-ml High Five culture with 10 ml of either $bG\beta_1$ or wild-type $G\beta_1$, 10 ml of γ_2 , and 5 ml of 6His α_2 baculovirus and incubate with continuous shaking (125 rpm) for 60 h at 27°.

2.1.3. Purification of $G\beta\gamma$ or $bG\beta\gamma$

Prior to harvesting the infected cells determine the cell density to be sure that the cells have not grown after infection. If they have not, it is unlikely that the cells were infected and either the virus is not of sufficient MOI or the dextran was not sufficiently washed away. It should be noted, however, that the cell number may increase by up to 20%.

Harvest cells by centrifugation at 2500 rpm for 20 min (Beckman centrifuge, JA10 rotor). Discard the supernatant, resuspend the cell pellet with 20 ml PBS, PTT, and 0.1 mM EDTA and EGTA, and transfer equal volumes to two 50-ml conical Falcon tubes. Pellet the cells at 3 to 4000 rpm (Sorvall RT6000B) for 20 min at 4°. Pour off the supernatant and freeze the pellet in liquid N₂ and store in -70° or suspend in 15 ml of cold lysis buffer

(50 mM HEPES, pH 8.0, 3 mM MgCl₂, 0.1 mM EDTA, 100 mM NaCl, 10 mM β ME, 10 μ M GDP, PI [as described in the PLC section]). If you are using a pellet that was stored at -70° , suspend the pellet in warm lysis buffer.

It is important to note that the biotinylated tail is sensitive to proteolytic cleavage, producing nonbiotinylated $\beta\gamma$. Therefore, this purification requires the inclusion of all protease inhibitors throughout the protocol (with the exception of SBTI being excluded where indicated later), whereas wild-type $\beta\gamma$ only needs the basic PTT protease inhibitor cocktail. All buffers are made fresh the day of purification and are prechilled to 4° prior to use unless otherwise indicated.

Freeze/thaw cells four times in liquid N₂. Dilute to 100 ml/liter starting culture with cold lysis buffer and centrifuge at 35,000 rpm in a Ti45 rotor for 30 min. Discard the supernatant. Suspend the membranes by adding 5 ml membrane wash buffer (50 mM HEPES, pH 8.0, 3 mM MgCl₂, 50 mM NaCl, 10 mM β ME, 10 μ M GDP, PI) to the pellet and dislodge the pellet with a glass pipette. Transfer two pellets to a glass homogenizer and make a homogeneous solution by grinding the pellets with the pestle. Bring the volume to 80 ml with wash buffer and repeat the centrifugation and homogenization steps. Add cholate to 1% and extract the membranes for 1 h with stirring at 4° . Pellet the extracted membranes by centrifugation at 35,000 rpm in a Ti45 rotor for 45 min. Save the supernatant and either snap-freeze for future processing or continue. Dilute the supernatant with 5 volumes buffer A1 (20 mM HEPES, pH 8.0, 100 mM NaCl, 0.5% C₁₂E₁₀, 1 mM MgCl₂, 10 mM β ME, 10 μ M GDP, PI). If a precipitate forms the supernatant must be centrifuged to remove the particulate matter; otherwise the Ni-NTA column will run exceedingly slow. Save 200 μ l as "load" for later analysis. Load extracted supernatant by gravity onto a 4-ml Ni-NTA column (prewashed with "buffer A1") at 0.5 ml/min overnight, making sure the column does not run dry. Save an aliquot of the flow through for the gel. Wash the column with 200 ml buffer A2 (20 mM HEPES, pH 8.0, 1 mM MgCl₂, 100 mM NaCl, 0.5% C₁₂E₁₀, 10 mM β ME, 10 μ M GDP, PI, 5 mM imidazole). All washes should be collected separately and saved for additional analysis. Warm the column to room temperature for 15 min and wash with 12 ml of buffer A2 (20 mM HEPES, pH 8.0, 1 mM MgCl₂, 300 mM NaCl, 0.5% C₁₂E₁₀, 10 mM β ME, 10 μ M GDP, PI, 5 mM imidazole) at 30° (warm up buffer in water bath). Wash the column with 5 ml of buffer A3 (20 mM HEPES, pH 8.0, 1 mM MgCl₂, 300 mM NaCl, 10 mM β ME, 10 μ M GDP, PI [no SBTI], 5 mM imidazole, 1% *n*-octyl- β -D-glucopyranoside [OG]). Elute the column three times with 4 ml EB1 (20 mM HEPES, pH 8.0, 50 mM MgCl₂, 50 mM NaCl, 30 mM AlCl₃, 10 mM NaF, 10 μ M GDP, 1% OG, 5 mM imidazole, PI) at 30° . Elute the column with 1 column volume of EB2 (20 mM HEPES, pH 8.0, 50 mM MgCl₂, 50 mM NaCl, 30 mM AlCl₃, 10 mM NaF, 10 μ M GDP, 1%

cholate, 5 mM imidazole, PI [no SBTI], 5 mM imidazole, 1% cholate) at 30°. Repeat for a total of five elutions with EB2. Do not pool the elutions. Analyze the load, flow through, wash, and elution fractions (10 μ l each) on a 12% SDS-PAGE gel followed by staining with Coomassie blue. In this procedure, endogenously expressed insect $G\beta\gamma$ subunits will also bind the 6His-tagged $G\alpha$ subunit and could copurify as a contaminant of the $G\beta\gamma$ preparation. The insect β subunit can be distinguished from the expressed $G\beta\gamma$ based on molecular weight where it runs slightly higher ($\sim$ 1 kDa higher) than wild-type $G\beta 1$ and lower than $b\beta$, which runs at approximately 39 to 40 kDa. The EB1 wash results in selective elution of the insect $G\beta\gamma$ such that EB2 elutions contain only expressed $G\beta\gamma$. The eluted expressed $G\beta\gamma$ contains cholate, which is deleterious to some assays so it is washed out in the subsequent hydroxyapatite processing of the preparation.

To concentrate, $bG\beta\gamma$ or wild-type $\beta\gamma$ is pooled from the EB2 elutions with any fractions containing insect $\beta\gamma$ excluded. Pooled fractions are loaded onto a Bio-Rad polyprep chromatography column packed with 0.5 ml hydroxyapatite (HAP) Bio-Gel HTP gel (Bio-Rad) preequilibrated with 5 ml HAP buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM DTT, and 1% OG). The flow through is discarded and the column is washed with 2 ml of HAP buffer followed by five 0.5-ml elutions with HAP buffer containing 200 mM KP_i , pH 8.0. Fractions are analyzed for protein concentration by Amido black, and peak fractions are aliquoted and frozen in liquid N_2 prior to storage at -80° . The yield of protein from this procedure is generally 1 to 2 mg/liter of starting culture.

2.2. PLC β assay

The assay for measurement of PLC activity and G-protein-regulated PLC activity utilizes small unilamellar phospholipid vesicles (SUVs) of defined lipid composition containing the substrate PIP_2 . The lipid composition has been optimized empirically to obtain significant stimulation by G-protein subunits and minimal amounts of PLC protein. Fluorescence resonance energy transfer and large unilamellar vesicle centrifugation-based assays indicate that PLC β isoforms have the intrinsic capacity to bind to the phospholipid vesicle surface such that when mixed with SUVs the enzyme binds to the surface of the vesicle, which is not altered by binding of the G protein (Romoser *et al.*, 1996; Runnels *et al.*, 1996). $G\beta\gamma$ subunit stocks require the presence of detergent above the critical micellar concentration (CMC) to maintain solubility, but in the assay, $G\beta\gamma$ subunits are added to the reaction such that the detergent is diluted well below the CMC. Under these conditions the $G\beta\gamma$ is not stable in aqueous solution and at least a fraction of it “finds” the vesicle surface in a position to interact with the vesicle-bound PLC. Other investigators have used an approach to

incorporate $G\beta\gamma$ into the vesicles by gel filtration that works well, but is more laborious (Myung *et al.*, 1999).

The components of the assay are prepared in four separate solutions (lipid vesicles, G protein, PLC, and Ca^{2+}) separately and mixed together. This allows for each of these components to be varied separately and can be further divided if inhibitors are to be tested. The total assay volume is 60 μ l and the component solutions are prepared such that 20 μ l of vesicle solution, 20 μ l of PLC solution, 10 μ l of G-protein solution, and 10 μ l of Ca^{2+} solution are added to each reaction tube. The PLC, either in the presence or in the absence of G protein, absolutely requires Ca^{2+} for activity. In the assay, Ca^{2+} in the mid- to high nanomolar range is supplied in a Ca-EGTA buffer system. First we describe how each of these component solutions is prepared followed by the assay itself.

2.2.1. Preparation of lipid vesicles

The volume of lipid vesicle solution needed for each set of assays is based on the total number of assays using 20 μ l vesicle solution/assay. All reactions are performed at least in duplicate. The final concentration of the lipids in the assay is 50 μ M PIP₂ (brain phosphatidylinositol 4,5- biphosphate; stock 1 mM, Avanti Polar-Lipids Inc.) and 200 μ M PE (liver L- α -phosphatidylethanolamine; stock 13 mM, Avanti Polar-Lipids Inc.) so the concentration in the lipid vesicle solution will be 150 μ M PIP₂ and 600 μ M PE, as the vesicles will be diluted threefold into the final 60- μ l assay volume. Calculate the amount of [³H]PIP₂ (Dupont/New England Nuclear) needed to give 6000 to 8000 cpm/assay. In general this can be calculated based on the total volume lipid vesicles needed multiplied by 0.03. The lipids are stored in chloroform solution at -20° in glass vials. Before the experiment, vials containing the lipids are warmed to room temperature in a desiccator before opening to prevent absorption of moisture. Transfer the amounts of lipid stock solutions in chloroform calculated to a 10-ml glass Pyrex tube using a 50- to 100- μ l glass Hamilton syringe. Rinse the syringe at least five times with chloroform between transfers of each of the different lipids. Dry down the lipid mixture under N₂ for 15 min. Connect a flexible hose between a regulator on a tank of nitrogen gas and a glass Pasteur pipette mounted on a ring stand. Lower the glass pipette about one-fourth of the way down the tube containing the lipids. Slowly turn on the flow of nitrogen until the surface of the chloroform solution is moving gently and allow to dry at this setting for 15 to 30 min. The dried lipids will form a film at the bottom of the tube. Add the calculated volume of sonication buffer (for 1 ml of sonication buffer: 500 μ l of 2 $\times$ assay buffer [100 mM HEPES, pH 7.2, 6 mM EGTA, 160 mM KCl], 10 μ l 0.1 M DTT, 490 μ l of water) and sonicate in a bath sonicator for 5 min. The sonicated vesicles should form a homogeneous translucent suspension with no particulate matter. Determine the number of CPM in 20 μ l of the lipid solution by liquid scintillation counting. This will

indicate how much radioactivity is being added to each assay and serves as an indicator that the vesicles were sonicated successfully. There should be between 4000 and 8000 CPM in 20 μ l of vesicle solution. If the CPM is significantly lower than this, something may have gone wrong in the vesicle preparation. Store the lipid vesicles on ice until ready to be added to the PLC reaction. Fresh vesicles are prepared each day.

2.2.2. Purified PLC β enzyme solution

The PLC β enzyme is used at a final concentration of 0.25 to 5 ng per reaction, depending on the type of the assay. See the section on assay optimization to determine how much PLC to include in each assay. Determine the number of reactions that will be performed to calculate the volume of PLC solution needed for the assay, given that each assay will get 20 μ l of PLC solution. For G $\beta\gamma$ activation of PLC β , make the PLC enzyme in 1 $\times$ assay buffer (50 mM HEPES, pH 7.2, 3 mM EGTA, 80 mM KCl) plus 3 mM DTT and 3 mg/ml bovine serum albumin (BSA). Add the purified PLC β such that 0.25 to 5 ng is added in the 20 μ l of solution that is added to each reaction. For G α_q activation of PLC β , the enzyme is prepared in a similar fashion except that AlF_4^- (10 $\times$ AlF_4^- solution: 100 μ l of 1 M NaF, 30 μ l of 10 mM AlCl_3 , 870 μ l of water) is added from the 10 $\times$ stock such that it is 2.5 $\times$ the final concentration in the reaction; once everything is mixed the final concentration of AlF_4^- is 1 $\times$. For example, for 10 reactions you need 200 μ l plus a little extra so the mixture would be made as follows for a $\beta\gamma$ activation assay: 125 μ l 2 $\times$ assay buffer, 7.5 μ l 100 mg/ml BSA, 7.5 μ l 0.1 M DTT, 62.5 ng PLC, and H_2O to 250 μ l total volume for a final of 5 ng PLC/assay. For an assay of PLC activation by G α_q , 62.5 μ l of 10 $\times$ AlF_4^- would be substituted for 62.5 μ l of the H_2O .

2.2.3. Purified G-protein solutions

The G $\beta\gamma$ stock solution should be concentrated enough so that when diluted into the assay the final concentration of OG in the assay is 0.1%. In general, the concentration of OG is 0.1% in the assays but as long as all the assay tubes contain the same amount of OG concentrations lower than 0.1% can be used. Dilute the G $\beta\gamma$ as needed in $\beta\gamma$ blank before adding to the incubation buffer. The $\beta\gamma$ blank is the same as the solution in which the $\beta\gamma$ protein is stored (HAP elution buffer), and the amount of OG that will be added to each reaction will depend on the amount of $\beta\gamma$ or α_q blank added because both contain 1% OG. The diluted G $\beta\gamma$ is mixed with 2 $\times$ incubation buffer (100 mM HEPES, pH 7.2, 6 mM EGTA, 2 mM EDTA, 200 mM NaCl, 10 mM MgCl_2), DTT, and H_2O so that the incubation buffer is 1 $\times$ (50 mM HEPES, pH 7.2, 3 mM EGTA, 1 mM EDTA, 100 mM NaCl, 5 mM MgCl_2) 1 mM DTT, and the G $\beta\gamma$ and OG are 6 $\times$ their final concentration in the assay. All tubes should get an equal volume of $\beta\gamma$ solution even if the concentration of G $\beta\gamma$ is varied. The concentration

range that we usually use is 3 to 300 nM $\beta\gamma$ with saturation occurring at 100 nM. A control reaction is always performed where a volume of $\beta\gamma$ blank solution is added to the incubation buffer equal that containing the $\beta\gamma$ subunit protein to control for detergent or other solution artifacts.

For assays of $G\alpha_q$ -dependent activation of PLC, dilute the stock $G\alpha_q$ into α_q blank solution if necessary prior to addition of the incubation buffer. The α_q blank may or may not contain OG. OG is not an obligate requirement for the $G\alpha_q$ activation of PLC β . Mix $G\alpha_q$ in $1\times$ incubation buffer at $6\times$ the final concentration in the assay with 1 mM DTT and $1\times$ AlF_4^- . The final concentration of $G\alpha_q$ in the assay should be between 1 and 60 nM. A control reaction is always performed where a volume of $\beta\gamma$ blank solution is added to the incubation buffer to equal that containing the $G\beta\gamma$ subunit protein to control for detergent or other solution artifacts. AlF_4^- is included in the reaction mixture for $G\alpha_q$ activation.

2.2.4. Calcium solution

Calcium is necessary for PLC activity and the catalytic reaction is initiated by its addition. A good starting free calcium concentration is ≈ 150 nM final so the calcium solution for this would contain 90 μ l 0.1 M $CaCl_2$, 500 μ l $2\times$ assay buffer, 400 μ l H_2O , and 10 μ l 0.1 M DTT. Free Ca^{2+} ions are controlled in the reactions using a Ca^{2+} -EGTA buffer system. Programs such as Maxchelator can be found on the Internet to perform calculation of free Ca^{2+} at different concentrations of total calcium, pH, and EGTA conditions. The assay conditions described here are pH 7.2 and contain 3.0 mM EGTA and 0.67 mM free Mg^{2+} .

2.2.5. PLC assay

Add 20 μ l of sonicated phospholipid vesicles to individual 5-ml polypropylene tubes (VWR). Add 10 μ l of G-protein solutions to the vesicles. Incubate the reaction tubes in an ice bath for 30 min. Add 20 μ l of PLC β solution to all tubes. Initiate the reaction by adding 10 μ l of calcium solution to the reaction tubes, resulting in a final volume of 60 μ l. Transfer the rack of tubes from the ice bath to a 30° water bath to initiate the enzyme reaction. At least two blank reactions containing PLC but no G protein are left on ice to determine the amount of PLC reaction-independent presence of aqueous ^3H present in the $[^3H]PIP_2$ stock. Incubate for 5 to 30 min and transfer back to the ice bath. Add 200 μ l ice-cold 10% TCA to each tube, including the blanks, to terminate the reaction. Add 100 μ l 10 mg/ml BSA to each tube and vortex. Remove precipitated proteins and lipids by centrifuging for 5 min at 3000 rpm. Pipette out 300 μ l of the supernatant with a bent pipette tip and transfer to a 6-ml capacity scintillation vial. Tilt the pellet so that it is up and direct the bent tip so that it will only touch the supernatant. While removing the supernatant, care has to be taken to ensure that the bent tip of the pipette tip does not touch the pellet. Add 4 ml of scintillation fluid,

shake, and then place in a scintillation counter. Measure the [^3H]IP $_3$ released by liquid scintillation counting for 5 min.

2.2.6. Calculations

Each assay gets 3 nmol of PIP $_2$. The CPM from counting of the lipid vesicles is divided by 3 nmol to give the specific radioactivity in CPM/nmol. Enzymatic specific activity is (nmol/mg PLC/min) = (assay CPM – blank CPM)/specific radioactivity (CPM/nmol)/mg PLC/minutes of reaction. Fold activation by G protein is calculated by dividing the specific activity in the presence of G protein by the specific activity in the absence of G protein.

2.3. PLC assay optimization

In the previous section, conditions were presented that should yield significant $G\beta\gamma$ -dependent activation of PLC activity. When the assay is first established in a laboratory it should be optimized to achieve the specific desired result because different preparations of G protein at different concentrations or different preparations of PLC may affect the outcome of the assay. Initial experiments should first determine the optimal concentrations of PLC β , $G\beta\gamma$, OG, and calcium in the final reaction mixture to obtain measurable basal PLC β enzymatic activity and to minimally achieve a 3-fold $G\beta\gamma$ -induced activation (relative to basal) with 5- to 10-fold being more optimal. As a starting point, it is important to ensure that the [^3H]IP $_3$ produced is within the linear range of the assay. Generally it is important to keep the total PIP $_2$ hydrolyzed to less than 20% of the PIP $_2$ in the assay.

Determining the optimal concentrations of PLC β is also important. High concentrations of PLC β 2 can result in elevated basal PIP $_2$ hydrolysis, reducing the dynamic range of the assay. In general we use small amounts of input (i.e., nanograms) PLC protein for studies intended to evaluate the modulation of enzyme activity. It is important to have measurable basal enzyme activity for two reasons: (1) the activation by G protein is often expressed as fold activation over basal. Thus it is important to have an accurate measure of basal activity to calculate the fold activation accurately. (2) In many cases this assay is used to evaluate potential effects of inhibitors or mutations that affect the interaction between the G protein and the phospholipase. Agents targeted to block G-protein-PLC interactions that directly affect basal PLC activity in the absence of G protein are generally discarded as nonspecific. In addition, mutations in PLC that strongly affect basal PLC activity are difficult to analyze for their effects on G-protein-dependent regulation and are often not analyzed further.

The concentration of calcium has a profound effect on basal PLC β activity. Free Ca $^{2+}$ ions are controlled in the reactions using a Ca $^{2+}$ -EGTA buffer system as discussed earlier. Calcium is necessary for PLC activity, and the catalytic reaction is initiated by its addition; however, too much

free calcium results in high basal activity, resulting in reduced G-protein-stimulated PLC β activity. Conversely, if the concentration of calcium is too low the basal activity will be too low to be measured accurately. If low calcium is used, either more PLC can be added or the reaction times can be extended to get appropriate activities. A good starting point is a 15-min reaction at 30° with 0.5 ng PLC β 2 or 2 ng PLC β 3 and 150 nM free Ca²⁺. In general, because PLC β 3 has very low G-protein-independent activity, basal activity can be difficult to measure, but the result of this is that the fold activation of this protein by G protein can be quite high (10- to 50-fold). PLC β 2, however, has quite high G-protein-independent activity and the fold activation by G $\beta\gamma$ is lower (5- to 10-fold).

2.4. Application of the PLC assay to evaluate peptide/small molecule modulation of G $\beta\gamma$ -dependent PLC activity

We have analyzed the effects of small molecules, peptides, mutants of G $\beta\gamma$, and mutants of PLC in the assay for PLC activity described earlier. One area of recent emphasis has been to evaluate G $\beta\gamma$ -dependent PLC β enzymatic activity in the presence of peptides or small molecules.

Using optimized assay conditions, small molecule or peptide mediated inhibition of G $\beta\gamma$ -dependent PLC β activity can be assayed. For these experiments we use subsaturating amounts of 30 to 100 nM G $\beta\gamma$ because high G $\beta\gamma$ protein concentrations will necessitate that greater concentrations of peptide/small molecule be used to achieve inhibition. Therefore, it is important to use the minimal amount of G $\beta\gamma$ possible to achieve measurable activation (at least threefold). Calculate the working stock concentration necessary for the desired inhibitor concentration in the assay (60 μ l volume) using an aliquot small enough so that 1% vehicle concentration (dimethyl sulfoxide) is not exceeded in the assay. We start small molecule/peptide screens by first using a single concentration to determine if any inhibition is seen. We generally do not exceed a concentration of 100 μ M small molecule in any of our assays to minimize nonspecific effects on the lipid vesicles. One approach is to add the small molecule from a concentrated stock solution to the assay once all of the reaction components, except for the Ca²⁺ solution, have been assembled. Alternately, the compounds/inhibitors can be preincubated with the G protein in the G-protein incubation solution at a 6 $\times$ concentration followed by dilution into the assay. Always include a vehicle control and test the small molecule for effects on basal and G $\beta\gamma$ -dependent PLC β activity. Testing the small molecule on basal enzymatic PLC activity is crucial to demonstrating that the compound is acting on G $\beta\gamma$ and not nonspecifically on PLC as discussed. If the compound is effective the IC₅₀/EC₅₀ can be determined.

Basal PLC activity must be evaluated in the presence of the peptide/small molecule to provide some insight into specificity and nonspecific

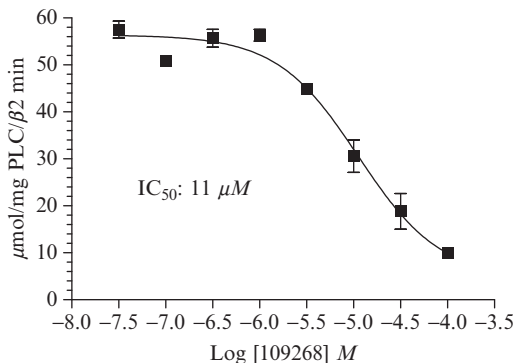


Figure 3.1 Examination of effects of compound NSC109268 on 100 nM $G\beta_1\gamma_2$ -dependent regulation of PLC β 2. The PLC assay was performed as described in the text for 12 min with 0.25 ng PLC in each assay. Free Ca^{2+} was 300 nM. Each point represents duplicate determinations. Effects of the compound on basal enzymatic activity were tested only at the highest concentration of compound: basal = 15 μ mol/mg PLC/min; basal + 100 μ M NSC109268 = 13 μ mol/mg PLC/min.

inhibitory (or potentiating) effects of the peptides/small molecules. Therefore, it is important to examine the small molecule effects on basal PLC activity, as well as $G\beta\gamma$ -dependent activation of PLC β enzymatic activity. An example of the analysis of effects of a compound that binds to $G\beta\gamma$ on $G\beta\gamma$ -dependent PLC β 2 regulation is shown in Fig. 3.1. In this experiment, compound NSC109268 inhibited $G\beta\gamma$ -dependent PLC β 2 activation in a dose-dependent manner but did not significantly affect PLC β 2 basal activity. This strongly suggests that the compound is interfering with $G\beta\gamma$ -PLC β interactions. This assay could be complemented and strengthened with one of the $G\beta\gamma$ -PLC β binding assays described later.

2.5. Evaluation of $G\beta\gamma$ -PLC binding

The G-protein-dependent regulation of the PLC assay provides a functional assessment of protein-protein interactions. As a complementary analysis to strengthen the interpretation of the results, direct binding of G protein to PLC interactions can be assessed. For example, inhibition of PLC enzymatic activity in the presence of specific small molecules/peptides is presumably because of alterations in the interaction between $G\beta\gamma$ and PLC. However, one could argue that decreased alterations in G-protein-dependent PIP₂ hydrolysis could be because of alterations in lipid vesicle properties that change G-protein-dependent regulation. Similarly, mutation of PLC β amino acids that inhibit $G\beta\gamma$ -dependent regulation in the PLC assay could, in theory, result in an alteration in lipid interactions that, for some reason, selectively inhibit G-protein regulation. There is no strong evidence that this occurs but direct binding analysis could reinforce the conclusions. We developed two assays to

measure direct interactions based on affinity-based precipitation (pull down) and another based on size exclusion chromatography.

2.5.1. Pull-down assay

We can directly evaluate the protein–protein interaction between $G\beta\gamma$ and PLC using purified proteins to perform pull-down experiments. We use $G\beta\gamma$ that includes the biotin acceptor sequence at the amino terminus of $G\beta$ allowing site-specific biotinylation. Advantages of the biotinylation system have been discussed. TetraLink Tetrameric Avidin Resin (Promega) is a high-capacity matrix (>30 nmol biotin/ml) used to bind and immobilize biotinylated proteins from complex mixtures. The high affinity of the avidin–biotin interaction results in efficient capture of biotinylated $G\beta\gamma$ with sufficient stability under a wide variety of wash conditions.

To prepare a 50% slurry of avidin resin, gently mix the stock bottle of avidin resin to suspend the matrix, remove 0.5 ml, and transfer to a 15-ml conical tube. Centrifuge the slurry at 500g for 5 min and carefully discard the supernatant. Wash the matrix with 10 ml ice-cold binding buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 1.2 mM $MgCl_2$, 0.1% $C_{12}E_{10}$, 1 mM DTT, 150 mM NaCl, 10 μ M GDP) by inversion. Centrifuge at 500g for 5 min, carefully discard the supernatant, and repeat the wash steps two more times. Following the final wash add 0.3 ml cold binding buffer to the beads and mix to produce a 50% avidin resin slurry. The slurry is good for at least 2 weeks when stored at 4°. Mix the slurry well before each use.

In microfuge tubes, mix 30 nM $bG\beta_{1\gamma_2}$ and 30 nM $PLC\beta_2$ in 200 μ l of binding buffer and incubate at 4° for 2 h to overnight. If inhibitors are to be tested, include them at the desired concentration. Set up control incubations without $bG\beta\gamma$ or without $PLC\beta_2$. Add 40 μ l of 50% avidin resin slurry and incubate at 4° for 2 h with gentle mixing. Centrifuge at 1000g for 1 min at 4° and carefully discard all the supernatant with a needle connected to a vacuum. Wash the matrix with 500 μ l of binding buffer and pellet the matrix at 1000g for 1 min. Carefully remove the supernatant by vacuum aspiration using a 23-gauge needle. Wash two more times and discard the supernatant. Add 20 μ l of 2 $\times$ SDS sample loading buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, 12.5% bromophenol blue) to the pellet, boil, and load 10 μ l onto a 12% SDS-PAGE gel for immunoblotting. Also include loading input controls (usually 1/20 of the supernatant) to show how much PLC or $G\beta\gamma$ was used in the assay. Transfer resolved proteins to nitrocellulose and block with 3% BSA (in PBS-0.1% Tween [PBST]) for 1 h at room temperature. Wash the blocked membrane with PBST once for 15 min and two additional times for 5 min, changing the PBST between each wash. Add primary antibodies (for anti- $G\beta$ blotting, use B600 at a dilution of 1:6000; for anti- $G\alpha$ blotting, use anti- $G\alpha$ antibody from Oncogene at a dilution of 1:1000; for anti- $PLC\beta_2$ blotting, use B520 at a dilution of 1:2000) and incubate at room

temperature for 2 h. Remove primary antibody solution and wash once for 15 min and two additional times for 5 min each with PBST. Incubate the membrane with 1:8000 dilution of HRP-conjugated goat antirabbit (Bio-Rad) secondary antibody in 5% milk PBST for 1 h at room temperature. Remove the secondary antibody and wash the membrane once for 15 min and four additional times for 5 min each with PBST. Add 2 ml of each of the ECL reagents onto membrane and incubate at room temperature for 5 min. Expose the immunoblot to film and ideally perform quantitative analysis of the blot with a quantitative chemiluminescence imaging system with a charge-coupled device camera.

2.5.2. Gel filtration chromatography

We also developed a gel filtration method to measure the formation of G-protein complexes with target molecules. One application of this assay has been the measurement of $G\beta\gamma$ binding to $PLC\beta$. For these experiments, tandem Superdex 75/200 columns (GE Healthcare) are coupled to a Pharmacia FPLC system developed based on published procedures from Tesmer *et al.* (2005) and Tall and Gilman (2005). The columns are connected in series with the Superdex 75 column first followed by the Superdex 200 column and are equilibrated with gel filtration buffer, which is identical to the binding buffer described earlier (20 mM HEPES, pH 8.0, 1 mM EDTA, 1.2 mM $MgCl_2$, 0.1% $C_{12}E_{10}$, 1 mM DTT, 150 mM NaCl, 10 mM GDP) for 2 h with a flow rate of 0.4 ml/min.

$G\beta_{1\gamma_2}$ and $PLC\beta_2$ (100 nM each) are incubated in 500 μ l gel filtration buffer plus PI at 4° for 2 h. Separate incubations are performed with $G\beta\gamma$ alone and $PLC\beta_2$ alone. The mixture is applied to preequilibrated columns and resolved at a flow rate of 0.4 ml/min at 4° and 1-ml fractions are collected. An aliquot of each fraction (20 μ l) is analyzed by SDS-PAGE on a 12% polyacrylamide gel and visualized by silver staining. Formation of a complex between $G\beta\gamma$ and $PLC\beta_2$ results in elution of both $G\beta\gamma$ and PLC in the same fraction at a predicted molecular weight higher than either protein component alone. The elution positions of the proteins in this system are highly reproducible. When $PLC\beta_2$ and $G\beta\gamma$ are mixed they consistently elute at 23 ml compared to 24 ml for $PLC\beta_2$ alone and 27 ml for $G\beta\gamma$ alone. Protein concentrations are intentionally kept as low as possible while still allowing detection by silver staining. Low concentrations are used to be confident that the complexes that result are not an artifact of nonspecific interactions induced at an artificially high protein concentration.

2.5.3. Example binding analysis of a $PLC\beta$ mutant predicted to interfere with $G\beta\gamma$, $PLC\beta_2$ interactions

We have investigated determinants on both $G\beta\gamma$ and $PLC\beta$ that contribute to $G\beta\gamma$ regulation of $PLC\beta$. A series of results from these analyses, outlined briefly here, indicate that interactions between the catalytic domain of

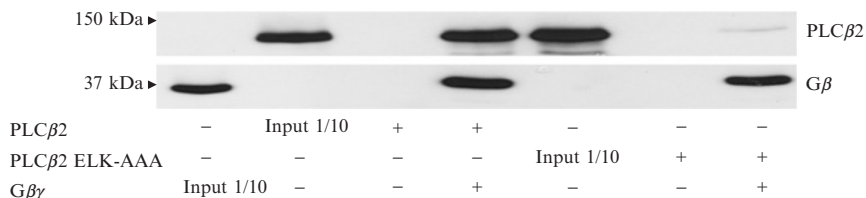


Figure 3.2 A PLCβ2 catalytic domain mutant binds weakly to Gβγ. Thirty nanomolar of either wild-type PLCβ2 or PLCβ2 (ELK-AAA) was mixed with 30 nM bGβ_{1γ2}, and the pull-down assay was performed as described.

PLCβ2 and Gβγ are important for PLCβ regulation by Gβγ. A fragment of the catalytic domain of PLCβ2 blocks βγ-dependent PLC activation in intact COS cells and binds Gβγ *in vitro* (Kuang *et al.*, 1996). In the PLC assay described here, a peptide representing the α₅ helix in the catalytic TIM domain of PLCβ2 (N20K) was shown to inhibit Gβγ-dependent PLC activation (Sankaran *et al.*, 1998). Chemical cross-linking assays demonstrated direct binding of this catalytic domain peptide to Gβγ (Sankaran *et al.*, 1998). Mutation of amino acids E574, L575, and K576 to alanine (ELK-AAA) in PLCβ2 corresponding to specific amino acids in the peptide inhibits Gβγ-dependent activation of PLCβ2 in the PLC enzyme assay (Bonacci *et al.*, 2005). These data strongly suggest that functional interactions between Gβγ and this region of the catalytic domain are involved in Gβγ-dependent regulation of PLCβ2. A possible interpretation of PLC mutant data is that this mutation alters interactions between PLCβ2 and the lipid surface in a way that obviates Gβγ-dependent regulation. To determine if the ELK-AAA mutation directly disrupts PLCβ–Gβγ interactions, a bGβγ pull-down assay was performed and binding of wild-type PLCβ2 was compared with PLCβ2 (E₅₇₄L₅₇₅K₅₇₆-AAA). Results in Fig. 3.2 show that PLCβ2 with the ELK-AAA mutation binds only poorly to Gβγ compared to wild-type Gβγ. This confirms, in combination with previously published mutagenesis data (Bonacci *et al.*, 2005), that this α helix in the catalytic domain of PLCβ2 is indeed a regulatory Gβγ-binding site. It also suggests that Gβγ binding to the PH domain is not sufficient to maintain a stable complex between PLCβ2 and Gβγ in solution.

3. CONCLUDING REMARKS

The ability to correlate the effects on PLCβ enzymatic activity with the protein–protein interaction between PLCβ and Gβγ is important. These studies can be extended further by including mutagenesis studies to determine which amino acids are necessary for small molecule binding in addition to protein interactions.

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REFERENCES

- Bonacci, T. M., Ghosh, M., Malik, S., and Smrcka, A. V. (2005). Regulatory interactions between the amino terminus of G-protein β g subunits and the catalytic domain of PLC β 2. *J. Biol. Chem.* **280**, 10174–10181.
- Bonacci, T. M., Mathews, J. L., Yuan, C., Lehmann, D. M., Malik, S., Wu, D., Font, J. L., Bidlack, J. M., and Smrcka, A. V. (2006). Differential targeting of G β g-subunit signaling with small molecules. *Science* **312**, 443–446.
- Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipase C- β 2 by G protein β gamma-subunits. *Nature* **360**, 684–686.
- Davis, T., Bonacci, T. M., Sprang, S. R., and Smrcka, A. V. (2005). Structural definition of a preferred protein interaction site in the G protein β 1 γ 2 heterodimer. *Biochemistry* **44**, 10593–10604.
- Dowal, L., Elliott, J., Popov, S., Wilkie, T. M., and Scarlata, S. (2001). Determination of the contact energies between a regulator of G protein signaling and G protein subunits and phospholipase C β 1. *Biochemistry* **40**, 414–421.
- Duffy, S., Tsao, K. L., and Waugh, D. S. (1998). Site-specific, enzymatic biotinylation of recombinant proteins in *Spodoptera frugiperda* cells using biotin acceptor peptides. *Anal. Biochem.* **262**, 122–128.
- Exton, J. H. (1996). Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. *Annu. Rev. Pharmacol. Toxicol.* **36**, 481–509.
- Harden, T. K., and Sondek, J. (2006). Regulation of phospholipase C isozymes by Ras superfamily GTPases. *Annu. Rev. Pharmacol. Toxicol.* **46**, 355–379.
- Jezyk, M. R., Snyder, J. T., Gershberg, S., Worthylake, D. K., Harden, T. K., and Sondek, J. (2006). Crystal structure of Rac1 bound to its effector phospholipase C- β 2. *Nat. Struct. Mol. Biol.* **13**, 1135–1140.
- Kozasa, T., and Gilman, A. G. (1995). Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits: Characterization of α_{12} and inhibition of adenylyl cyclase by α_2 . *J. Biol. Chem.* **270**, 1734–1741.
- Kuang, Y., Wu, Y., Smrcka, A., Jiang, H., and Wu, D. (1996). Identification of a phospholipase C β 2 region that interacts with G β g. *Proc. Natl. Acad. Sci. USA* **93**, 2964–2968.
- Lee, S. B., and Rhee, S. G. (1996). Molecular cloning, splice variants, expression, and purification of phospholipase C- δ 4. *J. Biol. Chem.* **271**, 25–31.
- Lee, S. B., Shin, S. H., Hepler, J. R., Gilman, A. G., and Rhee, S. G. (1993). Activation of phospholipase C- β 2 mutants by G protein α_q and β g subunits. *J. Biol. Chem.* **268**, 25952–25957.
- Myung, C. S., Paterson, A., Harden, T. K., and Garrison, J. C. (1999). Development of an assay for phospholipase C using column-reconstituted, extruded phospholipid vesicles. *Anal. Biochem.* **270**, 303–313.
- Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kozasa, T., and Neer, E. J. (1998). Sites important for PLC- β 2 activation by the G protein β g subunit map to the sides of the β propeller structure. *J. Biol. Chem.* **273**, 28298–28304.

- Rhee, S. G. (2001). Regulation of phospho-specific phospholipase C. *Annu. Rev. Biochem.* **70**, 281–312.
- Rhee, S. G., Kim, H., Suh, P.-G., and Choi, W. C. (1991). Multiple forms of phosphoinositide-specific phospholipase C and different modes of activation. *Biochem. Soc. Trans.* **19**, 337–341.
- Romoser, V., Ball, R., and Smrcka, A. V. (1996). Phospholipase C b2 association with phospholipid interfaces assessed by fluorescence resonance energy transfer: G protein bg subunit-mediated translocation is not required for enzyme activation. *J. Biol. Chem.* **271**, 25071–25078.
- Runnels, L. W., Jenco, J., Morris, A., and Scarlata, S. (1996). Membrane binding of phospholipases C-b 1 and C-b 2 is independent of phosphatidylinositol 4,5-bisphosphate and the α and bg subunits of G proteins. *Biochemistry* **35**, 16824–16832.
- Sankaran, B., Osterhout, J., Wu, D., and Smrcka, A. V. (1998). Identification of a structural element in phospholipase C b2 that interacts with G protein bg subunits. *J. Biol. Chem.* **273**, 7148–7154.
- Schaffner, W., and Weissmann, C. (1973). A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**, 502–514.
- Scott, J. K., Huang, S. F., Gangadhar, B. P., Samoriski, G. M., Clapp, P., Gross, R. A., Taussig, R., and Smrcka, A. V. (2001). Evidence that a protein-protein interaction ‘hot spot’ on heterotrimeric G protein bg subunits is used for recognition of a subclass of effectors. *EMBO J.* **20**, 767–776.
- Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997). Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu. Rev. Biochem.* **66**, 475–509.
- Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991). Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q . *Science* **251**, 804–807.
- Smrcka, A. V., and Sternweis, P. C. (1993). Regulation of purified subtypes of phosphatidylinositol specific phospholipase C b by G protein α and bg subunits. *J. Biol. Chem.* **268**, 9667–9674.
- Tall, G. G., and Gilman, A. G. (2005). Resistance to inhibitors of cholinesterase 8A catalyzes release of $G\alpha_i$ -GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/ $G\alpha_i$ -GDP complexes. *Proc. Natl. Acad. Sci. USA* **102**, 16584–16589.
- Taylor, S. J., and Exton, J. H. (1991). Two α subunits of the G_q class of G proteins stimulate phosphoinositide phospholipase C-b1 activity. *FEBS Lett.* **286**, 214–216.
- Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T., and Tesmer, J. J. G. (2005). Snapshot of activated G proteins at the membrane: The Gaq -GRK2-Gbg complex. *Science* **310**, 1686–1690.
- Waldo, G. L., Boyer, J. L., Morris, A. J., and Harden, T. K. (1991). Purification of an α 1f and G-protein β gamma-subunit-regulated phospholipase C-activating protein. *J. Biol. Chem.* **266**, 14217–14225.
- Wang, T., Dowal, L., El-Maghrabi, M. R., Rebecchi, M., and Scarlata, S. (2000). The pleckstrin homology domain of phospholipase C-b(2) links the binding of gbg to activation of the catalytic core. *J. Biol. Chem.* **275**, 7466–7469.

BIOCHEMICAL ANALYSIS OF PHOSPHOLIPASE D

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Contents

1. Introduction	50
2. Assay of Recombinant PLD <i>In Vitro</i>	52
2.1. Expression of recombinant PLD1	52
2.2. Purification of PLD1	52
2.3. Chromatography (6His)	52
2.4. Size-exclusion chromatography (Superdex 200)	53
2.5. Anion-exchange chromatography (Q-Sepharose)	55
2.6. Characteristics of purified, full-length PLD1	56
2.7. Purification of N-terminally truncated PLD1	56
2.8. Assay of phospholipase D activity <i>in vitro</i>	57
3. Regulated PLD1 Activity	59
4. Preparation of Activators of PLD1	60
4.1. Protein kinase C α (PKC α)	60
4.2. Myristoyl-ADP ribosylation factor 1	60
4.3. Purification of geranylgeranylated RhoA, Rac1, and Cdc42	60
5. Effects of Activators on PLD1 Activity	61
6. Synergy between PLD1 Activators	62
7. Binding of PLD1 to Phospholipid Vesicles	63
8. Kinetic Parameters of PLD1 Catalytic Activity	65
9. Kinetic Analyses of Synergistic Responses	69
10. Phosphatidylinositol 4,5-Bisphosphate is an Essential PLD1 Activator	69
11. <i>In Vivo</i> PLD Assay Using Radioisotopes	74
12. <i>In Vivo</i> PLD Assay Using Deuterated 1-Butanol	74
12.1. Materials and methods	75
12.2. Application	76
13. Fluorescent <i>In Vitro</i> PLD Assay	77
13.1. Preparation of lipid substrates	78

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13.2. Phospholipase D assay	79
14. Real-Time Diacylglycerol Lipase Assay	80
14.1. Synthesis of BD ₂ -DAG	80
14.2. Protocol for BD ₂ DAG preparation	82
14.3. Fluorescent assay for DAG-lipase activity	83
Acknowledgments	84
References	85

Abstract

Phospholipase D (PLD) is distributed widely in nature, being present in various isoforms in bacteria, protozoa, fungi, plants, and animals. It catalyzes the hydrolysis of phospholipids, primarily phosphatidylcholine (PC), into phosphatidic acid (PA) and the head group, choline. It also catalyzes a transphosphatidylation reaction in which water is replaced by a primary alcohol to yield a phosphatidyl alcohol. This reaction is exclusive to PLD and is employed as a specific assay for the enzyme in *in vivo* systems. When the purified enzyme is assayed *in vitro*, the release of choline from PC can be utilized. This chapter describes production of a recombinant mammalian isozyme of PLD (PLD₁) in baculovirus-infected insect cells and its purification. It also provides details of the assay procedure in the presence and absence of regulatory proteins *in vitro*. The assay of the enzyme in cells *in vivo* is also documented using labeling of endogenous PC by incubating the cells with ³H-labeled fatty acid. Details of the assay utilizing the transphosphatidylation reaction are presented. In this, 1-butanol is employed as the primary alcohol and [³H]phosphatidylbutanol is isolated by thin-layer chromatography of lipid extracts from the cells. A variation of this assay is described using deuterated 1-butanol (1-butanol-d₁₀) and detection of the synthesized deuterated phosphatidylbutanol species by mass spectrometry. Convenient alternative assays for PLD and diacylglycerol (DAG) lipase activity based on fluorescence are also described. Many of the materials for these assays are available commercially, with the exception of the fluorescently labeled DAG substrate, which can be synthesized enzymatically in a simple one-step procedure.

1. INTRODUCTION

Phospholipase D (PLD) is a membrane-associated enzyme that is widely distributed in eukaryotes and prokaryotes. Its principal substrate, at least in mammals, is phosphatidylcholine (PC), which it hydrolyzes to phosphatidic acid and choline. It also carries out a transphosphatidylation reaction in which H₂O is replaced by a primary alcohol to yield a phosphatidyl alcohol (Exton, 2002). This reaction is unique to PLD and can be used as a specific assay for the enzyme. Mammalian PLD exists in splice variants of two isoforms (PLD1 and PLD2) (Colley *et al.*, 1997; Hammond *et al.*, 1995). It contains four highly conserved sequences; two of these contain the

motif H(x)K(f)₄D, termed HKD, where f represents hydrophobic amino acids (Koonin, 1996; Ponting and Kerr, 1996). Both of these motifs are involved in the catalytic reaction and are therefore absolutely required for hydrolytic and transphosphatidylolation activities.

The enzymes contain tandem phox homology (PX) and pleckstrin homology (PH) domains in their N terminus (Hurley and Misra, 2000). These are involved in binding certain phosphoinositides and in membrane targeting. The enzymes are palmitoylated at two cysteine residues in the N terminus (Sugars *et al.*, 1999; Xie *et al.*, 2002) and can also be phosphorylated by protein kinase C in this region. However, deletion of the N terminus does not reduce catalytic activity (Park *et al.*, 1998). There is a "loop" region between the two HKD catalytic motifs. Its function is unknown. The extreme C-terminal KE(f)₃PfEfWT sequence is required for activity of the enzyme from animal species (Xie *et al.*, 2000). Phosphatidylinositol 4,5-bisphosphate (PIP₂) is essential for membrane binding and catalytic activity (Brown *et al.*, 1993; Henage *et al.*, 2006), and the lipid binds to a sequence of basic amino acids in the C-terminal catalytic subdomain (Sciorra *et al.*, 1999).

The functions of PLD relate to membrane/vesicle trafficking (endocytosis, exocytosis, and transport in the Golgi and across the plasma membrane), rearrangement of the actin cytoskeleton, superoxide generation, cellular proliferation, and apoptosis (Exton, 2002). PA is generally believed to be the second messenger involved, although changes in the lipid content of membranes could also play a role. PLD may have a physiological function through the further metabolism of PA to diacylglycerol (DAG) and lysophosphatidic acid.

The PLD1 isoform is regulated *in vitro* by members of the Rho and Arf families of small G proteins and by classical isoforms of protein kinase C. However, PLD2 shows little or no response to these factors (Hammond *et al.*, 1997). PLD1 is activated by many hormones, neurotransmitters, growth hormones, and cytokines *in vivo* acting through these G proteins and protein kinases. Protein kinase C interacts with PLD1 at multiple sites, but principally at the N terminus (Kook and Exton, 2005). The Rho proteins bind to the C terminus (Cai and Exton, 2001; Du *et al.*, 2000), but the site of interaction of the Arf proteins is undefined.

We describe here the assay of recombinant rat liver PLD1b *in vitro* in the presence or absence of small G proteins and protein kinase C either singly or in combination. The *in vivo* assay of the enzyme employing the transphosphorylation reaction (Walker and Brown, 2004) is also described.

Parts of this chapter are taken from the dissertation submitted to Vanderbilt University by Lee G. Henage as partial fulfillment of the requirements for the degree of Doctor of Philosophy. Some material is reproduced from the *Journal of Biological Chemistry* (Henage *et al.*, 2006) by permission of the journal.



2. ASSAY OF RECOMBINANT PLD *IN VITRO*

2.1. Expression of recombinant PLD1

Rat PLD1b cDNA (Park *et al.*, 1997) is inserted into a baculovirus transfer vector (pBlueBacHis2A, Invitrogen), modified to possess an N-terminal 6His-FLAG epitope. This transfer vector and linearized AcMNPV viral DNA (Invitrogen) are cotransfected into *Spodoptera frugiperda* (Sf21) cells by liposomal transfection (Cellfectin, Invitrogen) according to the manufacturer's instructions. Recombinant virus is isolated by three rounds of plaque selection and amplified in Sf21 cells adapted to suspension culture in *Trichoplusia ni* medium-formulation Hinks (TNM-FH) supplemented with 10% fetal bovine serum. Plasmid constructs and baculovirus DNA are sequenced to verify coding regions. PLD1 partitions to membrane and cytosolic fractions in Sf21 cells with 8 to 10% in the cytosolic fraction. Approximately 12% of total expressed PLD1 can be extracted by detergent. Because PLD activity is higher in soluble fraction than in the particulate fraction, it is likely that much of the overexpressed, insoluble PLD1 is misfolded or aggregated. Expression levels are equivalent, typically ≈ 0.2 to 0.3 mg/liter (where 1-liter cultures typically contain 2×10^6 cells). However, these conditions could be easily scaled without loss of expression.

2.2. Purification of PLD1

Full-length PLD is purified from detergent extracts of Sf21 cells. A phosphate buffer system is chosen based on its ability to extract peripheral membrane proteins (Hjelmeland, 1990). High ionic strength (0.4 M NaCl) is also useful in early purification steps, but has destabilizing effects on PLD in later purification steps. Because speed and temperature are critical to purification of this protein, rapid capture is achieved by affinity methods. Denaturing conditions (8 M urea) do not enhance purification. Furthermore, attempts to refold denatured PLD were unsuccessful and catalytic activity could not be recovered.

2.3. Chromatography (6His)

A 6His epitope is added to the N terminus of PLD1 for purification by metal-chelating resins. A number of chelating resins were tested (Ni-iminodiacetic acid, GE Biosciences; Ni-nitrilotriacetic acid-agarose, Qiagen; Co-carboxymethylaspartate, Clontech). While the other resins are superior when batch and expanded bed methods are used, the resin purchased from GE Biosciences is superior for fast protein liquid chromatography (FPLC). This resin tolerates low concentrations of reducing

Table 4.1 Purification of full-length PLD1 from baculovirus-infected Sf21 cells

Purification step	Volume (ml)	Total protein (mg/ml)	PLD (mg)	Purity (%)	Purification (fold)	Yield (%)
Detergent extract	8	9.4	0.33	< 1	1	100
NiIDA	4.5	0.12	0.28	52	130	85
Superdex 200	3	0.05	0.11	75	190	28
Q-Sepharose			0.04	80	200	12

agents (β -mercaptoethanol, dithiothreitol [DTT]) necessary to minimize PLD1 aggregation.

More than 90% of total 6His-PLD1 is captured from clarified lysates by chelating resins, and PLD1 is eluted efficiently by imidazole. A step gradient to 30 mM imidazole is used to elute nonspecifically bound proteins, followed by a linear imidazole gradient to elute PLD1 competitively. At pH 7.5, PLD1 elutes in a single peak with normal shape at ≈ 180 mM imidazole in a 50 mM sodium phosphate buffer with 250 mM NaCl, 1 mM MgCl_2 , 1% (w/v) *n*-octyl- β -D-glucopyranoside (β -OG), and 0.2 mM DTT (Fig. 4.1A). This separation is rapid; flow rates are limited only by the viscosity of detergent extracts at low temperatures. 6His-PLD is purified more than 100-fold with a minimal loss of protein (Table 4.1).

Because PLD is not stable in high concentrations of imidazole, pooled fractions are immediately desalted over Sephadex G-25 Superfine gel filtration columns (GE Biosciences) and exchanged to buffers containing HEPES, pH 7.5, 150 mM NaCl, 1% (w/v) β -OG (Calbiochem), and 1 mM DTT. Purified PLD1 is highly unstable in many solutions and precipitates readily at elevated temperatures or ionic strengths. Overnight storage at 4° often results in aggregated proteins that are not useful for further purification.

2.4. Size-exclusion chromatography (Superdex 200)

Phospholipase D elutes from gel-filtration columns in a heterogeneous manner, with the majority of active enzyme eluting near the void volume of the column (Fig. 4.1B). Consequently, estimates of molecular mass from sizing columns are often inaccurate and contradictory (80–400 kDa). Full-length PLD1 elutes at or near the void volume of every sizing column tested (Superdex 200, Superdex 75, Sephacryl S-300, Superose 8). Based on calibration curves for a XK 16/60 Superdex 200 column, PLD1 elutes with a predicted molecular mass of almost 1000 kDa. This anomalous molecular

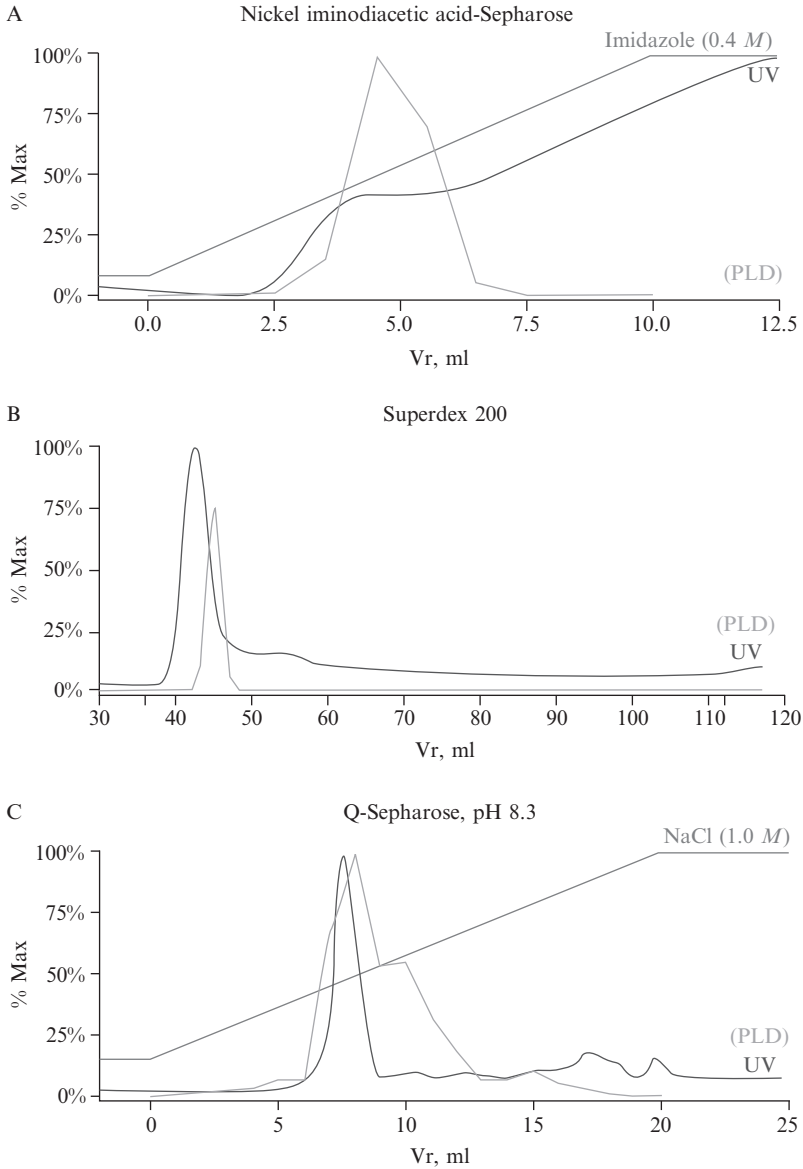


Figure 4.1 Purification of full-length PLD1: FPLC chromatograms. (A) Sf21 lysates were applied to Ni-charged chelating Sepharose. (B) Proteins were resolved by size-exclusion chromatography. (C) Anion-exchange chromatography on Q-Sepharose.

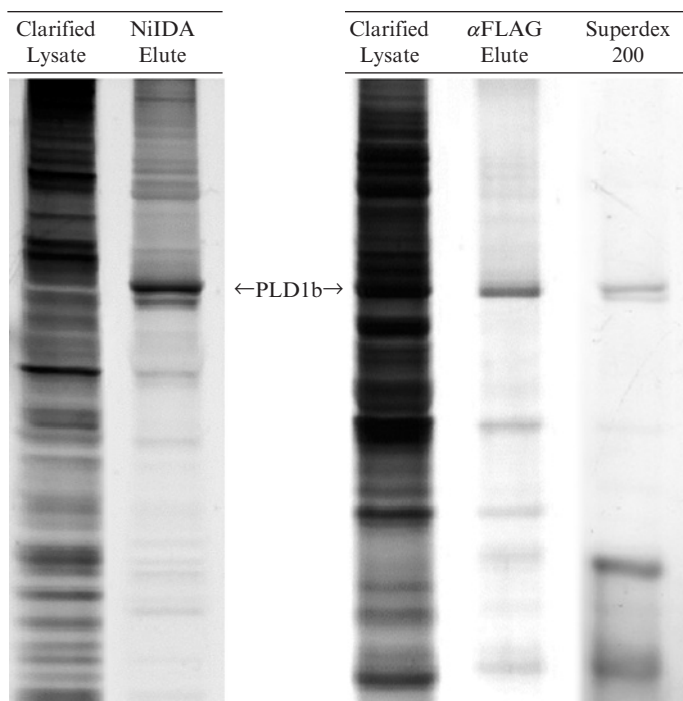


Figure 4.2 Purification of full-length PLD1: Eluted fractions. Lysates and peak fractions from chromatography were analyzed on silver-stained polyacrylamide (6%) gels.

mass is not likely the result of detergent micelles. Similar results are observed when PLD1 is purified in dilute detergent (e.g., β -OG at one-half its critical micelle concentration) or when detergent is substituted with 10 to 20% glycerol.

While most impurities are removed during size-exclusion chromatography, several major species coelute with PLD1. When analyzed by SDS-PAGE, a pair of bands at ≈ 75 and 55 kDa are found routinely with PLD (Fig. 4.2). Both correspond to major proteolytic fragments of PLD1 that are found in cell lysates (see later). Despite its anomalous behavior during size-exclusion chromatography, eluted PLD1 is highly active and does not appear to be aggregated.

2.5. Anion-exchange chromatography (Q-Sepharose)

Anion-exchange chromatography has been particularly useful in PLD purifications. Nearly every published purification scheme includes a weak anion-exchange step (i.e., diethylaminoethyl), a strong anion-exchange step

(i.e., quaternary ammonium), or both. Substantial purification gained by anion-exchange chromatographic separation is offset by low recovery in that step, typically less than 10%.

A strong anion exchanger, quaternary ammonium (Q)-Sephacrose, is useful in initial or intermediate purification steps. At pH 8 to 8.5, <30% of PLD1 adsorbs Q-Sephacrose media. Binding can be enhanced slightly by reapplying the sample to the column multiple times. PLD1 elutes in a major peak at ≈ 0.4 M NaCl and in several minor peaks at higher salt concentrations (Fig. 4.1C). Heterogeneous behavior is observed even when highly purified PLD (i.e., 6His/Superdex 200-purified PLD) is resolved by anion-exchange chromatography. PLD1 requires immediate buffer exchange after elution from Q-Sephacrose columns to stabilize the enzyme by reducing high salt concentrations.

2.6. Characteristics of purified, full-length PLD1

Purified PLD1 is highly active when reconstituted with lipid substrates. Phospholipase activity requires PIP_2 and is inhibited by detergents. Low salt (<150 mM NaCl) and reducing agents (DTT, β -mercaptoethanol) stabilize the enzyme in solution without inhibitory effects on enzymatic activity. Purified PLD1 is stable for several days at 4° and more than 1 year at -80° . The *pI* of purified PLD1 is determined to be approximately 7.5 by isoelectric focusing, equal to the reported pH optimum for enzymatic activity. PLD1 often appears as a doublet on SDS-polyacrylamide gels, with the major band at a predicted 115 kDa.

2.7. Purification of N-terminally truncated PLD1

PLD1.d311, an N-terminally truncated PLD1, with amino acids 312 to 1036, retains full catalytic activity, but a diminished response to protein kinase C (Henage *et al.*, 2006). However, it can be produced in larger quantities than full-length PLD1 using similar expression and purification systems. Its higher yield makes it useful for *in vitro* studies of the kinetics of the enzyme in the presence of various concentrations of activators.

To produce this truncated enzyme, PLD1.d311 is amplified from rPLD1b (Park *et al.*, 1997) and subcloned into pENTRdTOPO (Invitrogen) in frame with an N-terminal 6His/maltose-binding domain (*maltE*) fusion protein amplified from pSV282. PLD1b.d311 is transferred to pDEST8 baculovirus expression vectors by homologous recombination using Gateway methods (Invitrogen). Baculovirus encoding the PLD1.d311 construct is generated in Sf21 insect cells using the Bac-to-Bac system (Invitrogen) and screened for optimal expression conditions. Plasmid constructs and baculovirus DNA are sequenced to verify coding regions. Adherent Sf21 cultures (1×10^9 cells) are infected at a multiplicity of infection of 0.1 to 0.5 in

TNM-FH media with 10% fetal bovine serum. After 72 h, cells are harvested by centrifugation and resuspended in 20 ml lysis buffer A. Recombinant PLD1.d311 protein (131 kDa) is purified as described earlier for full-length rPLD1b. Purified protein elutes from HiTrap chelating HP NiIDA columns at 170 mM imidazole in a linear imidazole gradient. Pooled fractions are immediately desalted over a Sephadex G-25 Superfine gel filtration column (GE Biosciences) and exchanged to buffer B (30 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT). Aggregated protein and minor contaminants are resolved from the purified fractions by size-exclusion chromatography over a 16/60 Superdex 200-pg column (GE Biosciences). PLD1.d311 (>95% pure) elutes with a retention volume of 68 ml. Pooled fractions are concentrated to >1 mg/ml by ultrafiltration, frozen in 10% glycerol, and stored at -80° . Purified PLD1.d311 retains high enzymatic activity for 2 to 3 days at 4° and for >1 year at -80° .

2.8. Assay of phospholipase D activity *in vitro*

Enzyme activity is measured *in vitro* by established methods (Brown and Sternweis, 1995) by measuring the release of [*methyl*- ^3H]choline from [choline-*methyl*- ^3H]dipalmitoyl-PC (Perkin-Elmer Life Sciences). One to 10 nM PLD is reconstituted with phospholipid vesicle substrate. This is typically composed of 10 μM dipalmitoyl-PC, 100 μM PE (bovine liver), 6.2 μM PIP₂ (porcine brain), and 1.4 μM cholesterol, all from Avanti Polar Lipids. Lipid solutions are dried under a gentle stream of nitrogen and then resuspended in 100 mM HEPES, pH 7.5, 160 mM KCl, 6 mM EGTA, and 0.2 mM DTT. Small unilamellar vesicles (SUVs; 200 Å diameter) are prepared from lipid dispersions by repeated bath sonication (6 $\times$ 1-min intervals at 80 W).

All assays are conducted for 30 min at 37° in 50 mM HEPES, pH 7.5, 80 mM KCl, 3 mM EGTA, 0.1 mM DTT, 3.6 mM MgCl₂, 3.6 mM CaCl₂, and, when G proteins are present, 10 μM GTP γ S (Calbiochem). Reactions are stopped by the addition of trichloroacetic acid and bovine serum albumin (BSA). Free [*methyl*- ^3H]choline is separated from precipitated lipids and proteins by centrifugation and is analyzed by liquid scintillation counting. The enzymatic reactions are linear with time and protein concentration. Initial rates are determined from measurements between 5 and 25% PC hydrolysis. Data are presented as mean initial enzymatic rates $\pm$ standard error (nmol PC hydrolyzed/min/mg PLD) measured in 3 to 12 independent experiments performed in duplicate.

To allow steady-state kinetic characterization of PLD1 activity, it is necessary to identify assay conditions that allow accurate estimates of enzymatic rates. Vesicle concentrations are kept in excess of PLD concentrations (assuming ≈ 2500 phospholipids per vesicle). Because the lipid substrate forms an ordered bilayer structure, only the outer leaflet is available to PLD. Approximately 40% of the substrate partitions to the inner leaflet of

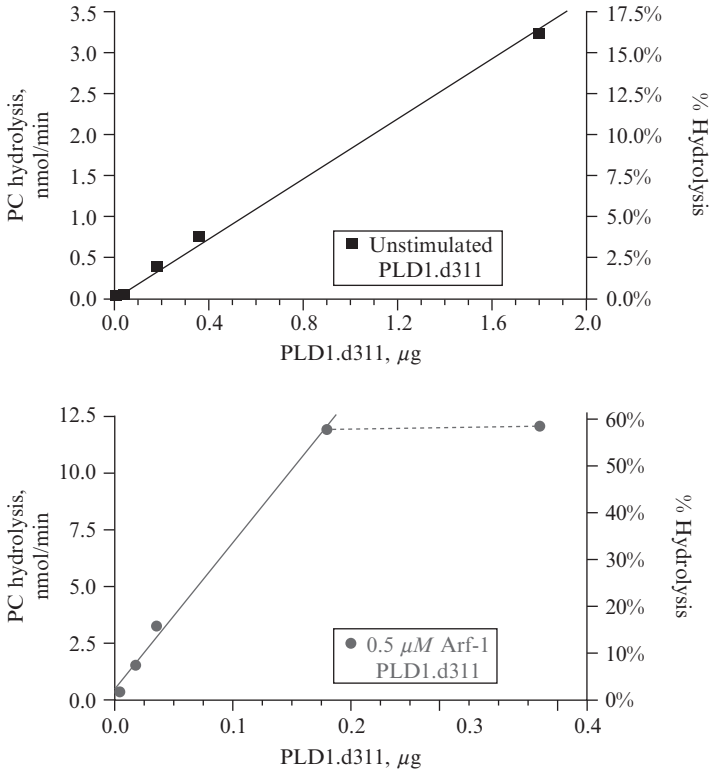


Figure 4.3 Linear range of *in vitro* activity assays. PLD1.d311 was added at the indicated amounts to reaction mixtures (60 μl) containing substrate vesicles. Phospholipase D activity was assayed by the standard *in vitro* method described in the text.

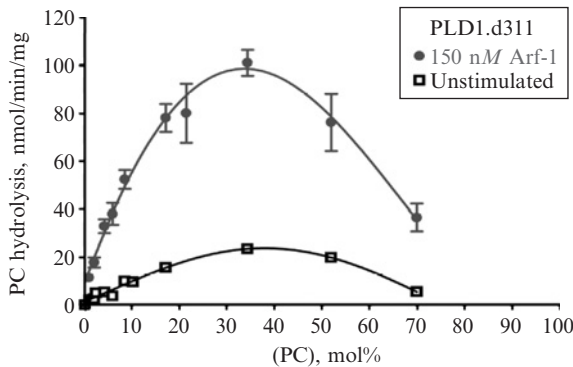


Figure 4.4 Substrate dependence of PLD1.d311 activity. Specific activity of PLD1.d311 was assayed with increasing surface concentrations of PC. Phospholipase activity was measured by the standard assay modified to vary the PC content of the lipid vesicles. Total lipid concentration was held constant at 116 μM, PIP₂ was 5 mol%, and the PC/PE ratio was adjusted to achieve the indicated PC concentration.

SUVs and is therefore inaccessible. In this assay, enzymatic rates become substrate limited when as little as 40 to 50% of the substrate is hydrolyzed. Reactions are linear with time and enzyme concentration over a limited range (Fig. 4.3). Initial rates are determined from measurements between 5 and 25% PC hydrolysis. In practice, a 100- to 200-fold range of enzymatic rates can be measured reliably.

PLD1.d311 exhibits saturation kinetics toward PC substrate. The surface concentration of PC is manipulated by adjusting the PE/PC ratio in vesicles containing 5 mol% PIP₂. PLD1.d311 activity exhibits classical Michaelis–Menten behavior in PE-dominated vesicles, but activity is reduced in PC-dominated vesicles (Fig. 4.4). A poor substrate for mammalian PLDs, PE enhances PLD activity toward PC (Nakamura *et al.*, 1996). In fact, PLD1 does not hydrolyze PC vesicles without the incorporation of other lipids (e.g., PIP₂, oleate). Kinetic assays are performed with substrate vesicles containing PC at less than 40 mol%.

3. REGULATED PLD1 ACTIVITY

To examine the enzymatic properties of purified PLD, standard *in vitro* methods (Brown and Sternweis, 1995) are used to assay activity toward the dipalmitoyl-PC substrate. PLD is reconstituted with purified effectors and lipid vesicles containing radiolabeled substrate. PLD1.d311 retains the full enzymatic activity of full-length PLD1 and is regulated by all classes of PLD activators (Fig. 4.5). PLD1.d311 exhibits slightly elevated basal activity in

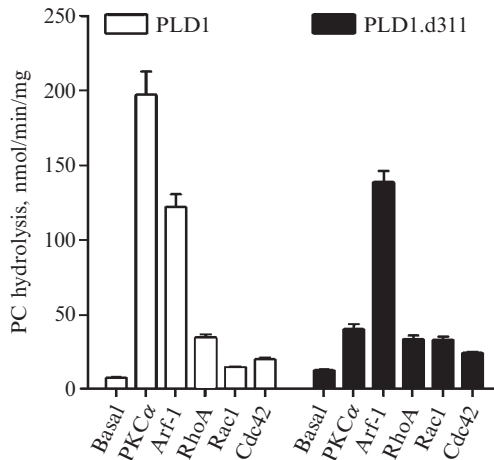


Figure 4.5 Regulated activity of PLD1 and PLD1.d311. Specific activities of the enzymes were determined using the standard *in vitro* assay with 5 to 10 nM enzyme in the presence of 10 μ M GTP γ S alone or in the presence of maximally effective concentrations of purified activators.

the absence of activators, similar to other N-terminally truncated PLD mutants (Park *et al.*, 1998; Sung *et al.*, 1999).



4. PREPARATION OF ACTIVATORS OF PLD1

4.1. Protein kinase C α (PKC α)

Protein kinase C α is expressed in baculovirus-infected Sf21 cells and is purified essentially as described for PKC β (Walker *et al.*, 2000).

4.2. Myristoyl-ADP ribosylation factor 1

Human Arf-1 is coexpressed with human N-myristoyltransferase 1 in BL21 (DE3) *Escherichia coli*. Four-liter cultures are grown in LB broth (150 μ g/ml carbenicillin and 50 μ g/ml kanamycin) at 37° and 250 rpm. At OD₆₀₀ = 0.7, myristic acid is added to 50 mg/liter. Expression is induced at OD₆₀₀ = 0.9 with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside. Cultures are incubated for another 3 h at 27° and harvested by centrifugation. Cells are resuspended in 10 ml lysis buffer (20 mM Tris-Cl, pH 8, 20 mM NaCl, 1 mM MgCl₂, 100 μ M GDP, 1 mM DTT, 10 mg/ml lysozyme, 2 mM phenylmethanesulfonyl fluoride [PMSF], complete protease inhibitor cocktail) and lysed by sonication (6 $\times$ 30-s pulses at 6 W on ice). Insoluble material is removed by centrifugation (40,000g for 30 min at 4°) and the lysate is clarified further (100,000g for 1 h at 4°). The supernatant is diluted to 50 ml in buffer C (20 mM Tris-Cl, pH 8, 20 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 100 μ M GDP, 1 mM DTT). The clarified lysate is applied to an 85-ml DEAE Sepharose FF column (GE Biosciences) and eluted at 110 mM NaCl in a linear NaCl gradient. Active fractions are identified by activation of PLD *in vitro* and concentrated to 5 ml by ultrafiltration. Recombinant Arf-1 is applied to a 26/60 Superdex 75 pg column (GE Biosciences) in buffer D (20 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM MgCl₂, 1 mM DTT). Purified (~95%) Arf-1 elutes with a retention volume of 196 ml. Active fractions are concentrated to 0.5 mg/ml by ultrafiltration, frozen in 5% glycerol, and stored at -80°.

4.3. Purification of geranylgeranylated RhoA, Rac1, and Cdc42

Baculoviruses encoding N-terminal His-tagged human RhoA, Rac1, and Cdc42 have been described previously (Walker and Brown, 2002). Adherent Sf21 cultures (3 $\times$ 10⁸) cells are infected at a multiplicity of infection of >1. After 72 h, cells are harvested by centrifugation and resuspended in lysis buffer B (50 mM sodium phosphate buffer, pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 1% [w/v] β -OG, 10 μ M GDP, 2 mM PMSF, complete protease inhibitor cocktail). Cells are disrupted by sonication 6 $\times$ 10 s at 6 W on ice.

The lysate is clarified by centrifugation at 100,000g at 6° for 60 min. Recombinant GTPases are purified over 1-ml HiTrap chelating HP NiIDA columns (GE Biosciences) and eluted with linear imidazole gradients. Active fractions (300–360 mM imidazole) are identified by activation of PLD *in vitro* and exchanged to a storage buffer containing 25 mM phosphate buffer, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 0.5% (w/v) β -OG over a Sephadex G-25 Superfine gel filtration column (GE Biosciences). Purified (>95%), highly active geranylgeranyl RhoA, Rac1, and Cdc42 are frozen in 5% glycerol and stored at –80°.

5. EFFECTS OF ACTIVATORS ON PLD1 ACTIVITY

Recent work has mapped major sites of interaction with PKC α to the extreme N terminus and PH domain of PLD1. Surprisingly, potent activation by PKC α , prepared as described elsewhere (Walker *et al.*, 2000), is still observed when these major PKC-binding sites are deleted from the N terminus of PLD1. The maximal PLD1.d311 response to PKC α is 16% of the maximal response of full-length PLD1 to PKC α (Fig. 4.5 and Table 4.2). Monomeric G-protein activators are equally effective toward PLD1 and PLD1.d311, both in terms of potency and in maximal activation. Full-length PLD1 and PLD1.d311 are both strongly activated by Arf-1•GTP γ S (Fig. 4.5). Arf-1 stimulates PLD1 activity in a concentration-dependent manner and its effects do not saturate at even 10 μ M Arf-1 (data not shown). Other activators are more than 30-fold more potent than Arf-1 (Fig. 4.6). No other activator equals the degree of activation elicited by Arf-1. Three members of the Rho GTPase subfamily potently

Table 4.2 Activation of PLD1 *in vitro* activity of purified PLD1 (1nM) in the presence of purified activators

Activator(s)	PLD1 (full-length)		PLD1.d311	
	EC ₅₀	Max	EC ₅₀	Max
	nM	nmol/min/mg	nM	nmol/min/mg
Unstimulated		7 $\pm$ 1		13 $\pm$ 1
PKCα	32 $\pm$ 1	204 $\pm$ 17	56 $\pm$ 22	34 $\pm$ 4
Arf-1		>500		>500
RhoA	29 $\pm$ 2	44 $\pm$ 5	67 $\pm$ 43	37 $\pm$ 4
Rac1	5 $\pm$ 2	17 $\pm$ 1	19 $\pm$ 19	31 $\pm$ 6
Cdc42	9 $\pm$ 4	23 $\pm$ 2	30 $\pm$ 20	23 $\pm$ 1

§Concentrations of Arf-1 were varied, RhoA or PKC α were held constant at 300 nM.

†Concentrations of PKC α are varied, RhoA was held constant at 300 nM, Arf-1 at 1 μ M.

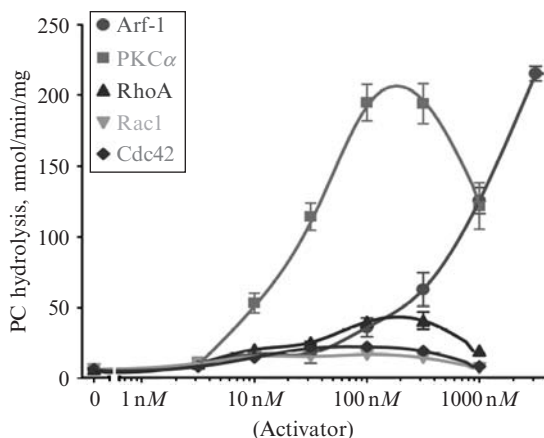


Figure 4.6 Allosteric activation of PLD1. Increasing concentrations of activators were reconstituted with 5 nM full-length PLD1. Initial rates of phospholipase D activity were measured using the standard *in vitro* assay.

stimulate PLD1, but with low maximal stimulation. GTP γ S-loaded RhoA, Rac1, or Cdc42 stimulates PLD1 and PLD1.d311 2- to 5-fold above basal activity (Table 4.2). Normal ($n_H \approx 1$) concentration-dependent effects are observed for each of the activators tested, although very high (μ M) concentrations of PKC α , RhoA, Rac1, or Cdc42 have reduced ability to activate PLD1 (Fig. 4.6).

6. SYNERGY BETWEEN PLD1 ACTIVATORS

At maximally effective concentrations, RhoA and PKC α stimulate PLD1 activity no more than PKC α alone. At lower PKC α concentrations, the combined effects of PKC and 100 nM RhoA are roughly equal to the sum of the effects of each activator alone (Fig. 4.7A). The combined effect of PKC α and Arf-1 exceed the additive effects of the activators alone, indicating synergy (Fig. 4.7A). Costimulation with Arf-1 enhances the maximal response to PKC α but does not change PKC α potency toward PLD1. Arf-1 (1 μ M) synergizes with PKC α to stimulate PLD1 100-fold above basal activity, more than twice the maximal response predicted for an additive effect.

Arf-1 also synergizes with RhoA to activate PLD1 (Fig. 4.7B). Arf-1 (1 μ M) and RhoA (100 nM) combine to enhance PLD1 activity almost 50-fold, twice the response predicted for an additive effect. Arf-1 exhibits synergistic relationships with all PLD1 effectors tested. Combinations without Arf-1 do not produce synergistic responses.

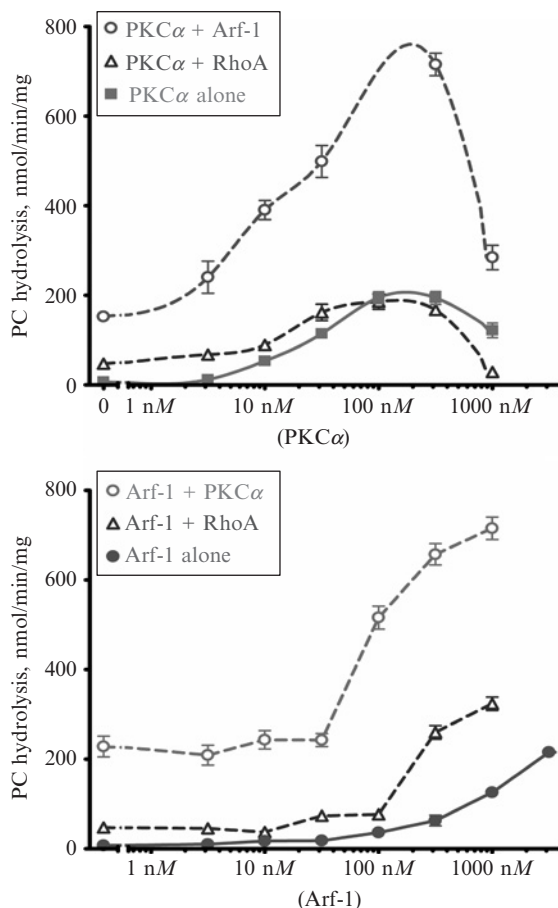


Figure 4.7 Synergistic activation of PLD1. Increasing concentrations of activators were reconstituted with full-length PLD1 (5 nM), and initial rates of phospholipase D activity were assayed by the standard method.

7. BINDING OF PLD1 TO PHOSPHOLIPID VESICLES

Previous studies have indicated that many PLD isozymes exhibit interfacial behavior (Chalif-Caspi *et al.*, 1998; Qin *et al.*, 2002; Yang and Roberts, 2003). Macroscopic Michaelis constants are dependent on interactions with substrate and with bulk lipid. Surface-dilution kinetic models account for aggregated substrates, describing both three-dimensional interactions with lipid interfaces and two-dimensional surface interactions with a specific substrate (Carman *et al.*, 1995). Because detergents strongly inhibit PLD1 *in vitro* (Brown and Sternweis, 1995; Hoer *et al.*, 2000; Jiang *et al.*, 2002; Nakamura *et al.*, 1996) and participate in the transphosphatidyl

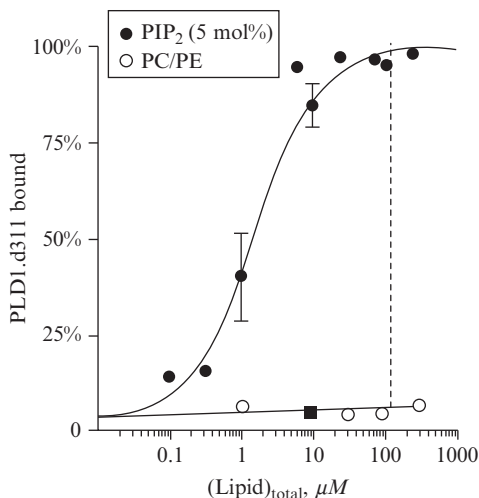


Figure 4.8 Binding of PLD1.d311 to phospholipid vesicles. Vesicles were prepared with and without PIP₂ (5 mol%). Bound enzyme was separated from free enzyme and measured as described in the text.

reaction as nucleophiles (Yang and Roberts, 2003), kinetic parameters cannot be calculated from mixed-detergent-lipid micelle experiments.

Association of PLD1 and the phospholipid interface is examined by measuring the binding of purified PLD1.d311 to sucrose-loaded phospholipid vesicles (Fig. 4.8). The binding assay is adopted, with minor modifications, from the procedure of Buser and McLaughlin (1998). Large unilamellar vesicles are prepared by extrusion (Lipex Biomembranes, Inc., Vancouver) of lipid dispersions through two 0.1- μ m polycarbonate filters (Nuclepore). Lipid composition is identical to standard *in vitro* assay preparations (see later) or modified to replace PIP₂ with PE. Vesicles are loaded with 176 mM sucrose, 50 mM HEPES, pH 7.5, 3 mM EGTA, 3 mM MgCl₂, and 3 mM CaCl₂. Sucrose-loaded vesicles are washed and resuspended in an isotonic buffer containing KCl. Sucrose-loaded vesicles are incubated with 10 nM PLD1.d311 for 30 min at 25° and sedimented by ultracentrifugation at 100,000g for 45 min at 20°. PLD1 present in supernatant (PLD_{sup}) and pellet (PLD_{pellet}) is estimated by immunoblot, and vesicle-associated PLD1.d311 is calculated according to Eq. (1):

$$\text{PLD}_{\text{vesicle}} = \frac{(\beta)\text{PLD}_{\text{pellet}}(\beta - 1)\text{PLD}_{\text{sup}}}{\alpha + \beta - 1} = \frac{(B_{\text{max}})[PL]_T}{K_s^A + [PL]_T} \quad (1)$$

where α is the fraction of sedimented vesicles determined by scintillation counting and β is the fraction of PLD immunoreactivity (horseradish

peroxidase [HRP]-conjugated α FLAG-M2 antibody, Sigma) in the supernatant fraction in the absence of lipid (accounts for PLD that precipitates without lipid). $[PL]_T$ is the total concentration of lipid, and K_s^A is a dissociation constant. Vesicle-bound PLD1.d311 is separated from the free enzyme by ultracentrifugation and analyzed by immunoblot (Buser and McLaughlin, 1998). The catalytic domain of PLD1 (PLD1.d311) displays high affinity for phospholipid surfaces.

The PIP_2 content of the vesicles is crucial for the binding interaction with PLD1.d311. For vesicles prepared with 5 mol% PIP_2 , we report a dissociation constant, K_s^A , of about $2 \mu M$ bulk lipid. Sucrose-loaded vesicles prepared without PIP_2 bind PLD1.d311 weakly. Prior reports demonstrated that other phosphoinositides can partially substitute for PIP_2 in promoting PLD association with lipid vesicles and that membrane association is dependent on conserved arginine residues within a C-terminal polybasic region (Du *et al.*, 2003; Sciorra *et al.*, 1999, 2001; Zheng *et al.*, 2002).

Data demonstrated that a limiting step in PLD1 catalysis (i.e., partitioning to the lipid interface) is saturated at $\approx 10 \mu M$ bulk lipid. Interestingly, conditions that promote maximal ($>98\%$) binding of PLD to phospholipid vesicles do not stimulate PLD activity to maximal rates. This indicates that activation of PLD1 involves effects on both bulk and interfacial binding steps. The *surface binding model* (Carman *et al.*, 1995) describes a two-step association of PLD1 with its substrate, where PLD1 nonspecifically binds the vesicle surface before specifically binding substrate. To examine subsequent interfacial events in PLD1 catalysis, the total lipid concentration is held constant at saturating levels ($116 \mu M$, $>98\%$ bound enzyme).

8. KINETIC PARAMETERS OF PLD1 CATALYTIC ACTIVITY

Kinetic parameters of PLD1 catalytic activity are determined by initial velocity experiments measuring dipalmitoyl-PC hydrolysis in PE-dominated vesicles as described earlier. Kinetic data are analyzed as follows.

Apparent dissociation constants and rate constants are determined from best-fit parameters by nonlinear regression (sum of squares) using Eq. (2) (Carman *et al.*, 1995):

$$\left. \frac{d[\text{choline}]}{dt} \right|_{(t \sim 0)} = \frac{k_{cat}[PL]_T[PC]_0[PLD]}{K_s^A K_m^B + K_m^B[PL]_T + [PL]_T[PC]_0} \quad (2)$$

Concentration–response data are fit to Eq. (3), where *MAX* and *MIN* refer to maximum and minimum rates, n_H is Hill's coefficient, and $[a]$ represents the concentration of an allosteric effector:

$$\frac{d[\text{choline}]}{dt} = \frac{MIN + (MAX - MIN)}{1 + 10^{(n_H \log EC_{50} - \log[a])}} \quad (3)$$

Synergy is expressed as a ratio, κ , of the response to combined activators relative to the responses to individual activators [Eq. (4)]. The degree of activation, ε (IUPAC-IUBMB, 1982), describes the increase in PLD catalytic rate due to the effects of individual activators, a and b , or activators in combination, a, b .

$$\kappa = \frac{\varepsilon_{(a,b)}}{\varepsilon_{(a)} + \varepsilon_{(b)}} \quad (4)$$

Calculations are performed using Prism v4.0 (GraphPad software).

Phospholipase activity is dependent on the surface concentration of substrate (Fig. 4.9 and Table 4.3), and experimental data fit Michaelis–Menten relationships by least squares and Eadie–Hofstee analyses ($r^2 > 0.7$). All reported kinetic constants are determined by nonlinear regression analysis. The interfacial Michaelis constant, K_m^B , describes two-dimensional surface interactions between PLD and its substrate, PC.

Apparent K_m^B values for dipalmitoyl-PC are about 33 mol% (39 μM) in the absence of protein activators, similar to the reported value of 42 μM for PLD purified from bovine kidney (Nakamura *et al.*, 1996). A $V_{\max}$ value of 32 nmol PC hydrolyzed $\text{min}^{-1}\text{mg}^{-1}$ PLD1 was obtained for the unstimulated enzyme. PLD1.d311 was only slightly activated, with a $V_{\max}$ of 46 nmol $\text{min}^{-1}\text{mg}^{-1}$.

PLD1 activators enhance catalytic efficiency, k_{cat}/K_m , both by reducing K_m^B and by enhancing catalytic potential, k_{cat} (Table 4.3). PLD1 activators can be discriminated based on their effects on these kinetic parameters. PKC α has profound effects on PLD1 kinetics, enhancing catalytic efficiency 75-fold. A *mixed activator*, PKC α produces dramatic effects on both K_m^B and k_{cat} . A maximal rate ($V_{\max}$) of 453 nmol $\text{min}^{-1}\text{mg}^{-1}$ is calculated for PKC α -stimulated PLD1. The PLD1.d311 maximal rate is not enhanced by PKC α . Kinetic analyses reveal that PKC α regulates the catalytic efficiency of PLD1.d311 via *binding activation* (IUPAC-IUBMB, 1982).

While marked effects on K_m^B remain, deletion of N-terminal PLD1 domains are reflected in a loss of PKC α effects on k_{cat} . While full effects of PKC α on PLD1 activity require both N- and C-terminal domains, C-terminal interactions with PKC α have marked effects on PLD1.d311 catalysis (Tables 4.2 and 4.3). These experiments provide new insights into PKC α regulation of PLD1.

Arf-1•GTP γ S is equally effective toward PLD1 and PLD1.d311. In both cases, Arf-1 enhances PLD catalytic efficiency with only minor effects on K_m^B . A *catalytic activator* (IUPAC-IUBMB, 1982), Arf-1 increases PLD1

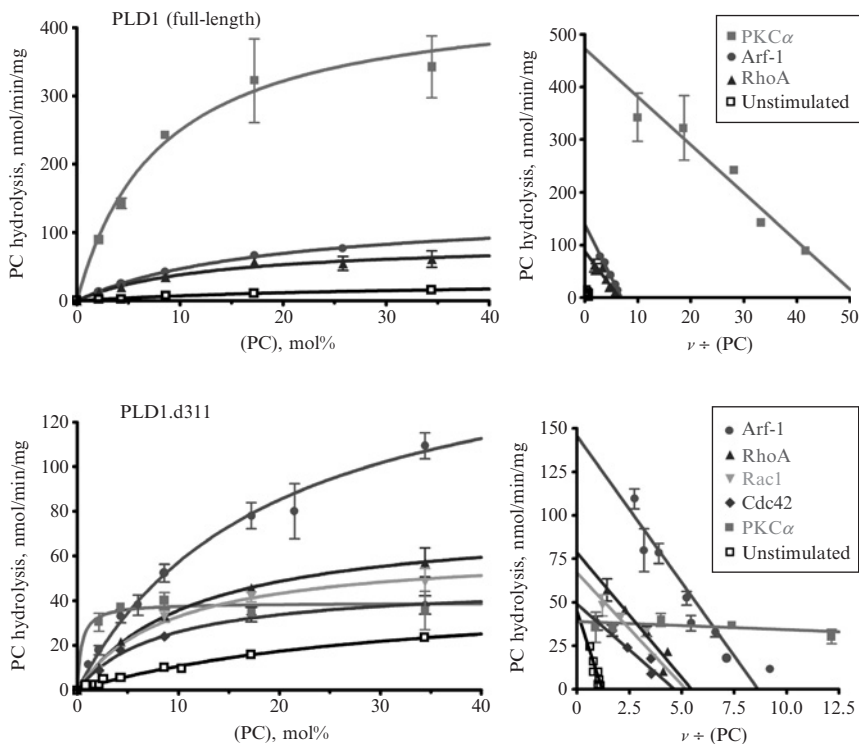


Figure 4.9 Kinetic properties of PLD activity in the presence and absence of activators. Specific activities of PLD1 and PLD1.d311 were assayed with increasing concentrations of PC using the standard assay. (Right) Eadie-Hofstee linear transformations of data. Activities were measured in the presence of $10 \mu\text{M}$ GTP γS alone (unstimulated) or with 300 nM PKC α , 150 nM Arf-1, 300 nM RhoA, 300 nM Rac1, or 300 nM Cdc42.

catalytic potential (k_{cat}) in a concentration-dependent manner. The maximal rate of PLD is increased 4-fold at 150 nM Arf-1, whereas K_m^B values are reduced only modestly, $\approx 40\%$ (Table 4.3). k_{cat} values are enhanced more than 10-fold at $10 \mu\text{M}$ Arf-1 (data not shown). To allow direct comparisons between activators and to compare synergistically activated conditions, Arf-1 concentrations are set at 150 nM throughout these experiments. These conditions allow accurate estimates of initial enzymatic rates where PLD activity is within the linear range of the assay (PC hydrolysis between 5 and 25% of total substrate).

Rho family GTPases regulate PLD1 catalysis via allosteric interactions that promote *binding activation*. It is understood that RhoA activates PLD1 through direct interaction with a C-terminal PLD catalytic subdomain (Cai and Exton, 2001; Du *et al.*, 2000; Sung *et al.*, 1999). Data show that RhoA-dependent activation of PLD1 is not altered by deletion of N-terminal domains (Table 4.3). Maximally effective concentrations of

Table 4.3 Kinetic properties of PLD activity. Effects of allosteric activators on K_m and K_{cat} of purified PLD1 towards PC

Activator(s)	PLD1 (<i>full-length</i>)			PLD1.d311		
	K_m^B	k_{cat}	k_{cat}/K_m^B	K_m^B	k_{cat}	k_{cat}/K_m^B
	<i>mol%</i>	<i>min</i> ⁻¹	<i>mol%</i> ⁻¹ <i>min</i> ⁻¹	<i>mol%</i>	<i>min</i> ⁻¹	<i>mol%</i> ⁻¹ <i>min</i> ⁻¹
Unstimulated	33 ± 12	4 ± 1	0.1	32 ± 8	6 ± 1	0.2
PKCα	6 ± 4	56 ± 10	9	< 1	5 ± 1	11
Arf-1	19 ± 6	16 ± 3	0.8	19 ± 4	22 ± 2	1.2
RhoA	13 ± 3	11 ± 1	0.8	11 ± 3	10 ± 1	0.9
Rac1				9 ± 2	8 ± 1	0.9
Cdc42				8 ± 2	6 ± 1	0.8
Arf-1 + PKCα				< 1	26 ± 2	48
Arf-1 + RhoA				2 ± 1	27 ± 2	12
Arf-1 + Rac1				4 ± 2	22 ± 3	5
Arf-1 + Cdc42				3 ± 2	22 ± 3	6
PKCα + RhoA				1 ± 1	6 ± 1	6
PKCα + Rac1				1 ± 2	6 ± 1	6
PKCα + Cdc42				2 ± 1	7 ± 1	4

Values were calculated as described in the text.

RhoA•GTP γ S enhance the catalytic efficiencies of PLD1 and PLD1.d311 equally. The primary kinetic effect of RhoA is on the apparent interfacial Michaelis constant, K_m^B . Effects of closely related GTPases, Rac1 and Cdc42, were also examined (see Fig. 4.9B). Like RhoA, Rac1 and Cdc42 are *binding activators*. K_m^B values were reduced 3- to 4-fold while k_{cat} values remained essentially unchanged. *Binding activators* stimulate PLD1 activity to limiting rates even at low concentrations of PC, shifting the substrate-dependence curve leftward without increasing the maximal rate.

9. KINETIC ANALYSES OF SYNERGISTIC RESPONSES

Kinetic analyses of PLD1.311 activity reveal that synergistic responses are hybrid responses, composed of the kinetic properties of each activator. Arf-1 and PKC α combine to activate PLD1.311 with catalytic activator effects on k_{cat} and binding activator effects on K_m . The substrate-dependence curve is shifted leftward, reflecting a 50-fold reduction in K_m^B , and vertically, reflecting a 4-fold increased k_{cat} (see Fig. 4.9 and Table 4.3). Moreover, the combined effects of PKC and Arf on K_m^B values are equivalent to the effects of PKC alone, and the effects of combined activators on k_{cat} values are equivalent to the effects of Arf alone. Together, these effects lead to cooperative effects on catalytic efficiency (k_{cat}/K_m) and lead to synergy between these activators.

Rho GTPases does not synergize with PKC α . Kinetic properties of PKC α -stimulated PLD1.d311 are not changed significantly by the addition of RhoA, Rac1, or Cdc42 to the reaction mixture (Fig. 4.10 and Table 4.3). Arf-1 synergizes with RhoA, Rac1, and Cdc42 (Fig. 4.10 and Table 4.3). Arf-1 contributes enhanced catalytic potential, whereas Rho GTPases contribute reduced K_m^B values. Shown in Fig. 4.10B, simultaneous stimulation by Arf-1 and RhoA leads to a synergistic response with properties of each activator. K_m^B values are reduced 14-fold and k_{cat} is enhanced 4-fold. PLD1.d311 initial rates reach levels nearly 3-fold greater than can be explained by additive effects of RhoA and Arf-1. Synergy between Arf-1 and Rac1 or Cdc42 is slightly less robust. Combinations of Arf-1 and Rac1 (see Fig. 4.10C) or Arf-1 and Cdc42 (Fig. 4.10D) stimulate PLD1.d311 to levels 2-fold greater than can be explained by additive effects.

10. PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE IS AN ESSENTIAL PLD1 ACTIVATOR

The activity of purified PLD1.d311 was measured over a range of PIP₂ concentrations; the parameters of the assay are described in relevant figure legends. In the absence of PIP₂, PLD1.d311 is inactive and does not respond to

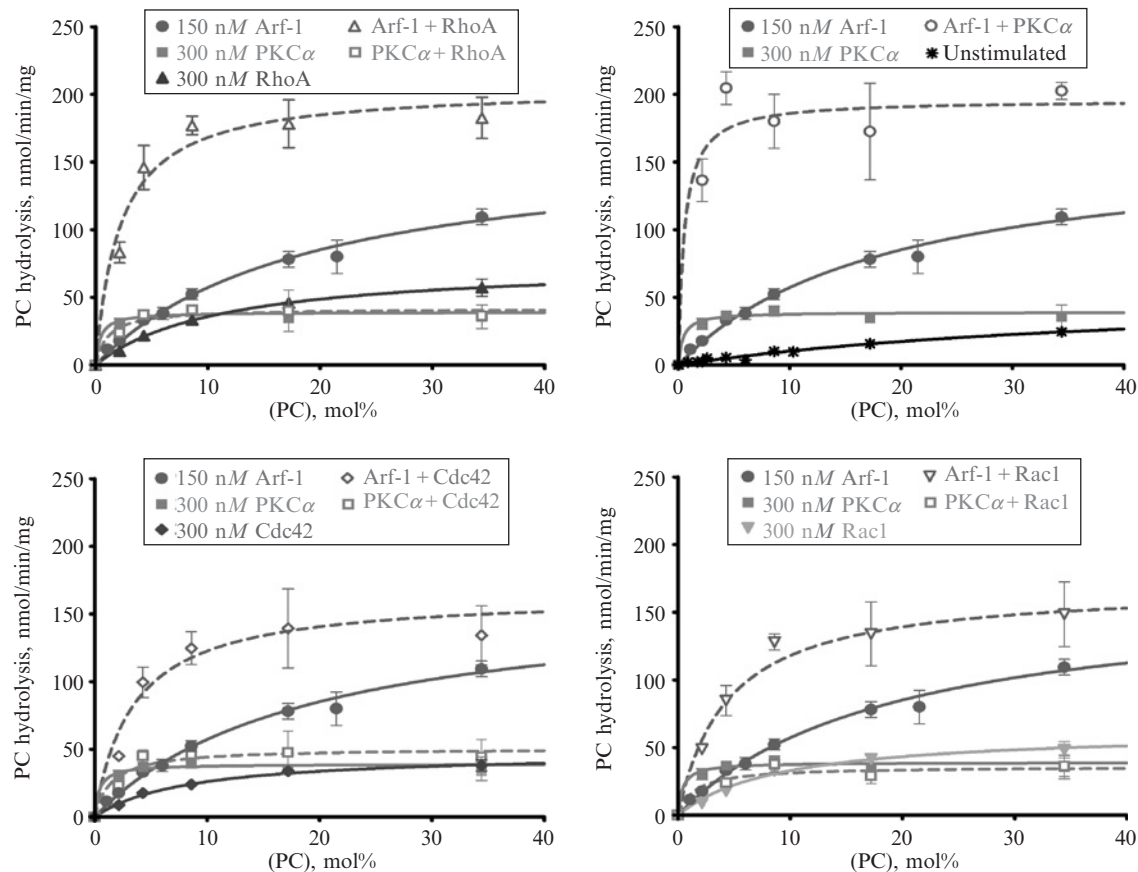


Figure 4.10 Dependence on Arf-1 of the synergistic activation of PLD1. Substrate dependence of PLD1.d311 activity was examined as described in the legend to Fig. 4.9.

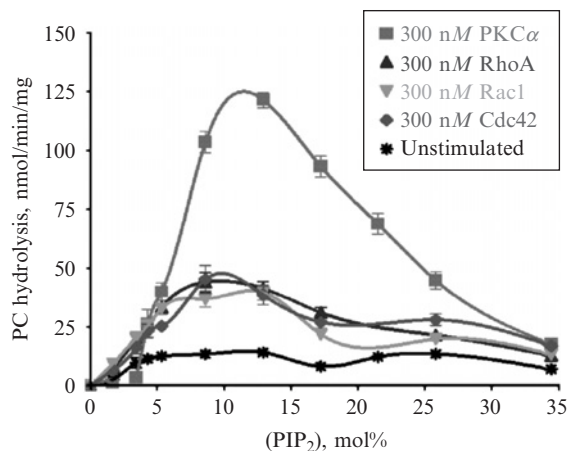


Figure 4.11 Dependence on PIP₂ of PLD1 activity. Specific activity of PLD1.d311 was assayed with increasing concentrations of PIP₂ using the standard assay. Concentrations of additions were as in Fig. 4.9.

activators. PIP₂ stimulates PLD1.d311 activity in a concentration-dependent manner (Fig. 4.11). In the absence of protein activators, PLD activity is detected at less than 2 mol% PIP₂ and achieves maximal velocity at ≈ 5 mol % PIP₂. Shown in Fig. 4.8, binding of PLD1.d311 to phospholipid vesicles requires PIP₂, and binding is essentially complete ($>98\%$) for vesicles prepared with 5 mol% PIP₂. Together, these data are consistent with the interpretation that PIP₂ activates PLD1.d311 catalysis by recruiting the enzyme to the surface of the membrane. Regulated PLD activity is steeply dependent on PIP₂, which potentiates the effects of all PLD1 activators. Maximal enzymatic rates are achieved at 8 to 12 mol% PIP₂, and concentrations greater than 35 mol% render PLD1.d311 unresponsive to activators. At optimal concentrations of PIP₂, effects of PKC α and Rho GTPases are nearly additive. At other concentrations of PIP₂, PKC α effects dominate.

PLD.d311 activity is stimulated four- to sixfold above basal activity at 150 nM Arf-1, with a PIP₂-dependence profile very similar to the profile of unstimulated PLD1.d311 (Fig. 4.12A and C). Arf-1 strongly synergizes with all other activators (see Fig. 4.12C). PLD1 activators synergistically activated PLD1.d311 only under optimal PIP₂ concentrations. Synergy between Arf-1 and PKC α is greatest at 8 mol% PIP₂, whereas synergy between Arf-1 and Rho GTPases is greatest at ≈ 5 mol%.

The proposed kinetic interpretation of synergism between PLD1 activators has several important corollaries. At limiting concentrations of substrate ($[PC]_0 \ll K_m$), the initial velocity of the PLD-catalyzed reaction is directly proportional to the initial substrate concentration ($v_0 \approx [PC]_0 V_{\max}/K_m$). This relationship is consistent with observations that synergy between

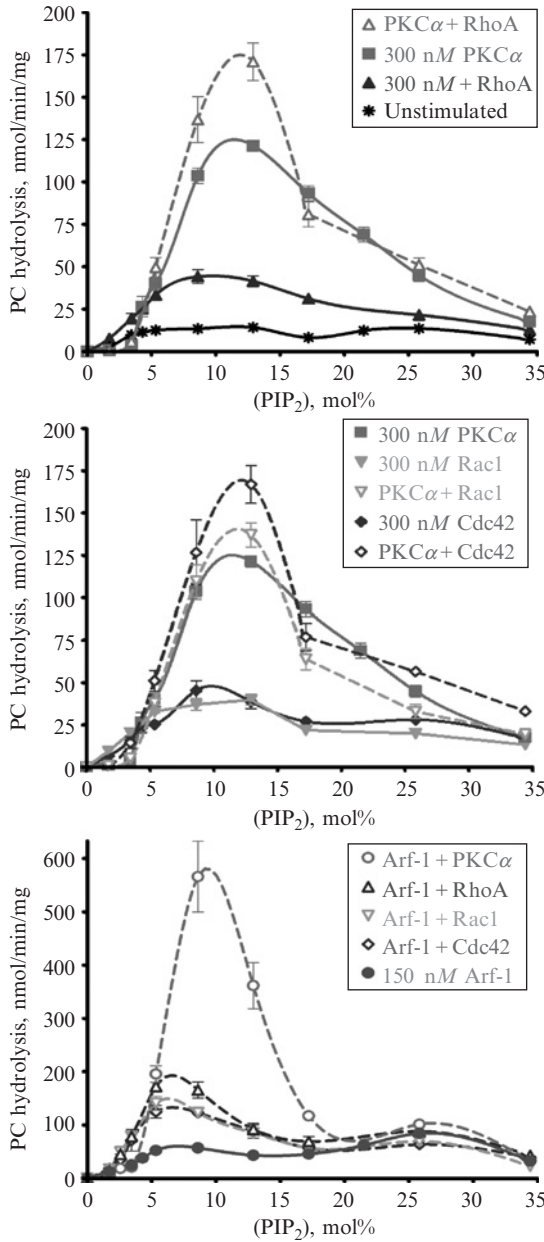


Figure 4.12 Dependence of synergy on both Arf-1 and PIP₂. Phospholipase activity of PLD1.d311 was assayed with increasing concentrations of PIP₂ and with activators as described in Fig. 4.11.

activators is pronounced at low PC concentrations. At high substrate concentrations ($[PC]_0 \gg K_m$), the reaction displays first-order kinetics and approaches maximal velocity ($v_0 \approx V_{\max}$). Under such conditions, *binding activators* have little effect on enzymatic rates and minimal capacity to synergize with other activators.

Protein kinase C α does not interact with Rho, Rac, or Cdc42 to produce synergistic responses ($\kappa = 1$, not shown). The coactivation index κ is a ratio of the responses to combined activators relative to the sum of the responses to individual activators: $\varepsilon_{(a,b)} = \kappa(\varepsilon_{(a)} + \varepsilon_{(b)})$ (Henage *et al.*, 2006). Combinations including Arf-1 lead to synergy ($\kappa > 1$) only when PC and PIP₂ concentrations are optimal (Fig. 4.13). Synergistic activation is related to differences in K_m values between individual activators; synergy

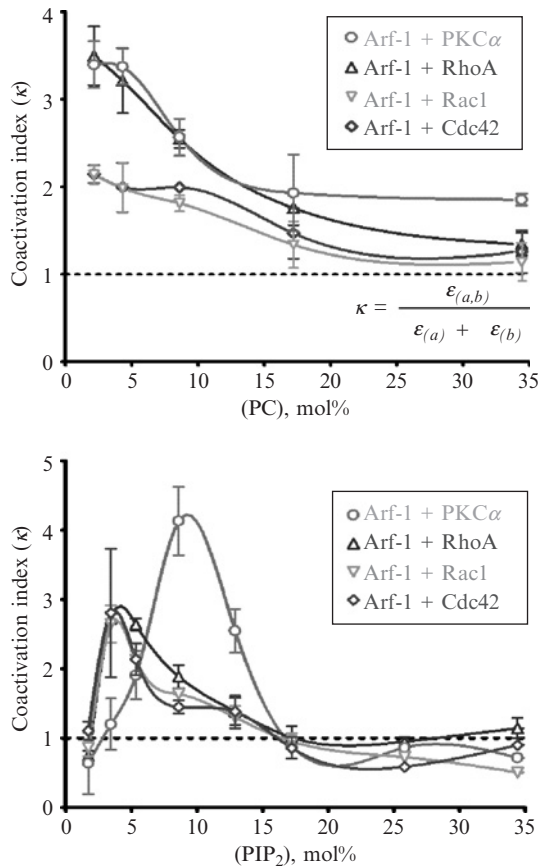


Figure 4.13 Analysis of synergistic effects of PLD1 activators. Synergy was assessed by measuring the coactivation index κ as described in the text. This is a ratio of the response to combined activators relative to the sum of the responses to individual activators.

is greatest at substrate concentrations less than K_m for Arf-1-stimulated PLD (≈ 19 mol% PC). Consequently, synergy is greatest at the lowest substrate concentrations tested (see Fig. 4.13A).

11. *In Vivo* PLD ASSAY USING RADIOISOTOPES

A traditional method for measurement of PLD activity in cells is through the use of radioisotope-based labeling of lipid precursors through incorporation of ^3H fatty acids. For a detailed description of the methodology, see Walker and Brown (2004). In this method either the conventional PLD product, phosphatidic acid, or the product of the transphosphatidylation reaction involving a primary alcohol (see later) can be measured. Typically, labeled free fatty acids are incorporated into cells. We have obtained satisfactory results using various ^3H fatty acids, including myristic, palmitic, oleic, and arachidonic acids. The optimal precursor depends to some extent on the cell type. As an illustration, Fig. 4.14 shows activation of PLD activity in 1321N1 astrocytomas. Parental (wild type) or stably transfected cells expressing either P2Y1 or P2Y2 purinergic receptors (a kind gift from Rob Nicholas and Ken Harden at UNC-CH) were shown to activate intracellular PLD in response to an appropriate ligand. The calcium ionophore A23187 was included as a positive control. Relative quantitation can be obtained through various methods, including scintillation counting or imaging programs.

12. *In Vivo* PLD ASSAY USING DEUTERATED 1-BUTANOL

The transphosphatidylation reaction is exclusive to PLD and is widely used to measure its activity (Bocckino *et al.*, 1987). This unique property of the enzyme has been utilized to specifically detect PLD activity in cells and to decouple PLD signaling from PA production. Thus, in the presence of a primary alcohol, hydrolysis stops at the phosphatidyl alcohol, whereas in the presence of water, PA can be metabolized further to DAG by phosphatidate phosphohydrolase/lipid phosphate phosphohydrolase or can be subsequently converted to lysophosphatidic acid. This section describes a new method utilizing mass spectrometry analysis and deuterated 1-butanol to measure endogenous PLD activity by its transphosphatidylation reaction. Different from the traditional radiolabeling PLD activity assay, cells are treated with primary (protonated) butanol (1-BuOH) and deuterated BuOH (1-BuOH- d_{10}) along with the agonist, and the formation of phosphatidylbutanol (PtdBuOH) is detected by mass spectrometric analysis. The production of PtdBuOH- d_9 is confirmed by the 9-Da shift in the peaks m/z

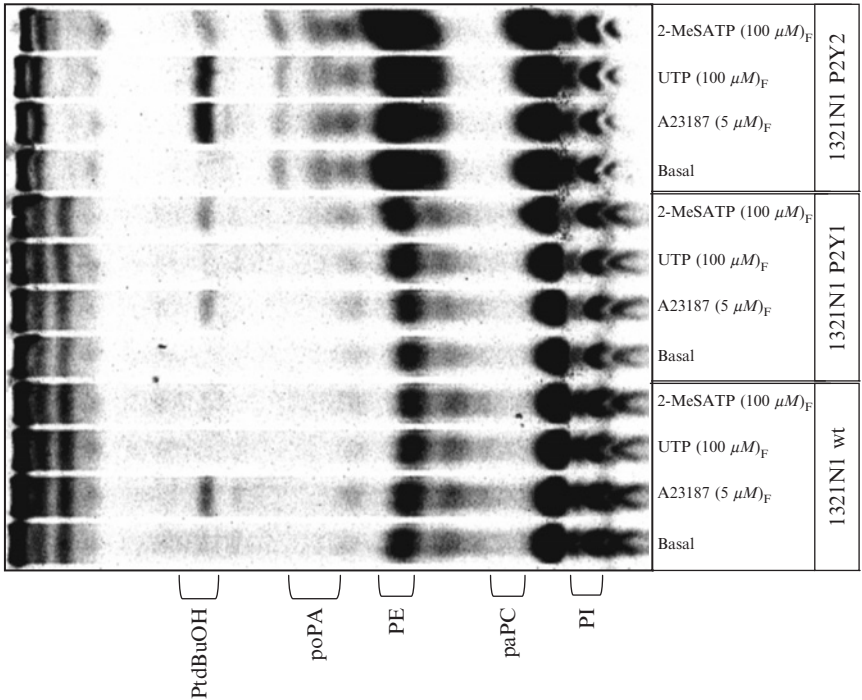


Figure 4.14 *In vivo* PLD activity assay in 1321N1 astrocytomas. 1321N1 astrocytoma cells were seeded in six-well plates (Corning Incorporated, Corning, NY) at a density of 5.0×10^5 cells per well using Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (10%) and antimycotic/antibacteria (1%). At 85% cell confluence (serum-deprived 24 h), 5 μ Ci tritiated myristic acid with bovine serum albumin [0.25 mg/ml]_F in serum-free DMEM was applied for 20 h. Labeling medium was removed and the cells were washed with serum-free DMEM. Treatment with UTP [100 μ M]_F, 2-methyl(thio)-ATP [100 μ M]_F, or calcium ionophore A23187 [5 μ M]_F in the presence of 1-butanol (0.3%) occurred for 15 min. Lipids were extracted using the method of Bligh and Dyer (1959), spotted on a TLC plate, and resolved using the solvent system described by Hajra and Agranoff (1998) and Martin *et al.* (1991) [CHCl_3 : CH_3OH :acetic acid:acetone:water, 10:2:2:4:1(v/v)]. The TLC plate was then exposed to a Phosphorimager tritium screen for 68 h and stained with I_2 to visualize standards.

corresponding to PtdBuOH when samples are treated with 1-BuOH- d_{10} instead of 1-BuOH.

12.1. Materials and methods

1-BuOH and 1-BuOH- d_{10} are from EM Science (EM Industries, Inc., NJ) and Acros Organics (Morris Plains, NJ). All solvents used for extraction or mass spectrometry are of HPLC grade or better, purchased from EMD Chemicals (Gibbstown, NJ).

Cells are incubated in the presence of an agonist, an agonist plus 0.3% 1-butanol, and an agonist plus 0.3% 1-BuOH- d_{10} for the desired times. At the end of the stimulation, plates are placed on ice and media aspirated. Cells are then washed with 2 ml of ice-cold phosphate-buffered saline (PBS) (for a 60-mm plate) and PBS aspirated. Reactions are stopped by the addition of 0.8 ml 0.1 N HCl:methanol (1:1), and cells are scraped and transferred into cold numbered Eppendorf microcentrifuge tubes. Glycerophospholipids are extracted by adding 0.4 ml of cold chloroform and vortexed for 1 min to mix. Phase separation is achieved on a benchtop centrifuge (5 min at 4°, 18,000g). The lower (organic layer) is transferred using a Hamilton syringe to a new tube, and solvent is evaporated in a vacuum centrifuge (Labconco Centrивap Concentrator, Kansas City, MO). The resulting lipid film is dissolved in 90 μ l of MS solvent (9:1 methanol:chloroform) and subjected to direct infusion mass spectrometry on a Finnigan TSQ Quantum triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA).

12.2. Application

This assay uses mass spectrometry to measure the changes in the PLD reaction product PtdBuOH. For detailed description of the mass spectrometry methods, see Ivanova *et al.* (2007). Unlike in TLC, multiple PtdBuOH species are detected by this method. Because the peaks of PtdBuOH overlap with the peaks of PA in mass spectrum, by using 1-BuOH- d_{10} and 1-BuOH we can distinguish and confirm the formation of PtdBuOH following the 9-Da shift in the peaks of PtdBuOH produced by deuterated BuOH compared to the protonated PtdBuOH.

The method measures the PLD activity in P2Y₆ receptor-expressing 1321N1 cells (Fig. 4.15). In the control sample (see Fig. 4.15A) and the sample treated with UDP and *tert*-BuOH (PLD enzyme does not utilize secondary or tertiary alcohols as nucleophiles) (see Fig. 4.15B), no PtdBuOH peaks are present. In samples treated with UDP and 1-BuOH for 1 min (see Fig. 4.15C), the peaks of 30:1 PtdBuOH at m/z 673, 32:1 PtdBuOH at m/z 701, 32:2 PtdBuOH at m/z 699, 34:1 PtdBuOH at m/z 729, and 34:2 PtdBuOH at m/z 727 are observed. All of these peaks are isobaric with peaks corresponding to PA (m/z 673 – 34:1 PA; m/z 701 – 32:1 PA; m/z 699 – 32:2 PA; m/z 729 – 34:1 PA, and m/z 727 – 34:2 PA). In the UDP and 1-BuOH- d_{10} treated samples, the peaks of 30:1 PtdBuOH- d_9 [P(D)BuOH at m/z 682, 32:1 P(D)BuOH at m/z 710, 32:2 P(D)BuOH at m/z 708, 34:1 P(D)BuOH at m/z 738, and 34:2 P(D)BuOH at m/z 736] are detected. All of the observed PtdBuOH peaks shifted by 9 Da (see Fig. 4.15D), thus confirming their identity as PtdBuOH and not PA. Following the formation of PtdBuOH and PtdBuOH- d_9 peaks “in tandem” by mass spectrometry allows discrimination between PA and PtdBuOH synthesis as a result of PLD activation.

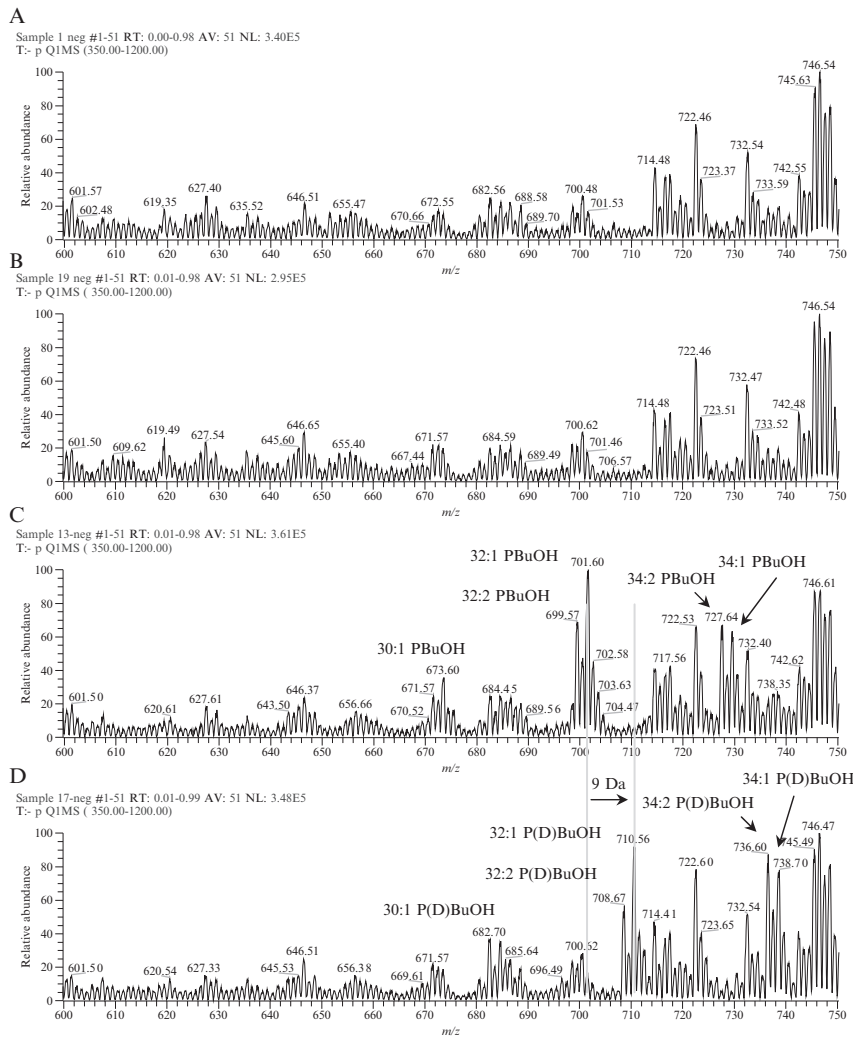


Figure 4.15 Negative ionization mass spectra of phospholipid extracts from P2Y₆ receptor expressing 1321N1 cells, extracted, and analyzed under identical conditions. (A) Control sample. (B) Sample treated with UDP and *tert*-BuOH. (C) Sample treated with UDP and 1-BuOH. (D) Sample treated with UDP and 1-BuOH-d₁₀.

13. FLUORESCENT *IN VITRO* PLD ASSAY

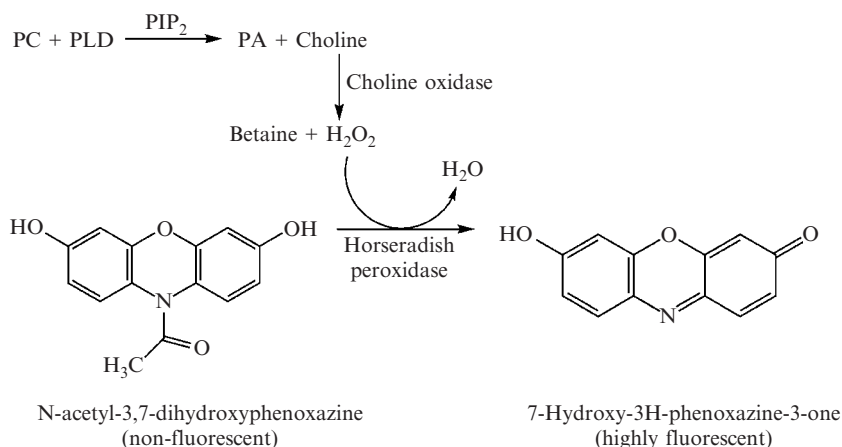
In an effort to develop nonradioactive methods of analysis of PLD-mediated PA production, a fluorescent *in vitro* assay was developed that utilizes commercially available reagents to measure PLD1.d311 activity.

These reagents, available as a kit from Invitrogen, can be used without modifications to measure PLD isoforms that do not require PIP_2 as a cofactor for PLD activity. We have modified the substrate and experimental conditions to adapt the assay for isoforms that do require PIP_2 for activity, such as PLD1.d311. The substrate in this modified assay consists of complex lipid vesicles with similar composition to that used in a traditional $[^3\text{H}]\text{PC}$ vesicle-based assay. This fluorescent assay effectively screens PLD1.d311 activity in the presence or absence of activators. While this does not replace radiolabeled methods of analysis for this or other PLD isoforms, it does provide a convenient alternative, especially in postpurification screening of fractions for activity. This allows for rapid detection of active protein, which, in the case of PLD, must be aliquoted and stored shortly after purification is complete. Unlike full-length PLD1, PLD1.d311 expresses at levels suitable for use in this fluorescent assay, which can be performed in 96-well plates.

In this enzymatically coupled assay, phospholipase D hydrolyzes PC in the presence of PIP_2 to yield PA and choline. Choline is then oxidized by choline oxidase to betaine and H_2O_2 . H_2O_2 , in the presence of HRP, oxidizes Amplex red in a 1:1 stoichiometry to generate fluorescent resorufin (7-hydroxy-3H-phenoxazin-3-one) (Scheme 4.1).

13.1. Preparation of lipid substrates

1. Prepare $1\times$ plus reaction buffer: 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 80 mM KCl, 6 mM MgCl_2 , and 6 mM CaCl_2 . Prepare $1\times$ reaction buffer as described earlier, without CaCl_2 and MgCl_2 .
2. Dry down lipids and prepare vesicles in $1\times$ reaction buffer at a final concentration of 100 μM PE (bovine liver), 6.2 μM PIP_2 (porcine brain),



Scheme 4.1 Fluorescent analysis of PLD activity.

1.4 μM cholesterol, and 10 μM dipalmitoyl PC (DpPC) (Avanti Polar Lipids) as described elsewhere (Henage *et al.*, 2006). Lipids may be aliquoted and stored under nitrogen in glass vials in chloroform (PE, cholesterol and DpPC); store PIP_2 in absolute ethanol. Lipids should be transferred using glass Hamilton syringes, as some plastic materials are not compatible with organic solvents.

3. Sonicate dried lipids for 3 min as described in Henage *et al.* (2006) in $1\times$ reaction buffer to concentrations listed previously and store at room temperature on a benchtop until ready to use.

13.2. Phospholipase D assay

1. For a 36-well assay run in a 96-well black plate for fluorescence, each well will contain 40 μl vesicles and 200 μl reaction mixture, for a total of 240 μl /well. This can be scaled as needed, but to no less than 120 μl total volume in the well.
2. Using the Amplex red phospholipase D assay kit, prepare stock solutions as follows. Amplex red stock: dissolve vial (0.26 mg) in 100 μl dimethyl sulfoxide (DMSO). This is enough for 100 assays and can be kept frozen at -20° in the dark. Protect solutions containing Amplex red from light before the assay is performed.
3. Immediately before use prepare stock 200 U/ml HRP by dissolving contents of HRP (component C) in 1.0 ml of $1\times$ plus reaction buffer (aliquot and freeze remaining at -20°).
4. Prepare 20 mM H_2O_2 (control).
5. Prepare 20 U/ml of choline oxidase by dissolving choline oxidase into plus $1\times$ reaction buffer. Aliquot remaining and store unused portion at -20° .
6. For a 36-well assay, prepare the working assay solution by combining 50 μl of Amplex red probe prepared earlier with 50 μl HRP, 50 μl choline oxidase, and 4.9 ml $1\times$ plus reaction buffer.
7. Keeping the plate on ice, add PLD1.d311 and any activators, and bring total volume to 80 μl with $1\times$ plus reagent buffer. It is important to note that because high concentrations of DTT cause spurious signals, DTT should be 1 mM or less in the assay. Also note that because this is a vesicle-based assay, detergents should be avoided, as they may disrupt vesicle structure.
8. Add 40 μl vesicles prepared earlier to each well.
9. Add 120 μl of working assay solution to each well, shake, or mix.
10. For reagent positive control, dilute 20 mM H_2O_2 to 10 μM in $1\times$ plus reaction buffer. This control gives an exceptionally high signal initially, which degrades quickly because of the nonenzymatic nature of the reaction. This control ensures the reagents are working appropriately (along with additional protein controls) and is used in place of PLD enzyme in the assay. BSA can be used as a negative control, and reagents alone are used for background measurement.

11. Incubate at 37° for 60 min or longer, taking readings at defined intervals with excitation at 530 to 560 nm and emission at 590 nm with a plate-based fluorescence reader. Adjust filters and sensitivity as required (Figs. 4.16 and 4.17).

14. REAL-TIME DIACYLGLYCEROL LIPASE ASSAY

Many conventional assays of lipid enzymes involve a time-delimited interaction between enzymes and substrates, followed by end point product analysis by TLC or LC/MS. These methods can be time-consuming and often lack sensitivity. Alternatives to lipid analysis with fluorescent methods are either indirect or require synthesis of substrates. In the current method, the use of commercially available starting materials combined with a simple, efficient enzymatic conversion to substrate results in a rapid method that allows measurement of DAG lipase activity in real time. DAG substrates with BODIPY groups on *sn*-1- and *sn*-2-acyl chains can be cleaved by lipoprotein lipase, demonstrating a robust signal resulting from this cleavage. The high degree of spectral overlap and high extinction coefficient for these fluorescent reporters allow detection of acyl chain hydrolysis with high sensitivity and can be measured in a convenient 96-well format.

Fluorescently labeled lipids can provide a sensitive tool to assay lipid-metabolizing enzymes and their activation kinetics (reviewed in Gupta *et al.*, 2003). The ability to synthesize labeled lipids with appropriate fluorescent groups is a prerequisite to probing lipid metabolism using fluorescence spectroscopy. In this example, fluorescent diacylglycerol analogs are generated and an assay is developed to investigate DAG lipase activity. This assay detects cleavage of acyl chains from DAG, shown here using bovine lipoprotein lipase (Fig. 4.18), after incorporation of labeled substrate into complex lipid vesicles. This approach will detect hydrolysis of acyl chains at either the *sn*-1 or the *sn*-2 position of the DAG backbone.

14.1. Synthesis of BD₂-DAG

Enzymatic synthesis of labeled DAG (BD₂DAG, 18b) from a commercially available fluorescently labeled PC molecule (BD₂PC, 18a) provides the DAG substrate needed for this assay. This synthesis relies on the ability of PC-specific phospholipase C (PLC) from *Bacillus cereus* to cleave the phosphodiester bond of PC to produce diacylglycerol (Scherer *et al.*, 2002). BD₂PC, 1, 2-bis(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine (Molecular Probes, WI) (50 μg) (Fig. 4.18A) is hydrolyzed to its DAG analog (BD₂DAG)(Fig. 4.18B) by treatment with PLC from *B. cereus* (0.1 mg) (Calbiochem, CA).

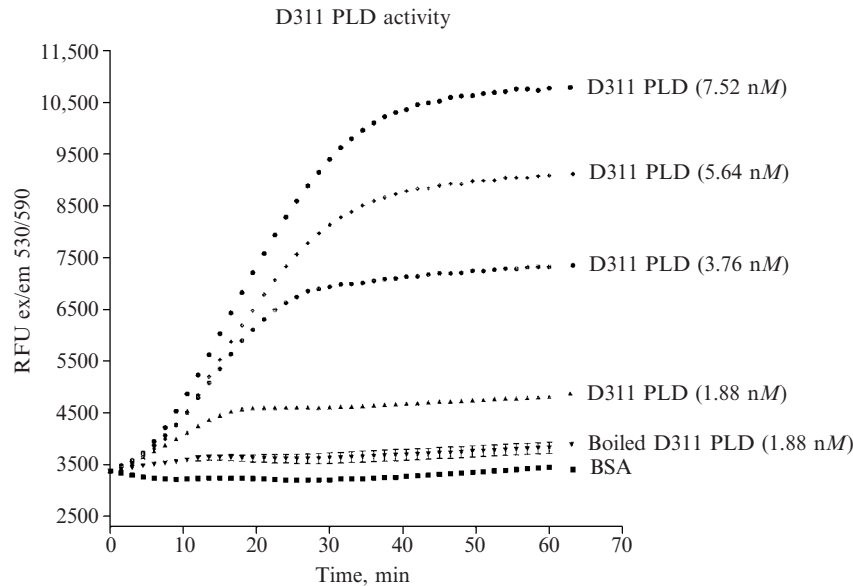


Figure 4.16 PLD1.d311 (D311 PLD) gives a dose-dependent increase in signal that is absent in samples containing boiled PLD or BSA alone, after subtraction of background in the absence of proteins. H_2O_2 gives a robust signal (off scale, not shown).

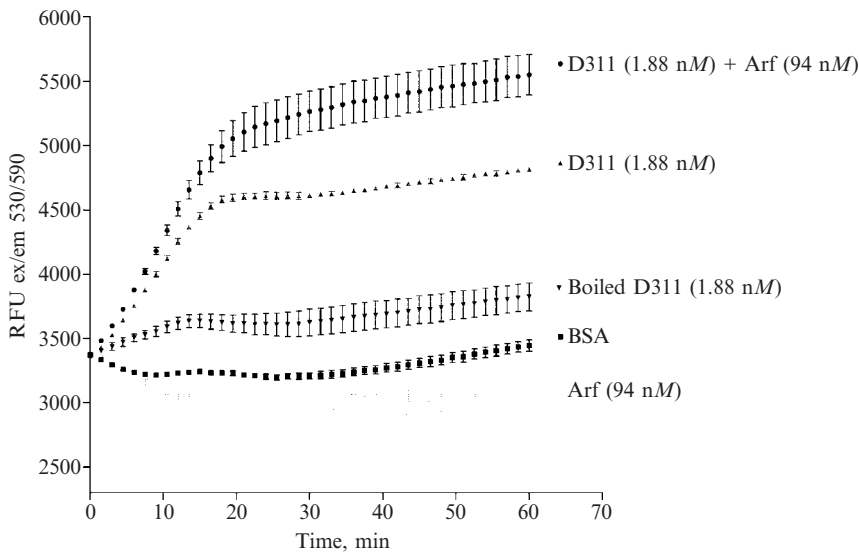


Figure 4.17 PLD1.d311 (D311 PLD) is stimulated by Arf, in contrast to the reaction in the presence of boiled PLD and Arf or in the presence of assay buffer alone.

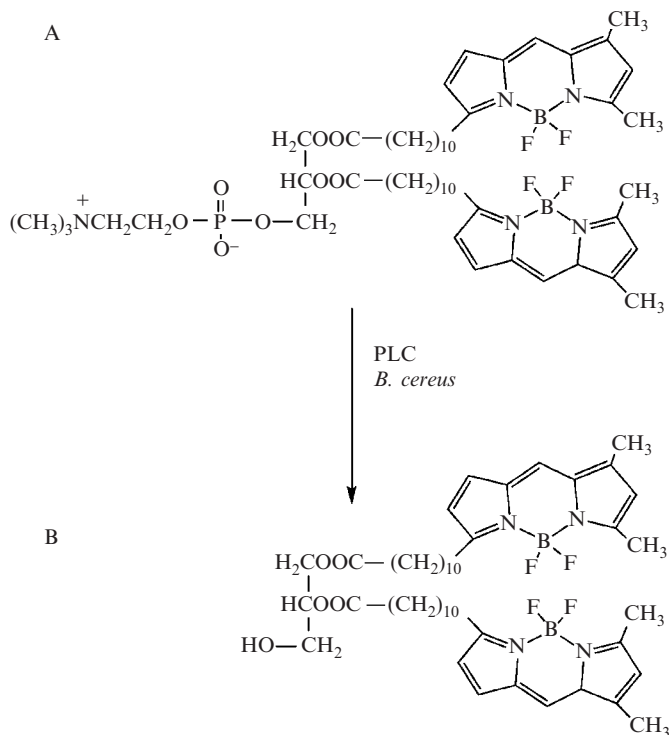


Figure 4.18 Synthesis of fluorescent DAG compounds. PLC from *B. cereus* cleaves BD₂-PC (A) to BD₂-DAG (B) with release of phosphocholine (not shown).

14.2. Protocol for BD₂DAG preparation

1. BD₂-PC (50 μg) in 10 μl DMSO is incubated at 30° with 0.1 mg PLC from *B. cereus* in 10 μl 20 mM Tris, 20 mM CaCl₂, pH 7.6, for 2 to 5 min as determined by disappearance of starting material and appearance of product on TLC as described later.
2. Organic extracts from the reaction are subjected to TLC on Watson K60F silica-coated plates, 2.5 $\times$ 7 cm, and visualized by ultraviolet (UV) irradiation.
3. Conversion from substrate (BD₂PC) to product (BD₂DAG) is confirmed by an increase in R_f for the fluorescent starting material after chromatography over silica in 4:1 CHCl₃:ethyl acetate. TLC is developed in 9:1:1 CHCl₃:CH₃OH:ethyl acetate.
4. When the reaction has gone to completion, evidenced by disappearance of starting material on TLC when visualized by UV irradiation, the reaction mixture is extracted and the organic layer is dried under nitrogen,

followed by storage at -20° . To check for completion, remove a small aliquot from the reaction mixture and extract into 4:1 CHCl_3 :ethyl acetate, applying a small amount of organic layer on the TLC plate (Fig. 4.19).

Fluorescence techniques are some of the most sensitive and convenient quantitative assays for real-time enzymatic analysis. For these studies, bovine lipoprotein lipase is used to hydrolyze DAG substrates.

14.3. Fluorescent assay for DAG-lipase activity

1. Vesicles are prepared by a 10-min sonication of L- α -phosphatidylcholine (Sigma-Aldrich) (0.3 mM) in 50 mM Tris, 50 mM NaCl, pH 7.5.
2. BD_2DAG (3 μM) is diluted 1:10 with PC vesicles and vortexed briefly.
3. Pipette 250 μl lipids containing BD_2DAG into wells. The reaction is incubated in a 96-well plate in the presence and absence of 0.1 μg

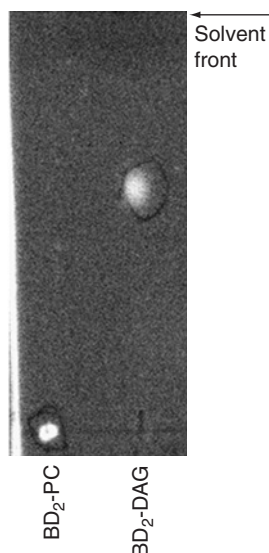


Figure 4.19 Substrate isolation. Aliquots of the reaction mixture were extracted into an equal volume of 4:1 CHCl_3 : CH_3OH , the aqueous layer was extracted again with 1 volume of CHCl_3 , and organic layers were combined. TLC analysis revealed substrate depletion, and conversion to product in the reaction mixture (evidenced by increase in R_f) was complete within 10 min. Organic layers extracted from the reaction mixture were dried under nitrogen gas and stored at -20°C , and a sample was subjected to mass spectrometry analysis (not shown). This analysis revealed that BD_2DAG was the major high molecular weight product from the reaction, with peaks corresponding to BD_2DAG (MW 863.5) and its sodium adduct (MW 898.5).

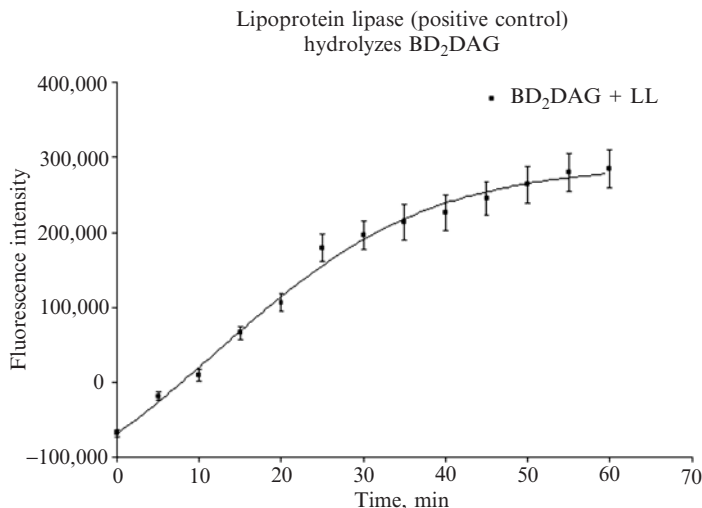


Figure 4.20 BD₂DAG in complex PC vesicles (soybean lipid, Sigma, St. Louis, MO) (Ruiz-Arguello *et al.*, 1998) and bovine lipoprotein lipase (Sigma) were incubated in a 96-well plate and fluorescence was measured at 5-min intervals over a 70-min period. The spectral overlap of this probe results in intramolecular self-quenching of fluorescence emission when the labeled DAG is intact; hydrolysis of the ester bond because of DAG lipase activity at the *sn*-1 or *sn*-2 position eliminates the interaction, resulting in increased fluorescence emission.

lipoprotein lipase (LL). Fluorescence emission is measured on a Perkin-Elmer Victor V multilabel plate reader with excitation/emission filters at 485 and 515 nm, respectively. Fluorescence intensity is expressed as the total emission less the average emission from vesicle control (prepared essentially the same as experimental samples in the absence of LL)(Fig. 4.20).

Fluorescent assays provide a convenient and robust measure of enzymatic activity without the drawbacks of radiolabeled assays and are an independent means to confirm activity from such labeled assays. The ability to measure activity at defined intervals allows for kinetic measurements in this direct, noncoupled assay of lipolytic activities.

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REFERENCES

- Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Bocchino, S. B., Wilson, P. B., and Exton, J. H. (1987). Ca^{2+} -mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS Lett.* **225**, 201–205.
- Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993). ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* **75**(6), 1137–1144.
- Brown, H. A., and Sternweis, P. C. (1995). Stimulation of phospholipase D by ADP-ribosylation factor. *Methods Enzymol.* **257**, 313–324.
- Buser, C. A., and McLaughlin, S. (1998). Ultracentrifugation technique for measuring the binding of peptides and proteins to sucrose-loaded phospholipid vesicles. *Methods Mol. Biol.* **84**, 267–281.
- Cai, S., and Exton, J. H. (2001). Determination of interaction sites of phospholipase D1 for RhoA. *Biochem. J.* **355**, 779–785.
- Carman, G. M., Deems, R. A., and Dennis, E. A. (1995). Lipid signaling enzymes and surface dilution kinetics. *J. Biol. Chem.* **270**, 18711–18714.
- Chalif-Caspi, V., Eli, Y., and Liskovitsch, M. (1998). Kinetic analysis in mixed micelles of partially purified rat brain phospholipase D activity and its activation by phosphatidylinositol 4,5-bisphosphate. *Neurochem. Res.* **23**(5), 589–599.
- Colley, W. C., Altshuler, Y. M., Sue-Ling, C. K., Copeland, N. G., Gilbert, D. J., Jenkins, N.A., Branch, K. D., Tsirka, S. E., Bollag, R. J., Bollag, W. B., and Frohman, M. A. (1997). Cloning and expression analysis of murine phospholipase D1. *Biochem. J.* **326**(3), 745–753.
- Du, G., Altschuller, Y. M., Kim, Y., Han, J. M., Ryu, S. H., Morris, A. J., and Frohman, M. A. (2000). Dual requirement for Rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. *Mol. Biol. Cell.* **11**(12), 4359–4368.
- Du, G., Altschuller, Y. M., Vitale, N., Huang, P., Chasserot-Golaz, S., Morris, A. J., Bader, M. F., and Frohman, M. A. (2003). Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. *J. Cell Biol.* **162**, 305–315.
- Exton, J. H. (2002). Phospholipase D: Structure, regulation and function. *Rev. Physiol. Biochem. Pharmacol.* **144**, 1–94.
- Gupta, R., Rath, P., Gupta, N., and Bradoo, S. (2003). Lipase assays for conventional and molecular screening: An overview. *Biotechnol. Appl. Biochem.* **37**, 63–71.
- Hajra, A. K., and Agranoff, B. W. (1998). Acyl dihydroxyacetone phosphate. *J. Biol. Chem.* **273**(7), 1617–1622.
- Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q. M., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. (1997). Characterization of two alternately spliced forms of phospholipase D1: Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C α . *J. Biol. Chem.* **272**, 3860–3868.
- Henage, L. G., Exton, J. H., and Brown, H. A. (2006). Kinetic analysis of a mammalian phospholipase D: Allosteric modulation by monomeric GTPases, protein kinase C, and polyphosphoinositides. *J. Biol. Chem.* **281**, 3408–3417.
- Hjelmeland, L. M. (1990). Removal of detergents from membrane proteins. *Methods Enzymol.* **182**, 277–282.
- Hoer, A., Cetindag, C., and Oberdisse, E. (2000). Influence of phosphatidylinositol 4,5-bisphosphate on human phospholipase D1 wild-type and deletion mutants: Is there

- evidence for an interaction of phosphatidylinositol 4,5-bisphosphate with the putative Pleckstrin homology domain? *Biochim. Biophys. Acta* **1481**, 189–201.
- Hurley, J. H., and Misra, S. (2000). Signaling and subcellular targeting by membrane-binding domains. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 49–79.
- IUPAC-IUBMB (1982). Nomenclature Committee of the International Union of Biochemistry (NC-IUB). Symbolism and terminology in enzyme kinetics. Recommendations 1981. *Eur. J. Biochem.* **128**, 281–291.
- Ivanova, P. T., Milne, S. B., Byrne, M. O., Xiang, Y., and Brown, H. A. (2007). Glycerophospholipid identification and quantitation by electrospray ionization mass spectrometry. *Methods Enzymol.* **432**, 21–59.
- Jiang, X., Gutowski, S., Singer, W. D., and Sternweis, P. C. (2002). Assays and characterization of mammalian phosphatidylinositol 4,5-bisphosphate-sensitive phospholipase D. *Methods Enzymol.* **345**, 328–334.
- Kook, S., and Exton, J. H. (2005). Identification of interaction sites of protein kinase C α on phospholipase D1. *Cell Signal.* **17**(11), 1423–1432.
- Koonin, E. V. (1996). A duplicated catalytic motif in a new superfamily of phosphohydrolases and phospholipid synthases that includes poxvirus envelope proteins. *Trends Biochem. Sci.* **21**, 242–243.
- Martin, A., Gomez-Munoz, A., Jamal, Z., and Brindley, D. N. (1991). Characterization and assay of phosphatidate phosphatase. *Methods Enzymol.* **197**, 553–556.
- Nakamura, S. I., Kiyohara, Y., Jinnai, H., Hitomi, T., Ogino, C., Yoshida, K., and Nishizuka, Y. (1996). Mammalian phospholipase D: Phosphatidylethanolamine as an essential component. *Proc. Natl. Acad. Sci. USA* **93**, 4300–4304.
- Park, S. K., Min, D. S., and Exton, J. H. (1998). Definition of the protein kinase C interaction site of phospholipase D. *Biochem. Biophys. Res. Commun.* **244**(2), 364–367.
- Park, S. K., Provost, J. J., Bae, C. D., Ho, W. T., and Exton, J. H. (1997). Cloning and characterization of phospholipase D from rat brain. *J. Biol. Chem.* **272**, 29263–29271.
- Ponting, C. P., and Kerr, I. D. (1996). A novel family of phospholipase D homologues that includes phospholipid synthases and putative endonucleases: Identification of duplicated repeats and potential active sites residues. *Protein Sci.* **5**(5), 914–922.
- Qin, C., Wang, C., and Wang, S. (2002). Kinetic analysis of Arabidopsis phospholipase Ddelta: Substrate preference and mechanism of activation by Ca^{2+} and phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **277**, 49685–49690.
- Ruiz-Arguello, M. B., Goni, F. M., and Alonso, A. (1998). Phospholipase C hydrolysis of phospholipids in bilayers of mixed lipid compositions. *Biochemistry* **37**, 11621–11628.
- Scherer, G. F., Paul, R. U., Holk, A., and Martinec, J. (2002). Down-regulation by elicitors of phosphatidylcholine-hydrolyzing phospholipase C and up-regulation of phospholipase A in plant cells. *Biochem. Biophys. Res. Commun.* **293**, 766–770.
- Sciorra, V. A., Hammond, S. M., and Morris, A. J. (2001). Potent direct inhibition of mammalian phospholipase D isoenzymes by calphostin-C. *Biochemistry* **40**, 2640–2646.
- Sciorra, V. A., Rudge, S. A., Prestwich, G. D., Frohman, M. A., Engelbrecht, J., and Morris, A. J. (1999). Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. *EMBO J.* **18**, 5911–5921.
- Sugars, J. M., Celtek, S., Manifava, M., Coadwell, J., and Ktistakis, N. T. (1999). Fatty acylation of phospholipase D1 on cysteine residues 240 and 241 determines localization on intracellular membranes. *J. Biol. Chem.* **274**, 30023–30027.
- Sung, T. C., Zhang, Y., Morris, A. J., and Frohman, M. A. (1999). Structural analysis of human phospholipase D1. *J. Biol. Chem.* **274**, 3659–3666.
- Walker, S. J., and Brown, H. A. (2002). Specificity of Rho insert-mediated activation of phospholipase D1. *J. Biol. Chem.* **277**, 26260–26267.
- Walker, S. J., and Brown, H. A. (2004). Measurement of G protein-coupled receptor-stimulated phospholipase D activity in intact cells. *Methods Mol. Biol.* **237**, 89–97.

- Walker, S. J., Wu, W. J., Cerione, R. A., and Brown, H. A. (2000). Activation of phospholipase D1 by Cdc42 requires the Rho insert region. *J. Biol. Chem.* **275**, 15665–15668.
- Xie, Z., Ho, W. T., and Exton, J. H. (2000). Conserved amino acids at the C-terminus of rat phospholipase D1 are essential for enzymatic activity. *Eur. J. Biochem.* **267**, 7138–7146.
- Xie, Z., Ho, W. T., and Exton, J. H. (2002). Functional implications of post-translational modifications of phospholipases D1. *Biochim. Biophys. Acta* **1580**(1), 9–21.
- Yang, H., and Roberts, M. F. (2003). Phosphohydrolase and transphosphatidylation reactions of two *Streptomyces* phospholipase D enzymes: Covalent versus noncovalent catalysis. *Protein Sci.* **12**, 2087–2098.
- Zheng, L., Shan, J., Krishnamoorthi, R., and Wang, X. (2002). Activation of plant phospholipase D β by phosphatidylinositol 4,5-bisphosphate: Characterization of binding site and mode of action. *Biochemistry* **41**, 4546–4553.

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MEASUREMENT OF AUTOTAXIN/ LYSOPHOSPHOLIPASE D ACTIVITY

Andrew J. Morris *and* Susan S. Smyth

Contents

1. Introduction	90
2. Overview of Methods for Determination of Autotaxin/LysoPLD Activity	93
3. Expression of V5-Tagged Autotaxin/LysoPLD in HEK293 Cells	94
4. Measurement of Autotaxin/LysoPLD Activity Using Radiolabeled Substrates	95
4.1. Source of reagents	95
4.2. Preparation of substrate	96
4.3. Assay buffer	96
4.4. Assay composition and incubation	96
4.5. Assay termination and product analysis	96
4.6. Kinetic analysis of recombinant autotaxin/lysoPLD using [¹⁴ C]lysoPC substrate	97
5. Measurement of Autotaxin/LysoPLD Activity Using Fluorogenic Substrates	98
5.1. Source of reagents and supplies	99
5.2. Preparation of substrate	100
5.3. Assay composition and incubation	100
5.4. Kinetic analysis of autotaxin/lysoPLD using FS-3	100
6. Concluding Comments	100
Acknowledgment	102
References	102

Abstract

Lysophosphatidic acid (LPA) is a bioactive lipid mediator present in the blood and other biological fluids at physiologically relevant concentrations. In the cardiovascular system, studies using *in vitro* and *in vivo* experimental models indicate that LPA stimulates platelet activation, differentiation and migration of

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vascular smooth muscle cells, and changes in vascular tone. A growing body of evidence suggests that aberrant production and actions of LPA could play an important role in atherothrombotic disease. Hydrolysis of lysophospholipids by the secreted plasma protein autotaxin/lysophospholipase D (lysoPLD) is a major mechanism for generation of LPA in the blood. This chapter describes methods for determining the activity of recombinant autotaxin/lysoPLD using radiolabeled and fluorogenic substrates.

1. INTRODUCTION

Lysophosphatidic acid (1-acyl 2 hydroxy glycerol 3-phosphate or LPA) is a bioactive phospholipid mediator that functions as an activator of cell surface (and possibly also intracellular) receptors to mediate a wide variety of cellular responses that include stimulation of cell growth, differentiation and alterations in cellular morphology, and motility (Moolenaar *et al.*, 2004). The best-characterized mechanisms for cell regulation by LPA involve activation of G-protein-coupled cell surface receptors, which include the widely studied LPA1, 2, and 3 receptors, as well as the more recently identified, structurally distinct, and presently less well-characterized LPA4 and LPA5 receptors (Ishii *et al.*, 2004; Lee *et al.*, 2006; Yanagida *et al.*, 2007). Studies using pharmacological agonists and antagonists of LPA receptors and inactivation of some of these LPA receptor genes in mice have identified physiological and pathophysiological roles for LPA signaling in tumor metastasis, cardiovascular regulation, reproduction, and neuropathic pain (Chun, 2005; Mills and Moolenaar, 2003; Siess and Tigyi, 2004). Understanding the role of LPA in cardiovascular physiology and pathophysiology is of particular interest because LPA is present in the blood at physiologically relevant levels and may be increased during platelet activation (Aoki, 2004). In experimental models, LPA regulates vascular tone and is a potent attractant and stimulus for inflammatory cells, including platelets, neutrophils, and monocytes. Because LPA accumulates in the oxidized low-density lipoprotein component of atherosclerotic plaques, it is attractive to speculate that release of LPA could play a role in platelet thrombus formation that follows plaque rupture, thereby contributing to acute myocardial infarction (Haseruck *et al.*, 2004).

Synthesis of LPA in biological systems has been suggested to be accomplished by at least three distinct pathways. The first of these involves acylation of glycerol 3-phosphate catalyzed by a fatty acyl coA-dependent acyltransferase. This pathway is clearly important for *de novo* synthesis of glycerophospholipids and triglyceride, but its role in the production of extracellular LPA is presently unclear. Second (Coleman, 2007), LPA can be formed through deacylation of phosphatidic acid by a selective phospholipase A₂ activity. Although enzymes that can catalyze this reaction *in vitro* have been reported,

their physiological role in the generation of LPA remains to be established (Aoki, 2004). The final and presently best-understood mechanism for formation of receptor-active LPA involves hydrolysis of lysophospholipids by lysophospholipase D (lysoPLD) (Fig. 5.1B). Activities of this type have been reported in mammalian cells and tissues, including the blood where the major lysoPLD responsible for the generation of LPA has been identified as a previously studied tumor cell-derived cytokine called autotaxin, hereafter referred to as autotaxin/lysoPLD (Umez-Goto *et al.*, 2002). Autotaxin/lysoPLD is a member of a family of enzymes broadly categorized as nucleotide pyrophosphatase/phosphodiesterases (NPPs) (Bollen *et al.*, 2000). These are cell surface ecto or, in some cases, secreted or glycosylphosphatidylinositol-anchored enzymes that exhibit a broad ability to catalyze indiscriminate hydrolysis of the phosphodiester bonds in a range of nucleotides and nucleotide derivatives. In this nomenclature, autotaxin/lysoPLD is termed NPP2 and there are five additional NPP family members. The domain structure of autotaxin/lysoPLD is shown in Fig. 5.1A. It is a single polypeptide with an N-terminal putative transmembrane domain, which may also function as a

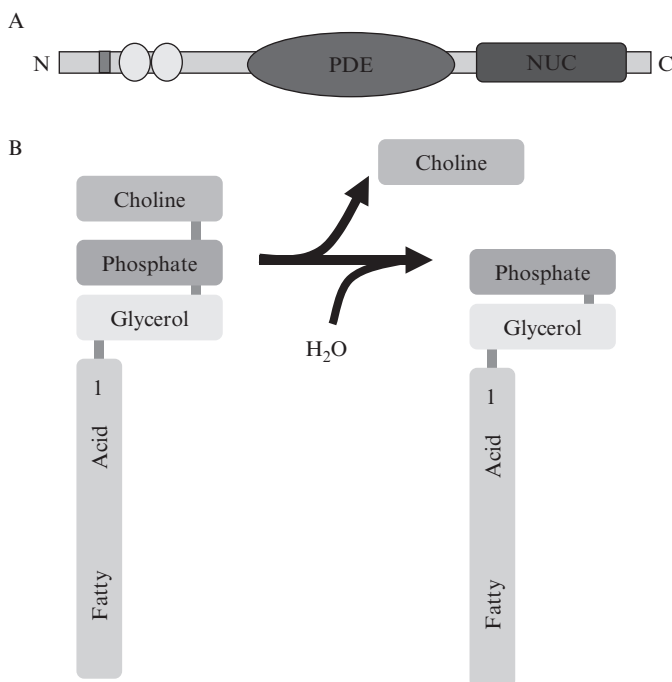


Figure 5.1 (A) Domain structure of autotaxin/lysoPLD. The putative transmembrane domain is shown in green, the somatomedin-like domains in yellow, and the phosphodiesterase (PDE) and nuclease (Nuc) domains are also indicated. (B) Reaction catalyzed by lysoPLD.

signal sequence specifying secretion of the protein (Jansen *et al.*, 2005). The N terminus also contains two somatomedin-like domains that have been suggested to be involved in dimerization, whereas the remainder of the protein contains sequence domains with homology to nucleases and phosphodiesterases, the latter of which is clearly involved in catalysis. Autotaxin/lysoPLD was originally isolated from conditioned medium of melanoma cells and shown to increase the motility of these and other cell types (Stracke *et al.*, 1992). In subsequent studies, overexpression of autotaxin/lysoPLD increased the metastatic potential of fibroblasts and promoted tumor angiogenesis in a xenograft model (Stracke *et al.*, 1993). Although autotaxin/lysoPLD is overexpressed in several cancers, which may indicate a pathophysiological role in tumorigenesis, other studies suggest that this enzyme plays a physiological function in adipogenesis, neurogenesis, and cardiovascular development and function (Savaskan *et al.*, 2007; Simon *et al.*, 2005; van Meeteren *et al.*, 2006). Most strikingly, inactivation of the autotaxin/lysoPLD gene in mice results in an early embryonic lethality characterized by a failure of neural crest formation and vascular defects identifying a critical role for the enzyme in early development of the cardiovascular and nervous systems (Tanaka *et al.*, 2006; van Meeteren *et al.*, 2006).

When first discovered, the potent effects of autotaxin/lysoPLD on cell growth and motility were perplexing because, although these effects were clearly dependent on the integrity of the phosphodiesterase catalytic site, they could not be readily explained by hydrolysis of the known nucleotide substrates of the enzyme (Clair *et al.*, 1997; MacDonald *et al.*, 1996). The discovery that autotaxin/lysoPLD could effectively hydrolyze lysophosphatidylcholine (lysoPC) to generate LPA provided a critical insight into the biological actions of the enzyme (Tokumura *et al.*, 2002; Umezū-Goto *et al.*, 2002). It is now clear that the effects of autotaxin/lysoPLD, at least on cells in culture, can be largely explained by the production of LPA because these effects are often enhanced by supplementation of culture medium with lysoPC and can be attenuated by genetic inactivation or pharmacological antagonism of LPA receptors (Hama *et al.*, 2004). *In vitro*, autotaxin/lysoPLD can also hydrolyze sphingosylphosphorylcholine (SPC) to generate sphingosine 1-phosphate (S1P), which is another bioactive lipid with a spectrum of signaling activities broadly similar to those of LPA (Clair *et al.*, 2003). Whereas lysoPC is present in the blood at concentrations well in excess of the measured K_m of autotaxin/lysoPLD for this substrate, levels of SPC are considerably lower, suggesting that this may not be a relevant substrate *in vivo*. Mice that are heterozygous for the wild-type and null allele of the autotaxin gene exhibit no obvious developmental phenotype but have reduced plasma levels of LPA (but not S1P) (van Meeteren *et al.*, 2006). This important finding implies that autotaxin/lysoPLD is a key component of a physiologically relevant pathway for the production of LPA in the blood and also suggests that, because levels of the lysoPC substrate are well

above the measured K_m of the enzyme, modulation of plasma levels of autotaxin/lysoPLD may be an important mechanism for the regulation of circulating levels of LPA.

Autotaxin/lysoPLD belongs to a family of six NPP enzymes. Although all of these enzymes can hydrolyze nucleotide phosphate substrates, only autotaxin/lysoPLD has activity against phospho- and sphingolipid substrates (Cimpean *et al.*, 2004). The most recently characterized member of this gene family, NPP6, exhibits phospholipase C activity against lysoPC *in vitro* (Sakagami *et al.*, 2005). However, this enzyme is not present in the blood and the biological relevance of its phospholipase activity remains undefined. Levels of the primary autotaxin/lysoPLD substrate in the blood, lysoPC, are high ($>100 \mu M$), which is close to and possibly exceeds the measured K_m of the enzyme for this substrate. However, circulating levels of LPA are much lower (generally in the range of 0.05 to $0.1 \mu M$). Although LPA is known to be actively dephosphorylated by cell surface phosphatases present in vascular cells, this finding has also been suggested to indicate that the activity of autotaxin/lysoPLD is suppressed in the blood. One mechanism for this effect may involve product inhibition of autotaxin/lysoPLD by LPA, which is a potent mixed (i.e., both competitive and noncompetitive) inhibitor of the hydrolysis of lysoPC by autotaxin/lysoPLD *in vitro* (van Meeteren *et al.*, 2005). This finding suggests the interesting possibility that LPA regulates its own biosynthesis through feedback inhibition of autotaxin/lysoPLD. This discovery also suggests an approach to develop small molecule inhibitors of autotaxin/lysoPLD, and it is possible that this approach may provide a unique avenue for the development of drugs to inhibit LPA production in the blood and possibly other settings (Baker *et al.*, 2006).

2. OVERVIEW OF METHODS FOR DETERMINATION OF AUTOTAXIN/LYSOPLD ACTIVITY

Because of the now clear importance of autotaxin/lysoPLD as a biologically relevant mechanism for the production of LPA in the blood and possibly other tissues and the well-established involvement of LPA in important physiological and pathophysiological processes, there has been considerable interest in the development of simple ways to detect and quantitate autotaxin/lysoPLD activity. These assays could be used to identify novel small molecule modulators of autotaxin/lysoPLD activity or to investigate how levels of the enzyme vary in various physiological and pathophysiological settings. In particular, a robust and reliable assay to detect and quantitate autotaxin/lysoPLD in the blood could be used to characterize animal models in which autotaxin/lysoPLD expression levels have been modulated or to investigate how levels of the enzyme are altered in disease

states where LPA could play an important role. The simplest way to measure the phosphodiesterase activity of autotaxin/lysoPLD is to use the chromogenic substrate *p*-nitrophenol thymidine 5'-monophosphate or even the simpler substrate bis(paranitrophenyl)phosphate (bis-pNPP) (Clair *et al.*, 1997; Gijbsers *et al.*, 2003). The obvious drawback of these substrates is that neither is specific for autotaxin/lysoPLD—they are readily hydrolyzed by other NPP family members and quite probably by other less specific phosphodiesterases, which makes them inappropriate for measurements of autotaxin/lysoPLD activity in anything other than experiments using purified preparations of the enzyme.

Several groups have described a coupled enzymatic assay for LPA that can be used to measure autotaxin/lysoPLD activity *in vitro*. In this assay, LPA is hydrolyzed by a specific lysophospholipase to generate glycerol 3-phosphate, which is then quantitated by an enzyme-coupled cycling reaction using glycerol 3-phosphate oxidase and glycerol 3-phosphate dehydrogenase with detection of hydrogen peroxide using colorimetric or fluorogenic reagents (Nakamura *et al.*, 2007; Tokumura *et al.*, 2007). The specificity of this assay is critically dependent on the selectivity of the lysophospholipase for LPA. Unfortunately, because the preparation of enzyme used is isolated from a microbiological source and has not been consistently available commercially, although clearly an effective way to measure both autotaxin/lysoPLD activity and levels of LPA in biological samples, this assay has not been widely adopted. Quantitation of LPA produced by autotaxin/lysoPLD *in vitro* can also be accomplished using liquid or gas chromatography/tandem mass spectrometry procedures, which have been developed for direct quantitation of LPA levels in biological samples (Georas *et al.*, 2007; Sutphen *et al.*, 2004). The equipment required is generally unavailable to most laboratories, and this type of assay is not readily suitable for analysis of large numbers of samples.

The following paragraphs describe radiochemical and fluorogenic substrate assays that can be used to quantitate autotaxin/lysoPLD activity in biological samples. The assays have been characterized using a recombinant autotaxin/lysoPLD preparation. We describe how to generate this preparation of the enzyme for use as an internal control when measuring autotaxin/lysoPLD activity in unknown samples.

3. EXPRESSION OF V5-TAGGED AUTOTAXIN/LYSOPLD IN HEK293 CELLS

Autotaxin/lysoPLD is expressed in HEK293 cells using a CMV-promoter vector (pcDNA—Invitrogen). The protein contains a C-terminal V5 tag to facilitate detection. To generate recombinant autotaxin/lysoPLD,

100-mm-diameter dishes of $\approx 50\%$ confluent cells are grown in complete DMEM containing 10% fetal bovine serum. The cells are transfected with $\sim 10 \mu\text{g}$ of pcDNA V5-autotaxin using lipofectamine and OPTI-MEM serum-free medium (Invitrogen). After 6 h the transfection medium is removed and replaced with complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. Because fetal bovine serum contains measurable levels of lysoPLD activity, to avoid interference with the recombinantly expressed enzyme, cells are washed twice in serum-free medium after a further 6 h and then cultured in serum-free medium for another 24 to 36 h. Western blotting experiments as shown in Fig. 5.2A reveal that most of the recombinant autotaxin/lysoPLD accumulates extracellularly, which may be a result of proteolytic cleavage close to the luminal side of the putative N-terminal transmembrane domain or, as has also been recently demonstrated, a result of this domain functioning as a signal sequence that specifies secretion of the protein. Note that the protein is glycosylated, which modestly decreases its electrophoretic mobility. Conditioned medium from these cells is used as a source of autotaxin/lysoPLD as described in the following assays.

4. MEASUREMENT OF AUTOTAXIN/LYSOPLD ACTIVITY USING RADIOLABELED SUBSTRATES

The simplest and most direct way to measure autotaxin/lysoPLD activity is to incubate preparations of the enzyme with radioactive lysoPC and monitor formation of LPA (Umezu-Goto *et al.*, 2002; van Meeteren *et al.*, 2006). To measure autotaxin/lysoPLD activity, this radiolabeled substrate is complexed with fatty acid-free bovine serum albumin (BSA) and incubated with the preparation of enzyme in a suitable assay buffer. Incubations are terminated by acidification and lipids are extracted with butanol before separation of radioactive product from unreacted substrate by thin-layer chromatography.

4.1. Source of reagents

Lyso palmitoyl phosphatidylcholine ($1[1-^{14}\text{C}]$ palmitoyl) is obtained from Perkin Elmer Life Sciences (specific radioactivity 40–60 mCi/mmol in 1:1 toluene ethanol). Unlabeled palmitoyl lysophosphatidylcholine and palmitoyl lysophosphatidic acid are obtained from Avanti Polar Lipids as CHCl_3 solutions. Fatty acid-free BSA is obtained from Sigma Aldrich. All other reagents used in this assay can be obtained from a variety of standard commercial sources.

4.2. Preparation of substrate

Radiolabeled lysoPC ($\approx 10\text{--}100,000$ dpm/assay) is combined with unlabeled lysoPC in sufficient quantities to give final assay concentrations in the range of 1 to 500 μM , and organic solvents are removed by evaporation. For standard measurements we use a substrate concentration of 1–10 μM . The lipids are resuspended in 10 mM Tris, pH 8.0, containing 1 mg/ml fatty acid-free BSA at a total lipid concentration of $10\times$ the final assay concentration by brief (≈ 30 s) bath sonication and repeated vortexing. Efficient resuspension of the radiolabeled substrate can be monitored by liquid scintillation counting.

4.3. Assay buffer

The assay buffer is prepared at $5\times$ final concentration. Final concentrations in the assay are 140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 50 mM Tris, pH 8.0.

4.4. Assay composition and incubation

The total assay volume is 100 μl comprising 20 μl $5\times$ assay buffer and 10 μl of $10\times$ radiolabeled substrate, and the remaining assay volume is composed of water or the source of enzyme. Assays can be conveniently conducted in 1.5-ml microcentrifuge tubes. Incubations are performed in a water bath at 37° for times up to 3 h.

4.5. Assay termination and product analysis

Assays are terminated by the addition of 20 μl of 0.2 M acetic acid and 55 μl of *n*-butanol. After thorough mixing and centrifugation the butanol phase is removed and the remaining water phase is extracted with an additional 55 ml of *n*-butanol. The pooled butanol phases are dried under vacuum. Reaction products are analyzed by thin-layer chromatography on silica gel plates (Whatman K-6), which are developed in a solvent system containing $\text{CHCl}_3/\text{MeOH}/\text{conc. acetic acid}/\text{H}_2\text{O}$ (50:30:8:4). The approximate R_f values in this solvent system for the LPA product of the enzyme and the lysoPC substrate are 0.9 and 0.25, respectively. Product and unreacted substrate can be visualized by autoradiography. Alternatively, reaction products can be “spiked” with ≈ 1 μg of palmitoyl LPA before drying and this can then be visualized by staining the plates with iodine vapor. For precise quantitation, the LPA and unreacted lysoPC can be excised from the plate by scraping and associated radioactivity quantitated by liquid scintillation counting.

4.6. Kinetic analysis of recombinant autotaxin/lysoPLD using [^{14}C]lysoPC substrate

As an example, Fig. 5.2B shows the substantial increase in lysoPLD activity in the culture medium of cells expressing V5-autotaxin/lysoPLD and data from an experiment in which the effect of substrate concentration on the activity of recombinant autotaxin/lysoPLD in medium conditioned by these cells was determined. The substrate concentration was varied in the range 10 to 500 μM and initial rates of autotaxin/lysoPLD activity monitored. In this

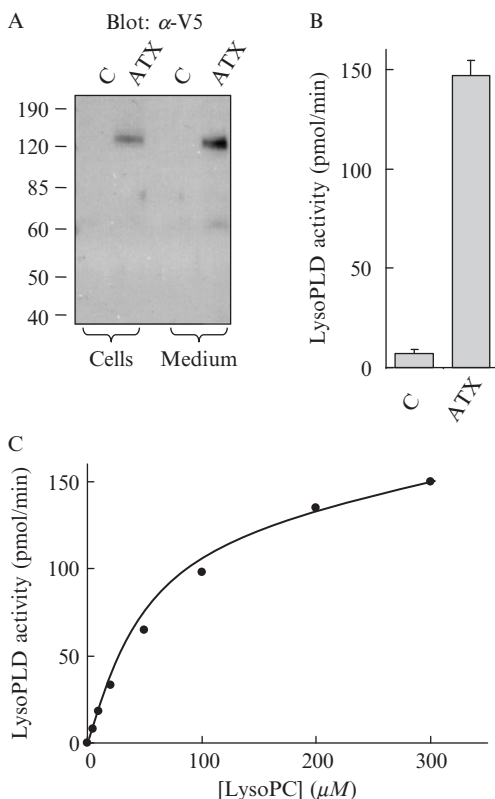


Figure 5.2 (A) HEK293 cells were transfected with a vector for expression of V5-tagged autotaxin (ATX) or a control vector (C), and proteins present in a whole cell fraction or culture medium were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. Note that in this experiment the material loaded on the gel represents 20% of the whole cell lysate but only 0.2% of the culture medium. (B) LysoPLD activity was determined in culture medium from control cells (c) or cells expressing V5-autotaxin (ATX). (C) Autotaxin/lysoPLD activity was determined at the indicated substrate concentrations using conditioned medium from V5-autotaxin expressing cells as the source of enzyme and radiolabeled lysoPC as substrate.

experiment the apparent K_m of autotaxin/lysoPLD for lysoPC was $\approx 100 \mu M$, which is consistent with previous reports (Gijlsbers *et al.*, 2003; van Meeteren *et al.*, 2006).

5. MEASUREMENT OF AUTOTAXIN/LYSOPLD ACTIVITY USING FLUOROGENIC SUBSTRATES

The development of fluorogenic substrates for autotaxin/lysoPLD offers an attractive strategy for a simple and potentially specific way to determine enzyme activity rapidly. CPF4 is a fluorescence resonance energy transfer (FRET)-based sensor of phosphodiesterase activity. CPF4 is a derivative of bisPNP in which the phenyl groups are conjugated to coumarin and fluorescein. Unreacted CPF4 exhibits a robust FRET signal, which is abolished when the substrate is hydrolyzed. Autotaxin/lysoPLD hydrolyzes CPF4 with high affinity ($K_m \approx 4 \mu M$) and a V_{max} that is only modestly lower than observed with lipid substrates. Interestingly, the K_m of autotaxin/lysoPLD for CPF4 is considerably lower than the K_m of the enzyme for bisPNP and moderately lower than the K_m for lysoPC, suggesting that the addition of fluorescent moieties to bisPNP significantly increases the affinity of this substrate for the enzyme (van Meeteren *et al.*, 2005). CPF4 is clearly a very useful substrate for enzymatic analysis of purified autotaxin/lysoPLD and could also be used to detect autotaxin/lysoPLD activity in serum-free conditioned culture medium isolated from cells expressing the enzyme. However, the usefulness of CPF4 for the determination of autotaxin/lysoPLD activity in biological fluids such as blood, which likely contain substrate-binding proteins and competing phosphodiesterase activities, remains to be determined.

FS-3 is a “second-generation” fluorogenic autotaxin/lysoPLD substrate. The molecule consists of an ethanoamido head group linked to a dabcyI “quencher” via the amino group. The hydroxyl group of the head group is conjugated to fluorescein via a phosphodiester bond and a polyethylene glycol linker. FS-3 is therefore a more “lipid-like” substrate than CP4. The rationale for design of this substrate is conceptually similar to that used to generate fluorogenic substrates for assays of a variety of other enzymes, including ceramidase, DNA ligase, and phospholipase A₂. In the unreacted substrate the fluorescein reporter is “silent” because of intramolecular FRET to the nonfluorescing quencher. Hydrolysis of the substrate by autotaxin/lysoPLD separates the dabcyI quencher from the fluorescein reporter, which becomes fluorescent. Figure 5.3A shows a schematic diagram of the hydrolysis of FS-3 by autotaxin/lysoPLD (Ferguson *et al.*, 2006). We later describe a method for the use of FS-3 to measure autotaxin/lysoPLD activity in conditioned medium from HEK293 cells expressing V5-autotaxin.

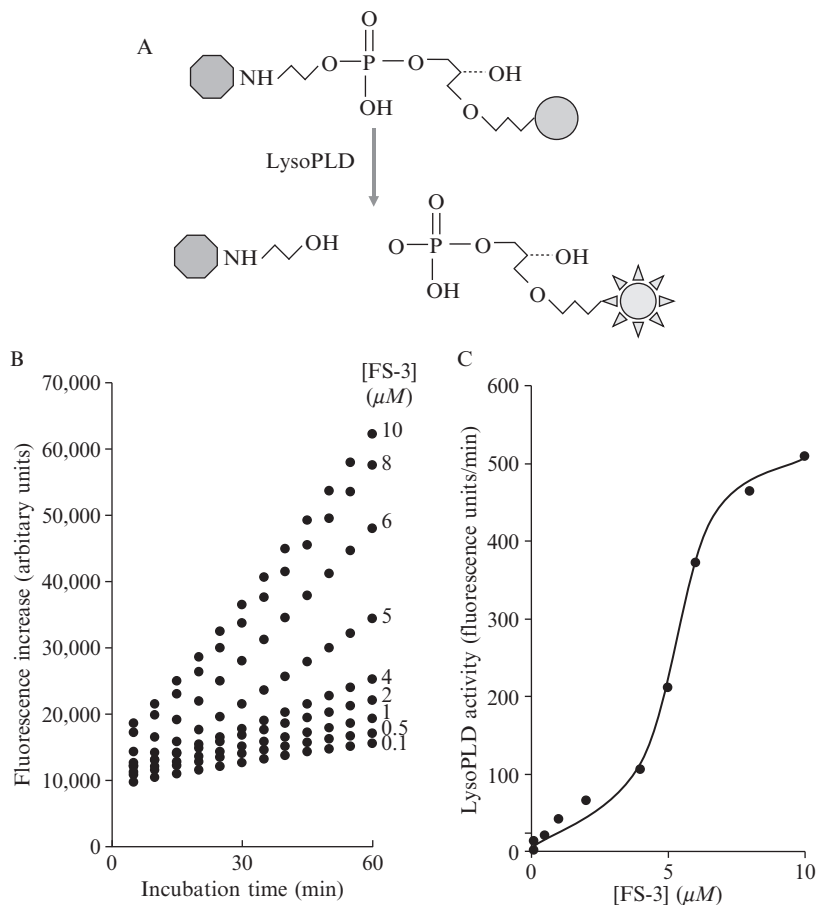


Figure 5.3 (A) Schematic of the structure of FS-3 and its hydrolysis by autotaxin/lysoPLD (adapted from Ferguson *et al.*, 2006). (B) The indicated concentrations of FS-3 were incubated with conditioned medium from V5-autotaxin expressing cells, and increases in FS-3 fluorescence were determined at the indicated times as described in the text. (C) Autotaxin/lysoPLD activity was determined as a function of increasing concentrations of FS-3 substrate.

5.1. Source of reagents and supplies

Assays are conducted in black 96-well microtiter plates (Fisher). FS-3 is from Echelon Biosciences, Inc., Salt Lake City, UT. Incubations are conducted in and monitored by a Biotek FLX-800 plate reading fluorimeter/shaker/incubator equipped with a 485/20-nm excitation filter and a 528/20-nm emission filter. Instrument control, data acquisition, and analysis are performed using KC-4 software obtained from the same manufacturer.

5.2. Preparation of substrate

FS-3 is obtained as a lyophilized solid. The substrate is soluble in water up to 5 mg/ml. A 50–100 μM stock solution of substrate is prepared in water and stored in aliquots in the dark at -20° .

5.3. Assay composition and incubation

Assays are conducted in a final volume of 100 μl comprising 20 μl 5 $\times$ assay buffer (prepared exactly as described in the preceding section) and 10 to 20 μl of FS-3 substrate to give a final concentration of 5 to 10 μM , and the remaining volume is taken up by water or the source of enzyme. Incubations are at 37° with intermittent shaking with the plate reader set to make fluorescence measurements every 5 to 10 min. Autotaxin/lysoPLD activity is quantitated by measuring the rate of increase in fluorescence at 528 nm with excitation at 485 nm.

5.4. Kinetic analysis of autotaxin/lysoPLD using FS-3

As an example, Fig. 5.3 shows data from an experiment in which autotaxin/lysoPLD activity was determined using conditioned medium from HEK293 cells expressing V5-autotaxin as the source of activity. In this experiment the concentration of FS-3 substrate was varied from 1 to 10 μM and reaction progress curves were monitored continuously. We found that autotaxin/lysoPLD activity showed a strong positive cooperativity with respect to substrate concentration. More needs to be done to understand this behavior, but one possibility is that it reflects a preference of the enzyme for a micellar or aggregated form of the substrate. Half-maximal enzyme activity was observed at approximately 4 μM , which is consistent with the apparent K_m of autotaxin/lysoPLD for this substrate reported by others (Ferguson *et al.*, 2006). As noted earlier, this illustrates that, as is the case with the fluorogenic substrate CP4, autotaxin/lysoPLD also exhibits a markedly higher affinity for these synthetic fluorogenic substrates than it does for lysophospholipid or colorimetric substrates such as pNPTMP or bis-pNPP.

6. CONCLUDING COMMENTS

The emerging role of autotaxin/lysoPLD as a major pathway for the generation of LPA and the involvement of this enzyme and tumorigenesis and cardiovascular development and function continue to stimulate the development of methods to detect and quantitate this enzyme. The

radiochemical assay described earlier is a relatively sensitive and specific way to detect and quantitate autotaxin/lysoPLD activity in biological samples. Unfortunately, because of the necessity of a thin-layer chromatography step, this assay is time-consuming to perform. A recently described assay that employs a fluorescent lysoPC analog (ADMAN-lysoPC) suffers from the same limitation (Uchiyama *et al.*, 2007), although direct imaging of these plates could provide a more rapid way to quantitate unreacted substrate and product than autoradiography and quantitation of radioactivity. Phosphatidylcholine-specific phospholipase D activity can be measured readily using radioactive substrates labeled in the choline head group. In these assays, release of water-soluble choline from the water-insoluble lipid substrate is monitored after organic solvent extraction or precipitation of unreacted substrate with trichloroacetic acid (Frohman *et al.*, 2000). Unfortunately, a head group-radiolabeled lysoPC is not available commercially and, although this lipid can be generated enzymatically (Luquain *et al.*, 2003), separation of the lysophospholipid substrate from the choline product cannot be achieved quantitatively using organic solvent extraction or acid precipitation. Consequently, there is a pressing need to develop more straightforward assays for this enzyme, particularly assays that do not require extraction or chromatographic steps with optical readouts that would be amenable to automation. Inhibition of autotaxin/lysoPLD activity by small molecules could be an effective new therapeutic approach for cancer and possibly cardiovascular disease. Alterations in LPA levels in the blood and other biological fluids such as ovarian cancer ascites fluid raise the possibility that autotaxin/lysoPLD levels are also increased, and some evidence shows that autotaxin/lysoPLD activity is higher in serum from women than from men. Simple and specific assays for autotaxin/lysoPLD are required to investigate these issues directly. The development of fluorogenic substrates for autotaxin/lysoPLD promises to provide a simple and rapid way to quantitate this enzyme. The finding that this enzyme has a higher affinity for these substrates than for lysophospholipids or *p*-nitrophenylphosphate esters suggests that this class of substrates may be very effective for this purpose. However, more work needs to be done to evaluate the extent to which other phosphodiesterases, competing activities, or substrate-binding proteins may limit the usefulness of these fluorogenic substrates for determinations of autotaxin/lysoPLD activity in complex biological samples. Preliminary experiments have found that FS-3 can be used to measure phosphodiesterase activities in human and mouse plasma and serum. In both cases, hydrolysis of the substrate can be inhibited by LPA, which is a hallmark feature of autotaxin/lysoPLD. It was also found that hydrolysis of FS-3 is elevated in the blood of mice that overexpress autotaxin/lysoPLD under control of the α_1 -antitrypsin promoter, which suggests that the activity measured using FS-3 is at least in part because of autotaxin/lysoPLD (unpublished observations). However, whereas recombinantly generated autotaxin/lysoPLD exhibits a very high

affinity for FS-3 (apparent $K_m \sim 4 \mu M$), FS-3 phosphodiesterase activity in human and mouse serum and plasma is not saturated at substrate concentrations up to $50 \mu M$, which suggests that substrate sequestration limits access to the enzyme. Consequently, although FS-3 can be used to detect autotaxin/lysoPLD activity in human and mouse blood, further work will be required to develop this assay for precise quantitation of the enzyme in the blood and other biological fluids.

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REFERENCES

- Aoki, J. (2004). Mechanisms of lysophosphatidic acid production. *Semin. Cell Dev. Biol.* **15**, 477–489.
- Baker, D. L., Fujiwara, Y., Pigg, K. R., Tsukahara, R., Kobayashi, S., Murofushi, H., Uchiyama, A., Murakami-Murofushi, K., Koh, E., Bandle, R. W., Byun, H. S., Bittman, R., *et al.* (2006). Carba analogs of cyclic phosphatidic acid are selective inhibitors of autotaxin and cancer cell invasion and metastasis. *J. Biol. Chem.* **281**, 22786–22793.
- Bollen, M., Gijsbers, R., Ceulemans, H., Stalmans, W., and Stefan, C. (2000). Nucleotide pyrophosphatases/phosphodiesterases on the move. *Crit. Rev. Biochem. Mol. Biol.* **35**, 393–432.
- Chun, J. (2005). Lysophospholipids in the nervous system. *Prostaglandins Other Lipid Mediat.* **77**, 46–51.
- Cimpean, A., Stefan, C., Gijsbers, R., Stalmans, W., and Bollen, M. (2004). Substrate-specifying determinants of the nucleotide pyrophosphatases/phosphodiesterases NPP1 and NPP2. *Biochem. J.* **381**, 71–77.
- Clair, T., Aoki, J., Koh, E., Bandle, R. W., Nam, S. W., Ptaszynska, M. M., Mills, G. B., Schiffmann, E., Liotta, L. A., and Stracke, M. L. (2003). Autotaxin hydrolyzes sphingomyelophosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate. *Cancer Res.* **63**, 5446–5453.
- Clair, T., Lee, H. Y., Liotta, L. A., and Stracke, M. L. (1997). Autotaxin is an exoenzyme possessing 5'-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities. *J. Biol. Chem.* **272**, 996–1001.
- Coleman, R. A. (2007). How do I fatten thee? Let me count the ways. *Cell Metab.* **5**, 87–89.
- Ferguson, C. G., Bigman, C. S., Richardson, R. D., van Meeteren, L. A., Moolenaar, W. H., and Prestwich, G. D. (2006). Fluorogenic phospholipid substrate to detect lysophospholipase D/autotaxin activity. *Org. Lett.* **8**, 2023–2026.
- Frohmman, M. A., Kanaho, Y., Zhang, Y., and Morris, A. J. (2000). Regulation of phospholipase D1 activity by Rho GTPases. *Methods Enzymol.* **325**, 177–189.
- Georas, S. N., Berdyshev, E., Hubbard, W., Gorshkova, I. A., Usatyuk, P. V., Saatian, B., Myers, A. C., Williams, M. A., Xiao, H. Q., Liu, M., and Natarajan, V. (2007). Lysophosphatidic acid is detectable in human bronchoalveolar lavage fluids at baseline and increased after segmental allergen challenge. *Clin. Exp. Allergy* **37**, 311–322.

- Gijssbers, R., Aoki, J., Arai, H., and Bollen, M. (2003). The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site. *FEBS Lett.* **538**, 60–64.
- Hama, K., Aoki, J., Fukaya, M., Kishi, Y., Sakai, T., Suzuki, R., Ohta, H., Yamori, T., Watanabe, M., Chun, J., and Arai, H. (2004). Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1. *J. Biol. Chem.* **279**, 17634–17639.
- Haseruck, N., Erl, W., Pandey, D., Tigyi, G., Ohlmann, P., Ravanat, C., Gachet, C., and Siess, W. (2004). The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: Involvement of P2Y1 and P2Y12 receptors. *Blood* **103**, 2585–2592.
- Ishii, I., Fukushima, N., Ye, X., and Chun, J. (2004). Lysophospholipid receptors: Signaling and biology. *Annu. Rev. Biochem.* **73**, 321–354.
- Jansen, S., Stefan, C., Creemers, J. W., Waelkens, E., Van, E. A., Stalmans, W., and Bollen, M. (2005). Proteolytic maturation and activation of autotaxin (NPP2), a secreted metastasis-enhancing lysophospholipase D. *J. Cell Sci.* **118**, 3081–3089.
- Lee, C. W., Rivera, R., Gardell, S., Dubin, A. E., and Chun, J. (2006). GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J. Biol. Chem.* **281**, 23589–23597.
- Luquain, C., Singh, A., Wang, L., Natarajan, V., and Morris, A. J. (2003). Role of phospholipase D in agonist-stimulated lysophosphatidic acid synthesis by ovarian cancer cells. *J. Lipid Res.* **44**, 1963–1975.
- MacDonald, N. J., Freije, J. M., Stracke, M. L., Manrow, R. E., and Steeg, P. S. (1996). Site-directed mutagenesis of nm23-H1: Mutation of proline 96 or serine 120 abrogates its motility inhibitory activity upon transfection into human breast carcinoma cells. *J. Biol. Chem.* **271**, 25107–25116.
- Mills, G. B., and Moolenaar, W. H. (2003). The emerging role of lysophosphatidic acid in cancer. *Nat. Rev. Cancer* **3**, 582–591.
- Moolenaar, W. H., van Meeteren, L. A., and Giepmans, B. N. (2004). The ins and outs of lysophosphatidic acid signaling. *Bioessays* **26**, 870–881.
- Nakamura, K., Ohkawa, R., Okubo, S., Tozuka, M., Okada, M., Aoki, S., Aoki, J., Arai, H., Ikeda, H., and Yatomi, Y. (2007). Measurement of lysophospholipase D/autotaxin activity in human serum samples. *Clin. Biochem.* **40**, 274–277.
- Sakagami, H., Aoki, J., Natori, Y., Nishikawa, K., Kakehi, Y., Natori, Y., and Arai, H. (2005). Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. *J. Biol. Chem.* **280**, 23084–23093.
- Savaskan, N. E., Rocha, L., Kotter, M. R., Baer, A., Lubec, G., van Meeteren, L. A., Kishi, Y., Aoki, J., Moolenaar, W. H., Nitsch, R., and Brauer, A. U. (2007). Autotaxin (NPP-2) in the brain: Cell type-specific expression and regulation during development and after neurotrauma. *Cell Mol. Life Sci.* **64**, 230–243.
- Siess, W., and Tigyi, G. (2004). Thrombogenic and atherogenic activities of lysophosphatidic acid. *J. Cell Biochem.* **92**, 1086–1094.
- Simon, M. F., Daviaud, D., Pradere, J. P., Gres, S., Guigne, C., Wabitsch, M., Chun, J., Valet, P., and Saulnier-Blache, J. S. (2005). Lysophosphatidic acid inhibits adipocyte differentiation via lysophosphatidic acid 1 receptor-dependent down-regulation of peroxisome proliferator-activated receptor gamma2. *J. Biol. Chem.* **280**, 14656–14662.
- Stracke, M., Liotta, L. A., and Schiffmann, E. (1993). The role of autotaxin and other motility stimulating factors in the regulation of tumor cell motility. *Symp. Soc. Exp. Biol.* **47**, 197–214.
- Stracke, M. L., Krutzsch, H. C., Unsworth, E. J., Arestad, A., Cioce, V., Schiffmann, E., and Liotta, L. A. (1992). Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J. Biol. Chem.* **267**, 2524–2529.

- Sutphen, R., Xu, Y., Wilbanks, G. D., Fiorica, J., Grendys, E. C., Jr., LaPolla, J. P., Arango, H., Hoffman, M. S., Martino, M., Wakeley, K., Griffin, D., Blanco, R. W., *et al.* (2004). Lysophospholipids are potential biomarkers of ovarian cancer. *Cancer Epidemiol. Biomark. Prev.* **13**, 1185–1191.
- Tanaka, M., Okudaira, S., Kishi, Y., Ohkawa, R., Iseki, S., Ota, M., Noji, S., Yatomi, Y., Aoki, J., and Arai, H. (2006). Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J. Biol. Chem.* **281**, 25822–25830.
- Tokumura, A., Kume, T., Fukuzawa, K., Tahara, M., Tasaka, K., Aoki, J., Arai, H., Yasuda, K., and Kanzaki, H. (2007). Peritoneal fluids from patients with certain gynecologic tumor contain elevated levels of bioactive lysophospholipase D activity. *Life Sci.* **80**, 1641–1649.
- Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, K., and Fukuzawa, K. (2002). Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J. Biol. Chem.* **277**, 39436–39442.
- Uchiyama, A., Mukai, M., Fujiwara, Y., Kobayashi, S., Kawai, N., Murofushi, H., Inoue, M., Enoki, S., Tanaka, Y., Niki, T., Kobayashi, T., Tigyi, G., *et al.* (2007). Inhibition of transcellular tumor cell migration and metastasis by novel carba-derivatives of cyclic phosphatidic acid. *Biochim. Biophys. Acta* **1771**, 103–112.
- Umezū-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G. B., Inoue, K., Aoki, J., and Arai, H. (2002). Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell Biol.* **158**, 227–233.
- van Meeteren, L. A., Ruurs, P., Christodoulou, E., Goding, J. W., Takakusa, H., Kikuchi, K., Perrakis, A., Nagano, T., and Moolenaar, W. H. (2005). Inhibition of autotaxin by lysophosphatidic acid and sphingosine 1-phosphate. *J. Biol. Chem.* **280**, 21155–21161.
- van Meeteren, L. A., Ruurs, P., Stortelers, C., Bouwman, P., van Rooijen, M. A., Pradere, J. P., Pettit, T. R., Wakelam, M. J., Saulnier-Blache, J. S., Mummery, C. L., Moolenaar, W. H., and Jonkers, J. (2006). Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. *Mol. Cell Biol.* **26**, 5015–5022.
- Yanagida, K., Ishii, S., Hamano, F., Noguchi, K., and Shimizu, T. (2007). LPA4/p2y9/GPR23 mediates rho-dependent morphological changes in a rat neuronal cell line. *J. Biol. Chem.* **282**, 5814–5824.

PLATELET-ACTIVATING FACTOR

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Contents

1. Introduction	105
2. Procedure	107
2.1. Reagents	107
2.2. Standard curves	108
2.3. Lipid extraction	109
2.4. Sample cleanup	112
2.5. Platelet-activating factor quantitation	113
Acknowledgments	115
References	115

Abstract

Platelet-activating factor (PAF) is a potent mediator that occurs at very low concentrations in cells and tissues. Accurate quantitation of PAF has always been difficult because of the physicochemical properties of PAF and its structural similarity to several much more abundant phospholipids. Numerous assays for PAF have been developed, all of which have their strengths and limitations. Herein, this chapter describes a high-pressure liquid chromatography (HPLC)–tandem mass spectrometry assay for PAF. Major strengths of the method are its sensitivity (detection limit = 1 pg) and selectivity. Another advantage is that, by using liquid instead of gas chromatography, sample derivatization is avoided. The limitations of the method are its use of expensive instrumentation and the requirement of performing two HPLC runs per sample. Detailed technical advice on application of the method to various types of samples is given.

1. INTRODUCTION

Platelet-activating factor (PAF) is a local mediator with proinflammatory, vasoactive, thrombotic, and other paracrine and autocrine actions. PAF has the chemical structure 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine

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(1-O-alkyl-2-acetyl-GPC) (Benveniste *et al.*, 1979; Blank *et al.*, 1979; Demopoulos *et al.*, 1979; Hanahan *et al.*, 1980). The alkyl moiety is composed almost entirely of hexadecyl (16:0), octadecyl (18:0), and octadecenyl (18:1) chains, although other alkyl chains also exist (Mueller *et al.*, 1984). PAF is among the most potent mediators known, evoking cellular responses at low picomolar concentrations (Bossant *et al.*, 1990; Demopoulos *et al.*, 1979). Because of such potency, and because of ubiquitous PAF acetylhydrolases that rapidly degrade PAF in cells and plasma, PAF generally occurs at very low concentrations in biological samples, presenting a significant technical challenge for quantitation. The biochemistry of PAF and its actions have been the subject of several reviews, including Snyder (1990), Stafforini *et al.* (2003), and Venable *et al.* (1993). A number of approaches for measuring PAF and its biosynthesis were reviewed earlier in Wykle *et al.* (1988).

Numerous studies on PAF have made use of metabolic radiolabeling for PAF measurement using known or suspected PAF metabolic precursors as radioactive tracers (Wykle *et al.*, 1988). These experimental approaches can offer a relatively straightforward and sensitive means of measuring PAF. When radiolabeled acetate is employed to follow PAF synthesis, 1-acyl-2-acetyl-GPC and 1-O-alk-1'-enyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine are also often labeled (Tessner and Wykle, 1987; Wykle *et al.*, 1988). Metabolic radiolabeling does not measure the absolute quantity of PAF in a sample, but is instead used to track relative increases and decreases in PAF with cell stimulation. However, this disadvantage is offset by the unique advantage of gaining information about the metabolic origins of PAF in the particular biological system under study (Chilton *et al.*, 1984; Mueller *et al.*, 1983; Nieto *et al.*, 1991).

A number of bioassays for PAF have been developed, which capitalize on the potency of PAF to achieve good sensitivity (Bossant *et al.*, 1990; Henson, 1990; Marathe *et al.*, 1999). The specificity of such assays for PAF is dependent on adequate chromatographic purification of PAF prior to assay, combined with the use of appropriate experimental controls. However, it should be remembered that there are situations where the relatively broad specificity of the PAF bioassay techniques is an advantage. For example, in some experiments it is PAF-like biological activity, rather than PAF *per se*, that is the desired end point (Marathe *et al.*, 1999). However, when the question is whether an observed biological activity is attributable to PAF, there is no substitute for the physicochemical assays.

The most sensitive and specific physicochemical assays for PAF are those based on mass spectrometry; the assay described here is based on a number of advances made since the earlier review (Wykle *et al.*, 1988). In these assays, a stable isotope-labeled PAF is added to the samples at the time of extraction as an internal standard. Samples are then processed by total lipid extraction, a sample cleanup step (preliminary fractionation of the

crude lipid extract to obtain a PAF-enriched fraction), and, finally, either high-pressure liquid chromatography (HPLC)–electrospray tandem mass spectrometry (LC-MS/MS) or derivatization and gas chromatography–mass spectrometry (GC-MS). The GC-MS techniques include the most sensitive PAF assays available (Yamada *et al.*, 1988). However, the requirement for derivatization makes these assays susceptible to losses, contamination, and artifacts (Haroldsen *et al.*, 1987, 1991; Tessner and Wykle, 1987). LC-MS/MS methods (Harrison *et al.*, 1999; Kita *et al.*, 2005; Owen *et al.*, 2005) enable mass spectrometric determination of PAF without the need for derivatization and can detect PAF at low picogram levels. The LC-MS/MS assays for PAF thus offer substantial advantages over other available assays; however, users should understand the potential pitfalls of LC-MS/MS for PAF. In a typical biological sample, PAF occurs alongside large molar excesses of other lipids with similar (although not identical) chromatographic and mass spectrometric properties. These potentially interfering lipids include formylated phosphatidylcholines, which coelute with PAF on normal-phase HPLC, and lysophosphatidylcholine (lysoPC), a portion of which coelutes with PAF on reversed-phase HPLC (Harrison *et al.*, 1999; Owen *et al.*, 2005). The following sections give recommendations on how to avoid these potential pitfalls and capitalize on the strengths of LC-MS/MS as a PAF assay.

2. PROCEDURE

2.1. Reagents

Internal standard:

1-O-hexadecyl-2-[$^2\text{H}_3$]acetyl-GPC (d3-PAF), prepared according to Clay (1990) or

1-O-[7',7',8',8'- $^2\text{H}_4$]hexadecyl-2-acetyl-GPC (d4-PAF; Cayman Chemical)

Unlabeled standards for generating standard curves:

1-O-hexadecyl-2-acetyl-GPC (16:0 PAF; Avanti Polar Lipids)

1-O-octadec-*cis*-9'-enyl-2-acetyl-GPC (18:1 PAF; Biomol)

1-O-octadecyl-2-acetyl-GPC (18:0 PAF; Avanti Polar Lipids)

Solvents for extraction:

Deionized or distilled water

200 proof anhydrous ethyl alcohol USP (Warner-Graham Co., Cockeysville, MD)

HPLC grade methanol and chloroform (Fisher)

Glacial acetic acid, ACS grade (Fisher)

Solvents for HPLC:

Methanol:water:acetonitrile (61:21:18, v/v/v) containing 1 mM ammonium acetate (solvent A)

Methanol containing 1 mM ammonium acetate (solvent B)
Methylene chloride:methanol:water (120:60:9, v/v/v) (solvent C)
Hexane, chloroform, and isopropanol
(Note all HPLC solvents are "GC2" grade from Burdick & Jackson.
Ammonium acetate crystals, ACS grade, are from J. T. Baker.)

2.2. Standard curves

For an internal standard, d3-PAF prepared according to Clay (1990) is preferred to the commercially available d4-PAF from Cayman, as the latter contains small but detectable quantities of unlabeled PAF. We use "Method 2" from Clay's paper and find that the synthesis and purification of d3-PAF are easy, and the quantity produced (≈ 10 mg) is sufficient to last several years when kept at -20° in tightly closed containers. After thin-layer chromatographic separation as recommended by Clay (1990), the d3-PAF band is visible without the need of sprays or other visualization techniques that can introduce contamination. The d3-PAF band is scraped from the plate, and the product is eluted from the silica with two washes of chloroform:methanol:water (2:5:1, v/v/v) (Christiansen, 1975). After evaporation of the solvent under a stream of nitrogen, the d3-PAF is redissolved in a known volume of chloroform:methanol (4:1, v/v). The exact concentration of this solution is measured using a colorimetric assay for total phosphorus content after perchloric acid digestion of duplicate aliquots containing 2 to 50 nmol (Rouser *et al.*, 1966). The yield of purified product is $\approx 99\%$. Use of the total phosphorus assay to measure exact concentration is also recommended for d4-PAF (if used) and the unlabeled 16:0, 18:1, and 18:0 PAF stock solutions.

A separate calibration curve must be prepared for each PAF molecular species to be measured. This is conveniently accomplished by preparing a mixture containing equal concentrations of 16:0, 18:1, and 18:0 PAF and adding various amounts of this mixture to vials containing a constant amount of internal standard. A convenient injection volume is 25 μ l. Standards are made up so that each 25 μ l contains 400 pg of internal standard and various amounts (1–1000 pg) of each unlabeled PAF molecular species. The solvent used is HPLC solvent A. For accuracy in pipetting nonaqueous solutions, disposable glass capillary pipettes or positive-displacement pipettors are preferred over Pipetman-type pipettors.

Calibration curve standards are analyzed by reversed-phase LC-MS/MS by injecting 25 μ l onto a Supelco Discovery C18 Bio-Wide Pore column, 1×50 mm, with 3- μ m particle size. The column is eluted at 100 μ l/min with a gradient of 100% solvent A to 100% solvent B over 5 min; hold at 100% B for 5 min; return to 100% A over 5 min; and reequilibrate the column at 100% A for at least 15 min. A splitting tee sends half of the column effluent to the electrospray source of the triple-quadrupole mass spectrometer (Waters

Table 6.1 Precursor/product ions for SRM detection of deuterated and unlabeled PAF molecular species

Species	Positive-ion mode (m/z)		Negative-ion mode (m/z)	
	Precursor ion	Product ion	Precursor ion	Product ion
d ₃ -PAF	527.2	185.0	−511.2	−62.0
d ₄ -PAF	528.2	184.0	−512.2	−59.0
16:0 PAF	524.2	184.0	−508.2	−59.0
18:1 PAF	550.2	184.0	−520.2	−59.0
18:0 PAF	552.2	184.0	−522.2	−59.0

Quattro II). The mass spectrometer is operated in selected-reaction monitoring (SRM) mode, and the reactions monitored are as shown in Table 6.1.

Note that the deuterium label affects fragmentation differently in d₃-PAF versus d₄-PAF, as the deuterium label is located on the acetyl moiety in d₃-PAF and on the alkyl moiety in d₄-PAF (Hsu and Turk, 2003). All reactions are monitored using a dwell time of 0.3 s. Nitrogen serves as the nebulizing gas and the drying gas, with a desolvation temperature of 250° and a source temperature of 80°. Both MS1 and MS2 are operated at a resolution setting of 13.5. Argon at 1.5 μ bar is the collision gas, with collision energy at 23 eV. With positive ionization, typical cone and capillary voltages are 38 V and 3.6 kV, respectively. In negative-ion mode, typical cone and capillary voltages are −60 V and −2.6 kV, respectively.

Calibration curves are plotted for 16:0, 18:1, and 18:0 PAF using chromatographic peak areas normalized to the internal standard peak area. When using d₄-PAF, the 18:0 PAF calibration curve may show a slight positive y intercept, reflecting the presence of a trace of 18:0 PAF in the internal standard (Fig. 6.1). In such cases, the 18:0 PAF response factors should be corrected by subtraction.

2.3. Lipid extraction

The PAF assay is carried out on total lipid extracts from biological samples. We use the lipid extraction method of Bligh and Dyer (1959), with various modifications depending on the sample type. For single-cell suspensions or liquid samples such as perfusion buffers, 2.5 ml of methanol containing 2.5% (v/v) glacial acetic acid is added for each milliliter of aqueous sample. Extractions are carried out in 15-ml polypropylene or 16 $\times$ 125-mm borosilicate glass test tubes. The internal standard (400 pg) is added in 50 μ l of ethanol. One milliliter of chloroform is then added and the mixture vortexed. At this point in the extraction, there should be a single liquid phase. Phase separation is achieved by adding 1 ml water, followed by 1 ml chloroform. After

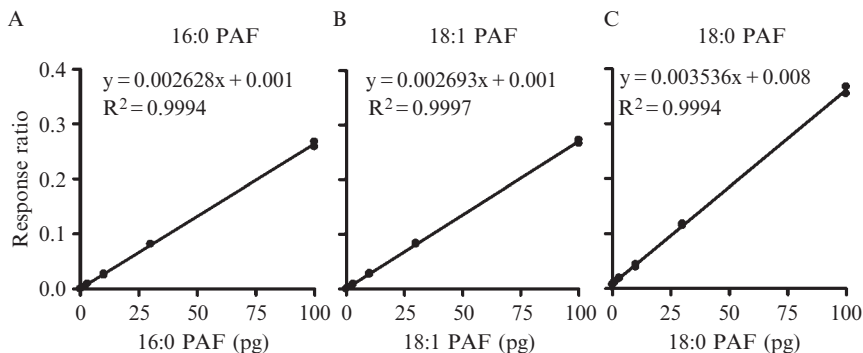


Figure 6.1 Calibration curves shown for 16:0 PAF (A), 18:1 PAF (B), and 18:0 PAF (C) were generated by serial dilution of a mixture containing equal masses of these three PAF molecular species. The indicated masses of unlabeled PAF species (0, 1, 3, 10, 30, and 100 pg) were coinjected with 400 pg of d4-PAF internal standard. Duplicate injections were performed at each PAF level. Response ratios were calculated as unlabeled PAF chromatographic peak area divided by internal standard chromatographic peak area. Reversed-phase LC-MS/MS conditions, using positive ionization, were as described in the text.

vortexing the mixture, it is then centrifuged for 10 min to cleanly separate the two phases. The lower (chloroform) phase is transferred to a new glass test tube, and the upper phase is reextracted with 2 ml chloroform. The pooled chloroform extracts are evaporated under a stream of nitrogen, and the lipid residue is dissolved in an appropriate solvent. If the number of cells in each sample is known with reasonable precision, then the final PAF measurement can be normalized to cell number. In the case of perfusion buffer, PAF is normalized to buffer volume. In other cases it may be desirable to measure total phosphorus (Rouser *et al.*, 1966) in duplicate aliquots of the lipid extract as an index of the amount of biological material present. If the phosphorus assay is used, then the dried lipid extract is dissolved in exactly 1 ml of chloroform:methanol (1:1, v/v); duplicate aliquots (typically 50 μ l) are removed from this solution, and the sample is again evaporated under nitrogen. The residues are dissolved in 125 μ l of solvent A and transferred to polypropylene autosampler vials for the cleanup procedure. If the samples must be stored overnight or longer prior to cleanup, then glass autosampler vials should be used and the samples kept at -20° .

For adherent cultured cells, cells are grown on 100- or 150-mm-diameter polystyrene culture dishes. Stimuli are typically added to serum-starved cells in a minimal volume of serum-free medium. In addition to being a potent stimulus in its own right, serum contains abundant PAF acetylhydrolase activity, complicating the interpretation of results. At the end of the stimulation period, the medium is removed, and cells are scraped into 2.5 ml of methanol containing 2.5% (v/v) acetic acid and 400 pg of internal standard.

The methanol and cells are transferred to a borosilicate glass 16×125 -mm test tube. Dishes are washed with 0.8 ml water, which is added to the extraction tubes, and the dishes are discarded. One milliliter of chloroform is added to the extraction tubes, and lipid extraction proceeds as described earlier for liquid samples. The phosphorus assay is especially useful in this type of experiment, as the cells are usually grown for some days after being added to the plate, so the exact cell number is not known at the time of stimulation. The stimulation medium may be analyzed as described previously for liquid samples, but typically the bulk of the PAF is cell associated. In our experience, cell lines capable of producing PAF will accumulate PAF maximally in response to a 10-min stimulation with $10 \mu\text{M}$ A23187 at 37° . In experiments measuring a "basal" PAF concentration in growing cells, the cells are not serum starved. Instead, upon reaching the desired confluence, cells are quickly washed twice with ice-cold normal saline (0.9% w/v), scraped into acidified methanol as described earlier, and the lipids extracted in the same way. The saline solution may be buffered with Tris or HEPES if desired, but not with phosphate if a phosphorus assay is planned.

For animal tissues, the specimen is weighed fresh and then disrupted. Disruption may be achieved by mincing the specimen as finely as possible with a razor blade, by freezing the sample in liquid nitrogen and pulverizing the frozen specimen in a chilled mortar, or by using a Polytron-type tissue homogenizer with an aqueous homogenization buffer. Ideally, lipid extraction should be performed immediately on the disrupted tissue to prevent degradation of PAF by PAF acetylhydrolase; however, homogenates can be snap frozen in liquid nitrogen and stored at -80° until the time of lipid extraction. In either case, the internal standard (400 pg) must be added at the earliest possible time in sample processing, but not at a time when live cells are present, as PAF is known to stimulate its own biosynthesis. Minced or pulverized tissues of up to 200 mg fresh weight are suspended in 0.8 ml of water, to which is added 2.5 ml of acidified methanol (2.5% acetic acid), 1 ml of chloroform, and the internal standard. All solvent volumes are doubled for specimens over 200 mg fresh weight. Lipid extraction from tissue homogenates may be scaled according to the aqueous homogenate volume. The mixture of disrupted tissue with solvents is incubated in a tightly capped glass test tube for 1 h at 60° to extract lipids. If other lipids less chemically stable than PAF are also to be analyzed in the extract, the mixture may instead be incubated for 1 h at room temperature or for 3 h at 4° with constant agitation. At the end of the extraction period, the solids are removed by centrifugation, and the phase separation and liquid-liquid extraction steps proceed as for the other sample types. The final PAF measurement may be normalized to specimen fresh weight or total phospholipid. It is also possible to dry the delipidated tissue solids, digest them in 0.5 M aqueous sodium hydroxide until clear, and assay duplicate aliquots for total protein using the Lowry method (Lowry *et al.*, 1951). However, total

protein assay, when applied to base-digested samples, is less accurate than the phosphorus assay applied to lipid extracts.

2.4. Sample cleanup

The major challenge of sample cleanup preparatory to PAF analysis by LC-MS/MS is to remove lysophosphatidylcholine (lysoPC) species, which are isobaric with the PAF species under study. In particular, 18:0 lysoPC is isobaric with 16:0 PAF and can be many times more abundant than PAF in samples. Samples are, therefore, submitted to cleanup by preparative normal-phase HPLC prior to PAF analysis. For small samples, such as 1×10^7 neutrophils or one dish of adherent cultured cells, a Thermo Hypersil silica column, 2.1×150 mm, with $5\text{-}\mu\text{m}$ particle size, is used. For larger samples, such as tissue specimens, a 4.6-mm-diameter column with $3\text{-}\mu\text{m}$ particle size is used. Thermo Hypersil silica guard cartridges are used with both columns. Both columns are eluted isocratically with solvent C using a flow rate of 0.3 ml/min for the smaller column and 1.2 ml/min for the larger column. Samples are injected in 100 μl of solvent C. A small part of the column effluent (5% for the small column and 0.7% for the large column) is diverted to the mass spectrometer using a splitting tee. Mass spectrometry parameters are as described earlier, except that the desolvation temperature is 150° . It is sometimes helpful to monitor phosphatidylcholine elution as m/z 760 $\rightarrow$ 184 and the sodium adduct of d3-PAF as m/z 549 $\rightarrow$ 490. PAF elutes after phosphatidylcholine and before lysoPC, typically around 6 to 9 min. Depending on column retentiveness and the lysoPC content of the samples, it may be necessary to extend the run time beyond 15 min or to increase the flow rate slightly (up to 0.4 ml/min for the small column and up to 1.44 ml/min for the large column) in order to make sure lysoPC has finished eluting by the end of each run. In some cases it may also be necessary to increase the water content of the solvent, from 9 to 10 parts water by volume, in order to hasten lysoPC elution.

Platelet-activating factor fractions are collected by hand for the first few samples, while monitoring PAF and lysoPC elution using the mass spectrometer. It should be kept in mind that there may be a significant difference in solvent delay times between high-flow and low-flow sides of the splitter; such time differences should be measured (for example, with an injection of acetone) and taken into account for fraction collection. The mass spectrometer is then placed in standby mode, and the entire column effluent is directed to an automatic fraction collector interfaced with the HPLC. The fraction collector is programmed with start and end times of PAF elution for the current set of PAF samples. At the end of a sample set, the HPLC column is cleaned thoroughly with methanol or with chloroform: isopropanol (1:1, v/v) and then equilibrated with hexane for storage.

2.5. Platelet-activating factor quantitation

The collected PAF fractions are evaporated under a stream of nitrogen and redissolved in 35 μl of solvent A. For PAF analysis, 25 μl is injected on the Supelco Discovery Bio-Wide Pore C18 column, using the same conditions described earlier for the standard curve. During the last 15 min of each run, the column effluent is diverted to waste to prevent the introduction of large amounts of sphingomyelin into the electrospray source. The amount of each PAF molecular species is calculated from its integrated chromatographic peak area, normalized to that of the internal standard.

Platelet-activating factor is usually detected using positive ionization; as little as 1 pg injected on the column can be detected in this way (signal/noise = 6), whereas we find that negative ionization requires 100 pg (signal/noise = 3.5). Others, using different instrumentation, report detecting 5 pg of PAF using negative ionization (Kita *et al.*, 2005). Response curves are linear at least up to 1 ng of PAF injected.

With samples containing particularly high lysoPC concentrations, or in case of problems with the sample cleanup step, some residual lysoPC may remain in the PAF fractions. Such residual lysoPC, when present, appears in the reversed-phase chromatograms as peaks in the PAF channels, eluting just after the PAF species (Fig. 6.2). This phenomenon is of concern, as under the solvent conditions used here, lysoPC tends to approach its equilibrium between 1-acyl-2-lyso and 1-lyso-2-acyl regioisomers, such that the 2-acyl isomer, which coelutes with PAF, represents roughly 10% of the total lysoPC. If the 1-acyl-2-lyso-GPC peak eluting after the PAF peak is no larger than the PAF peak, then a reasonably accurate estimate of the true PAF peak area can be obtained by subtracting 11% of the 1-acyl-2-lyso-GPC peak area from the “PAF” (actually PAF + 1-lyso-2-acyl-GPC) peak area. However, if the 1-acyl-2-lyso-GPC peak is larger than the PAF peak, it is advisable to repurify the PAF fractions on normal-phase HPLC.

A notable exception to the rule regarding the identity of peaks eluting just after PAF is seen in the 18:1 PAF channel, where, in some types of samples, 18:1 PAF elutes as two peaks (Owen *et al.*, 2005). In such samples, the earlier and larger 18:1 PAF peak coelutes with authentic 1-O-octadec-*cis*-9'-enyl-2-acetyl-GPC, whereas the later, smaller peak may represent 1-O-octadec-*trans*-11'-enyl-2-acetyl-GPC, that is, an 18:1 PAF isomer where the fatty alcohol substituent is vaccenyl alcohol rather than oleoyl alcohol. LysoPC interference should be suspected in the 18:1 PAF channel when the second peak accounts for more than one-third of the total area of the two PAF peaks.

For samples containing sufficiently high PAF concentrations to allow detection in negative-ion mode, lysoPC interference presents much less of a concern, as PAF and lysoPC give rise to different product ions. Depending on sample composition, this greater specificity of detection may even allow

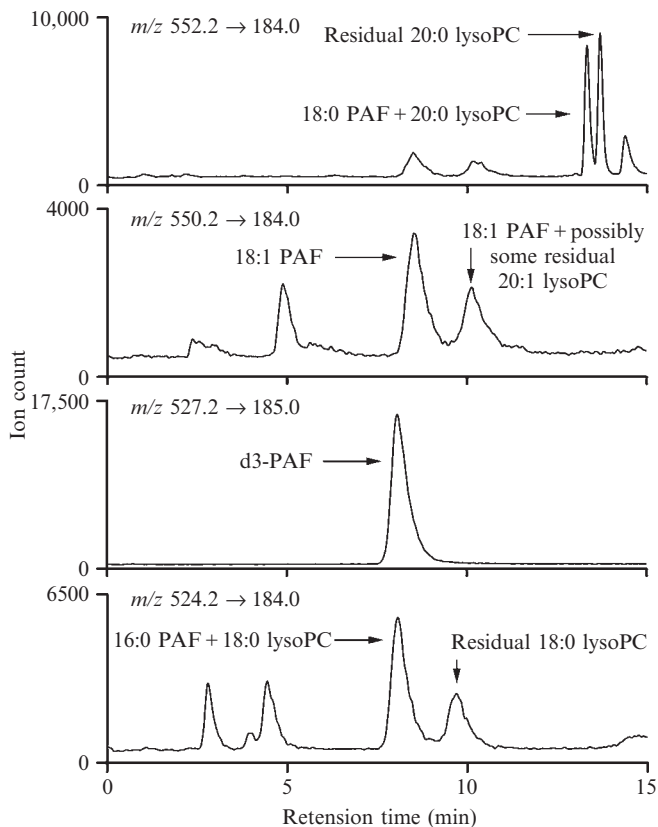


Figure 6.2 Effect of residual lysoPC on PAF quantitation. Cultured cells from one 100-mm-diameter tissue culture dish were extracted without stimulation and assayed for PAF as described in the text. Briefly, the total lipid extract was separated using normal-phase HPLC using a Thermo Hypersil 2.1×150 -mm silica column with $5\text{-}\mu\text{m}$ particle size, eluted isocratically at $300\text{ }\mu\text{l/min}$ with methylene chloride:methanol:water (120:60:9). A splitting tee diverted 5% of the column effluent to the mass spectrometer for monitoring elution of the d3-PAF internal standard. The fraction containing d3-PAF was collected in a glass test tube, evaporated under a stream of nitrogen, and dissolved in $35\text{ }\mu\text{l}$ of methanol:water:acetonitrile (61:21:18, v/v/v) containing 1 mM ammonium acetate. PAF was analyzed by reversed-phase LC-MS/MS using a 1×50 -mm Supelco Discovery Bio-Wide C 18 column eluted at $100\text{ }\mu\text{l/min}$ with a gradient of 100% A to 100% B over 5 min, held at 100% B for 5 min, and returned to 100% A over 5 min. Half of the effluent was directed to the mass spectrometer, which was operated in SRM mode with positive ionization. The chromatogram shows m/z 552.2 $\rightarrow$ 184.0 (18:0 PAF; top trace), m/z 550.2 $\rightarrow$ 184.0 (18:1 PAF; second trace), m/z 527.2 $\rightarrow$ 185.0 (d3-PAF; third trace), and m/z 524.2 $\rightarrow$ 184.0 (16:0 PAF; bottom trace). Residual 18:0 lysoPC not completely removed during normal-phase HPLC sample cleanup appears in the m/z 524.2 $\rightarrow$ 184.0 (bottom) trace. The 16:0 PAF peak area should be corrected for the portion of 18:0 lysoPC which coelutes with 16:0 PAF, as detailed in the text. Similarly, residual 20:0 lysoPC elutes just after the 18:0 PAF peak (top trace), but a portion of this lysoPC species also coelutes with the 18:0 PAF. The situation with 18:1 PAF (second trace) is more complex. The lysoPC species that is isobaric with 18:1 PAF (20:1 lysoPC) is generally less abundant

sample cleanup methods less laborious than preparative HPLC to be used. However, even in negative-ion mode, PAF and lysoPC have the same precursor ion m/z , and if lysoPC is present in great enough molar excess over PAF, it can still interfere in the same manner (although to a lesser extent) as described earlier for positive ionization. Thus, for some types of samples, investigators using negative-ion detection for PAF still find it necessary to use normal-phase HPLC for sample cleanup (Kihara *et al.*, 2005).

than the other lysoPC species of interest; moreover, 18:1 PAF elutes as two peaks even in the absence of lysoPC contamination because of the presence of different 18:1 PAF isomers, which have the double bond of the alkyl chain in different positions. The chromatogram was processed using two rounds of Savitzky-Golay smoothing with a window of three scans (3.96 s).

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REFERENCES

- Benveniste, J., Tence, M., Varenne, P., Bidault, J., Boulet, C., and Polonsky, J. (1979). [Semi-synthesis and proposed structure of platelet-activating factor (P.A.F.): PAF-acether an alkyl ether analog of lysophosphatidylcholine]. *C. R. Seances Acad. Sci. D* **289**, 1037–1040.
- Blank, M. L., Snyder, F., Byers, L. W., Brooks, B., and Muirhead, E. E. (1979). Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. *Biochem. Biophys. Res. Commun.* **90**, 1194–1200.
- Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Bossant, M. J., Ninio, E., Delautier, D., and Benveniste, J. (1990). Bioassay of paf-acether by rabbit platelet aggregation. *Methods Enzymol.* **187**, 125–130.
- Chilton, F. H., Ellis, J. M., Olson, S. C., and Wykle, R. L. (1984). 1-O-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine: A common source of platelet-activating factor and arachidonate in human polymorphonuclear leukocytes. *J. Biol. Chem.* **259**, 12014–12019.
- Christiansen, K. (1975). Lipid extraction procedure for *in vitro* studies of glyceride synthesis with labeled fatty acids. *Anal. Biochem.* **66**, 93–99.
- Clay, K. L. (1990). Quantitation of platelet-activating factor by gas chromatography-mass spectrometry. *Methods Enzymol.* **187**, 134–142.
- Demopoulos, C. A., Pinckard, R. N., and Hanahan, D. J. (1979). Platelet-activating factor: Evidence for 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J. Biol. Chem.* **254**, 9355–9358.
- Hanahan, D. J., Demopoulos, C. A., Liehr, J., and Pinckard, R. N. (1980). Identification of platelet activating factor isolated from rabbit basophils as acetyl glycerol ether phosphorylcholine. *J. Biol. Chem.* **255**, 5514–5516.

- Haroldsen, P. E., Clay, K. L., and Murphy, R. C. (1987). Quantitation of lyso-platelet activating factor molecular species from human neutrophils by mass spectrometry. *J. Lipid Res.* **28**, 42–49.
- Haroldsen, P. E., Gaskell, S. J., Weintraub, S. T., and Pinckard, R. N. (1991). Isotopic exchange during derivatization of platelet activating factor for gas chromatography-mass spectrometry. *J. Lipid Res.* **32**, 723–729.
- Harrison, K. A., Clay, K. L., and Murphy, R. C. (1999). Negative ion electrospray and tandem mass spectrometric analysis of platelet activating factor (PAF) (1-hexadecyl-2-acetyl-glycerophosphocholine). *J. Mass Spectrom.* **34**, 330–335.
- Henson, P. M. (1990). Bioassay of platelet-activating factor by release of [^{3} H]serotonin. *Methods Enzymol.* **187**, 130–134.
- Hsu, F. F., and Turk, J. (2003). Electrospray ionization/tandem quadrupole mass spectrometric studies on phosphatidylcholines: The fragmentation processes. *J. Am. Soc. Mass Spectrom.* **14**, 352–363.
- Kihara, Y., Ishii, S., Kita, Y., Toda, A., Shimada, A., and Shimizu, T. (2005). Dual phase regulation of experimental allergic encephalomyelitis by platelet-activating factor. *J. Exp. Med.* **202**, 853–863.
- Kita, Y., Takahashi, T., Uozumi, N., and Shimizu, T. (2005). A multiplex quantitation method for eicosanoids and platelet-activating factor using column-switching reversed-phase liquid chromatography-tandem mass spectrometry. *Anal. Biochem.* **342**, 134–143.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
- Marathe, G. K., Davies, S. S., Harrison, K. A., Silva, A. R., Murphy, R. C., Castro-Faria-Neto, H., Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1999). Inflammatory platelet-activating factor-like phospholipids in oxidized low density lipoproteins are fragmented alkyl phosphatidylcholines. *J. Biol. Chem.* **274**, 28395–28404.
- Mueller, H. W., O'Flaherty, J. T., and Wykle, R. L. (1983). Biosynthesis of platelet activating factor in rabbit polymorphonuclear neutrophils. *J. Biol. Chem.* **258**, 6213–6218.
- Mueller, H. W., O'Flaherty, J. T., and Wykle, R. L. (1984). The molecular species distribution of platelet-activating factor synthesized by rabbit and human neutrophils. *J. Biol. Chem.* **259**, 14554–14559.
- Nieto, M. L., Venable, M. E., Bauldry, S. A., Greene, D. G., Kennedy, M., Bass, D. A., and Wykle, R. L. (1991). Evidence that hydrolysis of ethanolamine plasmalogens triggers synthesis of platelet-activating factor via a transacylation reaction. *J. Biol. Chem.* **266**, 18699–18706.
- Owen, J. S., Wykle, R. L., Samuel, M. P., and Thomas, M. J. (2005). An improved assay for platelet-activating factor using HPLC-tandem mass spectrometry. *J. Lipid Res.* **46**, 373–382.
- Rouser, G., Siakotos, A. N., and Fleischer, S. (1966). Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids* **1**, 85–86.
- Snyder, F. (1990). Platelet-activating factor and related acetylated lipids as potent biologically active cellular mediators. *Am. J. Physiol.* **259**, C697–C708.
- Stafforini, D. M., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (2003). Platelet-activating factor, a pleiotropic mediator of physiological and pathological processes. *Crit. Rev. Clin. Lab. Sci.* **40**, 643–672.
- Tessner, T. G., and Wykle, R. L. (1987). Stimulated neutrophils produce an ethanolamine plasmalogen analog of platelet-activating factor. *J. Biol. Chem.* **262**, 12660–12664.
- Venable, M. E., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1993). Platelet-activating factor: A phospholipid autocoid with diverse actions. *J. Lipid Res.* **34**, 691–702.
- Wykle, R. L., O'Flaherty, J. T., and Thomas, M. J. (1988). Platelet-activating factor. *Methods Enzymol.* **163**, 44–54.
- Yamada, K., Asano, O., Yoshimura, T., and Katayama, K. (1988). Highly sensitive gas chromatographic-mass spectrometric method for the determination of platelet-activating factor in human blood. *J. Chromatogr.* **433**, 243–247.

QUANTITATIVE MEASUREMENT OF PHOSPHATIDYLINOSITOL 3,4,5-TRISPHOSPHATE

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Contents

1. Introduction	118
2. Measuring Levels of Radioactively Labeled Phosphoinositides in Isolated Cells	120
2.1. Preparation of monomethylamine reagent	121
2.2. Radiolabeling of cells with [^{32}P]P _i and stimulation with agonists	121
2.3. Extraction of cellular lipids	121
2.4. Deacylation of extracted lipids	122
2.5. High-performance liquid chromatography (HPLC) separation of deacylated lipids	122
3. Measuring PtdIns(3,4,5)P ₃ by Protein–Lipid Overlay	122
3.1. Preparation of recombinant GRP1 PH domain	123
3.2. Stimulation of neutrophils and extraction of cellular lipids	125
3.3. Neomycin bead-based purification of total PIs	126
3.4. Protein–lipid overlay	126
4. Conclusions	126
Acknowledgments	128
References	128

Abstract

The activation of class I phosphoinositide 3-kinases (PI3Ks) by cell surface receptors represents the initiation of a large and complex signaling network that couples many growth factors, antigens, and inflammatory stimuli to important cellular responses, such as cell growth, survival, and movement. The most direct measurement of class I PI3K activity in cells is the rate of production of its lipid product, phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃]. This chapter describes in detail two approaches used to estimate the levels of

PtdIns(3,4,5)P₃ in cells. One approach uses radiotracer labeling of cells, lipid extraction, deacylation, and subsequent quantitation of phosphoinositides by anion-exchange high-performance liquid chromatography. The second approach uses a novel, nonradioactive assay in which the cellular lipids are extracted, phosphoinositides are enriched through binding to a neomycin matrix, dried onto a nitrocellulose membrane, and PtdIns(3,4,5)P₃ quantified by a protein–lipid overlay approach using a GRP1 PH domain probe.

1. INTRODUCTION

The class I phosphoinositide 3-kinase (PI3K) signaling pathway is now accepted to be one of the most prevalent signaling pathways used by cell surface receptors to control cellular events. Receptors for a huge variety of extracellular ligands, including growth factors, hormones, adhesion molecules, neurotransmitters, antigens, and a variety of inflammatory stimuli, are known to use this pathway to regulate numerous cellular responses (Vanhaesebroeck *et al.*, 2001; Wymann and Pirola, 1998). These cellular responses include complex, coordinated events such as cell growth, division, survival, and movement (Engelman *et al.*, 2006; Hawkins *et al.*, 2006). Further, recent appreciation that several components of the class I PI3K signaling pathway are common oncogenes (e.g., the PI3K α isoform itself) or tumor suppressors (e.g., PTEN) has attracted interest in trying to inhibit this pathway as a novel approach to cancer therapy (Cully *et al.*, 2006; Shaw and Cantley, 2006; Stephens *et al.*, 2005). Thus from both an academic and a clinical point of view, there is a need to accurately quantify the activity of this signaling pathway in cells and tissues.

It is now generally accepted that class I PI3Ks are enzymes that phosphorylate the 3-OH of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] resident in the inner leaflet of the plasma membrane to form phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] (Stephens *et al.*, 1993). PtdIns(3,4,5)P₃ can be dephosphorylated at the 5 position (by the SHIP family of phosphatases; Kalesnikoff *et al.*, 2002) or 3 position (by the PTEN family of phosphatases; Gericke *et al.*, 2006) to form phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P₂] or PtdIns(4,5)P₂, respectively (see Fig. 7.1 for a summary of the major routes of metabolism of phosphoinositides). The rapid rises in PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ are the main signals generated by this pathway and act as membrane-captive messengers that control a plethora of intracellular events by binding directly and specifically to several so-called effector proteins in cells. These effector proteins are often predominantly resident in the cytosol of resting cells but translocate in varying quantities to the PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ generated at the plasma membrane by virtue of domains that bind PtdIns(3,4,5)P₃ and/or PtdIns

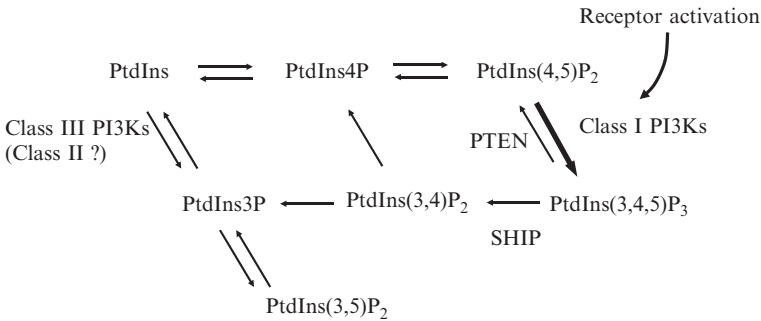


Figure 7.1 The major pathways of phosphoinositide metabolism.

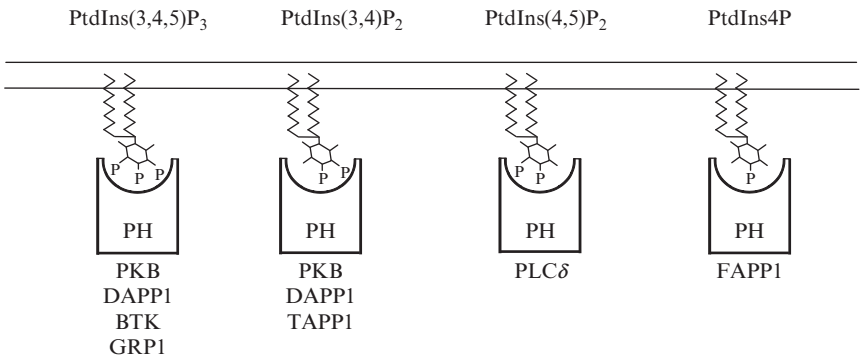


Figure 7.2 Examples of PH domain-containing effectors of phosphoinositide signaling pathways and their relative binding specificities.

(3,4)P₂ directly and specifically (Cullen *et al.*, 2001; Hurley, 2006; Lemmon, 2003). The most well-established domains that perform this role are a subset of PH domains [see Fig. 7.2 for some examples of PH domain-containing effectors of phosphoinositide signaling, including those with specificity for PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₂].

The most direct measure of the activation of class I PI3Ks is the rate of production of PtdIns(3,4,5)P₃. Unfortunately, quantifying the levels of PtdIns(3,4,5)P₃ (which, even in cases of extreme stimulation of the PI3K pathway, only amount to a maximum of 5% of polyphosphoinositides) is perceived as technically demanding. More often the activity of class I PI3Ks has been estimated indirectly, for example, by the appearance of PI3K activity or protein in antiphosphotyrosine or antisignaling complex protein-directed immunoprecipitates. The problem with this type of approach is that it is a qualitative rather than quantitative measure, as the activity status of the PI3K in isolated signaling complexes is not proportional to its activity in the cellular

environment. This approach is also only useful with class IA PI3Ks, which are activated by recruitment into protein tyrosine kinase-directed signaling complexes and not with class IB PI3Ks, which are activated by transient association with heterotrimeric G proteins (Hawkins *et al.*, 2006).

More recently, activity of the class I PI3K pathway is usually assessed by even more indirect measures, such as the activity status of established downstream effectors, for example, the specific phosphorylation of the serine/threonine kinase AKT/PKB or the subcellular localization of green fluorescent protein (GFP)-tagged PH domain probes. While these approaches have proved to be extremely useful (see Donahue *et al.*, 2007), they make the assumption that these readouts are proportional to the levels of PtdIns(3,4,5)P₃, which is likely only to apply to specific ranges and contexts of class I PI3K activation. Furthermore, the involvement of PI3K signaling in a cellular response is often inferred only from the effect of potent inhibitors of this pathway (e.g., wortmannin, LY294002) and not by specifically measuring its activation (Hawkins *et al.*, 2006). This chapter describes two protocols for directly measuring PtdIns(3,4,5)P₃ levels in cells that should be accessible to most laboratories, which, it is hoped, will encourage more direct measurement of the activity of this important signaling pathway.

2. MEASURING LEVELS OF RADIOACTIVELY LABELED PHOSPHOINOSITIDES IN ISOLATED CELLS

This methodology allows direct quantitation of the levels of several phosphoinositides in cells, generally phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 4-phosphate (PtdIns4P), PtdIns(3,4)P₂, PtdIns(4,5)P₂, and PtdIns(3,4,5)P₃. As with most radiolabeling studies, the extent to which the radiolabeled species measured is a true reflection of the unlabeled species is an issue, the extent of which depends on many factors, including pooling and equilibration times to isotopic equilibrium. Several studies have successfully labeled the phosphoinositides of cells in culture to isotopic equilibrium with [³H]inositol in *myo*-inositol-depleted culture medium (e.g., Barker *et al.*, 2004). Disadvantages of this approach are the high cost of the [³H]inositol and the long periods of labeling (4 to 6 days). Other studies have radiolabeled phosphoinositides in cells by incubating them with [³²P]P_i in P_i-free media (e.g., Auger *et al.*, 1989; Carter *et al.*, 1994; Jackson *et al.*, 1992; Stephens *et al.*, 1990). Advantages of this approach are the relatively low cost of the [³²P]P_i and the relatively short periods of time required to radiolabel the monoesterified phosphates of the polyphosphoinositides to “pseudo isotopic equilibrium” with the [³²P]P_i and [γ -³²P]ATP pools inside cells (45–90 min; Creba *et al.*, 1983; King *et al.*, 1989), allowing its use with primary cells. A protocol used successfully to

measure ³²P-labeled PtdIns3P, PtdIns4P, PtdIns(3,4)P₂, PtdIns(4,5)P₂, and PtdIns(3,4,5)P₃ in [³²P]P_i-labeled cells undergoing activation of class I PI3K is described (e.g., Condliffe *et al.*, 2005; Jackson *et al.*, 1992; Stephens *et al.*, 1990).

2.1. Preparation of monomethylamine reagent

Methylamine is a highly noxious gas and care should be taken to avoid inhalation. This process should be carried out under a good fume hood. Prepare the reagent by slowly bubbling methylamine gas (we use a Fluka cylinder and regulator) into a mixture of MeOH/H₂O/*n*-butanol [4/3/1 (v/v)] on dry ice until the final volume increases to 1.625× original (be aware that the mixture heats up as methylamine is added). Aliquot the reagent quickly into glass vials (e.g., 10-ml aliquots into glass scintillation vials) and store at −80°. Warm aliquots to room temperature and use immediately.

2.2. Radiolabeling of cells with [³²P]P_i and stimulation with agonists

Label cells with [³²P]P_i in phosphate-free balanced salts solution (variables are cell dependent, but try 0.5 mCi/ml, 70 min at 37°, 2.5 × 10⁷ cells/ml). Wash cells in [³²P]P_i-free medium at room temperature (e.g., three times with 20× cell volumes: think about radiochemical hazard!).

Resuspend cells in balanced salts at approximately 2.5 × 10⁷ cells/ml. Stimulate 150 μl aliquots ± 20 μl hormones at 37° (if possible in glass bottles; e.g., Cam Lab, Microcap, 5 ml volume). Terminate incubations by addition of 750 μl CHCl₃/MeOH/H₂O (32.6%:65.3%:2.1%, v/v) to produce a homogeneous primary extraction phase (samples can be stored at −20° at this stage).

2.3. Extraction of cellular lipids

Separate phases by the addition of (a) 725 μl CHCl₃ (containing 10 μg Folch lipids, e.g., Sigma B1502; this can also contain [³H]PtdIns(4,5)P₂ to act as an internal chromatographic and extraction marker) and (b) 170 μl 2 M HCl, 10 mM tetrabutylammonium sulfate (to give a final ratio of aqueous/MeOH/CHCl₃ of 3/4/8). Vortex and centrifuge to separate phases (1000g, 5 min).

Remove lower phase carefully into 2-ml Eppendorf tubes (use siliconized Gilson tips and equilibrate tips in CHCl₃ before use) already containing 0.708 ml synthetic upper phase (prepare freshly by mixing 1 M HCl, 25 mM Na₂EDTA, pH 7.0, 5 mM tetrabutylammonium sulfate/MeOH/CHCl₃ in proportions 3/4/8, centrifuge to separate phases, and store in

glass). Mix phases and centrifuge (microfuge, 5 min). Carefully remove lower phase into clean 2-ml Eppendorf tubes [Note: more efficient extraction of $\text{PtdIns}(3,4,5)\text{P}_3$ can be achieved by reextracting both the initial upper phase and the synthetic upper phase, sequentially, with an aliquot (1.108 ml) of synthetic lower phase.] Dry *in vacuo* (can be stored at this stage at -80°).

2.4. Deacylation of extracted lipids

Deacylate dry lipids by adding 200 μl of monomethylamine reagent (use an aliquot freshly thawed to room temperature and use immediately). Warm tubes to 53° for 5 min (place heavy plate on tubes to avoid caps blowing off). Vortex tubes vigorously and then return to a 53° water bath for a further 25 min. Cool samples to room temperature and dry *in vacuo*.

Add 0.5 ml H_2O and 0.6 ml petroleum ether (bp $40\text{--}60^\circ$)/*n*-butanol/ethyl formate [4/20/1 (v/v)]. Vortex and microfuge (5 min). Carefully remove upper organic phase and discard. Wash lower, water-soluble phase with a further 0.6 ml of petroleum ether/butanol/ethyl formate mix. Vortex, centrifuge, and discard upper phase as described earlier. Dry lower phase and interface *in vacuo*.

2.5. High-performance liquid chromatography (HPLC) separation of deacylated lipids

Redissolve pellet (bath sonicate briefly, vortex) in 2.0 ml H_2O filter ($0.45\ \mu\text{m}$) and analyze ^{32}P -deacylated lipids by HPLC: use Whatman Partisphere 5SAX column, 12.5 cm, flow 1.0 ml/min, gradient H_2O (A) vs 1.25 M NaH_2PO_4 (B), e.g., 0 min, 0% B; 1 min, 1% B; 30 min, 6% B; 31 min, 15% B; 60 min, 25% B; 61 min, 33% B; 80 min, 60% B; 81 min, 100% B. Collect 0.5-min fractions and add scintillant [check if it will dissolve salt in fractions; we add 0.5 ml $\text{MeOH}/\text{H}_2\text{O}$ (1:1, v/v) for fractions 1–130 and 0.5 ml H_2O for fractions 130–180, plus 3.5 ml Packard “Ultima Gold” scintillant]. An example of typical HPLC separations of ^{32}P -labeled phosphoinositides derived with this protocol is shown in Fig. 7.3.

3. MEASURING $\text{PtdIns}(3,4,5)\text{P}_3$ BY PROTEIN-LIPID OVERLAY

This methodology is based on the use of a recombinant PH domain with high specificity for $\text{PtdIns}(3,4,5)\text{P}_3$ as a probe to measure its concentration in a cellular lipid extract that had been subjected to a neomycin-based purification of total phosphoinositides (PIs). In theory, this approach

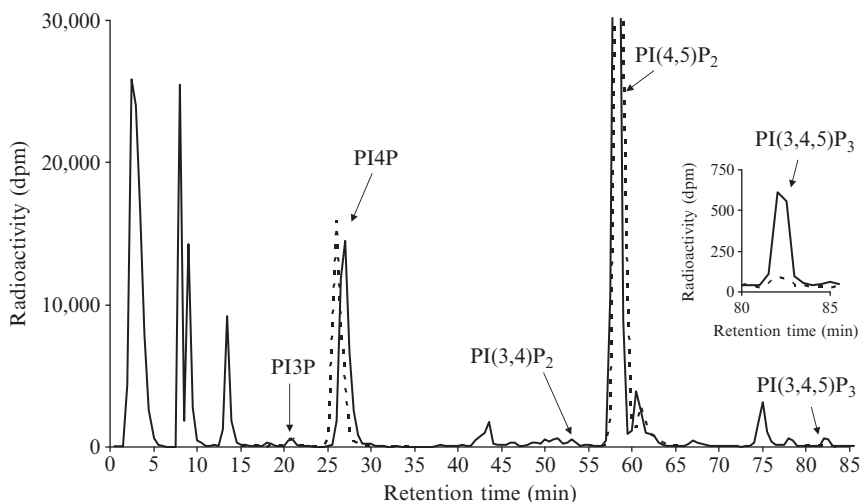


Figure 7.3 Examples of ion-exchange chromatography of deacylation products derived from lipid extracts of either control (dotted line) or fMLP (solid line)-stimulated ³²P-labeled mouse neutrophils. [³²P]_i-labeled neutrophils (0.5 mCi ml⁻¹, 2.5 × 10⁷ cells ml⁻¹) from mice bone marrow were stimulated or not with fMLP (10 μM) for 10 s (for details, see Condliffe *et al.*, 2005). Phosphoinositide levels were quantified by deacylation, HPLC, and scintillation counting. Relative proportions of the phosphoinositides quantified in these separations are shown in Table 7.1.

could be used for any PIs for which a PH domain with sufficient specificity exists. However, to our knowledge this approach has only been used to measure PtdIns(4,5)P₂ from cell lines (Divecha *et al.*, 2002) and PtdIns(3,4,5)P₃ from isolated neutrophils (Guillou *et al.*, 2007). This section describes use of the GRP1 PH domain to measure class I PI3K activation and the accumulation of PtdIns(3,4,5)P₃ in neutrophils. The GRP1 PH domain is known to be highly specific for PtdIns(3,4,5)P₃ (Venkateswarlu *et al.*, 1998). It has been used in other assays for PtdIns(3,4,5)P₃ (Furutani *et al.*, 2006; Gray *et al.*, 2003) and as a probe to visualize PtdIns(3,4,5)P₃ in cells by live imaging (Gray *et al.*, 1999) and electron microscopy (Lindsay *et al.*, 2006).

3.1. Preparation of recombinant GRP1 PH domain

We prepare a recombinant version of the GRP1 PH domain fused to a C-terminal GFP tag and to an N-terminal 6× histidine tag from baculovirus-infected Sf9 cells. A mutant version of the GRP1 PH domain unable to interact with phosphoinositides is obtained by site-directed mutagenesis changing K273 to A. After infection with the relevant virus, Sf9 cells are grown under standard conditions. Cells are harvested into ice-cold

Table 7.1 Relative proportions of different PIs quantified by radioactive counting of fractions separated by ion-exchange chromatography in Fig. 7.1

	Total radioactivity (dpm)		Radioactivity (%)		Relative proportion of PI (%) (corrected for the number of monoesterified phosphate groups)	
	Control	fMLP	Control	fMLP	Control	fMLP
PtdIns(3)P	919	997	0.522	0.591	0.84	0.95
PtdIns(4)P	39,996	38,641	22.707	22.894	37.20	37.49
PtdIns(3,4)P ₂	502	560	0.285	0.332	0.23	0.27
PtdIns(4,5)P ₂	134,636	127,410	76.439	75.489	61.70	60.92
PtdIns(3,4,5)P ₃	83	1172	0.047	0.694	0.03	0.37

0.41% KCl, 2.66% sucrose, 20 mM MgCl₂, and 8 mM NaH₂PO₄ (pH 6.2, 25°). Cells are then washed and frozen in liquid N₂ and stored at -80°.

The 6× histidine tag containing proteins is purified using a metal-ion chelation column (Talon, Clontech). Cell pellets are thawed and sonicated into 0.1 M NaCl, 50 mM sodium phosphate (pH 8.0, 4°), 10 mM Tris-HCl (pH 8.0, 4°), 1 mM MgCl₂, and antiproteases (10 µg/ml each of pepstatin A, aprotinin, leupeptin, antipain, and bestatin and 0.1 mM phenylmethylsulfonyl fluoride). A 120,000g cytosolic fraction of 100 ml is prepared and supplemented with additional Tween 20 and betaine [0.05 (w/v) and 1%, respectively] and loaded onto a column of Talon resin (10 ml). After loading, the column is washed sequentially with 20 column volumes each of buffers A, B, C, and D. Buffer A contains 50 mM sodium phosphate (pH 8.0, 4°), 10 mM Tris-HCl (pH 8.0, 4°), 0.15 M NaCl, 1% betaine, and Tween 20 (0.5%, w/v). Buffer B contains buffer A plus Triton X-100 (1%, w/v). Buffer C contains buffer A but pH 7.1 and 4°. Buffer D contains buffer A but pH 7.5 and contains Tween 20 (0.02%, w/v), ethylene glycol (0.05%, v/v), and 1 mM MgCl₂. The Talon resin is then washed with 8 column volumes of buffer E (which contains buffer D supplemented with 10 mM imidazole, pH 7.5) and the GRP1 PH domain eluted in buffer F (which contains buffer D supplemented with 70 mM imidazole, pH 7.5, final concentration). Typically, 1-ml fractions are immediately collected, supplemented with 1 mM dithiothreitol and 1 mM EGTA, frozen in liquid nitrogen, and stored at -80°. A small volume protein assay is carried out on these fractions, and samples are also run on SDS-PAGE gels to confirm the purity and yield of the probe (typically 3 mg of recombinant protein per liter of Sf9 culture).

3.2. Stimulation of neutrophils and extraction of cellular lipids

Resuspend cells in balanced salts at approximately 1.4×10^7 cells/ml. Stimulate 150 µl aliquots $\pm$ 20 µl hormones at 37° (in clean 2-ml Eppendorf tubes). Terminate incubations by the addition of 750 µl CHCl₃/MeOH/H₂O (32.6%:65.3%:2.1%, v/v) to produce a homogeneous primary extraction phase (samples can be stored at -20° at this stage).

Separate phases by addition of (a) 725 µl CHCl₃ and (b) 170 µl 2 M HCl, 10 mM tetrabutylammonium sulfate (to give a final ratio of aqueous/MeOH/CHCl₃ of 3/4/8). Vortex and centrifuge to separate phases (1000g, 5 min).

Remove lower phase carefully into 2-ml Eppendorf tubes (use siliconized Gilson tips and equilibrate tips in CHCl₃ before use) already containing 0.708 ml synthetic upper phase (prepare freshly by mixing 1 M HCl, 25 mM Na₂EDTA, pH 7.0, 5 mM tetrabutylammonium sulfate/MeOH/CHCl₃ in proportions 3/4/8, centrifuge to separate phases, and store in glass). Mix phases and centrifuge (1000g, 5 min). Carefully remove lower phase into clean 1.5-ml Eppendorf tubes. Dry samples *in vacuo*.

3.3. Neomycin bead-based purification of total PIs

Dissolve dry lipid extract in 500 μ l of a mixture containing 100 ml chloroform, 200 ml methanol, 3.2 ml 2 M ammonium formate, and 16.8 ml H₂O. Add the equivalent of 10- μ l packed neomycin beads (Palmer, 1981; Shacht, 1978) and mix end over end on a rotating wheel for 20 min. Wash beads three times with the same chloroform, methanol, and ammonium formate mixture.

Elute PIs by adding 950 μ l of methanol/chloroform/HCl 2.4 M (500:250:200), spin beads down (1000g, 30 s), and transfer the supernatant to a new clean 2-ml Eppendorf tube. Add chloroform (750 μ l) and water (125 μ l), vortex vigorously, and centrifuge (10,000g, 5 min). Collect the lower phase containing purified PIs and dry *in vacuo*.

3.4. Protein–lipid overlay

The protein–lipid overlay approach method has been described extensively by Kavran *et al.* (1998) and Dowler *et al.* (2000, 2002).

Dissolve purified PIs in 3 μ l of chloroform/methanol/HCl 12 M (200:100:1) and spot onto a reinforced nitrocellulose membrane (Hybond C-Extra, Amersham). Block nonspecific binding sites by incubating the membranes overnight at 4° with the GFP-tagged GRP1 PH domain at 0.5 μ g/ml in Tris-buffered saline (TBS) containing 2% bovine serum albumin (BSA) and 0.05% Tween 20.

Wash the membranes in TBS/0.05% Tween 20 three times for 10 min and incubate with the primary antibody (polyclonal anti-GFP) diluted in TBS containing 2% BSA and 0.05% Tween 20.

Wash the membrane three times in TBS/0.05% Tween 20 and incubate with antirabbit antibody conjugated to horseradish peroxidase (HRP; Santa Cruz) diluted in TBS containing 2% BSA and 0.05% Tween 20. Wash three times in TBS/0.05% Tween 20 and visualize using ECL (Amersham) or a similar type of HRP chemiluminescent detection system. Quantify by direct visualization of the chemiluminescence using a charge-coupled device camera (Image Reader LAS-1000; Fuji film). Perform densitometry and subtract background with appropriate software (AIDA Image Analyzer). An example of an fMLP-induced accumulation of PtdIns(3,4,5)P₃ in mouse neutrophils quantified by this protocol is provided in Fig. 7.4.

4. CONCLUSIONS

This chapter presented two distinct approaches for quantitative measurements of PtdIns(3,4,5)P₃. The first approach is time-consuming (approximately 1 week of 50% effort to analyze 12–18 samples), depends

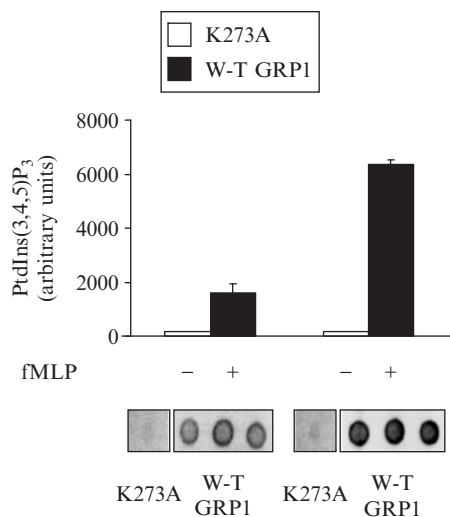


Figure 7.4 Example of protein–lipid overlay-based analysis of PtdIns(3,4,5)P₃ accumulation in mouse neutrophils. Neutrophils (2×10^6) prepared from mice bone marrow were primed with TNF α (500 U/ml) and stimulated with fMLP (10 μ M) for 10 s. Total lipids were extracted, and PIs were purified using neomycin beads and spotted onto nitrocellulose filters for protein–lipid overlay using GFP-GRP1 PH or GFP-(K273A)-GRP1 as the primary probes (for details, see Guillou *et al.*, 2007). Data were analyzed by densitometry and are presented as means $\pm$ standard deviation.

on radioactive labeling, and requires HPLC. However, we have found this assay to give very accurate and reproducible results, particularly if PtdIns(3,4,5)P₃ levels are normalized between experiments to control levels of PtdIns(4,5)P₂. Furthermore, it allows not only quantification of PtdIns(3,4,5)P₃, but also other polyphosphorylated phosphoinositides. The second approach does not require radiolabeling and allows several samples to be processed in parallel (approximately 2 days to analyze 12 samples). PtdIns(3,4,5)P₃ levels are normalized to the number of cells from which the total lipids were initially extracted; although we have found this adequate, we have generally found that estimates of PtdIns(3,4,5)P₃ levels are not as accurate as with the radiolabeling approach. It also requires some specific materials that are not yet widely available commercially (such as the GRP1 PH domain probe and the neomycin beads) and, thus far, we have only used it to measure PtdIns(3,4,5)P₃ in human and mouse neutrophils stimulated by agonists binding to G-protein-coupled receptors (Guillou *et al.*, 2007), which characteristically produce high levels of PtdIns(3,4,5)P₃. It would appear, however, that this new technique offers great potential for improvement at the level of initial phosphoinositide enrichment, the choice of surface for lipid presentation, and the choice of probe (including the use of probes selective for other phosphoinositides) and, in principle, it appears

applicable for measuring PtdIns(3,4,5)P₃ levels in lipid extracts under conditions where radiolabeling would be extremely difficult, for example, working with animal tissues.

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REFERENCES

- Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**, 167–175.
- Barker, C. J., Wright, J., Hughes, P. J., Kirk, C. J., and Michell, R. H. (2004). Complex changes in cellular inositol phosphate complement accompany transit through the cell cycle. *Biochem. J.* **380**, 465–473.
- Carter, A. N., Huang, R., Sorisky, A., Downes, C. P., and Rittenhouse, S. E. (1994). Phosphatidylinositol 3,4,5-trisphosphate is formed from phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets. *Biochem. J.* **301**, 415–420.
- Condliffe, A. M., Davidson, K., Anderson, K. E., Ellson, C. D., Crabbe, T., Okkenhaug, K., Vanhaesebroeck, B., Turner, M., Webb, L., Wymann, M. P., Hirsch, E., and Ruckle, T., *et al.* (2005). Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood* **106**, 1432–1440.
- Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Mitchell, R. H., and Kirk, C. J. (1983). Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca²⁺-mobilizing hormones. *Biochem. J.* **212**, 733–747.
- Cullen, P. J., Cozier, G. E., Banting, G., and Mellor, H. (2001). Modular phosphoinositide-binding domains: Their role in signalling and membrane trafficking. *Curr. Biol.* **11**, 882–893.
- Cully, M., You, H., Levine, A. J., and Mak, T. W. (2006). Beyond PTEN mutations: The PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat. Rev. Cancer* **6**, 4–92.
- Divecha, N., Roefs, M., Los, A., Halstead, J., Bannister, A., and D'Santos, C. (2002). Type I PIP kinases interact with and are regulated by the retinoblastoma susceptibility gene product-pRB. *Curr. Biol.* **12**, 582–587.
- Donahue, A. C., Kharas, M. G., and Fruman, D. A. (2007). Measuring phosphorylated Akt and other phosphoinositide 3-kinase-regulated phosphoproteins in primary lymphocytes. *Methods Enzymol* **434**, Chap. 8 (this volume).
- Dowler, S., Currie, R. A., Campbell, D. G., Deak, M., Kular, G., Downes, C. P., and Alessi, D. R. (2000). Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. *Biochem. J.* **351**, 19–31.
- Dowler, S., Kular, G., and Alessi, D. R. (2002). Protein lipid overlay assay. *Sci. STKE* **129**, PL6.
- Engelman, J. A., Luo, J., and Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat. Rev. Genet.* **7**, 606–619.

- Furutani, M., Itoh, T., Ijuin, T., Tsujita, K., and Takenawa, T. (2006). Thin layer chromatography-blotting, a novel method for the detection of phosphoinositides. *J. Biochem.* **139**, 163–170.
- Gericke, A., Munson, M., and Ross, A. H. (2006). Regulation of PTEN phosphatase. *Gene* **374**, 1–9.
- Gray, A., Olsson, H., Batty, I. H., Priganica, L., and Downes, C. P. (2003). Nonradioactive methods for the assay of phosphoinositide 3-kinases and phosphoinositide phosphatases and selective detection of signaling lipids in cell and tissue extracts. *Anal. Biochem.* **313**, 234–245.
- Gray, A., Van der Kaay, J., and Downes, C. P. (1999). The pleckstrin homology domains of protein kinase B and GRP1 (general receptor for phosphoinositides-1) are sensitive and selective probes for the cellular detection of phosphatidylinositol 3,4-bisphosphate and/or phosphatidylinositol 3,4,5-trisphosphate *in vivo*. *Biochem. J.* **344**, 929–936.
- Guillou, H., Lécureuil, C., Anderson, K. E., Suire, S., Ferguson, G. J., Ellson, C. D., Gray, A., Divecha, N., Hawkins, P. T., and Stephens, L. R. (2007). Use of the GRP1 PH domain as a tool to measure the relative levels of PtdIns(3,4,5)P₃ through a protein-lipid overlay approach. *J. Lipid Res.* **48**, 726–732.
- Hawkins, P. T., Anderson, K. E., Davidson, K., and Stephens, L. R. (2006). Signalling through class I PI3Ks in mammalian cells. *Biochem. Soc. Trans.* **34**, 647–662.
- Hurley, J. H. (2006). Membrane binding domains. *Biochim. Biophys. Acta* **1761**, 805–811.
- Jackson, T. R., Stephens, L. R., and Hawkins, P. T. (1992). Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. *J. Biol. Chem.* **267**, 16627–16636.
- Kalesnikoff, J., Lam, V., and Krystal, G. (2002). SHIP represses mast cell activation and reveals that IgE alone triggers signaling pathways which enhance normal mast cell survival. *Mol. Immunol.* **38**, 1201–1206.
- Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998). Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. *J. Biol. Chem.* **273**, 30497–30508.
- King, C. E., Hawkins, P. T., Stephens, L. R., and Michell, R. H. (1989). Determination of the steady-state turnover rates of the metabolically active pools of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in human erythrocytes. *Biochem. J.* **259**, 893–896.
- Lemmon, M. A. (2003). Phosphoinositide recognition domains. *Traffic* **4**, 201–213.
- Lindsay, Y., McCoull, D., Davidson, L., Leslie, N. R., Fairservice, A., Gray, A., Lucocq, J., and Downes, C. P. (2006). Localization of agonist-sensitive PtdIns(3,4,5)P₃ reveals a nuclear pool that is insensitive to PTEN expression. *J. Cell. Sci.* **119**, 5160–5168.
- Palmer, F. B. (1981). Chromatography of acidic phospholipids on immobilized neomycin. *J. Lipid Res.* **22**, 1296–1300.
- Shacht, J. (1978). Purification of polyphosphoinositides by chromatography on immobilized neomycin. *J. Lipid Res.* **19**, 1063–1067.
- Shaw, R. J., and Cantley, L. C. (2006). Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* **441**, 424–430.
- Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1990). Pathway of phosphatidylinositol (3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature* **351**, 33–39.
- Stephens, L. R., Jackson, T. R., and Hawkins, P. T. (1993). Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate: A new intracellular signalling system? *Biochim. Biophys. Acta* **1179**, 27–75.
- Stephens, L., Williams, R., and Hawkins, P. (2005). Phosphoinositide 3-kinases as drug targets in cancer. *Curr. Opin. Pharmacol.* **5**, 357–365.

- Vanhaesebroeck, B., Leever, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535–602.
- Venkateswarlu, K., Gunn-Moore, F., Oatey, P. B., Tavaré, J. M., and Cullen, P. J. (1998). Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. *Biochem. J.* **335**, 139–146.
- Wymann, M. P., and Pirola, L. (1998). Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta* **1436**, 127–150.

MEASURING PHOSPHORYLATED AKT AND OTHER PHOSPHOINOSITIDE 3-KINASE-REGULATED PHOSPHOPROTEINS IN PRIMARY LYMPHOCYTES

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Contents

1. Overview	132
1.1. Phosphoinositide 3-kinase (PI3K) introduction	132
1.2. PI3K signaling in B lymphocytes	133
2. Choosing a Downstream Readout: General Considerations	134
2.1. Downstream readout: Akt phosphorylation	134
2.2. Downstream readout: mTOR activation	135
2.3. Downstream readout: Phosphorylation of Erk	136
3. Protocols for Detection of PI3K-Regulated Phosphoproteins by Immunoblot	137
3.1. Choice of inhibitors	137
3.2. Stimulation of primary B lymphocytes	138
3.3. Harvest, lysis, and SDS-PAGE	139
3.4. Immunoblotting	139
3.5. Interpretation	141
4. Protocols for Detection of Phosphoproteins by Flow Cytometry	142
4.1. Cell type discrimination by surface marker staining	143
4.2. Inhibitor treatment, stimulation, and harvest of primary B lymphocytes	144
4.3. Phosflow detection of pAkt and pErk	144
4.4. Phosflow detection of pS6	145
4.5. Data analysis and interpretation	145
5. Discussion	147
Acknowledgments	150
References	150

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Abstract

Phosphoinositide 3-kinase (PI3K) is a lipid kinase whose activation is crucial for many biological functions in multiple cell types. One research area of particular interest for basic biologists and drug developers is PI3K signaling in lymphocytes. Inhibitor studies and PI3K mutants have demonstrated that PI3K is required for development, activation, proliferation, differentiation, and survival of B lymphocytes, as well as optimal activation and proliferation of T lymphocytes. As the actual products of PI3K can be difficult to measure, the field has often adopted the practice of examining the activation of downstream effectors of PI3K, with the most common readout being phosphorylation of Akt. This chapter discusses key pathways influenced by PI3K signaling and the advantages and caveats of using activation of these pathways as indicators of PI3K activity. In addition, we provide traditional immunoblotting methods of assaying PI3K-dependent pathway activation, as well as more recent flow cytometry-based approaches (termed “phosflow”). Although we describe assays optimized for B lymphocytes, these methods are easily adapted to T lymphocytes and other leukocyte cell types.

1. OVERVIEW

1.1. Phosphoinositide 3-kinase (PI3K) introduction

The PI3K family of lipid kinases phosphorylates the 3-hydroxyl position on the inositol head group of phosphatidylinositol (PtdIns) lipid species embedded in the plasma membrane. There are multiple classes of PI3K, defined primarily by mode of activation and substrate selectivity (for detailed reviews, see Deane and Fruman, 2004; Vanhaesebroeck *et al.*, 2001). Class IA and class IB PI3K are the only classes able to generate the second-messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃]. Cellular proteins containing modular domains that bind PtdIns(3,4,5)P₃ are recruited to the plasma membrane following class I PI3K activation. Chemical inhibitors of PI3K (wortmannin or LY294002) block murine B- and T-cell activation and proliferation, demonstrating the importance of this kinase to lymphocyte function. Class IA PI3K is generally activated downstream of tyrosine kinase receptors in lymphocytes, whereas class IB PI3K is activated downstream of G-protein-coupled receptors. Both class IA and IB PI3Ks exist as heterodimers of a catalytic and a regulatory subunit. There are now numerous mouse strains with targeted deletion of one or more class I PI3K catalytic or regulatory subunit (Deane and Fruman, 2004; Fruman, 2007). Most of these have interesting immune phenotypes; however, much work remains to be done to understand the impact of these knockouts on PI3K signaling mediated by diverse receptors on lymphocytes. One purpose of this chapter is to provide standardized protocols for assessing PI3K-signaling

output in lymphocytes lacking PI3K family members or treated with isoform-selective inhibitors that are emerging from pharmaceutical company screens (Barber *et al.*, 2005; Bilancio *et al.*, 2006).

1.2. PI3K signaling in B lymphocytes

Phosphoinositide 3-kinase is activated in B lymphocytes downstream of multiple receptors, including the B-cell antigen receptor (BCR), CD40, Toll-like receptors (TLRs), and numerous cytokine receptors (Fig. 8.1). In B-cell lines such as WEHI-231 or A20, BCR-mediated PI3K activation is characterized by transient increases in $\text{PtdIns}(3,4,5)\text{P}_3$ levels in the membrane, usually accompanied by a delayed increase in phosphatidylinositol 3,4-bisphosphate [$\text{PtdIns}(3,4)\text{P}_2$] levels (Astoul *et al.*, 1999; Gold and Aebersold, 1994). For quantitation of PI3K kinase activity, the levels of these two PtdIns species can be measured directly via thin-layer

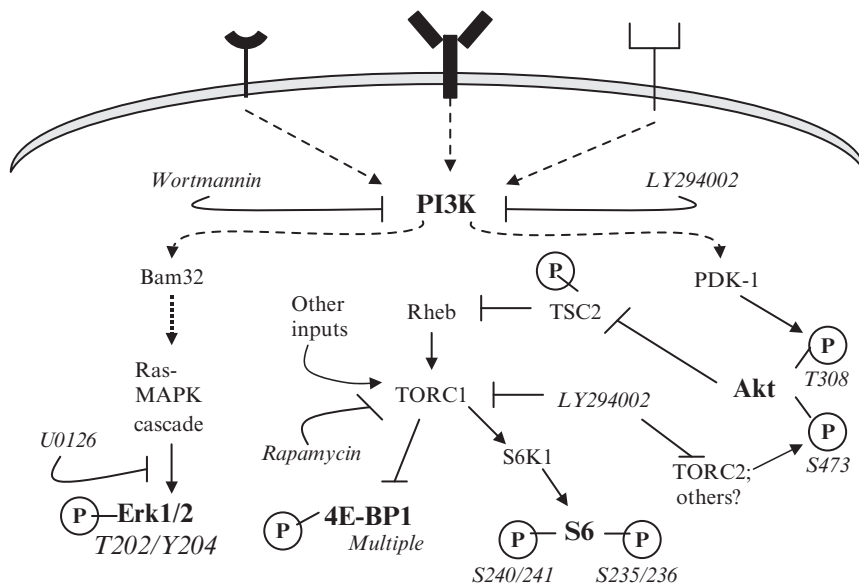


Figure 8.1 Readouts of PI3K activity. PI3K is activated downstream of many receptors in lymphocytes, including the antigen receptor, CD40, TLRs, and multiple cytokines. PI3K activity, through production of 3-phosphorylated phosphoinositides (not shown), leads in turn to the activation of other pathways and the phosphorylation of targets that can be used as readouts of PI3K activation, as described in the text. Protein names in bold represent downstream readouts discussed herein; italicized residues are phosphorylation sites analyzed. Note that S6 residues S240/241 are shown; these sites are PI3K dependent in some systems and can be assayed by immunoblot but not phospho in our hands. Hatched arrows represent unknown intermediates or steps omitted for simplicity. “Other inputs” leading to activation of the TORC1 (mTOR-Raptor) complex include serum components, amino acids, and glucose, as described in the text.

chromatography (TLC) or high-performance liquid chromatography (see Guillou *et al.*, 2007). However, these assays require specialized equipment and large amounts of radioactivity. Moreover, labeling of primary lymphocytes requires a prohibitively large cell number for adequate detection and is complicated by the rapid death of these cells *ex vivo*. Other techniques for direct measurement of $\text{PtdIns}(3,4,5)\text{P}_3$ have been described but these methods have questionable sensitivity and/or specificity when applied to primary lymphocytes (see Section 5). These considerations have led to a search for alternative approaches for ascertaining whether PI3K has been activated. Engagement of each of the receptors mentioned earlier activates a unique suite of pathways. However, many of the downstream effectors of PI3K are conserved between these pathways. For this reason, it is possible to make use of common PI3K targets as readouts of its activation. Although these approaches provide only an indirect measurement of PI3K-signaling output, quantitative data can be obtained and, in many contexts, the results are quite informative. The following sections discuss various PI3K-regulated phosphoproteins before presenting specific protocols for measuring their phosphorylation.



2. CHOOSING A DOWNSTREAM READOUT: GENERAL CONSIDERATIONS

2.1. Downstream readout: Akt phosphorylation

The most established PI3K target in many systems is the serine/threonine kinase Akt (Deane and Fruman, 2004; Fruman, 2004). With an ever-increasing number of known substrates, Akt appears to be crucial for survival and proliferation of B cells following BCR engagement (Yusuf *et al.*, 2004) and is involved in signaling following stimulation through other receptors as well. Activation of Akt requires phosphorylation of two important sites: threonine 308 (T308) in the activation loop and serine 473 (S473) (Fig. 8.1). T308 is phosphorylated by PDK-1 (Alessi *et al.*, 1997; Stephens *et al.*, 1998), whereas several kinases might mediate phosphorylation of S473. One likely S473-kinase is the mammalian target of rapamycin (mTOR) complex 2 (TORC2; see later) (Sarbasov *et al.*, 2005). Both of these phosphorylation events appear to be PI3K dependent, as there is little or no phosphorylation at these sites in unstimulated cells, and mitogen-stimulated Akt phosphorylation is blocked by pharmacological inhibitors of PI3K and reduced in lymphocytes from various PI3K knockout mouse strains (Bilancio *et al.*, 2006; Clayton *et al.*, 2002; Deane *et al.*, 2007; Glassford *et al.*, 2005; Hess *et al.*, 2004; Okkenhaug *et al.*, 2002; Suzuki *et al.*, 2003; Vigorito *et al.*, 2004). Furthermore, Akt phosphorylation is observed downstream of receptors that activate either class IA PI3K

(e.g., antigen receptors, CD40, cytokine receptors, TLRs) or class IB (e.g., chemokine receptors) (Andjelic *et al.*, 2000; Guo and Rothstein, 2005; Ortolano *et al.*, 2006; Venkataraman *et al.*, 1999; Vivarelli *et al.*, 2004). For these reasons, the degree of phosphorylation of Akt is an excellent correlate of PI3K activity in cells. As emphasized later, samples treated with global PI3K inhibitors such as wortmannin are useful specificity controls by revealing any PI3K-independent “background” signal in various types of assays. It is important to stress that activation of Akt does not mean that all PI3K-dependent responses will be activated to a similar extent. For example, PI3K-dependent Ca^{2+} mobilization in B cells occurs downstream of the BCR and chemokine receptors, but not via CD40 or most cytokine receptors that nevertheless trigger Akt phosphorylation.

2.2. Downstream readout: mTOR activation

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that functions in one of two complexes: the rapamycin-sensitive mTOR complex-1 (TORC1) containing the adaptor Raptor and the rapamycin-insensitive mTOR complex-2 containing the adaptor Rictor (TORC2) (Fig. 8.1). These complexes exhibit differential regulation and are responsible for the phosphorylation of different substrates. Activation of mTOR in lymphocytes is crucial for mediating the protein synthesis and cell size increases required for proliferation (Richardson *et al.*, 2004). Indeed, the drug rapamycin was first studied as an immunosuppressant and has been in clinical use for this purpose for many years. The best known substrates of TORC1 are 4E-BP1 and S6K, with S6K activation by mTOR leading to phosphorylation of the ribosomal protein S6. S6K and/or S6 is phosphorylated in primary B cells following stimulation with several mitogens, including cross linking anti-IgM antibodies (Abs), lipopolysaccharide (LPS), and anti-CD40+IL-4 (Bilancio *et al.*, 2006; Donahue and Fruman, 2003; Hess *et al.*, 2004). PI3K-dependent activation of TORC1 is mediated by Akt through phosphorylation of the tuberous sclerosis complex (TSC2) (Manning *et al.*, 2002). TSC2 is a negative regulator of TORC1 function via its negative regulation of the Rheb GTPase and is rendered inactive by phosphorylation on Akt-dependent sites (Richardson *et al.*, 2004; Tee *et al.*, 2003; Zhang *et al.*, 2003).

TORC1 activation occurs somewhat downstream from PI3K and is not as direct a readout as Akt phosphorylation (Fig. 8.1). TORC1 is also regulated by factors in serum, and TORC1 activity generally requires the presence of nutrients, most importantly amino acids and glucose (Kam and Exton, 2004; Wullschlegler *et al.*, 2006). The role of PI3K in these signaling inputs to TORC1 is not entirely clear and appears to vary among cell contexts. For example, in some B-cell tumor lines, TORC1 activity maintained by nutrients appears entirely independent of PI3K and occurs in the

absence of Akt phosphorylation (Wlodarski *et al.*, 2005). It is also important to consider that nutrient-dependent TORC1 activity requires the wortmannin-sensitive class III PI3K enzyme in some cellular systems, not class I PI3K (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005). BCR-mediated phosphorylation of S6K, S6, and 4E-BP1 in splenic B cells is substantially blocked by wortmannin and by a selective class IA inhibitor (Bilancio *et al.*, 2006; Donahue and Fruman, 2007), indicating that class IA PI3K signaling plays a major role in this particular pathway. However, inhibition of TORC1 signaling by PI3K inhibitors is rarely as complete as that seen with direct mTOR inhibition by rapamycin. With some stimuli, for example, LPS, TORC1 activation in B cells appears largely PI3K independent (Donahue and Fruman, 2007). The caveat that TORC1 activation is often not completely dependent on PI3K activation is important to remember when interpreting data involving mTOR signaling and emphasizes the importance of including parallel samples treated with rapamycin (total inhibition) and wortmannin (to define the PI3K-dependent component). However, as excellent antibodies (Abs) exist for detection of S6K, S6, or 4E-BP1 phosphorylation, this pathway can be readily used as a reliable readout of PI3K activity in systems where PI3K is known to contribute to TORC1 activation.

A final word of caution for mTOR analyses relates to the PI3K inhibitor LY294002. Like wortmannin, this compound blocks enzyme activity of most PI3K catalytic isoforms. However, LY294002 is a direct inhibitor of both TOR complexes at concentrations normally used to inhibit PI3K (Brunn *et al.*, 1996; Knight *et al.*, 2006). Indeed, LY294002-treated B cells have considerably lower TORC1 signaling than cells treated with wortmannin or a class IA inhibitor (Bilancio *et al.*, 2006; Wlodarski *et al.*, 2005). At higher concentrations (i.e., 100 nM), wortmannin also can inhibit TOR kinase activity directly. Therefore, LY294002 should be avoided in studies of PI3K-dependent mTOR signaling, and wortmannin should be used at concentrations below 100 nM.

2.3. Downstream readout: Phosphorylation of Erk

Phosphoinositide 3-kinase is also involved in the activation of the Erk MAP kinase cascade in B cells through a pathway that may involve the PtdIns(3,4) P₂-binding protein Bam32 and/or PI3K-dependent Ras activation (Han *et al.*, 2003; Jacob *et al.*, 2002). Ras-mediated Raf and Mek activation downstream of the BCR leads to phosphorylation of Erk1/2, and this phosphorylation is mostly blocked by pretreatment of unfractionated B cells with PI3K inhibitors (Bilancio *et al.*, 2006; Hess *et al.*, 2004; Jacob *et al.*, 2002). Good antibodies also exist for phosphorylated Erk1/2, making this pathway another reliable readout of PI3K in some systems. Appropriate controls include samples treated with Mek inhibitors (which generally give

total inhibition of pErk) and samples treated with wortmannin (to define the PI3K-dependent fraction of the signal). However, Erk phosphorylation is not triggered (or is delayed) downstream of many receptors that mediate acute activation of Akt or mTOR. This stresses the importance of choosing relevant downstream readouts in a particular system.

3. PROTOCOLS FOR DETECTION OF PI3K-REGULATED PHOSPHOPROTEINS BY IMMUNOBLOT

For the pathways discussed earlier, reliable immunoblotting protocols have been used by many investigators to examine the phosphorylation state of PI3K targets. Phospho-specific Abs for Akt (S473 or T308), Erk1/2 (T202/Y204), and S6 (S235/236) detect these proteins only when they are phosphorylated on the indicated sites. Although some laboratories have used phospho-specific antibodies to S6 kinase 1 (S6K1) and 4E-BP1 (Bilancio *et al.*, 2006; Prabhu *et al.*, 2007; Wlodarski *et al.*, 2005), we have had more success detecting phosphorylation-dependent mobility shifts of these proteins during SDS-PAGE separation that can be detected by immunoblot with an Ab directed against total protein (Donahue and Fruman, 2003, 2007). The degree to which these phosphorylation events depend on PI3K activation can be assessed by incubation of a sample of cells with PI3K pharmacological inhibitors prior to stimulation. The immunoblot approach is most informative in cases where cell populations are homogeneous and abundant, such as lymphoma cell lines. When using primary lymphocytes, it can be difficult to obtain a sufficient number of cells to achieve a good signal-to-noise ratio, especially if the experiment calls for many conditions to be tested. In addition, heterogeneity in signaling responses among cell subtypes in mixed populations will be indistinguishable. This masking of heterogeneous responses, as well as the sometimes prohibitively large number of cells required for immunoblotting, is addressed in our discussion of flow cytometry-based assays.

3.1. Choice of inhibitors

Inhibitors are becoming available that will allow researchers to study the requirement for specific PI3K catalytic isoforms. For the purposes of this chapter, however, we discuss the standard pan-catalytic PI3K inhibitors LY294002 and wortmannin and the mTOR inhibitor rapamycin. LY294002, originally identified by the Lilly Corporation, binds reversibly to PI3K and is stable in culture and continues to inhibit over several days. In contrast, the naturally occurring compound wortmannin binds irreversibly but has a chemical half-life in culture of roughly 2 h. Both compounds are

light sensitive and can have off-target effects at higher concentrations. LY294002 inhibits the kinase activity of mTOR at a similar concentration to what is used for PI3K inhibition and must therefore be used with care and not in experiments examining mTOR targets. Although wortmannin can also have off-target effects, there appear to be effective concentrations at which the inhibitor is PI3K selective (i.e., ≤ 100 nM).

1. LY294002 and rapamycin should be diluted in 100% ethanol to a stock concentration of 10 mM, kept sterile and light protected, and stored at -80° in a vial with an O-ring cap to prevent evaporation of the solvent. Incubate cells at 5 to 10 μ M (for LY294002) or 10 nM (rapamycin) for 15 min at 37° prior to stimulation and compare with cells treated with a similar dilution of ethanol vehicle. An intermediate dilution in culture medium is usually necessary to achieve an accurate working concentration of rapamycin. It is important to note that rapamycin should not be used as a negative control inhibitor for other PI3K downstream readouts (e.g., pAkt), as mTOR inhibition can affect these pathways indirectly (Wullschleger *et al.*, 2006).
2. Wortmannin should be diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. As repeated freeze/thaw cycles decrease the efficacy of the inhibitor, single-use aliquots of 2 to 3 μ l should be stored in light-protected tubes at -80° . An intermediate dilution in culture medium is usually necessary to achieve an accurate working concentration. Incubate cells at 25 to 100 nM for at least 15 min at 37° prior to stimulation and compare with cells treated with a similar dilution of DMSO vehicle.

3.2. Stimulation of primary B lymphocytes

We have found that detection of the phosphorylated proteins described herein requires at least 1.5 to 2×10^6 primary B lymphocytes per sample. If possible, 5×10^6 cells should be used for detection of pAkt (also for analysis of primary T cells). Purified B cells are obtained from murine spleen by magnetic separation as described previously (Donahue and Fruman, 2003). When using cell lines such as A20, detection is feasible using 0.5 to 1×10^6 cells. Cells should be at a concentration of 1×10^6 /ml and mixed with an equal volume of a warmed (37°) solution containing $2 \times$ final concentration of stimulus for a final concentration of 5×10^5 cells/ml. Note that the final concentration of any drugs present during the pretreatment period is also reduced by half; this is more relevant for reversible inhibitors such as LY294002 than for the covalent irreversible inhibitor wortmannin. Cell stimulations may take place in tissue culture plates in a 37° incubator or in microcentrifuge tubes in a 37° water bath. We find that although the use of microcentrifuge tubes requires multiple tubes for each sample, this approach

makes for a more efficient harvest of cells and therefore causes less variation in stimulation time from sample to sample, especially when treating for very short time periods (e.g., 1 min).

3.3. Harvest, lysis, and SDS-PAGE

Standardized procedures for cell harvest, lysis, SDS-PAGE, and immunoblot are available from many sources, including Coico (2006). Many investigators have used such methods or slight variations to detect PI3K-regulated phosphoproteins. The following sections present our own protocol while highlighting some important considerations for working with primary lymphocytes and phosphoproteins.

Pellet cells for 5 min at 160g (about 1100 rpm in a typical table-top centrifuge) for 5 min and wash twice with cold phosphate-buffered saline (PBS) to remove abundant serum proteins (i.e., albumin) and excess stimulus. If necessary, combine cells from each samples' multiple tubes during this washing process. Aspirate the supernatant following the final wash, disperse the cell pellet by flicking vigorously, and either snap-freeze cells and store at -20° or continue directly. Lyse with Triton X-100 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol) containing protease and phosphatase I and II inhibitor cocktails (Sigma) at 1:100 dilution. Vortex and incubate lysates on ice for 10 min, vortexing roughly every 5 min. Spin lysates for 5 min at top speed in a microcentrifuge at 4° to pellet nuclei, and carefully transfer supernatants to fresh tubes. Add an equal volume of $2\times$ SDS-PAGE sample buffer and heat lysates for at least 5 min at 95° . Lysates can then be stored frozen or immediately loaded onto an SDS-PAGE gel. If determination of protein concentration is desired, remove a small volume of the lysates prior to addition of the $2\times$ sample buffer.

We find that the phosphoproteins described herein are of sufficiently different size (pAkt ≈ 60 kDa; pErk $\approx 42/44$ kDa; pS6 ≈ 32 kDa; 4E-BP1 $\approx 15\text{--}20$ kDa) that when resolved on a large 12% SDS-PAGE gel, their separation makes detection of all of them possible in a single immunoblot. Transfer proteins onto a filter support (standard nitrocellulose is adequate in our experience) and stain with Ponceau S to visualize protein and aid in cutting the membrane into sections, each containing the region around one protein of interest, for immunoblotting.

3.4. Immunoblotting

All of the primary Abs recommended here are obtained from Cell Signaling Technology and are detected using the same immunoblotting protocol: pAkt (S473) rabbit monoclonal Ab (mAb) or pAkt (T308) rabbit mAb, pErk1/2 or p44/p42 (T202/Y204) rabbit mAb, pS6 (S235/236) rabbit polyclonal Ab or mAb, and total 4E-BP1 rabbit polyclonal Ab. These and other key reagents described in this chapter are listed in Table 8.1.

Table 8.1 Reagents and suppliers

Reagent	Supplier
pAkt (S473) rabbit mAb	Cell Signaling Technologies
pAkt (T308) rabbit mAb	Cell Signaling Technologies
pErk1/2 (T202/Y204) rabbit mAb	Cell Signaling Technologies
pS6 (S235/236) rabbit polyclonal Ab	Cell Signaling Technologies
pS6 (S235/236) rabbit mAb	Cell Signaling Technologies
4E-BP1 rabbit polyclonal Ab	Cell Signaling Technologies
Goat anti-rabbit IgG-A488 secondary Ab	Invitrogen (Molecular Probes)
Goat anti-rabbit IgG-FITC secondary Ab	Sigma-Aldrich
Goat anti-rabbit IgG-HRP	Promega
Goat anti-mouse IgG-HRP	Promega
CytoFix/CytoPerm kit	BD Biosciences
Methanol-free formaldehyde (16%)	Polysciences Inc
SuperSignal West Pico chemiluminescent substrate kit	Pierce
Restore Western blot stripping buffer	Pierce

Rock the membrane in blocking solution (5% nonfat dry milk in TBST: 100 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Tween 20) for at least 1 h at room temperature or for longer periods at 4°. Despite reports that milk contains phosphatases that diminish the signal in antiphosphoprotein immunoblots, this has not been our experience for the target proteins described herein, and milk is in fact the blocking solution recommended by Cell Signaling. Then incubate the membrane with primary Ab at a 1:1000 dilution in a 5% bovine serum albumin (BSA)/TBST solution overnight at 4° on a nutator. Wash the membrane with TBST four times, 5 min each, and then add secondary Ab. Horseradish peroxidase-conjugated goat antirabbit IgG secondary Ab (Promega) should be used at a dilution of 1:10,000 in TBST and incubated for 1 h at room temperature on a shaker. Wash four more times with TBST, 5 min each. Visualize bound Ab by enzyme-linked chemiluminescence (ECL; Pierce SuperSignal West Pico chemiluminescence kit) and expose to film.

Equivalent protein loading can be demonstrated by probing the appropriate stripped and reblocked membrane section with primary Ab directed against total Akt protein (Cell Signaling, rabbit polyclonal Ab) or total S6 protein (Cell Signaling, rabbit or mouse mAbs). Antibodies to other proteins (e.g., β -actin; Sigma) can be used as loading controls. However, care should be used in titrating the primary antibody in order to achieve detection in the linear range of the film used for chemiluminescent detection. If the signal is too strong, a shortened secondary Ab incubation (e.g., 30 min) or a twofold dilution of the ECL solution can help. Membranes can

be stripped quickly and easily with Restore Western blot stripping buffer (Pierce) per the accompanying instructions.

3.5. Interpretation

A typical result for cytoplasmic extracts from purified murine B cells stimulated via BCR cross linking is shown in Fig. 8.2. In the case of the phospho-specific Abs (pAkt, pS6, and pErk1/2), stimulation through the BCR causes a robust increase in band intensity, representing phosphorylation at the targeted site. That these phosphorylation events are dependent on PI3K is demonstrated by the loss of signal in lanes containing lysates of cells pretreated with wortmannin. A low level of signal is sometimes detected in unstimulated cells and can be influenced by incubation time and composition of the media (Donahue and Fruman, 2007). It is helpful to remember that PI3K is activated by diverse extracellular stimuli and it is important to determine the basal level of signaling in a particular experimental system. Of note, the general conditions described here are also sufficient for detecting phosphorylation of Akt substrates, including TSC2 and FOXO proteins, using commercially available antibodies selective for Akt phosphorylation sites (Hess *et al.*, 2004; Yusuf *et al.*, 2004).

The Ab used for detection of 4E-BP1 here recognizes total protein, and interpretation of phosphorylation levels is dependent on migration of the

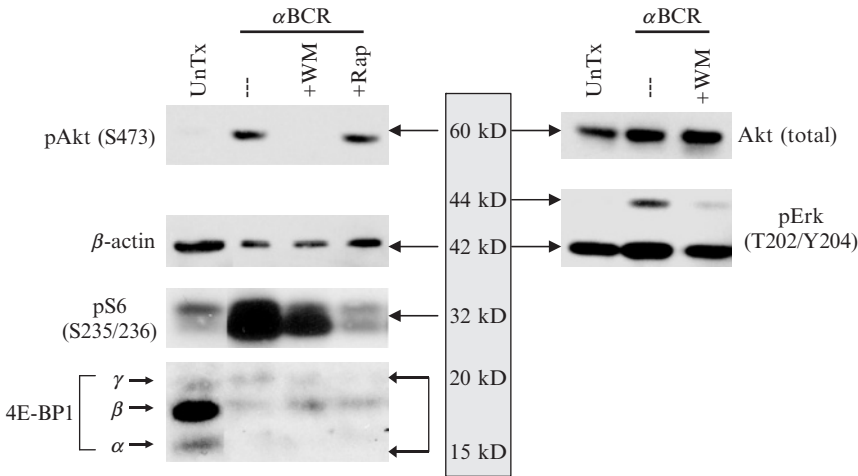


Figure 8.2 Immunoblot detection of PI3K-dependent phosphorylation events. Following BCR cross linking in purified primary B lymphocytes, phosphorylation of multiple PI3K readouts can be visualized on a single immunoblot. Representative experiments using 1.5 to 2×10^6 B cells resolved on 11 to 12% gels are shown. Relative sizes are shown in the center. Carefully cut membranes allow detection of pAkt (S473 or T308), pErk (T202/Y204), pS6 (S235/236), and 4E-BP1 (multiple sites). Loading controls, including total Akt or β -actin, can be detected following stripping of the blot.

protein: the most heavily phosphorylated species of 4E-BP1, labeled γ in Fig. 8.2, migrates the slowest, while the least phosphorylated species, labeled α , migrates fastest. A third, intermediately phosphorylated β form of 4E-BP1 can be detected migrating between the α and the γ forms. A stimulated sample in which 4E-BP1 is highly phosphorylated will show greater signal intensity in the top band when compared to an unstimulated or inhibitor-treated sample, which will show greater intensity in the middle and/or lowest band (Fig. 8.2).

Interpretation of immunoblotting with phospho-specific Abs requires confirmation that a greater or lesser signal intensity in one lane compared to another is because of differential phosphorylation levels and not simply because of a greater or lesser amount of total protein. Thus the inclusion of a loading control in any such experiment is crucial to the credibility of the results. In the case of 4E-BP1 or other proteins where phosphorylation is assessed by the ratio of bands with different migration distances, equivalent loading is less important.

4. PROTOCOLS FOR DETECTION OF PHOSPHOPROTEINS BY FLOW CYTOMETRY

Lymphocytes obtained from lymphoid organs of a mouse or peripheral blood of a human look homogeneous under a light microscope but are remarkably heterogeneous in gene expression and function. Within the general categories of T lymphocyte and B lymphocyte there are numerous subsets that have evolved to fulfill different roles in the adaptive immune system. Evidence is accumulating that differential signaling occurs in distinct subsets even when the same receptor is engaged (Benschop *et al.*, 1999, 2001; Li *et al.*, 2001; Tanaka *et al.*, 2003). Thus, immunoblot analysis of populations such as “total B cells” or “CD4⁺ T cells” examines a heterogeneous population, and cell types present in greater numbers will mask the response of the less numerous subsets. This is of special concern when comparing cells from individuals that have different ratios of these subsets. For example, many PI3K mutant mouse strains have altered lymphocyte development and exhibit altered percentages of T- or B-cell subsets compared to wild-type mice (Deane and Fruman, 2004; Donahue and Fruman, 2004; Koyasu, 2004; Okkenhaug and Vanhaesebroeck, 2003). Although it is possible to obtain relatively pure populations of individual subsets, this requires additional manipulations that can alter the response of the target cell type and often yields fewer cells than are needed for immunoblot analysis. These concerns call for a method that allows discrimination of these different subsets within unfractionated populations, and flow cytometry-based protocols have been established to that end.

Several laboratories (Chow *et al.*, 2001; Fleisher *et al.*, 1999; Krutzik *et al.*, 2005; Perez and Nolan, 2002) have developed methods to analyze the phosphorylation state of proteins of interest via flow cytometry (here abbreviated FACS for fluorescence-activated cell sorting). Several antibody supply companies (e.g., BD Biosciences, Cell Signaling) have developed and optimized antibodies for this approach, which has been termed “phosflow” or sometimes “phosphoflow.” In addition to allowing discrimination of different populations of cells within a single sample via staining with Abs directed against surface markers, the phosflow method requires far fewer cells than immunoblotting. Despite the lower cell number requirements, statistically robust data are obtained as a consequence of the collection of hundreds or thousands of individual cell events per subset analyzed. The approach also avoids potential postlysis artifacts that can occur in traditional methods, including but not limited to the action of cellular phosphatases and proteases. Heterogeneous cell populations can be stimulated en masse, and the responses of specific cell types within a sample can be quantitated and compared. This section presents two protocols for phosflow analysis of PI3K downstream readouts, describing the detection of pAkt, pErk, and pS6 by FACS.

4.1. Cell type discrimination by surface marker staining

A central consideration in phosflow analysis is choosing when to stain cells with antibodies to cell surface markers. Ideally, staining should take place after cell stimulation and fixation so that the binding of antibodies does not initiate signals that could confound interpretation of the results. However, some epitopes are masked or diminished by fixation. One approach is to stimulate cells followed by rapid cooling to 4° without fixation and then staining at this temperature before fixation. A caveat with this method is the potential for ongoing signaling (or phosphatase action) even at reduced temperatures. We have found that in many cases, staining chilled cells at the beginning of the experiment, before warming and addition of experimental stimuli, yields results that are equivalent to those obtained with cells stimulated before staining (data not shown). However, the surface markers used to distinguish the cell type(s) of interest should be chosen carefully, primarily to avoid those surface proteins known to initiate signaling when bound by specific Ab. An additional advantage of this approach is the ability to stain a batch of cells in bulk before splitting into different tubes for stimulation. This reduces time, antibody costs, and variability in staining. Staining of nonfixed cells before stimulation is a requirement when performing live cell-signaling measurements by FACS, for example, measurement of Ca²⁺ mobilization where signaling is measured in real time (Hess *et al.*, 2004).

As with most FACS experiments, markers should also be unambiguous whenever possible, providing clear delineation between populations. Be sure to test each chosen Ab, comparing staining of fixed cells to unfixed

cells, as we have found that some Ab-binding events do not survive the fixation process. In some cases this failure is fluorophore dependent, and the Ab will work if conjugated to a different fluorophore or if biotinylated and revealed with a streptavidin-conjugated secondary Ab. We incubate the cells with the FACS Abs for at least 15 min in Hanks' balanced salt solution on ice. Wash the cells at around 1100 rpm, resuspend them in media to 1×10^6 /ml, and aliquot into plates, microcentrifuge tubes, or even capped FACS tubes.

4.2. Inhibitor treatment, stimulation, and harvest of primary B lymphocytes

Pretreat cells for 15 min with PI3K inhibitors or vehicle and then stimulate the cells with a $2 \times$ volume of warmed mitogen as described earlier. Following stimulation, immediately transfer the cells to FACS tubes if necessary, centrifuge the cells at 1100 rpm for 5 min at 4° , and continue with the appropriate protocol.

4.3. Phosflow detection of pAkt and pErk

After harvest and centrifugation, resuspend the cells to 1×10^6 /ml in $1 \times$ PBS, add 16% MeOH-free formaldehyde (PolySciences) to a final concentration of about 2%, vortex briefly, and then incubate for 10 min in 37° water bath to fix the cells, then chill for 1 min on ice before spinning. When doing cell surface staining on fixed cells, this is the point after which cells are washed and stained with the appropriate antibodies. Spin the cells, decant the supernatant and resuspend in 100 μ l incubation buffer (IB; $1 \times$ PBS, 0.5% BSA). Slowly add 90 μ l ice-cold 100% MeOH while vortexing vortex briefly, and incubate on ice for 30 min to permeabilize the cells. Wash twice with 1 ml IB resuspend in IB, at about 50 μ l/ 1×10^6 cells, and incubate for 15 min at room temperature. Then add primary phospho-specific Ab and incubate for 60 min at room temperature. We use the same phospho-specific Abs for Akt (S473) and Erk (T202/Y204) in phosflow as for immunoblotting. It is essential to determine the optimal concentrations of both primary and secondary Abs empirically for each cell type, beginning in the range of 1:50. Choose the concentrations that give the greatest increase in fluorescence intensity between stimulated and unstimulated or inhibitor-treated cells. We have found that for primary splenic B cells, 1:50 is the optimal dilution for both pAkt (S473) and pErk (T202/Y204) Abs. Wash the cells with 1 ml IB, resuspend in 100 μ l/ 1×10^6 cells volume of secondary Ab, at the empirically determined dilution in IB, and incubate for 30 min at room temperature. For this protocol we make use of a goat anti-rabbit IgG secondary Ab conjugated to Alexa-488 (A488; Molecular Probes/Invitrogen); we have found the optimal dilution for primary B lymphocytes

to be 1:1000. Wash a final time with 1 ml IB and then resuspend to 1×10^6 /ml for acquisition. During preparation of the proofs of this article, we have improved the reliability of pAkt detection by including an amplification step. For a secondary antibody, we now use biotinylated donkey anti-rabbit (Jackson Immuno Research; 1:300 dilution). After 30 min, we wash again with 1 ml IB, then resuspend cells in streptavidin-APC (1:300 in 100 μ l of IB). After 20 min at 4°, continue with final wash and acquisition.

4.4. Phosflow detection of pS6

After harvest and centrifugation, resuspend the cells in 250 μ l CytoFix/CytoPerm fixation buffer (Becton-Dickinson kit) and incubate 20 min on ice. Wash once with 500 μ l staining buffer (SB; $1 \times$ PBS, 1% fetal bovine serum, 0.09% sodium azide, pH 7.4–7.6). When doing cell surface staining on fixed cells, this is the point after which cells are washed and stained with the appropriate antibodies. Wash twice with 500 μ l $1 \times$ perm/wash buffer (Becton-Dickinson kit) to permeabilize the cells. Resuspend after the final wash in 200 μ l $1 \times$ perm/wash buffer and add the same primary anti-pS6 Ab used for immunoblotting, at the empirically determined concentration. We use 0.3 μ l (1:600 dilution) of pS6 Ab per sample for splenic B cells. Incubate the cells for 20 min on ice, wash with 500 μ l $1 \times$ perm/wash, and resuspend in 200 μ l $1 \times$ perm/wash. Add the goat antirabbit IgG-FITC (Sigma) secondary Ab or the goat anti-rabbit IgG-A488 secondary Ab used earlier at the empirically determined concentration and incubate for 20 min on ice. For this protocol we prefer the FITC-conjugated Ab and use 0.3 μ l (1:600) per sample. Wash a final time with 500 μ l $1 \times$ perm/wash and resuspend in a 1×10^6 volume of $1 \times$ perm/wash for acquisition.

4.5. Data analysis and interpretation

An overlay of representative histograms for each Ab described is shown in Fig. 8.3. The PI3K dependence of phosphorylation of each protein is shown by the leftward shift of the peak when cells were pretreated with the inhibitor wortmannin. Where possible, additional inhibitors were used to demonstrate complete inhibition and give a background level of phospho-specific Ab binding, and in lieu of isotype controls, which often are not available for these sorts of experiments. As a control for pS6 we use the TORC1 inhibitor rapamycin (10 ng/ml), and for pErk we use the MEK inhibitor U0126 (10 μ M; from 10 mM stock concentration in ethanol). We find that in primary B cells, the peak shift of pAkt in stimulated cells is modest, but highly reproducible. The degree of separation between unstimulated and stimulated samples can be increased by optimizing the time period of cell “resting” at 37° before stimulation. A 1-h rest period works well. pErk and pS6 give more robust peak shifts, although these can also be

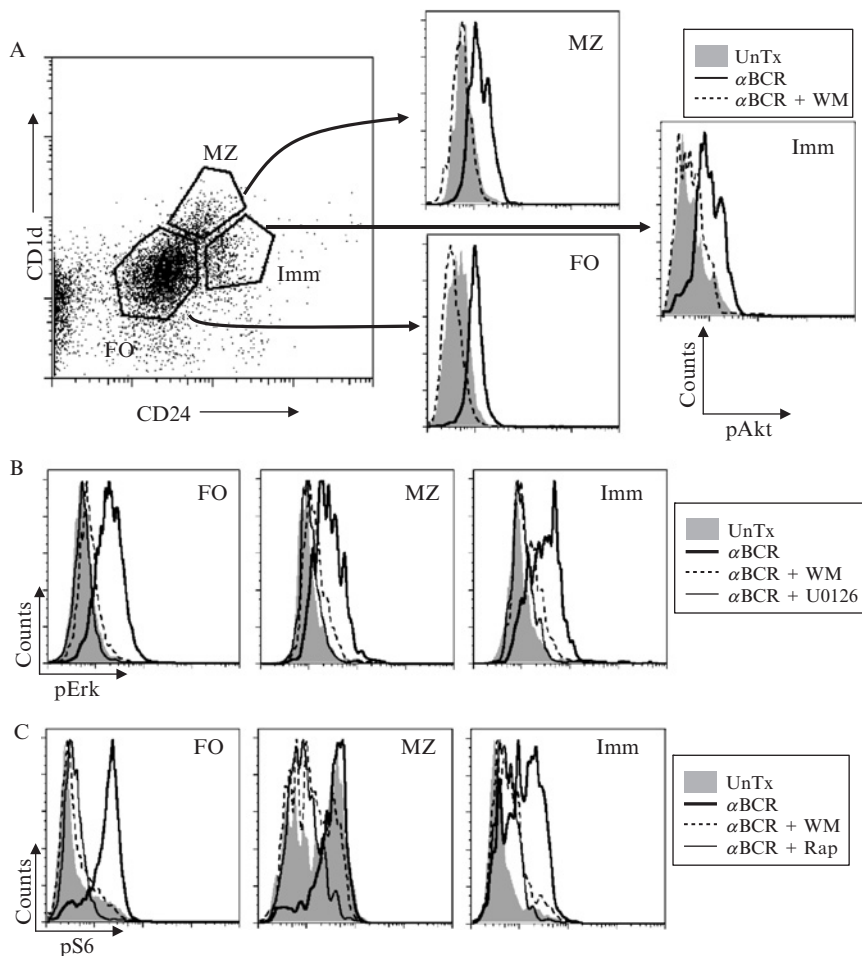


Figure 8.3 Phosflow detection of PI3K-dependent phosphorylation events. A representative experiment depicting analysis of splenic B-cell developmental subsets is shown. Total splenocytes are stained for surface markers CD24 and CD1d as shown in A, allowing differentiation among follicular cells (FO; CD24^{lo}/CD1d^{lo}), marginal zone cells (MZ; CD24^{int}/CD1d^{hi}), and the combined immature subsets (Imm; CD24^{hi}/CD1d^{int}) (Lyubchenko *et al.*, 2005). Cells are then stimulated via BCR cross linking, fixed, permeabilized, and incubated with the same phospho-specific Abs used for detection by immunoblot. (A) Histograms at right represent mean fluorescence intensity (MFI) of pAkt (S473) in FO, MZ, and immature cells. Note that stimulated cells display an increase in pAkt levels as shown by the rightward shift of the stimulated histogram (heavy line) when compared to unstimulated cells (filled histogram). This increase in pAkt levels is blocked by pretreatment with wortmannin (WM; dotted line), as evidenced by the leftward shift in the peak. The peak shift with pAkt (S473) is modest but highly reproducible. (B) Phosflow detection of pErk (T202/Y204) in BCR-stimulated B-cell subsets. The increase in MFI in stimulated cells (heavy line) with respect to unstimulated cells (filled histogram) indicates that Erk is phosphorylated in these cells,

influenced by the length of the rest period before stimulation (Donahue and Fruman, 2007). In splenic T cells, signal intensities are somewhat different, with pAkt detection more robust and pS6 less robust than in B cells. When analyzing data across several experiments, it must be kept in mind that settings vary from day to day in FACS experiments, which means that fluorescence intensity (MFI) values can also vary. It may be necessary, therefore, to calculate fold increases in MFI, with respect to unstimulated cells or some other control, in order to minimize the level of variation between experiments. When choosing gates to delineate separate subsets, it is critical to be conservative (i.e., edges of gates/regions should be well separated). Although restricting the gates reduces the number of cellular events analyzed, it increases the fidelity of subset discrimination. Robust data for even rare populations can be obtained by acquiring an appropriately large number of total cells.

5. DISCUSSION

Measuring PI3K-signaling activity in cells is a frequent goal of investigators in academic and pharmaceutical laboratories. There are a number of methods available to quantitate the PI3K lipid products themselves, and some of these are discussed in Guillou *et al.* (2007). For investigators studying PI3K signaling in primary lymphocytes, the available assays for measuring PtdIns(3,4,5)P₃ and other phosphoinositides directly are, as discussed later, insufficiently sensitive or selective to be readily applied in these cell types. Here we have provided protocols for measuring PI3K-dependent protein phosphorylation events as surrogate readouts of PI3K-signaling activity. We suggest that immunoblotting approaches are best applied to homogeneous cell populations such as lymphoma cell lines, whereas

whereas the PI3K dependence of this response is demonstrated by cells pretreated with wortmannin (dotted line). Pretreatment with the MEK inhibitor U0126 (thin line), which abolishes Erk phosphorylation in this system, establishes a baseline level of fluorescence and demonstrates specificity. (C) Phosflow detection of pS6 (S235/236) in BCR-stimulated B-cell subsets. FO and immature cells show low levels of pS6 in unstimulated cells with robust increases following BCR stimulation, whereas unstimulated MZ cells exhibit high levels of pS6, only slightly lower than those seen after activation. Further, wortmannin pretreatment blocks S6 phosphorylation almost completely in FO and immature cells, but less completely in MZ cells (Donahue and Fruman, 2007). It is the ability to detect these differences in rare subpopulations such as MZ cells that makes the phosflow method so powerful and important. Use of the mTOR inhibitor rapamycin (Rap) demonstrates specificity of the Ab by establishing a fluorescence background level that is TORC1 independent. Note that for each phosphorylation event measured, the cellular response is heterogeneous even within a given subset; that is, the histograms are not uniform. This is most apparent for cells in the marginal zone and immature gates, suggesting further phenotypic categories within these subsets.

FACS-based phosflow assays are particularly useful for analysis of primary lymphocytes where cell numbers are limiting and discrimination of signaling states among subsets is desired.

The phosflow approach carries additional advantages for studying PI3K signaling in lymphocytes, or indeed any signaling event that can be measured by this technique. Using flow cytometers capable of detecting multiple fluorophores, it is possible to analyze samples stained simultaneously for different phosphorylation events. Thus, relationships between different signaling events can be determined (Sachs *et al.*, 2005) and the aggregate signaling states of cellular subsets can be defined (Krutzik *et al.*, 2005; Perez and Nolan, 2002). Throughput can be enhanced and costs reduced by “bar coding” samples with different concentrations of fluorophores such as CFSE prior to pooling and bulk staining/analysis (Krutzik and Nolan, 2006).

One of the potential disadvantages of the phosflow approach is that it is difficult to prepare a “loading control,” that is, to normalize a phospho-specific signal to the total concentration of the target protein. The FACS approach circumvents loading issues in part because the same amounts of cell events are acquired even when total cell recovery differs among samples. Thus, when comparing the same cell type under two different treatment conditions, the total concentration of the target protein will generally be the same on a per cell basis. However, when comparing two different cell subsets, concentration of the target protein might differ. There are antibodies available to the nonphospho forms of the proteins described here (Akt, Erk, S6). However, most of these are polyclonal and the corresponding nonimmune sera are not as ideal as isotype controls for monoclonal antibodies. Nevertheless, the specific signal provided by phosphoAbs can be determined (even polyclonals) by using a cell sample treated with the appropriate pathway inhibitor. Hence, we favor the approach of calculating the change in MFI (Δ MFI) or the MFI fold change between samples $\pm$ inhibitor and then comparing these values between experimental samples. In some cases it might be desirable to compare the percentages of cells above a particular fluorescence threshold rather than the MFI of the entire population. This approach applies more when only a subset of cells responds to the stimulus and the background of nonshifted cells dampens the detection of signal differences.

Another potential issue with phosflow is the ability of some phosphoAbs to recognize multiple proteins in a manner sensitive to the pathway inhibitor. For example, anti-pAkt might have some affinity for other phosphoproteins whose phosphorylation is wortmannin sensitive. For this reason, it is important to determine the overall specificity of a given antibody by probing an immunoblot containing whole cell extracts of the target cell type before and after stimulation $\pm$ inhibitor (and visualizing a full range of molecular weights). The phosflow Abs described here are all highly specific when tested in lymphocytes by this approach (data not shown). There might be other antibodies that detect several bands whose phosphorylation is

wortmannin sensitive. However, if one is only looking for a general readout of PI3K pathway activity, it might be less important to study a single phosphoprotein than to study an aggregate “PI3K-dependent” signal.

As mentioned earlier, some investigators have used monoclonal antibodies directed to $\text{PtdIns}(3,4,5)\text{P}_3$ to quantitate this lipid in primary lymphocytes and cell lines by FACS (Anzelon *et al.*, 2003; Perez *et al.*, 2002, 2003; Sachs *et al.*, 2005). The obvious attraction of this approach is to measure the direct product of class I PI3K rather than a surrogate downstream readout. The commonly used antibody, available from Echelon Biosciences, appears to work quite well for fluorescence microscopy of adherent cells (Chen *et al.*, 2002; Niswender *et al.*, 2003). However, despite a significant effort in our laboratory and by several immunologist colleagues, we have been unable to validate this antibody for detection of $\text{PtdIns}(3,4,5)\text{P}_3$ generation in primary B or T cells. In some cases, the fluorescent signal decreases after cell activation, whereas in other cases there is an increased signal but it is not LY-sensitive. A representative experiment is shown in Fig. 8.4, which

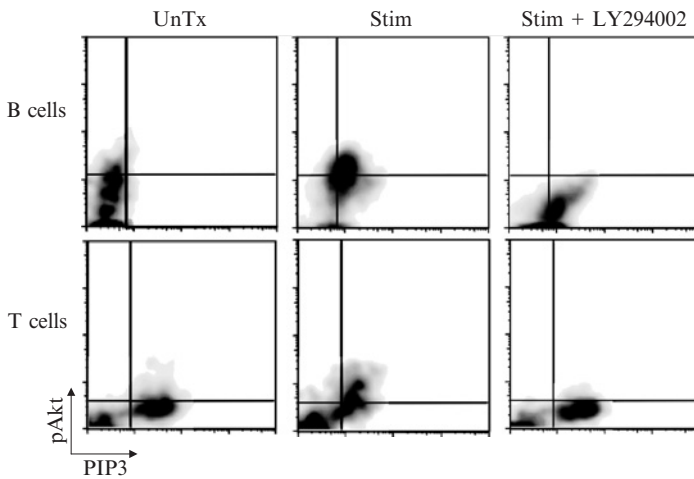


Figure 8.4 A monoclonal antibody to $\text{PtdIns}(3,4,5)\text{P}_3$ does not reveal specific increases in activated mature lymphocytes. Lymph node cells were incubated with unlabeled hamster mAb anti-CD3 to bind the T-cell antigen receptor complex. Cells were then aliquoted, warmed, and either left unstimulated (UnTx) or stimulated (stim) with either anti-IgM (B cells) or goat anti-hamster (T cells) to cross link the antigen receptors in the presence or absence of LY294002. After 1 min, cells were fixed, permeabilized, and stained with mouse mAb anti- $\text{PtdIns}(3,4,5)\text{P}_3$ (PIP3)-biotin and rabbit anti-pAkt, along with rat anti-B220-PE to differentiate between B and T cells. Cells were washed and then stained with secondary antibodies streptavidin-APC and anti-rabbit-Alexa488. Note that in B cells, the increase in $\text{PtdIns}(3,4,5)\text{P}_3$ is not LY-sensitive, whereas the increase in pAkt is blocked by this PI3K inhibitor. In T cells, the increase in pAkt is accompanied by a decrease in apparent detection of $\text{PtdIns}(3,4,5)\text{P}_3$. Similar results were obtained with different fixation/permeabilization protocols, concentrations of antibodies, and activation times.

depicts a two-color experiment in which the detection of pAkt in activated B and T cells increases in parallel to a LY-insensitive increase in PtdIns(3,4,5)P₃ (B cells) or a decrease in PtdIns(3,4,5)P₃ detection (T cells). To our knowledge, none of the published reports using this antibody in mature resting lymphocytes or Jurkat cells has shown data on the wortmannin/LY sensitivity of the observed fluorescence shifts (Anzelon *et al.*, 2003; Perez *et al.*, 2002, 2003; Sachs *et al.*, 2005). It may be that the antibody can detect PtdIns(3,4,5)P₃, as suggested by the enhanced signal in PTEN-deficient B cells (Anzelon *et al.*, 2003), but that it cross reacts with other cellular epitopes that mask the PI3K-dependent changes under physiological conditions of cell stimulation. The specificity of the antibody might also be cell type-dependent, as studies of neutrophils have suggested that robust, LY294002-dependent changes in fluorescence can be measured readily (Kuan *et al.*, 2006).

Other assay systems are available for measuring PtdIns(3,4,5)P₃ in cell extracts without radioactive labeling of the cells (Downes *et al.*, 2003). The specificity of these methods appears quite strong; however, it is not yet clear whether the approaches are sensitive enough to detect PtdIns(3,4,5)P₃ starting from reasonable cell numbers of primary lymphocytes. It is important to mention that elegant imaging techniques can be used to visualize the dynamics of PI3K lipid production and localization in primary lymphocytes and cell lines. The general approach is to use transfection or transgenesis to introduce green fluorescent protein-linked PH domain probes selective for PtdIns(3,4,5)P₃ or other phosphoinositides. Live cell fluorescence imaging can then be employed to examine the subcellular localization of the probes, with redistribution to the membrane an indication of lipid production (Costello *et al.*, 2002; Harriague and Bismuth, 2002). However, these approaches tend to be used more for qualitative assessments of lipid production as well as kinetic and spatial analyses. Until the development of PtdIns(3,4,5)P₃ quantitation assays of sufficient sensitivity and selectivity for lymphocytes, the downstream readouts described herein provide convenient correlates of PI3K-signaling output.

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REFERENCES

- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr. Biol.* **7**, 261–269.

- Andjelic, S., Hsia, C., Suzuki, H., Kadowaki, T., Koyasu, S., and Liou, H. C. (2000). Phosphatidylinositol 3-kinase and NF-kappa B/Rel are at the divergence of CD40-mediated proliferation and survival pathways. *J. Immunol.* **165**, 3860–3867.
- Anzelon, A. N., Wu, H., and Rickert, R. C. (2003). Pten inactivation alters peripheral B lymphocyte fate and reconstitutes CD19 function. *Nat. Immunol.* **4**, 287–294.
- Astoul, E., Watton, S., and Cantrell, D. (1999). The dynamics of protein kinase B regulation during B cell antigen receptor engagement. *J. Cell Biol.* **145**, 1511–1520.
- Barber, D. F., Bartolome, A., Hernandez, C., Flores, J. M., Redondo, C., Fernandez-Arias, C., Camps, M., Ruckle, T., Schwarz, M. K., Rodriguez, S., Martinez, A. C., Balomenos, D., *et al.* (2005). PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. *Nat. Med.* **11**, 933–935.
- Benschop, R. J., Brandl, E., Chan, A. C., and Cambier, J. C. (2001). Unique signaling properties of B cell antigen receptor in mature and immature B cells: Implications for tolerance and activation. *J. Immunol.* **167**, 4172–4179.
- Benschop, R. J., Melamed, D., Nemazee, D., and Cambier, J. C. (1999). Distinct signal thresholds for the unique antigen receptor-linked gene expression programs in mature and immature B cells. *J. Exp. Med.* **190**, 749–756.
- Bilancio, A., Okkenhaug, K., Camps, M., Emery, J. L., Ruckle, T., Rommel, C., and Vanhaesebroeck, B. (2006). Key role of the p110delta isoform of PI3K in B-cell antigen and IL-4 receptor signaling: Comparative analysis of genetic and pharmacologic interference with p110delta function in B cells. *Blood* **107**, 642–650.
- Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., Jr., and Abraham, R. T. (1996). Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J.* **15**, 5256–5267.
- Byfield, M. P., Murray, J. T., and Backer, J. M. (2005). hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J. Biol. Chem.* **280**, 33076–33082.
- Chen, R., Kang, V. H., Chen, J., Shope, J. C., Torabinejad, J., DeWald, D. B., and Prestwich, G. D. (2002). A monoclonal antibody to visualize PtdIns(3,4,5)P(3) in cells. *J. Histochem. Cytochem.* **50**, 697–708.
- Chow, S., Patel, H., and Hedley, D. W. (2001). Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: Potential for pharmacodynamic monitoring of signal transduction inhibitors. *Cytometry* **46**, 72–78.
- Clayton, E., Bardi, G., Bell, S. E., Chantry, D., Downes, C. P., Gray, A., Humphries, L. A., Rawlings, D., Reynolds, H., Vigorito, E., and Turner, M. (2002). A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation. *J. Exp. Med.* **196**, 753–763.
- Coico, R. (ed.) (2006). “Current Protocols in Immunology,” Vol. 5. Wiley, New York.
- Costello, P. S., Gallagher, M., and Cantrell, D. A. (2002). Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. *Nat. Immunol.* **3**, 1082–1089.
- Deane, J. A., and Fruman, D. A. (2004). Phosphoinositide 3-kinase: Diverse roles in immune cell activation. *Annu. Rev. Immunol.* **22**, 563–598.
- Deane, J. A., Kharas, M. G., Oak, J. S., Stiles, L. N., Luo, J., Moore, T. I., Ji, H., Rommel, C., Cantley, L. C., Lane, T. E., and Fruman, D. A. (2007). T cell function is partially maintained in the absence of class IA phosphoinositide 3-kinase signaling. *Blood* **109**, 2894–2902.
- Donahue, A. C., and Fruman, D. A. (2003). Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. *J. Immunol.* **170**, 5851–5860.
- Donahue, A. C., and Fruman, D. A. (2004). PI3K signaling controls cell fate at many points in B lymphocyte development and activation. *Semin. Cell Dev. Biol.* **15**, 183–197.

- Donahue, A. C., and Fruman, D. A. (2007). Distinct signaling mechanisms activate the target of rapamycin in response to different B cell stimuli. *Eur. J. Immunol.*, in press.
- Downes, C. P., Gray, A., Watt, S. A., and Lucocq, J. M. (2003). Advances in procedures for the detection and localization of inositol phospholipid signals in cells, tissues, and enzyme assays. *Methods Enzymol.* **366**, 64–84.
- Fleisher, T. A., Dorman, S. E., Anderson, J. A., Vail, M., Brown, M. R., and Holland, S. M. (1999). Detection of intracellular phosphorylated STAT-1 by flow cytometry. *Clin. Immunol.* **90**, 425–430.
- Fruman, D. A. (2004). Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. *Curr. Opin. Immunol.* **16**, 314–320.
- Fruman, D. A. (2007). The role of class I phosphoinositide 3-kinase in T-cell function and autoimmunity. *Biochem. Soc. Trans.* **35**(Pt. 2), 177–180.
- Glassford, J., Vigorito, E., Soeiro, I., Madureira, P. A., Zoumpoulidou, G., Brosens, J. J., Turner, M., and Lam, E. W. (2005). Phosphatidylinositol 3-kinase is required for the transcriptional activation of cyclin D2 in BCR activated primary mouse B lymphocytes. *Eur. J. Immunol.* **35**, 2748–2761.
- Gold, M. R., and Aebersold, R. (1994). Both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase products are increased by antigen receptor signaling in B cells. *J. Immunol.* **152**, 42–50.
- Guillou, H., Stephens, L., and Hawkins, P. T. (2007). Quantitative measurement of phosphatidylinositol 3,4,5-trisphosphate. *Methods Enzymol.* **434**, Chap. 7 (this volume).
- Guo, B., and Rothstein, T. L. (2005). B cell receptor (BCR) cross-talk: IL-4 creates an alternate pathway for BCR-induced ERK activation that is phosphatidylinositol 3-kinase independent. *J. Immunol.* **174**, 5375–5381.
- Han, A., Saijo, K., Mecklenbrauker, I., Tarakhovsky, A., and Nussenzweig, M. C. (2003). Bam32 links the B cell receptor to ERK and JNK and mediates B cell proliferation but not survival. *Immunity* **19**, 621–632.
- Harriague, J., and Bismuth, G. (2002). Imaging antigen-induced PI3K activation in T cells. *Nat. Immunol.* **3**, 1090–1096.
- Hess, K. L., Donahue, A. C., Ng, K. L., Moore, T. I., Oak, J., and Fruman, D. A. (2004). Frontline: The p85alpha isoform of phosphoinositide 3-kinase is essential for a subset of B cell receptor-initiated signaling responses. *Eur. J. Immunol.* **34**, 2968–2976.
- Jacob, A., Cooney, D., Pradhan, M., and Coggeshall, K. M. (2002). Convergence of signaling pathways on the activation of ERK in B cells. *J. Biol. Chem.* **277**, 23420–23426.
- Kam, Y., and Exton, J. H. (2004). Role of phospholipase D1 in the regulation of mTOR activity by lysophosphatidic acid. *FASEB J.* **18**, 311–319.
- Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., Balla, T., Weiss, W. A., *et al.* (2006). A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* **125**, 733–747.
- Koyasu, S. (2004). Role of class IA phosphoinositide 3-kinase in B lymphocyte development and functions. *Biochem. Soc. Trans.* **32**, 320–325.
- Krutzik, P. O., Clutter, M. R., and Nolan, G. P. (2005). Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. *J. Immunol.* **175**, 2357–2365.
- Krutzik, P. O., and Nolan, G. P. (2006). Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat. Methods* **3**, 361–368.
- Kuan, Y. H., Lin, R. H., Lin, H. Y., Huang, L. J., Tsai, C. R., Tsao, L. T., Lin, C. N., Chang, L. C., and Wang, J. P. (2006). Activation of phosphoinositide 3-kinase and Src family kinase is required for respiratory burst in rat neutrophils stimulated with artocarpol A. *Biochem. Pharmacol.* **71**, 1735–1746.

- Li, X., Martin, F., Oliver, A. M., Kearney, J. F., and Carter, R. H. (2001). Antigen receptor proximal signaling in splenic B-2 cell subsets. *J. Immunol.* **166**, 3122–3129.
- Lyubchenko, T., dal Porto, J., Cambier, J. C., and Holers, V. M. (2005). Coligation of the B cell receptor with complement receptor type 2 (CR2/CD21) using its natural ligand C3dg: Activation without engagement of an inhibitory signaling pathway. *J. Immunol.* **174**, 3264–3272.
- Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol. Cell* **10**, 151–162.
- Niswender, K. D., Gallis, B., Blevins, J. E., Corson, M. A., Schwartz, M. W., and Baskin, D. G. (2003). Immunocytochemical detection of phosphatidylinositol 3-kinase activation by insulin and leptin. *J. Histochem. Cytochem.* **51**, 275–283.
- Nobukuni, T., Joaquin, M., Roccio, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J., and Thomas, G. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc. Natl. Acad. Sci. USA* **102**, 14238–14243.
- Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S. E., Salpekar, A., Waterfield, M. D., Smith, A. J., and Vanhaesebroeck, B. (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* **297**, 1031–1034.
- Okkenhaug, K., and Vanhaesebroeck, B. (2003). PI3K-signalling in B- and T-cells: Insights from gene-targeted mice. *Biochem. Soc. Trans.* **31**, 270–274.
- Ortolano, S., Hwang, I. Y., Han, S. B., and Kehrl, J. H. (2006). Roles for phosphoinositide 3-kinases, Bruton's tyrosine kinase, and Jun kinases in B lymphocyte chemotaxis and homing. *Eur. J. Immunol.* **36**, 1285–1295.
- Perez, O. D., Kinoshita, S., Hitoshi, Y., Payan, D. G., Kitamura, T., Nolan, G. P., and Lorens, J. B. (2002). Activation of the PKB/AKT pathway by ICAM-2. *Immunity* **16**, 51–65.
- Perez, O. D., Mitchell, D., Jager, G. C., South, S., Murriel, C., McBride, J., Herzenberg, L. A., Kinoshita, S., and Nolan, G. P. (2003). Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nat. Immunol.* **4**, 1083–1092.
- Perez, O. D., and Nolan, G. P. (2002). Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat. Biotechnol.* **20**, 155–162.
- Prabhu, S., Saadat, D., Zhang, M., Halbur, L., Fruehauf, J. P., and Ong, S. T. (2007). A novel mechanism for Bcr-Abl action: Bcr-Abl-mediated induction of the eIF4F translation initiation complex and mRNA translation. *Oncogene* **26**, 1188–1200.
- Richardson, C. J., Schalm, S. S., and Blenis, J. (2004). PI3-kinase and TOR: PIKTORing cell growth. *Semin. Cell Dev. Biol.* **15**, 147–159.
- Sachs, K., Perez, O., Pe'er, D., Lauffenburger, D. A., and Nolan, G. P. (2005). Causal protein-signaling networks derived from multiparameter single-cell data. *Science* **308**, 523–529.
- Sarbasov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098–1101.
- Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* **279**, 710–714.
- Suzuki, H., Matsuda, S., Terauchi, Y., Fujiwara, M., Ohteki, T., Asano, T., Behrens, T. W., Kouro, T., Takatsu, K., Kadowaki, T., and Koyasu, S. (2003). PI3K and Btk

- differentially regulate B cell antigen receptor-mediated signal transduction. *Nat. Immunol.* **4**, 280–286.
- Tanaka, Y., Bi, K., Kitamura, R., Hong, S., Altman, Y., Matsumoto, A., Tabata, H., Lebedeva, S., Bushway, P. J., and Altman, A. (2003). SWAP-70-like adapter of T cells, an adapter protein that regulates early TCR-initiated signaling in Th2 lineage cells. *Immunity* **18**, 403–414.
- Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003). Tuberous sclerosis complex gene products, Tuberlin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* **13**, 1259–1268.
- Vanhaesebroeck, B., Leever, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535–602.
- Venkataraman, C., Shankar, G., Sen, G., and Bondada, S. (1999). Bacterial lipopolysaccharide induced B cell activation is mediated via a phosphatidylinositol 3-kinase dependent signaling pathway. *Immunol. Lett.* **69**, 233–238.
- Vigorito, E., Bardi, G., Glassford, J., Lam, E. W., Clayton, E., and Turner, M. (2004). Vav-dependent and vav-independent phosphatidylinositol 3-kinase activation in murine B cells determined by the nature of the stimulus. *J. Immunol.* **173**, 3209–3214.
- Vivarelli, M. S., McDonald, D., Miller, M., Cusson, N., Kelliher, M., and Geha, R. S. (2004). RIP links TLR4 to Akt and is essential for cell survival in response to LPS stimulation. *J. Exp. Med.* **200**, 399–404.
- Wlodarski, P., Kasprzycka, M., Liu, X., Marzec, M., Robertson, E. S., Slupianek, A., and Wasik, M. A. (2005). Activation of mammalian target of rapamycin in transformed B lymphocytes is nutrient dependent but independent of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, insulin growth factor-I, and serum. *Cancer Res.* **65**, 7800–7808.
- Wullschleger, S., Loewith, R., and Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471–484.
- Yusuf, I., Zhu, X., Kharas, M. G., Chen, J., and Fruman, D. A. (2004). Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. *Blood* **104**, 784–787.
- Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol.* **5**, 578–581.

REGULATION OF PHOSPHATIDYLINOSITOL 4-PHOSPHATE 5-KINASE ACTIVITY BY PARTNER PROTEINS

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Contents

1. Introduction	156
2. Protocols	158
2.1. Preparation of PIP5K for <i>in vitro</i> activity and interaction assays	158
2.2. Preparation of PIP5K activators	159
2.3. <i>In vitro</i> assay of PIP5K activity	161
2.4. Assay for <i>in vitro</i> interactions of PIP5K γ 661 with β 2 adaptin and talin head	164
2.5. Assay for <i>in vivo</i> interaction of endogenous PIP5K γ 661 and β 2 adaptin	165
Acknowledgments	166
References	166

Abstract

The remarkably versatile phospholipid, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], plays crucial roles in signal transduction, actin cytoskeleton reorganization, clathrin-dependent endocytosis, and regulation of membrane morphology. In mammalian cells, PI(4,5)P₂ is synthesized predominantly by phosphatidylinositol 4-phosphate [PI(4)P] 5-kinase (PIP5K) through phosphorylation of PI(4)P at the D-5 position of the inositol ring. PIP5K is composed of three isoforms, PIP5K α , β , and γ , and three splicing variants of the γ isozyme. Although the PIP5K γ splicing variant PIP5K γ 661 appears to be very specifically activated by talin, which plays a crucial role in focal adhesion formation, and the adaptor complex AP-2, the regulation of activities of other PIP5K isoforms is not fully understood at present. To understand the activation mechanism and the physiological function specific to each PIP5K isozyme, it is required to

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identify a specific activator of each PIP5K isozyme. This chapter describes common assays used to measure interaction and activation of PIP5K isozymes by activators thus far identified. In addition, procedures for preparation of PIP5K isozymes and activators are described.

1. INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] plays crucial roles in signal transduction, actin cytoskeleton reorganization, clathrin-dependent endocytosis, and regulation of membrane morphology (Di Paolo and De Camilli, 2006; Itoh and Takenawa, 2002; Janetopoulos and Devreotes, 2006; Yin and Janmey, 2003). One of the best understood functions of PI(4,5)P₂ is to serve as a precursor of two well-characterized second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), which are produced by PI-specific phospholipase C (Berridge, 1987): DAG activates Ca²⁺/phospholipid-dependent conventional protein kinase C (Nishizuka, 1984) and IP₃ releases Ca²⁺ from the endoplasmic reticulum to increase the concentration of intracellular-free Ca²⁺ (Berridge and Irvine, 1984). PI(4,5)P₂ also functions as a substrate for PI 3-kinases to generate PI 3,4,5-trisphosphate (Auger *et al.*, 1989; reviewed by Cantley *et al.*, 1991), which is a key player in mitogenic and antiapoptotic signaling pathways (Wymann and Pirola, 1998).

Events regulated directly by PI(4,5)P₂ are mediated through interaction with specific protein targets such as actin-binding proteins (Yin and Janmey, 2003), clathrin-dependent endocytic proteins, for example, AP-2 clathrin adaptor complex, AP180, epsin, and amphiphysin (Di Paolo and De Camilli, 2006; Itoh and Takenawa, 2004), and signal transducing proteins and enzymes with pleckstrin homology, phox homology, and epsin N-terminal homology domains, such as phospholipase D and ARF GAP (Brown *et al.*, 1995; Colley *et al.*, 1997; Hammond *et al.*, 1997; Itoh and Takenawa, 2002; Randazzo, 1997). Thus, PI(4,5)P₂ is a remarkably versatile phospholipid.

In mammalian cells, PI(4,5)P₂ is synthesized predominantly by phosphatidylinositol 4-phosphate [PI(4)P] 5-kinase (PIP5K) through phosphorylation of PI(4)P at the D-5 position of the inositol ring (reviewed by Toker, 1998). PIP5K is composed of three isoforms, PIP5K α , β , and γ (Ishihara *et al.*, 1996, 1998; Loijens and Anderson, 1996), and three splicing variants of the γ isozyme (Giudici *et al.*, 2004; Ishihara *et al.*, 1998): PIP5K γ 635, which encodes 635 amino acid residues; PIP5K γ 661, which contains an additional 26 amino acids at the C terminus; and PIP5K γ 687, which contains an additional 26 amino acids inserted before the C-terminal 26 amino acid residues of PIP5K γ 661. PIP5K γ , especially PIP5K γ 661, is highly expressed in the brain (Ishihara *et al.*, 1998; Wenk *et al.*, 2001) and concentrates at

neuronal synapses (Wenk *et al.*, 2001), whereas PIP5K α and β are expressed ubiquitously (Ishihara *et al.*, 1996; Loijens and Anderson, 1996; Wenk *et al.*, 2001), suggesting that PIP5K γ might play a role in synaptic functions and the other PIP5K isozymes in common cellular functions. A report using knock-out mice elegantly demonstrated that PIP5K γ does in fact have a critical function in synapses (Di Paolo *et al.*, 2004). Specific functions of the other PIP5K isozymes have not yet been clarified.

The regulation of mammalian PIP5K activity is complex and not fully understood at present. Each PIP5K isozyme may be activated differently, although the exact degree of the difference remains to be clarified. It was initially reported that the small GTPases RhoA and Rac1 increased levels of PI(4,5)P₂ in the lysate of fibroblasts (Chong *et al.*, 1994) and permeabilized platelets (Hartwig *et al.*, 1995), suggesting that they function as PIP5K activators. However, the failure to observe activation of recombinant PIP5K by RhoA and Rac1 in an *in vitro* system suggested that the stimulatory event reported in the cell lysates was not direct (Honda *et al.*, 1999), which was supported further by a finding that the RhoA effector protein ROCK activates PIP5K in N1E-115 cells (Yamazaki *et al.*, 2002). We and others have since provided evidence that another small GTPase, ARF, activates PIP5K directly (Honda *et al.*, 1999; Jones *et al.*, 2000). However, there does not appear to be specificity for this stimulation: all three PIP5K isozymes can be activated by ARF in the *in vitro* system to a comparable extent (Honda *et al.*, 1999). Thus, it remains to be clarified if any of the PIP5K isozymes are activated differentially by specific isoforms of ARF in physiological settings. In contrast, the PIP5K γ splicing variant PIP5K γ 661 appears to be very specifically activated by talin, which plays a crucial role in focal adhesion formation (Di Paolo *et al.*, 2002; Ling *et al.*, 2002). Talin activates PIP5K γ 661 by binding to its C-terminal 26 amino acid tail in a manner that is controlled by the signaling-regulated phosphorylation of PIP5K γ 661 at Tyr 644 (Ling *et al.*, 2003). It has been found that adaptor complex AP-2, which is a component of the endocytic machinery required for clathrin-dependent endocytosis, also interacts with and activates PIP5K γ 661 via its subunit β 2 adaptin, which in turn triggers clathrin-dependent synaptic vesicle endocytosis in mouse hippocampal neurons (Nakano-Kobayashi *et al.*, 2007). Interestingly, the binding site on PIP5K γ 661 again lies within the C-terminal 26 amino acid tail and is regulated by phosphorylation; however, the key site of phosphorylation that enables AP-2 complex binding is Ser 645, which is different than that required for the talin interaction, and the dephosphorylated form, rather than the phosphorylated form, is the one that interacts productively with its partner protein.

This chapter describes common assays used to measure interaction and activation of PIP5K isozymes by activators thus far identified. In addition, procedures for preparation of PIP5K isozymes and activators are described.

2. PROTOCOLS

2.1. Preparation of PIP5K for *in vitro* activity and interaction assays

A purification method for endogenous mammalian PIP5K based on column chromatography has been described previously (Jenkins *et al.*, 1994; Moritz *et al.*, 1990). However, it has not been established how well, if at all, the procedure separates the individual isozymes. To address this issue and to generate large amounts of the individual enzymes, we instead express individual isoforms tagged with a FLAG epitope that facilitates their purification. After expression in *Escherichia coli* or mammalian tissue culture cells, the recombinant proteins can be recovered by affinity chromatography using anti-FLAG M2 antibody-conjugated agarose resin (Sigma).

1. The bacterial expression vectors for FLAG-tagged PIP5K isozymes (pFLAG-MAC-PIP5Ks) were constructed in our laboratory and are available upon request. These plasmids should be transformed into the BL21(DE3) pLys strain (or an equivalent).
2. FLAG-PIP5K protein expressed is induced by the addition of 0.3 mM of isopropyl- β -D-thiogalactoside (IPTG) to 200 ml of *E. coli* culture when the OD at 600 nm of the culture becomes 0.3. After incubation at 30° for 2.5 h, *E. coli* are pelleted by centrifugation at 300g for 5 min at 4°, resuspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol [DTT], 150 mM NaCl, 0.5% Nonidet P-40 [NP-40], and protease inhibitors), sonicated by an ultrasonic disruptor equipped with a microtip (output 6.0, duty 60) for 3 min at 4°, and centrifuged at 100,000g for 20 min at 4°.
3. The supernatant (35 ml) is then incubated with 0.1 ml of anti-FLAG M2 antibody-conjugated agarose resin at 4° for 2 h with gentle rotation. The resin is batch washed five times with 10 ml of the lysis buffer supplemented with 0.5 M NaCl and three times with 10 ml of buffer consisting of 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM MgCl₂, and 0.004% (w/v) NP-40 by centrifugation at 300g for 5 min at 4°.
4. Finally, the FLAG-PIP5K protein is eluted from the resin using 100 μ l of 0.2 mg/ml FLAG peptide twice, snap frozen in liquid nitrogen in aliquots, and stored at -80° until use.

This protocol generates about 10 μ g PIP5K that is approximately 30% full-length protein. Much of the remainder of the preparation contains C-terminally truncated, non-full-length protein that results from incomplete extension of translation of the mammalian cDNA in this prokaryotic expression system. Further optimization of the expression conditions using *E. coli* optimized for mammalian expression, such as the Rosetta strain, or less robust

induction conditions (less IPTG, lower temperatures, and shorter time periods) could be used to improve the efficiency of translation of full-length protein products, albeit at the cost of lower levels of expression.

Although the bacterially purified FLAG-tagged PIP5K protein suffices for enzymatic characterization, PIP5Ks purified from mammalian cultured cells are required to study the interaction with and activation by partner proteins, as PIP5Ks are phosphorylated by several protein kinases, including PKA (Park *et al.*, 2001), FAK (Ling *et al.*, 2003), and Cdk5 (Lee *et al.*, 2005), and the phosphorylation status modulates the interaction with its activators (Lee *et al.*, 2005; Nakano-Kobayashi *et al.*, 2007). To generate PIP5Ks using a mammalian expression system, we transiently overexpress FLAG-tagged PIP5K isozymes in HEK293T cells by transfection of the mammalian expression vectors pcDNA3-FLAG-PIP5Ks and purify.

In general, HEK293T cells, which are plated in a 15-cm dish at 6×10^4 cells/cm², are transfected with 20 μ g of pcDNA3-FLAG-PIP5Ks by the calcium phosphate method and are incubated at 37° for 18 to 24 h. Cells are then harvested, lysed in 2 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 10 mM KCl, 1% Triton S-100), and then centrifuged at 10,000g for 1 h at 4°. Expressed PIP5Ks in the supernatant are purified using the procedure described earlier for recovery of FLAG-PIP5K after expression in *E. coli*.

This procedure yields 0.5 to 1.0 μ g of full-length proteins with approximately 95% purity. If large quantities of protein are needed, or such protein production is needed frequently, generation of inducible cell lines (e.g., Tet regulated) would be a more efficient way to produce recombinant PIP5K routinely.

2.2. Preparation of PIP5K activators

2.2.1. Preparation of ARF

Myristoylation of the small GTPase ARF at its N-terminal glycine residue is absolutely required for regulation of its downstream effectors (Brown *et al.*, 1993), including PIP5K. We prepare myristoylated ARF by purification from bovine (or porcine) brain cytosol (Honda *et al.*, 1999), following it during the purification process by Western blotting with an anti-ARF antibody. ARF purified from brains contains primarily the isozyme ARF1, accompanied by a small amount of ARF3.

1. Bovine (or porcine) brains (300–400 g) are minced, homogenized with a Dounce homogenizer in 300 ml of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]), filtered through two layers of cheesecloth, and centrifuged at 100,000g for 30 min at 4°. The supernatant (cytosol) is snap frozen in liquid nitrogen and stored at –80° until use.

2. Cytosol proteins (approximately 800 mg protein) are precipitated with 25% saturation of ammonium sulfate and centrifuged at 10,000g for 30 min at 4°. To the supernatant is added 60% saturation of ammonium sulfate, and the protein pellet obtained by centrifugation at 10,000g for 30 min at 4° is dissolved in 100 ml of 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, and 1 mM EDTA.
3. After being dialyzed three times against 5 liters of buffer A for each 2 to 3 h at 4°, the sample is loaded onto a DEAE Sephacel column (150-ml bed volume) and proteins are eluted with a linear gradient of 0 to 0.4 M NaCl in buffer A.
4. The ARF protein, which is eluted at 0.1 M NaCl, is concentrated to 10 ml using Amicon Centriprep YM-10 and then subjected to gel filtration chromatography on a Sephacryl S-300 HR column (300-ml bed volume) in buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 1 μM GDP, and 50 mM NaCl).
5. The ARF protein, which is eluted at the position of molecular mass of 20 kDa from the Sephacryl S-300 HR, is loaded onto a hydroxylapatite column (10-ml bed volume) and eluted with a linear gradient of 0 to 120 mM potassium phosphate, pH 7.5, in 20 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.1 mM PMSF, and 1 μM GDP. The ARF fraction should elute at 35 mM potassium phosphate.
6. After an equal volume of 4 M ammonium sulfate is added to the ARF fraction, the protein is further subjected to chromatography on a phenyl-Toyopearl 650M column (5-ml bed volume) and eluted with a reverse linear gradient of 2–0 M ammonium sulfate. The ARF fraction should elute at 0.6 M ammonium sulfate.
7. Finally, the ARF fraction is concentrated and gel filtrated on a Superdex 75 HR 10/30 column (24-ml bed volume) using buffer B.

Alternatively, myristoylated ARF1 can be prepared by expression in and purification from *E. coli*, as long as *N*-myristoyltransferase is coexpressed (Duronio *et al.*, 1990). However, because only 5 to 20% of the ARF1 expressed in *E. coli* becomes myristoylated under these conditions, the potency of bacterially purified ARF1 as an activator of PIP5K is much lower than that of endogenous ARF1 purified from brains (Honda *et al.*, 1999).

1. *E. coli* BL21(DE3) cells are cotransformed with pET-20b-ARF1, which is an expression vector for C-terminally hexahistidine-tagged ARF1 (ARF1-His), and pBB131, an expression vector for *N*-myristoyltransferase (Duronio *et al.*, 1990). The transformant is grown in LB medium at 37°. To induce the expression of myristoylated ARF1-His, IPTG and myristic acid are added to make final concentrations of 0.4 and 0.2 mM, respectively, when the OD at 600 nm becomes ≈1.0.
2. After 2 h of culture, ARF1-His is solubilized as described earlier for PIP5K and is then purified by chromatography on a ProBond

nickel-chelating column (Invitrogen) using 300 mM imidazole as the elution buffer, as recommended by the manufacturer.

3. After removal of imidazole by dialysis, the purified ARF-His is stored at -80° until use. A 500-ml culture of the transformant usually yields 1 to 5 mg of ARF1-His with a purity of $>90\%$.

2.2.2. Preparation of the head domain of talin

Talin specifically interacts with and activates PIP5K γ 661 via its head domain, which corresponds to amino acids 1 to 435 (Di Paolo *et al.*, 2002; Ling *et al.*, 2002). The portion of the cDNA needed to express the head domain is amplified from total RNA of HeLa cells using RT-PCR, sequenced, and subcloned into the bacterial expression vector pGEX-6P-1 (GE Healthcare BioSciences; available upon request). The plasmid should be transformed into BL21(DE3) pLys strain (or an equivalent). The glutathione S-transferase (GST)-fused talin head is expressed in *E. coli* via IPTG induction, lysed in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5% NP-40, and protease inhibitors and purified with glutathione-Sepharose 4B beads using the manufacturer's protocol. The procedures for protein induction and lysis are basically followed by the protocol for the bacterial expression of PIP5Ks described earlier: in this case, the protein is induced at 20° for 20 h to avoid protein degradation. Because high concentrations of detergent in the reaction mixture strongly suppress PIP5K activity, the beads to which the GST-talin head domain protein is bound should be washed extensively with buffer to remove the NP-40 before eluting the protein. This protocol yields about 300 to 500 μ g GST-talin head with approximately 95% purity.

2.2.3. Preparation of β 2 adaptin

PIP5K γ 661 also specifically interacts with β 2 adaptin via its Ear domain, which corresponds to amino acids 700 to 951. cDNAs encoding full-length mouse β 2 adaptin (Takatsu *et al.*, 2001) and the Ear domain (which is amplified by PCR with the full-length cDNA as template) are inserted into pGEX-6P-1, and the GST fusion proteins are expressed in *E. coli* and purified as described earlier for talin.

2.3. *In vitro* assay of PIP5K activity

To assay PIP5K activity as stimulated by activators *in vitro*, purified recombinant PIP5K isozymes are reconstituted with purified endogenous or recombinant activators in the presence of the PIP5K substrate PI(4)P and [γ - ^{32} P]ATP, and generation of the product [γ - ^{32} P]PI(4,5)P $_2$ is measured as described next.

1. Samples (50 μ l) containing PIP5K isozymes are incubated at 37° for 25 min in 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM MgCl₂, 0.004% (w/v) NP-40 (see “Notes and reagents”), 50 μ M PI(4)P, 50 μ M [γ -³²P]ATP (1 μ Ci/assay), either 40 μ M GTP γ S or GDP, and 10 to 50 μ M phosphatidic acid (PA) (see “Notes and reagents”).
2. The reaction is terminated by adding 400 μ l of a stop solution consisting of chloroform/methanol/11.7 N HCl (200:400:1, by volume).
3. [³²P]phospholipids are extracted according to the methods of Bligh and Dyer (1959): 100 μ l of chloroform, 100 μ l of 200 mM KCl, and 5 mM EDTA are added to the samples, vortexed vigorously, and centrifuged at 10,000g for 2 min, which separates the mixture into organic and aqueous phases.
4. The organic phase, which contains [³²P]phospholipids, is transferred to a new tube.
5. To achieve greater recovery of [³²P]phospholipids, reextraction is performed by adding 100 μ l of chloroform to the aqueous phase and collecting the organic phase after centrifugation at 10,000g for 2 min. The organic phase is combined with the previously extracted organic solution of [³²P]phospholipids.
6. After addition of 100 μ l of the synthetic upper layer solution (see “Notes and reagents”) to the collected organic solution, the mixture is vortexed and centrifuged at 10,000g for 2 min, and the separated organic phase is transferred to a new tube.
7. The collected organic solution, which contains the extracted [³²P]phospholipids, is dried under nitrogen gas and then dissolved in 50 μ l of chloroform/methanol (6:1, by volume).
8. The [³²P]phospholipid sample is spotted on a Merck silica gel 60 plate (10-cm height, 1-cm slot) impregnated with 1.2% potassium oxalate and separated by thin-layer chromatography (TLC) by developing in the solvent system of chloroform/methanol/acetone/water/acetic acid (7:5:2:2:2, by volume). Five micrograms of PI(4,5)P₂ in chloroform is also applied as a standard for PI(4,5)P₂.
9. After drying the plate, the PI(4,5)P₂ standard is visualized with iodine vapor. The [³²P]PI(4,5)P₂ is quantitated using a BAS2500 Bioimaging analyzer (Fuji Photo Film, Tokyo, Japan) or autoradiography.

The result of synergistic activation of PIP5K α by ARF1 and PA performed according to the procedure described earlier is shown in Fig. 9.1.

Notes and reagents

Very low concentrations of the detergent NP-40 are required for ARF stimulation of PIP5K activity. However, high concentrations of detergent suppress PIP5K activity.

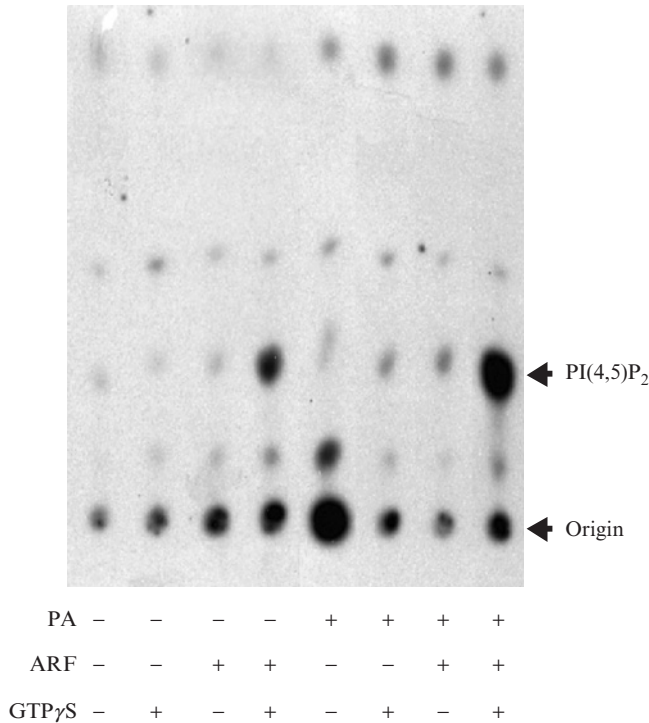


Figure 9.1 Synergistic activation of PIP5K α by the small GTPase ARF and the phospholipid PA. Bacterially purified recombinant PIP5K α was incubated with 300 nM of ARF1 purified from bovine brain and 50 μ M of PA at 37° for 25 min under the conditions described in the text. After lipids were extracted and separated by TLC, the [^{32} P] phospholipids were visualized by autoradiography.

Preparation of phospholipids: Phospholipids in chloroform are dried under nitrogen gas and sonicated in 20 mM HEPES-NaOH, pH 7.5, in a bath-type sonicator.

The synthetic upper layer: 1 volume of 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 10 mM MgCl₂, 8 volumes of the stop solution, 2 volumes of chloroform, and 2 volumes of 200 mM KCl, 5 mM EDTA are mixed and centrifuged at 10,000 rpm for 5 min to separate into organic and aqueous phases and the aqueous phase is used as the synthetic upper layer.

Under the conditions used in our laboratory, in which the PI(4)P substrate is the only phospholipid present in the liposomes, PA synergistically activates PIP5K with ARF (Honda *et al.*, 1999). However, it has been reported that when liposomes are composed of both PI(4)P and phosphatidylcholine, PA is inhibitory for ARF stimulation of PIP5K activity (Jones *et al.*, 2000).

2.4. Assay for *in vitro* interactions of PIP5K γ 661 with β 2 adaptin and talin head

As described earlier, PIP5K γ 661 specifically interacts with β 2 adaptin of the AP-2 complex and talin via Ear and head domains, respectively. *In vitro* interaction of PIP5K γ 661 with these molecules is examined according to the following protocol, and the results obtained are shown in Fig. 9.2.

1. Bacterially expressed GST- β 2 adaptin, GST- β 2 adaptin Ear domain, or GST-talin head domain (30 pmol of each protein) is bound to 10 μ l of glutathione-Sepharose beads (GE Healthcare BioSciences).

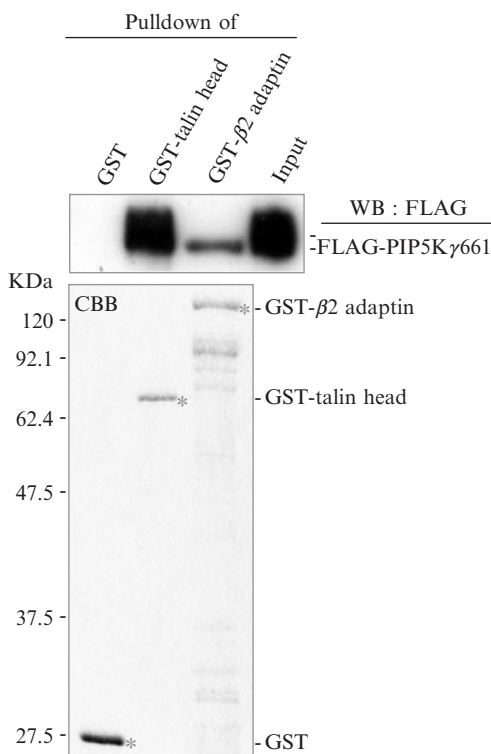


Figure 9.2 *In vitro* interaction of PIP5K γ 661 with β 2 adaptin and talin head. Bacterially purified GST-talin head and β 2 adaptin were incubated at 4° for 2 h with the lysate of HEK293T cells overexpressing FLAG-PIP5K γ 661. GST-tagged activators were pulled down, and coprecipitated FLAG-PIP5K γ 661 was detected by Western blotting with the anti-FLAG M2 antibody. The lower panel represents CBB staining of purified GST-tagged activators. Notes: β 2 adaptin can interact with the lower band of PIP5K γ 661, which is the dephosphorylated form, whereas the talin head interacts with both lower and upper bands.

2. HEK293T cells (total $1-2 \times 10^6$ cells) expressing FLAG-PIP5K γ 661 are lysed in 0.4 ml of buffer consisting of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 10 mM KCl, 1% Triton X-100, 5 mM NaF, 2 mM Na₃VO₄, 4 mM Na₂P₂O₇, and protease inhibitors and centrifuged at 17,000g for 10 min. The supernatant is transferred to a new tube.
3. Beads immobilized with GST-fusion proteins (PIP5K γ 661 activators) (10 μ l) are incubated at 4° for 2 h with 0.4 ml of the supernatant of HEK293T cells containing FLAG-PIP5K γ 661.
4. After the immobilized beads are washed with the buffer used for the lysis of HEK293T cells and centrifuged at 300g for 1 min, coprecipitated PIP5K γ 661 is detected by Western blotting using an anti-PIP5K γ monoclonal antibody (BD Biosciences) or an anti-FLAG M2 antibody.

2.5. Assay for *in vivo* interaction of endogenous PIP5K γ 661 and β 2 adaptin

We found that PIP5K γ 661 is expressed abundantly in mouse hippocampal neurons (Nakano-Kobayashi *et al.*, 2007). *In vivo* interaction of PIP5K γ 661 with β 2 adaptin in the mouse brain can be detected according to the following procedure.

1. Three mouse brains dissected from postnatal day (P)5 ICR mice are homogenized in 3 ml of buffer consisting of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, and 1 mM PMSE. The brain homogenate (800 μ g protein) is then resuspended in 1 ml of the lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 10 mM KCl, 1% Triton X-100, 5 mM NaF, 2 mM Na₃VO₄, 4 mM Na₂P₂O₇, and protease inhibitors and centrifuged at 15,000g for 20 min. The supernatant is transferred to a new tube.
2. Endogenous PIP5K γ 661 in mouse brain extract is immunoprecipitated with 10 μ g of the anti-PIP5K γ polyclonal antibody, which was raised against a peptide corresponding to the 622 to 635 amino acid region in our laboratory.
3. The coprecipitated AP-2 complex is detected by Western blotting using anti- α adaptin and anti- β adaptin antibodies (BD Biosciences).

We also found that interaction of PIP5K γ 661 with the AP-2 complex increases in mouse hippocampal neurons upon depolarizing stimulation (Nakano-Kobayashi *et al.*, 2007). This interaction can be detected by the following procedure, and results obtained are shown in Fig. 9.3.

1. Ten hippocampi dissected from embryonic day (E)17.5 ICR mice are gently pipetted in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) and treated with 10 U ml⁻¹ papain and 100 U ml⁻¹ DNase in DMEM at 37° for 20 min. The dissociated hippocampal neurons are

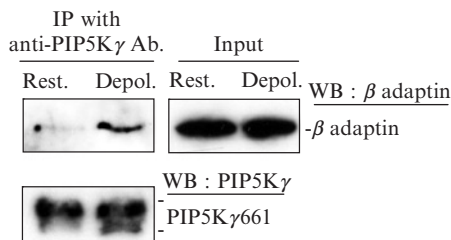


Figure 9.3 Interaction of PIP5K γ 661 with β adaptin in mouse hippocampal neurons upon depolarizing stimulation. Mouse hippocampal neurons cultured for 19 days *in vitro* were stimulated with or without 45 mM KCl for 2 min, and the PIP5K γ was immunoprecipitated using the anti-PIP5K γ antibody. Immunoprecipitated PIP5K γ 661 and coimmunoprecipitated β adaptin were detected by Western blotting with the anti-PIP5K γ and β -adaptin antibodies, respectively. Note: Upon depolarizing stimulation, the amount of the dephosphorylated form of PIP5K γ and the interaction with β adaptin increase.

cultured in neurobasal media (Invitrogen) supplemented with B-27 (Invitrogen), 0.5 mM L-glutamine and penicillin/streptomycin.

- Hippocampal neurons are cultured for 17 to 21 days *in vitro* on a 60-mm dish and stimulated with or without 45 mM KCl for 2 min.
- After lysis of hippocampal neurons with the lysis buffer used for the preparation of brain lysate as described earlier, endogenous PIP5K γ 661 is immunoprecipitated with the anti-PIP5K γ antibody. The coprecipitated AP-2 complex is detected by Western blotting using an anti- β -adaptin antibody (BD Biosciences).

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REFERENCES

- Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**, 167–175.
- Berridge, M. J. (1987). Inositol trisphosphate and diacylglycerol: Two interacting second messengers. *Annu. Rev. Biochem.* **56**, 159–193.

- Berridge, M. J., and Irvine, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321.
- Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993). ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* **75**, 1137–1144.
- Brown, H. A., Gutowski, S., Khan, R. A., and Sternweis, P. C. (1995). Partial purification and characterization of Arf-sensitive phospholipase D from porcine brain. *J. Biol. Chem.* **270**, 14935–14943.
- Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. *Cell* **64**, 281–302.
- Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* **79**, 507–513.
- Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altshuler, Y., Barsagi, D., Morris, A. J., and Frohman, M. A. (1997). Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7**, 191–201.
- Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651–657.
- Di Paolo, G., Moskowitz, H. S., Gipson, K., Wenk, M. R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R. M., Ryan, T. A., and De Camilli, P. (2004). Impaired PtdIns (4,5)P₂ synthesis in nerve terminals produces defects in synaptic vesicle trafficking. *Nature* **431**, 415–422.
- Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zoncu, R., Voronov, S., Chang, S., Guo, J., Wenk, M. R., and De Camilli, P. (2002). Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. *Nature* **420**, 85–89.
- Duronio, R. J., Jackson-Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S., and Gordon, J. I. (1990). Protein N-myristoylation in *Escherichia coli*: Reconstitution of a eukaryotic protein modification in bacteria. *Proc. Natl. Acad. Sci. USA* **87**, 1506–1510.
- Giudici, M. L., Emson, P. C., and Irvine, R. F. (2004). A novel neuronal-specific splice variant of type I phosphatidylinositol 4-phosphate 5-kinase isoform gamma. *Biochem. J.* **379**, 489–496.
- Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. (1997). Characterization of two alternately spliced forms of phospholipase D1: Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C- α . *J. Biol. Chem.* **272**, 3860–3868.
- Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A., and Stossel, T. P. (1995). Thrombin receptor ligation and activated Rac uncouple actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82**, 643–653.
- Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999). Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521–532.

- Ishihara, H., Shibasaki, Y., Kizuki, N., Katagiri, H., Yazaki, Y., Asano, T., and Oka, Y. (1996). Cloning of cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol-4-phosphate 5-kinase. *J. Biol. Chem.* **271**, 23611–23614.
- Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T., and Oka, Y. (1998). Type I phosphatidylinositol-4-phosphate 5-kinases: Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. *J. Biol. Chem.* **273**, 8741–8748.
- Itoh, T., and Takenawa, T. (2002). Phosphoinositide-binding domains: Functional units for temporal and spatial regulation of intracellular signalling. *Cell Signal.* **14**, 733–743.
- Itoh, T., and Takenawa, T. (2004). Regulation of endocytosis by phosphatidylinositol 4,5-bisphosphate and ENTH proteins. *Curr. Top. Microbiol. Immunol.* **282**, 31–47.
- Janetopoulos, C., and Devreotes, P. (2006). Phosphoinositide signaling plays a key role in cytokinesis. *J. Cell Biol.* **174**, 485–490.
- Jenkins, G. H., Fiset, P. L., and Anderson, R. A. (1994). Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.* **269**, 11547–11554.
- Jones, D. H., Morris, J. B., Morgan, C. P., Kondo, H., Irvine, R. F., and Cockcroft, S. (2000). Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment. *J. Biol. Chem.* **275**, 13962–13966.
- Lee, S. Y., Voronov, S., Letinic, K., Nairn, A. C., Di Paolo, G., and De Camilli, P. (2005). Regulation of the interaction between PIPKI gamma and talin by proline-directed protein kinases. *J. Cell Biol.* **168**, 789–799.
- Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W., and Anderson, R. A. (2002). Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. *Nature* **420**, 89–93.
- Ling, K., Doughman, R. L., Iyer, V. V., Firestone, A. J., Bairstow, S. F., Mosher, D. F., Schaller, M. D., and Anderson, R. A. (2003). Tyrosine phosphorylation of type I gamma phosphatidylinositol phosphate kinase by Src regulates an integrin-talin switch. *J. Cell Biol.* **163**, 1339–1349.
- Loijens, J. C., and Anderson, R. A. (1996). Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family. *J. Biol. Chem.* **271**, 32937–32943.
- Moritz, A., De Graan, P. N., Ekhardt, P. F., Gispen, W. H., and Wirtz, K. W. (1990). Purification of a phosphatidylinositol 4-phosphate kinase from bovine brain membranes. *J. Neurochem.* **54**, 351–354.
- Nakano-Kobayashi, A., Yamazaki, M., Unoki, T., Hongu, T., Murata, C., Taguchi, R., Katada, T., Frohman, M. A., Yokozeki, T., and Kanaho, Y. (2007). Role of activation of PIP5Kgamma661 by AP-2 complex in synaptic vesicle endocytosis. *EMBO J.* **26**, 1105–1116.
- Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693–698.
- Park, S. J., Itoh, T., and Takenawa, T. (2001). Phosphatidylinositol 4-phosphate 5-kinase type I is regulated through phosphorylation response by extracellular stimuli. *J. Biol. Chem.* **276**, 4781–4787.
- Randazzo, P. A. (1997). Functional interaction of ADP-ribosylation factor 1 with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **272**, 7688–7692.
- Takatsu, H., Futatsumori, M., Yoshino, K., Yoshida, Y., Shin, H. W., and Nakayama, K. (2001). Similar subunit interactions contribute to assembly of clathrin adaptor complexes and COPI complex: Analysis using yeast three-hybrid system. *Biochem. Biophys. Res. Commun.* **284**, 1083–1089.

- Toker, A. (1998). The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Cell Biol.* **10**, 254–261.
- Wenk, M. R., Pellegrini, L., Klenchin, V. A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T. F., and De Camilli, P. (2001). PIP kinase I gamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. *Neuron* **32**, 79–88.
- Wymann, M. P., and Pirola, L. (1998). Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta* **1436**, 127–150.
- Yamazaki, M., Miyazaki, H., Watanabe, H., Sasaki, T., Maehama, T., Frohman, M. A., and Kanaho, Y. (2002). Phosphatidylinositol 4-phosphate 5-kinase is essential for ROCK-mediated neurite remodeling. *J. Biol. Chem.* **277**, 17226–17230.
- Yin, H. L., and Janmey, P. A. (2003). Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* **65**, 761–789.

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BIOCHEMICAL ANALYSIS OF INOSITOL PHOSPHATE KINASES

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Contents

1. Introduction	172
2. Experimental Methods	174
2.1. IP kinase expression constructs	174
2.2. Expression and purification of IP kinases	175
2.3. Enzymatic generation of IPs for use as high-performance liquid chromatography (HPLC) standards and IP kinase substrates	175
2.4. Analysis of inositol phosphates by thin-layer chromatography (TLC)	177
2.5. Kinetic analysis of human IHPK1	178
2.6. Purification of inositol phosphates	179
2.7. Analysis of PP-IP ₅ by proton-decoupled ³¹ P NMR	180
3. Conclusions	182
Acknowledgments	183
References	183

Abstract

Lipid-derived inositol phosphates (IPs) are a complex group of second messengers generated by the sequential phosphorylation of inositol 1,4,5-trisphosphate (IP₃). Synthetic pathways leading from IP₃ to the formation of inositol tetrakisphosphate IP₄, inositol pentakisphosphate IP₅, inositol hexakisphosphate IP₆, and inositol pyrophosphates PP-IPs have been elucidated in eukaryotes from yeast to human. Studies have attributed a variety of cellular functions to IPs, highlighting the importance of understanding how the pathways for their synthesis are regulated. This chapter summarizes experimental techniques for the

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biochemical characterization of the key inositol phosphate kinases IPKs necessary for producing the diverse array of IP species.

1. INTRODUCTION

Phosphorylation of the 6-carbon *myo*-D-inositol ring provides a scaffold for the generation of a variety of sterically unique signaling molecules (Berridge and Irvine, 1989; Irvine and Schell, 2001; Majerus, 1992; Shears, 1998). There are two general classes of phosphorylated inositols: (1) lipids, commonly referred to as phosphatidylinositol phosphates or phosphoinositides (PIPs), and (2) water-soluble inositol phosphates (IPs). Many of the soluble IPs found in cells have been shown to be “lipid derived” and arise from metabolism of inositol 1,4,5-trisphosphate (IP₃), a product of PI(4,5)P₂ hydrolysis by phospholipase C. Conversion of IP₃ by a series of inositol phosphate kinases (IPKs) and inositol phosphate phosphatases (IPPs) produces over 20 IPs among which are a group of “higher” phosphorylated species: inositol tetrakisphosphate (IP₄), inositol pentakisphosphate (IP₅), inositol hexakisphosphate (IP₆), and inositol pyrophosphates (PP-IPs) (Shears, 2004; York, 2006). Higher IPs have been shown to participate in a variety of cellular functions, including regulation of transcription, mRNA export, telomere length, chromatin remodeling, and endocytosis (reviewed in Bennett *et al.*, 2006; Shears, 2004; York, 2006). Studies demonstrating that kinases IPMK/IPK2 and IPK1 are essential for the proper development of mice and zebrafish serve to emphasize the biological importance of the synthesis of IP₅ and IP₆ in cellular adaptation (Frederick *et al.*, 2005; Sarmah *et al.*, 2005; Verbsky *et al.*, 2005a).

Synthesis of most of the higher IPs is executed by evolutionarily conserved IPKs (Shears, 2004; York, 2006). In yeast, the synthetic pathway for higher IPs is composed of four known IP kinases (Fig. 10.1). The synthesis of I(1,3,4,5,6)P₅ from I(1,4,5)P₃ is carried out by *IPK2/ARG82*, which sequentially phosphorylates the 6- and 3-positions on the inositol ring (Odom *et al.*, 2000; Saiardi *et al.*, 2000a). *IPK1* then phosphorylates the 2-position of I(1,3,4,5,6)P₅ to generate IP₆ (Ives *et al.*, 2000; York *et al.*, 1999). In addition, there are two inositol pyrophosphate synthase IPS that modify IP substrates to form PP-IPs. The first is *Kcs1p*, which converts IP₆ to generate PP-IP₅ (Saiardi *et al.*, 1999, 2000b). *Kcs1p* also utilizes other IP substrates, most notably IP₅ to form PP-IP₄ (Seeds *et al.*, 2005; York *et al.*, 2005). A second IPS activity, designated *IDS1/IPS1*, was identified as an IP₆ kinase activity present in *kcs1* null yeast (Seeds *et al.*, 2005; York *et al.*, 2005), and its molecular identity has been reported (Mulugu *et al.*, 2007).

In plants and metazoans, the higher IP synthetic pathways are more complex with the presence of six classes of IPKs, some of which have multiple

A second higher IP synthetic pathway has been reported in most metazoans, which does not appear to exist in budding yeast or fruit flies (Majerus, 1992; Shears, 1998). In this pathway, $I(1,4,5)P_3$ is first converted to $I(1,3,4,5)P_4$ by a family of three IP_3 3-kinases (IP3KA, IP3KB, and IP3KC) or possibly IPK2/IPMK (Fig. 10.1) (Choi *et al.*, 1990; Dewaste *et al.*, 2003; Takazawa *et al.*, 1990). An IPP 5-phosphatase hydrolyzes $I(1,3,4,5)P_4$ to generate $I(1,3,4)P_3$ (Majerus, 1992; Shears, 1989), which is then phosphorylated by an IP_3 5/6 kinase (ITPK1) to generate $I(1,3,4,6)P_4$ (Shi *et al.*, 2003; Verbsky *et al.*, 2005b; Wilson and Majerus, 1996; Yang and Shears, 2000). The 5-kinase activity of IPK2/IPMK can then generate $I(1,3,4,5,6)P_5$ from $I(1,3,4,6)P_4$ (Chang *et al.*, 2002; Fujii and York, 2005; Stevenson-Paulik *et al.*, 2002), and the IP_5 2-kinase activity of Ipk1 produces IP_6 (Fujii and York, 2005; Verbsky *et al.*, 2002, 2005a). The increased complexity of the higher IP pathway in plants and metazoans no doubt enables more signaling capacity in these cells.

This chapter discusses techniques used commonly in our laboratory for the biochemical characterization of IP synthetic pathways. This covers expression and purification of IP kinases, use of these recombinant enzymes for the generation of radiolabeled IP substrates and standards, and application of recombinant enzyme and substrates into kinetic assays. The chapter also describes techniques for the purification and ^{31}P nuclear magnetic resonance (NMR) analysis of milligram quantities of enzymatically prepared IPs and PP-IPs.

2. EXPERIMENTAL METHODS

2.1. IP kinase expression constructs

The cDNA for *Arabidopsis thaliana* IPK2 β (atIPK2 β) is obtained by polymerase chain reaction (PCR) amplification from an *Arabidopsis* cDNA pool (Stevenson-Paulik *et al.*, 2002). The cDNA for *A. thaliana* IPK1 (atIPK1) is identified as cDNA clone M40H3 and is obtained from the *Arabidopsis* Biological Resource Center (Stevenson-Paulik *et al.*, 2005). The cDNA for *Drosophila melanogaster* $I(1,4,5)P_3$ 3-kinase (dmIP3K) and human IHPK1 (hIHPK1) are identified as EST clones SD19941 and 646420, respectively, and are from Research Genetics (Seeds *et al.*, 2004, 2005). Each of these cDNA is amplified by PCR and subcloned into the glutathione S-transferase (GST) fusion vector pGEX-KG (Guan and Dixon, 1991). The cDNA for human $I(1,3,4)P_3$ 5/6-kinase (hITPK1) in the expression vector pQE30 was the gift of Dr. Stephen Shears (NIEHS, RTP, NC USA). The cDNA for human $I(1,4,5)P_3$ type I 5-phosphatase (h5-Ptase) was the gift of Dr. Philip Majerus (Washington University, St. Louis, MO). The expressed protein for hITPK1 and h5-Ptase carries hexahistidine tags (H_6 tags) at the N terminus for purification.

2.2. Expression and purification of IP kinases

Expression constructs are transformed into BL21DE3 cells, and freshly transformed colonies are inoculated into 50 ml LB media with 100 $\mu\text{g/ml}$ ampicillin and grown overnight at 37°. Saturated cultures are spun down, and the cell pellet is washed twice with LB. Pellets are resuspended in 20 ml LB, and 4 ml of the resuspended pellets is inoculated per 1 liter of LB/100 $\mu\text{g/ml}$ ampicillin. Cultures are grown at 37° until OD₆₀₀ reaches 0.6, at which time isopropyl- β -D-thiogalactoside is added to a final concentration of 200 μM . Cultures are placed back at 37° and harvested after 3 to 4 h. Cells are pelleted by centrifugation, and cell pellets are flash frozen and stored at -80° until needed.

Cell pellets are resuspended at a volume of 20 ml/liter of original culture in either (GST-fusions proteins) 25 mM Tris-HCl (pH 8.0), 350 mM NaCl, 1 mM dithiothreitol (DTT), 150 μM phenylmethylsulfonyl fluoride (PMSF), 25 μM tosyl-L-phenylalanyl chloromethyl ketone (TPCK), and 25 μM tosyl-L-lysine chloromethyl ketone (TLCK) or (H₆-tag proteins) 25 mM Tris-HCl (pH 8.0), 350 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol, 150 μM PMSF, 25 μM TPCK, and 25 μM TLCK. Cells are lysed using a high-pressure homogenizer, and cellular debris is removed by centrifugation.

Clarified cell extracts are loaded onto either glutathione-Sephadex columns or nickel-agarose columns that have been equilibrated with either GST-wash buffer (25 mM Tris-HCl [pH 8.0], 350 mM NaCl, 1 mM DTT) or H₆-tag wash buffer (25 mM Tris-HCl [pH 8.0], 350 mM NaCl, 10 mM 2 mercaptoethanol, 10 mM imidazole [pH 8.0]) at a ratio of 2 ml resin/liter of original culture. Columns are washed with 50 bed volumes of wash buffer, and recombinant proteins are eluted from the column in 0.5 bed volume fractions of elution buffer. The buffer used for elution of GST-fusion proteins is 25 mM Tris-HCl (pH 8.0), 350 mM NaCl, 1 mM DTT, and 10 mM glutathione, and the elution buffer for H₆-tagged proteins is 25 mM Tris-HCl (pH 8.0), 350 mM NaCl, 10 mM 2-mercaptoethanol, and 250 mM imidazole (pH 8.0). Fractions from the purifications are evaluated by SDS-PAGE, and those containing protein are pooled and dialyzed in 25 mM Tris-HCl (pH 8.0), 350 mM NaCl, and 1 mM DTT. Following dialysis, protein samples are aliquoted into tubes, flash frozen in liquid nitrogen, and stored at -80°.

2.3. Enzymatic generation of IPs for use as high-performance liquid chromatography (HPLC) standards and IP kinase substrates

This section outlines synthetic routes for a variety of radiolabeled inositol phosphates. When conducting kinetic analysis of IP kinases, the use of radiolabeled IPs is preferred over using radiolabeled ATP for tracking the

progression of the kinase reaction. This is because the K_m of IP kinases for ATP are in the millimolar range. Additionally, radiolabeled IPs are important tools for determining the identity of IPs produced in cells labeled metabolically with [*myo*- ^3H]inositol (reviewed in Stevenson-Paulik *et al.*, 2006). However, the only radiolabeled IPs that are readily available commercially are [^3H]I(1,4,5) P_3 and [^3H]I(1,3,4,5) P_4 (which can be purchased from Perkin Elmer [Wellesley, MA], among other vendors). Other radiolabeled IPs need to be generated in the laboratory, and the most straightforward route to doing this is enzymatic. Unlabeled IPs used in this section are from Cell Signals (Columbus, OH). Routes of enzymatic synthesis of radiolabeled IPs are summarized in Table 10.1. Following synthesis, labeled IPs can be stored in aliquots at -80° .

[^3H]I(1,3,4) P_3 is made by dephosphorylating the 5-phosphate of [^3H]I(1,3,4,5) P_4 by h5-Ptase. Nine hundred nanograms of recombinant h5-Ptase is incubated in 50 μl of kinase buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 10 mM MgCl_2) with 70 nM [^3H]I(1,3,4,5) P_4 at 37° for 30 min. The reaction is quenched by heating the reaction tube for 1 min in a boiling water bath.

[6- ^{32}P]I(1,4,5,6) P_4 is generated by phosphorylation of the 6-position of I(1,4,5) P_3 by atIPK2 β . Five hundred nanograms of recombinant GST-atIPK2 is incubated in 50 μl of kinase buffer with 5 μM I(1,4,5) P_3 and 3.4 μCi [γ - ^{32}P]ATP (6000 Ci/mmol, final ATP concentration is 70 nM) at 37° for 20 min. The reaction is terminated by boiling for 1 min. As the 6- and 3-kinases of activities of atIPK2 are ordered with the phosphorylation of the 6-position of I(1,4,5) P_3 occurring first, these conditions prevent the generation of IP $_5$ and instead result in the formation of I(1,4,5,6) P_4 . We note that the use of *S. cerevisiae* IPK2 will yield the same results. However, because the order of the 3- and 6-kinase reactions with rat IPK2 is reversed and will generate I[1,3,4,5] P_4 under these conditions, caution should be taken when using IPK2 from different species to generate I(1,4,5,6) P_4 .

Table 10.1 Enzymatic synthesis of radiolabeled inositol phosphates

Product	Starting substrate	Enzyme
[^3H]I(1,3,4) P_3	[^3H]I(1,3,4,5) P_4	h5-Ptase
[6- ^{32}P]I(1,4,5,6) P_4	I(1,4,5) P_3	atIPK2
[3- ^{32}P]I(1,3,4,5) P_4	I(1,4,5) P_3	dmIP3K β
[6- ^{32}P]I(1,3,4,6) P_4	I(1,3,4,5) P_3	h1TPK1, h5-Ptase
[6- ^{32}P]I(1,3,4,5,6) P_5	I(1,3,4,5) P_3	atIPK2
[2- ^{32}P]I(1,2,3,4,5,6) P_6	I(1,3,4,5,6) P_5	atIPK1
[^{32}P]PP-IP $_4$	I(1,3,4,5,6) P_5	hIP6K1
[^{32}P]PP-IP $_5$	IP $_6$	hIP6K1

$[3\text{-}^{32}\text{P}]\text{I}(1,3,4,5)\text{P}_4$ is generated from $\text{I}(1,4,5)\text{P}_3$ by phosphorylation of the 3-position using recombinant dmIP3K β . Seven and a half micrograms of dmIP3K β is incubated in 50 μl kinase buffer with 5 μM $\text{I}(1,4,5)\text{P}_3$ and 3.4 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction is allowed to proceed at 37° for 30 min and is terminated by boiling for 1 min.

$[6\text{-}^{32}\text{P}]\text{I}(1,3,4,6)\text{P}_4$ is generated from $\text{I}(1,3,4)\text{P}_3$ by phosphorylation of the 6-position by hITPK1. 5-Ptase is included in this reaction to eliminate any $\text{I}(1,3,4,5)\text{P}_4$ that may arise from the 5-kinase activity of hITPK1. Five hundred nanograms of GST-hITPK1 and 200 ng of 5-Ptase are incubated in 50 μl kinase buffer with 5 μM $\text{I}(1,3,4)\text{P}_3$ and 3.4 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction is placed at 37° for 30 min and is terminated by boiling for 1 min.

$[3\text{-}^{32}\text{P}]\text{I}(1,3,4,5,6)\text{P}_5$ is generated from $\text{I}(1,3,4,5)\text{P}_4$ by phosphorylation of the 6-position by atIPK2 β . Five hundred nanograms of GST-atIPK2 β is incubated in 50 μl of kinase buffer with 5 μM $\text{I}(1,3,4,5)\text{P}_4$ and 3.4 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at 37°. The reaction is quenched by boiling for 1 min.

$[2\text{-}^{32}\text{P}]\text{IP}_6$ is generated from $\text{I}(1,3,4,5,6)\text{P}_5$ by phosphorylation of the 2-position using atIPK1. Five hundred nanograms of GST-atIPK1 is incubated in 50 μl kinase buffer with 5 μM $\text{I}(1,3,4,5,6)\text{P}_5$ and 3.4 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at 37°. The reaction is quenched by boiling for 1 min.

$[^{32}\text{P}]\text{PP-IP}_4$ and $[^{32}\text{P}]\text{PP-IP}_5$ are synthesized from IP_5 and IP_6 , respectively, by phosphorylation using hIHPK1. Nine hundred nanograms of recombinant GST-hIHPK1 is incubated in 50 μl of kinase buffer with 3.4 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and either 5 μM IP_5 or 5 μM IP_6 . The reaction is allowed to proceed for 30 min at 37° and is terminated by boiling for 1 min.

2.4. Analysis of inositol phosphates by thin-layer chromatography (TLC)

Thin-layer chromatography using polyethyleneimine-cellulose sheets (PEI-TLC) provides a convenient route of analysis of ^{32}P -labeled IPs generated by *in vitro* kinase assays. We utilize a TLC buffer system adapted from those described by Ryu *et al.* (1987) and Voglmaier *et al.* (1996) consisting of 1.02 M KH_2PO_4 , 0.64 M K_2HPO_4 , and 1.84 M HCl. This buffer system allows for the separation of IPs ranging from IP_4 to IP_7 (Fig. 10.2). Kinase reactions are typically carried out in 50 mM HEPES (pH 7.5), 50 mM KCl, 10 mM MgCl_2 , 1 to 10 mM ATP, 50,000 to 300,000 cpm ^{32}P -labeled IP substrate, and an appropriate concentration of unlabeled IP substrate (0.5–40 μM). Reaction volumes are typically 10 μl , and reactions are initiated by the addition of 10 to 100 ng of enzyme. Reactions are incubated at 37° for 10 to 60 min and are halted by the addition of 1 μl TLC tank buffer. Samples are spotted 3 μl at a time onto cellulose PEI-F flexible TLC sheets (J. T. Baker, Phillipsburg, NJ), allowing the sample to dry between each spot to avoid spreading. Blowing hot air over the spot after each application typically

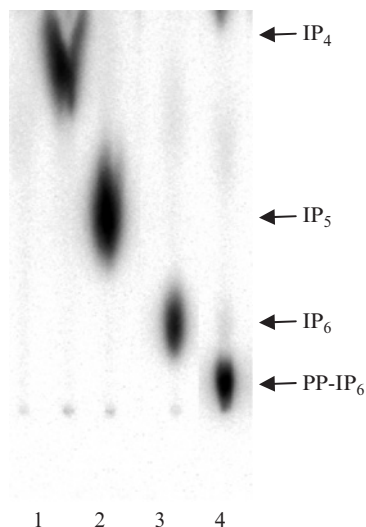


Figure 10.2 Separation of inositol phosphates by PEI-cellulose TLC. ^{32}P -labeled IPs were generated enzymatically and were resolved using PEI-TLC. One hundred thousand counts per minute of each IP were spotted onto PEI-TLC sheets and were resolved using $1.02\text{ M KH}_2\text{PO}_4/0.64\text{ M K}_2\text{HPO}_4/1.84\text{ M HCl}$. Plates were dried and visualized by phosphor imaging. Samples were (1) $[^{32}\text{P}]\text{IP}_4$, (2) $[^{32}\text{P}]\text{IP}_5$, (3) $[^{32}\text{P}]\text{IP}_6$, and (4) $[^{32}\text{P}]\text{PP-IP}_5$.

accelerates the drying process. The walls of the TLC tanks are lined with filter paper, and buffer is added to the tanks and allowed to equilibrate overnight so that the atmosphere of the tank becomes saturated. The TLC sheets are developed until the solvent front reaches the top of the sheet, air dried, and exposed to a phosphor imager screen. IPs resolved on the TLC sheet are analyzed using a 4500 SI phosphor imager (Molecular Dynamics).

2.5. Kinetic analysis of human IHPK1

Kinetic assays are performed using purified recombinant GST-IHPK1 with IP_5 and IP_6 as substrates (Fig. 10.3). The expected products for each substrate are PP-IP_4 and PP-IP_5 , respectively. Reactions are performed in $10\text{ }\mu\text{l}$ of 125 mM HEPES (pH 7.5), 15 mM MgCl_2 , and 6 mM ATP . Three hundred thousand counts per minute of $[6\text{-}^{32}\text{P}]\text{IP}_5$ or $[2\text{-}^{32}\text{P}]\text{IP}_6$ are diluted to final concentrations of 1.25 to $40\text{ }\mu\text{M}$ with unlabeled IP_5 or IP_6 , respectively. Reactions are initiated by the addition of 30 ng of IHPK1, allowed to proceed for 10 min at 37° , and then quenched by the addition of $1\text{ }\mu\text{l}$ of TLC tank buffer. Samples are spotted onto PEI-TLC sheets, and the sheets are developed, dried, and analyzed using a phosphor imager. The GST-hIHPK1 exhibited a K_m of $20.6\text{ }\mu\text{M}$ and a V_{max} of

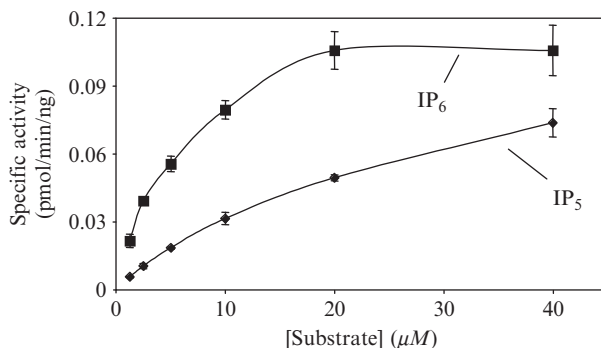


Figure 10.3 Kinetic analysis of human IHPK1. Saturation curves were generated for GST-hIHPK1 using [^{32}P]IP₅ and [^{32}P]IP₆ as substrates. Reactions were performed in 10 μl of 125 mM HEPES (pH 7.5), 15 mM MgCl₂, and 6 mM ATP for 10 min at 37°, and the reaction was quenched by the addition of 1 μl of TLC tank buffer. Reactions were spotted onto PEI-TLC sheets, and the respective products PP-IP₄ and PP-IP₅ were resolved from substrate and quantified by phosphor imaging. Reactions were performed in triplicate.

0.070 pmol/min/ng for IP₅ and a K_m of 6.3 μM and a V_{max} of 0.094 pmol/min/ng for IP₆.

2.6. Purification of inositol phosphates

While milligram quantities of many unlabeled IPs are available commercially, that is not the case for IP pyrophosphates. We have developed a protocol for the enzymatic production and purification of pp-IP₅ (Fig. 10.4): 500 μM IP₆ (Sigma, St. Louis, MO) is incubated with 500 mM ATP and 500 μg of recombinant GST-hIHPK1 for 24 h at 37° in 10 ml of 50 mM HEPES (pH 7.5), 100 mM KCl, and 2 mM MgCl₂. IP₆ and the product from the large-scale GST-hIHPK1 reaction are purified using an IonPac AS7 anion-exchange column (Dionex, Sunnyvale, CA) that has been equilibrated with 10 mM methyl piperazine, pH 4.0. IPs are eluted from the column using a linear gradient of 0 to 100% 1 M NaNO₃/10 mM methyl piperazine (pH 4.0) and a flow rate of 0.3 ml/min. Fractions are collected, and phosphate in the fractions is detected by a colorimetric assay (Stevenson-Paulik *et al.*, 2005). Five microliters of each fraction is mixed with 100 μl of a solution of 0.015% (w/v) FeCl₃ and 0.15% (w/v) sulfosalicylic acid. This reagent has an absorbance maximum at 550 nm, which is sensitive to phosphate. Thus elution of IP₆ and PP-IP₅ from the column is evident by the decreases observed in the absorbance at 550 nm (Fig. 10.4). Analysis of the HPLC purification demonstrated that the conversion of IP₆ to PP-IP₅ went to completion, and we were successful in obtaining several milligrams of purified PP-IP₅. We expect that

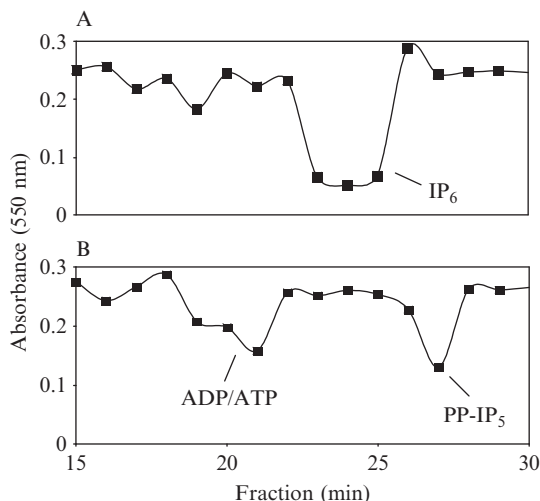


Figure 10.4 Purification of IP₆ and PP-IP₅ by HPLC. PP-IP₅ was produced enzymatically from IP₆ using recombinant GST-IHPK1. IP₆ (A) and PP-IP₅ (B) were purified by HPLC using an IonPac AS7 anion-exchange column. IPs were eluted using a linear gradient of 0 to 100% 1 M NaNO₃/10 mM methyl piperazine (pH 4.0), and the elution of phosphate species was detected by adding 5 μ l of each fraction to a solution of 0.015% (w/v) FeCl₃ and 0.15% (w/v) sulfosalicylic acid. This reagent exhibits a decrease in absorbance at 550 nm in the presence of phosphate; valleys in the HPLC tracing therefore represent elution of purified IPs. Fractions containing IP₆, PP-IP₅, and ADP/ATP from the kinase reaction are noted on the tracings.

this method will also be suitable for the production of PP-IP₄ by substituting IP₅ for IP₆ as substrate for the kinase reaction.

2.7. Analysis of PP-IP₅ by proton-decoupled ³¹P NMR

³¹P NMR provides a powerful tool for the analysis of the positions of phosphates and chemical nature of IP and PP-IP molecules, provided one has milligram quantities necessary for analysis. We obtained 2 mg of chemically synthesized I(3,4,5,6)P₄ and I(1,3,4,5,6)P₅ as a generous gift of Cell Signals, Inc. and we HPLC purified milligram amounts of IP₆ (purchased from Sigma Chemical) and IHPK1 produced PP-IP₅ as described earlier. These samples are subjected to proton-decoupled phosphorus NMR (Fig. 10.5). Two milligrams of each IP is prepared in 1 M sodium nitrate in H₂O buffer and examined at 25° using 0.6 ml in 5-mm NMR tubes. The pH of each sample is adjusted into the range between pH 11.1 and 11.9 by the addition of sodium hydroxide. I(3,4,5,6)P₄ is analyzed at pH 11.9, I(1,3,4,5,6)P₅ at pH 11.8, IP₆ at pH 11.1, and PP-IP₅ at pH 11.5.

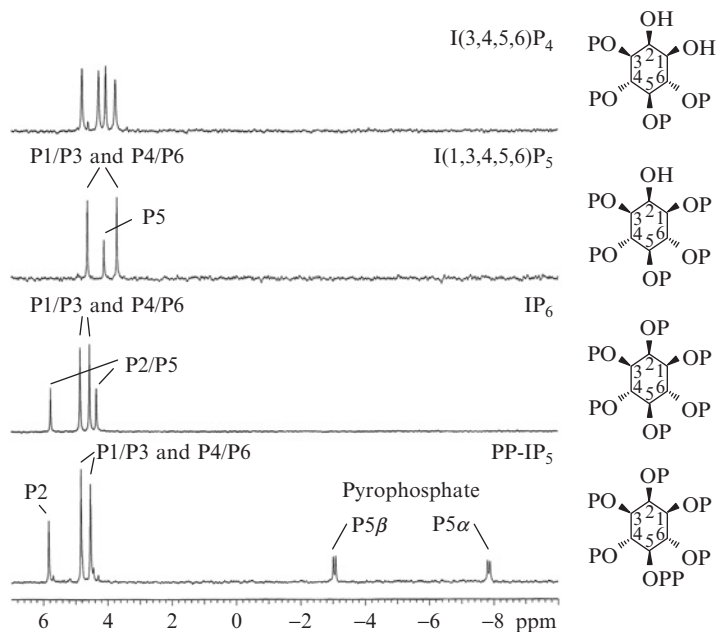


Figure 10.5 Analysis of PP-IP₅ by ³¹P NMR. HPLC-purified I(3,4,5,6)P₄, I(1,3,4,5,6)P₅, IP₆, and PP-IP₅ were analyzed by proton-decoupled phosphorous NMR. A schematic of each IP is depicted to the right of the spectrum, including the predicted structure of the PP-IP₅ produced by IHPK1. Assignment of phosphate positions in each spectrum is noted. We did not assign specific peaks to the P1/P3 and P4/P6 phosphate groups in this analysis.

NMR analysis is performed at the NMR Center at Duke University Medical Center.

To begin, we analyzed several IP standards. One of the important aspects of interpreting NMR studies of *D-myo*-inositol and certain phosphorylated derivatives relates to the chemical equivalency of phosphates due to their orientation relative to a mirror axis of symmetry lying between the 2- and the 5-ring positions. In the case of one of the standards, I(3,4,5,6)P₄, all the phosphates are chemically distinct, thus four singlet peaks with unique chemical shifts in the NMR spectrum are observed (Fig. 10.5, top trace). In contrast, for I(1,3,4,5,6)P₅, phosphorylation of the 1-position introduces chemical equivalency at the P1/P3 phosphates, rendering the chemical shifts of these phosphates indistinguishable. Likewise, the P4/P6 phosphates are also indistinguishable, but because they are adjacent to the P5 phosphate, they have a unique chemical shift as compared to the P1/P3 pair. The NMR spectrum reflects this, and three singlet peaks are observed with an intensity ratio of 2:1:2; the two large peaks represent the P1/P3 and P4/P6 pairs and the small peak the P5 phosphate (Fig. 10.5, second trace from top).

In IP₆, the P1/P3 and P4/P6 are again two equivalent pairs of phosphates whose chemical shifts are influenced by the new chemically distinct P2 phosphate. Therefore, the spectrum of IP₆ has four peaks with intensity ratios of 1:2:2:1 (Fig. 10.5, third trace from top). This is consistent with the presence of six monophosphate groups, two nonequivalent P2 and P5, and two equivalent pairs of P1/P3 and P4/P6 phosphates.

In the case of the product of the IHPK1 reaction, the PP-IP₅ exhibited a spectrum that had five monophosphate peaks with intensity ratios of 1:2:2 and pyrophosphate that splits into two upstream phosphorous–phosphorous–coupled doublets, corresponding to the β - and α -phosphates (Fig. 10.5, bottom trace). The 1:2:2 peak ratios for the monophosphate groups indicate the presence of just one nonequivalent monophosphate (either P2 or P5) and retention of the P1/P3 and P4/P6 equivalent monophosphate pairs. It is interesting to note that pH has a dramatic effect on the chemical shifts of this product as we have performed the analysis elsewhere at pH 5.8 (Mulugu *et al.*, 2007). Because single-dimensional experiments are not able to resolve which of the nonequivalent peaks are modified (see Laussmann *et al.*, 1996, 1998; Mulugu *et al.*, 2007), we have determined the position of the pyrophosphate to be P5 by two additional lines of evidence: (1) the proton-decoupled phosphorus NMR spectrum of the IHPK1 product performed at pH 5.8 is nearly identical to that of a known 5-PP-I(1,2,3,4,6)P₅ spectrum (Laussmann *et al.*, 1998; Mulugu *et al.*, 2007) and (2) the biochemical evidence that I(1,3,4,5,6)P₅ is a substrate for IHPK1, indicating that a P2 phosphate is not a prerequisite for activity.

3. CONCLUSIONS

This chapter described methods for the preparation of recombinant inositol phosphate kinases, the use of these enzymes in preparing radiolabeled IPs for use as standards and enzyme substrates, and deployment of these substrates in kinetic assays. Additionally, we described a method for the purification of milligram quantities of enzymatically generated IPs and how ³¹P NMR can be used to evaluate novel phosphorylation events on the inositol phosphate backbone. Now that many of the inositol phosphate kinases involved in IP synthetic pathways have been identified, important questions remain on how these pathways are regulated in order to generate cellular profiles of inositol phosphates that participate in specific signaling networks. As we expect that at least some regulation of these pathways will come in the form of posttranslational regulation of the IP kinases themselves, biochemical analysis of IP kinase activity will remain an important tool to understand the relevance of potential modifications on IP kinase enzymatic activity.

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REFERENCES

- Bennett, M., Onnebo, S. M., Azevedo, C., and Saiardi, A. (2006). Inositol pyrophosphates: Metabolism and signaling. *Cell. Mol. Life Sci.* **63**, 552–564.
- Berridge, M. J., and Irvine, R. F. (1989). Inositol phosphates and cell signalling. *Nature* **341**, 197–205.
- Chang, S. C., Miller, A. L., Feng, Y., Wentte, S. R., and Majerus, P. W. (2002). The human homolog of the rat inositol phosphate multikinase is an inositol 1,3,4,6-tetrakisphosphate 5-kinase. *J. Biol. Chem.* **277**, 43836–43843.
- Choi, K. Y., Kim, H. K., Lee, S. Y., Moon, K. H., Sim, S. S., Kim, J. W., Chung, H. K., and Rhee, S. G. (1990). Molecular cloning and expression of a complementary DNA for inositol 1,4,5-trisphosphate 3-kinase. *Science* **248**, 64–66.
- Dewaste, V., Moreau, C., De Smedt, F., Bex, F., De Smedt, H., Wuytack, F., Missiaen, L., and Erneux, C. (2003). The three isoenzymes of human inositol-1,4,5-trisphosphate 3-kinase show specific intracellular localization but comparable Ca²⁺ responses on transfection in COS-7 cells. *Biochem. J.* **374**, 41–49.
- Frederick, J. P., Mattiske, D., Wofford, J. A., Megosh, L. C., Drake, L. Y., Chiou, S. T., Hogan, B. L., and York, J. D. (2005). An essential role for an inositol polyphosphate multikinase, Ipk2, in mouse embryogenesis and second messenger production. *Proc. Natl. Acad. Sci. USA* **102**, 8454–8459.
- Fujii, M., and York, J. D. (2005). A role for rat inositol polyphosphate kinases rIPK2 and rIPK1 in inositol pentakisphosphate and inositol hexakisphosphate production in rat-1 cells. *J. Biol. Chem.* **280**, 1156–1164.
- Guan, K., and Dixon, J. E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**, 262–267.
- Holmes, W., and Jögl, G. (2006). Crystal structure of inositol phosphate multikinase 2 and implications for substrate specificity. *J. Biol. Chem.* **281**, 38109–38116.
- Irvine, R. F., and Schell, M. J. (2001). Back in the water: The return of the inositol phosphates. *Nat. Rev. Mol. Cell Biol.* **2**, 327–338.
- Ives, E. B., Nichols, J., Wentte, S. R., and York, J. D. (2000). Biochemical and functional characterization of inositol 1,3,4,5,6-pentakisphosphate 2-kinases. *J. Biol. Chem.* **275**, 36575–36583.
- Laussmann, T., Eujen, R., Weisshuhn, C. M., Thiel, U., and Vogel, G. (1996). Structures of diphospho-myo-inositol pentakisphosphate and bisdiphospho-myo-inositol tetrakisphosphate from *Dictyostelium* resolved by NMR analysis. *Biochem. J.* **315**(Pt. 3), 715–720.
- Laussmann, T., Hansen, A., Reddy, K. M., Reddy, K. K., Falck, J. R., and Vogel, G. (1998). Diphospho-myo-inositol phosphates in *Dictyostelium* and *Polysphondylium*: Identification of a new bisdiphospho-myo-inositol tetrakisphosphate. *FEBS Lett.* **426**, 145–150.
- Majerus, P. W. (1992). Inositol phosphate biochemistry. *Annu. Rev. Biochem.* **61**, 225–250.

- Mulugu, S., Bai, W., Fridy, P. C., Bastidas, R. J., Otto, J. C., Dollins, D. E., Haystead, T. A., Ribeiro, A. A., and York, J. D. (2007). A conserved family of enzymes that phosphorylate inositol hexakisphosphate. *Science* **316**, 106–109.
- Nalaskowski, M. M., Deschermeier, C., Fanick, W., and Mayr, G. W. (2002). The human homologue of yeast ArgR111 protein is an inositol phosphate multikinase with predominantly nuclear localization. *Biochem. J.* **366**, 549–556.
- Odom, A. R., Stahlberg, A., Wenthe, S. R., and York, J. D. (2000). A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. *Science* **287**, 2026–2029.
- Ryu, S. H., Lee, S. Y., Lee, K. Y., and Rhee, S. G. (1987). Catalytic properties of inositol trisphosphate kinase: Activation by Ca^{2+} and calmodulin. *FASEB J.* **1**, 388–393.
- Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000a). Inositol polyphosphate multikinase (ArgR111) determines nuclear mRNA export in *Saccharomyces cerevisiae*. *FEBS Lett.* **468**, 28–32.
- Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000b). The inositol hexakisphosphate kinase family: Catalytic flexibility and function in yeast vacuole biogenesis. *J. Biol. Chem.* **275**, 24686–24692.
- Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999). Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. *Curr. Biol.* **9**, 1323–1326.
- Saiardi, A., Nagata, E., Luo, H. R., Sawa, A., Luo, X., Snowman, A. M., and Snyder, S. H. (2001a). Mammalian inositol polyphosphate multikinase synthesizes inositol 1,4,5-trisphosphate and an inositol pyrophosphate. *Proc. Natl. Acad. Sci. USA* **98**, 2306–2311.
- Saiardi, A., Nagata, E., Luo, H. R., Snowman, A. M., and Snyder, S. H. (2001b). Identification and characterization of a novel inositol hexakisphosphate kinase. *J. Biol. Chem.* **276**, 39179–39185.
- Sarmah, B., Latimer, A. J., Appel, B., and Wenthe, S. R. (2005). Inositol polyphosphates regulate zebrafish left-right asymmetry. *Dev. Cell* **9**, 133–145.
- Seeds, A. M., Bastidas, R. J., and York, J. D. (2005). Molecular definition of a novel inositol polyphosphate metabolic pathway initiated by inositol 1,4,5-trisphosphate 3-kinase activity in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 27654–27661.
- Seeds, A. M., Sandquist, J. C., Spana, E. P., and York, J. D. (2004). A molecular basis for inositol polyphosphate synthesis in *Drosophila melanogaster*. *J. Biol. Chem.* **279**, 47222–47232.
- Shears, S. B. (1989). The pathway of myo-inositol 1,3,4-trisphosphate phosphorylation in liver: Identification of myo-inositol 1,3,4-trisphosphate 6-kinase, myo-inositol 1,3,4-trisphosphate 5-kinase, and myo-inositol 1,3,4,6-tetrakisphosphate 5-kinase. *J. Biol. Chem.* **264**, 19879–19886.
- Shears, S. B. (1998). The versatility of inositol phosphates as cellular signals. *Biochim. Biophys. Acta* **1436**, 49–67.
- Shears, S. B. (2004). How versatile are inositol phosphate kinases? *Biochem. J.* **377**, 265–280.
- Shi, J., Wang, H., Wu, Y., Hazebroek, J., Meeley, R. B., and Ertl, D. S. (2003). The maize low-phytic acid mutant *lpa2* is caused by mutation in an inositol phosphate kinase gene. *Plant Physiol.* **131**, 507–515.
- Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., and York, J. D. (2005). Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. *Proc. Natl. Acad. Sci. USA* **102**, 12612–12617.
- Stevenson-Paulik, J., Chiou, S. T., Frederick, J. P., dela Cruz, J., Seeds, A. M., Otto, J. C., and York, J. D. (2006). Inositol phosphate metabolomics: Merging genetic perturbation with modernized radiolabeling methods. *Methods* **39**, 112–121.
- Stevenson-Paulik, J., Odom, A. R., and York, J. D. (2002). Molecular and biochemical characterization of two plant inositol polyphosphate 6-/3-/5-kinases. *J. Biol. Chem.* **277**, 42711–42718.

- Takazawa, K., Lemos, M., Delvaux, A., Lejeune, C., Dumont, J. E., and Erneux, C. (1990). Rat brain inositol 1,4,5-trisphosphate 3-kinase: Ca^{2+} -sensitivity, purification and antibody production. *Biochem. J.* **268**, 213–217.
- Verbsky, J., Lavine, K., and Majerus, P. W. (2005a). Disruption of the mouse inositol 1,3,4,5,6-pentakisphosphate 2-kinase gene, associated lethality, and tissue distribution of 2-kinase expression. *Proc. Natl. Acad. Sci. USA* **102**, 8448–8453.
- Verbsky, J. W., Chang, S. C., Wilson, M. P., Mochizuki, Y., and Majerus, P. W. (2005b). The pathway for the production of inositol hexakisphosphate in human cells. *J. Biol. Chem.* **280**, 1911–1920.
- Verbsky, J. W., Wilson, M. P., Kisseleva, M. V., Majerus, P. W., and Wente, S. R. (2002). The synthesis of inositol hexakisphosphate: Characterization of human inositol 1,3,4,5,6-pentakisphosphate 2-kinase. *J. Biol. Chem.* **277**, 31857–31862.
- Voglmaier, S. M., Bembenek, M. E., Kaplin, A. I., Dorman, G., Olszewski, J. D., Prestwich, G. D., and Snyder, S. H. (1996). Purified inositol hexakisphosphate kinase is an ATP synthase: Diphosphoinositol pentakisphosphate as a high-energy phosphate donor. *Proc. Natl. Acad. Sci. USA* **93**, 4305–4310.
- Wilson, M. P., and Majerus, P. (1996). Isolation of inositol 1,3,4-trisphosphate 5/6-kinase, cDNA cloning and expression of the recombinant enzyme. *J. Biol. Chem.* **271**, 11904–11910.
- Xia, H. J., Brearley, C., Elge, S., Kaplan, B., Fromm, H., and Mueller-Roeber, B. (2003). Arabidopsis inositol polyphosphate 6-/3-kinase is a nuclear protein that complements a yeast mutant lacking a functional ArgR-Mcm1 transcription complex. *Plant Cell* **15**, 449–463.
- Yang, X., and Shears, S. B. (2000). Multitasking in signal transduction by a promiscuous human $\text{Ins}(3,4,5,6)\text{P}(4)$ 1-kinase/ $\text{Ins}(1,3,4)\text{P}(3)$ 5/6-kinase. *Biochem. J.* **351**(Pt. 3), 551–555.
- York, J. D. (2006). Regulation of nuclear processes by inositol polyphosphates. *Biochim. Biophys. Acta* **1761**, 552–559.
- York, J. D., Odom, A. R., Murphy, R., Ives, E. B., and Wente, S. R. (1999). A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. *Science* **285**, 96–100.
- York, S. J., Armbruster, B. N., Greenwell, P., Petes, T. D., and York, J. D. (2005). Inositol diphosphate signaling regulates telomere length. *J. Biol. Chem.* **280**, 4264–4269.

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ANALYSIS OF PHOSPHOINOSITIDES AND THEIR AQUEOUS METABOLITES

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Contents

1. Introduction	188
2. Cell Sample Extraction	191
2.1. Acidified “Bligh & Dyer”	194
2.2. Neutral extraction	196
3. Lipid Phase: TLC, HPLC Separation, and Desalting	197
3.1. TLC	197
3.2. Indirect Partisil 10 SAX HPLC-deacylated PIs analysis	199
3.3. Direct Econosphere NH ₂ HPLC	201
3.4. Post-Econosphere desalting	202
4. Aqueous Phase: HPLC Separation, Desalting, and Scintillant Extraction	203
4.1. Partisil 10 SAX HPLC-phosphate	203
4.2. Partisil 10 SAX HPLC-formate	206
4.3. Nucleodex β-OH HPLC	206
4.4. Further Partisil HPLC systems	207
4.5. Desalting	209
4.6. Scintillant extraction	211
5. Chemical Identification	212
5.1. Periodate oxidation	212
5.2. Acidified butanol	214
6. ESI-MS/MS Identification	214
6.1. GPIs in general	215
6.2. GroPIns ₄ P versus MePIns ₄ P	216
7. Standards	219
7.1. InsP _(n-1)	219
7.2. LysoPtdIns ₄ P	221
7.3. GroPIns ₅ P	222

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7.4. Cyclic IPs	224
7.5. MePIns ₄ P	225
8. Final Considerations	226
Acknowledgments	227
References	227

Abstract

Many and various experimental techniques have been developed to fully analyze the intracellular signaling pathways of membrane phosphoinositides and their water-soluble derivatives. This chapter concentrates mainly on the range of lipid-derived, water-soluble signaling molecules that can be produced in cells from these membrane phosphoinositides, for which we and others have proposed biological roles. These include lysophosphatidylinositol, produced via phospholipase A_{1/2} activities on phosphatidylinositol; cyclic inositol phosphates, produced via phosphatidylinositol/lysophosphatidylinositol-specific phospholipase C activities; and glycerophosphoinositols, produced via lysophospholipase A_{2/1} activities on their corresponding lysophosphoinositides. While the methodologies described in this chapter are aimed more specifically at the separation, identification, and quantification of monophosphorylated glycerophosphoinositols and other similarly charged inositol-containing products of the membrane phosphoinositides in cell extracts, they can be equally well applied to the full range of lysophosphoinositides, glycerophosphoinositols, inositol phosphates, and further inositol-containing water-soluble products of the phosphoinositides (e.g., cyclic inositol phosphates, methylphosphoinositol phosphates).

1. INTRODUCTION

In recent years, the role of membrane phosphoinositides (PIs) has extended well beyond their initial intracellular signaling pathways (Payraastre *et al.*, 2001; Toker, 2002) and now includes modulation of cytoskeleton organization, membrane trafficking, and apoptosis (Cantley, 2002; Cockcroft and De Matteis, 2001; Cremona and De Camilli, 2001; De Matteis *et al.*, 2002; Simonsen *et al.*, 2001; Takenawa and Itoh, 2001; Yin and Janmey, 2003); PIs are also known to have an increasing functional relevance in the nucleus (Irvine, 2002, 2003; Martelli *et al.*, 1999). While some of these intracellular events can arise via the formation of *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Ins145P₃) by receptor-stimulated, PI-specific PLC (PI-PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns45P₂) (Berridge, 1993; Nishizuka, 1995), many now appear to be mediated more directly via a selection of modular domains found within PI-binding proteins (Itoh and Takenawa, 2002; McLaughlin *et al.*, 2002). We and others have also proposed biological roles for a number of the water-soluble products of PIs, including lysophosphatidylinositol (lysoPtdIns),

produced via phospholipase A_{1/2} (PLA_{1/2}) activities on PtdIns; cyclic inositol phosphates (cIPs), produced via PI/lysoPtdIns-specific PLC activities; and glycerophosphoinositols (GPIs), produced via lysoPLA_{2/1} activities on their corresponding lysophosphoinositides (LPIs) (see Corda *et al.*, 2002 and references therein).

This chapter provides a profile of the use of a collection of methodological techniques that either have become standard in the field of inositol lipids and inositol phosphates or have been more recently developed to answer specific questions regarding the metabolic processing and hormonal stimulation of the full range of PIs and their aqueous derivatives that can be found in cells (Figs. 11.1 and 11.2). As such, [³H]inositol equilibrium labeling has been by far the most used protocol for following the main aspects of intracellular signaling through PIs during acute receptor/modulator stimulation of cells, and this technique has provided us with essential knowledge of many aspects of PIs and their aqueous derivatives (e.g., see Figs. 11.1 and 11.2). However, it is worth noting here that mass spectrometry techniques are now becoming ever more powerful in their use for the identification of complex molecular structures. Indeed, following on from the development of electrospray-ionization mass spectrometry (ESI-MS) techniques for the detection and identification of the phospholipids in general (for a discussion of quantitative compositional data, see Koivusalo *et al.*, 2001; for a review, see Pulfer and Murphy, 2003), there has been particular recent emphasis toward the use of combinatorial approaches based around mass spectrometry in the study of what has become known as lipidomics and the cellular lipidome, with both computational (Forrester *et al.*, 2004; Lu *et al.*, 2005) and biological (Han and Cheng, 2005; Han and Gross, 2003, 2005; Han *et al.*, 2004, 2005; Hermansson *et al.*, 2005; Houjou *et al.*, 2005; Ivanova *et al.*, 2004; Wenk, 2005) approaches. The wide applications of these lipidomics studies are fully covered in other chapters of this volume.

However, the average laboratory that wishes to dabble in, or indeed to focus on, PIs and their aqueous derivatives will continue to have limited access to the mainstream lipidomics approaches described elsewhere in this volume. Furthermore, the preparation of samples for mass spectrometry analysis still involves the use of at least a cell extract, and in many cases the integration of mass spectrometry with further sample processing. Therefore, despite the strength of these new analytical mass spectrometry techniques, there remains the need for the still widely used standard biochemical and chemical first approaches detailed herein. Thus we follow from the initial cell extraction choices to the need to separate these lipid and aqueous inositol-containing compounds in the cell extracts. Here, the main thin-layer chromatography (TLC)/high-performance liquid chromatography (HPLC) separation techniques then lead us to the identification of these inositol-containing compounds through a number of approaches, including our own mass spectrometry analyses. Finally, the descriptions of the production of a diverse range of

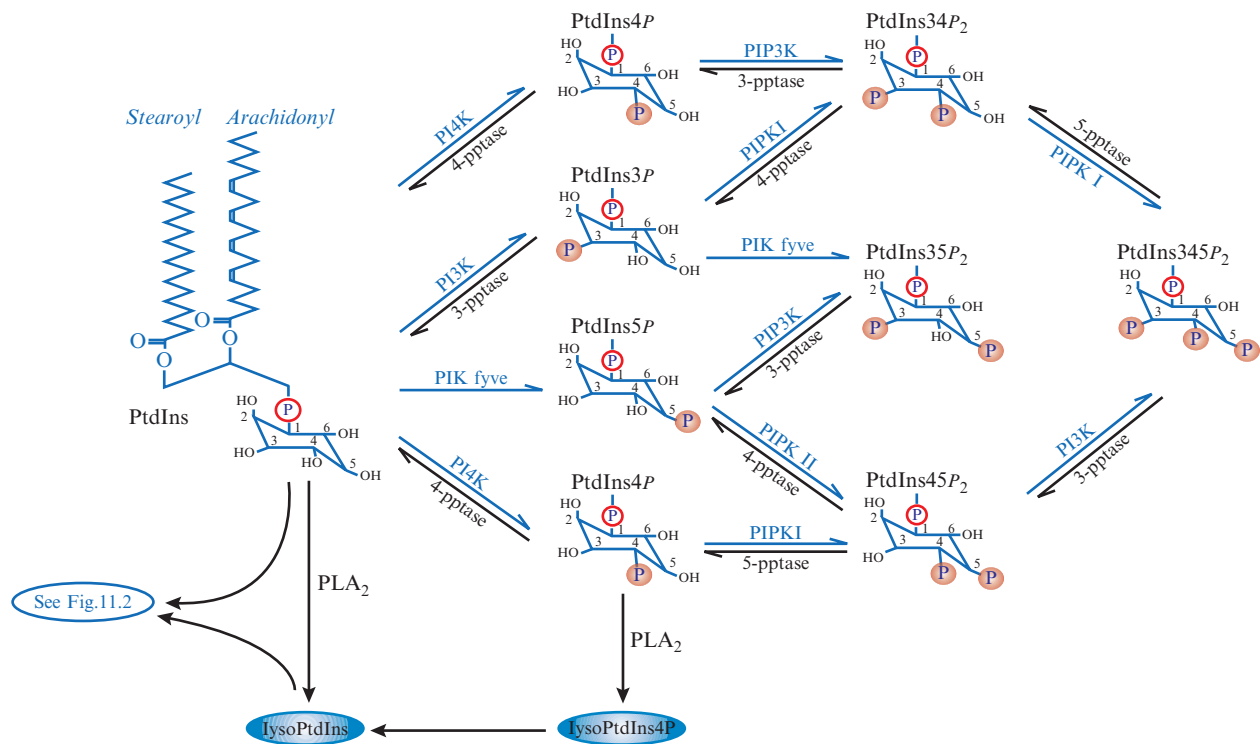


Figure 11.1 Cellular phosphoinositides and their enzymatic interrelationships. The repetition of PtdIns4P is solely for representational convenience.

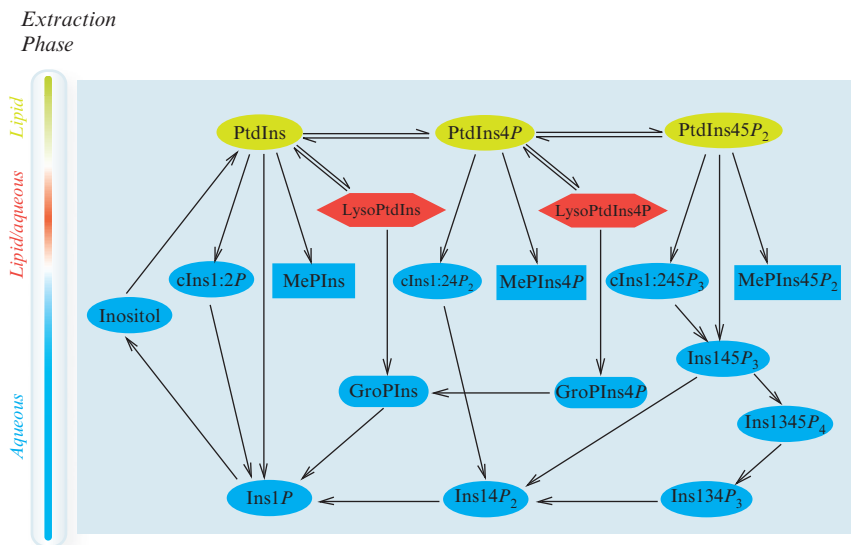


Figure 11.2 Metabolic interrelationships between the classical signaling phosphoinositides and their major aqueous derivatives in tissues and cell extractions.

specific and essential standards in the laboratory from relatively low-cost starting materials are described to complement these approaches.

2. CELL SAMPLE EXTRACTION

For any system for extraction of PIs and their water-soluble derivatives the initial consideration is whether the cIPs (see Figs. 11.2–11.4) or the actual intracellular levels of Ins1P, Ins14P₂, and Ins145P₃ are to be analyzed. Although numerous studies have indeed included the cIPs (see Section 7.4), the main extraction procedure used is the “acidified Bligh & Dyer” (Bligh and Dyer, 1959). The inclusion of acid promotes the separation of the water-soluble products into an aqueous (methanol/water) phase, and the lipids into an organic (chloroform/methanol) phase, with particular effects on the relative separation of the lysophosphoinositides (LPIs). Under acidic extraction conditions, the cIPs are hydrolyzed (compare Fig. 11.3A with Fig. 11.3B), producing Ins1P, Ins14P₂, and Ins145P₃, thus masking the actual cellular levels of these IPs. Brief reference is also made to two alternative methodologies, and for neutral extraction conditions, see Section 2.2.

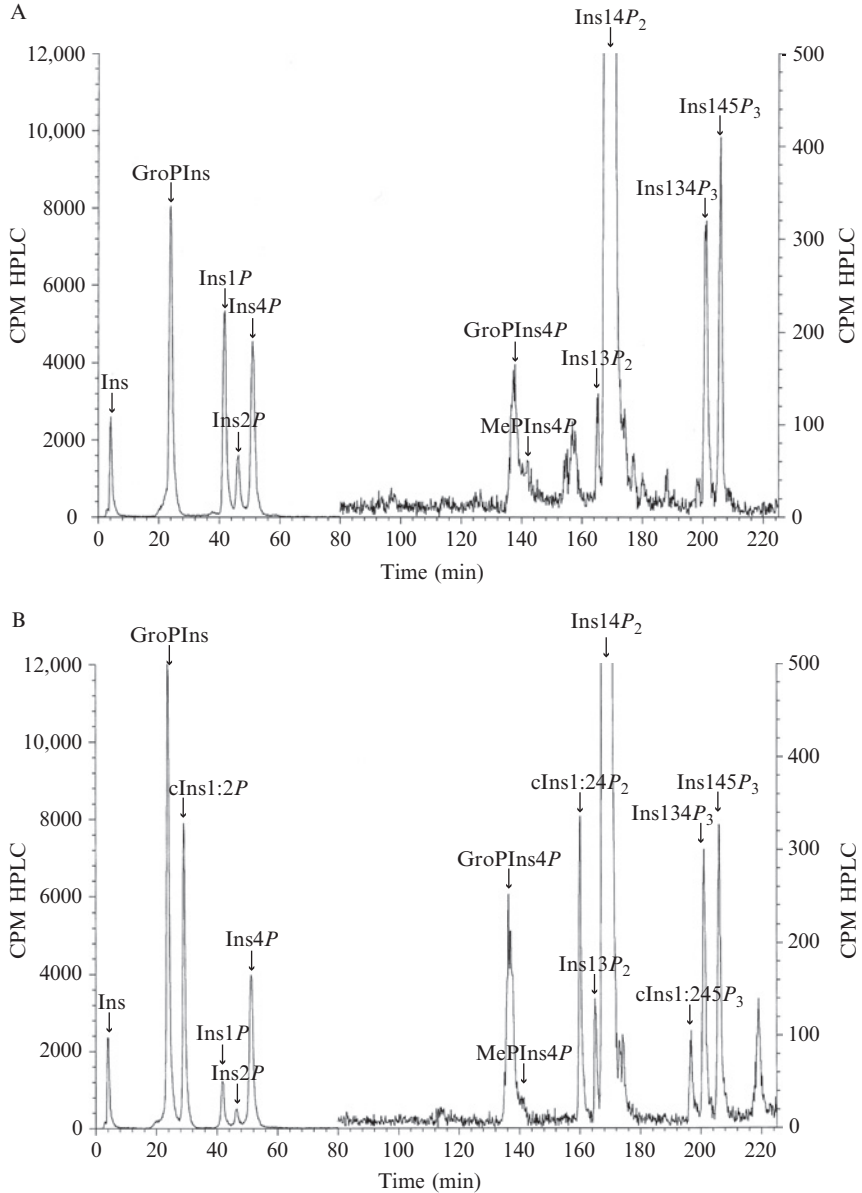


Figure 11.3 Phosphate Partisil 10 SAX HPLC separation (see Section 4.1) of aqueous components from [³H]inositol-labeled FRT-Fibro-Ha cells under stimulation (A23187; 1 μ M; 15 min) following acid (A) and neutral (B) “Bligh & Dyer” extraction conditions (see Section 2). This reveals the higher levels of Ins1P, in particular, produced under acid extraction conditions that arise from the acid hydrolysis of the cIns1:2P seen under neutral extraction conditions.

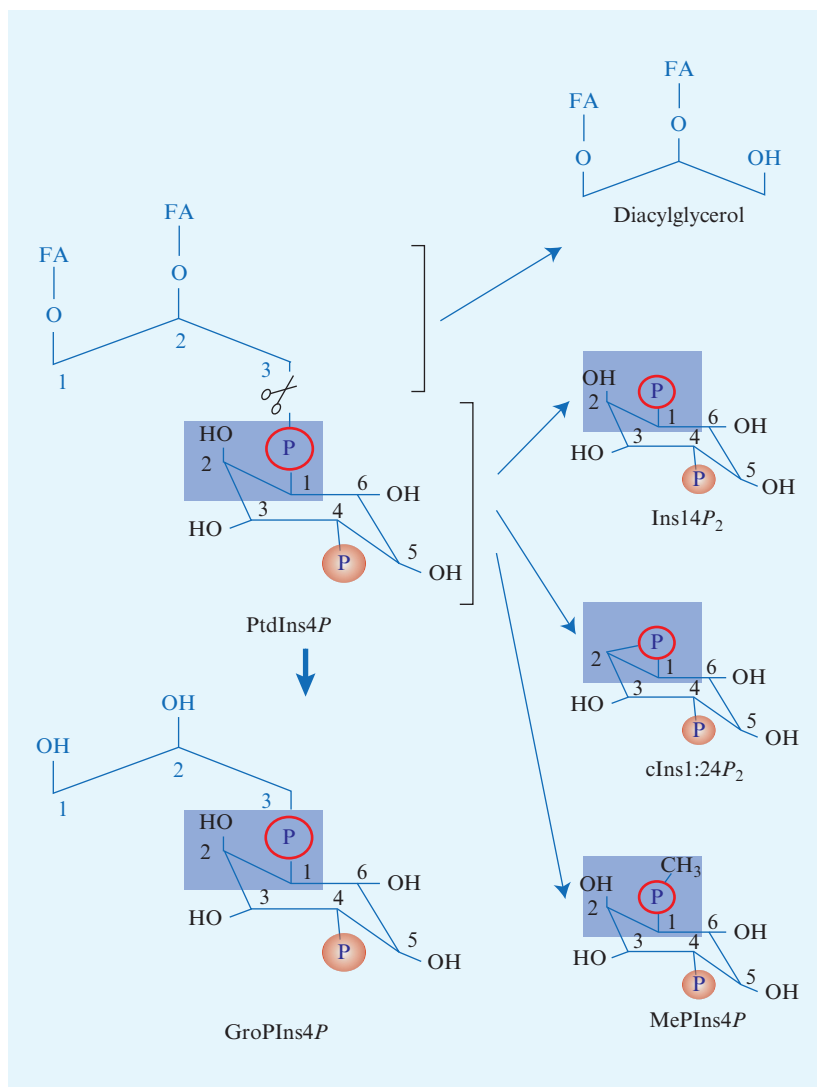


Figure 11.4 Structural variations that can arise from PtdIns4P either in cells or through specific extraction conditions. The liberation of diacylglycerol via either phospholipase C activity or acid hydrolysis can be accompanied by three specific forms of the inositol head group: Ins14P₂, cIns1:24P₂, and MePIns4P. The (lyso)phospholipase A_{1/2} activity will liberate GroPIns4P (via the production of lysoPtdIns4P) (see also Fig. 11.2). FA: *sn*-1 and *sn*-2 fatty acids, generally reported as stearoyl and arachidonyl, respectively, in living cells (see also Fig. 11.1).

2.1. Acidified “Bligh & Dyer”

The acidified Bligh & Dyer extraction can be used under various incubation termination conditions and can also be useful for later analytical stages where there is the need to clean up either aqueous or lipid components of an analysis. This procedure separates the lipid and aqueous components by bringing the extraction to a standard chloroform/methanol/water (plus HCl) ratio to provide a two-phase extraction (lipids in the lower phase, aqueous components in the upper phase). While different groups use slightly different ratios (giving different relative upper-phase and lower-phase volumes), approximately the same volumes of chloroform and methanol should be used, with around half the volume of water. Our standard final ratio for all uses is 1:1:0.5 (chloroform:methanol:water [~ 0.3 N HCl]): 1.25 ml each of chloroform and methanol, 600 μ l water, and 13 μ l concentrated HCl. For cell “killing” and extraction, the acid is combined with the methanol (e.g., methanol/1 N HCl; 1:1, v/v; see later), and no further acid is added. Ratios of up to 1:1:0.9 are also used, and diluted HCl can be used directly as the aqueous component (0.1–1.0 N HCl). This two-phase extraction can also be carried out without acid, resulting in the loss of some of the more charged lipid components to the aqueous phase, particularly in the case of lysoPtdIns ($\approx 40\%$ can remain in the upper, aqueous phase in the absence of acid; see also Section 2.2).

We routinely use cultured cells prepared in 12-well plates for experiments (see also Section 7.5), with the chloroform in the acidified Bligh & Dyer extraction added later due to the use of tissue culture plastics. To terminate incubations, the medium is aspirated rapidly (with suitable disposal if radio-labels are present), and 1 ml methanol/1 N HCl (1:1, v/v) at -20° is added to each well. The cells should be immediately scraped to release them from the plastic (nonsterile standard plastic “cell scraper”), as delayed scraping can change the aqueous ^3H -labeling profile considerably (unpublished data). Transfer the samples to 10-ml test-tubes (chloroform-resistant; e.g., polypropylene, polyethylene), and add 850 μ l -20° methanol:water at 7.5:1 (v/v). Transfer the washes to their respective tubes, and add 1.25 ml chloroform. Vigorously vortex the tubes and leave them for 30 min at room temperature (vortexing occasionally for complete extraction); the phases are then allowed to separate under gravity. If these volumes are used for larger scale extractions (using more cells or tissue pieces), the samples may need to be centrifuged (benchtop, 5 min, 300g, room temperature) to pack the cell debris better at the interface between the two phases.

The upper phases can then be transferred to new tubes, taking care not to disturb the interface and introducing cell debris protein into the samples for HPLC analysis, as this can distort, and even move, the peaks produced. Lyophilize the upper (aqueous) phases and resuspend them in 1.1 ml water, vortex briefly, and filter them (low volume retention filters: 13-mm, 0.2- μ m, nylon syringe filters). One milliliter can then be applied to the relevant Partisil 10 SAX HPLC system under use (see Section 4).

For the lower lipid phase, carefully remove the interface (or remove a standard fixed volume of the lower phase through the interface, dependent on the amount of cell debris), and evaporate the lower phase under a stream of N_2 ; the dried lipid-phase samples can be stored under nitrogen at -20° for later analysis (Section 3).

While this acidified Bligh & Dyer extraction covers most needs, the use of chloroform can sometimes be problematic should a laboratory carry out numerous extractions and not have the use of a fume hood. Therefore, there are two main perchloric acid (PCA)-based incubation termination and extraction systems that can be recommended and are briefly described here (for more details, see Stephens and Downes, 1990).

For the first PCA protocol, following aspiration of the medium, ice-cold 5% PCA is added, the cells are scraped, and the samples are transferred to plastic test tubes. For the potassium-perchlorate-based system of extraction, vortex the PCA-stopped samples and keep them on ice for 30 min. Remove the cell debris by centrifugation (benchtop, 10 min, 800g, 4°), and transfer the supernatant to a clean tube (the lipid fraction in the pellet can be washed and analyzed as indicated later and in Section 3). The perchlorate is removed by precipitation through neutralizing to pH 6.0 to 7.5 with the addition of either KOH or $KHCO_3$, depending on the standard protocols of various groups: for example, (i) add a previously established fixed volume of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 2 M KOH, 10 mM EDTA for a final pH of 6.0 (Stephens and Downes, 1990) or (ii) add a previously established fixed volume of 50% saturated $KHCO_3$ to provide a final pH of 7.5 (Munnik *et al.*, 1998). Following either addition, after 60 min on ice, the precipitated potassium perchlorate is removed by centrifugation (benchtop, 10 min, 800g, 4°); note that by keeping everything at 4° , the potassium perchlorate that remains in solution is minimized (see Stephens and Downes, 1990). Store the supernatant at -20° for further processing by HPLC (see Section 4).

The alternative PCA-based extraction is from the original method of Sharps and McCarl (1982), and has also been reported in Downes *et al.* (1986) and Stephens and Downes (1990). Briefly, incubations are stopped by direct addition to the incubations of an equal volume of ice-cold 10% PCA; scrape/mix vigorously and transfer the samples to plastic test tubes. After 30 min on ice, pellet the cell debris by centrifugation (benchtop, 10 min, 800g, 4°) and transfer the supernatant to a clean tube (microcentrifuge tubes can be used here; the lipid fraction in the pellet can be washed and analyzed as indicated later and in Section 3). To extract the supernatant, add Na_2EDTA to ≈ 2 mM and then at least 300 μ l of a freshly prepared 1:1 mixture (v/v) of tri-*n*-octylamine/1,1,2-trichlorotrifluoroethane (Freon) per 200 μ l 10% PCA added (i.e., 750 μ l for a 500- μ l initial incubation volume). Vigorously mix the samples and briefly microcentrifuge (2–5 s, 12,000g, room temperature); the upper of the resulting three phases contains the aqueous components of the extraction at a pH of ≈ 6 and can be removed to clean tubes for further analysis.

Finally, a trichloroacetic acid (TCA) extraction has also been widely used, although because of the repeated diethyl ether washing and the relative simplicity of the other extraction procedures (Bligh & Dyer, PCA based), this is now rarely used. For completeness, the following protocol was our standard for cells cultured in 12-well plates (Falasca and Corda, 1994; Falasca *et al.*, 1995). Rapidly aspirate the medium, and terminate the incubations by the addition of 1 ml ice-cold 10% TCA to each well. Immediately scrape the cells from the surface of the well and leave the plate at 4° for 10 min. Transfer the samples to plastic test tubes and pellet the cell debris by centrifugation (benchtop, 10 min, 300g, 4°). Transfer the resulting supernatant to new tubes (the lipid fraction in the pellet can be washed and analyzed as indicated later and in Section 3), and resuspend and wash the pellet six times with 6 ml water-saturated diethyl ether. After the final wash, neutralize the aqueous sample by adding enough Tris to take the pH to 7.0 to 7.5.

For the lipid fraction in these PCA/TCA-based extractions, the pellets can be resuspended in chloroform:methanol (2:1, v/v) and put through an acidified Bligh & Dyer extraction at the standard ratios (chloroform/methanol/water; 1.25:1.25:0.6, plus 13 μ l concentrated HCl; see earlier). After removal of the upper phase, the lower phase is taken to dryness under a N₂ stream, and the lipids are ready for further processing (see Section 3).

2.2. Neutral extraction

The simplest neutral extraction procedure parallels the standard acidified Bligh & Dyer extraction (Section 2.1), but without inclusion of the acid. Here the cell incubations are terminated with -20° methanol, and the procedure detailed earlier for the acidified Bligh & Dyer is followed (Section 2.1), with no addition of acid. It should also be noted that this termination of incubations with methanol alone can lead to the production of appreciable quantities of methylphosphoinositols (MePIPs; see later) in some cells.

Again, there is also an alternative protocol that is based on the original method described by Slater *et al.* (1973) for the extraction of adenine nucleotides from mitochondria (see, for example, Hawkins *et al.*, 1987). Briefly, small volume incubations (250 μ l) are stopped by the direct addition of 4° chloroform/methanol (1:2, v/v; 930 μ l). For cell culture plastics, kill the cells with 1 ml methanol at -20° and immediately scrape them; again, note that delayed scraping of the cells can change the aqueous ³H-labeling profile considerably (unpublished data). Transfer the samples to suitable, chloroform-resistant, tubes (polypropylene, polyethylene) and add 500 μ l chloroform. Chill the samples on solid CO₂ and dry them under vacuum. To the dried samples, add 250 μ l water and 750 μ l 10 mM Na₂EDTA/5 mM KH₂PO₄, pH 6.8, followed by 1.0 ml of phenol/chloroform/isoamyl alcohol (38:24:1, v/v/v; phenol heated gently to its melting point prior to use).

Mix the samples vigorously and then centrifuge them to separate the phases (benchtop, 5 min, 300g, room temperature). The upper phase contains the aqueous components with a 90 to 98% recovery of standards. A final alternative has also been described for 250- μ l aqueous cell extracts: add 750 μ l ice-cold EDTA/ KH_2PO_4 , pH 6.8, directly to the samples, followed by 330 μ l phenol/chloroform/isoamyl alcohol at 38:24:1 (v/v/v; Stephens *et al.*, 1988a). As before, mix the samples vigorously and centrifuge them (benchtop, 5 min, 300g, room temperature) to separate the phases; the upper phase contains the aqueous components.

3. LIPID PHASE: TLC, HPLC SEPARATION, AND DESALTING

3.1. TLC

The standard TLC methods used for the analysis of either whole lipid extracts or PI extracts from cells have been based around various different modifications of essentially the same system (Schacht and Agranoff, 1974). For most needs, any of the variously modified standard TLC methodologies can be used for the analysis of radiolabeled PIs from the organic phases of cell extracts (Bligh and Dyer, 1959; Folch *et al.*, 1957; Hara and Radin, 1978). The protocol described here is one of many variations, with further available in the literature (e.g., Bird, 1994a,b, 1998; Munnik *et al.*, 1994; Singh and Jiang, 1995; Zhang and Buxton, 1998).

Aluminum-backed silica gel TLC plates (e.g., silica gel 60 F₂₅₄) are used for the standard TLC separation of lipid samples (Gonzalez-Sastre and Folch-Pi, 1968; Munnik *et al.*, 1994). Pretreat them briefly (soak for 80 s) in 1% potassium oxalate, 2 mM EDTA prepared in 50% ethanol; leave to dry at room temperature and store the dry pretreated TLC plates in their box for later use. This oxalate/EDTA pretreatment is an absolute requirement for the use of this system for the PIs as it sequesters the small amounts of calcium present that interfere with their migration in this system (Gonzalez-Sastre and Folch-Pi, 1968). Although not essential for standard TLC analysis, if glass-backed silica plates are used, the separation of PIs can be improved (although at greater expense) by also oven treating the plates for at least 30 min at 115° immediately before use (Gonzalez-Sastre and Folch-Pi, 1968; Munnik *et al.*, 1994).

Before loading the samples on the pretreated TLC plates, add $\approx$ 120 ml of the running solvent to a standard glass TLC tank, seal it, and allow the atmosphere inside the tank to equilibrate for at least 30 min. The running solvent of chloroform/methanol/4 M NH_4OH (54:42:12, v/v) is based on the original system of Schacht and Agranoff (1974). Also, to limit over-running of the solvent front at the sides of the plate (“smile effect”), scrape

off 0.5 cm of the silica from the plate up each side edge, making sure that this does not interfere with the running of the outside samples (leave at least 1.0 cm between sample positions and new edges of the silica).

Following resuspension of the dried lipid extract (from Section 2; in 150–200 μl of either chloroform:methanol [2:1, v/v] or, for cleaner TLC spotting, in chloroform alone), load the samples in their predetermined positions (pencil marks) along a line drawn 1.5 to 2.0 cm above the bottom of the plate (the origin; to position the loaded samples above the running solvent). Up to eight samples can be loaded per standard TLC plate for optimal use and separation, as the loading of too many samples can result in cross contamination and prevent a scanner from correctly defining the specific lanes. Load the samples slowly (pipette tip always in contact with the silica) under a stream of hot air (hair dryer) to rapidly evaporate the solvent and to obtain small and controlled “spotting” of the samples.

Once fully loaded, position the plate in the solvent in the tank (the sample spots must be above the solvent or sample will be lost to the solvent), cover the tank again, and run the TLC for ≈ 2 h, after which time the solvent front will be within 2.5 cm of the top of the plate. In positioning the plate inside the tank, for uniform running of the samples, make sure it is tilted slightly backwards in the tank ($\geq 10^\circ$ off the vertical, with samples spotted on the upper face); if the plate is positioned badly (or moves during the run), the samples will run more slowly in the upper parts of the silica on the plate.

After completion, remove the TLC plate and allow it to dry in the air (under a fume hood) before scanning. There are various scanning possibilities on the market, and we ourselves have used a linear analyzer with a position-sensitive gas flow (90% argon/10% methane) proportional counter tube (e.g., Berthold/Raytest), with 10 min of data collection being sufficient for standard experimental conditions. More recently, we have used a new type of scanner (Fuji BAS1800 II), which involves an initial period in the dark for the TLC plate and the imaging plate, with scanning being done on the imaging plate.

To process the lipid samples on the TLC plate further (e.g., for confirmation of identification by an alternate method), the lipid bands can be initially defined via a scanner system, with their delineation in pencil. The silica in the individual bands can then be scraped and the lipids extracted by soaking the silica in chloroform:acidified methanol (2:1, v/v). The acidified methanol is provided with 0.1 N HCl by adding concentrated HCl (≈ 12 N) directly to methanol, thus keeping the introduction of water to a minimum. However, while good recoveries are obtained for PtdIns and lysoPtdIns ($>90\%$), despite the improvement gained by acidification, this silica extraction provides minimal extraction of PIPs and PIP₂s (≈ 20 and $\approx 5\%$, respectively). Furthermore, repeated extractions and pooling of samples to increase the amounts of the PIPs and PIP₂s extracted will result in degradation problems during drying down of the resulting extract (see later), through the concentration of acid and water following the initial

removal of chloroform and methanol. Following this extraction of lipids from the silica (vortex; 10 min at room temperature), transfer the solvent (from the silica pieces) to a clean tube and dry it under a stream of N_2 ; the dried, TLC-purified lipid samples can be stored under N_2 at -20° for later processing. Such reduced recoveries are, however, not seen if the samples are processed immediately for deacylation (Section 3.2), and thus the lipid-containing silica can be scraped directly into the methylamine reagent without prior extraction.

To accurately quantify and compare the amounts of [^3H]inositol-labeled PIs on these TLC plates, some potential problems need to be taken into consideration. In our hands, quantification of the [^3H]inositol-labeled lipids using the Fuji scanner (BAS1800 II) gave anomalous ratios of the PIs when compared to deacylation and quantification by aqueous HPLC (Section 3.2). This was confirmed by scraping and counting of the same TLC spots using a scintillation counter. Thus, while it is possible to obtain high counting efficiencies (10–15%) using such dedicated scanners (using long scanning times), the efficiencies of counting of the different lipid types within a single TLC plate can vary (unpublished data). Under our scanning conditions, we obtained a PtdIns:lysoPtdIns:PtdIns4P:PtdIns45P₂ relative counting efficiency ratio, to lysoPtdIns at 1.00 ($\pm$ SE) as the lowest, of 1.81 ($\pm$ 0.10):1.00 ($\pm$ 0.06):1.91 ($\pm$ 0.13):1.52 ($\pm$ 0.14), respectively. Therefore, while PtdIns and PtdIns4P are counted at similar efficiencies in this system, there are significant differences between their counting efficiencies and those of both PtdIns45P₂ ($p < 0.05$) and lysoPtdIns ($p < 0.0001$). These differences potentially arise from a combination of the low energy of the ^3H radiolabel, the thickness of the silica, and the way in which the individual lipids run within the silica layer. This thus illustrates the need to check relative efficiencies of detection not only across different experimental samples, but also within the same samples across the different lipids.

Finally, direct ESI tandem mass spectrometry (ESI-MS/MS) analyses of unprocessed total lipid extracts from rat liver (DeLong *et al.*, 2001) have shown that the phospholipid processing needed for the standard separation and identification approaches, including TLC and HPLC, can often lead to changes in the molecular species composition of the samples themselves. For the present TLC considerations in particular, this study noted both the oxidation of lipid samples (particularly during drying of TLC plates) and the ability for different molecular (acyl group) species of a given phospholipid to migrate differently within a single phospholipid “spot” on a TLC plate (DeLong *et al.*, 2001).

3.2. Indirect Partisil 10 SAX HPLC-deacylated PIs analysis

Despite the higher initial and running costs, an online HPLC analysis system of these lipid samples provides great advantages: (i) better defined separation characteristics of the individual components; (ii) increased sensitivity of

detection, from $\approx 2\%$ efficiency for ^3H on TLC scanners up to $\approx 25\%$ for online scintillation counting; (iii) quicker throughput of sample analysis through automation of HPLC analyses; and (iv) an easier collection system for further processing of individual components following their separation.

However, the combination of the hydrophobic diacylglycerol moiety and the highly charged nature of the inositol head group of the PIs means that the design of HPLC systems for their direct analysis has been limited. Therefore, their separation, identification, and quantification have generally been achieved indirectly, through deacylation of the [^3H]inositol-labeled lipids in the organic phase of the standard acidified Bligh & Dyer extraction (Section 2.1), producing their corresponding [^3H]GPIs (see later). These can then be analyzed as an aqueous extract on one of the many Partisil-based, $(\text{NH}_4)_2\text{PO}_4$, pH 3.8, gradient HPLC systems (Section 4). This can also be combined with a deglyceration with sodium periodate (periodate oxidation; Section 5.1) to produce the corresponding [^3H]IPs, again for separation and analysis on standard phosphate Partisil HPLC systems (Section 4). A similar, but nonradioactive, methodology has also been proposed, whereby the GPIs are detected by suppressed conductivity measurements (Nasuhoglu *et al.*, 2002).

The methylamine deacylation protocol generally uses lower-phase samples from standard acidified Bligh & Dyer extractions (Bligh and Dyer, 1959; Section 2.1) that have been dried under a stream of N_2 , although it can also be applied directly to the lipid-containing silica scraped from TLC plates (Section 3.1) and to the water-washed pellets from the PCA- and TCA-based extractions (Section 2.1).

The methylamine reagent is prepared as a 2-ml mix of aqueous methylamine (40%; 720 μl), methanol (940 μl), *n*-butanol (180 μl), and water (160 μl) (i.e., 180:235:45:40, v/v/v/v, respectively); this is a slight modification of the original methylamine reagent reported due to the previous use of 30% aqueous methylamine with methanol and butanol (at 43:46:11, v/v/v, respectively). Add 1 ml of this mixture to the dried/washed lipid sample, mix well, and incubate in a closed tube for 50 min at 52° . Then evaporate the contents of the tube to dryness under vacuum centrifugation and add 500 μl water to resuspend the deposit. To this, add 700 μl of an extraction mixture containing *n*-butanol, light petroleum 40 to 60° and ethyl formate (20:4:1, v/v/v), and mix well. After brief centrifugation (benchtop, 5 min, 300g, room temperature) to settle the resulting two phases, remove and discard the upper phase (containing the transacylated methylamine and any nondeacylated lipids). Add a further 500 μl of this extraction mixture and mix and centrifuge as before. Remove and discard the upper phase again, and lyophilize the lower phase. For standard cell samples, this procedure produces quantitative recoveries ($\geq 98\%$) of the GPIs from their respective PIs, with minimal levels of IPs formed. However, should this deacylation be used with samples containing very low

chemical amounts of the PIs (e.g., using a purchased standard of [^3H] PtdIns4*P* to produce a [^3H]GroPIns4*P* standard), there will be greater levels of degradation seen (>30%), with the formation of the corresponding IP_s and small amounts of MePIns4*P*. The dried samples can be kept at -20° for later processing, via resuspension in 1.1 ml water, filtering (0.22 μm), and analysis by phosphate Partisil 10 SAX HPLC (Section 4.1).

Here, the deacylation of PIs automatically loses the LPIs to the same GPI pool as their corresponding PIs. Therefore, it may be preferable to analyze cellular PI pools by TLC (Section 3.1) or use a specifically designed lipid HPLC system, as described in the next section (Section 3.3).

3.3. Direct Econosphere NH₂ HPLC

We have designed a lipid separation HPLC system that was modified from that originally described by Morris *et al.* (1995) for the bulk purification of PtdIns4*P* and PtdIns45*P*₂ from the Folch fraction of bovine brain lipids. They used an Econosphere NH₂ 5U HPLC column with a linear 0 to 1 *M* ammonium acetate gradient in chloroform/methanol/water (20:9:1, v/v). Our modifications to this system include the following.

- i. Substitution of chloroform in the running buffer with methylene chloride (dichloromethane; CH₂Cl₂), at the same ratio. This improves the efficiency of online ^3H detection (Packard FLO ONE A-525) from $\approx 8\%$ with CHCl₃ to $\approx 27\%$ with CH₂Cl₂ at a flow rate ratio of sample to scintillant (e.g., Ultima Flow AP, Perkin Elmer) of 1:4 (v/v).
- ii. Improvement of the separation characteristics using a nonlinear gradient system for this Econosphere NH₂ 5U HPLC column: buffer A, methylene chloride/methanol/water (20:9:1, v/v); buffer B, buffer A plus 1 *M* ammonium acetate; gradient (as % buffer B): 0 to 1 min, 0%; 1 to 3 min, 0 to 10%; 3 to 18 min, 10 to 30%; 18 to 43 min, 30 to 100%, 43 to 48 min, 100%; 48 to 50 min, 100 to 0%; 50 to 65 min, 0%.

As shown in Fig. 11.5A, this system provides well-separated peaks of a standard [^3H]inositol-labeled cellular lipid extract, with elution times of PtdIns, 14 to 15 min; lysoPtdIns, 19 to 20 min (double peak); PtdIns4*P*, 34 min; PtdIns45*P*₂, 40 min; and PtdIns345*P*₃, 46 min (not shown). In cells with higher PtdIns:lysoPtdIns ratios, baseline separation of these two lipids can be lost, and the doublet lysoPtdIns peak potentially represents different fatty acid content/positions on the glycerol backbone (length, saturation; *sn*-1, *sn*-2) of lysoPtdIns from various cell sources.

Use of a mixed PtdIns4*P*/lysoPtdIns4*P* standard (75:25) demonstrated that on this lipid HPLC system, lysoPtdIns4*P* coelutes with PtdIns45*P*₂ (see Fig. 11.5A and B). Thus we modified this gradient further (same buffers A and B) to (%B): 0 to 1 min, 0%; 1 to 3 min, 0 to 10%; 3 to 18 min, 10 to

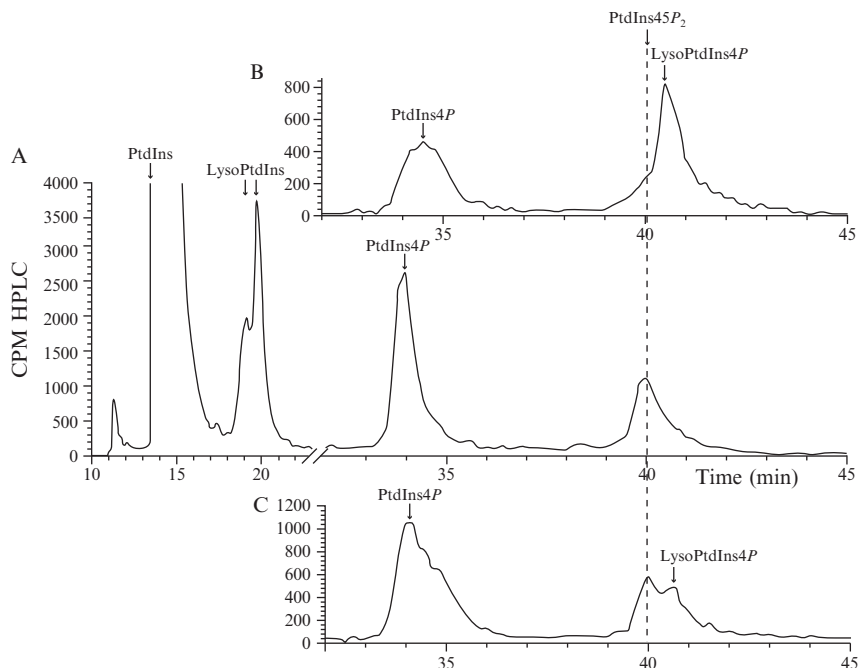


Figure 11.5 Econosphere NH₂ HPLC separation (see Section 3.3) of the lipid components pooled from various [³H]inositol-labeled cells. (A) Standard gradient demonstrating good separation of the main phosphoinositides from cells and the double peak of lysoPtdIns that is often seen on this system. (B) Detail of this standard gradient following bee venom PLA₂ treatment of PtdIns4P, which results in the formation of lysoPtdIns4P. The absence of PtdIns45P₂ in this sample allows the lysoPtdIns4P elution peak to be clearly seen. (C) Detail of an extended gradient with a combined sample from A and B.

20%; 18 to 33 min, 20 to 68%; 33 to 53 min, 68 to 100%; 53 to 58 min, 100%; 58 to 60 min, 100 to 0%; 60 to 75 min, 0%. Although this gradient does not provide baseline separation between lysoPtdIns4P and PtdIns45P₂, it does allow some separation between the 40-min PtdIns45P₂ peak and the 41-min lysoPtdIns4P peak (see Fig. 11.5B and C) and has confirmed our TLC analyses indicating detectable levels of lysoPtdIns4P in specific cell types under certain conditions (e.g., FRT-Fibro-Ha, RBL cells; unpublished data).

3.4. Post-Econosphere desalting

For the preparation of purified pools of PIs following this Econosphere lipid HPLC separation (Section 3.3), the eluate pools can be desalted via the use of methanol/2 M NaCl. For every 6 ml of pooled sample (containing

ammonium acetate) add 2.2 ml methanol, 3.4 ml 2 M NaCl, at 4°, and mix thoroughly. Allow the phases to settle and remove (aspirate) the upper phase and reextract the lower phase with 4 ml methanol, 3.6 ml 2 M NaCl per 6 ml starting pool; mix thoroughly again. Combine the two lower phase extractions and dry under N₂. Store under N₂ at -20° for later use. We have used this system more specifically for the desalting of PtdIns and lysoPtdIns fractions, as the lower phase of the first extraction contains ≈99% of the PtdIns, and the second extraction allows for a better recovery of lysoPtdIns (≈75% overall). For PtdIns4P in particular, we obtained a much reduced recovery of ≈70% overall, with a better PtdIns45P₂ recovery of ≈70% overall.

4. AQUEOUS PHASE: HPLC SEPARATION, DESALTING, AND SCINTILLANT EXTRACTION

There have been many Partisil/Partisphere-5/10 SAX/WAX-based, (NH₄)₂PO₄ gradient HPLC systems used for the analysis of water-soluble products of [³H]inositol-labeled cell extracts (for early review, see Dean and Beaven, 1989). Many of these use personally modified gradient systems to serve specific analytical needs, and they have been coupled to further analytical techniques for the definite identification of each peak obtained (e.g., Stephens *et al.*, 1988a,b, 1989, 1991a). This section provides our recommended systems that have been designed more specifically for full separation of the various IP_n and GPIP/P₂ isomers (phosphate/formate Partisil 10 SAX, Nucleodex β-OH), along with some examples of other specifically designed systems (Partisil 5 WAX/5-PAC). Furthermore, with the need for further, post-HPLC, processing of many samples, we also provide some standard desalting and scintillation extraction methodologies.

4.1. Partisil 10 SAX HPLC–phosphate

Prior to our specific investigations into the signaling significance of the GPIP_ns, these compounds had an early importance through the extraction (Section 2) and deacylation (Section 3.2) of a range of ³H/¹⁴C/³²P-labeled PIs from cells. Thus, we have used phosphate Partisil 10 SAX (strong anion exchange) HPLC to separate and identify not just GroPIns3P and GroPIns4P, but also GroPIns5P, GroPIns34P₂, GroPIns35P₂, and GroPIns45P₂. However, to achieve good baseline separation between GroPIns4P and GroPIns5P and the GPIP₂s, an extended phosphate Partisil 10 SAX HPLC gradient should be used.

In earlier work on GPIs (Falasca and Corda, 1994; Falasca *et al.*, 1995, 1997), the “GroPIns4P” peak that eluted with the standard GroPIns4P

(produced by deacylation of PtdIns4P; Section 3.2) was sometimes seen as a broadened peak. This original standard phosphate Partisil 10 SAX HPLC gradient (120 min; Falasca and Corda, 1994; Falasca *et al.*, 1995) was run at 1 ml/min in water (buffer A) and 1 M ammonium phosphate, pH 3.35 (phosphoric acid) (buffer B). To investigate further this GroPIns4P peak in particular, the gradient was initially extended to 150 min (Meijer *et al.*, 2001) (still at 1 ml/min; as % buffer B): 0 to 45 min, 0.0 to 1.5%; 45 to 46 min, 1.5 to 2.4%; 46 to 80 min, 2.4 to 4.5%; 80 to 81 min, 4.5 to 6.0%; 81 to 141 min, 6.0 to 35.0%; 141 to 142 min, 35 to 100%; 142 to 147 min, 100%; 147 to 150 min, 100 to 0%; 150 to 180 min, 0% wash. This revealed two "GroPIns4P" peaks. The first peak then coeluted with the GroPIns4P standard, while the use of a GroPIns5P standard (Section 7.3) produced a third, later peak that also coeluted with GrPIns4P on the original 120-min phosphate Partisil 10 SAX HPLC analysis.

As a brief illustration of the analytical procedures linked to the identification of this unknown HPLC peak, short periodate treatment (Section 5.1) produced Ins14P₂ from the first peak (GroPIns4P) and Ins15P₂ from the last peak (GroPIns5P), as expected; the middle peak was resistant to the short periodate treatment, but produced Ins14P₂ on acidified butanol treatment (Section 5.2). Thus stimulation of some cells with both EGF and A23187 resulted in an increase in the levels of both GroPIns4P (Corda *et al.*, 2002) and this compound. Through a series of further analyses, which included the use of ESI-MS/MS (see Section 6.2), this was shown to be the methylphosphoryl derivative of Ins14P₂, that is, MePIns4P (see Figs. 11.2–11.4), and its production was linked to the use of –20° methanol to terminate incubations. Use of acidified methanol (methanol:1 N HCl, 1:1, v/v; –20°; see Section 2.1) to terminate incubations resulted in a minimal and constant amount of MePIns4P in the cell extractions, thus allowing the interference of this compound in our HPLC analyses of the receptor-stimulated pathways of GroPIns4P production in cells to be removed.

To better separate these three closely eluting [³H]inositol-labeled compounds, we sought to improve this system further. Of note, to maintain sufficient separation of the GPIP_ns and the higher IP_ns (InsP₃s and above) isomers, the slope of the earlier sections of the gradient needs to be maintained (do not use a steeper initial gradient), as if this section is shortened, this leads to a loss of separation of these later-eluting compounds. On this basis, we further extended this gradient to 220 min (maintaining the 1-ml/min flow rate) for full separation of the polyphosphorylated isomers of both the GPIPs and the IPs, as follows (as % buffer B; see Fig. 11.3): 0 to 140 min, 0.0 to 2.9%; 140 to 195 min, 2.9 to 30%; 195 to 200 min, 30 to 100%; 200 to 205 min, 100%; 205 to 206 min, 100 to 0%; 206 to 225 min, 0% wash.

Here, buffer B (1 M ammonium phosphate, pH 3.35, with phosphoric acid) needs to be prepared beforehand. For a 1.0 liter final volume, add 132.06 g ammonium phosphate to ≈800 ml water and leave stirring until it

appears to have dissolved. Using 85% phosphoric acid, an initial ≈ 70 ml can be added slowly, with continual stirring. Finally, continue to add 85% phosphoric acid until a pH of 3.35 is reached, with the last adjustments made drop by drop. After adjusting the final volume to 1.0 liter, this buffer B is vacuum filtered ($0.45\ \mu\text{m}$) before use; similarly, the water used as buffer A should be vacuum filtered ($0.45\ \mu\text{m}$).

Our full HPLC system also includes short guard columns for each main HPLC column used. These are easily replaced when pressure rises in the system indicate the need, and they serve to provide a longer useful life for the main HPLC columns, despite the filtering ($0.22\ \mu\text{m}$) of all HPLC buffers that do not come directly from HPLC-grade suppliers. Our full HPLC system is also set up for online radiolabel detection via a Packard FLO ONE A-525 detector. This has a number of advantages over the need to collect the HPLC eluate in fractions (usually every 1 min) in scintillation vials for the addition of high salt scintillant and standard β counting in a scintillation counter. There are thus now a number of high salt scintillants that can be used directly for online ^3H detection, with scintillant to eluate ratios of 3:1 to 4:1 usually providing online counting efficiencies of up to 25%. While these efficiencies can be improved upon with standard scintillation counting (up to 50%), where low-activity samples can be counted for longer times, the extra time, effort, and scintillant use are generally best avoided by use of an online radiolabel detection system.

Certain conditions should be fulfilled to consider HPLC coelution with known standards as satisfactory full identification of a given peak. This can be seen by the original coelution of GroPIns4*P*, GroPIns5*P*, and/or MePIns4*P* on our 120-min gradient, in particular. Thus we routinely use the 220-min phosphate Partisil 10 SAX HPLC gradient when the area of the elution profile of the GPIPs is under consideration, and more specifically when the identification of GroPIns4*P* in different cellular systems is required. Thus the coelution of GroPIns4*P* with the deacylation product (see Section 3.2) of either purchased or cell-derived [^3H]PtdIns4*P* is accompanied by peak collection and short periodate treatment (see Section 5.1) for its positive identification. For the latter, Ins14*P*₂ is produced from GroPIns4*P*, Ins15*P*₂ from GroPIns5*P*, and MePIns4*P* is resistant to this treatment (see Section 5.1).

Therefore, while peak coelution with the appropriate standards (see also Section 7) can generally be used as a suitable guide to peak identification on HPLC analysis, this should be double-checked with at least one of the standard procedures also described here (see Sections 5 and 6). For such further analyses, the peak(s) of interest needs to be pooled from the HPLC separation. In its simplest form, this can be achieved by collection of the elution time of interest in 1-ml samples by directing the eluate into suitable tubes in an automatic sampler, set for 1 min/sample for a 1-ml/min HPLC run. Small volumes (10–50 μl ; depending on radiolabel input) can be

transferred to scintillation vials and counted to determine the distribution around the peak of interest, and hence the samples that can be pooled for any specific peak. Once pooled, these samples can be desalted (see Section 4.5) prior to processing for further identification procedures (see Sections 5 and 6).

It is also possible to collect the eluate from an HPLC run following online scintillation counting, pooling the relevant sample times as they exit from the online counting cell. While this can be problematic sometimes (dependent on scintillant used and purity needs) and is thus not ideal, successful scintillant-extraction protocols have been developed and used (see Section 4.6).

4.2. Partisil 10 SAX HPLC–formate

A useful modification of Partisil 10 SAX HPLC is a formate-based buffer system. This has the advantage of providing samples that can be lyophilized directly after pH adjustment, although complete removal of the ammonium formate is not achieved routinely. A further disadvantage is that the full separation of the higher IP_n s isomers (beyond the IP_3 s, in particular) is essentially lost.

Our gradient on this system that we have used primarily for partial purification of specifically selected IP s and $GPIP$ s uses water (buffer A) and 1.5 M ammonium formate, pH 3.37 (formic acid) (buffer B), with the following gradient (as % buffer B): 0 to 120 min, 0 to 35%; 120 to 145 min, 35 to 100%; 145 to 150 min, 100%; 150 to 151 min, 100 to 0%; 151 to 176 min, 0% wash. Buffer B is prepared in a similar fashion to that for the phosphate Partisil 10 SAX system given earlier, and the system is run at 1 ml/min, with 1-min samples collected for liquid scintillation counting, using a fraction collector. After pooling the peak fractions, the pH is carefully adjusted to ≈ 7 by the addition of triethylamine. Here, it is advisable to initially test the pH adjustment of a similarly pooled fraction either before or after the pool of interest to obtain a feel for the volume of triethylamine needed, noting that the HPLC buffer has little buffering power once neutrality is reached, and passed. The ammonium formate can then be largely removed by directly lyophilizing these pooled fractions for at least 24 h (dependent on the condition of lyophilizer; a clean lyophilizer that can pull a good vacuum is essential).

4.3. Nucleodex β -OH HPLC

We have developed a further analytical HPLC technique that distinguishes between the full range of inositol-containing compounds up to and including the IP_{25} , again, through our interest in the $GPIP$ fractions from Partisil HPLC analysis. This uses a Nucleodex β -OH HPLC system (Berrie *et al.*, 2002)

(again, with guard column) that does not solely function on anion-exchange principles, thus providing an elution profile that has proved useful for identification and further purification of these compounds. The potential benefits of this system can be seen from an analysis of the single Partisil 10 SAX GroPIs peak on this Nucleodex system (shown not to be pure; Fig. 11.6A and B) and from the reverse elution profile obtained (see later; Berrie *et al.*, 2002).

The gradient system is essentially linear over the elution range and is based on acetonitrile (buffer A) and acetonitrile/ethanol/acetic acid/water/1 M ammonium acetate/200 mM sodium hydrogen phosphate (180/34/22/220/60/10, v/v; buffer B), with a flow rate of 0.8 ml/min. The 200 mM sodium phosphate stock should be filtered before use (0.22 μ m), and it is essential for elution of the IPs (without altering the inositol and GroPIs elution times), thus indicating the combined reversed-phase and ionic elution obtained. Because of the aqueous nature of the samples, the gradient is started at 35% buffer B directly, 200 μ l of which can be used to resuspend lyophilized samples for application to the system. The gradient used is as follows (as % buffer B): 0 to 5 min, 35%; 5 to 85 min, 35 to 75%; 85 to 87 min, 75 to 100%; 87 to 90 min, 100 to 35%; 90 to 105 min, 35% wash. Inositol elutes at 15 min, and with GroPIs and Ins14P₂ eluting at 30 and 43 min (the most phosphorylated sample we have routinely used on this system; see Fig. 11.6B and C), respectively, this relatively fast (considering the standard Partisil 10 SAX HPLC) elution profile requires sampling at 10- to 15-s intervals for full baseline separation of the components if a fraction collector is used. Although inconvenient, this also provides small volume elution pools for the individual components indicated in Fig. 11.6B and C. Should online scintillation counting be available, the closeness of elution of the peaks from this system indicates that a small volume online analysis cell is needed.

The full significance of this elution profile in comparison with that of the standard Partisil 10 SAX HPLC systems is that while GroPIs again elutes between inositol and the IP₁s, the orders of elution within the IP₁s (Ins4P, 31 min; Ins2P, 32 min; Ins1P, 33 min) and between MePIs4P (36.5 min) and GroPIs4P (40.5 min) are the reverse of those on the standard anion-exchange Partisil 10 SAX HPLC (e.g., compare Fig. 11.6C and Fig. 11.3). As well as providing further purification of samples, this Nucleodex β -OH HPLC system also provides a further approach to peak separation and identification.

4.4. Further Partisil HPLC systems

For some more specific IP separations, other Partisil systems have also been used. One example can be seen with the separation of inositol tetrakisphosphates (IP₄s [Stephens *et al.*, 1988b]), with the following Partisil 5 WAX (weak anion exchange) system. A 12.5-cm Partisil 5 WAX column is pre-equilibrated with water (50 ml at 1 ml/min) prior to sample addition in 1 ml

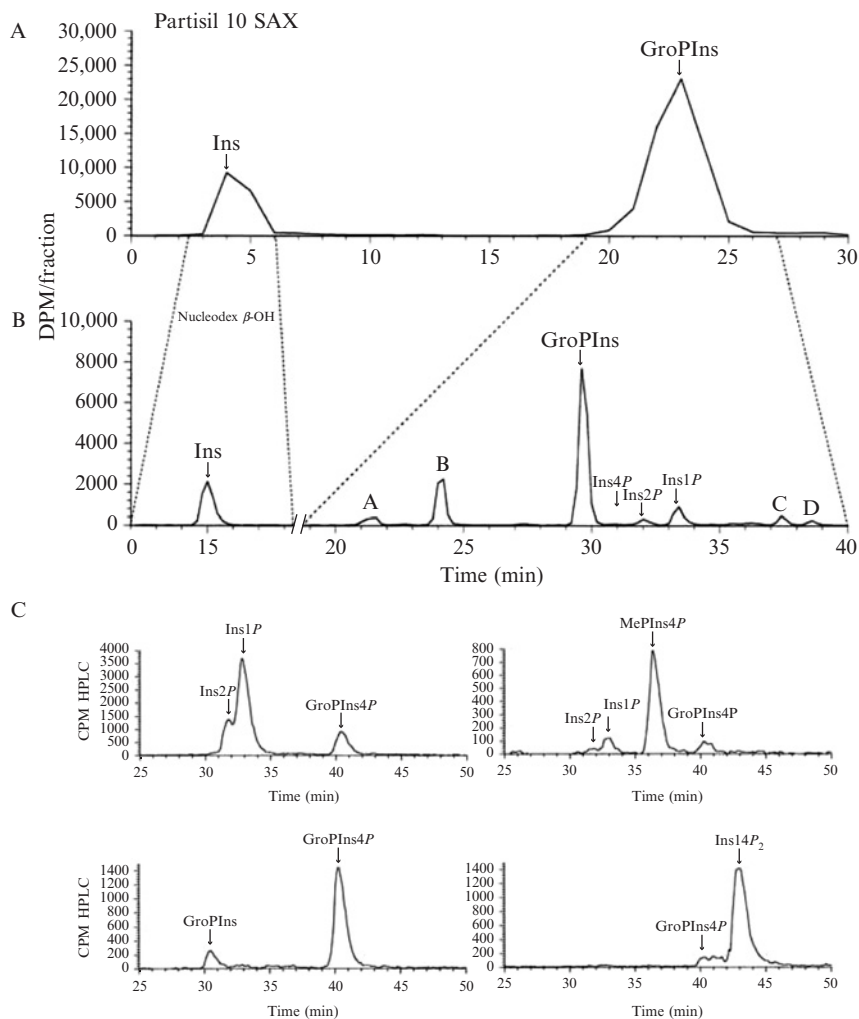


Figure 11.6 Use of the Nucleodex β -OH HPLC system (see Section 4.3) for further analysis of aqueous derivatives of phosphoinositides. (A) Detail of the first 30 min of the Partisil 10 SAX HPLC separation of aqueous components from [^{3} H]inositol-labeled FRTL5 thyroid cells (see Section 4.1). (B) Nucleodex β -OH HPLC separation showing the combined elution profiles of the inositol and "GroPIns" peaks from the Partisil 10 SAX HPLC separation shown in A. While inositol elutes as a single early peak on the Nucleodex system, the Partisil "GroPIns" peak is seen to be separated further into a number of compounds, with GroPIns representing some 90% of the whole, which is variable according to the cell type. The identities of peaks A, B, C, and D remain to be ascertained. (C) Nucleodex β -OH HPLC separation of a series of known standards, demonstrating the reverse elution order for the IP₁s and between MePIns4P and GroPIns4P, as compared to their Partisil elution profile (see text and Fig. 11.3). A, B: Reproduced from Berrie *et al.* (2002), with permission from Elsevier.

water. The gradient uses water (buffer A) and 0.25 M ammonium phosphate, pH 3.2 (with phosphoric acid; buffer B), at a flow rate of 1 ml/min (as % buffer B): 0 to 5 min, 0%; 5 to 6 min, 0 to 60%; 6 to 80 min, 60%; 80 to 81 min, 60 to 100%; 81 to 90 min, 100%; 90 to 91 min, 100 to 0%. Under these gradient conditions, a baseline separation of Ins1346P₄, D-Ins1345P₄, and L-Ins1456P₄ is achieved (in this order) after 50 to 60 min, although the % B required to maintain these elution times (and baseline separation) needs to be reduced with use of the column (Stephens *et al.*, 1988b).

This same Partisil 5 WAX column has also been used with a triethylamine/formic acid gradient for final purification of some of the lesser phosphorylated IPs (<IP₃) and GPIs (up to the GPI_{P2S}) (Stephens *et al.*, 1988b). This uses water (buffer A) and 2 M triethylamine, pH 4.0 (with formic acid; buffer B), and essentially a linear gradient of 0 to 100% buffer B over 40 min. As with the use of formate (Section 4.2), the fractions are lyophilized directly, although it has only proven to be useful with InsP₃s and below for full quantitative elution (Stephens *et al.*, 1988b).

To investigate InsP₁s more specifically, a Partisil 5-PAC system has proven useful for their separation (Stephens *et al.*, 1988b). This uses a 25 × 0.46-cm column (plus guard column) pre-equilibrated in propan-2-ol (100 ml at 0.5 ml/min) and then water (100 ml at 0.75 ml/min). The sample is then applied, and water (buffer A) and 1.2 M sodium acetate/acetic acid, pH 3.6 (buffer B), are used as a linear gradient (after 5 min of 0% buffer B) from 0 to 50% buffer B over 45 min.

4.5. Desalting

Throughout the various analyses needed for these aqueous products of PIs, many procedures obtain samples that contain various salts at relatively high concentrations (mainly phosphate). This is not a problem if this is the final procedure in an analysis. However, as many samples can be processed further, we provide here the details of our two main aqueous sample desalting procedures; for the desalting of lipid samples, see Section 3.4.

To desalt samples from various phosphate-containing HPLC gradients (see Sections 4.1 and 4.4), our standard method is to remove the phosphate while transferring the sample into an ammonium formate-based form. Again, this is because a clean and efficient lyophilizer can remove most of the ammonium formate directly; the principle is also used for the elution of samples from Partisil 10 SAX HPLC with an ammonium formate gradient (Section 4.2). Thus to desalt a sample pool from the standard phosphate Partisil 10 SAX HPLC gradient separation (Section 4.1), first adjust the pH to ≈7 by the addition of triethylamine (practice with a similar HPLC-derived pool before using on any precious samples). Then dilute the sample at least fivefold (v/v) in water and add it to a Dowex AG-1 × 8 (200–400 mesh, formate form) column (200-μl columns can be used because of

the low chemical quantities after the initial HPLC separation). Following a 5-ml water wash of the column, elute the samples with the necessary ammonium formate/formic acid mixture(s) (see later), pH adjust them with triethylamine to ≈ 7 (practice first), and lyophilize them directly to provide the largely desalted extraction from the HPLC buffer (or scintillant, see Section 4.6). Note again the need for a well-functioning lyophilizer.

For elution of IP_s, use (as ammonium formate/formic acid) IP_{1s}, 0.2 M/0.1 M (10 ml); IP_{2s}, 0.4 M/0.1 M (10 ml); IP_{3s}, 0.8 M/0.1 M (10 ml); IP_{4s}, 1.05 M/0.1 M (10 ml); and IP_{5s}, 2.0 M/0.1 M (6 ml). For elution of GPIs, use GroPI_{ns}, 0.18 M ammonium formate/5 mM Na₂B₄O₇ (15 ml); and as ammonium formate/formic acid: GPI_{Ps}, 0.3 M/0.1 M (10 ml); GPI_{P2s}, 0.8 M/0.1 M (12 ml); and GroPI_{nsP3}, 1.0 M/0.1 M (8 ml).

To avoid the need for a well-functioning lyophilizer for the removal of ammonium formate in these and other procedures detailed earlier, two further similar Dowex-based desalting procedures can be used. The first of these takes these pH-adjusted (triethylamine) ammonium formate/0.1 M formic acid samples from the Dowex AG-1 $\times$ 8 columns and forms the Li⁺ salts of the IP/GPI being processed. Thus ammonium formate is removed by LiCl desalting (Grado and Ballou, 1961): dilute the sample 5-fold in water and add it to a 200- μ l Dowex AG-1 $\times$ 8 (200–400 mesh, hydroxide form) column; elute with 3 ml 1 M LiCl. Dry the eluate under vacuum and remove the LiCl by repeated washing of the dried residue with dry ethanol. The second desalting technique here (Stephens *et al.*, 1988b) starts directly from the pH-adjusted (triethylamine), phosphate Partisil 10 SAX HPLC samples. Dilute them 10-fold in water and apply them to 300- μ l Dowex AG-1 $\times$ 8 (200–400 mesh, formate form) columns. Remove the inorganic phosphate using 10 ml 0.2 M triethylamine (pH 4.0 with formic acid) and then elute the InsP_{2–4} fraction with 2 $\times$ 2 ml 2 M triethylamine (pH 3.8 with formic acid). Elute the InsP₅ fraction (of specific interest for Stephens *et al.*, 1988b) with 2 $\times$ 2 ml 2 M ammonium formate/0.1 M formic acid. Both of these fractions can then be lyophilized directly (Stephens *et al.*, 1988b); also note that for quantitative recovery in water of trace quantities of the InsP_{5s} through this procedure (where the samples contained triethylamine), the use of polypropylene vials was necessary, as opposed to glass.

With the use of Dowex AG-1 $\times$ 8 (200–400 mesh) being common through most of these desalting techniques, it should be noted that the various ionic forms of this Dowex can be generated (and regenerated) as columns as follows (per resin volume, using a flow rate of 2 ml/min/cm³):

- i. OH[−] to Cl[−]: conversion, 2 volumes 1 N HCl; rinse, 4 volumes deionized water; completion, pH > 4.5.
- ii. OH[−] to formate: conversion, 2 volumes 1 N formic acid; rinse, 4 volumes deionized water; completion, pH > 4.5.

- iii. Cl^- to OH^- : conversion, 20 volumes 1 N NaOH; rinse, 4 volumes deionized water; completion, $\text{pH} < 9$.
- iv. Cl^- to formate: convert to OH^- (iii) and then to formate (ii).

Finally, we have developed a relatively rapid standard desalting procedure for larger sample pools from phosphate Partisil 10 SAX HPLC. This is particularly useful, as it leaves a minimal salt residue and provides recoveries of 70 to 80% for GroPIs and InsP_1 s, 60 to 70% for GPIPs, and $\approx 50\%$ for InsP_2 s. However, for GPIP_2 s and IP_3 s and above, the recoveries are very low ($\leq 10\%$) and some salt remains in the samples. This desalting procedure is based on the knowledge that methanol addition to the standard phosphate buffer used for Partisil 10 SAX HPLC results in precipitation of the phosphate (and hence repeated washing is needed if an HPLC system is being changed between a Partisil 10 SAX column for aqueous phase separation and an Econosphere column for lipid phase separation) and that propanol has been used in a basic pH background for paper chromatography desalting. Thus our standard procedure here is as follows: for every 1-ml pooled fraction from the phosphate gradient of a Partisil 10 SAX HPLC analysis, add 12 ml propanol:33% NH_4OH (5:4, v/v). Mix well and leave for 10 min at room temperature before centrifuging to pack down the resulting precipitate (benchtop, 5 min, 800g, room temperature). Filter the supernatant ($0.22\ \mu\text{m}$) before further processing. Resuspend the pellet in 500 μl water for every 1-ml pooled fraction and add a further 6 ml propanol:33% NH_4OH (5:4, v/v). Take this reextraction through the same procedure as before and combine and lyophilize the two filtered supernatants. When resuspended in water, a fine precipitate is usually seen, which can be removed by a further filtration step ($0.22\ \mu\text{m}$).

4.6. Scintillant extraction

Samples that have been run through HPLC with online scintillation counting (or indeed from full scintillation counting) can also be extracted. For example, this might be necessary to perform further analytical steps in the identification of unknown peaks following HPLC separation and scintillation counting. As such, the scintillants used here will generally be of the “high salt” type because of the salt concentrations needed for the aqueous anion-exchange HPLC systems used (Sections 4.1–4.4). The following protocol illustrates some of the considerations involved, and it is based on one originally used by Stephens *et al.* (1988b) and Stephens and Downes (1990).

For every 1 ml of a “standard” high salt scintillant (containing scintillation fluid/water/methanol/HPLC eluant, in various ratios depending on the scintillant, the system used, and the elution time of the gradient), add 300 μl chloroform. After addition of 500 μl water/ml scintillant, it may also be necessary to add up to around 300 μl methanol/ml scintillant to promote

the formation of two clear phases upon centrifugation (benchtop, 2 min, 300g, room temperature). The partitioning of the detergents present in the scintillant is aided by the addition of salt, which is usually in the form of the HPLC buffer, to maintain better sample uniformity. This will be dependent on the area of the HPLC gradient where the sample has come from (IP_4 s and above should not need extra salt), with the aim of providing ≈ 50 mM salt [≈ 35 μ l/ml scintillant of 1.25 M $(NH_4)_2HPO_4$; pH 3.8 with H_3PO_4]. Once this has been mixed vigorously and centrifuged (benchtop, 2 min, 300g, room temperature), dilute the upper phase ≈ 10 -fold with water, adjust the pH to ≈ 7 with triethylamine, and add the sample to a 2-ml column of Dowex AG-1 $\times$ 8 (200–400 mesh, formate form). The sample can then be eluted as detailed for desalting in Section 4.5.

It should also be stressed that as there are a number of differently based scintillants on the market, we have used variations on this extraction theme that were individually designed and tested according to the scintillant system used. The general concept is thus to provide a two-phase chloroform/methanol/water extraction system (as for Bligh & Dyer extractions [Bligh and Dyer, 1959]; Section 2) and to at least initially follow the partitioning of the compound(s) of interest through use of a radiolabel.

5. CHEMICAL IDENTIFICATION

To reliably identify peaks on HPLC analysis of specific samples, coelution with a known standard will be the first approach to be taken. However, should such a standard not be available or not coelute precisely with a given peak (and there are no standard conditions for its analysis by approaches such as mass spectrometry), chemical approaches for full identification can then be followed. This section gives details of the main useful techniques in this respect: for details for the chemical dephosphorylation of the IP s in particular, see Section 7.1.

5.1. Periodate oxidation

The standard periodate oxidation methodology for IP s and GPI s was originally modified from Brown and Stewart (1966) and is to confirm the identification of the aqueous products of the PI s. It is based on the ability of carbon–carbon bonds to be cleaved by sodium periodate only if they are flanked by free hydroxyl groups (Brown and Stewart, 1966; Grado and Ballou, 1961). The sensitivities of the GPI s to this treatment have shown that this cleavage occurs initially at the glycerol of the GPI s (short periodate treatment; 30 min), producing the equivalent IP s, and then later within the inositol ring, providing there are suitable sites for cleavage (long periodate treatment; 36 h).

Thus the short periodate treatment (see also Stephens *et al.*, 1988a) is generally used to remove the “glycerol backbone” from the GPIs (see Section 5.2 for a simple alternative). It can also be used as an analytical procedure for the identification of, for example, coeluting peaks on phosphate Partisil 10 SAX HPLC, such as GroPIIns4P, GroPIIns5P, and MePIIns4P: the GPIPs will produce Ins14P₂ and Ins15P₂, respectively, while MePIIns4P is resistant to this treatment. Briefly, lyophilize the sample in a small (2 ml) dark-glass vial. Resuspend it in 250 μ l water, 250 μ l 20 mM sodium periodate, pH 5.5 (nitrogen bubbled prior to use); incubate in the dark for 30 min at 25°. Then add 14 μ l of a 1:10 dilution in water of 99.5% ethylene glycol solution (a 5-fold excess over the sodium periodate) to remove the remaining sodium periodate; incubate for a further 30 min in the dark at 25°. Next add 1.8 ml freshly prepared 1% aqueous dimethylhydrazine solution (pH 4.5 with formic acid) to remove the aldehydes; incubate for a further 4 h at 25°. Finally, dilute the sample to 5 ml with water and apply it to an 8-ml column of Bio-Rad AG-50W $\times$ 8 (200–400 mesh, hydrogen form) to remove excess dimethylhydrazine. Pool the flow-through plus the first 4 ml water wash (following sampling for scintillation counting to determine peak eluate samples), adjust the pH to $\approx$ 7 with triethylamine, and lyophilize. Resuspend the sample in water before applying it to the selected HPLC system (Section 4) for identification of the [³H] InsP₂s produced (or for confirmation of resistance to this treatment).

As indicated earlier, the long periodate treatment (36 h) will both remove the glycerol moiety of the GPIs and rupture the inositol ring in all of the available positions (carbon–carbon bonds flanked by free hydroxyl groups). This procedure parallels that for the short treatment, with the initial 30-min sodium periodate incubation in the dark at 25° being extended to 36 h, and it allows the IPs to be referred to as periodate resistant or periodate sensitive. For the GPIP_ns and MePIP_ns that can be obtained from deacylation (Section 3.2) and acidified methanol treatment (Section 7.5), respectively, and that can be obtained from the PIP_ns found in living cells (phosphorylated in the 3, 4, 5, 34, 35, 45, or 345 positions), only those deriving from PtdInsP₃ will be resistant to this long periodate treatment (because of the phosphate group that is also provided at position 1 on the inositol ring). This is of note for GPIPs identification: that is, while the short periodate treatment will allow differentiation between the products of the almost coeluting GroPIIns4P, MePIIns4P, and GroPIIns5P, under long periodate treatment the disappearance of a peak in this region of a phosphate or formate Partisil 10 SAX HPLC separation (see Debetto *et al.*, 1999) cannot be used as proof of the presence of a GPIP (GroPIIns4P or GroPIIns5P), as MePIIns4P will also be “removed.”

The long periodate treatment was also used initially to determine the stereochemical configurations of the IPs that are produced during agonist stimulation of cells, via the resulting polyol production, separation, and

identification (as first adapted by Irvine *et al.*, 1984). The full background details of this approach can be found in Stephens and Downes (1990).

5.2. Acidified butanol

We have designed an acidified butanol treatment of GPIs that is a relatively simple and quick way to avoid the need for their periodate oxidation (Section 5.1) for their corresponding IP_s. Generally using volumes = 1 ml, slowly add concentrated HCl ($\approx 12\text{ N}$) to butanol at a ratio of 1:4 (v/v), while mixing gently. Heat this acidified butanol to 100° in a boiling water bath and add the GPI sample. After a further 1 min in the boiling water bath, cool and lyophilize the solution. This treatment removes the glycerol moiety from the GPIs and can be used to either produce a known IP_n from a known GPIP_n or, alternatively, to produce an identifiable IP_n from an unknown GPIP_n.

6. ESI-MS/MS IDENTIFICATION

Most mass spectrometry studies have been used in combination with one or more of the standard analytical techniques described here, such as TLC and HPLC. This is similar to the use of gas chromatography and nuclear magnetic resonance, which are also often combined with mass spectrometry. As such, these combinatorial approaches are becoming ever more powerful for the identification of complex molecular structures.

For the PIs themselves, fast atom bombardment mass spectrometry (FAB-MS) (Sherman *et al.*, 1985), matrix-assisted laser desorption and ionization/time of flight mass spectrometry (MALDI-TOF-MS) (Muller *et al.*, 2001; Schiller *et al.*, 1999), and ESI-MS/MS (Hsu and Turk, 2000) have all been used to define their structural determinants. For biological samples, MALDI-TOF-MS has been used for PtdIns in murine brain extracts (Berry *et al.*, 2004) and ESI-MS for PtdInsP and PtdInsP₂ in total lipid extracts from cultured cells and rat brain (Wenk *et al.*, 2003). The full biological applicability of these mass spectrometry techniques has also expanded more recently with analyses of the acyl chain contents of PtdIns during its synthesis and transfer via the PtdIns transfer protein in tissue and cultured cells (Hunt *et al.*, 2004; Postle *et al.*, 2004); ESI-MS has also been applied to the analysis of lysoPtdIns in biological fluids due to an emerging role for this lysolipid as a potential marker for ovarian cancer (Shen *et al.*, 2001; Sutphen *et al.*, 2004; Xiao *et al.*, 2000, 2001).

Various aqueous extraction and mass spectrometry analyses have also been applied to a number of biological systems, including the identification of cIPs (Mandal *et al.*, 2002; Shayman *et al.*, 1986; Wilson *et al.*, 1985a,b),

Ins145P₃ (Portilla and Morrison, 1986), and the diphospho derivatives of InsP₄s and InsP₅s (Stephens *et al.*, 1993). Of particular interest, there has also been a more recent report of structural distinctions among the IP_n isomers that can be revealed through high-energy and low-energy collisional-activated dissociation under ESI-MS/MS analysis (Hsu *et al.*, 2003). However, with the high phosphate buffers used routinely for the separation of IPs, GPIs, and their isomers (Section 4.1), this has limited the use of mass spectrometry for their measurement in biological samples. Similarly, from the lipid analysis side, mass spectrometry approaches have more generally been applied to direct cell extracts without extensive treatments being followed.

6.1. GPIs in general

We have used ESI-MS/MS for the identification of GPIs as standards and GroPIIns in cell extracts following their separation on a novel Nucleodex β -OH-based HPLC system that allows the samples to be applied directly to ESI-MS/MS (Berrie *et al.*, 2002; Dragani *et al.*, 2004). With the use of a standard solution containing a mixture of GroPIIns, GroPIIns4P, and GroPIIns45P₂, this provided baseline separation of these three GPIs. However, there is a much reduced detection sensitivity to GroPIIns4P and GroPIIns45P₂ (15- and 30-fold, respectively), which, when coupled to the greatly reduced concentrations of these GPIs in cells (a maximum of a few μ M vs 50–900 μ M for GroPIIns [Berrie *et al.*, 2002]), means that in biological samples it has only been possible to reliably detect and quantify intracellular GroPIIns concentrations (see Berrie *et al.*, 2002).

We achieved this after the standard acidified Bligh & Dyer extraction (Section 2.1) of cultured cells grown to confluence on 8.5-cm petri dishes. Detach the cells with trypsin/EDTA (using a minimal volume of 2–3 ml/petri dish) and wash twice in 10 ml HBSS⁺⁺. To allow the later conversion of ESI-MS/MS mass determinations of GroPIIns into intracellular concentrations, a small sample of the washed cell suspension is taken for cell counting and determination of cell volume (e.g., Coulter counter). After final pelleting of the cells (benchtop, 5 min, 300g, room temperature), add 5 ml methanol (–20°), followed by 4 ml water, 5.5 ml chloroform, and 66 μ l concentrated HCl (acidified Bligh & Dyer; Section 2.1). Mix and aliquot the upper phase (1 ml) for lyophilizing. Resuspend the dried samples in 100 μ l water and apply 20- μ l aliquots to the HPLC-ESI-MS/MS system (see also Berrie *et al.*, 2002; Dragani *et al.*, 2004).

This HPLC-MS/MS analysis was performed at room temperature using a micro-LC pump system and a Nucleodex β -OH HPLC column (plus guard column). Injection of standards and samples was performed automatically via an autosampler (thermostated at 4°) with a 20- μ l injection loop (see also Berrie *et al.*, 2002; Dragani *et al.*, 2004). Separation of the GPI

standards, GroPIIns, GroPIIns4P, and GroPIIns45P₂, and of GroPIIns in cell samples was achieved using a flow rate of 0.7 ml/min, with a binary linear gradient of 20 mM ammonium formate (solvent A) and acetonitrile (solvent B): from 20 to 56% solvent A in 12 min, followed by a 10-min system reequilibration with 20% solvent A (Berrie *et al.*, 2002; Dragani *et al.*, 2004).

The Nucleodex β -OH column eluate entered directly into a triple-quadrupole mass spectrometer (via the micro-LC pump system) through a Turbo IonSpray interface operated in negative ESI mode (see also Berrie *et al.*, 2002; Dragani *et al.*, 2004). The mass spectrometer was initially calibrated using polypropylene glycol as standard, and the mass spectrometry parameters for each compound were optimized by direct infusion of each standard in the mobile phase using a make-up system. Analyses were performed using the highly specific multiple reaction monitoring mode for which a “precursor-to-product ion” pair of each analyte was monitored. The ion pair used for GroPIIns detection and quantification (against standard samples of known GroPIIns concentrations) was precursor ion $\rightarrow$ product ion m/z of 333 $\rightarrow$ 153 amu. The standard levels of GroPIIns4P and GroPIIns45P₂ were monitored with the ion pairs m/z 413 $\rightarrow$ 241 amu and m/z 493 $\rightarrow$ 395 amu, respectively. This methodology showed a high sensitivity toward GroPIIns (limit of detection $\approx$ 10 ng/ml; with intracellular concentrations of 50–900 μ M; Berrie *et al.*, 2002).

In principle, this system can also be used for the separation and detection of IPs, particularly as they often have relatively high intracellular concentrations and show higher levels of fragmentation under ESI-MS (unpublished data). However, this HPLC system provides separation only up to the IP₃s, and therefore the system would need modifying for the higher charged IP_ns.

6.2. GroPIIns4P versus MePIIns4P

For ESI-MS/MS analysis of MePIIns4P in comparison with GroPIIns4P (see also Section 4.1), these compounds were produced as standards *in vitro* using acidified methanol treatment of PtdIns4P (see Section 7.5). Following an acidified Bligh & Dyer extraction (Section 2.1) after 60 min of this treatment of PtdIns4P, the aqueous phase was analyzed by infusion in negative-ion mode ESI-MS, with the resulting fragmentation patterns in the negative ions in the m/z 100–480 range given in Fig. 11.7A. This ESI-MS spectrum was obtained on a quadrupole instrument, interfaced with a Turbo IonSpray source and operated in the negative-ionization mode. Infusions were made in methanol/water (1:1, v/v) with 0.1% formic acid at a flow rate of 5 μ l/min. The instrument conditions were as follows: ion spray voltage, -440 V; orifice voltage, -50 V; ring voltage, -250 V; collision cell gas, N₂ at 2.2×10^{-5} Torr; collision energy, 24 eV.

To obtain further information on the structures of some of these peaks in the initial mass spectrometry analysis (Fig. 11.7A), they can be subjected to

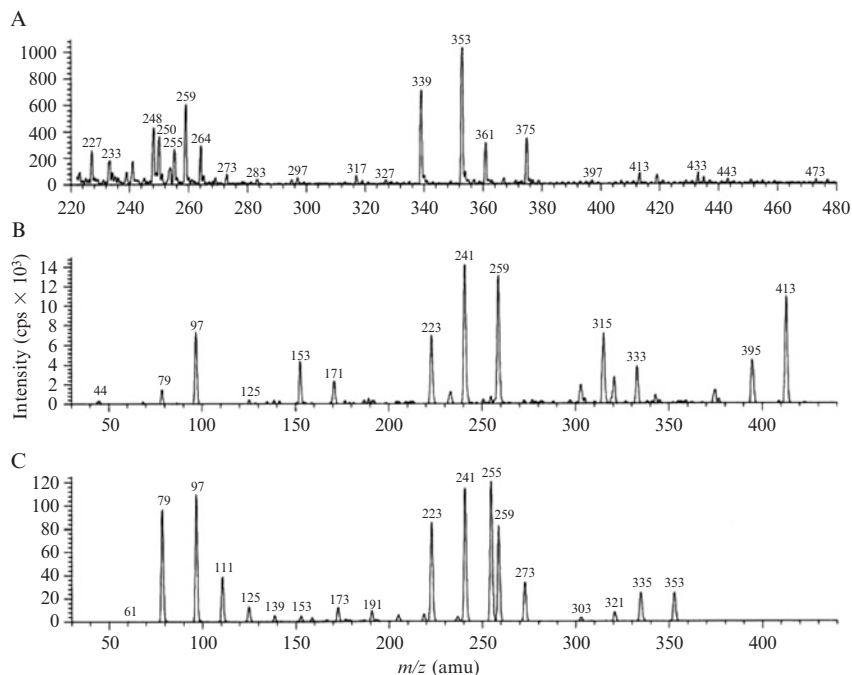


Figure 11.7 ESI-MS spectra obtained from infusion of a 60-min sample of an aqueous phase “acidified Bligh & Dyer” extraction of acidic methanol treatment of PtdIns4P (methanol/1 N HCl, 50°, 60 min). (A) Negative-ion full scan mass spectrometry spectrum of this 60-min aqueous extract. (B) Negative-ion MS/MS spectrum of the m/z 413 ion in A (arising from GroPIns4P). (C) Negative-ion MS/MS spectrum of the m/z 353 ion in A (arising from MePIns4P). See Fig. 11.8 for fragmentation analysis of the m/z 413 and 353 negative ions and text for details (Section 6.2).

negative-ion ESI-MS/MS analysis using the triple quadrupole potential of these machines, allowing the fragmentation of selected peaks within a sample. Figure 11.7B and C show this further analysis for the m/z 413 and 353 negative ions seen in Fig. 11.7A, respectively. As indicated in Fig. 11.8A and B by the deduced fragmentation patterns from Fig. 11.7B and C, respectively, the main fragments seen for negative ion at m/z 413 correspond to those of GroPInsP, whereas those at m/z 353 confirm that this is a very different fragmentation pattern, representing MePInsP. Although the ability to designate the individual positions of the phosphate groups is lost here, as the starting material was PtdIns4P, the only positions possible should be the 1-phosphate and the 4-phosphate on the inositol ring. Similarly, the fragmentation pattern of negative ion at m/z 353 does not allow the specific designation of the position of the methyl group in MePIns4P; this could be on the 1-phosphate, as indicated in Fig. 11.8B, or on the 2-hydroxide of the inositol ring.

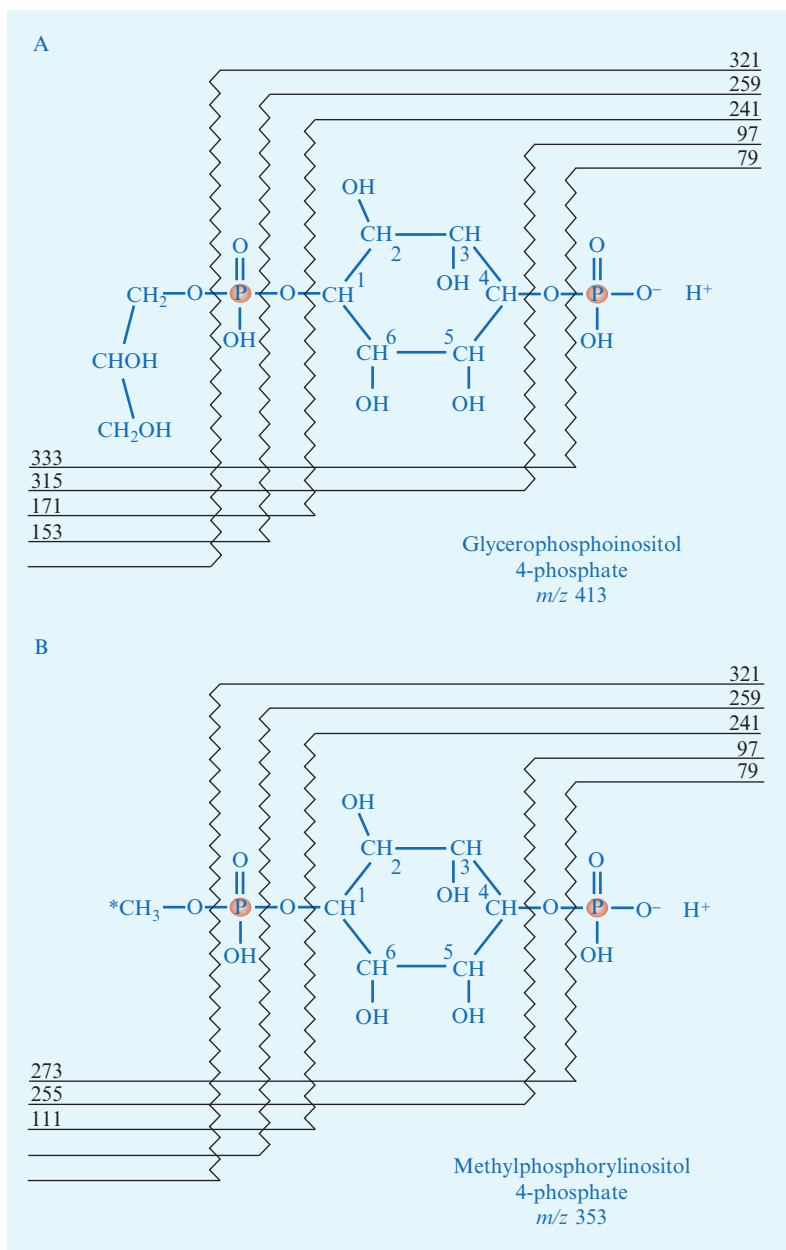


Figure 11.8 Main fragmentation profiles of the *m/z* 413 and 353 negative ions seen in Fig. 11.7 produced from GroPIIns4P and MePIIns4P, respectively (see also Section 6.2). *The positioning of the methyl group of MePIIns4P is shown as being on the 1-phosphate, although it could also be positioned on the 2-hydroxide of the inositol ring.

Certain negative ions are common between these two ESI-MS/MS analyses: from InsP_2 (m/z 321), InsP (m/z 241, 259), and phosphate (m/z 79, 97). Alternatively, there are also those that are specific for GroPInsP (from GroPInsP , m/z 394, 413; from GroPIns , m/z 315, 333; from GroP , m/z 153, 171) and for MePInsP (from MePInsP , m/z 334, 353; from MePIns , m/z 255, 273; from MeP , m/z 111).

This thus demonstrates how the use of different ESI-MS/MS profiles can be used for determination of the main structural groups of these, and similar, compounds. At the same time, the identification of MePIns4P also included the analysis of nonradiolabeled samples from cells by negative-ion ESI-MS/MS. This required larger quantities of the cell-derived MePIns4P for analysis, as its levels in cell extracts were generally much lower than those of GroPIns4P . Here, the use of large-scale incubations and 30% methanol to “stimulate” the cells (Section 7.5) produces large levels of MePIns4P (and some MePIns45P_2). Although this did allow the production of enough MePIns4P for its m/z 333 negative ion to be seen in our ESI-MS/MS analysis of the cell extract, any system that is used more routinely with living cell samples needs to be better defined and standardized, as we ourselves have done in the case of our analysis of GroPIns levels in cultured cells (Berrie *et al.*, 2002; Dragani *et al.*, 2004).

7. STANDARDS

Although a range of specific standards are now available commercially, many compounds that can be equilibrium radiolabeled in cells with [^3H] inositol still remain too expensive, too difficult to produce, or too little used in either their radiolabeled or their unlabeled forms. Hence, it can be useful to produce specific compounds as standards for HPLC and MS analyses in particular and to be aware of the various ways that such standards can be produced with the technology and chemistry available in the average “phosphoinositide laboratory.” To illustrate this point, we provide specific details here for the production of multiple IP_n isomers and four of the more “unusual” standards.

7.1. $\text{InsP}_{(n-1)}$

The chemical removal of phosphates from any given IP_n standard can be used for the production of less phosphorylated $\text{IP}_{(n-1)}$ standards, while the pattern of the products from an unknown IP_n can provide information about its identification. This procedure will also result in the formation of IPs from GPIPs , and thus as an identification technique it needs to be approached with caution. As an example of this protocol, as initially

described by Stephens *et al.* (1988b), this section describes production of the full range of IP_1 s and IP_2 s from a known [^3H]inositol-labeled $Ins145P_3$ standard (purchased), with the consequent confirmation of the products. Of note, with the low levels of radioactivity that are actually needed for standards in HPLC analyses, the use of random chemical dephosphorylation of a single purchased IP_n standard can remove the need for the purchase of the full range of less phosphorylated radiolabeled standards.

Mix $\approx 100,000$ dpm of a purchased sample of [^3H] $Ins145P_3$ with 1.0 mg phytic acid in 100 μ l water; the phytic acid ($InsP_6$) is included as a carrier for the very low chemical amount of $Ins145P_3$, thus avoiding potentially large losses to glassware or plasticware during the procedure. It is advisable to initially clean the sample: add this solution to a 200- μ l column of Dowex AG-50W $\times$ 8 (200–400 mesh, ammonium form); once it has entered the column, elute it with 100- μ l aliquots of water. Collect and pool the first 400 μ l of eluate (containing the sample). After lyophilizing this pool, resuspend it in 500 μ l 10 M NH_3 solution and transfer the sample to a sealed vial (aluminum crimp seal, PTFE/silicon septa, borosilicate glass; sealed Carius tube [Stephens *et al.*, 1988a] or a carefully sealed equivalent); place this vial in a sand bath at 110° for ≈ 30 h. This sample is then lyophilized, and the chemical dephosphorylation of the [^3H] $Ins145P_3$ can be followed through extended phosphate Partisil 10 SAX HPLC (Section 4.1). As shown in Fig. 11.9, under these conditions about 30% of the [^3H]

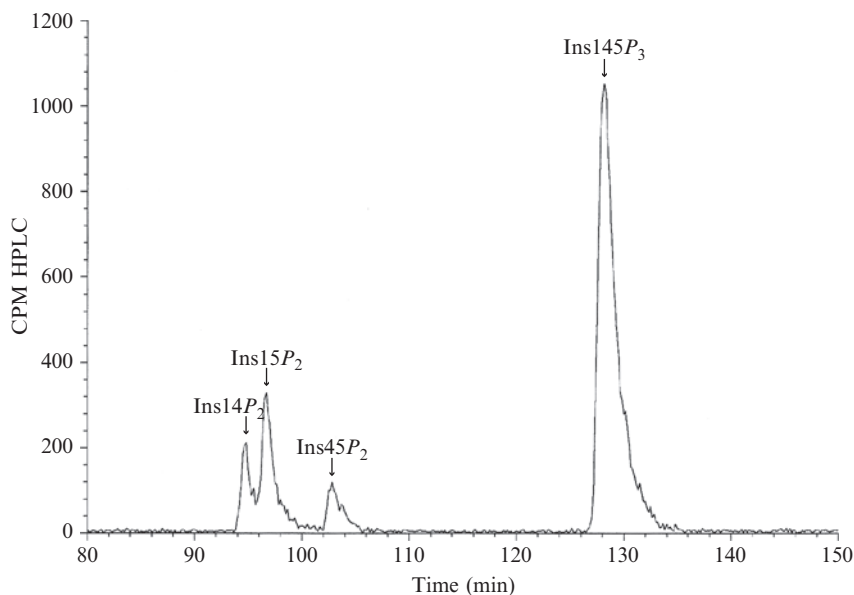


Figure 11.9 Detail of Partisil 10 SAX HPLC separation following 10 M NH_3 solution treatment of a [^3H]inositol-labeled $Ins145P_3$ standard for 30 h at 110° (see Section 7.1).

Ins145P₃ is converted into the full range of possible IP₂s (Ins14P₂, Ins15P₂, Ins45P₂), giving three peaks that elute at 95, 97, and 103 min under our HPLC system. The first of these coeluted precisely with our Ins14P₂ standard (purchased and from cells). To further identify the last of these peaks (103 min), the collected sample was put through the same chemical dephosphorylation for a further 25 h, yielding a broad double peak (39.0–40.0 min) that essentially coeluted with the Ins4P standard and which contained no Ins1P (32.0–33.0 min) (not shown). This had thus produced Ins4P and Ins5P, identifying the 103-min peak as Ins45P₂, and hence the 97-min peak as Ins15P₂. Similar dephosphorylation of the second IP₂ peak confirmed this profile (yielding Ins1P and Ins5P). This procedure can thus produce the full range of IP₁ and IP₂ standards that include phosphate groups in the 1, 4, and 5 positions. The same protocol can be used starting from the higher phosphorylated IP_ns, with Stephens *et al.* (1988b) noting that for obtaining their full pattern of IP₁s, the incubation at 110° will need to be carried out for times beyond 100 h.

Alkaline conditions can be used instead should this high-temperature, NH₃-based incubation prove problematic. As detailed by Stephens *et al.* (1988b) for the dephosphorylation of InsP₆, resuspend the sample in 50 mM Na₂HPO₄/NaOH, pH 10.5, and heat it to 120° in an autoclave. After 3 h, about 15 to 20% of the InsP₆ will be dephosphorylated to mainly give an approximately equal range of IP₅s. Before further processing this mixture through HPLC for the separation of the resulting four chromatographically distinct IP₅s, take the pH to ≈7 with formic acid and add EDTA to 5 to 10 mM. This procedure can then be repeated for the chemical dephosphorylation of the full range of the various IP_ns.

7.2. LysoPtdIns4P

Generally there is no need to actively monodeacylate PtdIns to obtain lysoPtdIns, as the lipid phases from cell extracts contain usable levels of lysoPtdIns (≈6–8% of the PI pool from [³H]inositol-labeled cells; see also Fig. 11.5) that can be separated from PtdIns both on TLC (Section 3.1) and using our standard Econosphere NH₂ lipid HPLC system (Section 3.3). However, if there is the need to produce lysoPtdIns4P either as a standard or as a substrate, this can be achieved using bee venom PLA₂ and PtdIns4P under the following conditions, which can also be used for the production of any of the LPIs from their respective PIs.

Aliquot the necessary amount of purchased or cell-extracted [³H] PtdIns4P into a clean glass tube and dry under a stream of N₂. Add 540 μl incubation buffer (50 mM Tris, pH 8.8 [±5 mM sodium cholate]), and sonicate (bath or probe, to disperse the light lipid deposit into the buffer; vortexing should be sufficient if sodium cholate is included). Then add 10 mM CaCl₂ (6 μl from 1 M stock) and 100 U/ml bee venom PLA₂

(60 μ l from 1000 U/ml purchased stock) to give a final incubation volume of 600 μ l. Leave this at 37° for $\approx$ 4 h, during which time $\approx$ 50% of the [^{3} H] PtdIns4*P* should be converted to [^{3} H]lysoPtdIns4*P*. This sample can then be put directly through an acidified Bligh & Dyer extraction (Section 2.1), with the maintenance of the standard chloroform:methanol:water:concentrated HCl ratio for a two-phase split (1.25:1.25:0.6:0.01) by the addition of 1.25 ml chloroform, 1.25 ml methanol, and 13 μ l concentrated HCl. After vigorous mixing and then standing at room temperature for $\approx$ 10 min, allow the phases to settle under gravity. Remove the upper phase to a clean tube (this will also contain some of the lysoPtdIns4*P* produced) and evaporate the lower lipid phase to dryness under a stream of N₂. Separate the [^{3} H]PtdIns4*P* and [^{3} H]lysoPtdIns4*P* on the short-gradient Econosphere HPLC system for the PIs (as there is no PtdIns45*P*₂ present; see Fig. 11.5A and Section 3.3) and collect and pool the peaks of [^{3} H]PtdIns4*P* and [^{3} H]lysoPtdIns4*P* (detected through online counting or sampling and liquid scintillation counting) for desalting (Section 3.4).

7.3. GroPIns5*P*

Due to either the absence of or the presence of very low levels of PtdIns5*P* in cells, the production of a radiolabeled GroPIns5*P* standard by deacylation (Section 3.2) of the parent lipid, PtdIns5*P*, from cells is not generally possible. To produce a radiolabeled GroPIns5*P* standard ourselves to identify the deacylation product from a PI that was believed to be PtdIns5*P* produced by the unicellular, biflagellate alga *Chlamydomonas moewusii* (Meijer *et al.*, 2001), the following procedure was used.

The starting material for the production of ^{32} P-labeled GroPIns5*P* is [^{32} P]PtdIns35*P*₂. This was produced by ^{32} P-labeled *Chlamydomonas* cells that were stressed osmotically (Meijer *et al.*, 1999), although this can be replaced by either purchased [^{3} H]PtdIns35*P*₂, where possible, or from radiolabeled PtdIns35*P*₂ extracted from cells such as osmotically stressed yeast (Dove *et al.*, 1997) or some mammalian cell lines (dependent on the level of stimulation of its production; see also Michell *et al.*, 2006). The radiolabeled PtdIns35*P*₂ is initially deacylated as part of the lipid pool using methylamine deacylation (Section 3.2); this is the standard method for the removal of fatty acid chains from LPIs and PIs.

Following deacylation of the [^{32} P]PtdIns35*P*₂, lyophilize the full lower phase ($\approx$ 400 μ l), resuspend it in water (1.1 ml), and apply the filtered (0.22 μ m) sample to the phosphate Partisil 10 SAX HPLC (1.0 ml; Section 4.1); the peak that corresponds to the known elution time of GroPIns35*P*₂ is collected. The identity of this [^{32} P]GroPIns35*P*₂ produced can be confirmed through a combination of dephosphorylation and deglyceration of the products via periodate oxidation (Section 5.1) or acidified butanol treatment (Section 5.2). The [^{32} P]GroPIns35*P*₂ pool is desalted

using a 2-ml Dowex AG1 $\times$ 8 (200–400 mesh, formate form) column (Section 4.5), eluting the [^{32} P]GroPIns35P₂ in 2-ml aliquots of 1.0 M ammonium formate/0.1 M formic acid. Pool the peak fractions (fractions 2, 3, 4; 6 ml), adjust the pH to 6 to 7 with triethylamine, and lyophilize thoroughly. Resuspend in water, and specifically dephosphorylate the 3,5-bisphosphorylated inositol head group of the [^{32} P]GroPIns35P₂ in the 3-position by a modification of the 5-phosphatase selectivity of human erythrocyte (red blood cell; RBC) ghosts through the inclusion of EDTA in the incubation (Stephens *et al.*, 1991b; Whiteford *et al.*, 1997). This is an enzyme preparation obtained from human RBC ghosts, which we have prepared as follows.

To a suitable volume of fresh human blood, add the necessary anticoagulant (e.g., per 6 volumes blood, add 1 volume ACD anticoagulant: 2.5 g trisodium citrate, 1.5 g citric acid, 2.0 g glucose, to 100 ml in water at 4°), and pellet the RBCs by centrifugation (benchtop, 5 min, 1100g, 4°). After aspiration of the supernatant (plasma) and the buffy coat, wash the RBCs twice in 150 mM NaCl, 1.5 mM HEPES, pH 7.2 (to original volume; centrifuge as before). After this second wash, resuspend the RBCs to the same volume in citrate buffer (50 mM HEPES, pH 7.2, 57.5 mM trisodium citrate, 5 mM sodium pyruvate, 5 mM inosine, 1 mM adenine, 10 mM glucose), and after a final centrifugation (as before), store the packed RBCs at 4° overnight or process them further immediately (at this stage, they can also be radiolabeled with [^{32} P]P_i for production of ^{32} P-labeled PIPs; see Harden *et al.*, 1987, 1988). Resuspend 1 volume packed RBCs in $\approx$ 15 volume RBC lysis buffer: 20 mM Tris, pH 7.1, 1 mM EDTA. After 30 min on ice, centrifuge (15 min, 12,000g, 4°) and then aspirate and discard the supernatant; resuspend the pellets in a further 15 volumes RBC lysis buffer. Following centrifugation as before and aspiration of the supernatant, loosely resuspend the RBC ghost pellet by swirling the tube so that upon tipping of the tube, the dark red pellet can also be removed by aspiration without loss of RBC ghosts. This procedure is repeated for a further three washes of the RBC ghosts in RBC lysis buffer, with the final wash of the pelleted RBC ghosts in 20 mM Tris/acetate buffer, pH 5.5. Loosely resuspend the final pellet by swirling, and aliquot and freeze at -20° for later use.

Resuspend 500 μ l of these packed RBC ghosts in 1 ml EDTA buffer (12.5 mM HEPES, pH 7.0, 1 mM EGTA, 5 mM EDTA), pellet by centrifugation (microcentrifuge, 5 min, 12,000g, 4°), and wash twice in EDTA buffer. After final resuspension in 500 μ l EDTA buffer, use 150 μ l in the final 300 μ l radiolabeled GroPIns35P₂ dephosphorylation incubation in EDTA buffer. After 5 h at 37°, stop the incubation by addition of an equal volume (300 μ l) of ice-cold (4°) 10% PCA (Section 2.1). Following 30 min on ice, centrifuge the sample (10 min, 17,000g, 4°) to remove the RBC ghost pellet and neutralize the supernatant (to pH 7.5 with 50% KHCO₃). After a further 60 min on ice, remove the precipitate again by

centrifugation as before and use the supernatant directly as a spiked radio-labeled GroPIns5P standard with the extended phosphate Partisil 10 SAX HPLC (150 min; Section 4.1). Under these conditions, where the elution times of both GroPIns3P and GroPIns4P are known, this [^{32}P]GroPIns5P standard will elute some 1.0 to 2.0 min after the [^3H]GroPIns4P standard (Meijer *et al.*, 2001). Upon further extension of the HPLC gradient beyond that of Meijer *et al.* (2001), to 220 min (Section 4.1), it is possible to demonstrate three essentially independent peaks in this region, GroPIns4P, MePIns4P, and GroPIns5P (in that order), that all coelute on the shorter (120 min) phosphate Partisil 10 SAX HPLC gradients originally used by ourselves, and as also used by a number of other laboratories.

7.4. Cyclic IPs

^{32}P -labeled cIns1:245P₃ can be produced from ^{32}P -labeled RBC ghosts (see Section 7.3) along with [^{32}P]Ins145P₃ (see also Harden *et al.*, 1987, 1988). However, if the need is for a range of cIPs or for significant chemical quantities, we have used the following protocol for the production of microgram quantities of cIns1:24P₂ and cIns1:245P₃ (Auchus *et al.*, 1987).

Dissolve 40 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbamide hydrochloride in 40 ml water and add 400 μl triethylamine (pH to ≈ 11.4); add about 150 μl concentrated HCl to take the pH to 8.2. Dissolve the Ins14P₂ or Ins145P₃ for cyclization in water (1 mg in 200 μl) and then add it to this prepared solution; when needed, the radiolabeled tracer(s) should also be added at this stage. The solution is then pH adjusted back to 8.2 with triethylamine, and the volume is taken to a final 50 ml with water. After 3 h with stirring at room temperature, add 800 μl 4 M ammonium formate to quench this reaction mixture and then lyophilize. For the production of a standard, a sample can be taken for HPLC analysis prior to lyophilizing and applied directly to the HPLC column to determine the extent of cyclization obtained.

The sample is then resuspended in 800 μl water and desalted using our propanol/ammonium hydroxide protocol described in Section 4.5. Briefly, add 9 ml propanol:33% NH₄OH (5:4, v/v), mix well, centrifuge (bench-top, 5 min, 800g, room temperature), and filter (0.22 μm) the supernatant. Resuspend the pellet from the centrifugation in 100 μl water and add a further 6 ml propanol:33% NH₄OH (5:4, v/v); reextract as before. Finally, combine the two filtered supernatants (15 ml) and lyophilize; the recoveries at this stage of the overall standards (cyclic plus noncyclic) should be $\approx 80\%$ for the bisphosphate and $\approx 50\%$ for the trisphosphate.

Although the reported levels of cyclization of Ins14P₂ and Ins145P₃ through this protocol are 25% (Auchus *et al.*, 1987), we ourselves obtained around 5 and 15%, respectively. To improve on these final yields, the Ins14P₂ and Ins145P₃ can be recycled through this cyclization protocol

following separation from their relevant cIP and desalting. To achieve this, resuspend each postcyclization sample in water and apply it batch wise to a standard Partisil 10 SAX analytical column. Collect the separated cIP and IP peaks, desalt them through the propanol/ammonium hydroxide desalting (Section 4.5), and reuse them through this cyclization protocol. For confirmation of the cIP structures for their elution times on the HPLC, some of the samples should be acid treated (or simply put through an acidified Bligh & Dyer extraction; Section 2.1) to confirm their hydrolysis to their respective IPs (Ins14P₂ for cIns1:24P₂, and Ins145P₃ for cIns1:245P₃). Similarly, once the elution times of these cIPs have been established, further ³H-labeled standards can be produced by direct stimulation of cells (see, for example, Fig. 11.3) and collecting and desalting of the relevant fractions from the HPLC of the aqueous cell extract following a neutral extraction procedure (Section 2.2). Routine confirmation of the acid-labile nature of these cIP peaks should be carried out for full confirmation of their identification.

7.5. MePIIns4P

As indicated earlier (Sections 4.1, 6.2), with the extension of the gradient of our standard phosphate Partisil 10 SAX HPLC system (Section 4.1), the “GroPIIns4P region” of the elution profile was shown to contain the previously essentially coeluting standards of GroPIIns4P, MePIIns4P, and GroPIIns5P. While the identity of the GroPIIns5P peak was achieved through the production of a ³²P-labeled GroPIIns5P standard (Section 7.2), identification of the MePIIns4P, and its further phosphorylated equivalent, MePIIns45P₂, required further analytical procedures and the production of their radiolabeled standards.

MePIIns45P₂ has been previously produced *in vitro* by the acidified methanol treatment of both its cIP equivalent, cIns1:245P₃ (Lips *et al.*, 1988), and its “parent” lipid, PtdIns45P₂ (Brown *et al.*, 1988). We produced MePIIns, MePIIns4P, and MePIIns45P₂ with the lipid approach, both as standards (using the [³H]inositol-labeled PIs) and in microgram quantities (using the unlabeled PIs). Briefly, aliquot 100 μg of each of the unlabeled PIs into separate clean glass tubes and add the respective [³H]inositol-labeled PI standard if needed; dry under N₂. Add methanol in ≈1 N HCl (12:1, v/v, methanol/concentrated HCl) and incubate at 50°. This *in vitro* system can produce [³H]inositol-labeled MePIIns, MePIIns4P, and MePIIns45P₂, which can also be further verified according to their resistance to short periodate treatment (Section 5.1), the incorporation of [¹⁴C]methanol during their production *in vitro* (unpublished data), and their negative-ion ESI-MS/MS fragmentation profiles (Section 6.2).

We have also defined cell culture conditions for the production of these MePIPs as standards. This was achieved through the use of [³H]

inositol-labeled FRT-Fibro-Ha cells (H-Ras-transformed fibroblasts; although any cell type should suffice) and direct treatment with methanol. This followed our standard conditions for [^3H]inositol equilibrium labeling, stimulation, extraction, and analysis of cells in 12-well plates. The FRT-Fibro-Ha cells are routinely grown in 8.5-cm-diameter petri dishes in Coon's medium supplemented with glutamine, penicillin, streptomycin, and 5% calf serum, with the addition of gentamycin for maintenance of the transformation. Detach the cells with trypsin/EDTA and pellet them by centrifugation (benchtop, 5 min, 300g, room temperature); after a wash in growth medium, plate the cells out at a suitable concentration for 60 to 80% confluence in the 12-well plates after 36 to 48 h. Once at >60% confluence, radiolabeling of these cells is carried out (add $\approx 10 \mu\text{Ci}$ [^3H]inositol/well) for 36 to 48 h in 1 ml M199 medium supplemented with L-glutamine, penicillin, streptomycin, and 2% calf serum. Following this radiolabeling period, aspirate the medium (with suitable radiolabel disposal), wash the cells twice with 1 ml HBSS $^{++}$, and add 700 μl HBSS $^{++}$ followed by 300 μl methanol. Incubate the cells in this 30% methanol for 2 min at 37 $^\circ$ (which provides the maximal stimulation of MePIIns4P production while maintaining viable, living cells; they will be fixed rapidly at 40% methanol) and kill and extract the samples as described in Section 2.1, through the acidified Bligh & Dyer extraction. Under these conditions, the main MePIP produced is MePIIns4P, along with relatively high levels of MePI45P $_2$.

8. FINAL CONSIDERATIONS

With the birth of the “-omics” approaches to lipid analysis, the use of techniques such as mass spectrometry has opened the possibilities of fully investigating the field of the PIs and their aqueous derivatives. It should be noted that the first approaches to the identification of an unknown PI or PI derivative in a cell are still generally based on the more rapid, routine, and economical analytical protocols described here. Similarly, some of these protocols are needed for the processing of samples prior to mass spectrometry. As we ourselves achieved with our ESI-MS/MS approach for the quantification of intracellular levels of GroPIIns (Section 6.1; Berrie *et al.*, 2002; Dragani *et al.*, 2004), this might just involve linking of the standard acidified Bligh & Dyer extraction (Section 2.1) and a new HPLC separation system to the mass spectrometer. We foresee that in the coming years this complementarity between cell sample handling and extraction and final lipid detection will increase, with mass spectrometry probably becoming a routine procedure. However, until this is achieved, most investigators will remain dependent on the various chemical and biochemical analyses included herein.

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REFERENCES

- Auchus, R. J., Kaiser, S. L., and Majerus, P. W. (1987). Synthesis of inositol 1,2-(cyclic)-4,5-trisphosphate. *Proc. Natl. Acad. Sci. USA* **84**, 1206–1209.
- Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–325.
- Berrie, C. P., Dragani, L. K., van der Kaay, J., Iurisci, C., Brancaccio, A., Rotilio, D., and Corda, D. (2002). Maintenance of PtdIns45P2 pools under limiting inositol conditions, as assessed by liquid chromatography-tandem mass spectrometry and PtdIns45P2 mass evaluation in Ras-transformed cells. *Eur. J. Cancer* **38**, 2463–2475.
- Berry, G. T., Buccafusca, R., Greer, J. J., and Eccleston, E. (2004). Phosphoinositide deficiency due to inositol depletion is not a mechanism of lithium action in brain. *Mol. Genet. Metab.* **82**, 87–92.
- Bird, I. M. (1994a). Analysis of cellular phosphoinositides and phosphoinositols by extraction and simple analytical procedures. *Methods Mol. Biol.* **27**, 227–248.
- Bird, I. M. (1994b). Analysis of cellular phosphoinositides and phosphoinositols by high-performance liquid chromatography. *Methods Mol. Biol.* **27**, 249–277.
- Bird, I. M. (1998). Phosphoinositidase C activation assay. III. HPLC analysis of cellular phosphoinositides and phosphoinositols. *Methods Mol. Biol.* **105**, 25–45.
- Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Brown, D. M., and Stewart, J. C. (1966). The structure of triphosphoinositide from beef brain. *Biochim. Biophys. Acta* **125**, 413–421.
- Brown, J. E., Rudnick, M., Letcher, A. J., and Irvine, R. F. (1988). Formation of methyl-phosphoryl inositol phosphates by extractions that employ methanol. *Biochem. J.* **253**, 703–710.
- Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–1657.
- Cockcroft, S., and De Matteis, M. A. (2001). Inositol lipids as spatial regulators of membrane traffic. *J. Membr. Biol.* **180**, 187–194.
- Corda, D., Iurisci, C., and Berrie, C. P. (2002). Biological activities and metabolism of the lysophosphoinositides and glycerophosphoinositols. *Biochim. Biophys. Acta* **1582**, 52–69.
- Cremona, O., and De Camilli, P. (2001). Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci.* **114**, 1041–1052.
- Dean, N. M., and Beaven, M. A. (1989). Methods for the analysis of inositol phosphates. *Anal. Biochem.* **183**, 199–209.
- Debetto, P., Cargnelli, G., Antolini, M., Bova, S., Trevisi, L., Varotto, R., and Luciani, S. (1999). Alpha1-adrenoceptor-mediated formation of glycerophosphoinositol 4-phosphate in rat heart: Possible role in the positive inotropic response. *Biochem. Pharmacol.* **58**, 1437–1446.
- DeLong, C. J., Baker, P. R., Samuel, M., Cui, Z., and Thomas, M. J. (2001). Molecular species composition of rat liver phospholipids by ESI-MS/MS: The effect of chromatography. *J. Lipid Res.* **42**, 1959–1968.

- De Matteis, M., Godi, A., and Corda, D. (2002). Phosphoinositides and the golgi complex. *Curr. Opin. Cell Biol.* **14**, 434–447.
- Dove, S. L., Joung, J. K., and Hochschild, A. (1997). Activation of prokaryotic transcription through arbitrary protein-protein contacts. *Nature* **386**, 627–630.
- Downes, C. P., Hawkins, P. T., and Irvine, R. F. (1986). Inositol 1,3,4,5-tetrakisphosphate and not phosphatidylinositol 3,4-bisphosphate is the probable precursor of inositol 1,3,4-trisphosphate in agonist-stimulated parotid gland. *Biochem. J.* **238**, 501–506.
- Dragani, L. K., Berrie, C. P., Corda, D., and Rotilio, D. (2004). Analysis of glycerophosphoinositol by liquid chromatography–electrospray ionisation tandem mass spectrometry using a beta-cyclodextrin-bonded column. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **802**, 283–289.
- Falasca, M., Carvelli, A., Iurisci, C., Qiu, R. G., Symons, M. H., and Corda, D. (1997). Fast receptor-induced formation of glycerophosphoinositol-4-phosphate, a putative novel intracellular messenger in the Ras pathway. *Mol. Biol. Cell* **8**, 443–453.
- Falasca, M., and Corda, D. (1994). Elevated levels and mitogenic activity of lysophosphatidylinositol in k-ras-transformed epithelial cells. *Eur. J. Biochem.* **221**, 383–389.
- Falasca, M., Silletta, M. G., Carvelli, A., Di Francesco, A. L., Fusco, A., Ramakrishna, V., and Corda, D. (1995). Signalling pathways involved in the mitogenic action of lysophosphatidylinositol. *Oncogene* **10**, 2113–2124.
- Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–509.
- Forrester, J. S., Milne, S. B., Ivanova, P. T., and Brown, H. A. (2004). Computational lipidomics: A multiplexed analysis of dynamic changes in membrane lipid composition during signal transduction. *Mol. Pharmacol.* **65**, 813–821.
- Gonzalez-Sastre, F., and Folch-Pi, J. (1968). Thin-layer chromatography of the phosphoinositides. *J. Lipid Res.* **9**, 532–533.
- Grado, C., and Ballou, C. E. (1961). Myo-inositol phosphates obtained by alkaline hydrolysis of beef brain phosphoinositide. *J. Biol. Chem.* **236**, 54–60.
- Han, X., and Cheng, H. (2005). Characterization and direct quantitation of cerebroside molecular species from lipid extracts by shotgun lipidomics. *J. Lipid Res.* **46**, 163–175.
- Han, X., and Gross, R. W. (2003). Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: A bridge to lipidomics. *J. Lipid Res.* **44**, 1071–1079.
- Han, X., and Gross, R. W. (2005). Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom. Rev.* **24**, 367–412.
- Han, X., Yang, J., Cheng, H., Ye, H., and Gross, R. W. (2004). Toward fingerprinting cellular lipidomes directly from biological samples by two-dimensional electrospray ionization mass spectrometry. *Anal. Biochem.* **330**, 317–331.
- Han, X., Yang, K., Cheng, H., Fikes, K., and Gross, R. W. (2005). Shotgun lipidomics of phosphoethanolamine-containing lipid molecular species in biological samples after one-step *in situ* derivatization. *J. Lipid Res.* **46**, 1548–1560.
- Hara, A., and Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **90**, 420–426.
- Harden, T. K., Hawkins, P. T., Stephens, L., Boyer, J. L., and Downes, C. P. (1988). Phosphoinositide hydrolysis by guanosine 5'-[gamma-thio]triphosphate-activated phospholipase C of turkey erythrocyte membranes. *Biochem. J.* **252**, 583–593.
- Harden, T. K., Stephens, L., Hawkins, P. T., and Downes, C. P. (1987). Turkey erythrocyte membranes as a model for regulation of phospholipase C by guanine nucleotides. *J. Biol. Chem.* **262**, 9057–9061.
- Hawkins, P. T., Berrie, C. P., Morris, A. J., and Downes, C. P. (1987). Inositol 1,2-cyclic 4,5-trisphosphate is not a product of muscarinic receptor-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis in rat parotid glands. *Biochem. J.* **243**, 211–218.

- Hermansson, M., Uphoff, A., Kakela, R., and Somerharju, P. (2005). Automated quantitative analysis of complex lipidomes by liquid chromatography/mass spectrometry. *Anal. Chem.* **77**, 2166–2175.
- Houjou, T., Yamatani, K., Imagawa, M., Shimizu, T., and Taguchi, R. (2005). A shotgun tandem mass spectrometric analysis of phospholipids with normal-phase and/or reverse-phase liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 654–666.
- Hsu, F. F., and Turk, J. (2000). Characterization of phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate by electrospray ionization tandem mass spectrometry: A mechanistic study. *J. Am. Soc. Mass Spectrom.* **11**, 986–999.
- Hsu, F. F., Turk, J., and Gross, M. L. (2003). Structural distinction among inositol phosphate isomers using high-energy and low-energy collisional-activated dissociation tandem mass spectrometry with electrospray ionization. *J. Mass Spectrom.* **38**, 447–457.
- Hunt, A. N., Skippen, A. J., Koster, G., Postle, A. D., and Cockcroft, S. (2004). Acyl chain-based molecular selectivity for HL60 cellular phosphatidylinositol and of phosphatidylcholine by phosphatidylinositol transfer protein alpha. *Biochim. Biophys. Acta* **1686**, 50–60.
- Irvine, R. F. (2002). Nuclear lipid signaling. *Sci. STKE* **2002**, RE13.
- Irvine, R. F. (2003). Nuclear lipid signalling. *Nat. Rev. Mol. Cell. Biol.* **4**, 349–360.
- Irvine, R. F., Letcher, A. J., Lander, D. J., and Downes, C. P. (1984). Inositol trisphosphates in carbachol-stimulated rat parotid glands. *Biochem. J.* **223**, 237–243.
- Itoh, T., and Takenawa, T. (2002). Phosphoinositide-binding domains: Functional units for temporal and spatial regulation of intracellular signalling. *Cell. Signal.* **14**, 733–743.
- Ivanova, P. T., Milne, S. B., Forrester, J. S., and Brown, H. A. (2004). LIPID arrays: New tools in the understanding of membrane dynamics and lipid signaling. *Mol. Interv.* **4**, 86–96.
- Koivusalo, M., Haimi, P., Heikinheimo, L., Kostinen, R., and Somerharju, P. (2001). Quantitative determination of phospholipid compositions by ESI-MS: Effects of acyl chain length, unsaturation, and lipid concentration on instrument response. *J. Lipid Res.* **42**, 663–672.
- Lips, D. L., Bross, T. E., and Majerus, P. W. (1988). Isolation of 1-monomethylphosphoinositol 4,5-bisphosphate [a product of methanolysis of inositol 1,2-(cyclic)-4,5-trisphosphate] from Swiss mouse 3T3 cells. *Proc. Natl. Acad. Sci. USA* **85**, 88–92.
- Lu, Y., Hong, S., Tjonahen, E., and Serhan, C. N. (2005). Mediator-lipidomics: Databases and search algorithms for PUFA-derived mediators. *J. Lipid Res.* **46**, 790–802.
- Mandal, S., Richardson, V. J., Banoub, J., and Church, J. G. (2002). Identification of myo-inositol 1,2-cyclic monophosphate by electrospray tandem mass spectrometry, a major constituent of EGF-stimulated phosphoinositide turnover in MDA 468 cells. *Exp. Cell Res.* **277**, 131–138.
- Martelli, A. M., Capitani, S., and Neri, L. M. (1999). The generation of lipid signaling molecules in the nucleus. *Prog. Lipid Res.* **38**, 273–308.
- McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002). PIP(2) and proteins: Interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 151–175.
- Meijer, H., Diveka, N., van den Ende, H., Musgrave, A., and Munnik, T. (1999). Hyperosmotic stress induces rapid synthesis of phosphatidyl-D-inositol 3,5-bisphosphate in plant cells. *Planta* **208**, 294–298.
- Meijer, H. J., Berrie, C. P., Iurisci, C., Divecha, N., Musgrave, A., and Munnik, T. (2001). Identification of a new polyphosphoinositide in plants, phosphatidylinositol 5-monophosphate (PtdIns5P), and its accumulation upon osmotic stress. *Biochem. J.* **360**, 491–498.
- Michell, R. H., Heath, V. L., Lemmon, M. A., and Dove, S. K. (2006). Phosphatidylinositol 3,5-bisphosphate: Metabolism and cellular functions. *Trends Biochem. Sci.* **31**, 52–63.

- Morris, A. J., Rudge, S. A., Mahlum, C. E., and Jenco, J. M. (1995). Regulation of phosphoinositide-3-kinase by G protein beta gamma subunits in a rat osteosarcoma cell line. *Mol. Pharmacol.* **48**, 532–539.
- Muller, M., Schiller, J., Petkovic, M., Oehrl, W., Heinze, R., Wetzker, R., Arnold, K., and Arnhold, J. (2001). Limits for the detection of (poly-)phosphoinositides by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS). *Chem. Phys. Lipids* **110**, 151–164.
- Munnik, T., Irvine, R. F., and Musgrave, A. (1994). Rapid turnover of phosphatidylinositol 3-phosphate in the green alga *Chlamydomonas eugametos*: Signs of a phosphatidylinositol 3-kinase signalling pathway in lower plants? *Biochem. J.* **298**, 269–273.
- Munnik, T., Irvine, R. F., and Musgrave, A. (1998). Phospholipid signalling in plants. *Biochim. Biophys. Acta* **1389**, 222–272.
- Nasuhoglu, C., Feng, S., Mao, J., Yamamoto, M., Yin, H. L., Earnest, S., Barylko, B., Albanesi, J. P., and Hilgemann, D. W. (2002). Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. *Anal. Biochem.* **301**, 243–254.
- Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**, 484–496.
- Payraastre, B., Missy, K., Giuriato, S., Bodin, S., Plantavid, M., and Gratacap, M. (2001). Phosphoinositides: Key players in cell signalling, in time and space. *Cell. Signal.* **13**, 377–387.
- Portilla, D., and Morrison, A. R. (1986). Bradykinin-induced changes in inositol trisphosphate mass in MDCK cells. *Biochem. Biophys. Res. Commun.* **140**, 644–649.
- Postle, A. D., Dombrowsky, H., Clarke, H., Pynn, C. J., Koster, G., and Hunt, A. N. (2004). Mass spectroscopic analysis of phosphatidylinositol synthesis using 6-deuteriated-myo-inositol: Comparison of the molecular specificities and acyl remodelling mechanisms in mouse tissues and cultured cells. *Biochem. Soc. Trans.* **32**, 1057–1059.
- Pulfer, M., and Murphy, R. C. (2003). Electrospray mass spectrometry of phospholipids. *Mass Spectrom. Rev.* **22**, 332–364.
- Schacht, J., and Agranoff, B. W. (1974). Stimulation of hydrolysis of phosphatidic acid by cholinergic agents in guinea pig synaptosomes. *J. Biol. Chem.* **249**, 1551–1557.
- Schiller, J., Arnhold, J., Benard, S., Muller, M., Reichl, S., and Arnold, K. (1999). Lipid analysis by matrix-assisted laser desorption and ionization mass spectrometry: A methodological approach. *Anal. Biochem.* **267**, 46–56.
- Sharps, E. S., and McCarl, R. L. (1982). A high-performance liquid chromatographic method to measure ³²P incorporation into phosphorylated metabolites in cultured cells. *Anal. Biochem.* **124**, 421–424.
- Shayman, J. A., Auchus, R. J., and Morrison, A. R. (1986). Bradykinin-induced changes in myo-inositol 1,2-(cyclic)phosphate in rabbit papillary collecting tubule cells. *Biochim. Biophys. Acta* **888**, 171–175.
- Shen, Z., Wu, M., Elson, P., Kennedy, A. W., Belinson, J., Casey, G., and Xu, Y. (2001). Fatty acid composition of lysophosphatidic acid and lysophosphatidylinositol in plasma from patients with ovarian cancer and other gynecological diseases. *Gynecol. Oncol.* **83**, 25–30.
- Sherman, W. R., Ackermann, K. E., Bateman, R. H., Green, B. N., and Lewis, I. (1985). Mass-analysed ion kinetic energy spectra and B1E-B2 triple sector mass spectrometric analysis of phosphoinositides by fast atom bombardment. *Biomed. Mass Spectrom.* **12**, 409–413.
- Simonsen, A., Wurmser, A. E., Emr, S. D., and Stenmark, H. (2001). The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* **13**, 485–492.
- Singh, A. K., and Jiang, Y. (1995). Quantitative chromatographic analysis of inositol phospholipids and related compounds. *J. Chromatogr. B Biomed. Appl.* **671**, 255–280.

- Slater, E. C., Rosing, J., and Mol, A. (1973). The phosphorylation potential generated by respiring mitochondria. *Biochim. Biophys. Acta* **292**, 534–553.
- Stephens, L., Hawkins, P. T., Carter, N., Chahwala, S. B., Morris, A. J., Whetton, A. D., and Downes, P. C. (1988a). L-myo-inositol 1,4,5,6-tetrakisphosphate is present in both mammalian and avian cells. *Biochem. J.* **249**, 271–282.
- Stephens, L., Radenberg, T., Thiel, U., Vogel, G., Khoo, K. H., Dell, A., Jackson, T. R., Hawkins, P. T., and Mayr, G. W. (1993). The detection, purification, structural characterization, and metabolism of diphosphoinositol pentakisphosphate(s) and bisdiphosphoinositol tetrakisphosphate(s). *J. Biol. Chem.* **268**, 4009–4015.
- Stephens, L. R., and Downes, C. P. (1990). Product-precursor relationships amongst inositol polyphosphates: Incorporation of [^{32}P]Pi into myo-inositol 1,3,4,6-tetrakisphosphate, myo-inositol 1,3,4,5-tetrakisphosphate, myo-inositol 3,4,5,6-tetrakisphosphate and myo-inositol 1,3,4,5,6-pentakisphosphate in intact avian erythrocytes. *Biochem. J.* **265**, 435–452.
- Stephens, L. R., Hawkins, P. T., Barker, C. J., and Downes, C. P. (1988b). Synthesis of myo-inositol 1,3,4,5,6-pentakisphosphate from inositol phosphates generated by receptor activation. *Biochem. J.* **253**, 721–733.
- Stephens, L. R., Hawkins, P. T., and Downes, C. P. (1989). An analysis of myo-[^3H]inositol trisphosphates found in myo-[^3H]inositol prelabelled avian erythrocytes. *Biochem. J.* **262**, 727–737.
- Stephens, L. R., Hawkins, P. T., Stanley, A. F., Moore, T., Poyner, D. R., Morris, P. J., Hanley, M. R., Kay, R. R., and Irvine, R. F. (1991a). myo-inositol pentakisphosphates: Structure, biological occurrence and phosphorylation to myo-inositol hexakisphosphate. *Biochem. J.* **275**, 485–499.
- Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991b). Pathway of phosphatidylinositol (3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature* **351**, 33–39.
- Sutphen, R., Xu, Y., Wilbanks, G. D., Fiorica, J., Grendys, E. C., Jr., LaPolla, J. P., Arango, H., Hoffman, M. S., Martino, M., Wakeley, K., Griffin, D., Blanco, R. W., et al. (2004). Lysophospholipids are potential biomarkers of ovarian cancer. *Cancer Epidemiol. Biomark. Prev.* **13**, 1185–1191.
- Takenawa, T., and Itoh, T. (2001). Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochim. Biophys. Acta* **1533**, 190–206.
- Toker, A. (2002). Phosphoinositides and signal transduction. *Cell. Mol. Life Sci.* **59**, 761–779.
- Wenk, M. R. (2005). The emerging field of lipidomics. *Nat. Rev. Drug Discov.* **4**, 594–610.
- Wenk, M. R., Lucast, L., Di Paolo, G., Romanelli, A. J., Suchy, S. F., Nussbaum, R. L., Cline, G. W., Shulman, G. I., McMurray, W., and De Camilli, P. (2003). Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat. Biotechnol.* **21**, 813–817.
- Whiteford, C. C., Brearley, C. A., and Ulug, E. T. (1997). Phosphatidylinositol 3,5-bisphosphate defines a novel PI 3-kinase pathway in resting mouse fibroblasts. *Biochem. J.* **323**, 597–601.
- Wilson, D. B., Bross, T. E., Sherman, W. R., Berger, R. A., and Majerus, P. W. (1985a). Inositol cyclic phosphates are produced by cleavage of phosphatidylphosphoinositols (polyphosphoinositides) with purified sheep seminal vesicle phospholipase C enzymes. *Proc. Natl. Acad. Sci. USA* **82**, 4013–4017.
- Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A. N., Rubin, L. J., and Brown, J. E. (1985b). Isolation and characterization of the inositol cyclic phosphate products of polyphosphoinositide cleavage by phospholipase C: Physiological effects in permeabilized platelets and *Limulus* photoreceptor cells. *J. Biol. Chem.* **260**, 13496–13501.

- Xiao, Y., Chen, Y., Kennedy, A. W., Belinson, J., and Xu, Y. (2000). Evaluation of plasma lysophospholipids for diagnostic significance using electrospray ionization mass spectrometry (ESI-MS) analyses. *Ann. N. Y. Acad. Sci.* **905**, 242–259.
- Xiao, Y. J., Schwartz, B., Washington, M., Kennedy, A., Webster, K., Belinson, J., and Xu, Y. (2001). Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: Comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. *Anal. Biochem.* **290**, 302–313.
- Yin, H. L., and Janmey, P. A. (2003). Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* **65**, 761–789.
- Zhang, L., and Buxton, I. L. (1998). Measurement of phosphoinositols and phosphoinositides using radio high-performance liquid chromatography flow detection. *Methods Mol. Biol.* **105**, 47–63.

COMBINATION OF C₁₇ SPHINGOID BASE HOMOLOGUES AND MASS SPECTROMETRY ANALYSIS AS A NEW APPROACH TO STUDY SPHINGOLIPID METABOLISM

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Contents

1. Introduction	234
2. Mass Spectrometry Analysis	235
3. Ceramide Synthase	236
4. <i>In Vitro</i> Ceramide Synthase Method	236
5. Sphingosine Kinase	237
6. <i>In Vitro</i> Sphingosine Kinase Method	238
7. In Cells Labeling with C ₁₇ Sphingoid Base	239
Acknowledgments	240
References	240

Abstract

In recent years, sphingolipid metabolites ceramide, sphingosine, and sphingosine-1-phosphate have emerged as important second messengers in addition to their role as precursors of biomembrane components. The investigation of these sphingolipid metabolites requires the development of new, more sensitive methods for assaying the enzymes involved in their production. This chapter describes the utilization of mass spectrometry technology in combination with nonnaturally occurring C₁₇ sphingoid bases in the *in vitro* assays of two of the enzymes of the sphingolipid pathway, ceramide synthase and sphingosine kinase. These new *in vitro* methods provide high sensitivity and extreme accuracy even when crude extracts are used as enzyme sources.

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1. INTRODUCTION

Several sphingolipid metabolites, particularly ceramide, sphingosine, and sphingosine-1-phosphate, in addition to their role as precursors or breakdown products of key structural components of biological membranes, have been identified as important bioactive molecules that regulate cell growth and death (Hannun and Obeid, 2002; Merrill, 2002; Spiegel and Milstien, 2002). This discovery emphasizes the need for developing more efficient and sensitive methods for characterizing the enzymes of sphingolipid metabolic pathways. Until now, the majority of the methods used to measure the activities of most enzymes in the sphingolipid pathway have relied on the use of radioactive substrates. While using radioactivity leads to sensitive methodology, it does not escape the hazards of routinely handling radioactivity, which can lengthen procedures as a consequence of regulations and special care required for such handling. In addition, the substrates and products of sphingolipid enzymes are, for the most part, highly hydrophobic and detection requires separation via thin-layer chromatography, which has limitations when it comes to separation of species with similar mobilities.

Mass spectrometry (MS) methodology, as discussed in this chapter, is an efficient and sensitive means for measuring even small changes in sphingolipid species. The electrospray ionization (ESI) MS methodology, coupled with high-performance liquid chromatography, allows generation of intact molecular ions of molecules directly from solutions. In addition to the sensitivity and selectivity of MS, our new approach utilizes synthetic sphingolipids containing a sphingoid-base backbone that is 17 carbons in length in lieu of the naturally occurring mammalian sphingoid bases, sphingosine (2-amino-1,3-dihydroxyoctadecene) and dihydrosphingosine (2-amino-1,3-dihydroxyoctadecane), which are 18 carbons in length (Fig. 12.1). This elegant approach allows for extremely accurate measurement of the products and/or the substrates of the enzymes of the sphingolipid

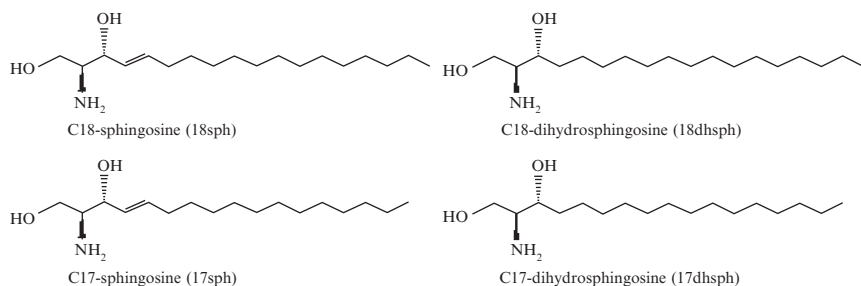


Figure 12.1 Sphingoid bases with 17 and 18 carbon atoms.

pathway even in crude cell homogenates. In addition to the *in vitro* enzyme activity methods, the MS approach, when used in conjunction with sphingoid bases with C₁₇ backbones, is a powerful tool for cellular analysis of sphingolipids. This method analyzes simultaneously C₁₇ and C₁₈ sphingolipid species, which is not available with traditional methods. It also provides detailed information about their composition so that the particular sphingolipid metabolic pathway can be analyzed without being obscured by endogenous sphingolipids. This chapter focuses on the application of these approaches to a novel method to study the *in vitro* and cellular activities of two sphingolipid pathway enzymes, namely ceramide synthase and sphingosine kinase (SK).

2. MASS SPECTROMETRY ANALYSIS

The described ESI/MS/MS analyses of sphingolipids, containing the C₁₇ backbone sphingoid base, are developed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction monitoring positive ionization mode (Bielawski *et al.*, 2006). The analyzed samples are fortified with 50 pmol of an internal standard, *N*-palmitoyl-D-erythro-C₁₃ sphingosine, and extracted with ethyl acetate/isopropanol/water (60/30/10, v/v) solvent system. After evaporation and reconstitution in 100 μ l of methanol, samples are injected on the HP1100/Thermo Finnigan TSQ 7000 LC/MS system and gradient eluted from the BDS Hypersil C8, 150 $\times$ 3.2 mm, 3- μ m particle size column, with 1 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and internal standards are integrated and processed using the Xcalibur software system. Quantitative analysis is based on the calibration curves generated by spiking an artificial matrix, bovine serum albumin, with known amounts of the target analyte synthetic standards (C₁₇ backbone sphingosine, C₁₇ backbone dihydrosphingosine, C₁₆ and C_{24:1} ceramides with C₁₇ backbone sphingoid base), and an equal amount, as for the test samples, of the internal standard. The target analyte/internal standard peak area ratios are plotted against analyte concentration. The target analyte/internal standard peak area ratios from the samples are similarly normalized to the internal standard and compared to the calibration curves, using a linear regression model. Quantitation of target ceramides with C₁₇ sphingoid backbone, for which no synthetic standards are available, is accomplished as follows: the calibration curve of C₁₇/C₁₆ ceramide is used for C₁₇/C₁₄ ceramide, C₁₇/C₁₈ ceramide, and C₁₇/C_{18:1} ceramide; the calibration curve of C₁₇/C_{24:1} ceramide is used for quantification of C₁₇/C₂₀ and C₁₇/C₂₄ ceramide.

3. CERAMIDE SYNTHASE

Ceramide synthases catalyze the biosynthesis of dihydroceramide or ceramide from a sphingoid base and fatty acid CoA. Depending on the source of the sphingoid base, members of this family of enzymes can be involved in the *de novo* synthesis or recycling of ceramide. Ceramide synthases can utilize all four isomers of the sphingoid bases dihydrosphingosine and sphingosine, that is, D-threo-, D-erythro-, L-threo, and L-erythro, but with different specificity. The preferred substrate is the D-erythro- isomer (Nikolova-Karakashian *et al.*, 1997). The enzymes also exhibit different specificities toward the chain length of the fatty acid CoA substrate. Studies have revealed that this fatty acid chain length specificity is due to the presence of different isoforms of the enzyme. The different isoforms of ceramide synthase are encoded by the Lag/LASS gene family. The Lag/LASS family includes genes from all eukaryotic organisms and some bacteria (Venkataraman and Futerman, 2002). In all studied eukaryotes, there is more than one paralogue of Lag/LASS in a genome, for example, in baker's yeast genome there are two catalogues, in *Arabidopsis* genome there are three, and in all studied mammalian genomes there are six. All Lag/LASS proteins have five to seven predicted transmembrane domains, and all Lag/LASS homologues have a characteristic Lag1p motif, which is necessary for their ceramide synthase activity. In addition, some of the homologues contain a homeodomain region, but its function is yet to be determined (Pewzner-Jung *et al.*, 2006; Spassieva *et al.*, 2006). Lag/LASS proteins have been localized in the endoplasmatic reticulum and in the nuclear envelope (Mizutani *et al.*, 2005; Riebeling *et al.*, 2003; Venkataraman *et al.*, 2002). The localization data corroborate with earlier enzymology studies, which show that the main ceramide synthase activity is in microsomal fractions (Hirschberg *et al.*, 1993; Morell and Radin, 1970). Therefore, for the *in vitro* ceramide synthase method currently described, if purified protein is not available, microsomes are recommended as an enzyme source.

4. IN VITRO CERAMIDE SYNTHASE METHOD

The pH optimum of ceramide synthase is pH 7.5 (Sribney, 1966). *In vitro* reactions performed in phosphate buffer show higher activities as compared to *in vitro* reactions performed in Tris buffer, and ceramide synthase activity is not dependent on metal ions (Morell and Radin, 1970; Sribney, 1966). The reaction mix, usually 100 μ l, contains 25 mM potassium phosphate buffer (pH 7.4). The C₁₇ backbone sphingoid base substrates (dihydrosphingosine or sphingosine) are hydrophobic, and therefore special care has to be taken for their delivery into the reaction mix. The sphingoid base substrate can be

delivered by using delipidated bovine serum albumin (BSA) as the carrier. However, BSA can influence the partitioning of the sphingoid base into microsomes, which can affect the rate of the reaction. An alternative delivery method involves incorporating the sphingoid base substrate into liposomes, thus providing a controlled lipid environment for the *in vitro* reaction (Wang and Merrill, 2000). The sphingoid base substrate can also be delivered without using a carrier. In the latter case, an aliquot of concentrated (0.1 mM) ethanol stock solution of the sphingoid base is placed in a reaction tube (an Eppendorf microcentrifuge tube) and dried under a stream of nitrogen. The reaction buffer is then added followed by vigorous vortexing for at least 1 min and sonication for 2 min (water bath sonicator). The stock solution of very long-chain fatty acid CoAs can be in 0.1% Zwittergent (Guillas *et al.*, 2003) or, alternatively, digitonin (0.1% final concentration) can be used in the reaction mixes, when very long chain base fatty acid CoA are substrates (Mizutani *et al.*, 2005). Delivery of the middle and the long chain fatty acid CoAs is simpler in that they are water soluble. Stock solutions of 500 μ M can be prepared, aliquoted, and stored at -20° . The final concentration of the C₁₇ backbone sphingoid base and the fatty acid CoA in the reaction mix can vary, but higher concentrations of one of the substrates relative to the other one can inhibit the activity (unpublished observation). The optimal final concentration is between 5 and 20 and 10 and 75 μ M for sphingoid base and fatty acid CoA, respectively. The reaction mix, containing the buffer, both substrates, and an appropriate amount of water to adjust for the final volume, is warmed for 5 min at 37° . The reaction is started by adding the enzyme, usually as microsomes (20–50 μ g protein), and incubation is carried out for 15 min at 37° . The reaction is stopped by pipetting the reaction mix into the extraction solvent already supplemented with internal standard for MS analysis. Lipids are then extracted and subjected to ESI/MS/MS analysis as described earlier.

5. SPHINGOSINE KINASE

Sphingosine kinases are highly conserved enzymes that catalyze the biosynthesis of sphingosine to sphingosine-1-phosphate and the biosynthesis of dihydrosphingosine to dihydrosphingosine-1-phosphate. The enzyme has been purified or cloned from a variety of sources, including *Saccharomyces cerevisiae* (Nagiec *et al.*, 1998), *Arabidopsis thaliana* (Imai and Nishiura, 2005), *Drosophila* (Herr *et al.*, 2004), rat (Imamura *et al.*, 2001), mouse (Kohama *et al.*, 1998), and human (Liu *et al.*, 2000; Pitson *et al.*, 2000). SKs from different organisms share similar biochemical proprieties and display similar kinetic parameters. In general, K_m values for ATP are ≈ 25 to 100 μ M, whereas those for D-erythro-sphingosine and D-erythro-dihydrosphingosine are in the range of 2 to 12 μ M. Two mammalian isoforms have been

identified through molecular cloning, designated SK1 and SK2. The two isoforms possess a high degree of sequence similarity although they are quite different in size (43 and 65 kDa, respectively), tissue distribution, developmental expression, and chromosomal location (chromosomes 17 and 19, respectively). SK1 and SK2 possess five regions in their polypeptide sequence, designated C1 to C5, that show very high sequence identity across all of the known SKs. SK2 possesses two additional polypeptide regions, one at the N terminus and another within the central region of the sequence. Moreover, a number of apparently alternatively spliced isoforms of both human SK1 and SK2 have been identified, although the molecular basis for their existence is not currently known (Billich *et al.*, 2003). The splice variants display similar activity and substrate specificity. SK1 and SK2 differ in their expression pattern. The highest expression of SK1 is reported in lung and spleen, while the highest expression of SK2 is found in liver and heart (Kohama *et al.*, 1998; Liu *et al.*, 2000). One of the major distinctions between SK1 and SK2 is the variation in their substrate specificities. Although both enzymes use D-erythro-sphingosine and D-erythro-dihydrosphingosine efficiently, SK2 has much greater activity than SK1 against phytosphingosine (Liu *et al.*, 2000; Pitson *et al.*, 2000) and the artificial substrate ω -biotinyl D-erythro-sphingosine (Roberts *et al.*, 2004). SK1 does not have obvious membrane-anchoring or -docking sequences and therefore appears to be a cytosolic enzyme. However, cell exposure to various growth factors and agonists is often accompanied by SK1 translocation to the plasma membrane, which correlates with its activation (Johnson *et al.*, 2002; Pitson *et al.*, 2003). SK2 can be found in the nucleus and cytoplasm, although localization to the nucleus is cell type dependent (Igarashi *et al.*, 2003). A putative nuclear export signal in the SK2 sequence has been discovered. In addition, Inagaki and colleagues (2003) found two functional nuclear export signal sequences in the middle region of SK1. Moreover, it has been shown that platelet-derived growth factor can induce nuclear localization of SK1 (Kleuser *et al.*, 2001). The *in vitro* enzymatic assay described in this chapter is for quantifying SK1 and SK2 activities in total cell lysate. However, because of the different localization of these enzymes, it is worth analyzing SK activity in subcellular fractionations.

6. *IN VITRO* SPHINGOSINE KINASE METHOD

Cells are usually grown to 70 to 80% confluency, washed twice with an appropriate volume of cold phosphate-buffered saline, and collected by centrifugation at 3000g for 5 min. The pellets are then resuspended in two different buffers, known to selectively favor SK1 or SK2. The buffer for SK1 contains 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM

deoxyypyridoxine, 15 mM NaF, 1 mM β -mercaptoethanol, 1 mM sodium orthovanadate, 40 mM β -glycerophosphate, 10% glycerol, 0.5% Triton X-100, 10 mM KCl, and 1.5 mM semicarbazide and is supplemented with protease inhibitor cocktail. The SK2 buffer contains the same components as the buffer for SK1, except 0.5% Triton X-100 is replaced with 200 mM KCl (Billich *et al.*, 2003). The cell lysates are sonicated and centrifuged at 2500g for 15 min to remove unbroken cells, and protein concentrations are determined in the supernatants. The optimal protein concentration for the *in vitro* SK enzyme assay (ranging from 20 to 80 μ g) must be determined independently for each cell type and experimental condition. The supernatants can be stored at -80° for several weeks, but longer term storage results in loss of activity. The substrate D-erythro-C₁₇-sphingosine must be delivered as a BSA complex (Olivera *et al.*, 2000). Briefly, appropriate volumes of 4 mg/ml delipidated BSA and 50 mM D-erythro-C₁₇-sphingosine for a final concentration of 1 mM are mixed drop-wise in a glass tube and subsequently sonicated for 1 or 2 min. The solution can be cloudy, but no matter should be visible. At the time of SK assay, conical glass tubes are placed on ice, and suitable volumes of cell test extracts are added. A sufficient SK1 or SK2 buffer is added to bring the total volume to 180 μ l and then 10 μ l of 1 mM D-erythro-C₁₇-sphingosine-BSA complex (final concentration 50 μ M) is added. Tubes are vortexed gently, the reactions are started by the addition of 10 μ l of freshly prepared 20 mM ATP in 200 mM MgCl₂ (final concentration 1 mM), and subsequently incubated for 30 min at 37 $^{\circ}$ in a water bath. The reactions are stopped by the addition of 20 μ l of 1 N HCl. Fifty picomoles of internal standards for MS analysis is added to the reaction mix, and lipids are extracted and subjected to ESI/MS/MS analysis as described earlier.

7. IN CELLS LABELING WITH C₁₇ SPHINGOID BASE

Sphingoid bases with C₁₇ backbone are efficient and easy to handle molecular probes. They are suitable for pulse or long-term metabolic labeling of sphingolipids in the cells. Aliquots of concentrated ethanol solutions of C₁₇ sphingoid bases can be added directly to the growth media alone or in combination with other treatments. The final concentration of the C₁₇ sphingoid base probe in media depends on the cell type used in the experiment. However, it is important to note that micromolar amounts of a sphingoid base can have an effect on the cells, which can complicate the interpretation of results. After labeling, cells are washed (e.g., with phosphate buffer saline), and extraction solvent fortified with internal standard (N-palmitoyl-D-erythro-C₁₃ sphingosine) is added to cell pellets. Sphingolipid extraction and MS analysis are performed as described earlier.

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REFERENCES

- Bielawski, J., Szulc, Z. M., Hannun, Y. A., and Bielawska, A. (2006). Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Methods* **39**, 82–91.
- Billich, A., Bornancin, F., Devay, P., Mechtcheriakova, D., Urtz, N., and Baumruker, T. (2003). Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J. Biol. Chem.* **278**, 47408–47415.
- Guillas, I., Jiang, J. C., Vionnet, C., Roubaty, C., Uldry, D., Chuard, R., Wang, J., Jazwinski, S. M., and Conzelmann, A. (2003). Human homologues of LAG1 reconstitute acyl-CoA-dependent ceramide synthesis in yeast. *J. Biol. Chem.* **278**, 37083–37091.
- Hannun, Y. A., and Obeid, L. M. (2002). The ceramide-centric universe of lipid-mediated cell regulation: Stress encounters of the lipid kind. *J. Biol. Chem.* **277**, 25847–25850.
- Herr, D. R., Fyrst, H., Creason, M. B., Phan, V. H., Saba, J. D., and Harris, G. L. (2004). Characterization of the *Drosophila* sphingosine kinases and requirement for Sk2 in normal reproductive function. *J. Biol. Chem.* **279**, 12685–12694.
- Hirschberg, K., Rodger, J., and Futerman, A. H. (1993). The long-chain sphingoid base of sphingolipids is acylated at the cytosolic surface of the endoplasmic reticulum in rat liver. *Biochem. J.* **290**(Pt 3), 751–757.
- Igarashi, N., Okada, T., Hayashi, S., Fujita, T., Jahangeer, S., and Nakamura, S. (2003). Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. *J. Biol. Chem.* **278**, 46832–46839.
- Imai, H., and Nishiura, H. (2005). Phosphorylation of sphingoid long-chain bases in *Arabidopsis*: Functional characterization and expression of the first sphingoid long-chain base Kinase gene in plants. *Plant Cell Physiol.* **46**, 375–380.
- Imamura, T., Ohgane, J., Ito, S., Ogawa, T., Hattori, N., Tanaka, S., and Shiota, K. (2001). CpG island of rat sphingosine kinase-1 gene: Tissue-dependent DNA methylation status and multiple alternative first exons. *Genomics* **76**, 117–125.
- Inagaki, Y., Li, P. Y., Wada, A., Mitsutake, S., and Igarashi, Y. (2003). Identification of functional nuclear export sequences in human sphingosine kinase 1. *Biochem. Biophys. Res. Commun.* **311**, 168–173.
- Johnson, K. R., Becker, K. P., Facchinetti, M. M., Hannun, Y. A., and Obeid, L. M. (2002). PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane: Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA). *J. Biol. Chem.* **277**, 35257–35262.
- Kleuser, B., Maceyka, M., Milstien, S., and Spiegel, S. (2001). Stimulation of nuclear sphingosine kinase activity by platelet-derived growth factor. *FEBS Lett.* **503**, 85–90.
- Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998). Molecular cloning and functional characterization of murine sphingosine kinase. *J. Biol. Chem.* **273**, 23722–23728.
- Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000). Molecular cloning and functional characterization

- of a novel mammalian sphingosine kinase type 2 isoform. *J. Biol. Chem.* **275**, 19513–19520.
- Merrill, A. H., Jr. (2002). *De novo* sphingolipid biosynthesis: A necessary, but dangerous, pathway. *J. Biol. Chem.* **277**, 25843–25846.
- Mizutani, Y., Kihara, A., and Igarashi, Y. (2005). Mammalian Lass6 and its related family members regulate synthesis of specific ceramides. *Biochem. J.* **390**, 263–271.
- Morell, P., and Radin, N. S. (1970). Specificity in ceramide biosynthesis from long chain bases and various fatty acyl coenzyme A's by brain microsomes. *J. Biol. Chem.* **245**, 342–350.
- Nagiec, M. M., Skrzypek, M., Nagiec, E. E., Lester, R. L., and Dickson, R. C. (1998). The LCB4 (YOR171c) and LCB5 (YLR260w) genes of *Saccharomyces* encode sphingoid long chain base kinases. *J. Biol. Chem.* **273**, 19437–19442.
- Nikolova-Karakashian, M., Vales, T. R., Wang, E., Menaldino, D. C., Alexander, C., Goh, J., Loitta, D. C., and Merrill, A. H., Jr. (1997). In "Sphingolipid-Mediated Signal Transduction, Molecular Biology Intelligence Unit" (Y. A. Hannun, ed.), p. 159. R. G. Landes Co.
- Olivera, A., Barlow, K. D., and Spiegel, S. (2000). Assaying sphingosine kinase activity. *Methods Enzymol.* **311**, 215–223.
- Pewzner-Jung, Y., Ben-Dor, S., and Futerman, A. H. (2006). When do Lasses (longevity assurance genes) become CerS (ceramide synthases)? Insights into the regulation of ceramide synthesis. *J. Biol. Chem.* **281**, 25001–25005.
- Pitson, S. M., D'Andrea, R. J., Vandeleur, L., Moretti, P. A., Xia, P., Gamble, J. R., Vadas, M. A., and Wattenberg, B. W. (2000). Human sphingosine kinase: Purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem. J.* **350**(Pt 2), 429–441.
- Pitson, S. M., Moretti, P. A., Zebol, J. R., Lynn, H. E., Xia, P., Vadas, M. A., and Wattenberg, B. W. (2003). Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J.* **22**, 5491–5500.
- Riebeling, C., Allegood, J. C., Wang, E., Merrill, A. H., Jr., and Futerman, A. H. (2003). Two mammalian longevity assurance gene (LAG1) family members, *trh1* and *trh4*, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *J. Biol. Chem.* **278**, 43452–43459.
- Roberts, J. L., Moretti, P. A., Darrow, A. L., Derian, C. K., Vadas, M. A., and Pitson, S. M. (2004). An assay for sphingosine kinase activity using biotinylated sphingosine and streptavidin-coated membranes. *Anal. Biochem.* **331**, 122–129.
- Spassieva, S., Seo, J. G., Jiang, J. C., Bielawski, J., Alvarez-Vasquez, F., Jazwinski, S. M., Hannun, Y. A., and Obeid, L. M. (2006). Necessary role for the Lag1p motif in (dihydro) ceramide synthase activity. *J. Biol. Chem.* **281**, 33931–33938.
- Spiegel, S., and Milstien, S. (2002). Sphingosine 1-phosphate, a key cell signaling molecule. *J. Biol. Chem.* **277**, 25851–25854.
- Sribney, M. (1966). Enzymatic synthesis of ceramide. *Biochim. Biophys. Acta* **125**, 542–547.
- Venkataraman, K., and Futerman, A. H. (2002). Do longevity assurance genes containing Hox domains regulate cell development via ceramide synthesis? *FEBS Lett.* **528**, 3–4.
- Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Sullards, M. C., Merrill, A. H., Jr., and Futerman, A. H. (2002). Upstream of growth and differentiation factor 1 (*uog1*), a mammalian homolog of the yeast longevity assurance gene 1 (*LAG1*), regulates N-stearoyl-sphinganine (C18-[dihydro]ceramide) synthesis in a fumonisins B1-independent manner in mammalian cells. *J. Biol. Chem.* **277**, 35642–35649.
- Wang, E., and Merrill, A. H., Jr. (2000). Ceramide synthase. *Methods Enzymol.* **311**, 15–21.

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MEASUREMENT OF MAMMALIAN SPHINGOSINE-1-PHOSPHATE PHOSPHOHYDROLASE ACTIVITY *IN VITRO* AND *IN VIVO*

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Contents

1. Introduction	244
2. Principle	249
3. Measurement of SPP Activity in Cell Lysates	249
3.1. Preparation of cell lysates	249
3.2. Preparation of labeled S1P	250
3.3. <i>In vitro</i> SPP assay	251
4. Measurement of SPP Activity in Live Cells	252
4.1. Measurement of S1P uptake and hydrolysis in nonpermeabilized cells	252
4.2. TLC of sphingoid base phosphates	253
Acknowledgments	253
References	253

Abstract

Sphingolipid metabolites have emerged as key players in diverse processes including cell migration, growth, and apoptosis. Ceramide and sphingosine typically inhibit cell growth and induce apoptosis, while sphingosine-1-phosphate (S1P) promotes cell growth, inhibits apoptosis, and induces cell migration. Thus, enzymes that regulate the levels of these sphingolipid metabolites are of critical importance to understanding cell fate. There are two known mammalian isoforms of S1P phosphohydrolases (SPP1 and SPP2) that reversibly degrade S1P to sphingosine. This chapter discusses the importance of SPPs and describes

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assays that can be used to measure the activity of these two specific S1P phosphohydrolases in cells and cell lysates.

1. INTRODUCTION

The interconvertible sphingolipid metabolites, ceramide, sphingosine, and sphingosine-1-phosphate (S1P), have emerged as potent signaling molecules that regulate a wide variety of cellular functions. Ceramide and sphingosine are important regulatory components of stress responses, inducing growth arrest and apoptosis (Ogretmen and Hannun, 2004; Reynolds *et al.*, 2004). In contrast, S1P has been implicated in cellular proliferation, survival, and motility (Maceyka *et al.*, 2002; Saba and Hla, 2004; Spiegel and Milstien, 2003). S1P is produced by phosphorylation of sphingosine catalyzed by two sphingosine kinase isoenzymes (SphK1 and SphK2) and is a ligand for a family of five G-protein-coupled receptors (S1PRs), termed S1P₁₋₅. In many cases, activation of these S1PRs appears to involve “inside-out” signaling whereby growth factors, cytokines, or cross linking of IgE receptors stimulates cytosolic SphK and induces its translocation to the plasma membrane where its substrate sphingosine resides (Spiegel and Milstien, 2003). This activation process produces S1P that may be secreted from specific types of cells, perhaps through the ABC transporter ABCC1 (Mitra *et al.*, 2006), to stimulate S1PRs in an autocrine or paracrine manner. While activation of certain S1PRs by S1P is clearly involved in cell motility, angiogenesis, and lymphocyte trafficking (Goparaju *et al.*, 2005; Liu *et al.*, 2000; Rosen and Goetzl, 2005; Rosenfeldt *et al.*, 2001; Seitz *et al.*, 2005), intracellularly generated S1P can enhance cell growth and survival independently of cell surface S1PRs (Kohno *et al.*, 2006; Olivera *et al.*, 2003; Van Brocklyn *et al.*, 1998), acting through as yet unknown intracellular effectors. Whereas cell stresses, such as tumor necrosis factor- α , irradiation, and anticancer drugs, induce accumulation of ceramide leading to apoptosis, many other stimuli, particularly growth and survival factors, activate SphK1, resulting in accumulation of S1P and consequent suppression of ceramide-mediated apoptosis (Cuvillier *et al.*, 1996; Spiegel and Milstien, 2003). It has been suggested that the dynamic balance between intracellular S1P vs sphingosine and ceramide, and the consequent regulation of opposing signaling pathways, is an important factor that determines whether cells survive or die (Cuvillier *et al.*, 1996). This “sphingolipid rheostat” has important clinical implications for cancer treatment (Ogretmen and Hannun, 2004; Reynolds *et al.*, 2004) and is evolutionarily conserved, as it also plays a role in regulation of stress responses of yeast (Ogretmen and Hannun, 2004; Saba and Hla, 2004).

Much attention has been focused on the enzymes that produce S1P, yet there is growing evidence that S1P signaling is also controlled by enzymes

that degrade it. Levels of S1P can be decreased by irreversible cleavage by S1P lyase (SPL). SPL is highly conserved in eukaryotes and has been shown to play a role in development and stress responses in a variety of species (Saba and Hla, 2004). SPL overexpression decreases S1P and elevates stress-induced ceramide generation and apoptosis (Reiss *et al.*, 2004). Moreover, SPL expression is induced upon DNA damage and its knockdown protects cells from apoptosis (Oskouian *et al.*, 2006). Interestingly, the food colorant 2-acetyl-4-tetrahydroxybutylimidazole, which has been shown to induce lymphopenia in mice, is an SPL inhibitor that can increase S1P levels more than 100-fold in lymphoid tissues where its concentration is normally low. This suggests that lymphocyte egress from lymph nodes may be mediated by S1P gradients that are established by S1P lyase activity and that the endoplasmic reticulum (ER)-localized SPL can somehow regulate external S1P levels (Schwab *et al.*, 2005).

Sphingosine-1-phosphate can also potentially be reversibly dephosphorylated by a variety of other nonspecific phosphohydrolases. However, there are two S1P phosphohydrolases (SPPs) in yeast (Lbp1p/Ysr2p/Lcb3p and Lbp2p/Ysr3p) (Le Stunff *et al.*, 2002b) and in mammals (SPP1 and SPP2) (reviewed in Le Stunff *et al.*, 2004b), suggesting the importance of SPPs in S1P metabolism (Table 13.1). SPPs are integral membrane proteins with eight predicted transmembrane spans (Kihara *et al.*, 2003) and are localized mainly to the ER (Le Stunff *et al.*, 2002a,b,c; Mandala *et al.*, 1998, 2000; Mao *et al.*, 1997; Ogawa *et al.*, 2003). SPPs share three distinct homology domains with the type 2 lipid phosphate phosphohydrolase (LPP) family and each of these domains is essential for activity (Kihara *et al.*, 2003; Le Stunff *et al.*, 2002b). There are three LPP isoenzymes in mammalian cells, which are primarily plasma membrane proteins (Brindley, 2004; McDermott *et al.*, 2006). Compared to SPPs, LPPs have a wide substrate specificity and have similar activities with S1P, ceramide-1-phosphate, and glycerolphospholipids, such as lysophosphatidic acid and phosphatidic acid. LPPs are characterized by a lack of requirement for bivalent cations and insensitivity to *N*-ethylmaleimide (NEM) inhibition. In contrast, SPP1 and SPP2 only dephosphorylate sphingoid base phosphates and not ceramide-1-phosphate or other phospholipids (Le Stunff *et al.*, 2002c; Mandala *et al.*, 1998; Ogawa *et al.*, 2003) (Table 13.1).

Using different genetic approaches, Lbp1 and Lbp2 were independently cloned from yeast (Mandala *et al.*, 1998; Mao *et al.*, 1997). Deletion of both phosphatase genes resulted in increased survival in response to heat shock (Mandala *et al.*, 1998; Mao *et al.*, 1997; Skrzypek *et al.*, 1999), suggesting that phosphorylated sphingoid bases play a protective role in stress responses. Conversely, overexpression of Lbp1p made yeast cells more sensitive to heat stress and induced their arrest in the G₁ phase of the cell cycle, consistent with the observation that increased S1P promotes cell cycle progression in mammalian cells (Olivera *et al.*, 1999). Further work on

Table 13.1 Comparison of lipid phosphate phosphohydrolases^a

	SPP1	SPP2	LPP
Molecular mass (aa)	48.9 (441)	44.7 (399)	≈33 (≈300)
Predicted TMD	8	8	6
Substrate specificity:			
S1P	+	+	+
Dihydro-S1P	+	+	+
Phyto-S1P	+	n.d.	+
Lysophosphatidic acid	—	—	+
Phosphatidic acid	—	—	+
Ceramide-1-phosphate	Inhib.	n.d.	+
Mg ⁺² dependent	—	—	—
NaF inhibition	+	—	+
Orthovanadate inhibition	+	+	+
Triton X-100 inhibition	+	+	—
Cellular localization	Mainly ER	Mainly ER	PM, internal membranes
NEM sensitive	+	n.d.	+ / —

^a Various properties of human SPP1, SPP2, and LPP family members are compared. Molecular masses are in kDa. TMD, transmembrane spans; ER, endoplasmic reticulum; PM, plasma membrane; NEM, *N*-ethylmaleimide; n.d., not determined.

Lbp1p revealed its role in sphingolipid synthesis. Deletion of Lbp1p prevented incorporation of exogenous sphingosine in sphingolipids (Mandala *et al.*, 1998; Mao *et al.*, 1997). Later it was shown that a specific sphingosine kinase, Lcb4p, was also required for the incorporation of sphingosine into sphingolipids, suggesting that sphingoid bases must first be phosphorylated and then dephosphorylated before being salvaged back into sphingolipids (Funato *et al.*, 2003). Interestingly, SPP1 partially complements deletion of Lbp1p in yeast, suggesting that these two proteins are functional homologues (Mandala *et al.*, 2000).

SPP1 from mice and humans contains 430 and 441 amino acids, respectively, with 78% identity and 84% similarity. SPP2 has 39% identity and 70% similarity to S1PP1. SPP1 mRNA is nearly ubiquitously expressed in mammalian tissues, with pronounced expression in the kidney (Johnson *et al.*, 2003; Mandala *et al.*, 2000), whereas SPP2 has more limited expression and is expressed primarily in kidney and heart. Although members of the type 2 lipid phosphohydrolase family, such as LPPs and SPPs, are usually insensitive to thiol-alkylating agents such as NEM (Brindley and Waggoner, 1998),

murine SPP1 was inhibited by NEM (Le Stunff *et al.*, 2002c), while the yeast homolog was not (Mandala *et al.*, 1998). However, other thiol-reactive agents did not inhibit SPP1 (Le Stunff *et al.*, 2002c), suggesting that NEM may alkylate a noncysteine residue of SPP1. Indeed, the yeast Lpp1 is also NEM sensitive, suggesting that NEM sensitivity is not a consistent criterion for classification (Furneisen and Carman, 2000). Further characterization revealed that SPP1 has a K_m for S1P of 38.5 μM and a physiological pH optimum (7.5) (Le Stunff *et al.*, 2002c). SPP1 is also inhibited by sphingosylphosphorylcholine and ceramide-1-phosphate, suggesting that it binds but does not hydrolyze these substrates. Importantly, NaF inhibits SPP1 (Le Stunff *et al.*, 2002c) but not SPP2 (Ogawa *et al.*, 2003), and thus can be used to differentiate between them.

SPP1 overexpression in NIH 3T3 cells decreased S1P levels and induced apoptosis (Mandala *et al.*, 2000) and also increased ceramide levels, particularly when cells were fed exogenous S1P (Le Stunff *et al.*, 2002a). The increase in ceramide was attributed to increased salvage of sphingosine into ceramide, as inhibition of ceramide synthase with fumonisin B1, but not inhibition of *de novo* ceramide synthesis with myriocin, reduced ceramide accumulation (Le Stunff *et al.*, 2002a). Thus, SPP1 apparently has an important role in regulating sphingolipid synthesis.

The yeast Lbp1p has all three conserved domains facing the luminal side of the ER. In agreement, Lbp1p activity was not affected by incubation of yeast cells with protease unless detergent was used to permeabilize the plasma membrane (Kihara *et al.*, 2003). Like LPPs, SPPs are also thought to have their active site on the ecto-cytoplasmic or luminal side of the membrane (Brindley, 2004). This presents an intriguing paradox because the SPP substrate S1P is made by SphKs on the cytosolic side of the membrane. Yet, when S1P was added to the cytosolic side of ER preparations, it was efficiently degraded by SPPs (Kihara *et al.*, 2003). Moreover, S1P added to mammalian cells is not readily dephosphorylated by SPP1 unless the plasma membrane is first permeabilized with digitonin (Le Stunff *et al.*, 2004a), indicating that SPP1 can catalyze the dephosphorylation of S1P when it is presented on the cytosolic side of the ER. Thus, either S1P can rapidly intercalate and flop in ER membranes or an ATP-independent transport mechanism exists in the ER to flop S1P. Alternatively, the SPP1 active site may be imbedded within the membrane and have access to substrates on one or both sides of the membrane. Nevertheless, very low levels of SPP1 expression and activity have also been detected in the plasma membrane fraction and, in concordance, a small increase in ceramide was detected in the plasma membrane fraction after incubating cells with S1P (Le Stunff *et al.*, 2002a), suggesting that SPP1 might also have some ecto-phosphatase activity. The biological significance of this activity has not yet been explored.

As might be expected, SPP1 can also function to antagonize S1P signaling. Knockdown of SPP1 expression with small interfering RNA

(siRNA) elevated S1P levels (Johnson *et al.*, 2003). SPP1 knockdown also potentiated S1P-dependent activation of cyclooxygenase 2 and prostaglandin production (Pettus *et al.*, 2003), suggesting a role for SPP1 in regulating inflammatory responses. Additionally, siRNA directed against SPP1 protected cancer cells from chemotherapy-induced apoptosis (Johnson *et al.*, 2003). Intriguingly, it was also shown that knockdown of SPP1 increased secretion of S1P. Regulation of S1P secretion by SPP1 could have direct consequences for cell signaling, as it was subsequently observed that SPP1 downregulation potentiated the “inside out,” autocrine signaling of growth factors to S1P receptors involved in chemotaxis, whereas overexpression of SPP1 inhibited it (Le Stunff *et al.*, 2004a). Hence, SPP1 can degrade S1P produced in response to growth factors before it is secreted, paralleling the results observed with SPL inhibition, which alters S1P gradients that regulate lymphocyte trafficking (Schwab *et al.*, 2005). Interestingly, marked downregulation of expression of SPL as well as SPP1 and SPP2 in colorectal carcinomas compared to normal adjacent tissues has been reported, further supporting the importance of S1P metabolism in cancer (Oskouian *et al.*, 2006).

It should be noted that in yeast, Lbp1 and Lbp2 are not functionally redundant as they cannot complement each other, suggesting that even though they both localize to the ER, they are involved in partially overlapping but distinct physiological pathways (Funato *et al.*, 2003; Mandala *et al.*, 1998; Mao *et al.*, 1997). It is still unclear whether SPP1 and SPP2 have distinct, overlapping, or redundant functions in mammalian cells. Nonetheless, transcription of SPP2, but not SPP1, has been shown to be induced by inflammatory stimulation in a variety of cells and to be upregulated in patients with psoriasis, an inflammatory skin disease (Mechtcheriakova *et al.*, 2007). SPP2, but not SPP1, was further shown to be required for the production of the proinflammatory cytokines interleukin (IL)-1 β and IL-8 in response to tumor necrosis factor- α (Mechtcheriakova *et al.*, 2007).

As mentioned earlier, treatment of SPP-1-overexpressing cells with S1P, but not with dihydro-S1P, increased all ceramide species, particularly the long-chain ceramides. This was not due to inhibition of ceramide metabolism to sphingomyelin or monohexosylceramides but rather to the inhibition of ER-to-Golgi trafficking, determined with the fluorescent ceramide analog DMB-ceramide. In agreement, fumonisins B1 prevented S1P-induced elevation of all ceramide species and corrected the defect in ER transport of ceramide. Importantly, protein trafficking from the ER to the Golgi, determined with vesicular stomatitis virus ts045 G protein fused to green fluorescent protein, was also inhibited in SPP-1-overexpressing cells in the presence of S1P but not in the presence of dihydro-S1P. These data suggest that SPP-1 not only regulates ceramide levels in the ER, but by doing so influences the anterograde membrane transport of both sphingolipids and proteins from the ER to the Golgi.

2. PRINCIPLE

To date, little is known about the regulation of SPPs and whether they are constitutively active. Because there are no identifiable phosphorylation sites or protein interaction domains in SPP1 or SPP2, only measurements of their enzymatic activities can provide information about their activation. SPP activity assays are straightforward, although care needs to be taken to rule out ubiquitous nonspecific phosphatases. The specificity of *in vitro* SPP assays can be increased by purifying membranes to remove cytosolic phosphohydrolases, using a neutral pH where lysosomal phosphohydrolases are not active, and by adding EDTA to inhibit Mg^{+2} -dependent phosphohydrolases. SPP activity can be measured in cell lysates or in whole cells that have had their plasma membrane permeabilized with digitonin to allow S1P access to the ER, where the majority of both SPP1 and SPP2 reside. The relative contributions of SPP1 and SPP2 to total S1P phosphohydrolase activity can be determined by measuring the enzymatic activity in the absence and presence of NaF, which inhibits SPP1 (Le Stunff *et al.*, 2002c) but not SPP2 (Ogawa *et al.*, 2003). Importantly, results from enzymatic assays that implicate SPP1 or SPP2 should be confirmed by downregulating their expression with specific siRNAs targeted to one of the SPPs.

3. MEASUREMENT OF SPP ACTIVITY IN CELL LYSATES

3.1. Preparation of cell lysates

Because SPP1 and SPP2 are integral membrane proteins that are mainly localized to the ER, they can be partially purified from other cytosolic phosphohydrolases by differential centrifugation (Le Stunff *et al.*, 2002a). To this end, adherent cells in a six-well tissue culture plate are washed with cold phosphate-buffered saline and then scraped on ice in 1 ml of buffer A (100 mM HEPES [pH 7.5], 10 mM EDTA, 1 mM dithiothreitol, and protease inhibitor cocktail). The cells are lysed by multiple freeze-thaw cycles or by sonication. Centrifugation at 1500g for 5 min at 4° removes unbroken cells and debris. The supernatant is then centrifuged at 100,000g for 1 h at 4° to pellet internal and plasma membranes. The membrane pellet is gently washed and homogenized in 200 μ l buffer A, and protein concentration is determined by the Bradford assay. The membrane fraction can be solubilized with detergent if immunoprecipitation is to be performed. Care should be taken in the selection of detergents, as Triton X-100 inhibits SPP1 (Mandala *et al.*, 2000) and SPP2 (Ogawa *et al.*, 2003). Active, overexpressed SPP1 has been immunoprecipitated successfully in buffer containing 0.1% Tween 20 (Johnson *et al.*, 2003; Mandala *et al.*, 2000).

3.2. Preparation of labeled S1P

The choice of substrate dictates the purification and quantification procedures. The most convenient assays use radiolabeled S1P as substrate, prepared from either ^3H -labeled sphingosine or dihydrosphingosine or by phosphorylation of unlabeled sphingoid base with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Johnson *et al.*, 2003; Le Stunff *et al.*, 2002a; Mandala *et al.*, 1998; Ogawa *et al.*, 2003). Although fluorescent S1P analogs are now available commercially (Avanti Polar Lipids), whether or not they are substrates for SPPs has not yet been reported. ^{32}P -labeled sphingoid base phosphates have the advantage of being relatively inexpensive and simple to prepare. ^{32}P S1P can be synthesized using lysates from HEK 293 cells overexpressing SphK1 (Van Brocklyn and Spiegel, 2000). Differential solvent extraction is a rapid and effective method to purify labeled S1P (Giussani *et al.*, 2006; Mitra *et al.*, 2006; Olivera *et al.*, 2000; Van Brocklyn and Spiegel, 2000). An alkaline chloroform–methanol extraction is used to separate S1P (aqueous phase) from unreacted sphingosine (organic phase). After acidification of the aqueous phase, S1P can be extracted into chloroform–methanol, leaving unused ^{32}P ATP as well as any ^{32}Pi in the aqueous phase. Because S1P is the only labeled product in the organic phase, the radioactivity can be counted directly as a measure of the S1P concentration, although purity should be routinely confirmed by thin-layer chromatography (TLC; see later).

1. Combine the following in a siliconized glass tube: 12 μl 1 M MgCl_2 , 65 μl 1 mM sphingosine in 5% Triton X-100, 30 μl $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 mCi/ml; PerkinElmer), and 10–100 μg lysate from HEK 293 cells overexpressing SphK1. Adjust the volume to 1 ml with sphingosine kinase buffer (20 mM Tris [pH 7.4], 1 mM EDTA, 0.5 mM 4-deoxypyridoxine, 1 mM β -mercaptoethanol, 1 mM orthovanadate, 40 mM β -glycerophosphate, 10% glycerol, and protease inhibitor cocktail).
2. Mix and incubate for 60 min at 37°.
3. Stop the reaction by adding 1 ml chloroform, 1 ml methanol, and 100 μl 3 M NaOH.
4. Cover and vortex for 30 s and then centrifuge for 10 min at 1500g to separate the phases.
5. Transfer the upper aqueous phase to a siliconized glass tube.
6. Add 1 ml chloroform and 200 μl concentrated HCl.
7. Cover and vortex for 30 s and then centrifuge for 10 min at 1500g to separate the phases.
8. Remove the upper aqueous phase and discard.
9. Dry the lower organic phase containing the labeled sphingoid base under a stream of nitrogen.
10. Confirm purity of sample by TLC (see later).

11. Count an aliquot of the labeled S1P to calculate specific activity.
12. Appropriate care should be taken when handling and disposing of radioactivity.

3.3. *In vitro* SPP assay

Care should be taken in the choice of S1P delivery solvent, as some detergents inhibit SPP activity (Mandala *et al.*, 2000; Ogawa *et al.*, 2003). Both SPP1 and SPP2 are fully active when substrates are provided complexed to bovine serum albumin (BSA). Moreover, detergents such as Triton X-100 may stimulate the activity of broad specificity LPPs, complicating the assay of S1P-specific phosphohydrolase activity (Le Stunff *et al.*, 2002b). Typically, unlabeled S1P and [^{32} P]S1P, prepared as described earlier, are mixed to adjust the specific activity, dried under a stream of nitrogen in a siliconized glass tube, and then resuspended by sonication with 0.3% defatted BSA (Sigma-Aldrich, St. Louis, MO) in buffer A to a final concentration of 10 μ M (Johnson *et al.*, 2003; Le Stunff *et al.*, 2002c). The amounts of cell membranes required for the assay vary depending on cell type, and usually 4 to 20 μ g is added. As discussed earlier, NaF can be added to distinguish the individual contributions of SPP1 and SPP2 activities. SPP activity is expressed as nanomoles S1P degraded/min/mg protein. When using [^{3} H]S1P prepared from [^{3} H]sphingosine as a substrate, we have found that the aforementioned differential solvent extraction procedure separates >95% of S1P from sphingosine (Mitra *et al.*, 2006). Alternatively, if care is taken to prevent inorganic phosphate contamination, although sensitivity is much lower, it is possible to use unlabeled S1P and quantify the released inorganic phosphate (McDermott *et al.*, 2006; Walsh and Bell, 1992).

1. Add the following to a siliconized glass tube: 20 μ l of cell membranes (4 μ g total protein for HEK 293 cells or HUVECs) and 180 μ l 10 μ M [^{32} P]S1P (100,000 cpm) in 0.3% BSA in buffer A.
2. Incubate at 37° for 30 min.
3. Stop the reaction by adding 500 μ l water-saturated butanol and 300 μ l 1.5 M KCl.
4. Vortex vigorously and then centrifuge for 10 min at 1500g to separate the phases.
5. Aspirate the lower, aqueous phase and discard (in appropriate radioactive waste).
6. Wash the upper phase 3 $\times$ with 500 μ l 1.5 M KCl, discarding the lower, aqueous phases.
7. Count 100 μ l of the upper phase containing the remaining [^{32} P] S1P in 4 ml scintillant (Ultima Gold, PerkinElmer) as a measure of dephosphorylation.

4. MEASUREMENT OF SPP ACTIVITY IN LIVE CELLS

It is also possible to determine SPP activity in cells by incubating cells with [^{32}P]S1P complexed to BSA. Unless the cells are permeabilized first with digitonin to allow rapid entry into the cells, dephosphorylation of S1P is probably due to ectophosphohydrolase activity of LPPs or nonspecific phosphatase activity (Le Stunff *et al.*, 2004a). Much greater S1P dephosphorylation was noted in digitonin-permeabilized cells and was increased greatly when SPP1 was overexpressed (Le Stunff *et al.*, 2004a), consistent with the finding that SPP1 is mainly localized to the ER but not the plasma membrane (Le Stunff *et al.*, 2002a; Ogawa *et al.*, 2003).

1. Plate 5×10^5 cells per well in a six-well tissue culture dish.
2. Permeabilize by incubating the cells with 50 μM digitonin for 10 min.
3. Confirm efficient digitonin permeabilization by the uptake of trypan blue.
4. Add 10 μM [^{32}P]S1P (100,000 cpm/reaction) in 0.3% BSA to the cells.
5. Incubate at 37° for 30 min.
6. Extract the lipids from 1 ml of medium with 2.7 ml of a mixture of chloroform/methanol/HCl (100/200/1, v/v).
7. Separate the phases by adding 1.2 ml chloroform and 1.2 ml 2 M KCl.
8. Vortex and then centrifuge for 10 min at 1500g.
9. Remove the organic phase to a siliconized glass tube and dry under a stream of nitrogen.
10. Resuspend in chloroform/methanol (19/1) and resolve by TLC.

In this assay, the loss of [^{32}P]S1P is quantified by phosphorimaging of the TLC plate. Alternatively, the alkaline/acidic solvent extraction protocol can be used as described earlier to purify [^{32}P]S1P away from ^{32}P -labeled lipids and $^{32}\text{PO}_4$, and residual [^{32}P]S1P quantified by direct scintillation counting of the final solvent phase. TLC should be used to confirm that [^{32}P]S1P is the only labeled lipid in the final extract, as it is possible that liberated $^{32}\text{P}_i$ can be reincorporated into other lipids, particularly upon extended incubations (Giussani *et al.*, 2006). Moreover, when [^3H]S1P is used as a substrate, residual [^3H]S1P should be quantified by TLC as the products of digestion of S1P by SPL, hexadecenal and ethanolamine phosphate, would also be labeled and could potentially be incorporated back into lipids (Giussani *et al.*, 2006).

4.1. Measurement of S1P uptake and hydrolysis in nonpermeabilized cells

Although sphingoid base phosphates are not taken up by cells rapidly, they do accumulate these lipids over the course of several hours, measured both with ^{32}P labeling and with mass spectroscopy of unlabeled lipids (Giussani

et al., 2006). Interestingly, dihydrosphingosine-1-phosphate (dihydro-S1P) accumulates in cells at levels 5- to 10-fold higher than S1P (Giussani *et al.*, 2006), which is decreased by overexpression of SPP1. Thus, dihydro-S1P may normally be sequestered away from SPP1, as SPP1 has roughly equal *in vitro* activity with both S1P and dihydro-S1P (Le Stunff *et al.*, 2002a). It is possible that differential trafficking of S1P and dihydro-S1P to SPP1 may be the reason that S1P increases ceramide in cells, whereas dihydro-S1P does not (Le Stunff *et al.*, 2002a).

4.2. TLC of sphingoid base phosphates

S1P and dihydro-S1P can be separated efficiently from their precursors and potential contaminating phospholipids such as lysophosphatidic acid, phosphatidic acid, and ceramide-1-phosphate by chromatography on high-performance silica gel-coated TLC plates (Merck, Darmstadt). There are two commonly used solvent systems. The chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) system is more convenient and rapid (Le Stunff *et al.*, 2002c) and can also be used for the determination of other lipids, including ceramide-1-phosphate, phosphatidic acid, and glycerolipids. However, the R_f values for sphingoid base phosphates in this system are low (approximately 0.2), which may be problematic when there is a large amount of slower moving contaminants. The second, slower solvent system utilizing butanol/acetic acid/water (3:1:1, v/v) (Mechtcheriakova *et al.*, 2007) gives better separations (R_f of S1P is approximately 0.5). Lipids can be quantified directly on the TLC plate using a radiochromatogram scanner (for tritium; AR 2000, Bioscan, Washington, DC) or a phosphorimager (for ^{32}P) depending on the substrate. Alternatively, lipid standards run in an adjacent lane can be visualized with iodine, appropriate areas scraped, and lipids extracted from the silica and quantified by liquid scintillation counting.

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REFERENCES

- Brindley, D. N. (2004). Lipid phosphate phosphatases and related proteins: Signaling functions in development, cell division, and cancer. *J. Cell. Biochem.* **92**, 900–912.
- Brindley, D. N., and Waggoner, D. W. (1998). Mammalian lipid phosphate phosphohydrolases. *J. Biol. Chem.* **273**, 24281–24284.

- Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996). Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800–803.
- Funato, K., Lombardi, R., Vallée, B., and Riezman, H. (2003). Lcb4p is a key regulator of ceramide synthesis from exogenous long chain sphingoid base in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278**, 7325–7334.
- Furuse, J. M., and Carman, G. M. (2000). Enzymological properties of the LPP1-encoded lipid phosphatase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1484**, 71–82.
- Giussani, P., Maceyka, M., Le Stunff, H., Mikami, A., Lepine, S., Wang, E., Kelly, S., Merrill, A. H., Jr., Milstien, S., and Spiegel, S. (2006). Sphingosine-1-phosphate phosphohydrolase regulates endoplasmic reticulum-to-Golgi trafficking of ceramide. *Mol. Cell Biol.* **26**, 5055–5069.
- Goparaju, S. K., Jolly, P. S., Watterson, K. R., Bektas, M., Alvarez, S., Sarkar, S., Mel, L., Ishii, I., Chun, J., Milstien, S., and Spiegel, S. (2005). The S1P2 receptor negatively regulates platelet-derived growth factor-induced motility and proliferation. *Mol. Cell Biol.* **25**, 4237–4249.
- Johnson, K. R., Johnson, K. Y., Becker, K. P., Mao, C., and Obeid, L. M. (2003). Role of human sphingosine-1-phosphate phosphatase 1 in the regulation of intra- and extracellular sphingosine-1-phosphate levels and cell viability. *J. Biol. Chem.* **278**, 34541–34547.
- Kihara, A., Sano, T., Iwaki, S., and Igarashi, Y. (2003). Transmembrane topology of sphingoid long-chain base-1-phosphate phosphatase, Lcb3p. *Genes Cells* **8**, 525–535.
- Kohno, M., Momoi, M., Oo, M. L., Paik, J. H., Lee, Y. M., Venkataraman, K., Ai, Y., Ristimäki, A. P., Fyrist, H., Sano, H., Rosenberg, D., Saba, J. D., *et al.* (2006). Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation. *Mol. Cell Biol.* **26**, 7211–7223.
- Le Stunff, H., Galve-Roperh, I., Peterson, C., Milstien, S., and Spiegel, S. (2002a). Sphingosine-1-phosphate phosphohydrolase in regulation of sphingolipid metabolism and apoptosis. *J. Cell Biol.* **158**, 1039–1049.
- Le Stunff, H., Mikami, A., Giussani, P., Hobson, J. P., Jolly, P. S., Milstien, S., and Spiegel, S. (2004a). Role of sphingosine-1-phosphate phosphatase 1 in epidermal growth factor-induced chemotaxis. *J. Biol. Chem.* **279**, 34290–34297.
- Le Stunff, H., Milstien, S., and Spiegel, S. (2004b). Generation and metabolism of bioactive sphingosine-1-phosphate. *J. Cell. Biochem.* **92**, 882–899.
- Le Stunff, H., Peterson, C., Liu, H., Milstien, S., and Spiegel, S. (2002b). Sphingosine-1-phosphate and lipid phosphohydrolases. *Biochim. Biophys. Acta* **1582**, 8–17.
- Le Stunff, H., Peterson, C., Thornton, R., Milstien, S., Mandala, S. M., and Spiegel, S. (2002c). Characterization of murine sphingosine-1-phosphate phosphohydrolase. *J. Biol. Chem.* **277**, 8920–8927.
- Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., Liu, C. H., Hla, T., *et al.* (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* **106**, 951–961.
- Maceyka, M., Payne, S. G., Milstien, S., and Spiegel, S. (2002). Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochim. Biophys. Acta* **1585**, 193–201.
- Mandala, S. M., Thornton, R., Galve-Roperh, I., Poulton, S., Peterson, C., Olivera, A., Bergstrom, J., Kurtz, M. B., and Spiegel, S. (2000). Molecular cloning and characterization of a lipid phosphohydrolase that degrades sphingosine-1-phosphate and induces cell death. *Proc. Natl. Acad. Sci. USA* **97**, 7859–7864.
- Mandala, S. M., Thornton, R., Tu, Z., Kurtz, M. B., Nickels, J., Broach, J., Menzelev, R., and Spiegel, S. (1998). Sphingoid base 1-phosphate phosphatase: A key regulator of sphingolipid metabolism and stress response. *Proc. Natl. Acad. Sci. USA* **95**, 150–155.

- Mao, C., Wadleigh, M., Jenkins, G. M., Hannun, Y. A., and Obeid, L. M. (1997). Identification and characterization of *Saccharomyces cerevisiae* dihydrosphingosine-1-phosphate phosphatase. *J. Biol. Chem.* **272**, 28690–28694.
- McDermott, M. I., Sigal, Y. J., Crump, J. S., and Morris, A. J. (2006). Enzymatic analysis of lipid phosphate phosphatases. *Methods* **39**, 169–179.
- Mechtcheriakova, D., Wlachos, A., Sobanov, J., Kopp, T., Reuschel, R., Bornancin, F., Cai, R., Zemmann, B., Urtz, N., Stingl, G., Zlabinger, G., Woisetschlager, M., et al. (2007). Sphingosine 1-phosphate phosphatase 2 is induced during inflammatory responses. *Cell Signal.* **19**, 748–760.
- Mitra, P., Oskeritzian, C. A., Payne, S. G., Beaven, M. A., Milstien, S., and Spiegel, S. (2006). Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc. Natl. Acad. Sci. USA* **103**, 16394–16399.
- Ogawa, C., Kihara, A., Gokoh, M., and Igarashi, Y. (2003). Identification and characterization of a novel human sphingosine-1-phosphate phosphohydrolase, hSPP2. *J. Biol. Chem.* **278**, 1268–1272.
- Ogretmen, B., and Hannun, Y. A. (2004). Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* **4**, 604–616.
- Olivera, A., Barlow, K. D., and Spiegel, S. (2000). Assaying sphingosine kinase activity. *Methods Enzymol.* **311**, 215–223.
- Olivera, A., Kohama, T., Edsall, L. C., Nava, V., Cuvillier, O., Poulton, S., and Spiegel, S. (1999). Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J. Cell Biol.* **147**, 545–558.
- Olivera, A., Rosenfeldt, H. M., Bektas, M., Wang, F., Ishii, I., Chun, J., Milstien, S., and Spiegel, S. (2003). Sphingosine kinase type 1 induces G12/13-mediated stress fiber formation yet promotes growth and survival independent of G protein coupled receptors. *J. Biol. Chem.* **278**, 46452–46460.
- Oskouian, B., Sooriyakumaran, P., Borowsky, A. D., Crans, A., Dillard-Telm, L., Tam, Y. Y., Bandhuvula, P., and Saba, J. D. (2006). Sphingosine-1-phosphate lyase potentiates apoptosis via p53- and p38-dependent pathways and is down-regulated in colon cancer. *Proc. Natl. Acad. Sci. USA* **103**, 17384–17389.
- Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., Morrow, J., Chalfant, C. E., Obeid, L. M., and Hannun, Y. A. (2003). The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J.* **17**, 1411–1421.
- Reiss, U., Oskouian, B., Zhou, J., Gupta, V., Sooriyakumaran, P., Kelly, S., Wang, E., Merrill, A. H., Jr., and Saba, J. D. (2004). Sphingosine-phosphate lyase enhances stress-induced ceramide generation and apoptosis. *J. Biol. Chem.* **279**, 1281–1290.
- Reynolds, C. P., Maurer, B. J., and Kolesnick, R. N. (2004). Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Lett.* **206**, 169–180.
- Rosen, H., and Goetzl, E. J. (2005). Sphingosine 1-phosphate and its receptors: An autocrine and paracrine network. *Nat. Rev. Immunol.* **5**, 560–570.
- Rosenfeldt, H. M., Hobson, J. P., Maceyka, M., Olivera, A., Nava, V. E., Milstien, S., and Spiegel, S. (2001). EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. *FASEB J.* **15**, 2649–2659.
- Saba, J. D., and Hla, T. (2004). Point-counterpoint of sphingosine 1-phosphate metabolism. *Circ. Res.* **94**, 724–734.
- Schwab, S. R., Pereira, J. P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J. G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* **309**, 1735–1739.
- Seitz, G., Boehmler, A. M., Kanz, L., and Mohle, R. (2005). The role of sphingosine 1-phosphate receptors in the trafficking of hematopoietic progenitor cells. *Ann. N. Y. Acad. Sci.* **1044**, 84–89.

- Skrzypek, M. S., Nagiec, M. M., Lester, R. L., and Dickson, R. C. (1999). Analysis of phosphorylated sphingolipid long-chain bases reveals potential roles in heat stress and growth control in *Saccharomyces*. *J. Bacteriol.* **181**, 1134–1140.
- Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: An enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* **4**, 397–407.
- Van Brocklyn, J. R., Lee, M. J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D. M., Coopman, P. J. P., Thangada, S., Hla, T., and Spiegel, S. (1998). Dual actions of sphingosine-1-phosphate: Extracellular through the G_i-coupled orphan receptor edg-1 and intracellular to regulate proliferation and survival. *J. Cell Biol.* **142**, 229–240.
- Van Brocklyn, J. R., and Spiegel, S. (2000). Binding of sphingosine 1-phosphate to cell surface receptors. *Methods Enzymol.* **312**, 401–416.
- Walsh, J. P., and Bell, R. M. (1992). Diacylglycerol kinase from *Escherichia coli*. *Methods Enzymol.* **209**, 153–162.

A RAPID AND SENSITIVE METHOD TO MEASURE SECRETION OF SPHINGOSINE-1-PHOSPHATE

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Contents

1. Introduction	258
2. Measurement of S1P	259
2.1. Principle	259
2.2. Materials	259
2.3. Preparation of labeled S1P	260
2.4. Determination of recovery of [³ H]sphingosine and [³ H]S1P by differential extraction	260
2.5. Labeling of adherent cells with [³ H]sphingosine	260
2.6. Labeling of nonadherent cells with [³ H]sphingosine	261
2.7. Calculations	261
3. Conclusions and Perspectives	262
Acknowledgments	263
References	263

Abstract

The serum-borne, bioactive sphingolipid mediator, sphingosine-1-phosphate (S1P), regulates numerous important physiological and pathological processes, mainly acting through specific cell surface G-protein-coupled receptors. Although many mammalian cells can produce S1P, there is little information as to how it is secreted to reach its receptors. Progress in elucidating this mechanism has been hampered by the difficulty of measuring very low levels of S1P. This chapter describes a simple, rapid method to measure S1P export from cells. It also discusses the current knowledge of how S1P is exported out of cells and its physiological significance.

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1. INTRODUCTION

Sphingosine-1-phosphate (S1P) is now recognized as a pleiotropic lipid mediator of a wide variety of physiological processes important for the development of the nervous, cardiovascular, reproductive, and immune systems (Spiegel and Milstien, 2003). S1P also has pathophysiological actions associated with cancer, immunity, and allergy (Chun and Rosen, 2006; Milstien and Spiegel, 2006). S1P is formed from sphingosine by two sphingosine kinase (SphK) isoenzymes and is degraded by S1P phosphatases and S1P lyase. In contrast to S1P, which has been associated with growth and survival, its precursors, sphingosine and ceramide, have long been associated with cell growth arrest and apoptosis (Ogretmen and Hannun, 2004). Hence, it has been proposed that these interconvertible sphingolipid metabolites function as components of a rheostat regulating cellular processes in response to cellular and environmental cues (Spiegel and Milstien, 2003). SphK1 is a critical regulator of this rheostat, as it not only produces the progrowth and antiapoptotic S1P, it also reduces levels of proapoptotic ceramide and sphingosine.

Growth factors, cytokines, and cross linking of IgE receptors stimulate cytosolic SphK1, induce its translocation to the plasma membrane where its substrate sphingosine resides, and increase intracellular S1P (reviewed in Spiegel and Milstien, 2003). Intracellularly-generated S1P can enhance cell growth and survival independently of cell surface S1P receptors (Spiegel and Milstien, 2003), acting through as yet unknown intracellular effectors. Although the intracellular targets of S1P are not well defined, it is well established that many other actions of S1P are mediated by its binding to a family of five specific G-protein-coupled receptors (GPCRs), named S1P₁₋₅. These S1P receptors are coupled to various G proteins enabling them to regulate numerous signaling pathways. Of note, intracellularly generated S1P can also act in an autocrine/paracrine manner to activate S1P receptors present on the same cells that produce it (Hobson *et al.*, 2001) and this “inside-out” signaling by S1P is critical for many of its actions (Spiegel and Milstien, 2003). In this regard, administration of an S1P-specific monoclonal antibody to neutralize extracellular S1P was highly effective at inhibiting tumor growth and tumor angiogenesis in xenograft models of several types of human cancers (Visentin *et al.*, 2006).

Sphingosine-1-phosphate is present at relatively high levels in serum as well as in ascites fluid. However, its secretion has only been demonstrated in a few types of cells—platelets (Yatomi *et al.*, 1997), astroglial cells (Vann *et al.*, 2002), and mast cells (Jolly *et al.*, 2004). Nevertheless, all cells examined so far have been shown to express SphKs and at least one of the S1P receptors. Transport of S1P out of cells is an important issue as it impinges on its actions at the cell surface and possibly inside cells.

Membrane lipids do not spontaneously exchange across lipid bilayers because their polar head groups do not traverse the hydrophobic interior of the membrane readily and cells have evolved many special transporter proteins for this purpose. Studies suggest that S1P is exported from cells by ATP-binding cassette (ABC) transporters (Honig *et al.*, 2003; Kobayashi *et al.*, 2006; Mitra *et al.*, 2006). The large ABC transporter superfamily is made up of membrane proteins that utilize ATP hydrolysis to translocate a wide variety of substrates across membranes, including metabolic products, bioactive lipids, sterols, and drugs (van Meer and Lisman, 2002). For example, secretion of a platelet-activating factor is mediated by ABCB1 (Raggers *et al.*, 2001), ABCC4 has particular affinity for the transport of prostaglandins PGE₁ and PGE₂ (Reid *et al.*, 2003), and ABCC1 is a high-affinity leukotriene C₄ transporter (Wijnholds *et al.*, 1997). Using the method described in this chapter, we convincingly demonstrated that S1P produced intracellularly in mast cells is secreted independently of their degranulation and demonstrated that this is mediated by the ABC transporter ABCC1 (Mitra *et al.*, 2006). We have developed a simple, rapid, yet highly sensitive method to measure secretion of S1P from cultured cells that should facilitate studies of the mechanism of its secretion and aid in the identification of other potential specific S1P transporters.



2. MEASUREMENT OF S1P

2.1. Principle

Measurement of mass levels of S1P secreted from cells by the enzymatic method (Edsall and Spiegel, 1999) is time-consuming and limited by low sensitivity. Because [³H]sphingosine is readily taken up by cells and rapidly converted intracellularly to [³H]S1P and further metabolites, differential extraction of S1P from other sphingolipid metabolites can be used as a simple and rapid method to measure both secreted and intracellular levels of S1P.

2.2. Materials

Sphingosine is obtained from Biomol. D-*erythro*-[3-³H]Sphingosine (23 Ci/mmol) is from Perkin Elmer and stored at -20° in ethanol at 0.1 mCi/ml. As the amino group and the *trans* double bond enhance its susceptibility to oxidation, sphingosine and radiolabeled sphingosine are relatively unstable and glass surfaces accelerate their decomposition. Tissue-culture grade, fatty acid-free bovine serum is from Sigma. Silica G60 thin-layer chromatography (TLC) plates (10 × 20 cm) are from Merck. Scintillation fluid (Ultima Gold) is from Perkin Elmer.

2.3. Preparation of labeled S1P

The ^3H -labeled S1P standard is prepared with recombinant SphK1 as described previously using D-erythro-[3- ^3H]sphingosine as substrate (Olivera *et al.*, 2000). To check the purity of the [^3H]S1P, the product is separated by TLC with CHCl_3 /acetone/methanol/acetic acid/water (10/4/3/2/1, v/v) as solvent and either scanned and quantified with a Bioscan AR-2000 scanner or sprayed with En ^{3} Hance (Perkin Elmer) and visualized by autoradiography.

2.4. Determination of recovery of [^3H]sphingosine and [^3H]S1P by differential extraction

To determine recoveries, aliquots of [^3H]S1P and [^3H]sphingosine standards are mixed with 1 ml medium and then differentially extracted as described later. In agreement with previous results (Edsall and Spiegel, 1999; Paugh *et al.*, 2003), under alkaline solvent extraction conditions, S1P is negatively charged and partitions into the aqueous phase, whereas other neutral sphingolipids, including sphingosine, are retained in the organic phase. More than 95% of the radioactivity recovered from the aqueous phase is [^3H]S1P and 98.8% in the organic phase is [^3H]sphingosine as determined by TLC analysis and radioautography or radiochromatogram scanning.

2.5. Labeling of adherent cells with [^3H]sphingosine

1. To prepare [^3H]sphingosine-labeling solution for one six-well plate, add 27 μl [^3H]sphingosine and 4.5 μl nonradioactive sphingosine (2 mM stock in methanol) to a siliconized glass tube.
2. Evaporate the solvent under a stream of nitrogen and resuspend the sphingosine in 270 μl of appropriate cell culture medium containing 0.1% bovine serum albumin. Determine the specific activity of the [^3H]sphingosine-labeling mixture by counting a 1- μl aliquot.
3. Aspirate media from adherent cells and carefully wash the cell monolayers with phosphate-buffered saline (PBS). Add 1 ml of medium and 45 μl of sphingosine-labeling solution to each well (1.5 μM final concentration, 0.45 μCi).
4. Incubate cells for 10 min at 37° to label intracellular sphingosine pools and produce labeled S1P.
5. Aspirate off media, followed by two washes with ice-cold PBS. Discard.
6. Add 1 ml fresh medium and incubate cells at 37° for the desired secretion time.
7. Transfer the medium containing secreted S1P to 15-ml polypropylene centrifuge tubes and centrifuge at 1200 rpm for 5 min to pellet any cells.

8. Transfer the medium to conical glass tubes and extract by adding 1 ml each of methanol and chloroform and 100 μl of 3 *N* NaOH. Vortex for 1 min and centrifuge at 3000 rpm for 10 min to separate the aqueous and organic phases.
9. To extract cellular lipids (if desired), wash cell monolayers with cold PBS, add 500 μl of methanol, and incubate on ice for 10 min. Scrape the cells into conical glass tubes and briefly bath sonicate on ice to break cell clumps. Add 500 μl chloroform to each, vortex for 1 min, and incubate on ice for 20 min. Add 500 μl 1 *M* NaCl and 50 μl 3 *N* NaOH, and vortex for 1 min. Centrifuge at 3000 rpm for 10 min to separate the phases.
10. Pipette 100- μl aliquots of the aqueous and organic phases from the medium and the cell extracts into scintillation vials and measure the radioactivity by scintillation counting.
11. For normalization, total phospholipid phosphate content in 25- μl aliquots of the organic phases is measured as described (Van Veldhoven and Mannaerts, 1987).

2.6. Labeling of nonadherent cells with [^3H]sphingosine

1. The [^3H]sphingosine-labeling solution is prepared as described previously.
2. Aliquot 10^6 cells in 1 ml of media into 1.5-ml microcentrifuge tubes.
3. Add 45 μl of sphingosine-labeling solution to each tube.
4. Incubate for 10 min at 37° to label the intracellular sphingosine pools and produce labeled S1P.
5. Centrifuge at 1200 rpm for 5 min and carefully aspirate off media.
6. Suspend the pelleted cells in 1 ml ice-cold PBS, followed by centrifugation at 1200 rpm for 5 min and aspiration of the supernatant.
7. Add 1 ml fresh medium and incubate the cells at 37° for the desired secretion time.
8. Centrifuge at 1200 rpm for 5 min and carefully collect the supernatant for analysis of secreted S1P.
9. Extract and measure secreted and cellular [^3H]S1P exactly as described in steps 8–11 for adherent cells.

2.7. Calculations

The amount of cellular and secreted S1P is calculated from the specific activity of the [^3H]sphingosine-labeling solution. Divide the dpm of the sample aliquot (100 μl) by the specific activity in dpm/pmol of the labeling solution to yield pmol S1P/100 μl . The final volumes of the aqueous phase extracts of the medium and cells are 2 and 1 ml, respectively. To determine the total amount of S1P produced, multiply the pmol S1P/100 μl by 20 and 10, respectively, and express as pmol S1P secreted or produced per time of incubation.

3. CONCLUSIONS AND PERSPECTIVES

We have found that [^3H]sphingosine is rapidly taken up by most cells, including mast cells, HEK 293 cells, fibroblasts, and MCF-7 human breast cancer cells, and within 10 to 30 min, more than 70% disappear from the medium. Therefore, 10 to 30 min is selected as an appropriate labeling time. Secretion of SIP from many types of cells into the medium is detected readily

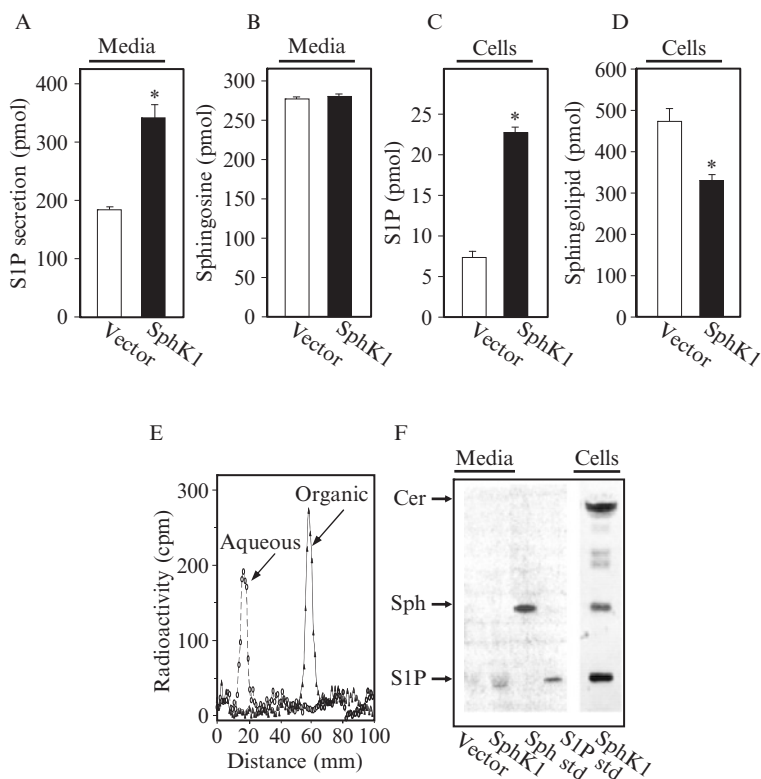


Figure 14.1 Effect of SphK1 on SIP formation and secretion. (A) RBL-2H3 cells stably transfected with vector (open bars) or SphK1 (filled bars) were incubated with [^3H] sphingosine ($1.5 \mu\text{M}$, $0.45 \mu\text{Ci}$) for 30 min. Labeled lipids were extracted differentially from media (A and B) and cells (C and D) into aqueous (A and C) and organic fractions (B and D) and quantified by scintillation counting. Data are means $\pm$ SD of duplicate determinations. * $P < 0.05$. (E) Labeled media lipids from aqueous (open circles) and organic (filled triangles) phases after solvent extractions were also separated by TLC and radioactivity determined with a Bioscan AR-2000 scanner. (F) Aqueous phases from media and total cell lipid extracts were dried under nitrogen, and lipids were dissolved in chloroform/methanol (1:2, v/v) and separated by TLC together with ^3H -labeled sphingosine and SIP standards. TLC plates were sprayed with En ^{3} Hance and exposed to X-ray film for 48 h at -80° . Arrows indicate migration of standards.

within 10 min and gradually increases thereafter to a maximum at around 120 min. Typical results of production and secretion of S1P by RBL 2H3 mast cells are shown in Fig. 14.1. In agreement with previous mass measurements (Jolly *et al.*, 2005), secreted [^3H]S1P, as well as cell-associated [^3H]S1P, is increased by overexpression of SphK1 (Figs. 14.1A and 14.1C). Expression of SphK1 did not affect uptake of [^3H]sphingosine (Fig. 14.1B). TLC analysis of lipids extracted from the media confirmed that the organic and aqueous phases each contained a single major radiolabeled product, [^3H]sphingosine and [^3H]S1P, respectively (Fig. 14.1E). In addition, TLC analysis confirmed that [^3H]S1P release into the medium was increased significantly by overexpression of SphK1 (Fig. 14.1F). [^3H]Sphingosine taken up by the cells was also partially converted to ceramide, the precursor of more complex sphingolipids (Fig. 14.1F). Furthermore, the content of ^3H -labeled sphingosine-based sphingolipids was decreased by overexpression of SphK1 (Fig. 14.1D), suggesting that [^3H]sphingosine is rapidly converted intracellularly by SphK1 to [^3H]S1P at the expense of incorporation into ceramide and other complex sphingolipids.

This rapid and robust method has been used to demonstrate that external stimuli, such as platelet-derived growth factor and cross linking of Fc ϵ RI, increase production and secretion of S1P from fibroblasts and mast cells, respectively, and that S1P is exported out of mast cells at least in part by the ABC transporter ABCC1 (Mitra *et al.*, 2006). This method should facilitate determination of S1P secretion from many types of cells, including cell lines and primary cells, in which S1P has been shown to play important roles, as well as elucidation of the mechanisms of its secretion.

ACKNOWLEDGMENTS

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REFERENCES

- Chun, J., and Rosen, H. (2006). Lysophospholipid receptors as potential drug targets in tissue transplantation and autoimmune diseases. *Curr. Pharm. Des.* **12**, 161–171.
- Edsall, L. C., and Spiegel, S. (1999). Enzymatic measurement of sphingosine 1-phosphate. *Anal. Biochem.* **272**, 80–86.
- Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Miltien, S., and Spiegel, S. (2001). Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science* **291**, 1800–1803.
- Honig, S. M., Fu, S., Mao, X., Yopp, A., Gunn, M. D., Randolph, G. J., and Bromberg, J. S. (2003). FTY720 stimulates multidrug transporter- and cysteinyl leukotriene-dependent T cell chemotaxis to lymph nodes. *J. Clin. Invest.* **111**, 627–637.
- Jolly, P. S., Bektas, M., Olivera, A., Gonzalez-Espinosa, C., Proia, R. L., Rivera, J., Miltien, S., and Spiegel, S. (2004). Transactivation of sphingosine-1-phosphate

- receptors by Fc ϵ RI triggering is required for normal mast cell degranulation and chemotaxis. *J. Exp. Med.* **199**, 959–970.
- Jolly, P. S., Bektas, M., Watterson, K. R., Sankala, H., Payne, S. G., Milstien, S., and Spiegel, S. (2005). Expression of SphK1 impairs degranulation and motility of RBL-2H3 mast cells by desensitizing S1P receptors. *Blood* **105**, 4736–4742.
- Kobayashi, N., Nishi, T., Hirata, T., Kihara, A., Sano, T., Igarashi, Y., and Yamaguchi, A. (2006). Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner. *J. Lipid Res.* **47**, 614–621.
- Milstien, S., and Spiegel, S. (2006). Targeting sphingosine-1-phosphate: A novel avenue for cancer therapeutics. *Cancer Cell* **9**, 148–150.
- Mitra, P., Oskeritzian, C. A., Payne, S. G., Beaven, M. A., Milstien, S., and Spiegel, S. (2006). Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc. Natl. Acad. Sci. USA* **103**, 16394–16399.
- Ogretmen, B., and Hannun, Y. A. (2004). Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* **4**, 604–616.
- Olivera, A., Barlow, K. D., and Spiegel, S. (2000). Assaying sphingosine kinase activity. *Methods Enzymol.* **311**, 215–223.
- Paugh, S. W., Payne, S. G., Barbour, S. E., Milstien, S., and Spiegel, S. (2003). The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS Lett.* **554**, 189–193.
- Raggers, R. J., Vogels, I., and van Meer, G. (2001). Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor. *Biochem. J.* **357**, 859–865.
- Reid, G., Wielinga, P., Zelcer, N., van der Heijden, I., Kuil, A., de Haas, M., Wijnholds, J., and Borst, P. (2003). The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal anti-inflammatory drugs. *Proc. Natl. Acad. Sci. USA* **100**, 9244–9249.
- Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: An enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* **4**, 397–407.
- van Meer, G., and Lisman, Q. (2002). Sphingolipid transport: Rafts and translocators. *J. Biol. Chem.* **277**, 25855–25858.
- Vann, L. R., Payne, S. G., Edsall, L. C., Twitty, S., Spiegel, S., and Milstien, S. (2002). Involvement of sphingosine kinase in TNF- α -stimulated tetrahydrobiopterin biosynthesis in C6 glioma cells. *J. Biol. Chem.* **277**, 12649–12656.
- Van Veldhoven, P. P., and Mannaerts, G. P. (1987). Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **161**, 45–48.
- Visentin, B., Vekich, J. A., Sibbald, B. J., Cavalli, A. L., Moreno, K. M., Matteo, R. G., Garland, W. A., Lu, Y., Yu, S., Hall, H. S., Kundra, V., Mills, G. B., *et al.* (2006). Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages. *Cancer Cell* **9**, 225–238.
- Wijnholds, J., Evers, R., van Leusden, M. R., Mol, C. A., Zaman, G. J., Mayer, U., Beijnen, J. H., van der Valk, M., Krimpenfort, P., and Borst, P. (1997). Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. *Nat. Med.* **3**, 1275–1279.
- Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., and Kume, S. (1997). Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J. Biochem.* **121**, 969–973.

CERAMIDE KINASE AND CERAMIDE-1-PHOSPHATE

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Contents

1. Introduction	266
2. Recombinant Expression and Kinetic Analysis of CERK	269
2.1. Principle	269
2.2. Reagents	270
2.3. Buffers	270
2.4. Procedures	270
3. <i>In Vitro</i> Kinetic Analysis of CERK Activity Using Mixed Micellar Assays	272
3.1. Principle	272
3.2. Reagents	275
3.3. Buffers	275
3.4. Procedures	275
4. Effective Delivery of C1P to Cells in Tissue Culture to Study Biological Effects	278
4.1. Principle	278
4.2. Reagents	280
4.3. Procedures	280
5. Analysis of Levels of Kinase-Derived C1P in Cells	281
5.1. Principle	281
5.2. Reagents	282
5.3. Procedure	283
6. Analysis of CERK Localization in Cells	284
6.1. Principle	284
6.2. Reagents	284
6.3. Procedure	284

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7. Analysis of CERK Function by siRNA-Mediated Manipulation of CERK Expression	286
7.1. Principle	286
7.2. Reagents	287
7.3. Procedure	287
8. Analysis of CERK mRNA Levels by Q-PCR	288
8.1. Principle	288
8.2. Reagents	288
8.3. Procedure	289
Acknowledgments	289
References	290

Abstract

It has been over a decade since the sphingolipid ceramide-1-phosphate (C1P) was described. Until recently, only sparse reports on possible biological functions for this lipid have been published. A large number of reports have now surfaced demonstrating distinct biological mechanisms regulated by C1P produced from ceramide kinase (CERK). In the following methods chapter, the methodologies for examining CERK function *in vitro* and in cells are outlined in detail. The methodologies for examining C1P levels and the use of exogenous C1P on cells to observe lipid specific effects on a particular biology are also detailed.

1. INTRODUCTION

The sphingolipid ceramide-1-phosphate (C1P) is a direct metabolite of ceramide produced in mammalian cells by the phosphorylation of ceramide by ceramide kinase (CERK) (Fig. 15.1). CERK was first described as a lipid kinase found in brain synaptic vesicles in 1989 by Bajjalieh and co-workers. This research group demonstrated that this activity was specific for the conversion of ceramide to C1P without effects on the closely related glycerol lipid, diacylglycerol (Bajjalieh *et al.*, 1989). Just months after this initial finding, Kolesnick and colleagues reported the existence of C1P in human leukemia (HL-60) cells (Dressler and Kolesnick, 1990). They demonstrated that during stimulation, C1P was produced from ceramide derived from sphingomyelin, but not from glycosphingolipids. Later the same year, Kolesnick and co-workers also reported a CERK activity distinguishable from diacylglycerol kinase (DGK) activity in HL-60 cells, verifying the findings of Bajjalieh and colleagues (Kolesnick and Hemer, 1990). To date, in mammalian cells, C1P has only been shown to be derived from the phosphorylation of ceramide by a specific CERK activity.

In 2002, the cDNA sequence for CERK was cloned by Sugiura and co-workers from Jurkat acute T-cell leukemic cells. The enzyme was found

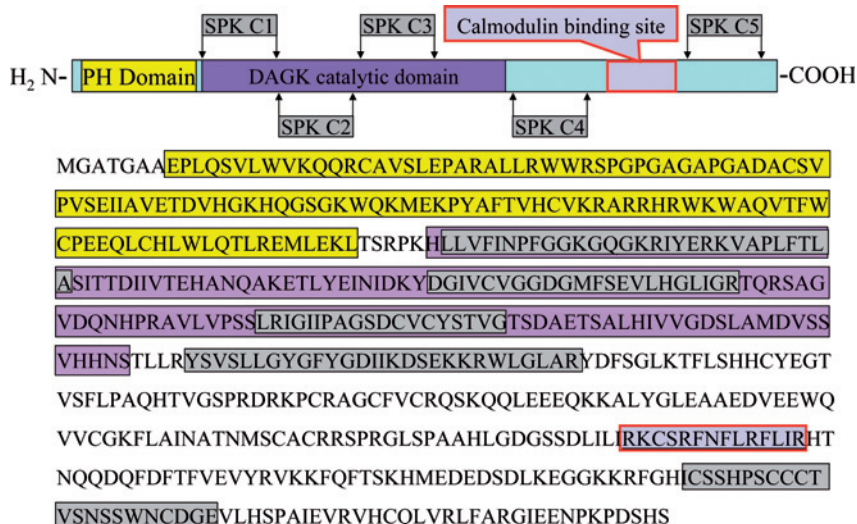


Figure 15.1 Important structural features of CERK. Schematic representation of the domain structure and conserved sequences of CERK with the corresponding primary structure. PH domain; pleckstrin homology domain (yellow), DAGK catalytic domain; diacylglycerol kinase catalytic domain (purple), SphK C1 to C5 conserved regions within CERK (gray), calmodulin-binding region (blue).

to be mainly expressed in heart, kidney, brain, and hematopoietic cells. Human CERK was also found to be a 537 amino acid protein closely related in structure and amino acid homology to sphingosine kinases 1 (SphK1) and 2 (SphK2). CERK was found to contain the five conserved domains (C1–C5) identified previously for SphK1 and 2. The first three of these domains, C1 to C3, are found within a putative DGK kinase domain (amino acids 126–342). The C4 region consists of a short 28 amino acid stretch designated the central homology region located just after the DGK catalytic domain, and the C5 region (amino acids 385–537) is located in the C terminus of the enzyme (Bajjalieh and Batchelor, 2000; Bajjalieh *et al.*, 1989; Sugiura *et al.*, 2002) (Fig. 15.1).

CERK also contains additional conserved regions across several species (*Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Oryza sativa*). These include a PH domain in its N terminus known to bind the β/γ subunit of heterotrimeric G proteins, phosphoinositol 4,5-bisphosphate, and phosphorylated tyrosine residues (Sugiura *et al.*, 2002). CERK also contains a calcium/calmodulin-binding motif of the 1–8–14 type B spanning residues 422 to 435 [(F/I/L/V/W) XXXXXX (F/A/I/L/V/W) XXXXX (F/I/L/V/W) with a net charge of 2^{+} to 4^{+}] (Sugiura *et al.*, 2002). These conserved domains have been shown to play a regulatory function for the enzyme. For example, Igarashi and co-workers and Bornancin and

colleagues have both demonstrated that the PH domain is required for the activity of CERK *in vitro* as well as proper localization of the enzyme in cells (Carre *et al.*, 2004; Kim *et al.*, 2006). Regarding the calmodulin-binding motif, Igarashi and colleagues demonstrated that calmodulin interacts with CERK and acts as a calcium “sensor” for the enzyme (Mitsutake and Igarashi, 2005). Thus, these conserved domains play distinct roles in regulating CERK activity *in vitro* and in cells.

In the past few years, a large number of reports have demonstrated distinct biological mechanisms regulated by C1P. For example, published findings from our laboratory demonstrated that C1P is a direct activator of cytosolic phospholipase A₂ (cPLA₂α) through interaction with the C2/CalB domain (Pettus *et al.*, 2004). These results, coupled with previous findings that CERK/C1P pathway is required for PLA₂ activation in response to calcium ionophore and cytokines (Pettus *et al.*, 2003), demonstrated that C1P is a “missing link” in the eicosanoid synthetic pathway. Mechanistic studies demonstrated that the interaction of C1P and cPLA₂α was very specific, as closely related lipids and metabolites were unable to activate cPLA₂α *in vitro* and in cells (Pettus *et al.*, 2004; Subramanian *et al.*, 2005). Furthermore, these studies demonstrated that C1P interacted with cPLA₂α in the C2 domain via a novel and previously undescribed interaction site. Finally, the studies also defined the mechanism of cPLA₂α activation: (1) decreasing the dissociation constant of cPLA₂α with membranes and (2) acting in a manner similar to a positive allosteric activator (Subramanian *et al.*, 2005). A role for CERK and its product, C1P, in a separate pathway of allergic/inflammatory signaling has also been reported in mast cells. Igarashi and colleagues demonstrated that treatment of RBL-2H3 cells or overexpression of CERK in these cells enhanced the degranulation induced by A23187 (Mitsutake *et al.*, 2004).

Another biological mechanism regulated by CERK and C1P was demonstrated by Shayman and co-workers. Specifically, they demonstrated that CERK was activated in the context of phagocytosis in neutrophils after challenging the cells with formyl peptide and antibody-coated erythrocytes (FMLP/EIgG) (Hinkovska-Galcheva *et al.*, 1998). Thus, these data demonstrated that C1P may play a distinct role in membrane fusion, possibly explaining the early finding that high levels of C1P are found in synaptic vesicles (Bajjalieh *et al.*, 1989).

The first report of a biological effect for C1P was by Gomez-Munoz and co-workers (1995). They demonstrated that short chain (not naturally found in cells) C1P induced DNA synthesis in rat-1 fibroblasts. Later, Gomez-Munoz *et al.* (1997) also demonstrated that treatment of T17 fibroblasts with natural ceramide-1-phosphate induced a potent increase in DNA synthesis and the levels of proliferating nuclear antigen. The most recent report was from the Gomez-Munoz (2006) laboratory demonstrating that C1P prevented cell death in bone marrow-derived macrophages after withdrawal of

macrophage colony-stimulating factor. Treatment of these cells with C1P effectively blocked the activation of caspases and prevented DNA fragmentation upon serum removal. In the same study, they also demonstrated that C1P treatment inhibited ceramide generation from acid sphingomyelinase (A-SMase). Finally, A-SMase was shown to be a direct target of C1P inducing inhibition of this enzyme (Gomez-Munoz *et al.*, 2004).

In conclusion, C1P and ceramide kinase have been shown to play significant biological roles in a variety of cell-signaling cascades. This chapter describes the methodologies used for examining CERK function and C1P-specific effects *in vitro* and tissue culture systems.



2. RECOMBINANT EXPRESSION AND KINETIC ANALYSIS OF CERK

2.1. Principle

Production of CERK of sufficient quantity and purity is essential for producing consistent and reproducible results for *in vitro* kinetic analysis. Currently, there are several ways to purify endogenous CERK. The first method was described by Bajjalieh *et al.* (1989) using membrane synaptic vesicles from rat brains. A second method involves extracting the membrane fractions that are generally high in CERK activity. Neither method produces CERK of sufficient purity or quantity for detailed kinetic analysis. In addition, there is currently an absence of an antibody for efficient immunoprecipitation of the enzyme. Our laboratory has demonstrated that the use of recombinant technology is an option available to obtain CERK of sufficient quantity and purity for *in vitro* kinase assays.

For large-scale production of recombinant CERK, baculoviral-mediated expression in insect cells is recommended for several reasons. First, the post-translational modifications of insect cells are comparable to that of mammalian cells (Kost *et al.*, 2005; Possee, 1997). This allows for CERK to be obtained in a form that resembles mammalian CERK. Second, insect cells can be cultured in large quantities, allowing for large-scale purification of CERK. Third, bacterially expressed CERK may be contaminated by non-specific lipid kinases due to aggregation unlike the use of baculovirus. Adenoviral-mediated expression of CERK is also the method of choice for the expression of CERK in human cell lines. However, adenoviral-mediated gene expression allows only for the transient expression of CERK, as the adenoviral genome does not get incorporated into the genome. Furthermore, large quantities of recombinant CERK from human cells are costly in comparison to insect cells.

2.2. Reagents

Sf-9 cells and SF-900 media are from Invitrogen Corporation. *Sf-9* cells are stored in liquid nitrogen, and media are stored at 4° until use. pBlueBac4.5/V5-His-TOPO vector, the pBlueBac4.5/V5-His-TOPO TA kit, pcDNA 3.1D/V5-His-TOPO, pcDNA 3.1 Directional TOPO expression kit and Back-n-Blue DNA are from Invitrogen Corporation and are stored at -20°. Cellfectin is also obtained from Invitrogen Corporation and is stored at 4°. The pTRE Shuttle vector and BD Adeno-X Tet-Off Expression system 1 are from Clontech and are stored at -20° until use. MOPS, KCl, glycerol, Triton X-100, imidazole, and phenylmethylsulfonyl fluoride (PMSF) are from Sigma-Aldrich and are stored at room temperature. Aprotinin, leupeptin, and pepstatin are also from Sigma-Aldrich. These are stored at -80°. Dithiothreitol (DTT) is also obtained from Sigma-Aldrich and, upon receipt, a 500 mM solution is made, aliquoted, and stored at -80°. Ni-NTA resin is obtained from Qiagen Corporation. XK columns are from Amersham Biosciences. Anti-6XHis and anti-V5 antibodies are from Santa Cruz Biotechnology and are stored at 4°.

2.3. Buffers

Cell lysis buffer: 10 mM MOPS, pH 7.2, 150 mM KCl, 10% glycerol, 0.5% Triton X-100, 0.001 mg/ml leupeptin, 0.01 mg/ml aprotinin, 0.005 mg/ml pepstatin, 1 mM PMSF

Buffer A for Ni-NTA His purification (Ni-NTA wash buffer): 10 mM MOPS, pH 7.2, 150 mM KCl, 10% glycerol

Buffer B for Ni-NTA His purification: 10 mM MOPS, pH 7.2, 150 mM KCl, 10% glycerol, 250 mM imidazole

Dialysis buffer: 10 mM MOPS, pH 7.2, 150 mM NaCl, 2 mM EGTA, 50% glycerol, 1 mM DTT

2.4. Procedures

2.4.1. Recombinant expression of CERK in insect cells

Ceramide kinase is polymerase chain reaction (PCR) amplified (high fidelity) from an A549 cDNA library and then cloned into the pBlueBac4.5/V5-His-TOPO vector using the pBlueBac4.5/V5-His-TOPO TA expression kit. The cDNA is verified by sequence analysis. To produce CERK baculovirus, this CERK plasmid is transfected into *s9* insect cells simultaneously with Back-n-Blue DNA using the Cellfectin transfection reagent in serum-free medium following the manufacturer's recommended protocol. Recombinant viral genome is produced through homologous recombination, leading to the production of infective baculoviral particles.

The recombinant virus is identified by the presence of blue plaques in the presence of X-gal. Viral particles from the individual blue plaques are used to infect new *sf9* cells growing in separate wells of 12-well plates. DNA is extracted from a portion of the resultant P0 viral stock and PCR amplified for 15 cycles (94°, 30 s; 58°, 30 s; 72°, 1 min) using the primer pair 5'-TCGTCTGTGTCGGCGGAGAT-3' and 5'-CTTTGCCACCAACC-CAGAACG-3' to verify the presence of the sequence of interest. The primers cross one exon/intron boundary, eliminating the possibility of amplifying contaminating genomic DNA from *Sf-9* cells. Presence of a 355-bp band upon gel electrophoresis of the PCR product verifies the presence of CERK in the viral genome. The remaining P0 viral stock from PCR-verified viral particles is then amplified twice to produce 1 liter of P2 viral stock. This viral stock is titered by end point dilution and is used to infect *sf9* cells at a density of 1.2 to 1.5×10^6 cells/ml in a 50-ml culture. Optimal transfection/expression is determined at 1 multiplicity of infection (MOI) of baculovirus for 72 h postinfection. Cells are harvested by centrifugation at 400g.

2.4.2. Recombinant expression of CERK adenovirus in mammalian cells

The ceramide kinase cDNA library is obtained from an A549 cDNA library using high-fidelity PCR and cloned into the pcDNA 3.1D/V5-His-TOPO vector using the pcDNA 3.1 Directional TOPO cloning kit. CERK expression in the adenoviral genome is carried out using the BD Adeno-X Tet-Off Expression system. CERK with V5 and 6XHis tags (on C terminus) is excised from the pcDNA vector using *Bam*HI and *Pme*I and is inserted into the pTRE shuttle vector. Recombinant CERK containing the adenoviral expression cassette is excised from the pTRE shuttle and ligated to Adeno-X viral DNA according to the manufacturer's protocol and amplified in *Escherichia coli*. After amplification in *E. coli*, the recombinant CERK adenovirus is obtained by transfection of the linearized adenoviral genome containing the recombinant CERK expression cassette into HEK 293 cells, and the recombinant adenoviral particles produced are harvested according to the manufacturer's protocol. Cotransfection of mammalian cells with these adenoviral particles along with the Adeno Tet-Off regulatory virus allows for transient overexpression of CERK in mammalian cells. Optimal transfection/expression is determined at 150 MOI each of Adeno-CERK and Adeno Tet-Off virus infecting 2×10^6 A549 cells growing in 15-cm plastic culture dishes in normal A549 media [Dulbecco's modified Eagle medium (DMEM)/RPMI containing 10% serum and 2% penicillin/streptomycin]. Cells are exposed to the virus for 48 h and then harvested for CERK purification.

2.4.3. Purification of CERK

Recombinant CERK expressed in either of the aforementioned methods can be purified using Ni-NTA affinity column chromatography. Large-scale purification of baculoviral-mediated CERK expression in *Sf-9* cells is recommended. The following protocol is used for large-scale purification of CERK from infected *Sf-9* cells. The cell paste is suspended in 10 volumes of lysis buffer, and cells are lysed by douncing 10 times per sample using a dounce homogenizer with a type B pestle. A 100-ml sample is used at a time and kept chilled on ice. The lysates are then centrifuged at 16,000g for 45 min at 4°. Simultaneously, 100 ml of Ni-NTA superflow resin is equilibrated in 5 column volumes of Ni-NTA wash buffer. The supernatant is then batch bound with the equilibrated Ni-NTA for 1.5 h at 4° with nutation. The resin is then transferred to a 5-cm XK column, and the column is washed until baseline absorbance at 280 nm with Ni-NTA wash buffer at 4 ml/min flow rate using fast protein liquid chromatography. Thereafter, the column is washed with a 19.5 mM imidazole wash buffer at 4 ml/min until baseline absorbance at 280 nm. CERK is eluted from the column using 250 mM imidazole in Ni-NTA wash buffer at a 5-ml/min flow rate collecting the elutions in fractions. The eluted fractions are analyzed by SDS-PAGE to assess the purification and by Western blotting to identify the presence of recombinant CERK using anti-His and anti-V5 antibodies. All fractions containing CERK are pooled, and DTT is added to a final concentration of 1 mM. The pooled samples are dialyzed against the dialysis buffer overnight at 4° with stirring using dialysis tubing with a 12 to 14,000 molecular weight exclusion. The dialyzed sample is centrifuged at 31,000g to remove any precipitant, and the total protein is quantified by the Bradford assay. The protocol yields recombinant CERK that is 60 to 70% (Fig. 15.2) pure with a specific activity of approximately 0.19 ($\mu\text{mol C1P}$ produced/min/mg protein) toward D-erythro-C_{16:0} ceramide (Fig. 15.3). Glycerol is added to the pooled samples to 50%. The samples are then aliquoted and stored at -80°. Under these conditions CERK is stable for approximately 1 year. *Note:* For mammalian cells the same protocol can be utilized, but a type A pestle or sonication is recommended for cell lysis. Furthermore, this is a 1-day procedure. The enzyme needs to be purified and stored at -80° as soon as possible for stability purposes.

3. *IN VITRO* KINETIC ANALYSIS OF CERK ACTIVITY USING MIXED MICELLAR ASSAYS

3.1. Principle

For the following assays, D-erythro-C_{16:0} ceramide and adenosine triphosphate (ATP) are routinely utilized for kinetic analysis. However, in terms of solubility, D-erythro-C_{18:1} ceramide is more soluble and better suited for

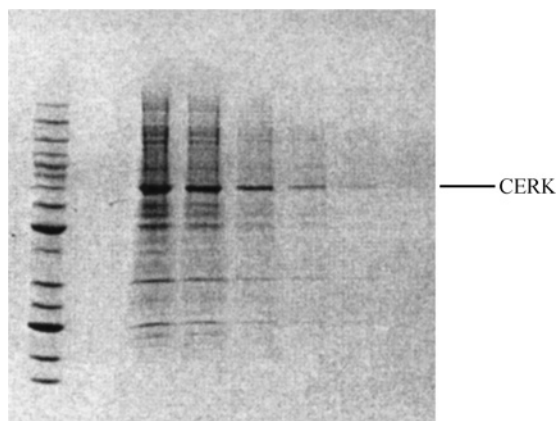


Figure 15.2 SDS-PAGE analysis of Ni-NTA purified CERK from *Sf-9* cells. Cell lysates were subjected to purification by 6XHis Ni-NTA purification. Pooled fractions with the highest levels of CERK were dialyzed overnight against the storage buffer. An aliquot of purified CERK was separated on a 10% SDS-PAGE gel followed by Coomassie staining to visualize the purity of the protein. Lane 1, BenchMark protein ladder; lane 2, 15 μ g protein; lane 3, 10 μ g protein; lane 4, 5 μ g protein; lane 5, 2.5 μ g protein.

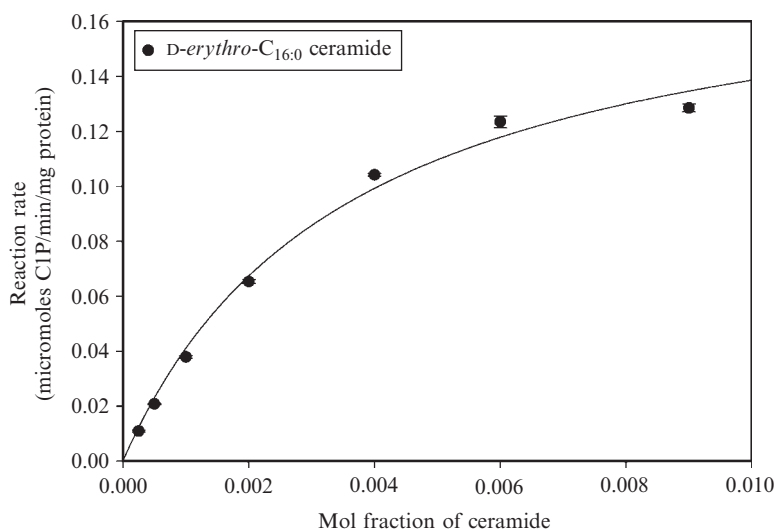


Figure 15.3 Surface dilution kinetics for CERK using Triton X-100 mixed micellar assay. Michaelis–Menten curve for *D-erythro-C*_{16:0} ceramide assayed at 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol%. The graph depicts micromoles of C1P produced per minute per milligram of CERK (baculovirus-expressed) versus the mole fraction of ceramide to obtain $V_{\max}$ and K_m^b . Data are presented as means SEM and are representative of three separate determinations.

measuring endogenous CERK activity *in vitro*. In the methods described here, the activity of CERK is assayed by measuring the levels of ceramide-1-phosphate formed using a detergent micelle system for substrate presentation. Because endogenous CERK acts on membrane lipids, the presentation of its substrate in this form compatible to a biological membrane is appropriate. When studying the activity of CERK, one has to take into account the diffusion of the enzyme in a three-dimensional cellular matrix (locating the membranes) and the diffusion in two dimensions (locating and utilizing its substrate in the membrane) bound to the membrane. Thus, the true kinetic behavior of the enzyme is best studied using a model that takes into account both bulk and surface diffusion. Surface dilution kinetics, first introduced in 1973 by Dennis, is an excellent means of studying this type of enzyme behavior *in vitro*. This technique can be coupled to both detergent micelles and lipid vesicles, but the use of vesicles as a mean of delivering the substrate is not suitable for the study of kinetics of CERK for several reasons. First, the number of substrate molecules per vesicle is not constant. Second, substrate molecules in the inner membrane are not accessible to the enzyme. Finally, mixed vesicles lack uniformity in size. Mixed micellar assays are better suited for the study of kinetics in being uniform in size with the same number of substrate molecules being present in each micelle. Thus, mixed micellar assays are the method of choice for *in vitro* assays when studying the kinetics of CERK.

β -Octylglucoside and Triton X-100 are the two published methods for the presentation of substrate to CERK as mixed micelles (Bajjalieh and Batchelor, 2000; Wijesinghe *et al.*, 2005). The β -octylglucoside-based method is the traditional method used in the assay of CERK (Bajjalieh and Batchelor, 2000). This system would be ideal for investigating substrates of ceramide. However, molecular modeling studies have shown micelles of β -octylglucoside to have an irregular surface (Garavito and Ferguson-Miller, 2001), thereby preventing equal presentation of all substrate molecules to the enzyme. This tends to be a drawback when studying the kinetics of a lipid kinase. Also, compared to Triton X-100, β -octylglucoside has a very high critical micellar concentration (CMC), thus the amount of detergent required to achieve CMC is about 100 times greater.

Compared to the β -octylglucoside method, Triton X-100 micelles provide several advantages. First, they provide an inert surface for CERK. Second, the size of the micelles is relatively independent of ionic strength and temperature within the physiological range (Lichtenberg *et al.*, 1983; Robson and Dennis, 1979, 1983). Third, mixed micelles up to 20 mol% phospholipids are similar in structure to pure Triton X-100 micelles, but proportionally larger (Lichtenberg *et al.*, 1983; Robson and Dennis, 1979, 1983). Finally, pure Triton X-100 micelles do not coexist with mixed micelles. Thus, Triton X-100 is an excellent method of choice in the assaying of CERK activity. Both the β -octylglucoside assay and the Triton X-100 assay are detailed.

3.2. Reagents

CERK is prepared as mentioned earlier using baculoviral-mediated expression followed by Ni-NTA 6XHis purification. Triton X-100 is from Pierce Biotechnology as a 10% solution (≈ 155 mM). D-*erythro*-C_{16:0} ceramide is from Avanti Polar Lipids. Upon receipt, a 0.82 mM solution is prepared by dissolving in CHCl₃ and is stored at -20° until use. Cardiolipin is also obtained as a 6.67 mM solution in CHCl₃ from Avanti Polar Lipids and is stored at -20° upon receipt. MOPS, NaCl, DETAPAC, and glycerol are from Sigma-Aldrich and are stored at room temperature. DTT is also from Sigma-Aldrich and, upon receipt, a 500 mM solution is made and stored at -80° . Thin-layer chromatography (TLC) plates are from VWR and are stored desiccated at room temperature. Borosilicate glass tubes (13 $\times$ 100 mm) are from Fisher Scientific.

3.3. Buffers

Reaction buffer for β -octylglucoside assay: 20 mM MOPS, pH 7.2, 50 mM NaCl, 1 mM DTT, 3 mM CaCl₂, 51.24 mM β -octylglucoside, 1 mM cardiolipin, and 0.2 mM diethylenetriaminepentaacetic acid

Reaction buffer for Triton X-100 assay: 20 mM MOPS, pH 7.2, 50 mM NaCl, 1 mM DTT, 3 mM CaCl₂, 1 mM cardiolipin, 0.2 mM diethylenetriaminepentaacetic acid

3.4. Procedures

3.4.1. Solubilization of ceramides

β -octylglucoside methods First described by Bajjalieh and co-workers (2000), this method utilizes β -octylglucoside as the micelle detergent. β -Octylglucoside is a nonionic detergent that forms micelles of 27 molecules each at a CMC of 23 to 25 mM. Micelle solubilization of ceramide in this method is carried out by sonicating ceramide in reaction buffer. The preparation of β -octylglucoside micelles for CERK assay is described next.

Twenty-five milligrams of cardiolipin is dried under a stream of dry nitrogen gas followed by dissolving in 3.333 ml water containing 256 mM β -octylglucoside and 1 mM DETAPAC to obtain a 5 mM cardiolipin solution. To prepare the reaction buffer, 160 μ l of this solution is mixed with MOPS, NaCl, CaCl₂, and DTT such that the final concentrations are 20 mM MOPS (pH 7.2), 50 mM NaCl, 1 mM DTT, 3 mM CaCl₂, 51.24 mM β -octylglucoside, 1 mM cardiolipin, and 0.2 mM DETAPAC. Ceramide (640 nmol) is dried down under a stream of dry nitrogen gas followed by micellar solubilization in 720 μ l of the aforementioned reaction buffer. A Misonix 3000 probe sonicator with a microtip probe (tip diameter of 3 mm) is used in the sonication (using three pulses of 1-min duration with 10-s intervals in between, on ice). Of this mixture, 180 μ l is used per reaction as explained later.

Triton X-100 method First described for CERK by Wijesinghe *et al.* (2005), this method uses the nonionic detergent Triton X-100 for the preparation of mixed micelles. Triton X-100 differs from β -octylglucoside in having 140 molecules per micelle and a CMC of 0.24 mM. The preparation of Triton X-100 mixed micelles containing D-*erythro*-C_{16:0} ceramide at its K_m^b (0.36 mol%) and cardiolipin is described next.

Ceramide (43.35 nmol) is dried down with 250 nmol cardiolipin under a stream of dry nitrogen gas. Triton X-100 (5.75 μ mol) in water is then added to the dried lipids such that the final volume is 500 μ l and the final concentrations are 8.67 μ M ceramide, 0.5 mM cardiolipin, and 11.5 mM Triton. A Misonix 3000 probe sonicator with a microtip probe (tip diameter of 3 mm) is used to sonicate this mixture. Sonication is carried out on ice using three pulses of 1-min duration with a 10-s resting interval (on ice) in between each pulse. Forty microliters of this mixture containing the mixed micelles is added to 140 μ l of reaction buffer (20 mM MOPS, pH 7.2, 50 mM NaCl, 1 mM DTT, 3 mM CaCl₂, and 0.2 mM diethylenetriaminepentaacetic acid) for a total volume of 180 μ l.

In both methods, care must be taken to prevent the ceramide solution from heating. This is achieved by carrying out the sonication on ice. Also, “frothing” of the mixture must be avoided at all costs. The mixed micelles, thus prepared, can be kept on ice up to a day. It is recommended that they not be stored for longer.

3.4.2. Ceramide kinase assay

Reactions are carried out in 13 $\times$ 100-mm borosilicate glass tubes. Micellar solubilized ceramide (180 μ l) in reaction buffer as described earlier is used in the reaction. Recombinant/purified ceramide kinase is diluted to 0.1 μ g/ μ l in lysis buffer (if using unpurified cell lysates from CERK over-expressed cells, the crude lysate is diluted 1 μ g/ μ l in lysis buffer). Ten microliters of the diluted CERK is added to each reaction, mixed by gently pipetting up and down and incubated at 37° for 10 min. The reaction is then started by the addition of 10 μ l of ATP from a stock solution of 20 mM such that the final concentration is 1 mM (the total reaction volume is 200 μ l). The mixture is incubated at 37° for 15 min and the reaction is stopped by the addition of 1.2 ml (1:1, v/v) CHCl₃:CH₃OH. Thereafter, 500 μ l of 1 M KCl in 20 mM MOPS (pH 7.2) is added, followed by vigorous vortexing and separation of phases by centrifugation at 250g.

3.4.3. Quantification of C1P produced

There are three different methods used in the quantification of C1P produced *in vitro* in the aforementioned reaction. They are quantification using TLC (Bielawska *et al.*, 2001), quantification by direct scintillation counting (Bektas *et al.*, 2003), and quantification using electrospray

ionization liquid chromatography tandem mass spectrometry (ESI-LC/MS/MS) (Merrill *et al.*, 2005). The first two methods involve labeling a portion of C1P produced using [γ - ^{32}P]ATP, and the reaction is started by adding a mixture of labeled and unlabeled ATP such that final concentration in the reaction is 0.05 μCi and 1 mM ATP. The TLC method, while being time-consuming and cumbersome, allows for the elimination of any other contaminating lipid species (radiolabeled) from contributing to the total radioactivity assayed producing for more accurate results. This technique is best suited for instances where tissues levels of active ceramide kinase are to be investigated. The direct scintillation counting method is a faster method of analyzing CERK activity. However, any other lipid radiolabeled by contaminating lipid kinases will also be present. Thus, this method is best suited for instances where the enzyme is highly expressed such that contributions from other lipid kinases are negligible or when using purified CERK.

Another method of quantifying CERK activity is ESI LC/MS/MS. The main advantage of the ESI LC/MS/MS method is the ability to assay C1P levels without using hazardous radioactive material. This allows for the reactions to be carried out on any laboratory bench without shielding. The high selectivity of the method allows for separation of C1P from other lipid phosphates, from the activity of other lipid kinases and lipid substrates. Thus, the technique is good for measuring CERK activity in tissues as well. However, in terms of sensitivity, the radiolabeled assays are currently superior to mass spectrometric assays.

Quantification by thin-layer chromatography An aliquot of the organic phase is spotted on a TLC plate, and lipids are separated in chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v). Radioactive bands are visualized with a phosphoimager (Bio-Rad), and [^{32}P]ceramide-1-phosphate is quantitated by densitometry or scintillation counting (see Table 15.1 for R_f values of various C1P species).

Quantification by direct scintillation counting After the reaction is stopped, the organic layer is washed four times using 500 μl of 1 M KCl in 20 mM MOPS (600 μl). The amount of C1P produced is quantified by counting the 100- μl organic phase using a Beckman-Coulter LS 6500 scintillation counter. The presence of C1P can be confirmed by TLC as detailed earlier.

Quantification using ESI LC/MS/MS To the organic phase obtained earlier, a known quantity of internal standard, ideally an unnatural C1P (e.g., C₁₂ C1P or C₁₇ C1P), is added. The organic phase is then dried, and the residue is brought up in sample buffer (Merrill *et al.*, 2005). The C1P produced can be identified and quantified by MRM using either

Table 15.1 R_f values for lipid standards resolved in solvent system: chloroform–acetone–methanol–acetic acid–water (10:4:3:2:1; v/v) (Bielawska *et al.*, 2001)

Standards	R_f value
D-E-C2:0 ceramide phosphate	0.36
D-E-C6:0 ceramide phosphate	0.49
D-E-C12:0 ceramide phosphate	0.58
D-E-C14:0 ceramide phosphate	0.61
D-E-C16:0/D-E-C18:0 ceramide phosphate	0.63
D-E-C24:0/D-E-C24:1 ceramide phosphate	0.68
D-E-C2:0 dihydroceramide phosphate	0.37
D-E-C6:0 dihydroceramide phosphate	0.53
D-E-C16:0/D-E-C18:0 dihydroceramide phosphate	0.68
A-OH-D-E-C6:0 ceramide phosphate	0.40
C26:0 phytoceramide phosphate	0.63
A-OH-C24:0 phytoceramide phosphate	0.51

$[M+H]^+$ (molecular ion) and m/z 264.4 (ceramide) ion pair in the positive ion mode or $[M-H]^-$ (molecular ion) and m/z 78.9 (phosphate) ion pair in the negative mode (Dr. Cameron Sullards and Dr. Alfred H. Merrill Jr., personal communication).

4. EFFECTIVE DELIVERY OF C1P TO CELLS IN TISSUE CULTURE TO STUDY BIOLOGICAL EFFECTS

4.1. Principle

It is now over a decade since C1P was first delivered to cells in culture. The first of these studies was performed using cell-permeable (short-chain C2-, C6-, or C8-) ceramides because these compounds were more water soluble than natural (long-chain) ceramides, and hence easier to mix with the culture medium. Addition of the short-chain C1Ps (dispersed in water by sonication) to rat-1 fibroblasts enhanced the incorporation of $[^3H]$ thymidine into DNA and increased cell number after 24 to 48 h of incubation (Gomez-Munoz *et al.*, 1995). However, natural (long-chain) C1P had no effect when presented in water to the fibroblasts. This prompted the design of an appropriate solvent that could facilitate the dispersion of this phospholipid and its interaction with cells in culture. In an earlier report, Hirabayashi and co-workers had established that a mixture of ethanol:dodecane (98:2, v/v) enhanced the dispersion of long-chain ceramides in culture medium and their uptake by cells (Ji *et al.*, 1995). Because C1P is a

polar lipid, ethanol was substituted by methanol to facilitate dispersion of the phospholipid in the solvent. Using this solvent mixture and EGFR T17 fibroblasts, it was confirmed that long-chain C1P also had mitogenic properties (Gomez-Munoz *et al.*, 1997). Similar to long-chain [^{14}C]ceramides in ethanol:dodecane (Zhang *et al.*, 1995), the uptake of [^{32}P]C1P in methanol:dodecane by the fibroblasts was greater than when [^{32}P]C1P was added directly to cells in water (Gomez-Munoz *et al.*, 1997). The ethanol dodecane system can also be used to deliver C1P effectively to cells. For example, Pettus and co-workers (2004) used low concentrations of C1P (up to $2.5\ \mu\text{M}$) in ethanol:dodecane (98:2, v/v) and demonstrated that C1P stimulates cPLA $_2\alpha$ directly in A549 cells (Pettus *et al.*, 2004). In agreement with these findings, Granado, Gomez-Munoz, and co-workers observed that C1P ($15\ \mu\text{M}$) dispersed in water interacted readily with NR8383 macrophages to induce arachidonic acid release (unpublished findings). This observation also contrasts with that of the Bornancin group who reported that C1P is only effective at stimulating cPLA $_2\alpha$ activity or arachidonic acid release when dispersed in ethanol:dodecane (Tauzin *et al.*, 2006). Also, the stimulation of arachidonic acid release in macrophages and A549 cells was specific for C1P, as other related phospholipids such as ceramide, S1P, or PA failed to do so (Pettus *et al.*, 2003; Granado and Gomez-Munoz, unpublished work). The observation that C1P can activate cPLA $_2\alpha$ in the absence of dodecane, or any other organic solvent, is also relevant because it discards any possible nonspecific interaction of the phospholipid with the organic compounds used for its delivery to cells in culture. In this regard, it should be emphasized that the stimulatory effect of C1P on proliferation of rat-1 fibroblasts and the inhibition of apoptosis in bone marrow-derived macrophages were all observed using C1P dispersed in water, in the absence of any organic solvent (Gomez-Munoz *et al.*, 1995, 2004, 2005).

Although mixtures of alcohol:dodecane (98:2, v/v) have proven to be effective at delivering long-chain ceramide or C1P to cells in culture, these solvents might cause unwanted effects when combined with relatively high concentrations of some lipids or lipid phosphates, such as ceramide, C1P, or PA. These lipids can cause toxic effects in cells when dispersed in ethanol or methanol:dodecane, although the extent of toxicity depends on cell type, time of exposure, and nature of the lipid. For example, concentrations up to $25\ \mu\text{M}$ C1P in methanol:dodecane, $30\ \mu\text{M}$ C2-ceramide, or $100\ \mu\text{M}$ long-chain ceramide in ethanol:dodecane were not toxic for EGFR T17 fibroblasts (Gomez-Munoz *et al.*, 1997), B16 melanoma cells (Komori, 1995), or Swiss 3T3 cells, respectively (Sasaki *et al.*, 1995). However, concentrations above $2.5\ \mu\text{M}$ C1P for raw 264.7 macrophages or above $5\ \mu\text{M}$ C1P for NR8383 macrophages and A549 cells were toxic for these cells after 6 h of incubation (unpublished work). In general, lipid phosphates seem to be more toxic for cells than nonphosphate-containing lipids when mixed with dodecane. A second potential problem when using dodecane on cells in

culture is its ability to interfere with fluorescent probes that are used commonly for cation image within cells. In particular, intracellular calcium mobilization could not be determined in the presence of dodecane in fibroblasts that were preloaded with Fura-2 AM (Gomez-Munoz *et al.*, 1997).

Other solvents used for delivery of C1P to cells in culture include dimethyl sulfoxide (DMSO) (Colina *et al.*, 2005; Hogback *et al.*, 2003), ethanol (Dragusin *et al.*, 2006), or chloroform:methanol (2:1, v/v) (Nakamura *et al.*, 2006). Whereas low concentrations of DMSO or ethanol are inert for most cell types, special attention should be paid when using chloroform on cells in culture because this solvent is a potent lipid solubilizer that can affect the integrity of the lipid bilayer of biological cell membranes. The following details the procedures used to deliver C1P to cells to examine specific biological effects attributed to C1P.

4.2. Reagents

C1P is from the lipidomics core of Medical University of South Carolina. Methanol and dodecane are from Sigma-Aldrich. A549 cells are from ATCC. Cell media (RPMI and DMEM) and fetal bovine serum (FBS) are from Invitrogen Corp; penicillin/streptomycin is from Biowhittaker Inc.

4.3. Procedures

4.3.1. Dispersion of short-chain C1P (C2, C6, and C8 C1P)

C1P is dissolved in ethanol to make a 10 mM solution. The required amount of C1P from this stock solution is dried down under N₂ gas. Water is added to the dried ceramide to the desired concentration. The solution is then sonicated on ice until a clear solution is obtained. This stock solution can be stored at -20°, but is recommended to be used as soon as possible.

4.3.2. Dispersion of long-chain C1P in methanol/dodecane or ethanol/dodecane

Methanol or ethanol and dodecane are mixed at a ratio of 98:2, followed by vortexing and prewarming at 37°. Meanwhile, C1P is dissolved in chloroform:methanol (1:1). The required volume is then dried down under N₂ gas. The prewarmed alcohol:dodecane mixture is added to the dried C1P such that the final concentration is 2.5 mM (a stock solution up to 10 mM can be made). This mixture is vortexed thoroughly and incubated at 37° for a further 20 min followed by further vortexing. The stock solution, thus created, is stored at -20°.

4.3.3. Addition onto cells for uptake

The stock solution of C1P in delivery medium is incubated at 37° followed by vortexing. C1P is diluted to the appropriate concentration in alcohol/dodecane solution and added to cells at a dilution of 1:1000. This concentration is recommended to prevent adverse effects on cells by the delivery medium itself. A sham control is always included at the same concentration as the treatment. Lipids of similar structure or closely related metabolites should also be included as specificity controls. The use of PA and ceramide with the same acyl chain composition as C1P and S1P are suggested as controls.

4.3.4. Conclusions

From the comments mentioned previously, we recommend not using dodecane for delivery of C1P to cells in culture unless low concentrations of the lipid are used ($\leq 1 \mu M$) with close monitoring of cell viability. Furthermore, biological responses that occur within 1 h are also preferable when utilizing this delivery system. Ideally, C1P should be vehicled in aqueous solutions if possible so as to avoid any side effects that might be generated when organic solvents are added to biological tissues or cells in culture. Use of controls containing lipids that closely resemble C1P (e.g., PA, ceramides, and S1P) are highly recommended in studies delineating a specific role for C1P in a biological function regardless of delivery system chosen.

5. ANALYSIS OF LEVELS OF KINASE-DERIVED C1P IN CELLS

5.1. Principle

To analyze C1P levels in tissue culture models, several methods exist. In one method, cells can be labeled with [^3H]palmitate or [^3H]sphingosine in culture, which are incorporated into the sphingolipid populations in cells. Another method is the use of mass spectrometry as described elsewhere (Lamour *et al.*, 2007). Finally, pulse labeling of cells in culture with [^{32}P]orthophosphate is another method of examining C1P formation. Our method of choice is the use of [^{32}P]orthophosphate to quantitate C1P levels derived from CERK for several reasons. First, the use of [^3H]palmitate or [^3H]sphingosine labels all sphingolipids, producing many phospholipids visualized on TLC. This effect makes the proper identification of C1P (even with the R_f values listed in Table 15.1) difficult. The use of these radioactive precursors also does not exclude C1P produced by other sources such as a SMase D or S1P acylase activity, which may exist in cells. Second,

mass spectrometry analysis of C1P levels requires expensive equipment and a high level of training/experience to undertake. Finally, ^{32}P labeling of cells is an inexpensive method of examining only kinase-derived C1P using a short-term pulse (approximately 2 h). In this method, we use [^{32}P]orthophosphate, in which endogenous ceramide is radiolabeled through phosphorylation by endogenous ceramide kinase. After extraction of lipids by the Bligh and Dyer (1959) method and base hydrolysis to remove glycerol lipids (e.g., phosphatidic acid), C1P is visualized with a TLC using specific standards. This method produces a sensitive and easily recognizable C1P band by TLC that can be quantified by densitometry or scintillation counting (Fig. 15.4).

5.2. Reagents

A549 cells are from ATCC, and media (RPMI and DMEM) and FBS are from Invitrogen Corporation. Penicillin/streptomycin is from Biowhittaker Inc. [^{32}P]Orthophosphate, 1 mCi (285.6 Ci/mg), is from Perkin Elmer (NEX 053) and is stored at 4° until use. Chloroform, methanol, HCl, acetone, acetic acid, and NaOH are from Fisher Scientific and are stored at room temperature until use. TLC plates are from VWR and are stored desiccated at room temperature. All glassware is from Fisher Scientific.

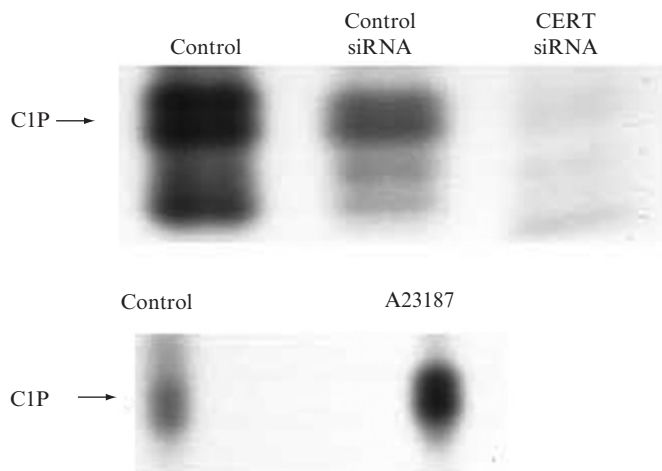


Figure 15.4 C1P levels analyzed by pulse labeling with [^{32}P]orthophosphate. (Top) Sequence-specific silencing of CERT was performed using small interfering RNA as described in Section 7. The effect of downregulation of CERT on C1P levels analyzed by pulse labeling is shown. (Bottom) A549 cells were treated with 10 μM of A23187 for 10 min. C1P was extracted by the Bligh and Dyer (1959) method followed by base hydrolysis as explained in the text and analyzed by TLC. The effect of A23187 on C1P levels is depicted.

5.3. Procedure

A549 cells are plated in a 10-cm dish at a concentration of 1×10^6 per dish in DMEM/RPMI containing 10% serum. The following day, cells are transfected with or without double-stranded RNA (for siRNA experiments, see Section 7). After a 48-h incubation, [^{32}P]orthophosphate (Perkin Elmer) is added at a concentration of 30 $\mu\text{Ci}/\text{ml}$ for 2 h. Media are replaced with fresh media containing 2% serum for 2 h. Cells are then treated with various agonists (e.g., 0.1 mM ATP for 45 min and 10 μM A23187 for 10 min) if warranted. Following treatment, the plates are placed on ice, and lipids are extracted using the Bligh and Dyer method (1959). Briefly, cells are washed with ice-cold phosphate-buffered saline (PBS) to remove excess media and scraped in 2 ml of ice-cold methanol. Cells in methanol are placed into a glass tube (13 $\times$ 100 mm). Extraction is started by adding 1 ml of chloroform followed by vigorous vortexing for 30 s. Eight hundred microliters of water is then added, followed again by vigorous vortexing. If the phases separate, a small amount of methanol (≈ 0.5 ml) is added to the tubes. In order to extract the maximum of lipids, the samples are then put at 4° overnight or at room temperature for 2 h, followed by centrifugation at 100g for 5 min to remove all of the cell debris. Of the supernatant, 3.5 ml is transferred to a new 13 $\times$ 100-mm glass tube, and 1 ml of chloroform and 1 ml of water are added to separate the phases. The sample is then vortexed and incubated at room temperature for 30 min. After centrifugation (400g for 5 min), the top layer is aspirated and the bottom organic phase is transferred into a new glass tube and dried under N_2 . Prior to drying under N_2 , a small sample (100 μl) is analyzed for total phosphate as described previously (Verdhoven and Mannaerts, 1986).

Following the Bligh and Dyer (1959) extraction, samples are subjected to base hydrolysis (Perry *et al.*, 2000). Lipids are resuspended in 1 ml of chloroform. Two hundred microliters of 2 N methanolic NaOH is then added to each sample (2 N NaOH dissolved in methanol) and incubated for 2 h at 37°. Samples are neutralized by adding 200 μl of 2 N HCl, 2 ml of methanol, and 600 μl of water. After vortexing, samples are incubated at room temperature for at least 30 min. To separate the organic and aqueous phases, 1 ml of chloroform and 1 ml of water are added followed by vigorous vortexing. After centrifugation (400g for 5 min), the lower organic phase is collected, transferred to a new 13 $\times$ 100-mm glass tube, and dried under N_2 .

For detection of C1P, samples are resuspended in chloroform:methanol (75:25, v/v) and spotted onto a 10 $\times$ 10-cm TLC plate (silica gel) (VWR). Lipids are separated using a chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1, v/v) solvent mixture. ^{32}P -labeled lipids are detected by exposing the plates onto an X-ray film or phosphoimaging screen (R_f value for long-chain C1P is 0.6) (Fig. 15.4).



6. ANALYSIS OF CERK LOCALIZATION IN CELLS

6.1. Principle

Several studies have now demonstrated the trafficking of CERK in response to cellular stress in cells. Thus, producing methodologies for examining the localization of CERK in the context of this cell biology is warranted. One method used to examine localization of CERK in cells is to add a green fluorescent protein (GFP) tag to the enzyme on the N terminus and express this protein ectopically in living cells. The GFP-labeled CERK can then be visualized by fluorescence confocal microscopy (Carre *et al.*, 2004). As with any protein overexpressed in cells, artifacts can arise as to localization simply because of the high level of the particular protein expressed under a viral promoter. Therefore, a method that can be used to examine the cellular localization of endogenous CERK is simply to use standard immunofluorescence techniques coupled to confocal microscopy. This technique can also be used to examine ectopically expressed CERK as well. In essence, the following method details the use of a specific antibody against CERK in conjunction with various antibodies to specific organelles to identify the subcellular compartments to which CERK localizes.

6.2. Reagents

A549 cells are from ATCC, and cell media (RPMI and DMEM) and FBS are from Invitrogen Corporation. Penicillin/streptomycin is from Biowhitaker Inc. Complete media are stored at 4° until use. Coverslips and slides are from Fisher Scientific. Formaldehyde and methanol are also from Fisher Scientific and are stored at room temperature. Glycine, sodium azide, and bovine serum albumin (BSA) are from Sigma-Aldrich and are stored at 4°. Triton X-100 is from Pierce Biotechnology. Glycerol is from Invitrogen Corporation.

6.3. Procedure

6.3.1. Confocal microscopy

For confocal microscopy, cells are seeded onto 22 × 22-mm coverslips in 35-mm-diameter plates in their appropriate media and incubated at 37° under standard incubator conditions overnight. Cells are washed twice with ice-cold PBS to remove excess protein and are then fixed on the coverslips. The fixative needs to be empirically tested and optimized for each antibody. One general fixation that works well for the primary antibodies designated in this section (specifically anti-CERK and anti-6XHis) is 3.7% formaldehyde. Formaldehyde is diluted from 37% liquid stock in PBS and samples

are fixed for 10 to 30 min at room temperature. After fixation, slides are washed twice in PBS containing 10 mM glycine and 0.2% sodium azide. Because formaldehyde fixation does not permeabilize the cells, another step containing 0.5% Triton X-100 in PBS for 3 min is necessary followed by two washes in PBS–glycine. Another good method that can be used to fix and permeabilize cells for the designated antibodies is the use of 100% cold methanol for 10 min at -20° . The slides are washed extensively after fixing, with PBS containing 10 mM glycine and 0.2% sodium azide. For primary staining, all the antibodies can be added individually or in conjunction with an organelle-specific antibody (e.g., GPP130) at the appropriate concentration (see later) in PBS–glycine with 1% BSA for 40 min at room temperature. The coverslips are placed in 50 μ l of diluted antibodies, placed in a drop on a Parafilm sheet. Secondary antibody staining is performed in the same manner as the primary antibody and incubated again for 40 min at room temperature. After a quick wash in PBS–glycine, coverslips are then mounted in 10 mM *n*-propagolate in 100% glycerol and are viewed using Leica confocal microscopy. Quantification of colocalization is accomplished as described using Zeiss LSM510 software (Manders *et al.*, 1993) (Fig. 15.5).

Note: Suggested controls are primary antibody only, secondary antibody only, and fixation only.

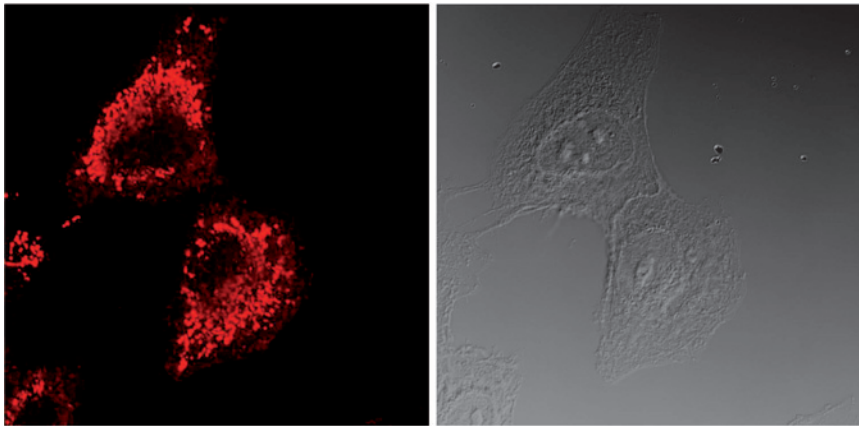


Figure 15.5 Localization of ceramide kinase in cells. For confocal microscopy, cells were seeded onto 22 $\times$ 22-mm coverslips in 35-mm-diameter plates in their appropriate media and incubated at 37 $^{\circ}$ under 5% CO₂ overnight. Cells were washed twice with PBS to remove excess protein and then fixed with 100% cold methanol for 10 min at -20° . Slides were washed extensively after fixing, with PBS containing 10 mM glycine and 0.2% sodium azide. Cells were incubated with a primary antibody, a mouse anti-CERK (1:1) for 40 min, and a secondary antibody, an antimouse antibody linked with Texas red (1:100). Coverslips were mounted in 10 mM *n*-propagolate in glycerol and viewed using Leica confocal microscopy. Data are representative of three different experiments.

6.3.2. Antibody dilutions used to localize CERK

Primary antibodies Rabbit anti-GPP130 (1:100) (Covance, PRB-144C, cis-Golgi marker), a mouse anti-CERK (1:1) (Exalpha), a rabbit anti-EEA1 (1:100) (Abcam Inc., ab2900, early endosome marker), a rabbit anti-TOM20 (1:100) (a gracious gift of Dr. B. Wattenberg, mitochondria marker), a rabbit anti-TGN46 (1:100) (Abcam Inc., *trans*-Golgi marker) a rabbit anticalreticulin (1:100) (Stressgen Bioreagents, endoplasmic reticulum marker), a rabbit anti-rab7 (1:100) (Sigma-Aldrich, early late endosome marker), a rabbit anti-cytoC (1:100) (Santa Cruz Biotechnology, mitochondria marker), and a goat anti-cPLA₂ α (1:100) (Santa Cruz Biotechnology).

Secondary antibodies FITC, or cy5-conjugated antirabbit antibody (1:200) (Jackson Immuno Research), Texas red (TR)-conjugated antimouse antibody (1:200) (Jackson Immuno Research), and/or FITC-conjugated antigoat antibody (1:200) (Jackson Immuno Research).

6.3.3. Adenovirus transfection

A549 cells (0.5×10^5) are seeded onto 22×22 -mm coverslips (Fisher) in 35-mm-diameter plates in their appropriate media (DMEM/RPMI containing 10% serum) and incubated at 37° under 5% CO₂ overnight. The following day, cells are transfected with adenovirus containing 6XHis-CERK alone or with another adenovirus (e.g., GFP-cPLA₂ α) at 150 and 40 MOI, respectively. Multiplicity of infection is calculated from the number of cells to be infected as well as the plaque-forming units of each virus. For transfection, media are removed from the A549 cells and replaced with 1 ml of fresh media containing the appropriate MOI of virus. After 48 h of incubation, cells can be treated with agonist (e.g., $10 \mu\text{M}$ A23187 for 10 min) or assayed immediately. Cells are washed twice with PBS to remove excess protein and then fixed on the coverslips with 100% cold methanol for 10 min at -20° . Slides are washed twice after fixing with PBS containing 10 mM glycine and 0.2% sodium azide. As described previously, transfected or control cells are then incubated for 40 min with the first antibody, a rabbit anti-6XHis (1:100) (Sigma-Aldrich), and after washing with PBS, the TR-conjugated antirabbit antibody (1:200) is added and incubated for 40 min at room temperature. After a quick wash in PBS-glycine, coverslips are mounted in 10 mM *n*-propagatate in glycerol and are viewed using Leica confocal microscopy.

7. ANALYSIS OF CERK FUNCTION BY siRNA-MEDIATED MANIPULATION OF CERK EXPRESSION

7.1. Principle

Several methods can be utilized to examine the function of a particular enzyme in cells. These include small molecule inhibitors, the use of gene knockout technology, and siRNA-mediated downregulation of a specific

target. In the case of CERK, no small molecule inhibitors are available, and a knockout mouse for ceramide kinase is not yet available. Thus, a rapid method used to study the function of CERK is the use of RNA interference technology (siRNA) to specifically downregulate CERK in cells. The following is a validated and published methodology for the use of siRNA to downregulate both mouse and human CERK in cells using specific siRNAs ($IC_{50} < 25 \text{ nM}$) (Lassus *et al.*, 2002). Use of this technology has allowed the examination of CERK function in specific biological processes (Pettus *et al.*, 2003).

7.2. Reagents

A549 cells are from ATCC, and cell media (RPMI and DMEM), as well as FBS and Optimen, are from Invitrogen Corporation; penicillin/streptomycin is from Biowhittaker Inc. Different sequences of siRNA and the transfection reagent Dharmafect 1 are from Dharmacon (Fisher Scientific). All these compounds are stored according to the manufacturer's instructions.

7.3. Procedure

Sequence-specific silencing of CERK is performed using sequence-specific small interfering RNA purchased from Dharmacon. One of the human CERK RNAi sequences starts 142 nucleotides from the start codon (5'-UGCCUGCUCUGUGCCUGUAdTdT-3' and 5'-UACAGGCA-CAGAGCAGGCAdTdT-3'); the second sequence we have been using is 5'-CCACUGACAUCAUCGUUACUdTdT-3' and 5'-GUAACGAU-GAUGUCAGUGGUdTdT-3'. The siRNA for mouse ceramide kinase is from Dharmacon, it contains four different sequences [sequences 1 (5'-GAACUGCGAUGGCGAAGUCdTdT-3') and 2 (5'-CAAUAGACGU-GUCCUCUGUdTdT-3') are the most efficient]. The sequence targets the accession number NM_145475. Our standard siRNA control is obtained from Dharmacon. Ten to 100 nM of these siRNA is transfected into the cells using Dharmafect 1 reagent, following the manufacturer's instructions. Briefly, A549 cells are plated at a concentration of 0.5×10^5 cells per well in six-well tissue culture plates. The following day, 4 μl of Dharmafect is mixed with 11 μl of Optimen for 10 min. This solution is then mixed with 10 μl of 10 μM of siRNA (for a final concentration of 100 nM siRNA) for 20 min to allow formation of the complexes. During this time, cells are washed once with Optimen to remove excess serum, and 800 μl of Optimen is added to each well. Two hundred microliters of the siRNA solution is then added drop wise onto the cells. After 4 h of incubation, 500 μl of 3X media (for 50 ml: 15 ml FBS, 3 ml penicillin/streptomycin, 32 ml Optimen) is added and cells are allowed to recover for 4 h. At the end of the recovery, this mixture is replaced by normal growth media containing 10%

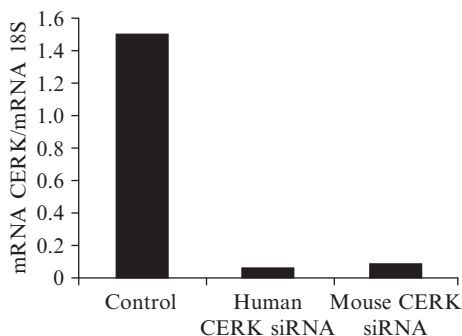


Figure 15.6 Q-PCR specific to ceramide kinase. Q-PCR was used to investigate the level of CERK expression in control cells and cells treated with human CERK siRNA and mouse CERK siRNA. Briefly, 0.5×10^5 A549 cells (for human siRNA sequence) and NIH 3T3 cells (for mouse siRNA sequence) were plated in a six-well dish and treated the following day with siRNA specific to CERK or control siRNA. Total RNA was extracted; reverse transcriptase PCR and Q-PCR were performed as described in Section 8. Arbitrary units depict the level of CERK expression normalized to the level of 18S ribosomal RNA expression.

serum. After incubation for 32 h, cells are analyzed by Western blotting using a specific antibody against CERK, by quantitative PCR (Q-PCR) (Fig. 15.6), or treated/analyzed in biological assays (e.g., PGE₂ levels).

8. ANALYSIS OF CERK mRNA LEVELS BY Q-PCR

8.1. Principle

Quantitative PCR is based on the detection of a fluorescent reporter molecule that increases as the PCR product accumulates with each cycle of amplification. This method allows either absolute or relative quantification of PCR product at the end of each amplification cycle. The fluorescent reporter used in this study was a molecule that binds double-stranded DNA, SYBR green. We have developed a Q-PCR method for ceramide kinase allowing us to study the quantity of transcript present in the cells. This is a superior method to our previously reported method of analyzing CERK mRNA levels by logarithmic reversed transcription polymerase chain reaction (RT-PCR) (Pettus *et al.*, 2003). The quantity of CERK is normalized with the quantity of 18S ribosomal RNA.

8.2. Reagents

A549 cells are from ATCC, and media (RPMI and DMEM) and FBS, as well as SSII reverse transcriptase kit and RNase H, are from Invitrogen Corporation. Penicillin/streptomycin is from Biowhittaker Inc. The

RNeasy kit is from Qiagen Inc. DYNAmo, SYBR green kit is from New England Biolabs Incorporation. 18S primers are from Ambion Incorporation. All these compounds are stored according to the manufacturers' instructions.

8.3. Procedure

8.3.1. RNA isolation

Total RNA from A549 cells treated with siRNA, as explained previously in Section 7, is isolated using the RNeasy kit (Qiagen Inc.) according to the manufacturer's protocol.

8.3.2. Reversed transcription polymerase chain reaction

One microgram of total RNA is reverse transcribed using Superscript II reverse transcriptase (Invitrogen Corporation) and oligo(dT) as the priming agent. After 50 min of incubation at 42°, reactions are stopped by heating at 70° for 15 min. Template RNA is then removed using RNase H (Invitrogen Corporation). This is the standard protocol provided by the manufacturer.

8.3.3. Q-PCR

The level of CERK mRNA is assayed using the DyNAmo kit from New England Biolabs according to the manufacturer's instructions. The master mix contained in this kit has all reagents needed for Q-PCR, including a hot start *Thermus brockianus* DNA polymerase, as well as the reaction buffer, dNTPs, salt, and so on. In conjunction with this kit, only template cDNA and specific primers are needed for this assay. For testing the human CERK, an upstream primer (5'-TCG TCT GTG TCG GCG GAG AT-3') and a downstream primer (5'-GAA ACG GTG GTT GGG TCT TGC-3') are used. For mouse CERK, the following primers are used: 5'-TAC ACA CAG ACA GCT ATG ATG-3' and 5'-ATG AAC ACA CAC AAT CTG TGG-3'. Primers are designed to amplify ceramide kinase crossing at least one exon/intron boundary to eliminate the possibility of amplifying genomic DNA. The reaction is amplified for 40 cycles using a T_m of 58° for both human and mouse CERK in an Applied Biosystems Q-PCR machine, 7900 HT sequence detection system.

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REFERENCES

- Bajjalieh, S., and Batchelor, R. (2000). Ceramide kinase. *Methods Enzymol.* **311**, 207–215.
- Bajjalieh, S. M., Martin, T. F., and Floor, E. (1989). Synaptic vesicle ceramide kinase: A calcium-stimulated lipid kinase that co-purifies with brain synaptic vesicles. *J. Biol. Chem.* **264**, 14354–14360.
- Bektas, M., Jolly, P. S., Milstien, S., and Spiegel, S. (2003). A specific ceramide kinase assay to measure cellular levels of ceramide. *Anal. Biochem.* **320**, 259–265.
- Bielawska, A., Perry, D. K., and Hannun, Y. A. (2001). Determination of ceramides and diglycerides by the diglyceride kinase assay. *Anal. Biochem.* **298**, 141–150.
- Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Carre, A., Graf, C., Stora, S., Mechtcheriakova, D., Csonga, R., Urtz, N., Billich, A., Baumruker, T., and Bornancin, F. (2004). Ceramide kinase targeting and activity determined by its N-terminal pleckstrin homology domain. *Biochim. Biophys. Res. Commun.* **26**, 1215–1219.
- Colina, C., Flores, A., Castillo, C., Garrido, M., del, R., Israel, A., DiPolo, R., and Benaim, G. (2005). Ceramide-1-phosphate induces Ca²⁺ mobilization in Jurkat T-cells by elevation of Ins(1,4,5)-P₃ and activation of a store-operated calcium channel. *Biochem. Biophys. Res. Commun.* **336**, 54–60.
- Dennis, E. A. (1973). Phospholipase A₂ activity towards phosphatidylcholine in mixed micelles: Surface dilution kinetics and the effect of thermotropic phase transitions. *Arch. Biochem. Biophys.* **158**, 485–493.
- Dragusin, M., Wehner, S., Kelly, S., Wang, E., Merrill, A. H., Jr., Kalff, J. C., and van Echten-Deckert, G. (2006). Effects of sphingosine-1-phosphate and ceramide-1-phosphate on rat intestinal smooth muscle cells: Implications for postoperative ileus. *FASEB J.* **20**, 1930–1932.
- Dressler, K. A., and Kolesnick, R. N. (1990). Ceramide 1-phosphate, a novel phospholipid in human leukemia (HL-60) cells: Synthesis via ceramide from sphingomyelin. *J. Biol. Chem.* **265**, 14917–14921.
- Garavito, R. M., and Ferguson-Miller, S. (2001). Detergents as tools in membrane biochemistry. *J. Biol. Chem.* **276**, 32403–32406.
- Gomez-Munoz, A. (2006). Ceramide 1-phosphate/ceramide, a switch between life and death. *Biochim. Biophys. Acta* **12**, 2049–2056.
- Gomez-Munoz, A., Duffy, P. A., Martin, A., O'Brien, L., Byun, H. S., Bittman, R., and Brindley, D. N. (1995). Short-chain ceramide-1-phosphates are novel stimulators of DNA synthesis and cell division: Antagonism by cell-permeable ceramides. *Mol. Pharmacol.* **47**, 833–839.
- Gomez-Munoz, A., Frago, L., Alvarez, L., and Varela-Nieto, I. (1997). Stimulation of DNA synthesis by natural ceramide 1-phosphate. *Biochem. J.* **325**, 435–440.
- Gomez-Munoz, A., Kong, J. Y., Parhar, K., Wang, S. W., Gangoi, P., Gonzalez, M., Eivemark, S., Salh, B., Duronio, V., and Steinbrecher, U. P. (2005). Ceramide-1-phosphate promotes cell survival through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway. *FEBS Lett.* **579**, 3744–3750.
- Gomez-Munoz, A., Kong, J. Y., Salh, B., and Steinbrecher, U. P. (2004). Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. *J. Lipid Res.* **45**, 99–105.
- Hinkovska-Galcheva, V. T., Boxer, L. A., Mansfield, P. J., Harsh, D., Blackwood, A., and Shayman, J. A. (1998). The formation of ceramide-1-phosphate during neutrophil phagocytosis and its role in liposome fusion. *J. Biol. Chem.* **273**, 33203–33209.
- Hogback, S., Leppimäki, P., Rudnas, B., Björklund, B., Slotte, J. P., and Tornquist, K. (2003). Ceramide 1-phosphate increases intracellular free calcium concentrations in

- thyroid FRTL-5 cells: Evidence for an effect mediated by inositol 1,4,5-trisphosphate and intracellular sphingosine 1-phosphate. *Biochem. J.* **370**, 111–119.
- Ji, L., Zhang, G., Uematsu, S., Akahori, Y., and Hirabayashi, Y. (1995). Induction of apoptotic DNA fragmentation and cell death by natural ceramide. *FEBS Lett.* **23** **358**, 211–214.
- Kim, T. J., Mitsutake, S., and Igarashi, Y. (2006). The interaction between the pleckstrin homology domain of ceramide kinase and phosphatidylinositol 4,5-bisphosphate regulates the plasma membrane targeting and ceramide 1-phosphate levels. *Biochem. Biophys. Res. Commun.* **342**, 611–617.
- Kolesnick, R. N., and Hemer, M. R. (1990). Characterization of a ceramide kinase activity from human leukemia (HL-60) cells: Separation from diacylglycerol kinase activity. *J. Biol. Chem.* **265**, 18803–18808.
- Komori, H., and Ito, M. (1995). Conversion of short-chain ceramides to short-chain ceramide GM3 in B16 melanoma cells. *FEBS Lett.* **374**, 299–302.
- Kost, T. A., Condreay, J. P., and Jarvis, D. L. (2005). Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat. Biotechnol.* **23**, 567–575.
- Lamour, N. F., Stahelin, R. V., Wijesinghe, D. S., Maceyka, M., Wang, E., Allegood, J. C., Merrill, A. H., Cho, W., and Chalfant, C. E. (2007). Ceramide kinase uses ceramide provided by ceramide transport protein: Localization to organelles of eicosanoid synthesis. *J. Lipid Res.* **48**, 1293–1304.
- Lassus, P., Rodriguez, J., and Lazebnik, Y. (2002). Confirming specificity of RNAi in mammalian cells. *Sci. STKE*. **147**, 113.
- Lichtenberg, D., Robson, R. J., and Dennis, E. A. (1983). Solubilization of phospholipids by detergents: Structural and kinetic aspects. *Biochim. Biophys. Acta* **737**, 285–304.
- Manders, E., Verbeek, F. J., and Aten, A. J. (1993). Measurement of co-localisation of objects in dual colour confocal images. *J. Microsc.* **169**, 375–382.
- Merrill, A. H., Jr., Sullards, M. C., Allegood, J. C., Kelly, S., and Wang, E. (2005). Sphingolipidomics: High-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods* **36**, 207–224.
- Mitsutake, S., and Igarashi, Y. (2005). Calmodulin is involved in the Ca^{+2} dependent activation of ceramide kinase as a calcium sensor. *J. Biol. Chem.* **280**, 40436–40441.
- Mitsutake, S., Kim, T. J., Inagaki, Y., Kato, M., Yamashita, T., and Igarashi, Y. (2004). Ceramide kinase is a mediator of calcium-dependent degranulation in mast cells. *J. Biol. Chem.* **279**, 17570–17577.
- Nakamura, H., Hirabayashi, T., Shimuzu, M., and Murayama, T. (2006). Ceramide 1-phosphate activates cytosolic phospholipase A2a directly and by PKC pathway. *Biochem. Pharmacol.* **71**, 850–857.
- Perry, D. K., Bielawska, A., and Hannun, Y. A. (2000). Quantitative determination of ceramide using diglyceride kinase. *Methods Enzymol.* **312**, 22–31.
- Pettus, B. J., Bielawska, A., Spiegel, S., Roddy, P., Hannun, Y. A., and Chalfant, C. E. (2003). Ceramide kinase mediates cytokine and calcium ionophore-induced arachidonic acid release. *J. Biol. Chem.* **278**, 38206–38213.
- Pettus, B. J., Bielawska, A., Subramanian, P., Wijesinghe, D. S., Maceyka, M., Leslie, C. C., Evans, J. H., Freiberg, J., Roddy, P., Hannun, Y. A., and Chalfant, C. E. (2004). Ceramide-1-phosphate is a direct activator of cytosolic phospholipase A₂. *J. Biol. Chem.* **279**, 11320–11326.
- Possee, R. D. (1997). Baculoviruses as expression vectors. *Curr. Opin. Biotechnol.* **8**, 569–572.
- Robson, R. J., and Dennis, E. A. (1979). Mixed micelles of sphingomyelin and phosphatidylcholine with nonionic surfactants: Effect of temperature and surfactant polydispersity. *Biochim. Biophys. Acta* **573**, 489–500.

- Robson, R. J., and Dennis, E. A. (1983). Micelles of nonionic detergents and mixed micelles with phospholipids. *Accts. Chem. Res.* **16**, 251–258.
- Sasaki, T., Hazeki, K., Hazeki, O., Ui, M., and Katada, T. (1995). Permissive effect of ceramide on growth factor-induced cell proliferation. *Biochem. J.* **311**, 829–834.
- Subramanian, P., Stahelin, R. V., Szulc, Z., Bielawska, A., Cho, W., and Chalfant, C. E. (2005). Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2 alpha and enhances the interaction of the enzyme with phosphatidylcholine. *J. Biol. Chem.* **280**, 17601–17607.
- Sugiura, M., Kono, K., Liu, H., Shimizugawa, T., Minekura, H., Spiegel, S., and Kohama, T. (2002). Ceramide kinase, a novel lipid kinase: Molecular cloning and functional characterization. *J. Biol. Chem.* **277**, 23294–23300.
- Tauzin, L., Graf, C., Sun, M., Rovina, P., Bouveyron, N., Jaritz, M., Winiski, A., Hartmann, N., Staedtler, F., Billich, A., Baumruker, T., Zhang, M., *et al.* (2006). Effects of ceramide-1-phosphate on cultured cells: Dependence on dodecane in the vehicle. *J. Lipid Res.* **48**, 66–76.
- Van Veldhoven, P. P., and Mannaerts, G. P. (1986). Coenzyme A in purified peroxisomes is not freely soluble in the matrix but firmly bound to a matrix protein. *Biochem. Biophys. Res. Commun.* **139**, 1195–1201.
- Wijesinghe, D. S., Massiello, A., Subramanian, P., Szulc, Z., Bielawska, A., and Chalfant, C. E. (2005). Substrate specificity of human ceramide kinase. *J. Lipid Res.* **46**, 2706–2716.

MEASUREMENT OF MAMMALIAN DIACYLGLYCEROL KINASE ACTIVITY *IN VITRO* AND IN CELLS

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Contents

1. Introduction	294
2. <i>In Vitro</i> Assay of DGK	295
2.1. Detergent micelles	295
2.2. Liposomes	296
2.3. Substrate	296
2.4. Enzyme	297
2.5. ATP	298
2.6. Assay conditions	298
2.7. Product isolation	299
2.8. Enzyme kinetics	299
3. Measuring DGK Activity in Subcellular Compartments	300
3.1. Nuclear isolation	300
3.2. Sucrose gradient centrifugation for isolation of nuclei	300
3.3. Membrane-depleted nuclei	301
4. Measuring DGK Activity in Cultured Cells	301
4.1. Label and harvest cells	302
4.2. Extract lipids	302
4.3. Separate lipids	302
5. Summary	303
References	303

Abstract

Diacylglycerol kinase (DGK) catalyzes the conversion of diacylglycerol to phosphatidic acid. Because both the lipid substrate and the product are important in regulation, this enzyme plays an important role in signal transduction. In mammals there are several isoforms of diacylglycerol kinase. Their activities

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can be evaluated *in vitro* as well as in intact cells. *In vitro* assays are based on measuring the incorporation of ^{32}P from ATP into diacylglycerol, resulting in the formation of labeled phosphatidic acid. Diacylglycerol with long acyl chains is insoluble in water and must be dispersed with detergent or incorporated into liposomes. Detergent-based assays are easier to perform and generally more precise; however, liposomes more closely resemble the organization of biological membranes and also allow for the testing of the modulation of enzyme activity by changes in the physical or chemical properties of the membrane. The micelle assay can also be used to measure DGK activity in cellular organelles after stimulation of intact cells to activate particular DGK isoforms. This will assess the translocation of DGK among different subcellular compartments. In this regard the plasma membrane and nucleus appear to be particularly important for the regulatory actions of these enzymes. Finally, one can also measure the DGK activity in whole cells that have been prelabeled with [^{32}P]phosphate and determine the incorporation of label into phosphatidic acid that can be extracted from the whole cell or from cellular organelles.

1. INTRODUCTION

Diacylglycerol kinase (DGK) is an enzyme that catalyzes the phosphorylation of diacylglycerol with ATP. The enzyme has been found in bacteria, plants, and higher organisms. Curiously, no form of DGK has been found in yeast or in the yeast genome. Bacteria express only one form of DGK that has no homology to the DGK in higher species. Bacterial DGK, unlike mammalian forms, is an integral membrane protein with several transmembrane domains. In order to assay the activity of this protein in membrane bilayers, procedures have been devised to reconstitute the protein into membranes in its native conformation. Novel procedures have been established to promote the correct folding of the bacterial DGK (Mi *et al.*, 2006). In addition, bacterial DGK phosphorylates ceramide as well as diacylglycerol, while the mammalian forms are specific for diacylglycerol. One exception is a mitochondrial DGK that has some activity against ceramide (Waggoner *et al.*, 2004). The isoforms of DGK in mammals have been studied extensively and are the focus of this chapter. The mammalian DGK family is composed of 10 isoforms, of which 9 have been identified and studied extensively, while the 10th one, DGK κ , has been identified recently (Imai *et al.*, 2005). Moreover, the occurrence of alternative splicing has been detected in five mammalian DGK genes (β , γ , δ , ζ , η) (Luo *et al.*, 2004) and probably occurs in other isoforms as well. Only the ϵ isoform of DGK has a hydrophobic segment, with the other isoforms being amphitropic enzymes, present in the cytosol as well as the membrane. Even DGK ϵ cannot be considered an integral membrane protein, as it can be partly extracted from membranes at high salt concentration (Walsh *et al.*, 1994). All mammalian DGK isoforms have a

conserved catalytic domain responsible for kinase activity. In addition, all DGKs have at least two cysteine-rich regions that are predicted to bind DAG so as to localize DGKs where DAG accumulates in the membrane. Most DGKs have other structural domains that likely have regulatory roles and which separate the isoforms into five families (Kanoh *et al.*, 2002; Sakane and Kanoh, 1997; Topham and Prescott, 1999; Van Blitterswijk and Houssa, 2000). The structural diversity and the tissue and cell-dependent expression patterns are unique for each isoform, suggesting that each member is regulated by a distinct mechanism and performs distinct functions in particular types of cells (Topham and Prescott, 2002).



2. IN VITRO ASSAY OF DGK

There are two alternative methods for assaying the activity of DGK, each having its own advantages and limitations. One method employs detergent micelles to solubilize all lipid components, whereas the other method uses liposomes. Micelle-based assays generally give less error because the micelles form spontaneously with a particular size and chemical composition and they exchange components rapidly. They are also convenient for determining the dependence of substrate concentration on the enzymatic rate, for example, in determining the kinetic constants for the enzyme-catalyzed reaction. However, micelle assays suffer from having different physical properties compared with lipid bilayers that make up biological membranes. Furthermore, the observed rates are frequently dependent on the nature of the detergent used. Nevertheless, depending on what questions are asked, micelle assays can be very useful. The alternative to micelles is to use liposomes. Not only do liposomes have lipids organized as a bilayer, resembling biological membranes, but they also allow comparisons among membranes enriched in particular lipid components. Lipids can also be added to micelle assays to make comicelles, but generally assays employing liposomes are more sensitive to changes in the chemical or physical properties of the lipid than micelles. However, if a particular lipid is required as a cofactor for an enzymatic process, this can usually be identified in a micelle assay.

2.1. Detergent micelles

A detergent must be chosen that does not denature the enzyme. Generally, nonionic or zwitterionic detergents are used, whereas strong anionic detergents such as sodium dodecyl sulfate should be avoided. The detergent should be at a concentration above its critical micelle concentration. The substrate diacylglycerol is mixed with any other lipid component that will

be present in the assay. This mixture, as a solution in chloroform:methanol (2:1), is made into a lipid film by solvent evaporation under a stream of nitrogen. For an arachidonoyl-specific form of DGK, it has been shown that assays with different detergents give different rates of phosphorylation, but the more rapid phosphorylation of arachidonoyl-containing forms of diacylglycerol is independent of the detergent used (Walsh *et al.*, 1994).

2.2. Liposomes

There are several procedures for making liposomes of varying size. Liposomes that are too small, such as liposomes formed by sonication, suffer from being less stable and having altered properties because of defects formed as a result of curvature strain. More appropriate liposomes are made by extrusion in which a lipid suspension is forced through a polycarbonate filter made with uniform pore sizes. Generally, filters with a 100-nm pore size are used to make large unilamellar vesicles (LUV). These filters are manufactured by the Nucleopore Company and are available with various pore sizes. However, filters with pore sizes greater than 200 nm often produce liposomes that are not strictly unilamellar, but have several concentric rings of bilayers. Several devices are available commercially to prepare LUV by extrusions. These devices tend to fall into two classes. There are small extruders that permit lipid suspensions to be forced past the nucleopore filter from one syringe to another by hand pressure. These devices are convenient and particularly useful for small volumes of 2 ml or less. Extrusion has to be performed on a lipid suspension that has undergone several freeze/thaw cycles to ensure full hydration of the lipid. The temperature for extrusion has to be above the gel-to-liquid crystalline phase transition temperature. It is less convenient to heat the hand-held extruder above room temperature. However, there are also barrel-type extruders attached to gas cylinders for pressurization as well as enclosed circulating systems. Both types are easily adoptable to higher temperatures as well as larger volumes.

2.3. Substrate

The substrates for DGK are 1,2-diacylglycerols. These lipids are available commercially. There are two concerns about their purity and stability. One is acyl migration producing 1,3-diacylglycerols. The lipids should be stored in a freezer, preferably under desiccation. The product should be tested periodically using thin-layer chromatography (TLC) to test for the presence of acyl chain migration. The other potential complication is the oxidation of 1,2-diacylglycerols having polyunsaturated acyl chains. These lipids are oxidized very rapidly by contact with oxygen in the air. This would of course also be true for phospholipids with polyunsaturated acyl chains that are included as components of the assay. There are several precautions that

can avoid lipid oxidation. Lipids and lipid films should always be stored under argon. Argon is used in preference to nitrogen because it is heavier than air and will not diffuse away as rapidly. However, the initial solvent evaporation to make a lipid film can be done with nitrogen. In addition, these lipids can be purchased as a solution in chloroform/methanol with butylated hydroxytoluene (BHT) added as an antioxidant. The BHT will evaporate during preparation of the lipid film. When the lipid film is hydrated, EDTA should be present in the buffer to chelate heavy metal contaminants that will promote lipid oxidation. Ideally, all preparations using lipids with polyunsaturated acyl chains should be done in a glove box with an inert gas atmosphere, although such a facility is not available in most laboratories.

2.4. Enzyme

Some isoforms of DGK have been purified from mammalian tissue. This procedure is laborious and costly and the amount of purified enzyme obtained is not large. Alternatively, several isoforms of DGK have been expressed in mammalian cell lines. This can be done using a plasmid with a tag that can be used for purification, such as a His tag. The epitope tag rarely interferes with the activity of the enzyme. Another advantage of overexpressing a single isoform is that the vast majority of the DGK activity in such a cell comes from the overexpressed isoform. Hence considerable information about a single isoform can be obtained even without purification of the enzyme. This is of particular advantage because of the lability of the enzyme activity during purification. However, if a crude source of enzyme is used, additional factors must be considered. It is important to determine the time course of the reaction to ensure that neither ATP nor phosphatidic acid (PA) is being converted by other enzymes. There may also be endogenous DAG with a different acyl chain composition from the substrate. If one has a high expression level of the DGK or one does a partial fractionation of a crude extract, these complications are less likely. In addition, however, consideration must be given for how the enzyme will be transferred from the lysed cell to the particle containing the DAG substrate that has been added to the assay. All isoforms of DGK have some tendency to bind to membranes since their substrate is a lipid. Hence, often a large fraction of the expressed DGK is associated with the insoluble cell pellet. This fraction of the enzyme can be solubilized readily with detergent and used in micelle-based enzyme assays. However, detergent solubilization cannot be used in liposome-based assays, as the detergent will also damage the liposomes. Hence for liposome-based assays the enzyme must first be solubilized without the use of detergents, for example, with a solution of higher salt concentration. The ease of solubilization varies according to the particular isoform under study. Some DGK isoforms partition between

soluble and membrane-bound forms, and a fraction of the enzyme can be obtained simply by centrifugation of a cell homogenate in an Eppendorf centrifuge. A larger amount of enzyme can be extracted with 1 *M* KCl. The volumes must be kept small so that addition of the salt-extracted enzyme to the assay mixture will not greatly perturb the ionic strength. However, there is one isoform of DGK that is not extracted efficiently with salt, that is, DGK ϵ , the only isoform of DGK with a stretch of hydrophobic residues predicted to be a transmembrane helix (Thirugnanam *et al.*, 2001). DGK ϵ has only been assayed using a micelle-based assay.

If one wishes to obtain the catalytic rate constant or to compare the maximal rate of enzyme catalysis among different forms of the enzyme, it is also important to determine the concentration of the enzyme. For purified enzyme preparations, simply using a method to determine protein concentration can do this. However, for impure mixtures the concentration of a particular DGK must be measured using immunological methods, such as Western blots or ELISA assays. To quantify the amount of immunoreactive material requires comparison with a standard of known concentration having similar immunoreactivity. Standards with epitope labels are available commercially and can be used for this purpose. When quantitation with antibodies is not required, comparative values of enzyme activity can be obtained by normalizing the activity values obtained to the total protein in cell lysates or subcellular fractions.

2.5. ATP

ATP is really the second substrate, in addition to DAG, used by DGK. Generally, the reaction is initiated by adding 20 μ l of 5.0 mM [γ - ^{32} P]ATP (50 μ Ci/ml) to 180 μ l of all of the other components, including DAG and DGK. The reaction rate is monitored by measuring the incorporation of ^{32} P into phosphatidic acid as a result of DGK-catalyzed phosphorylation of DAG. The concentration of ATP can also be varied, along with the specific activity of [γ - ^{32} P]ATP in order to obtain the Michaelis–Menten constants for this substrate.

2.6. Assay conditions

The final assay mixture contains 50 mM MOPS, pH 7.2, 100 mM NaCl, 5 mM MgCl $_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM ATP, the enzyme preparation, DAG, and other amphiphiles such as phospholipids and/or detergent, depending on the specific design of the assay. In addition, some DGK isoforms require cofactors, such as the addition of Ca $^{2+}$ for type I DGK isoforms, DGK α , DGK β , and DGK γ . The reaction is carried out for 10 min at 25° and is terminated by extraction of the lipid with the addition of 2 ml CHCl $_3$:CH $_3$ OH (1:1, v/v) containing 0.25 mg/ml dihexadecyl

phosphate. The length of incubation and the amount of enzyme can be varied as long as constant initial rates are measured in which the production of PA is proportional to both time and enzyme concentration. Background blanks should also be run in the absence of enzyme. In addition, with the use of enzyme preparations made from transfected cells, controls for endogenous DGK activity should be run using mock-transfected cells. Expression levels of the transfected plasmid are generally found to be sufficiently high that the enzyme activity from mock-transfected cells is only a few percent of the rate measured using transfected cells.

2.7. Product isolation

The solution of extracted lipids in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v) containing 0.25 mg/ml dihexadecyl phosphate is washed three times with 2 ml each of 1% HClO_4 and 0.1% H_3PO_4 in $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ (7:1, v/v). The volume of the final CHCl_3 phase is 0.80 ml. A 0.40-ml aliquot of the organic phase is dried at 50° for 2 h, and the incorporation of ^{32}P into phosphatidic acid is determined by Cerenkov counting. It is also possible to purify the phosphatidic acid using TLC prior to determining the radioactivity, as described briefly later in relation to cell studies. This additional step would also require an internal standard with a different radioisotope to account for recovery. Using purified enzymes or cell extracts from transfected cells with high DGK expression levels, we found that this additional step was not required and did not improve the precision of the assay. However, for measuring low, endogenous levels of DGK in a crude extract, further purification of PA is required because other lipid kinases will produce phosphorylated lipids that will be extracted into the organic solvent.

2.8. Enzyme kinetics

Analysis of the kinetics of DGK-catalyzed reactions can be complicated by the fact that two phases are present: the aqueous phase and the phase of the micelle or liposome containing the DAG substrate. This is an example of interfacial catalysis in which the meaning of the Michaelis–Menten constants V_{max} and K_{m} have to be interpreted with caution (Epand, 2005). The concentration of DAG that will affect the rate of reaction is that in the micelle or liposome rather than in the bulk of the solution. Thus K_{m} is expressed as mol% of the material in the amphiphilic aggregate rather than in the more common molar concentration units (Walsh *et al.*, 1994). Further deviations can arise if the DGK does not exchange rapidly between soluble and membrane phases. Slow exchange is less likely in micelle-based assays, but can cause significant deviations from simple Michaelis–Menten kinetics when liposomes are used. Analysis of this type of reaction is discussed in more detail elsewhere (Deems, 2000).



3. MEASURING DGK ACTIVITY IN SUBCELLULAR COMPARTMENTS

Diacylglycerol kinases regulate DAG signaling in numerous subcellular compartments, and the biological function of each DGK isotype appears to be distinct. The list of subcellular compartments where DGK activity or DGKs have been found includes, but is not limited to, the plasma membrane, the endoplasmic reticulum, the neuromuscular junction, the cytoskeleton, and even the nucleus. In fact, within the nucleus, DGKs appear to be confined to separate, distinct regions (Topham and Prescott, 2002). Evidence indicates that most DGKs are not constitutively active. Instead, they are activated by a variety of cofactors and/or post-translational modifications and then translocate to specific subcellular compartments (Topham and Prescott, 2002). Because only a fraction of each DGK isotype is likely activated by a given stimulus, measuring DAG kinase activity *in vitro* using whole cell extracts might underestimate changes in the activity of a DGK isotype. Interrogating DAG kinase activity in a given subcellular compartment requires purification of that compartment followed by the DAG kinase assays described earlier. In most cases, purification schemes are compatible with the DAG kinase assays or require simple modifications. As an example, the following protocol describes isolation of nuclei to measure nuclear DGK activity.

3.1. Nuclear isolation

A number of techniques have been described to isolate nuclei from cultured cells and intact tissue (Ledeen and Wu, 2004). Whole nuclei with intact membranes can be isolated without detergent by sucrose gradient centrifugation. Alternatively, membrane-depleted nuclei can be isolated using a much simpler technique of hypotonic lysis with a nonionic detergent. The particular protocol that one should use depends on the questions being asked, the requirement for rapid isolation, and the availability of equipment. Regardless of the technique used, it is crucial to assess the purity of the nuclear preparation. This is usually accomplished by light or electron microscopy and/or Western blotting for proteins known to be resident in specific subcellular compartments. For example, tubulin is often used as an extranuclear marker, Na/K-ATPase is a marker for the plasma membrane, and glucose-6-phosphatase is a marker for the endoplasmic reticulum.

3.2. Sucrose gradient centrifugation for isolation of nuclei

Wash cultured cells with cold phosphate-buffered saline (PBS) and then scrape into an appropriate volume (200 μ l/35-mm well diameter) of buffer A (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA).

The amount of buffer A can be scaled to the surface area of the culture dish, and the same volume of buffer should be used for resuspension throughout the protocol. Probe sonicate the cell suspension for 15 to 30 s or dounce homogenize the suspension 10 times with a loose pestle and then pellet nuclei at 650g for 5 min. Resuspend the pellet in buffer A and dounce homogenize 10 times with a tight pestle. Layer the nuclear suspension over an equal volume of 45% (w/w) sucrose in buffer A and centrifuge 30 min at 1700g. Resuspend the nuclear pellet in buffer B (10 mM Tris, pH 7.5, 10 mM NaCl, 10% sucrose, 1 mM MgCl₂) and dounce homogenize seven times with a tight pestle. Pellet nuclei at 650g for 5 min. Resuspend the final nuclear pellet in desired buffer and proceed to DGK assays described earlier. Further fractionation of the nuclei can be achieved using protocols that have been described (Ledeen and Wu, 2004).

3.3. Membrane-depleted nuclei

Wash cultured cells with cold PBS and then scrape into an appropriate volume of buffer C (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1% Triton X-100, 0.5 mM CaCl₂, 10 mM Pipes, 1.2 mM phenylmethylsulfonyl fluoride, pH 6.8). The amount of buffer should be determined empirically to yield an average cell density of 10⁷ cells/ml (usually about 400 μ l buffer C/35-mm well diameter). Incubate the suspension on ice for 7 min with gentle mixing. Pellet membrane-depleted nuclei at 650g for 5 min at 4°. Remove supernatant and wash the nuclear pellet twice with buffer D (50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5 mM CaCl₂, 10 mM Pipes, 1.2 mM phenylmethylsulfonyl fluoride, pH 6.8). Pellet at 650g for 5 min after adding wash. Resuspend pellet in desired buffer and proceed to DGK assay as described earlier. Further fractionation into different nuclear components can be achieved using protocols that have been described elsewhere (Payraastre *et al.*, 1992).

4. MEASURING DGK ACTIVITY IN CULTURED CELLS

The DGK assays described previously require disruption of cells. Determining DGK activity in cultured cells requires a radioactive labeling of the phosphatidic acid that is produced by the DGK reaction. Because DGKs are not typically constitutively active, these types of assays are best done in the presence of an agonist to activate the DGK. Like the *in vitro* DGK reactions, these assays are also compatible with cell fractionation to measure DAG kinase activity in different subcellular compartments. For example, to measure DGK activity in the nucleus, follow the label and harvest protocol given later, but harvest the cells in nuclear isolation buffer instead of methanol. Purify nuclei, and then resuspend nuclei in methanol and proceed to the lipid extraction step. Within the time frame in the

protocols described later, the assay will detect incorporation of ^{32}P into several lipids, including phosphatidic acid and several phosphatidylinositols, which are then distinguished by their migration on a thin-layer chromatography plate. The majority of PA that is measured in this assay is produced by DGKs. However, because activation of DGKs is often brief and the PA that is produced by the DGK reaction is converted into other lipids, the cells should be treated with agonist for less than 30 to 60 min. Additionally, because of the variable efficiency of lipid extractions, the assays should be repeated several times to ensure reproducibility.

4.1. Label and harvest cells

Starve cells for 1 to 2 h with appropriate medium and then add 10 to 50 μCi [^{32}P]orthophosphate per milliliter of cell culture medium for 1 h to allow uptake. Stimulate cells with chosen agonist for desired time periods and then wash twice with cold PBS. Harvest the cells by scraping into 1 ml of cold methanol. Add another 1 ml of methanol and scrape again. This amount of methanol is sufficient for well diameters up to 150 mm. Proceed immediately to extraction.

4.2. Extract lipids

Extract lipids using the method of Bligh and Dyer (1959) in glass screw cap tubes (Corning). Add 0.5 ml methanol, 1 ml 1 M NaCl, and 1.25 ml chloroform and then vortex. The solution should be a single phase. If it is not, add 200 μl methanol at a time and vortex until the solution is clear. Now add 1.25 ml of 1 M NaCl and 1.25 ml of chloroform. Vortex and then centrifuge the sample 5 min at 1500g to separate the phases. Remove the upper phase and discard. Wash the lower phase twice with 2 ml preequilibrated upper phase (PEU). Separate phases after each wash by centrifuging at 1500g for 5 min. Remove and discard the upper phase after separation. Make PEU by mixing 50 ml chloroform, 50 ml methanol, and 45 ml NaCl in a glass bottle. Shake the solution to mix and then allow it to settle into two phases. Use the upper phase for the washes. After the second PEU wash, remove the upper phase and transfer the lower phase to a 12 $\times$ 75-mm tube (Fisher), being careful not to transfer the remnant upper phase. Dry the lower phase under a stream of nitrogen and resuspend the sample in 200 to 500 μl of 9:1 chloroform:methanol (v:v) (the amount of solvent for resuspension will depend on the efficiency of ^{32}P labeling).

4.3. Separate lipids

Separate the lipids by TLC using chloroform:methanol:water:ammonium hydroxide (60:48:11:1.8) as a solvent. There will be several ^{32}P -labeled lipids and phosphatidic acid can be identified by running commercial PA on

the same TLC plate and then staining the lipids in an enclosed glass container with iodine crystals. The amount of radioactive PA can be determined by exposing the TLC plate either to a Phosphoimager screen or to standard film. If desired, the PA band can then be scraped and quantified further using liquid scintillation spectrometry.

5. SUMMARY

The assays described here for DGK are all based on the incorporation of ^{32}P into PA. This assay can be done *in vitro* with isolated DGK isoforms or with transfected cells overexpressing a particular isoform. This will allow determination of the properties and mode of action of a specific form of DGK. In addition, an aspect of DGK regulation is a consequence of the translocation of the enzyme among cellular compartments. This phenomenon can be studied before and after stimulation of a cell by subcellular fractionation and assaying for DGK activity in a manner similar to that used for *in vitro* assays. An alternative method for assessing the activation of DGK is done with short-term labeling of the cell with [^{32}P]phosphate, followed by stimulation of the cell and detection of [^{32}P]PA. This method can also be extended to determine the increase in PA synthesis in a particular subcellular organelle. Thus methods are available to study the activity of this family of important regulatory enzymes both *in vitro* and *in vivo*.

REFERENCES

- Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Deems, R. A. (2000). Interfacial enzyme kinetics at the phospholipid/water interface: Practical considerations. *Anal. Biochem.* **287**, 1–16.
- Epad, R. M. (2005). "Role of Membrane Lipids in Modulating the Activity of Membrane-Bound Enzymes," In "Structure of Biological Membranes" (P. L. Yeagle, Ed.). 2nd ed., pp. 499–509 CRC Press, Boca Raton, FL.
- Imai, S., Kai, M., Yasuda, S., Kanoh, H., and Sakane, F. (2005). Identification and characterization of a novel human type II diacylglycerol kinase, DGK kappa. *J. Biol. Chem.* **280**, 39870–39881.
- Kanoh, H., Yamada, K., and Sakane, F. (2002). Diacylglycerol kinases: Emerging downstream regulators in cell signaling systems. *J. Biochem. (Tokyo)* **131**, 629–633.
- Ledeen, R. W., and Wu, G. (2004). Nuclear lipids: Key signaling effectors in the nervous system and other tissues. *J. Lipid Res.* **45**, 1–8.
- Luo, B., Regier, D. S., Prescott, S. M., and Topham, M. K. (2004). Diacylglycerol kinases. *Cell. Signal.* **16**, 983–989.
- Mi, D., Kim, H. J., Hadziselimovic, A., and Sanders, C. R. (2006). Irreversible misfolding of diacylglycerol kinase is independent of aggregation and occurs prior to trimerization and membrane association. *Biochemistry* **45**, 10072–10084.

- Payraastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A. J., and Van Bergen en Henegouwen, P. M. (1992). A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. *J. Biol. Chem.* **267**, 5078–5084.
- Sakane, F., and Kanoh, H. (1997). Molecules in focus: Diacylglycerol kinase. *Int. J. Biochem. Cell Biol.* **29**, 1139–1143.
- Thirugnanam, S., Topham, M. K., and Epand, R. M. (2001). Physiological implications of the contrasting modulation of the activities of the ϵ and ζ isoforms of diacylglycerol kinase. *Biochemistry* **40**, 10607–10613.
- Topham, M. K., and Prescott, S. M. (1999). Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* **274**, 11447–11450.
- Topham, M. K., and Prescott, S. M. (2002). Diacylglycerol kinases: Regulation and signaling roles. *Thromb. Haemost.* **88**, 912–918.
- Van Blitterswijk, W. J., and Houssa, B. (2000). Properties and functions of diacylglycerol kinases. *Cell. Signal.* **12**, 595–605.
- Waggoner, D. W., Johnson, L. B., Mann, P. C., Morris, V., Guastella, J., and Bajjalieh, S. M. (2004). MuLK, a eukaryotic multi-substrate lipid kinase. *J. Biol. Chem.* **279**, 38228–38235.
- Walsh, J. P., Suen, R., Lemaitre, R. N., and Glomset, J. A. (1994). Arachidonoyl-diacylglycerol kinase from bovine testis: Purification and properties. *J. Biol. Chem.* **269**, 21155–21164.

LIPID PHOSPHATE PHOSPHATASES FROM *SACCHAROMYCES CEREVISIAE*

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Contents

1. Introduction	306
2. Preparation of Radiolabeled Substrates	307
3. Assay Methods	307
4. Growth of Yeast	308
5. Purification Procedure	308
5.1. Preparation of cell extract	308
5.2. Preparation of microsomal membranes	308
5.3. Preparation of Triton X-100 extract	308
5.4. DE53 (DEAE-cellulose) chromatography	309
5.5. Affi-Gel blue chromatography	309
5.6. Hydroxylapatite chromatography	309
5.7. Mono Q I chromatography	309
5.8. Mono Q II chromatography	310
5.9. Enzyme purity	310
5.10. Identification of <i>DPP1</i> and <i>LPP1</i> genes	310
6. Properties of <i>DPP1</i> - and <i>LPP1</i> -Encoded Lipid Phosphate Phosphatases	311
Acknowledgment	313
References	313

Abstract

DPP1-encoded and *LPP1*-encoded lipid phosphate phosphatases are integral membrane proteins in the yeast *Saccharomyces cerevisiae*. They catalyze the Mg^{2+} -independent dephosphorylation of bioactive lipid phosphate molecules such as diacylglycerol pyrophosphate and phosphatidate. These enzymes possess a three-domain lipid phosphatase motif that is localized to the hydrophilic surface of the membrane. The lipid phosphate phosphatase activities of *DPP1*-encoded and *LPP1*-encoded enzymes are measured by following the release of

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water-soluble radioactive inorganic phosphate from chloroform-soluble radioactive lipid phosphate substrate following a chloroform/methanol/water phase partition. The *DPP1*-encoded enzyme, commonly referred to as diacylglycerol pyrophosphate phosphatase, is purified from wild-type *S. cerevisiae* membranes by detergent solubilization with Triton X-100 followed by chromatography with DEAE-cellulose (DE53), Affi-Gel blue, hydroxylapatite, and Mono Q. The purification scheme yields an essentially homogeneous enzyme preparation that is stable for several years upon storage at -80° . The properties of the *DPP1*-encoded and *LPP1*-encoded lipid phosphate phosphatase enzymes are summarized.

1. INTRODUCTION

Lipid phosphate phosphatase in the yeast *Saccharomyces cerevisiae* catalyzes the Mg^{2+} -independent dephosphorylation of bioactive lipid phosphate molecules such as diacylglycerol pyrophosphate (DGPP) and phosphatidate (PA) (Furneisen and Carman, 2000; Toke *et al.*, 1998, 1999a; Wu *et al.*, 1996) (reactions 1 and 2):

1. Diacylglycerol pyrophosphate $\rightarrow$ Phosphatidate + P_i
2. Phosphatidate $\rightarrow$ Diacylglycerol + P_i

Essentially all of the lipid phosphate phosphatase activities in *S. cerevisiae* are encoded by the *DPP1* (Toke *et al.*, 1998) and *LPP1* (Toke *et al.*, 1999a) genes, with the former gene being the major contributor of this enzyme (Toke *et al.*, 1999a). The *DPP1*- and *LPP1*-encoded enzymes are integral membrane proteins with six transmembrane-spanning regions; they are localized to the vacuole (Han *et al.*, 2001, 2004) and Golgi (Huh *et al.*, 2003) compartments of the cell, respectively. The lipid phosphate phosphatase enzymes possess a three-domain lipid phosphatase motif that is localized to the hydrophilic surface of the membrane (Han *et al.*, 2004; Stukey and Carman, 1997; Toke *et al.*, 1998, 1999a,b). This catalytic motif consists of the consensus sequences KxxxxxxRP (domain 1)—PSGH (domain 2)—SRxxxxxHxxxD (domain 3). The conserved arginine residue in domain 1 and the conserved histidine residues in domains 2 and 3 are essential for catalytic activity (Stukey and Carman, 1997; Toke *et al.*, 1999b; Zhang *et al.*, 2000).

The substrate specificity of the lipid phosphate phosphatases suggests that these enzymes are involved in signaling events rather than in phospholipid synthesis (Brindley *et al.*, 2002; Sciorra and Morris, 2002). The lipid phosphate phosphatase enzymes may play a role in signal transduction by terminating signaling events of lipid phosphates. Because the products of the lipid phosphate phosphatases are also bioactive lipid molecules, they can

initiate signal transduction by producing signaling molecules. Thus, the regulation of lipid phosphate phosphatase activities is likely to modulate the balance of the signaling molecules that are substrates and products in their reactions. The *DPP1*- and *LPP1*-encoded PA phosphatase activities are not responsible for *de novo* lipid synthesis; the *PAH1*-encoded Mg^{2+} -dependent PA phosphatase is the responsible enzyme for this function in *S. cerevisiae* (Carman and Han, 2006; Han *et al.*, 2006).

The *DPP1*-encoded enzyme (Toke *et al.*, 1998), commonly referred to as DGPP phosphatase, has been purified to homogeneity by standard protein purification procedures (Wu *et al.*, 1996). We describe here the purification of the enzyme.

2. PREPARATION OF RADIOLABELED SUBSTRATES

$[\beta\text{-}^{32}\text{P}]\text{DGPP}$ is synthesized enzymatically from PA and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using *Catharanthus roseus* PA kinase as described by Wu *et al.* (1996). $^{32}\text{P}]\text{PA}$ is synthesized enzymatically from diacylglycerol using *Escherichia coli* diacylglycerol kinase (Walsh and Bell, 1986) as described by Lin and Carman (1989). The labeled substrates are purified by thin-layer chromatography on potassium oxalate-treated silica gel 60 plates using the solvent system chloroform/acetone/methanol/glacial acetic acid/water (50:15:13:12:4) (Wissing and Behrbohm, 1993).

3. ASSAY METHODS

Lipid phosphate phosphatase activities are measured for 20 min at 30° by following the release of water-soluble $^{32}\text{P}]\text{P}_i$ from chloroform-soluble $[\beta\text{-}^{32}\text{P}]\text{DGPP}$ (10,000 cpm/nmol) or $^{32}\text{P}]\text{PA}$ (10,000 cpm/nmol) (Wu *et al.*, 1996). The reaction mixture for DGPP phosphatase activity contains 50 mM citrate buffer (pH 5.0), 0.1 mM DGPP, 2 mM Triton X-100, 10 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. The reaction mixture for PA phosphatase activity contains 50 mM Tris-maleate buffer (pH 6.5), 0.1 mM PA, 1 mM Triton X-100, 2 mM Na_2EDTA , 10 mM 2-mercaptoethanol, and enzyme in a total volume of 0.1 ml. The reactions are terminated by the addition of 0.5 ml of 0.1 N HCl in methanol. Chloroform (1 ml) and 1 M MgCl_2 (1 ml) are added, the system is mixed, and the phases are separated by 2 min of centrifugation at 100g. Ecoscint H (4 ml) is added to a 0.5-ml sample of the aqueous phase, and radioactivity is determined by scintillation counting. All enzyme assays are conducted in triplicate. A unit of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product/min.

Specific activity is defined as units per milligram of protein. Protein concentration is determined by the method of Bradford (1976) using bovine serum albumin as the standard.

4. GROWTH OF YEAST

Strain *MATa ade5* (Culbertson and Henry, 1975), which shows normal regulation of phospholipid metabolism (Greenberg *et al.*, 1982; Klig *et al.*, 1985, 1988; Poole *et al.*, 1986), is used for purification of the *DPP1*-encoded DGPP phosphatase. Cultures are maintained on YEPD medium (1% yeast extract, 2% peptone, 2% glucose) plates containing 2% Bacto-agar. For enzyme purification, cells are grown in YEPD medium at 30° to late exponential phase, harvested by centrifugation, and stored at -80° as described previously (Fischl and Carman, 1983).

5. PURIFICATION PROCEDURE

All steps are performed at 5°.

5.1. Preparation of cell extract

The cell extract is prepared from 200 g (wet weight) of cells by disruption with glass beads with a Bead-Beater (Biospec Products) in buffer A (50 mM Tris-maleate [pH 7.0], 1 mM Na₂EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride) (Fischl and Carman, 1983). Unbroken cells and glass beads are removed by centrifugation at 1500g for 5 min.

5.2. Preparation of microsomal membranes

Microsomal membranes are isolated from the cell extract by differential centrifugation (Fischl and Carman, 1983). They are washed, resuspended in buffer B (50 mM Tris-maleate [pH 7.0], 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 20% glycerol, and 0.5 mM phenylmethanesulfonyl fluoride), and frozen at -80° until used for purification.

5.3. Preparation of Triton X-100 extract

Microsomal membranes are suspended in buffer B containing 1% Triton X-100 at a final protein concentration of 10 mg/ml. The suspension is incubated for 1 h on a rotary shaker at 150 rpm. After the incubation, the suspension is centrifuged at 100,000g for 1.5 h to obtain the Triton X-100 extract (supernatant).

5.4. DE53 (DEAE-cellulose) chromatography

A DE53 column (2.5×20.5 cm) is equilibrated with buffer C (50 mM Tris-maleate [pH 7.0], 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 20% glycerol, and 1% Triton X-100). The Triton X-100 extract is applied to the column at a flow rate of 60 ml/h. The column is washed with 1 column volume of buffer C followed by elution of DGPP phosphatase activity in 6-ml fractions with 9 column volumes of a linear NaCl gradient (0–0.25 M) in buffer C. The peak of DGPP phosphatase activity elutes from the column at the beginning of the NaCl gradient. The most active fractions containing activity are pooled and used for the next step in the purification scheme.

5.5. Affi-Gel blue chromatography

An Affi-Gel blue column (2.0×16 cm) is equilibrated with buffer C. The DE53-purified enzyme is applied to the column at a flow rate of 30 ml/h. The column is washed with 1 column volume of buffer C followed by 2 column volumes of buffer C containing 0.3 M NaCl. DGPP phosphatase activity is eluted from the column in 3.5-ml fractions with 10 column volumes of a linear NaCl gradient (0.3–0.9 M) in buffer C. The peak of DGPP phosphatase activity elutes from the column at a NaCl concentration from 0.3 to 0.4 M. The most active fractions are pooled, and the enzyme preparation is desalted by dialysis against buffer D (10 mM potassium phosphate [pH 7.0], 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 20% glycerol, and 1% Triton X-100).

5.6. Hydroxylapatite chromatography

A hydroxylapatite column (1.5×8.5 cm) is equilibrated with buffer D. The desalted Affi-Gel blue-purified enzyme is applied to the column at a flow rate of 20 ml/h. The column is washed with 1 column volume of buffer D, and DGPP phosphatase activity is eluted from the column in 2-ml fractions with 20 column volumes of a linear potassium phosphate gradient (10–150 mM) in buffer D. Two peaks of DGPP phosphatase activity elute from the column. The first peak (peak I) of activity elutes from the column at the beginning of the gradient, and the second peak (peak II) of activity elutes at a phosphate concentration of about 24 mM. The most active fractions from each peak are pooled and dialyzed against buffer C.

5.7. Mono Q I chromatography

A Mono Q column (0.5×5 cm) is equilibrated with buffer C. The hydroxylapatite-purified enzyme from peak I is applied to the column at a flow rate of 24 ml/h. The column is washed with 2 column volumes of

buffer C. DGPP phosphatase activity is eluted from the column in 1-ml fractions with 40 column volumes of a linear NaCl gradient (0–0.3 M) in buffer C. The peak of DGPP phosphatase activity elutes from the column at a NaCl concentration of about 0.12 M. Fractions containing activity are pooled and stored at -80° . The purified enzyme is stable for at least 5 years.

5.8. Mono Q II chromatography

A second Mono Q column (0.5×5 cm) is equilibrated with buffer C. The hydroxylapatite-purified enzyme from peak II is applied to the column at a flow rate of 24 ml/h. The column is washed with 2 column volumes of buffer C. DGPP phosphatase activity is eluted from the column in 1-ml fractions with 40 column volumes of a linear NaCl gradient (0–0.3 M) in buffer C. The peak of DGPP phosphatase activity elutes from the column at the beginning of the NaCl gradient. Fractions containing activity are pooled and stored at -80° . The purified enzyme is completely stable for at least 5 years.

5.9. Enzyme purity

Mono Q I chromatography of the hydroxylapatite peak I enzyme results in isolation of an apparent homogeneous protein preparation as shown by SDS-PAGE. The minimum subunit molecular mass of the purified protein is 34 kDa. Mono Q II chromatography of the hydroxylapatite peak II DGPP phosphatase results in isolation of a protein preparation that contains a major protein doublet migrating at a molecular mass of 34 kDa. This DGPP phosphatase preparation also contains some minor protein contaminants. Overall, the hydroxylapatite peak I DGPP phosphatase is purified 33,333-fold over the cell extract with an activity yield of 0.73% to a final specific activity of 150 $\mu\text{mol}/\text{min}/\text{mg}$ (Table 17.1). The hydroxylapatite peak II DGPP phosphatase is purified 24,666-fold over the cell extract to a final specific activity of 111 $\mu\text{mol}/\text{min}/\text{mg}$ with an activity yield of 4% (Table 17.1).

5.10. Identification of *DPP1* and *LPP1* genes

Protein sequencing analysis has shown that DGPP phosphatase enzymes purified from Mono Q I and from Mono Q II are the same. The differences in chromatographic behavior of the two DGPP phosphatase preparations might be because of a post-translational modification(s). However, studies to address this hypothesis have not been pursued. The N-terminal amino acid sequence (MNRVSIKTPFNIGAKWRLE) and two internal amino acid sequences (QPVEGLPLDTLFTAK and FPPIDDPLPFKPLMD) derived from the pure DGPP phosphatase enzyme have been used to identify the *DPP1* gene in the *Saccharomyces* Genome Database (Toke *et al.*, 1998).

Table 17.1 Purification of *DPP1*-encoded DGPP phosphatase from *Saccharomyces cerevisiae*^a

Purification step	Total units (μmol/min)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (–fold)
1. Cell extract	49.4	10,957.6	0.0045	100	1
2. Microsomes	27.4	2312.8	0.0118	55.5	2.6
3. Triton X-100	16.2	803.2	0.0201	32.8	4.5
4. DE53	5.6	15.41	0.363	11.3	80.6
5. Affi-Gel blue	5.5	2.62	2.10	11.1	466.6
6a. Hydroxylapatite	0.51	0.143	3.49	1.0	775.5
6b. Hydroxylapatite	2.36	0.185	12.7	4.7	2822
7. Mono Q I	0.36	0.0024	150	0.73	33,333
8. Mono Q II	2.0	0.018	111	4.0	24,666

^a Data from Wu *et al.* (1996).

The *LPP1* gene has been identified in the yeast database because its deduced protein sequence shows homology to the *DPP1*-encoded protein (Toke *et al.*, 1999a). The expression of *DPP1* and *LPP1* genes on multicopy plasmids in *S. cerevisiae* results in enrichment of their encoded enzymes of 10- and 13-fold, respectively (Toke *et al.*, 1998, 1999a). The expression of *DPP1* and *LPP1* in baculovirus-infected Sf9 insect cells provides 500- and 200-fold enrichments of the *DPP1*- and *LPP1*-encoded lipid phosphate phosphatase enzymes, respectively, when compared with that found in wild-type yeast cell extracts (Toke *et al.*, 1998, 1999a). Accordingly, the overexpression of *DPP1*- and *LPP1*-encoded lipid phosphate phosphatase enzymes either in yeast or in insect cells should facilitate the purification of these enzymes.

6. PROPERTIES OF *DPP1*- AND *LPP1*-ENCODED LIPID PHOSPHATE PHOSPHATASES

Properties of the *DPP1*-encoded lipid phosphate phosphatase have been examined using purified enzyme (Toke *et al.*, 1998, 1999a; Wu *et al.*, 1996), whereas properties of the *LPP1*-encoded lipid phosphate phosphatase have been examined using membranes isolated from Sf9 insect cells (Furneisen and Carman, 2000). These enzymes do not have a divalent

Table 17.2 Kinetic constants for *DPP1*- and *LPP1*-encoded lipid phosphate phosphatases

<i>DPP1</i> -encoded lipid phosphatase ^a					<i>LPP1</i> -encoded lipid phosphatase ^b			
Substrate or inhibitor	$V_{\max}$ (units ^c /mg)	K_m (mol%)	$V_{\max}/K_m$ (units/ mg/mol%)	K_i (mol%)	$V_{\max}$ (units ^c /mg)	K_m (mol%)	$V_{\max}/K_m$ (units/mg/ mol%)	K_i (mol%)
DGPP	172	0.55	313	0.35 ^d	0.244	0.07	3.5	0.12 ^d
PA	70	2.2	32	NI ^e	0.210	0.05	4.2	0.12 ^f

^a Data for the *DPP1*-encoded enzyme were taken from Wu *et al.* (1996).

^b Data for the *LPP1*-encoded enzyme were taken from Furneisen and Carman (2000). Because the *LPP1*-encoded enzyme is not pure, the specificity constants reported for the enzyme cannot be compared with those reported for the *DPP1*-encoded enzyme.

^c Unit of activity defined as $\mu\text{mol}/\text{min}$.

^d Inhibitor constant with respect to PA as a substrate.

^e NI, not inhibitory.

^f Inhibitor constant with respect to DGPP as a substrate.

cation requirement for activity (Furneisen and Carman, 2000; Wu *et al.*, 1996); this is consistent with the enzymes being members of the superfamily of enzymes possessing the lipid phosphatase motif (Han *et al.*, 2004; Stukey and Carman, 1997; Toke *et al.*, 1999b). The kinetic properties of the lipid phosphate phosphatase enzymes utilizing DGPP and PA are summarized in Table 17.2. The specificity constant ($V_{\max}/K_m$) of the *DPP1*-encoded enzyme for DGPP is 10-fold greater than that of PA (Wu *et al.*, 1996). DGPP is a very potent inhibitor of the PA phosphatase activity of the *DPP1*-encoded enzyme, whereas PA does not inhibit the DGPP phosphatase activity of the enzyme (Wu *et al.*, 1996). The specificity constant of the *LPP1*-encoded enzyme for PA is slightly higher (1.2-fold) than that for DGPP (Furneisen and Carman, 2000). DGPP and PA inhibit PA phosphatase and DGPP phosphatase activities, respectively, of the *LPP1*-encoded enzyme with equal potencies (Furneisen and Carman, 2000). The affinity (reflected in K_m value) of the *LPP1*-encoded enzyme for PA and DGPP as substrates is greater than the affinity of the *DPP1*-encoded enzyme for these substrates. Purified *DPP1*-encoded lipid phosphate phosphatase also utilizes lysoPA (Dillon *et al.*, 1996), phosphatidylglycerophosphate (Dillon *et al.*, 1996), sphingoid base phosphates (Dillon *et al.*, 1997), and isoprenoid phosphates (Faulkner *et al.*, 1999) as substrates. The *LPP1*-encoded lipid phosphate phosphatase will also utilize lysoPA as a substrate (Furneisen and Carman, 2000). Although these enzymes utilize a variety of lipid phosphate substrates, only DGPP and PA have been shown to be substrates *in vivo* (Toke *et al.*, 1999a).

DPP1- and *LPP1*-encoded enzymes differ with respect to their sensitivity to thioreactive compounds. In particular, the *LPP1*-encoded enzyme is potently inhibited by *N*-ethylmaleimide (Furneisen and Carman, 2000), whereas the *DPP1*-encoded enzyme is insensitive to this compound (Wu *et al.*, 1996). This difference in *N*-ethylmaleimide sensitivity may be based on the fact that the *LPP1*-encoded enzyme has 10 cysteine residues, whereas the *DPP1*-encoded enzyme contains only 3.

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REFERENCES

- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

- Brindley, D. N., English, D., Pilquill, C., Buri, K., and Ling, Z. C. (2002). Lipid phosphate phosphatases regulate signal transduction through glycerolipids and sphingolipids. *Biochim. Biophys. Acta* **1582**, 33–44.
- Carman, G. M., and Han, G. S. (2006). Roles of phosphatidate phosphatase enzymes in lipid metabolism. *Trends Biochem. Sci.* **31**, 694–699.
- Culbertson, M. R., and Henry, S. A. (1975). Inositol requiring mutants of *Saccharomyces cerevisiae*. *Genetics* **80**, 23–40.
- Dillon, D. A., Chen, X., Zeimet, G. M., Wu, W.-I., Waggoner, D. W., Dewald, J., Brindley, D. N., and Carman, G. M. (1997). Mammalian Mg^{2+} -independent phosphatidate phosphatase (PAP2) displays diacylglycerol pyrophosphate phosphatase activity. *J. Biol. Chem.* **272**, 10361–10366.
- Dillon, D. A., Wu, W.-I., Riedel, B., Wissing, J. B., Dowhan, W., and Carman, G. M. (1996). The *Escherichia coli* *pgpB* gene encodes for a diacylglycerol pyrophosphate phosphatase activity. *J. Biol. Chem.* **271**, 30548–30553.
- Faulkner, A. J., Chen, X., Rush, J., Horazdovsky, B., Waechter, C. J., Carman, G. M., and Sternweis, P. C. (1999). The *LPP1* and *DPP1* gene products account for most of the isoprenoid phosphatase activities in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274**, 14831–14837.
- Fischl, A. S., and Carman, G. M. (1983). Phosphatidylinositol biosynthesis in *Saccharomyces cerevisiae*: Purification and properties of microsome-associated phosphatidylinositol synthase. *J. Bacteriol.* **154**, 304–311.
- Furneisen, J. M., and Carman, G. M. (2000). Enzymological properties of the *LPP1*-encoded lipid phosphatase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1484**, 71–82.
- Greenberg, M., Goldwasser, P., and Henry, S. A. (1982). Characterization of a yeast regulatory mutant constitutive for inositol-1-phosphate synthase. *Mol. Gen. Genet.* **186**, 157–163.
- Han, G.-S., Johnston, C. N., and Carman, G. M. (2004). Vacuole membrane topography of the *DPP1*-encoded diacylglycerol pyrophosphate phosphatase catalytic site from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**, 5338–5345.
- Han, G.-S., Johnston, C. N., Chen, X., Athenstaedt, K., Daum, G., and Carman, G. M. (2001). Regulation of the *Saccharomyces cerevisiae* *DPP1*-encoded diacylglycerol pyrophosphate phosphatase by zinc. *J. Biol. Chem.* **276**, 10126–10133.
- Han, G.-S., Wu, W.-I., and Carman, G. M. (2006). The *Saccharomyces cerevisiae* lipin homolog is a Mg^{2+} -dependent phosphatidate phosphatase enzyme. *J. Biol. Chem.* **281**, 9210–9218.
- Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* **425**, 686–691.
- Klig, L. S., Homann, M. J., Carman, G. M., and Henry, S. A. (1985). Coordinate regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*: Pleiotropically constitutive *opi1* mutant. *J. Bacteriol.* **162**, 1135–1141.
- Klig, L. S., Homann, M. J., Kohlwein, S. D., Kelley, M. J., Henry, S. A., and Carman, G. M. (1988). *Saccharomyces cerevisiae* mutant with a partial defect in the synthesis of CDP-diacylglycerol and altered regulation of phospholipid biosynthesis. *J. Bacteriol.* **170**, 1878–1886.
- Lin, Y.-P., and Carman, G. M. (1989). Purification and characterization of phosphatidate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**, 8641–8645.
- Poole, M. A., Homann, M. J., Bae-Lee, M., and Carman, G. M. (1986). Regulation of phosphatidylserine synthase from *Saccharomyces cerevisiae* by phospholipid precursors. *J. Bacteriol.* **168**, 668–672.

- Sciorra, V. A., and Morris, A. J. (2002). Roles for lipid phosphate phosphatases in regulation of cellular signaling. *Biochim. Biophys. Acta* **1582**, 45–51.
- Stukey, J., and Carman, G. M. (1997). Identification of a novel phosphatase sequence motif. *Protein Sci.* **6**, 469–472.
- Toke, D. A., Bennett, W. L., Dillon, D. A., Chen, X., Oshiro, J., Ostrander, D. B., Wu, W.-I., Cremesti, A., Voelker, D. R., Fischl, A. S., and Carman, G. M. (1998). Isolation and characterization of the *Saccharomyces cerevisiae* *DPP1* gene encoding for diacylglycerol pyrophosphate phosphatase. *J. Biol. Chem.* **273**, 3278–3284.
- Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W. I., Voelker, D. R., and Carman, G. M. (1999a). Isolation and characterization of the *Saccharomyces cerevisiae* *LPP1* gene encoding a Mg^{2+} -independent phosphatidate phosphatase. *J. Biol. Chem.* **273**, 14331–14338.
- Toke, D. A., McClintick, M. L., and Carman, G. M. (1999b). Mutagenesis of the phosphatase sequence motif in diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *Biochemistry* **38**, 14606–14613.
- Walsh, J. P., and Bell, R. M. (1986). sn-1,2-diacylglycerol kinase of *Escherichia coli*: Mixed micellar analysis of the phospholipid cofactor requirement and divalent cation dependence. *J. Biol. Chem.* **261**, 6239–6247.
- Wissing, J. B., and Behrbohm, H. (1993). Diacylglycerol pyrophosphate, a novel phospholipid compound. *FEBS Lett.* **315**, 95–99.
- Wu, W.-I., Liu, Y., Riedel, B., Wissing, J. B., Fischl, A. S., and Carman, G. M. (1996). Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 1868–1876.
- Zhang, Q. X., Pilquil, C. S., Dewald, J., Berthiaume, L. G., and Brindley, D. N. (2000). Identification of structurally important domains of lipid phosphate phosphatase-1: Implications for its sites of action. *Biochem. J.* **345**(Pt. 2), 181–184.

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Author Index

A

Abraham, A., 18, 19, 20, 23
 Abraham, R. T., 136
 Ackerman, S. J., 18, 19, 20, 23
 Ackermann, K. E., 214
 Aebersold, R. H., 4, 133
 Agranoff, B. W., 75, 197
 Ahmadi, K., 118, 132
 Ai, Y., 244
 Akahori, Y., 291
 Albanesi, J. P., 200
 Alessi, D. R., 126, 134
 Alexander, C., 236
 Alex Brown, H., 2, 49
 Ali, M. H., 24
 Ali, S. M., 134
 Allegood, J. C., 236, 277
 Alonso, A., 84
 Altman, A., 142
 Altman, Y., 142
 Altshuller, Y. M., 50, 51, 65, 67, 156
 Alvarez, L., 268, 279, 280
 Alvarez, S., 244
 Alvarez-Vasquez, F., 236
 Amakasu, Y., 4
 Amano, K., 4, 7
 Amin, D., 4
 Ananthanarayanan, B., 18, 19, 20, 23
 Anderson, J. A., 142
 Anderson, K., 134
 Anderson, K. E., 118, 120, 121, 123, 127
 Anderson, R. A., 156, 157, 158, 159, 161
 Andjelic, S., 135
 Anelli, V., 233
 Antolini, M., 213
 Anzelon, A. N., 149, 150
 Aoki, J., 1, 2, 3, 4, 7, 90, 91, 92, 93, 94, 95, 98
 Aoki, S., 94
 Appel, B., 172
 Arai, H., 1, 2, 3, 4, 7, 91, 92, 93, 94, 95, 98
 Arango, H., 94, 214
 Arestad, A., 92
 Aridor, M., 4
 Arioka, M., 156, 157
 Arm, J. P., 16
 Armbruster, B. N., 171, 172
 Arnhold, J., 214
 Arnold, K., 214

Asano, O., 107
 Asano, T., 134, 156, 157
 Asazuma, N., 258
 Astoul, E., 133
 Aten, A. J., 285
 Athenstaedt, K., 306
 Atsumi, G., 4
 Auchus, R. J., 214, 224
 Auger, K. R., 120, 156
 Austen, K. F., 16
 Azevedo, C., 172

B

Backer, J. M., 136
 Bader, M. F., 65
 Bae, C. D., 52, 56
 Bae-Lee, M., 308
 Baer, A., 92
 Bagnati, R., 187
 Bai, W., 172, 173, 182
 Bairstow, S. F., 157, 159
 Bajjalieh, S., 266, 267, 268, 269, 274, 294
 Baker, D. L., 93
 Baker, P. R., 199
 Balboa, M. A., 16
 Balestieri, B., 16
 Ball, R., 37
 Balla, A., 136
 Balla, T., 136
 Ballantyne, C. M., 4
 Ballou, C. E., 210, 212
 Balomenos, D., 133
 Balsinde, J., 16
 Bandhuvula, P., 245, 248
 Bandle, R. W., 92, 93
 Bandoh, K., 2, 3, 4, 7
 Bannister, A., 123
 Banoub, J., 214
 Banting, G., 119
 Bao, S., 16
 Barak, L. S., 258
 Barber, D. F., 133
 Barbour, S. E., 260
 Bardi, G., 134
 Barker, C. J., 120, 203, 207, 209, 210, 211, 220, 221
 Barlow, K. D., 239, 250, 260
 Bar-Sagi, D., 156

- Bartolome, A., 133
 Baruch, A., 18
 Barylko, B., 200
 Baskin, D. G., 149
 Bass, D. A., 106
 Bastidas, R. J., 172, 173, 174, 179, 182
 Batchelor, R., 267, 274
 Bateman, R. H., 214
 Batty, I. H., 123
 Bauldry, S. A., 106
 Baumruker, T., 238, 239, 268, 279, 284, 290
 Beaven, M. A., 203, 244, 250, 251, 259, 263
 Becker, G. W., 18
 Becker, K. P., 238, 246, 248, 249, 250, 251
 Behrbohm, H., 307
 Behrens, T. W., 134
 Beijnen, J. H., 259
 Bektas, M., 244, 258, 263, 276
 Belinson, J., 214
 Bell, R. M., 251, 307
 Bell, S. E., 134
 Bembenek, M. E., 177
 Benaim, G., 280
 Benard, S., 214
 Ben-Dor, S., 236
 Bennett, M., 172
 Bennett, W. L., 306, 307, 310, 311, 313
 Bensadoun, A., 4
 Benschop, R. J., 142
 Benveniste, J., 106
 Berdyshev, E., 94
 Berger, R. A., 214
 Bergstrom, J., 245, 246, 247, 249, 251
 Berridge, M. J., 156, 172, 188
 Berrie, C. P., 187, 189, 196, 204, 206, 207, 208, 215, 216, 219, 222, 224, 226
 Berry, G. T., 214
 Berthiaume, L. G., 306
 Bex, F., 174
 Bezzine, S., 18
 Bi, K., 142
 Bidault, J., 106
 Bidlack, J. M., 31
 Bielawska, A., 235, 268, 274, 276, 278, 279, 283, 287, 288
 Bielawski, J., 233, 235, 236, 248
 Bigman, C. S., 98, 99, 100
 Bilancio, A., 133, 134, 135, 136, 137
 Billich, A., 238, 239, 268, 279, 284, 290
 Bingham, C. O. III, 16
 Bird, I. M., 197
 Bismuth, G., 150
 Bittman, R., 93, 268, 278, 279
 Bjorklund, B., 280
 Blackwood, A., 268
 Blanco, R. W., 94, 214
 Blank, M. L., 106
 Blenis, J., 135
 Blevins, J. E., 149
 Bligh, E. G., 3, 5, 10, 75, 109, 162, 191, 197, 200, 212, 282, 283, 302
 Bocckino, S. B., 74
 Bodennec, J., 236
 Bodin, S., 188
 Boehmle, A. M., 244
 Bogoy, M., 18
 Bohrer, A., 16
 Bokoch, G. M., 157
 Bollag, R. J., 50
 Bollag, W. B., 50
 Bollen, M., 91, 92, 93, 94, 98
 Bollinger, J., 18
 Bonacci, T. M., 30, 31, 34, 46
 Bondada, S., 135
 Bonventre, J. V., 16
 Boonstra, J., 301
 Borensztajn, J., 4
 Bornancin, F., 238, 239, 248, 253, 268, 284, 290
 Borowsky, A. D., 245, 248
 Borst, P., 259
 Bos, J. L., 136
 Bossant, M. J., 106
 Boulet, C., 106
 Bouveyron, N., 279
 Bouwman, P., 92, 95, 98
 Bova, S., 213
 Boxer, L. A., 268
 Boyer, J. L., 30, 223, 224
 Bradford, M. M., 308
 Bradoo, S., 80
 Brancaccio, A., 206, 207, 208, 215, 216, 219, 226
 Branch, K. D., 50
 Brandl, E., 142
 Brauer, A. U., 92
 Brearley, C., 173
 Brearley, C. A., 223
 Breton, M., 301
 Brewster, G., 120
 Brindley, D. N., 245, 246, 247, 268, 278, 279, 306, 313
 Broach, J., 245, 246, 247, 248, 250
 Broedl, U. C., 4
 Bromberg, J. S., 259
 Brooks, B., 106
 Brosens, J. J., 134
 Bross, T. E., 214, 225
 Brown, D. M., 212
 Brown, H. A., 30, 51, 56, 57, 59, 60, 61, 63, 73, 74, 79, 156, 189
 Brown, J. E., 214, 225
 Brown, K. O., 30, 31
 Brown, M. R., 142
 Brunn, G. J., 136
 Buccafusca, R., 214
 Bunce, M. W., 157, 161
 Burgett, S., 18

Buri, K., 306
 Buser, C. A., 64, 65
 Bushway, P. J., 142
 Buxton, I. L., 197
 Byers, L. W., 106
 Byfield, M. P., 136
 Byun, H. S., 93, 268, 278, 279

C

Cadwallader, K., 50, 51, 156
 Caffrey, J. J., 172, 173
 Cai, R., 248, 253
 Cai, S., 51, 67
 Cambier, J. C., 142, 146
 Cambillau, C., 3, 4
 Campbell, D. G., 126
 Camps, M., 30, 133, 134, 135, 136, 137
 Cantley, L. C., 118, 120, 134, 135, 156, 188
 Cantrell, D., 133
 Cantrell, D. A., 150
 Capitani, S., 188
 Cargnelli, G., 213
 Carman, G. M., 63, 65, 247, 305, 306, 307, 308, 310, 311, 312, 313
 Caron, M. G., 258
 Carozzi, A., 30
 Carpenter, C., 156
 Carpenter, C. L., 157
 Carre, A., 284
 Carriere, F., 3, 4
 Carroll, A. S., 306
 Carter, A. N., 120
 Carter, N., 197, 203, 213, 220
 Carter, R. H., 142
 Carvelli, A., 196, 203, 204
 Casey, G., 214
 Castillo, C., 280
 Castro-Faria-Neto, H., 106
 Cavalli, A. L., 258
 Cellek, S., 51
 Cerione, R. A., 60, 61
 Cestra, G., 157, 161
 Cetindag, C., 63
 Ceulemans, H., 91
 Chae, S. S., 244
 Chahwala, S. B., 197, 203, 213, 220
 Chalfant, C. E., 248, 265, 268, 274, 276, 279, 287, 288
 Chalif-Caspi, V., 63
 Chan, A. C., 142
 Chan, L., 3, 4
 Chang, L. C., 150
 Chang, S., 156, 157, 161
 Chang, S. C., 173, 174
 Chantry, D., 134
 Charnecki, S., 30
 Chasserot-Golaz, S., 65
 Chen, J., 134, 141, 149
 Chen, R., 149
 Chen, X., 306, 307, 310, 311, 313
 Chen, Y., 214
 Cheng, H., 189
 Chilton, F. H., 106
 Chiou, S. T., 171, 172, 173, 174, 176, 179
 Chiou, X. G., 18
 Cho, W., 15, 16, 18, 19, 20, 23, 268
 Choi, K. Y., 174
 Choi, S., 4
 Choi, S. Y., 4
 Choi, W. C., 30
 Chong, L. D., 157
 Chow, S., 142
 Christiansen, K., 108
 Christodoulou, E., 92, 93, 98
 Chuard, R., 237
 Chun, J., 90, 92, 244, 258
 Chung, H. K., 174
 Church, J. G., 214
 Cilingiroglu, M., 4
 Cimpean, A., 93
 Cioce, V., 92
 Cioffi, J. A., 4
 Clair, T., 92, 94
 Clapp, P., 31
 Clark, J. D., 18, 19, 20
 Clarke, H., 214
 Clay, K. L., 107, 108
 Clayton, E., 134
 Cline, G. W., 214
 Clutter, M. R., 142, 148
 Coadwell, J., 51, 134
 Cockcroft, S., 157, 163, 188, 214
 Coggeshall, K. M., 136
 Cohen, P., 134
 Coico, R., 139
 Coleman, R. A., 90
 Colina, C., 280
 Colley, W. C., 50, 156
 Condliffe, A. M., 121, 123
 Condreay, J. P., 269
 Connolly, T. M., 214
 Conzelmann, A., 237
 Cook, S., 50, 51, 156
 Cooney, D., 136
 Cooper, A. D., 4
 Coopman, P. J. P., 244
 Copeland, N. G., 50
 Corda, D., 187, 188, 189, 196, 203, 204, 206, 207, 208, 215, 216, 219, 226
 Corson, M. A., 149
 Coso, O. A., 244
 Costello, P. S., 150
 Cozier, G. E., 119
 Crabbe, T., 121, 123

Crans, A., 245, 248
 Creason, M. B., 237
 Creba, J. A., 120
 Creemers, J. W., 92
 Cremesti, A., 306, 307, 310, 311
 Cremona, O., 188
 Crump, J. S., 245, 251
 Csonga, R., 284
 Cui, Z., 199
 Culbertson, M. R., 308
 Cullen, P. J., 119, 123
 Cully, M., 118
 Currie, R. A., 126
 Cusson, N., 135
 Cuvillier, O., 244, 245
 Cyster, J. G., 245, 248

D

Daggy, B., 4
 dal Porto, J., 146
 D'Andrea, R. J., 237, 238
 Daniell, L., 156, 157
 Dann, S. G., 136
 Darrow, A. L., 238
 Das, S., 16, 18, 19, 20
 Daum, G., 306
 Daviaud, D., 92
 Davidson, K., 118, 120, 121, 123
 Davidson, L., 123
 Davies, S. S., 106
 Davis, T., 34
 Deak, M., 126
 Dean, N. M., 203
 Deane, J. A., 132, 134, 142
 Debetto, P., 213
 De Camilli, P., 156, 157, 159, 161, 188, 214
 Deems, R. A., 63, 65, 299
 De Francesco, A. L., 196, 203, 204
 De Graan, P. N., 158
 de Haas, M., 259
 del, R., 280
 de la Cruz, J., 176
 Delautier, D., 106
 Dell, A., 215
 DeLong, C. J., 199
 Delvaux, A., 174
 De Matteis, M. A., 188
 Demopoulos, C. A., 106
 Deng, C. X., 244
 Dennis, E. A., 16, 63, 65, 274, 290
 Derian, C. K., 238
 Deschermeier, C., 173
 de Silva, H. V., 4
 De Smedt, F., 174
 De Smedt, H., 174
 Devay, P., 238, 239, 268
 Devine, C. S., 160

Devreotes, P., 156
 DeWald, D. B., 18, 19, 21, 149
 Dewald, J., 306, 313
 Dewaste, V., 174
 Diaz, B. L., 16
 Dichek, H. L., 4
 Dickson, R. C., 237, 238, 245
 Dillard-Telm, L., 245, 248
 Dillon, D. A., 306, 307, 310, 311, 313
 Di Paolo, G., 156, 157, 159, 161, 214
 DiPolo, R., 280
 Divecha, N., 123, 127, 204, 222, 224
 Diveka, N., 222
 Dixon, J. E., 174
 Doan, K., 4
 Dohmae, N., 2, 3, 4, 7, 91, 92, 95
 Doi, T., 2, 3, 4, 7
 Dollins, D. E., 172, 173, 182
 Dombrowsky, H., 214
 Donahue, A. C., 120, 131, 134, 135, 136, 137, 138, 141, 142, 143, 145, 147
 Dorman, G., 177
 Dorman, S. E., 142
 Dosch, R., 18, 19, 20, 21, 22
 Doughman, R. L., 157, 159, 161
 Dove, S. K., 222
 Dove, S. L., 222
 Dowal, L., 30, 31
 Dowhan, W., 313
 Dowler, S., 126
 Downes, C. P., 120, 123, 126, 134, 150, 195, 196, 203, 207, 209, 210, 211, 214, 220, 221, 223, 224
 Downes, P. C., 197, 203, 213, 220
 Dragani, L. K., 206, 207, 208, 215, 216, 219, 226
 Dragusin, M., 280
 Drake, L. Y., 172, 173
 Dressler, K. A., 266
 Driscoll, P. C., 118, 132
 D'Santos, C., 123
 Du, G., 51, 65, 67
 Dubin, A. E., 90
 Duckworth, B., 156
 Duffy, P. A., 268, 278, 279
 Duffy, S., 34
 Dumont, J. E., 174
 Duronio, R. J., 160
 Duronio, V., 279
 Dyer, W. J., 3, 5, 10, 75, 109, 162, 191, 197, 200, 212, 282, 283, 302

E

Earnest, S., 200
 Eccleston, E., 214
 Edgar, B. A., 135
 Edsall, L., 237, 238, 244
 Edsall, L. C., 237, 238, 245, 258, 259, 260

- Eivemark, S., 279
 Ekhart, P. F., 158
 Elge, S., 173
 Eli, Y., 63
 Elliott, J., 30, 31
 Ellis, J. M., 106
 Ellson, C. D., 121, 123, 127
 El-Maghrabi, M. R., 30, 31
 Elson, P., 214
 Emery, J. L., 133, 134, 135, 136, 137
 Emr, S. D., 188
 Emson, P. C., 156
 Eng, G., 16
 Engelbrecht, J., 51, 65
 Engelman, J. A., 118
 English, D., 306
 Enoki, S., 101
 Epand, R. M., 298, 299
 Erdjument-Bromage, H., 134, 172
 Erl, W., 90
 Erneux, C., 174
 Ertl, D. S., 174
 Eujen, R., 182
 Evans, J. H., 16, 268, 279
 Evers, R., 259
 Exton, J. H., 2, 4, 30, 49, 50, 51, 52, 56, 60, 67, 73, 74, 79, 135
 Ezawa, I., 4
- F**
- Facchinetti, M. M., 238
 Fairservice, A., 123
 Falasca, M., 126, 196, 203, 204
 Falck, J. R., 182
 Falvo, J. V., 306
 Fanick, W., 173
 Farber, S. A., 18, 19, 20, 21, 22
 Farjot, G., 134
 Farr, A. L., 111
 Farris, F. J., 21, 22
 Faulkner, A. J., 313
 Feldman, M. E., 136
 Feng, L., 18, 19, 21
 Feng, S., 200
 Feng, Y., 173, 174
 Ferguson, C. G., 98, 99, 100
 Ferguson, G. J., 123, 127
 Ferguson-Miller, S., 274
 Fernandez-Arias, C., 133
 Ferrato, F., 4
 Fijneman, R. J., 16
 Fikes, K., 189
 Fiorica, J., 94, 214
 Firestone, A. J., 157, 159, 161
 Fischl, A. S., 306, 307, 308, 310, 311, 312, 313
 Fisette, P. L., 158
 Fitzsimonds, R. M., 157
- Flavell, R., 157
 Fleischer, S., 108, 110
 Fleisher, T. A., 142
 Floor, E., 266, 267, 268, 269
 Flores, A., 280
 Flores, J. M., 133
 Folch, J., 197
 Folch-Pi, J., 197
 Font, J. L., 31
 Forrester, J. S., 2, 189
 Frago, L., 268, 279, 280
 Frederick, J. P., 172, 173, 176
 Freiberg, J., 268, 279
 Freije, J. M., 92
 Fridy, P. C., 171, 172, 173, 182
 Friend, D. S., 16
 Frohman, M. A., 50, 51, 60, 65, 67, 101, 155, 156, 157, 159, 160, 163, 165
 Fromm, H., 173
 Fruehauf, J. P., 137
 Fruman, D. A., 120, 131, 132, 134, 135, 136, 137, 138, 141, 142, 143, 145, 147
 Frye, R. A., 173, 174, 179
 Fu, S., 259
 Fujii, M., 173, 174
 Fujita, T., 238
 Fujiwara, M., 134
 Fujiwara, Y., 93, 101
 Fukaya, M., 92
 Fukushima, N., 90
 Fukuzawa, K., 92, 94
 Funato, K., 246, 248
 Furneisen, J. M., 247, 306, 311, 312, 313
 Furutani, M., 123
 Fusco, A., 196, 203, 204
 Futatsumori, M., 161
 Futerma, A. H., 236
 Fyrst, H., 237, 244
- G**
- Gabreski, G., 4
 Gachet, C., 90
 Gaffney, P. R., 134
 Gallagher, M., 150
 Gallis, B., 149
 Galve-Roperh, I., 245, 246, 247, 249, 250, 251, 252, 253
 Gambhir, A., 188
 Gamble, J. R., 237, 238
 Gamboa, G. C., 18
 Gangadhar, B. P., 31
 Gangoito, P., 279
 Gao, X., 135
 Garavito, R. M., 274
 Gardell, S., 90
 Garland, W. A., 258
 Garrido, M., 280

Garrison, J. C., 38
 Gaskell, S. J., 107
 Geha, R. S., 135
 Gelb, M. H., 16, 18
 Georas, S. N., 94
 Gericke, A., 118
 Gerke, L. C., 306
 Gershberg, S., 30
 Ghomashchi, F., 18
 Ghosh, M., 30, 31, 46
 Giepmans, B. N., 90
 Gierschik, P., 30
 Gijsbers, R., 91, 93, 94, 98
 Gilbert, D. J., 50
 Gilbert, H., 16
 Gilman, A. G., 30, 31, 34, 45
 Gipson, K., 157
 Girffin, D., 214
 Gispén, W. H., 158
 Giudici, M. L., 156
 Giuriato, S., 188
 Giussani, P., 247, 248, 250, 252, 253
 Glassford, J., 134
 Glick, J. M., 4
 Glomset, J. A., 2, 3, 4, 294, 296, 299
 Goddeau, R. P., 16
 Godi, A., 188
 Goding, J. W., 92, 93, 98
 Goetzel, E. J., 244
 Goh, J., 236
 Gokoh, M., 245, 247, 249, 250, 251, 252
 Gold, M. R., 133
 Goldenberg, D. D., 136
 Goldwasser, P., 308
 Gomez-Munoz, A., 265, 268, 269, 278, 279
 Goni, F. M., 84
 Gonzalez, B., 136
 Gonzalez, M., 279
 Gonzalez-Espinosa, C., 258
 Gonzalez-Sastre, F., 197
 Gooden, T., 18
 Goparaju, S. K., 244
 Gordon, J. I., 160
 Gorshkova, I. A., 94
 Grado, C., 210, 212
 Graf, C., 268, 279, 284
 Gratacap, M., 188
 Gratton, E., 20
 Gray, A., 123, 127, 134, 150
 Graziani, A., 156
 Green, B. N., 214
 Greenberg, M., 308
 Greene, D. G., 106
 Greenwell, P., 172
 Greer, J. J., 214
 Grendys, E. C., Jr., 94, 214
 Gres, S., 92

Greten, H., 4
 Griffin, D., 94
 Gross, M. L., 215
 Gross, R. A., 31
 Gross, R. W., 2, 189
 Grosser, J., 4
 Grusby, M. J., 16
 Gu, Q. M., 50, 51, 156
 Guan, K., 174
 Guastella, J., 294
 Guertin, D. A., 134
 Guigne, C., 92
 Guillas, I., 237
 Guillou, H., 117, 123, 127, 134, 147
 Gulati, P., 136
 Gunn, M. D., 259
 Gunn-Moore, F., 123
 Guo, B., 135
 Guo, J., 157, 161
 Gupta, N., 80
 Gupta, R., 80
 Gupta, V., 245
 Gutkind, S., 244
 Gutowski, S., 51, 63, 156

H

Hadziselimovic, A., 294
 Haimi, P., 189
 Hajra, A. K., 75
 Halbur, L., 137
 Hall, H. S., 258
 Hall, M. N., 135, 138
 Halpern, M. E., 18, 19, 20, 21, 22
 Halstead, J., 123
 Hama, K., 91, 92, 95
 Hamano, F., 90
 Hammond, S. M., 50, 51, 65, 156
 Han, A., 136
 Han, D. K., 4
 Han, G.-S., 306, 307, 313
 Han, J. M., 51, 67
 Han, M. H., 3, 4
 Han, S. B., 135
 Han, W. K., 16
 Han, X., 2, 189
 Hanahan, D. J., 106
 Hanley, M. R., 203
 Hannun, Y. A., 234, 235, 236, 238, 244, 245, 246, 248, 258, 268, 276, 278, 279, 283, 287, 288
 Hansen, A., 182
 Hara, A., 197
 Harden, T. K., 30, 38, 223, 224
 Haroldsén, P. E., 107
 Harriague, J., 150
 Harris, G. L., 237

- Harrison, K. A., 106, 107
 Harsh, D., 268
 Hartmann, N., 279
 Hartwig, J. H., 157
 Haseruck, N., 90
 Hatsuzawa, K., 3, 4, 11
 Hattori, N., 237
 Hawkins, P. T., 117, 118, 120, 121, 123, 127,
 134, 147, 195, 196, 197, 203, 207, 209, 210,
 211, 213, 215, 220, 221, 223, 224
 Hayakawa, J., 2
 Hayashi, S., 238
 Haystead, T. A., 172, 173, 182
 Hazebroek, J., 174
 Hazeki, K., 279
 Hazeki, O., 279
 Heath, V. L., 222
 Hedley, D. W., 142
 Heikinheimo, L., 189
 Heinze, R., 214
 Hemer, M. R., 266
 Henage, L. G., 49, 51, 56, 73, 79
 Hendrickson, E. K., 18, 19, 20, 21, 22
 Hendrickson, H. S., 18, 19, 20, 21, 22
 Henry, S. A., 308
 Henson, P. M., 106
 Hepler, J. R., 30, 31
 Hermansson, M., 189
 Hernandez, C., 133
 Herr, D. R., 237
 Herzenberg, L. A., 149, 150
 Hess, K. L., 134, 135, 136, 141, 143
 Heucheroth, R. O., 160
 Hide, W. A., 3
 Higgs, H. N., 2, 3, 4
 Hilgemann, D. W., 200
 Hinkovska-Galcheva, V. T., 268
 Hirabayashi, T., 280
 Hirabayashi, Y., 291
 Hiramatsu, T., 2, 3, 4, 7
 Hirata, K., 4
 Hirata, T., 259
 Hirsch, E., 121, 123
 Hirschberg, K., 236
 Hisano, N., 258
 Hitomi, T., 59, 63, 66
 Hitoshi, Y., 149, 150
 Hjelmeland, L. M., 52
 Hla, T., 244, 245
 Ho, S. Y., 18, 19, 20, 21, 22
 Ho, W. T., 51, 52, 56
 Hobson, J. P., 244, 247, 248, 252, 258
 Hochschild, A., 222
 Hoer, A., 63
 Hoffman, M. S., 94, 214
 Hogan, B. L., 172, 173
 Hogback, S., 280
 Holers, V. M., 146
 Holk, A., 80
 Holland, S. M., 142
 Holmes, A. B., 134
 Holmes, W., 173
 Homanics, G. E., 4
 Homann, M. J., 308
 Honda, A., 157, 159, 160, 163
 Hong, S., 142, 189
 Hongu, T., 157, 159, 165
 Honig, S. M., 259
 Horazdovsky, B., 313
 Hoskin, J., 18
 Hosono, H., 2
 Houjou, T., 189
 Houssa, B., 295
 Howson, R. W., 306
 Hsia, C., 135
 Hsu, F. F., 109, 214, 215
 Hsu, V. W., 16
 Huang, L. J., 150
 Huang, P., 65
 Huang, R., 120
 Huang, S. F., 31
 Huang, Y., 245, 248
 Huang, Z., 16
 Hubbard, W., 94
 Huesken, D., 290
 Hughes, K. T., 223
 Hughes, P. J., 120
 Huh, W. K., 306
 Humphries, L. A., 134
 Hunt, A. N., 214
 Hurley, J. H., 51, 119
 Hwang, I. Y., 135
- I**
- Igarashi, N., 238
 Igarashi, Y., 236, 237, 238, 245, 247, 249, 250,
 251, 252, 258, 259, 268
 Ijuin, T., 123
 Ikeda, H., 94
 Ikuta, K., 16
 Imagawa, M., 189
 Imai, H., 237
 Imai, S., 294
 Imamura, T., 237
 Imperiali, B., 24
 Inagaki, Y., 238, 268
 Inoue, A., 2
 Inoue, K., 2, 3, 4, 7, 91, 92, 95
 Inoue, M., 2, 101
 Irvine, R. F., 156, 157, 163, 172, 188, 195, 197,
 203, 214, 223, 225
 Isakoff, S. J., 126
 Iseki, S., 92
 Ishida, M., 2, 3, 4, 7
 Ishida, T., 4

Ishihara, H., 156, 157

Ishii, I., 90, 244

Ishii, S., 16, 90, 115

Israel, A., 280

Ito, M., 279

Ito, S., 237

Itoh, T., 123, 156, 159, 188

IUPAC-IUBMB, 66

Iurisci, C., 187, 189, 203, 204, 206, 207, 208,
215, 216, 219, 222, 224, 226

Ivanova, P. T., 2, 189

Ives, E. B., 172

Iwaki, S., 245, 247

Iwamatsu, A., 3, 4, 11

Iyer, V. V., 157, 159

J

Jackson, T. R., 118, 120, 121, 215

Jackson-Machelski, E., 160

Jacob, A., 136

Jager, G. C., 149, 150

Jahangeer, S., 238

James, S. R., 134

Janetopoulos, C., 156

Janmey, P. A., 156, 157, 188

Jansen, S., 92

Jaritz, M., 279

Jarvis, D. L., 269

Jaye, M., 4

Jazwinski, S. M., 236, 237

Jeffery, D. A., 18

Jenco, J., 37, 156

Jenco, J. M., 50, 51, 156, 201

Jenkins, G. H., 158, 245, 246, 248

Jenkins, N. A., 50

Jensen, G. L., 4

Jezyk, M. R., 30

Ji, H., 134

Ji, L., 291

Jiang, H., 31, 46

Jiang, J. C., 236, 237

Jiang, X., 63

Jiang, Y., 197

Jin, W., 4, 16

Jinnai, H., 59, 63, 66

Joaquin, M., 136

Jogl, G., 173

Johnson, G. E., 3, 4

Johnson, I. D., 18, 19, 20, 21, 22

Johnson, K. R., 238, 246, 248, 249, 250, 251

Johnson, K. Y., 246, 248, 249, 250, 251

Johnson, L. B., 294

Johnston, C. N., 306, 313

Jolly, P. S., 244, 247, 248, 252, 258, 263, 276

Jones, D. H., 157, 163

Jonkers, J., 92, 95, 98

Joung, J. K., 222

K

Kadowaki, T., 134, 135

Kai, M., 294

Kaiser, S. L., 224

Takehi, Y., 93

Kakela, R., 189

Kalesnikoff, J., 118

Kalff, J. C., 280

Kam, Y., 135

Kanao, Y., 101, 155, 157, 159, 160, 163, 165

Kanaoka, Y., 16

Kang, L. H., 18

Kang, V. H., 149

Kanoh, H., 294, 295

Kanz, L., 244

Kanzaki, H., 94

Kapeller, R., 156

Kaplan, B., 173

Kaplin, A. I., 177

Kariya, Y., 92

Kasahara, K., 2, 3, 4, 7

Kasprzycka, M., 136, 137

Katada, T., 157, 159, 165, 279

Katagiri, H., 156, 157

Katayama, K., 107

Kato, M., 268

Katso, R., 118, 132

Kavran, J. M., 126

Kawai, N., 101

Kawamoto, K., 157, 159, 160, 163

Kawano, T., 45

Kay, R. R., 203

Kearney, J. F., 142

Kehrl, J. H., 135

Kelley, M. J., 308

Kelliher, M., 135

Kelly, S., 245, 250, 252, 253, 277, 280

Kennedy, A., 214

Kennedy, A. W., 214

Kennedy, M., 106

Kerr, I. D., 51

Kharas, M. G., 120, 131, 134, 141

Khoo, K. H., 215

Kihara, A., 236, 237, 245, 247, 249, 250, 251,
252, 259

Kihara, Y., 115

Kikuchi, K., 92, 93, 98

Kim, H., 30

Kim, H. J., 294

Kim, H. K., 174

Kim, J. H., 18, 19, 20, 23

Kim, J. W., 174

Kim, K. P., 16, 18, 19, 20

Kim, S. Y., 136

Kim, T. J., 268

Kim, Y., 51, 67

Kim, Y. J., 16, 18, 19, 20, 23

- King, C. E., 120
Kinoshita, S., 149, 150
Kirk, C. J., 120
Kishi, Y., 91, 92, 95
Kisseleva, M. V., 174
Kita, Y., 16, 107, 113, 115
Kitamura, R., 142
Kitamura, T., 149, 150
Kiyohara, Y., 59, 63, 66
Kizuki, N., 156, 157
Klein, D. E., 126
Klenchin, V. A., 156, 157
Kleuser, B., 238, 244
Klig, L. S., 308
Knight, Z. A., 136
Knopf, J. L., 18
Kobayashi, N., 259
Kobayashi, S., 93, 101
Kobayashi, T., 101
Kogure, K., 92
Koh, E., 92, 93
Kohama, T., 237, 238, 245, 267
Kohlwein, S. D., 308
Kohno, M., 244
Koivusalo, M., 189
Kolesnick, R. N., 244, 266
Komagata, Y., 16
Komori, H., 279
Kondo, H., 157, 163
Kong, J. Y., 269, 279
Kono, K., 237, 238, 267
Kook, S., 51
Koonin, E. V., 51
Kopp, T., 248, 253
Kost, T. A., 269
Koster, G., 214
Kostiainen, R., 189
Kotter, M. R., 92
Kouro, T., 134
Koyasu, S., 134, 135, 142
Kozasa, T., 30, 34, 45
Krawiec, J., 4
Krimpenfort, P., 259
Krishnamoorthi, R., 65
Kriz, R. W. I., 18
Kronmal, G. S., 4
Krutzik, P. O., 142, 148
Krutzsch, H. C., 92
Krystal, G., 118
Ktistakis, N. T., 51
Kuan, Y. H., 150
Kuang, Y., 31, 46
Kudo, I., 4, 16
Kuil, A., 259
Kular, G., 126
Kume, K., 16
Kume, S., 258
Kume, T., 94
Kundra, V., 258
Kundu, R. K., 4
Kurtz, M. B., 245, 246, 247, 248, 249, 250, 251
Kuwata, H., 4
Kwatia, M., 18, 19, 20, 23
- L**
- Laboda, H. M., 4
Lam, B. K., 16
Lam, E. W., 134
Lam, V., 118
Lambeau, G., 16, 18
Lamour, N. F., 265
Lander, D. J., 214
Lane, T. E., 134
LaPolla, J. P., 94, 214
Lassus, P., 287
Latimer, A. J., 172
Lauffenburger, D. A., 148, 149, 150
Laussmann, T., 182
Lavine, K., 172, 174
Lawrence, J. C., Jr., 136
Lazdunski, M., 18
Lazebnik, Y., 287
Lebedeva, S., 142
Le Calvez, C., 18
Lécureuil, C., 123, 127
Ledeen, R. W., 300
Lee, A., 126
Lee, C. W., 90
Lee, H. Y., 92, 94
Lee, K. Y., 177
Lee, M. J., 244
Lee, S. B., 30, 31
Lee, S. Y., 159, 174, 177
Lee, Y. M., 244
Leeper, N. J., 4
Lees, M., 197
Leevers, S. J., 118, 132
Lehmann, D. M., 29, 31
Lejeune, C., 174
Lemaitre, R. N., 294, 296, 299
Lemmon, M. A., 119, 126, 222
Lemos, M., 174
Lepine, S., 250, 252, 253
Leppinmaki, P., 280
Leslie, C. C., 16, 268, 279
Leslie, N. R., 123
Lester, R. L., 237, 245
Le Stunff, H., 245, 247, 248, 249, 250, 251, 252, 253
Letcher, A. J., 214, 225
Letinic, K., 157, 159, 161
Levine, A. J., 118
Lewis, I., 214
Li, E., 16
Li, P. Y., 238

Li, W. H., 3
 Li, X., 142
 Li, Y., 30
 Libby, P., 120, 156
 Lichtenberg, D., 274
 Lichtenbergova, L., 18
 Liehr, J., 106
 Lin, A. Y., 18
 Lin, C. N., 150
 Lin, H. Y., 150
 Lin, L. L., 18
 Lin, R. H., 150
 Lin, Y.-P., 307
 Lindsay, Y., 123
 Ling, K., 157, 159, 161
 Ling, Z. C., 306
 Liotta, D. C., 236
 Liotta, L. A., 92, 94
 Liou, H. C., 135
 Lips, D. L., 225
 Liskovitsch, M., 63
 Lisman, Q., 259
 Liu, C. H., 244
 Liu, H., 237, 238, 245, 251, 267
 Liu, M., 94
 Liu, X., 136, 137
 Liu, Y., 244, 306, 307, 311, 312, 313
 Loewith, R., 135, 136, 138
 Logsdon, M. N., 135
 Loijens, J. C., 156, 157
 Lombardi, R., 246, 248
 Lorens, J. B., 149, 150
 Los, A., 123
 Lowe, M., 4
 Lowry, O. H., 111
 Lu, Y., 189, 258
 Lubec, G., 92
 Lucast, L., 214
 Luciani, S., 213
 Lucocq, J., 123
 Lucocq, J. M., 150
 Luo, B., 294
 Luo, H. R., 173
 Luo, J., 118, 134
 Luo, X., 173
 Luquin, C., 101
 Lynch, K. J., 4
 Lynn, H. E., 238
 Lyubchenko, T., 146

M

Ma, K., 4
 Ma, Z., 16
 MacDonald, N. J., 92
 Maceyka, M., 238, 243, 244, 250, 252, 253, 268, 279
 Madureira, P. A., 134
 Maeda, N., 4
 Maehama, T., 157
 Mahlum, C. E., 201
 Majerus, P. W., 172, 173, 174, 214, 224, 225
 Majima, E., 92
 Mak, T. W., 118
 Maki, K., 16
 Makide, K., 2
 Malik, S., 30, 31, 46
 Manabe, K., 18, 19, 21
 Mandal, S., 214
 Mandala, S. M., 245, 246, 247, 248, 249, 250, 251, 253
 Manders, E., 285
 Manifava, M., 51
 Mann, P. C., 294
 Manna, D., 15
 Mannaerts, G. P., 261
 Manning, B. D., 135
 Manrow, R. E., 92
 Mansfield, P. J., 268
 Mao, C., 245, 246, 248, 249, 250, 251
 Mao, J., 200
 Mao, X., 259
 Marathe, G. K., 106
 Marchadier, D., 4
 Marian, A. J., 4
 Martelli, A. M., 188
 Martin, A., 268, 278, 279
 Martin, F., 142
 Martin, T. F., 156, 157, 266, 267, 268, 269
 Martinec, J., 80
 Martinez, A. C., 133
 Martino, M., 94, 214
 Marzec, M., 136, 137
 Massiello, A., 274, 276
 Mathews, J. L., 31
 Matloubian, M., 245, 248
 Matsuda, S., 134
 Matsuda, Y., 4, 7
 Matsumoto, A., 142
 Matteo, R. G., 258
 Mattis, D., 172, 173
 Maugeais, C., 4
 Maurer, B. J., 244
 Mayer, U., 259
 Mayr, G. W., 173, 215
 McBride, J., 149, 150
 McCarl, R. L., 195
 McClintick, M. L., 306, 313
 McClure, D., 18
 McCormick, F., 134
 McCoull, D., 123
 McCoy, M. G., 4
 McDermott, M. I., 245, 251
 McDonald, D., 135
 McIntyre, T. M., 106
 McLaughlin, S., 64, 65, 188

- McMurray, W., 214
 Mechtcheriakova, D., 238, 239, 248, 253, 268, 284, 290
 Mecklenbrauker, I., 136
 Meek, S. E., 134
 Meeley, R. B., 174
 Megosh, L. C., 172, 173
 Meijer, H., 222
 Meijer, H. J., 204, 222, 224
 Mel, L., 244
 Melamed, D., 142
 Mellor, H., 119
 Menaldino, D. C., 236
 Menzeleev, R., 244, 245, 246, 247, 248, 250
 Merrill, A. H., Jr., 234, 236, 237, 245, 250, 252, 253, 277, 280
 Mezo, A. R., 24
 Mi, D., 294
 Mi, Y., 244
 Michell, R. H., 120, 222
 Mikami, A., 247, 248, 250, 252, 253
 Millar, J. S., 4
 Miller, A. L., 173, 174
 Miller, D. J., 16
 Miller, M., 135
 Mills, G. B., 90, 91, 92, 95, 258
 Milne, S. B., 2, 189
 Milona, N., 18
 Milstien, S., 234, 237, 238, 243, 244, 245, 247, 248, 249, 250, 251, 252, 253, 257, 258, 259, 260, 263, 276
 Min, D. S., 51, 60
 Minekura, H., 267
 Misra, S., 51
 Missiaen, L., 174
 Missy, K., 188
 Mitchell, D., 149, 150
 Mitchell, R. H., 120
 Mitra, P., 244, 250, 251, 257, 259, 263
 Mitsutake, S., 238, 268
 Miyazaki, H., 157
 Miyazaki, J., 16
 Miyazaki, Y., 4
 Mizoguchi, T., 3, 4, 11
 Mizutani, Y., 236, 237
 Mochizuki, Y., 174
 Mohle, R., 244
 Mol, A., 196
 Mol, C. A., 259
 Moley, K., 16
 Momoi, M., 244
 Monajemi, H., 4
 Moolenaar, W. H., 90, 92, 93, 95, 98, 99, 100
 Moomaw, C. R., 51, 156
 Moon, K. H., 174
 Moore, T., 134, 135, 136, 141, 143, 203
 Moreau, C., 174
 Morell, P., 236
 Moreno, K. M., 258
 Moretti, P. A., 237, 238
 Morgan, C. P., 157, 163
 Morikawa, R., 1, 2, 3, 4, 7
 Moritz, A., 158
 Morris, A. J., 30, 37, 50, 51, 60, 65, 67, 89, 101, 156, 157, 159, 160, 163, 196, 197, 201, 203, 213, 220, 245, 251, 306
 Morris, J. B., 157, 163
 Morris, P. J., 203
 Morris, V., 294
 Morrison, A. R., 214, 215
 Morrow, J., 248
 Mosher, D. F., 157, 159
 Moskowitz, H. S., 157
 Moskowitz, M. A., 16
 Mueller, H. W., 106
 Mueller-Roeber, B., 173
 Muirhead, E. E., 106
 Mukai, M., 101
 Muller, M., 214
 Mullins, M. C., 18, 19, 20, 21, 22
 Mulugu, S., 171, 172, 173, 182
 Mummery, C. L., 92, 95, 98
 Munnik, T., 195, 197, 204, 222, 224
 Munson, M., 118
 Murakami, M., 4, 16
 Murakami-Murofushi, K., 93
 Murata, C., 157, 159, 165
 Murayama, T., 280
 Murofushi, H., 93, 101
 Murphy, R., 172
 Murphy, R. C., 2, 106, 107, 189
 Murray, D., 188
 Murray, J. T., 136
 Murriel, C., 149, 150
 Musgrave, A., 195, 197, 204, 222, 224
 Myers, A. C., 94
 Myung, C. S., 38
- N**
- Nagahama, M., 3, 4
 Nagai, Y., 2, 3, 4, 7
 Nagano, T., 92, 93, 98
 Nagase, T., 16
 Nagata, E., 173
 Nagiec, E. E., 237
 Nagiec, M. M., 237, 238, 245
 Nairn, A. C., 159
 Nakajima, K., 3, 4
 Nakamoto, K., 4
 Nakamura, H., 157, 159, 160, 163, 280
 Nakamura, K., 94
 Nakamura, S., 238
 Nakamura, S. I., 59, 63, 66
 Nakano-Kobayashi, A., 157, 159, 165

Nakashima, S., 50, 51, 156
 Nakatani, N., 16
 Nakatani, Y., 4
 Nakayama, K., 155, 157, 159,
 160, 161, 163
 Nalaskowski, M. M., 173
 Nam, S. W., 92
 Nasuhoglu, C., 200
 Natarajan, V., 94, 101
 Natori, Y., 93
 Natt, F., 136, 290
 Nava, V. E., 237, 238, 244, 245
 Neer, E. J., 30
 Nemazee, D., 142
 Neri, L. M., 188
 Ng, K. L., 134, 135, 136, 141, 143
 Nguyen, E., 18
 Nichols, J., 172
 Nickels, J., 245, 246, 247, 248, 250
 Nieto, M. L., 106
 Nievers, M., 301
 Niki, T., 101
 Nikolova-Karakashian, M., 236
 Ninio, E., 106
 Nishi, T., 259
 Nishikawa, K., 93
 Nishiura, H., 237
 Nishizuka, Y., 59, 63, 66, 156, 188
 Niswender, K. D., 149
 Nitsch, R., 92
 Nitz, M., 24
 Nobukuni, T., 136
 Nogami, M., 157, 159, 160, 163
 Noguchi, K., 90
 Noji, S., 92
 Nolan, G. P., 142, 148, 149, 150
 Nozawa, Y., 50, 51, 156
 Nussbaum, R. L., 214
 Nussenzweig, M. C., 136

O

Oak, J. S., 134, 135, 136, 141, 143
 Oatey, P. B., 123
 Obayashi, M., 157
 Obaid, L. M., 233, 234, 236, 238, 245, 246, 248,
 249, 250, 251
 Oberdisse, E., 63
 O'Brien, L., 268, 278, 279
 Odom, A. R., 172, 173, 174
 Oehrl, W., 214
 O'Flaherty, J. T., 106
 Ogawa, C., 245, 247, 249, 250, 251, 252
 Ogawa, T., 237
 Ogino, C., 59, 63, 66
 Ogretmen, B., 244, 258
 Oh, Y. S., 16, 18, 19, 20, 23
 Ohgane, J., 237

Ohkawa, R., 92, 94
 Ohlmann, P., 90
 Ohta, H., 92
 Ohteki, T., 134
 Ohto, T., 16
 Oka, Y., 156
 Okada, M., 94
 Okada, T., 238
 Okkenhaug, K., 121, 123, 133, 134, 135, 136,
 137, 142
 Okubo, S., 94
 Okudaira, S., 92
 Okuyama, H., 2
 O'Leary, E., 16
 Olins, P. O., 160
 Oliver, A. M., 142
 Olivera, A., 237, 238, 239, 244, 245, 246, 247,
 249, 250, 251, 258, 260
 Olson, E. S., 18, 19, 20
 Olson, S. C., 106
 Olsson, H., 123
 Olszewski, J. D., 177
 Ong, S. T., 137
 Onnebo, S. M., 172
 Oo, M., 244
 Ortolano, S., 135
 Osada, J., 4
 O'Shea, E. K., 306
 Oshiro, J., 306, 307, 310, 311, 313
 Oskieritzian, C. A., 244, 250, 251, 259, 263
 Oskouian, B., 245, 248
 Osterhout, J., 30, 31, 46
 Ostrander, D. B., 306, 307, 310, 311
 Ota, M., 92
 Otto, J. C., 171, 172, 173,
 176, 182
 Otvos, J. D., 4
 Ouchi, Y., 16
 Owen, J. S., 105, 107, 113
 Ozaki, Y., 258

P

Pack, M., 18, 19, 20, 21, 22
 Paik, J. H., 244
 Painter, G. G., 134
 Palmer, F. B., 126
 Pan, D., 135
 Panchenko, M. P., 30
 Pandey, D., 90
 Parhar, K., 279
 Park, S. J., 159
 Park, S. K., 51, 52, 56, 60
 Parker, P. J., 30, 118, 132
 Patel, H., 142
 Paterson, A., 38
 Paugh, S. W., 260
 Paul, R. U., 80

Payan, D. G., 149, 150
Payne, S. G., 244, 250, 251, 257, 258,
259, 260, 263
Payraastre, B., 188, 301
Pearce, W., 134
Pe'er, D., 148, 149, 150
Pellegrini, L., 156, 157, 161
Pereira, J. P., 245, 248
Perez, O. D., 142, 148, 149, 150
Perez, R., 16
Perrakis, A., 92, 93, 98
Perrone, M., 4
Perry, D. K., 276, 278, 283
Pesckett, E., 134
Pete, M. J., 2, 4
Peterson, C., 245, 246, 247, 249,
250, 251, 252, 253
Petes, T. D., 172
Petkovic, M., 214
Pettit, T. R., 92, 95, 98
Pettus, B. J., 248, 268, 279, 287, 288
Pewzner-Jung, Y., 236
Phan, V. H., 237
Phillips, M. C., 4
Piccolo, E., 187
Pigg, K. R., 93
Pilquill, C., 306
Pilquill, C. S., 306
Pinckard, R. N., 106, 107
Pirianov, G., 244
Pirola, L., 118, 156
Pitson, S. M., 237, 238
Plantavid, M., 188
Polonsky, J., 106
Ponting, C. P., 51
Poole, M. A., 308
Popov, S., 30, 31
Porcelli, A. M., 248
Portilla, D., 215
Possee, R. D., 269
Postle, A. D., 214
Poulton, S., 237, 238, 245, 246, 247,
249, 251, 258
Poyner, D. R., 203
Prabhu, S., 137
Pradere, J. P., 92, 95, 98
Pradhan, M., 136
Preininger, A. M., 49
Prescott, S. M., 106, 294, 295, 300
Prestwich, G. D., 18, 19, 21, 50, 51,
65, 98, 99, 100, 149, 156, 177
Priddle, H., 134
Priganica, L., 123
Proia, R. L., 258
Provost, J. J., 52, 56
Ptaszynska, M. M., 92
Pulfer, M., 2, 189
Pynn, C. J., 214

Q

Qi, R., 258
Qin, C., 63
Qiu, R. G., 203
Quertermous, T., 4
Quintana, L., 4

R

Radenberg, T., 215
Rader, D. J., 4
Radin, N. S., 197, 236
Rafter, J. D., 16, 18, 19, 20
Raggers, R. J., 259
Ramakrishna, V., 196, 203, 204
Ramanadham, S., 16
Ramesha, C. S., 18
Randall, R. J., 111
Randazzo, P. A., 156
Randolph, G. J., 259
Rathi, P., 80
Ravanat, C., 90
Rawlings, D., 134
Reames, D. L., 248
Rebecchi, M., 30, 31
Reddy, K. K., 182
Reddy, K. M., 182
Redondo, C., 133
Reese, C. B., 134
Regier, D. S., 294
Reichl, S., 214
Reid, G., 259
Reiss, U., 245
Reuschel, R., 248, 253
Reynolds, C. P., 244
Reynolds, H., 134
Rhee, H. J., 16, 18, 19, 20
Rhee, S. G., 30, 31, 174, 177
Ribeiro, A. A., 171, 172, 173, 182
Richardson, C. J., 135
Richardson, R. D., 98, 99, 100
Richardson, V. J., 214
Rickert, R. C., 149, 150
Riebeling, C., 236
Riedel, B., 306, 307, 311, 312, 313
Riezman, H., 236, 246, 248
Ristimaki, A. P., 244
Rittenhouse, S. E., 120
Rivera, J., 258
Rivera, R., 90
Roberts, J. L., 238
Roberts, M. F., 63, 64
Robertson, E. S., 136, 137
Robson, R. J., 274
Roccio, M., 136
Rocha, L., 92
Roddy, P., 268, 279, 287, 288
Rodger, J., 236

Rodriguez, J., 287
 Rodriguez, S., 133
 Roefs, M., 123
 Rogers, R. A., 16
 Roll, R., 156
 Romanelli, A. J., 214
 Rommel, C., 133, 134, 135, 136, 137
 Romoser, V., 37
 Rose, T. M., 18, 19, 21
 Rosebrough, N. J., 111
 Rosen, H., 244, 258
 Rosenberg, D., 244
 Rosenfeldt, H. M., 244, 258
 Rosing, J., 196
 Ross, A. H., 2, 4, 118
 Rothstein, T. L., 135
 Rotilio, D., 206, 207, 208, 215, 216, 219, 226
 Rouault, M., 18
 Roubaty, C., 237
 Rouser, G., 108, 110
 Roussel, A., 3, 4
 Roux, P. P., 135
 Rovina, P., 279
 Ru, B., 135
 Rubin, E. M., 4
 Rubin, L. J., 214
 Ruckle, T., 121, 123, 133, 134, 135, 136, 137
 Rudge, S. A., 51, 65, 201
 Rudnas, B., 280
 Rudnick, M., 225
 Ruiz-Arguello, M. B., 84
 Runnels, L. W., 37
 Rush, J., 313
 Ruurs, P., 92, 93, 95, 98
 Ryan, T. A., 157
 Ryu, S. H., 51, 67, 177

S

Saadat, D., 137
 Saatian, B., 94
 Saba, J. D., 237, 244, 245, 248
 Sabatini, D. M., 134
 Sabers, C., 136
 Sachs, K., 148, 149, 150
 Sadilek, M., 18
 Sahl, B., 279
 Saiardi, A., 172, 173
 Saijo, K., 136
 Saiki, N., 2
 Sakagami, H., 93
 Sakai, Y., 92
 Sakane, F., 294, 295
 Salh, B., 269, 279
 Salpekar, A., 134
 Samoriski, G. M., 31
 Samuel, M. P., 107, 113, 199
 Sanai, Y., 2, 3, 4, 7
 Sanchez, S. A., 20
 Sancho, S., 134
 Sanders, C. R., 294
 Sandquist, J. C., 173, 174
 Sankala, H., 263
 Sankaran, B., 30, 31, 46
 Sano, H., 244
 Sano, T., 245, 247, 259
 Sapirstein, A., 16
 Sarbassov, D. D., 134
 Sarkar, S., 244
 Sarmah, B., 172
 Sasaki, T., 157, 279
 Satake, Y., 16
 Sato, T., 2, 3, 4, 7
 Satoh, K., 258
 Saucedo, L. J., 135
 Saulnier-Blache, J. S., 92, 95, 98
 Savaskan, N. E., 92
 Sawa, A., 173
 Saxena, K., 30
 Scarlata, S., 30, 31, 37
 Schacht, J., 197
 Schaffner, W., 33
 Schaller, M. D., 157, 159
 Schalm, S. S., 135
 Schaloski, R. H., 16
 Scheer, A., 30
 Schell, M. J., 172
 Scherer, G. F., 80
 Schiffmann, E., 92
 Schiller, J., 214
 Schnabel, P., 30
 Schotz, M. C., 3
 Schrecker, O., 4
 Schwab, S. R., 245, 248
 Schwartz, B., 214
 Schwartz, M. A., 157
 Schwartz, M. W., 149
 Schwarz, M. K., 133
 Sciorra, V. A., 51, 65, 306
 Scott, J. K., 31
 Seeds, A. M., 172, 173, 174, 176
 Seitz, G., 244
 Sen, G., 135
 Seo, J. G., 236
 Serhan, C. N., 189
 Serunian, L. A., 120, 156
 Shacht, J., 126
 Shan, J., 65
 Shankar, G., 135
 Shankaranarayanan, A., 45
 Sharp, J. D., 18
 Sharps, E. S., 195
 Shaw, R. J., 118
 Shayman, J. A., 214, 268
 Shears, S. B., 172, 173, 174
 Shen, Z., 214

- Sherman, W. R., 214
Shi, J., 174
Shibasaki, Y., 156, 157
Shimada, A., 115
Shimizu, T., 16, 90, 107, 113, 115, 189
Shimizuogawa, T., 267
Shimoi, W., 4
Shimuzu, M., 280
Shin, H. W., 161
Shin, S. H., 31
Shiota, K., 237
Shope, J. C., 18, 19, 21, 149
Shulman, G. I., 214
Siakotos, A. N., 108, 110
Sibbald, B. J., 258
Siess, W., 90
Sigal, Y. J., 245, 251
Silletta, M. G., 196, 203, 204
Silva, A. R., 106
Sim, S. S., 174
Simon, M. F., 92
Simonsen, A., 188
Singer, A. G., 18
Singer, W. D., 30, 63
Singh, A., 101
Singh, A. K., 197
Skatrud, P. L., 18
Skippen, A. J., 214
Skolnik, E. Y., 126
Skrzypek, M. S., 237, 245
Slater, E. C., 196
Slaughter, C., 51, 156
Slice, L. W., 160
Sloane Stanley, G. H., 197
Slotte, J. P., 280
Slupianek, A., 136, 137
Smith, A. J., 134
Smith, T. F., 30
Smrcka, A. V., 29, 30, 31, 34, 37, 46
Smyth, S. S., 89
Snowman, A. M., 172, 173
Snyder, F., 106
Snyder, J. T., 30
Snyder, S. H., 172, 173, 177
Sobanov, J., 248, 253
Soeiro, I., 134
Soltoff, S. P., 120, 156
Somerharju, P., 189
Sondek, J., 30
Song, H., 16
Sonoda, H., 2, 3, 4, 7
Sooriyakumaran, P., 245, 248
Sorisky, A., 120
South, S., 149, 150
South, V., 4
Spana, E. P., 173, 174
Spassieva, S., 233, 236
Speigel, S., 244
Spencer, D. M., 16
Spiegel, S., 234, 237, 238, 239, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 257, 258, 259, 260, 263, 267, 276, 279, 287, 288
Sportsman, J. R., 18
Sprang, S. R., 34
Sribney, M., 236
Staedtler, F., 279
Stafforini, D. M., 106
Stahelin, R. V., 268
Stahlberg, A., 172
Stalmans, W., 91, 92, 93
Stanley, A. F., 203
Steeg, P. S., 92
Stefan, C., 91, 92, 93
Steinbrecher, U. P., 269, 279
Stenmark, H., 188
Stephens, L., 117, 134, 147, 197, 203, 213, 215, 220, 223, 224
Stephens, L. R., 118, 120, 121, 123, 127, 195, 203, 207, 209, 210, 211, 214, 220, 221, 223
Sternweis, P. C., 30, 31, 51, 57, 59, 63, 156, 313
Sternweis, P. M., 30
Stevenson-Paulik, J., 173, 174, 176, 179
Stewart, J. C., 212
Stiles, L. N., 134
Stingl, G., 248, 253
Stokoe, D., 134, 136
Stora, S., 268, 284
Stortelers, C., 92, 95, 98
Stossel, T. P., 157
Stracke, M. L., 92, 94
Stukey, J., 306, 313
Subramanian, P., 268, 274, 276, 279
Suchy, S. F., 214
Sue-Ling, C. K., 50
Suen, R., 294, 296, 299
Sugars, J. M., 51
Sugiura, M., 237, 238, 267
Suh, P.-G., 30
Suire, S., 123, 127
Sullards, M. C., 236, 277
Sultzman, L. A., 18
Sun, G. S., 4
Sun, M., 279
Sung, T. C., 60, 67, 156
Sutphen, R., 94, 214
Suzuki, H., 134, 135
Suzuki, R., 92
Symons, M. H., 203
Szulc, Z. M., 235, 268, 274, 276
- T**
- Tabata, H., 142
Tagaya, M., 3, 4, 11
Taguchi, R., 2, 3, 4, 7, 157, 159, 165, 189
Tahara, M., 94

Taheri, M. R., 16
 Taira, A., 91, 92, 95
 Takahashi, T., 107, 113
 Takakusa, H., 92, 93, 98
 Takanezawa, Y., 2, 3, 4, 7
 Takatsu, H., 161
 Takatsu, K., 134
 Takazawa, K., 174
 Takenawa, T., 123, 156, 159, 188
 Takeuchi, Y., 2
 Takio, K., 2, 3, 4, 7, 91, 92, 95
 Tall, G. C., 45
 Tam, Y. Y., 245, 248
 Tanaka, M., 92
 Tanaka, S., 237
 Tanaka, Y., 101, 142
 Tani, K., 3, 4, 11
 Tarakhovsky, A., 136
 Tasaka, K., 94
 Tashiro, F., 16
 Taussig, R., 31
 Tauzin, L., 279
 Tavaré, J. M., 123
 Taylor, L. A., 157
 Taylor, S. J., 30
 Taylor, S. S., 160
 Tee, A. R., 135
 Tempst, P., 134, 172
 Tence, M., 106
 Terauchi, Y., 134
 Tesmer, J. J. G., 45
 Tesmer, V. M., 45
 Tessner, T. G., 106, 107
 Thangada, S., 244
 Thiel, U., 182, 215
 Thirstrup, K., 4
 Thirugnanam, S., 298
 Thomas, D. M., 244
 Thomas, G., 136
 Thomas, M. J., 105, 106, 107, 113, 199
 Thornton, R., 245, 246, 247, 248, 249, 250, 251, 253
 Tigyi, G., 90, 101
 Timms, J., 118, 132
 Tjonahen, E., 189
 Toda, A., 115
 Toke, D. A., 306, 307, 310, 311, 313
 Toker, A., 156, 157, 188
 Tokumura, A., 92, 94
 Tominaga, K., 92
 Topham, M. K., 294, 295, 298, 300
 Torabinejad, J., 149
 Tornquist, K., 280
 Toth, B., 136
 Tozuka, M., 94
 Traynor-Kaplan, A., 157
 Trevisi, L., 213
 Tsai, C. R., 150

Tsao, K. L., 34
 Tsao, L. T., 150
 Tsirka, S. E., 50
 Tsujimoto, M., 1
 Tsujita, K., 123
 Tsukahara, R., 93
 Tu, Z., 245, 246, 247, 248, 250
 Turk, J., 16, 109, 214, 215
 Turner, M., 121, 123, 134
 Twitty, S., 258
 Tyler, A. N., 214

U

Uchiyama, A., 93, 101
 Uchiyama, S., 4
 Uematsu, S., 291
 Uesaki, S., 4
 Ui, M., 279
 Uldry, D., 237
 Ulug, E. T., 223
 Umezū-Goto, M., 91, 92, 95
 Unoki, T., 157, 159, 165
 Unsworth, E. J., 92
 Uozumi, N., 16, 107, 113
 Uphoff, A., 189
 Urtz, N., 238, 239, 248, 253, 268, 284
 Usatyuk, P. V., 94

V

Vadas, M. A., 237, 238
 Vail, M., 142
 Valentin, E., 16
 Vales, T. R., 236
 Valet, P., 92
 Vallée, B., 246, 248
 Van, E. A., 92
 Van Bergen en Henegouwen, P. M., 301
 Van Blitterswijk, W. J., 295
 Van Brocklyn, J. R., 244, 250
 Vandeleur, L., 237, 238
 van den Ende, H., 222
 van der Heijden, I., 259
 van der Kaay, J., 123, 206, 207, 208, 215, 216, 219, 226
 van der Valk, M., 259
 van Echten-Deckert, G., 280
 Vanek, P. G., 244
 Vanhaesebroeck, B., 118, 121, 123, 132, 133, 134, 135, 136, 137, 142
 van Leusden, M. R., 259
 van Meer, G., 259
 van Meeteren, L. A., 90, 92, 93, 95, 98, 99, 100
 Vann, L. R., 258
 van Rooijen, M. A., 92, 95, 98
 van Tilbeurgh, H., 3
 Van Veldhoven, P. P., 261
 Varela-Nieto, I., 268, 279, 280

Varenne, P., 106
 Varotto, R., 213
 Vekich, J. A., 258
 Venable, M. E., 106
 Venkataraman, C., 135
 Venkataraman, K., 236, 244
 Venkateswarlu, K., 123
 Verbeek, F. J., 285
 Verbsky, J., 172, 174
 Verbsky, J. W., 174
 Verger, R., 3, 4
 Verkleij, A. J., 301
 Vigorito, E., 134
 Vijnholds, J., 259
 Vionnet, C., 237
 Visentin, B., 258
 Vitale, N., 65
 Vivarelli, M. S., 135
 Voelker, D. R., 306, 307, 310, 311, 313
 Vogel, G., 182, 215
 Vogels, I., 259
 Voglmaier, S. M., 177
 Voronov, S., 157, 159, 161

W

Wabitsch, M., 92
 Wada, A., 238
 Wada, R., 244
 Wada, T., 156
 Wadleigh, M., 245, 246, 248
 Waechter, C. J., 313
 Waelkens, E., 92
 Waggoner, D. W., 246, 294, 313
 Wagner, D. S., 18, 19, 20, 21, 22
 Wakelam, M. J., 92, 95, 98
 Wakeley, K., 94, 214
 Waldo, G. L., 30
 Walker, S. J., 51, 60, 61, 74
 Walsh, J. P., 251, 294, 296, 299, 307
 Wang, C., 63
 Wang, E., 236, 237, 245, 250, 252, 253, 277, 280
 Wang, F., 244
 Wang, H., 174
 Wang, J., 188, 237
 Wang, J. P., 150
 Wang, L., 101
 Wang, S., 63
 Wang, S. W., 279
 Wang, T., 30, 31
 Wang, X., 65
 Washington, M., 214
 Wasik, M. A., 136, 137
 Watanabe, H., 157, 159, 160, 163
 Watanabe, M., 92
 Waterfield, M. D., 118, 132, 134
 Watt, S. A., 150
 Wattenberg, B. W., 237, 238
 Watterson, K. R., 244, 263
 Watton, S., 133
 Waugh, D. S., 34
 Webb, L., 121, 123
 Weber, G., 21, 22
 Webster, K., 214
 Wehner, S., 280
 Weintraub, S. T., 107
 Weiss, W. A., 136
 Weissshuhn, C. M., 182
 Weissman, J. S., 306
 Weissmann, C., 33
 Wenk, M. R., 156, 157, 161, 189, 214
 Wente, S. R., 172, 173, 174
 Wetzker, R., 214
 Whetton, A. D., 197, 203, 213, 220
 White, D. L., 18
 Whiteford, C. C., 223
 Widmer, S., 18, 19, 21
 Wiederrecht, G., 136
 Wielinga, P., 259
 Wijesinghe, D. S., 265, 268, 274, 276, 279
 Wijewickrama, G. T., 18, 19, 20, 23
 Wijnholds, J., 259
 Wilbanks, G. D., 94, 214
 Wilkie, T. M., 30, 31
 Williams, J., 136
 Williams, M. A., 94
 Williams, O., 136
 Williams, R., 118
 Wilson, D. B., 214
 Wilson, M. P., 174
 Wilson, P. B., 74
 Winiski, A., 279
 Wirtz, K. W., 158
 Wissing, J. B., 306, 307, 311, 312, 313
 Withers-Martinez, C., 3
 Wlachos, A., 248, 253, 268
 Wlodarski, P., 136, 137
 Wofford, J. A., 172, 173
 Wohltmann, M., 16
 Woisetschlager, M., 248, 253
 Wong, H., 3, 4
 Worthylake, D. K., 30
 Woscholski, R., 118, 132
 Wright, J., 120
 Wu, D., 30, 31, 46
 Wu, G., 300
 Wu, H., 149, 150
 Wu, M., 214
 Wu, W.-I., 305, 306, 307, 310, 311, 312, 313
 Wu, W. J., 60, 61
 Wu, Y., 31, 46, 174
 Wullschleger, S., 135, 138
 Wurmser, A. E., 188
 Wykle, R. L., 105, 106, 107, 113
 Wymann, M. P., 118, 121, 123, 156
 Wytack, F., 174

X

Xia, H. J., 173
 Xia, P., 237, 238
 Xiang, Y., 49
 Xiao, H. Q., 94
 Xiao, Y., 214
 Xie, Z., 51
 Xu, Y., 94, 214, 245, 248

Y

Yamada, K., 107
 Yamaguchi, A., 259
 Yamamoto, A., 4
 Yamamoto, M., 200
 Yamashita, T., 244, 268
 Yamatani, K., 189
 Yamazaki, M., 157, 159, 160, 163, 165
 Yamori, T., 91, 92, 95
 Yanada, K., 295
 Yanagida, K., 90
 Yang, H., 63, 64
 Yang, J., 189
 Yang, L., 258
 Yang, X., 174
 Yang, Y., 4
 Yasuda, K., 92, 94
 Yasuda, S., 294
 Yatomi, Y., 92, 94, 258
 Yazaki, Y., 156, 157
 Ye, H., 189
 Ye, X., 90
 Yin, H. L., 156, 188, 200
 Yokozeki, T., 155, 157, 159, 160, 163, 165
 Yonemoto, W., 160

Yoon, E. T., 18
 Yopp, A., 259
 York, J. D., 171, 172, 173, 174,
 176, 179, 182
 York, S. J., 172
 Yoshida, K., 59, 63, 66
 Yoshimura, T., 107
 Yoshino, K., 161
 You, H., 118
 Yu, S., 258
 Yuan, C., 29
 Yusuf, I., 134, 141

Z

Zaman, G. J., 259
 Zebol, J. R., 238
 Zeimet, G. M., 313
 Zelcer, N., 259
 Zemmann, B., 248, 253
 Zhang, G., 291
 Zhang, L., 197
 Zhang, M., 137, 278, 279
 Zhang, Q. X., 306
 Zhang, S. H., 4
 Zhang, Y., 60, 67, 101, 135
 Zheng, L., 65
 Zhou, J., 245
 Zhu, X., 134, 141
 Zimmerman, G. A., 106
 Zlabinger, G., 248, 253
 Zoncu, R., 157, 161
 Zoumpoulidou, G., 134
 Zunder, E. R., 136
 Zwartkruis, F. J., 136
 Zweifach, A., 16

Subject Index

A

- Acidified Bligh and Dyer extraction,
 - phosphoinositides, 194–196
- $\beta 2$ Adaptin, phosphatidylinositol 4-phosphate
 - 5-kinase interactions
 - coimmunoprecipitation, 165–166
 - glutathione *S*-transferase pull-down assay, 164–165
 - protein purification, 161
 - stimulation assays, 161–163
- ADP-ribosylating factor
 - phosphatidylinositol 4-phosphate 5-kinase interactions
 - protein purification, 159–161
 - stimulation assays, 161–163
 - phospholipase D activation studies with Arf1
 - assay, 61–62
 - kinetic analysis, 65–69
 - purification of protein, 60
 - synergistic effects, 62, 69
- Akt, phosphatidylinositol 3-kinase signaling
 - phosphoprotein assays in B cells
 - B cell stimulation, harvesting, and lysis, 138–139
 - flow cytometry detection of phosphoprotein
 - advantages and limitations, 148–150
 - B cell inhibitor treatment, stimulation, and harvesting, 144
 - data analysis and interpretation, 145, 147
 - overview, 142–143
 - phosphorylation detection, 144–145
 - surface marker staining for cell type discrimination, 143–144
 - inhibitor selection, 137–138
 - rationale, 134
 - Western blot and interpretation, 139–142
- ARF, *see* ADP-ribosylating factor
- Autotaxin
 - assays
 - colorimetric assay, 94
 - coupled assay, 94
 - fluorescence assay
 - incubation conditions and analysis, 100
 - kinetic analysis, 100
 - materials, 99
 - overview, 98
 - substrate preparation, 100

radioassay

- buffer, 96
- incubation conditions and analysis, 96
- kinetic analysis, 97–98
- materials, 95
- substrate preparation, 96
- domains, 91–92
- feedback inhibition, 93
- lysophosphatidic acid synthesis, 91–92
- overexpression in cancer, 92
- physiological function, 92
- prospects for study, 100–102
- recombinant protein expression in human
 - embryonic kidney cells, 94–95
- substrate specificity, 93

B

- Baculovirus–insect cell expression system
 - ceramide kinase expression, 270–271
 - $G\beta\gamma$ expression, 34–35
 - phospholipase $C\beta$ expression, 31–32
 - phospholipase D1 expression, 52
- B cell, phosphatidylinositol 3-kinase
 - signaling assays
 - Akt phosphorylation, 134
 - B cell stimulation, harvesting, and lysis, 138–139
 - extracellular signal-regulated kinase
 - phosphorylation, 136–137
 - flow cytometry detection of phosphoproteins
 - advantages and limitations, 148–150
 - Akt phosphorylation detection, 144–145
 - B cell inhibitor treatment, stimulation, and harvesting, 144
 - data analysis and interpretation, 145, 147
 - extracellular signal-regulated kinase
 - phosphorylation detection, 144–145
 - overview, 142–143
 - S6 phosphorylation detection, 146
 - surface marker staining for cell type discrimination, 143–144
 - inhibitor selection, 137–138
 - mammalian target of rapamycin activation, 135–136
 - overview, 133–134
 - Western blot and interpretation, 139–142

C

- Cdc42, phospholipase D activation studies
 assay, 61–62
 kinetic analysis, 65–69
 purification of protein, 60–61
 synergistic effects, 62, 69
- Ceramide kinase
 function, 266, 268–269
 isoforms, 236
 liquid chromatography–mass spectrometry
 assay using C17 sphingoid bases,
 236–237
 mixed micellar assays
 ceramide solubilization
 β -octylglucoside, 275
 Triton X-100, 276
 incubation conditions, 276
 liquid chromatography–tandem mass
 spectrometry, 277–278
 materials, 275
 principles, 272, 274
 scintillation counting, 277
 thin-layer chromatography, 276–277
 purification of recombinant enzyme
 chromatography, 272
 expression systems
 adenovirus, 271
 baculovirus–insect cell
 system, 270–271
 materials, 270
 principles, 269
 quantitative polymerase chain reaction of
 messenger RNA levels, 288–289
 RNA interference knockdown, 286–288
 structure, 267–268
 subcellular localization
 adenovirus transfection, 286
 antibody dilutions, 286
 confocal microscopy, 284–285
 materials, 284
 overview, 236
 principles, 284
 substrate specificity, 236
- Ceramide-1-phosphate
 ceramide kinase assay
 liquid chromatography–tandem mass
 spectrometry, 277–278
 scintillation counting, 277
 thin-layer chromatography, 276–277
 ceramide kinase product analysis in cells,
 281–283
 delivery to cells in tissue culture
 dispersion
 long-chain ceramide-1-phosphate, 280
 short-chain ceramide-1-phosphate, 280
 incubation conditions, 281

- materials, 280
 principles, 278–280
 functions, 268–269

CERK, *see* Ceramide kinase

Coimmunoprecipitation, phosphatidylinositol
 4-phosphate 5-kinase- β 2 adaptin
 interactions, 165–166

Confocal microscopy, ceramide kinase subcellular
 localization
 adenovirus transfection, 286
 antibody dilutions, 286
 confocal microscopy, 284–285
 materials, 284
 principles, 284

D

- DAG lipase, *see* Diacylglycerol lipase
- DAPC, *see* 1-O-(1-(6-Dimethylamino)
 naphthoylacetyl)-2-arachidonoyl-
 sn-glycero-3-phosphocholine
- DGK, *see* Diacylglycerol kinase
- Diacylglycerol kinase
 assays
 ATP initiation, 298
 cultured cells
 cell labeling and harvesting, 302
 extraction, 302
 principles, 301–302
 thin-layer chromatography, 302–303
 enzyme preparation, 297–298
 incubation conditions, 298–299
 kinetic analysis, 299
 liposome assays, 296
 micelle assays, 295–296
 product extraction, 299
 substrate preparation, 296–297
 function, 294
 isoforms, 294
 structure, 294–295
 subcellular compartment analysis
 membrane-depleted nuclei
 preparation, 301
 nuclear isolation, 300–301
- Diacylglycerol lipase, fluorescence real-time assay
 incubation conditions and analysis, 84–85
 overview, 60
 substrate synthesis, 80, 82–84
- 1-O-(1-(6-Dimethylamino)
 naphthoylacetyl)-2-arachidonoyl-
 sn-glycero-3-phosphocholine
 calcium-dependent cytosolic phospholipase A2
 assay, 21–22, 24–25
 synthesis, 24–25
- Diphosphoinositol pentakisphosphate, *see* Inositol
 phosphates
- DPPI1, *see* Lipid phosphate phosphatase

E

- Erk, *see* Extracellular signal-regulated kinase
- Extracellular signal-regulated kinase,
 phosphatidylinositol 3-kinase signaling
 phosphoprotein assays in B cells
 B cell stimulation, harvesting, and lysis,
 138–139
 flow cytometry detection of phosphoprotein
 advantages and limitations, 148–150
 B cell inhibitor treatment, stimulation, and
 harvesting, 144
 data analysis and interpretation, 145, 147
 overview, 142–143
 phosphorylation detection, 144–145
 surface marker staining for cell type
 discrimination, 143–144
 inhibitor selection, 137–138
 rationale, 136–137
 Western blot and interpretation,
 139–142

F

- Flow cytometry, phosphatidylinositol 3-kinase
 signaling assays in B cells
 advantages and limitations, 148–150
 Akt phosphorylation detection, 144–145
 B cell inhibitor treatment, stimulation, and
 harvesting, 144
 data analysis and interpretation, 145, 147
 extracellular signal-regulated kinase
 phosphorylation detection, 144–145
 overview, 142–143
 S6 phosphorylation detection, 146
 surface marker staining for cell type
 discrimination, 143–144

G

- Gel filtration chromatography
 phospholipase C β -G $\beta\gamma$ interaction analysis, 45
 phospholipase D1 purification, 53, 55
- Glycerophosphoinositols, *see* Phosphoinositides
- G protein–phospholipase C β interactions
 activation, 30
 activity assay
 calcium solution preparation, 40
 calculations, 41
 incubation conditions and liquid
 scintillation counting, 40–41
 lipid vesicle preparation, 38–39
 optimization, 41–42
 overview, 37–38
 protein solution preparation, 39–40
 binding regions, 31
 gel filtration chromatography, 45

- isoform sensitivity, 30
modulator evaluation, 42–43
mutant studies, 45–46
pull-down assay, 44–45
purification of proteins
 G $\beta\gamma$
 cell harvesting and lysis, 35–36
 chromatography, 36–37
 expression in baculovirus–insect cell
 system, 34–35
 phospholipase C β
 cell lysis, 32
 expression in baculovirus–insect cell
 system, 31–32
 heparin affinity chromatography, 33
 nickel affinity chromatography, 32–33
 structure of complex, 30
- G protein–phospholipase D interactions, *see*
 specific G proteins

H

- High-performance liquid chromatography
 inositol phosphates, 179–180, 207, 209
 mass spectrometry coupling, *see* Mass
 spectrometry
 phosphoinositides
 deacylated phosphoinositides, 122
 desalting, 202–203, 209–211
 direct Econosphere NH2 HPLC, 201–202
 Nucleodex β -OH HPLC, 206–207
 Partisil 5-PAC HPLC, 209
 Partisil 5 WAX HPLC, 207, 209
 Partisil 10 SAX HPLC
 formate gradient, 206
 indirect analysis of deacylated
 phosphoinositides, 199–201
 scintillant extraction, 211–212
 HPLC, *see* High-performance liquid
 chromatography

I

- Inositol phosphate kinases
 expression and purification
 recombinant proteins in *Escherichia coli*, 175
 vectors, 174
 human IHPK1 kinetic analysis, 178–179
 mammalian pathways, 172–174
 synthesis of inositol phosphate standards and
 substrates, 175–177
 types, 173
 yeast pathways, 172–173
- Inositol phosphates
 diphosphoinositol pentakisphosphate analysis
 by proton-decoupled phosphorous-31
 nuclear magnetic resonance, 180–182

Inositol phosphates (*cont.*)
 enzymatic synthesis for use as standards and
 substrates, 175–177
 high-performance liquid chromatography,
 179–180, 207, 209
 metabolism, 172–173, 189, 191
 thin-layer chromatography, 177–178
 IPs, *see* Inositol phosphates

L

Lipid phosphate phosphatase
 assay
 extraction and quantification, 307–308
 incubation conditions, 307
 substrate preparation, 307
 function, 306–307
 isoforms in yeast
 comparison of DPP1 and LPP1 properties,
 311–313
 overview, 306
 sequencing and identification, 310–311
 purification from yeast
 affinity chromatography, 309
 anion-exchange chromatography,
 309–310
 cell extract preparation, 308
 culture, 308
 hydroxylapatite chromatography, 309
 microsomal membrane preparation, 308
 purification table, 310–311
 Triton X-100 extract preparation, 308
 structure, 306
 Lipid phosphate phosphohydrolase
 isoforms, 245
 sphingosine-1-phosphate phosphohydrolase
 comparison, 245–246
 LPA, *see* Lysophosphatidic acid
 LPP, *see* Lipid phosphate phosphohydrolase
 LPP1, *see* Lipid phosphate phosphatase
 Lysophosphatidic acid, *see also* Autotaxin
 functions, 90
 receptors, 90
 synthetic pathways, 90–91
 Lysophosphatidylinositol 4-phosphate, *see*
 Phosphoinositides
 Lysophospholipase D, *see* Autotaxin

M

Mammalian target of rapamycin,
 phosphatidylinositol 3-kinase signaling
 phosphoprotein assay in B cells
 B cell stimulation, harvesting, and lysis,
 138–139
 inhibitor selection, 137–138
 rationale, 135–136
 Western blot and interpretation, 139–142

Mass spectrometry
 ceramide-1-phosphate quantification with
 liquid chromatography–tandem mass
 spectrometry, 277–278
 phosphoinositide electrospray
 ionization–tandem mass spectrometry
 glycerophosphoinositol 4-phosphate versus
 methylphosphoinositol 4-phosphate,
 216–217, 219
 glycerophosphoinositols, 215–216
 overview, 214–215
 standards
 cyclic inositol phosphates, 224–225
 glycerophosphoinositol 5-phosphate,
 222–224
 InsP(n-1), 219–221
 lysophosphatidylinositol 4-phosphate,
 221–222
 methylphosphoinositol 4-phosphate,
 225–226
 phospholipase A1 assay
 incubation conditions and analysis, 8, 10
 intracellular enzyme activity, 10–11
 materials, 6
 recombinant enzyme preparation, 6–8
 platelet-activating factor liquid
 chromatography–mass spectrometry
 assay
 cleanup of samples, 112
 lipid extraction, 109–112
 materials, 107–108
 overview, 106–107
 quantitative analysis, 113, 115
 running conditions, 113
 standard curves, 108–109
 sphingolipid analysis using liquid
 chromatography–mass spectrometry of
 C17 sphingoid bases
 cell labeling with C17 sphingoid base, 239
 ceramide synthase assay, 236–237
 overview, 234–235
 sphingosine kinase assay, 238–239
 Mitogen-activated protein kinase, *see*
 Extracellular signal-regulated kinase
 MS, *see* Mass spectrometry
 mTOR, *see* Mammalian target of rapamycin
 activation

N

Neomycin, bead-based purification of total
 phosphoinositides, 126
 NMR, *see* Nuclear magnetic resonance
 NPP2, *see* Autotaxin
 Nuclear magnetic resonance, diphosphoinositol
 pentakisphosphate analysis by proton-
 decoupled phosphorous-31 spectroscopy,
 180–182

P

- PAF, *see* Platelet-activating factor
- PCR, *see* Polymerase chain reaction
- Perchloric acid, Bligh and Dyer extraction of
phosphoinositides, 194–196
- Phosphatidylinositol 4,5-bisphosphate
functions, 156
phospholipase D activation studies, 69,71,73,74
synthesis, *see* Phosphatidylinositol
4-phosphate 5-kinase
- Phosphatidylinositol 3-kinase
activation assays, 119–120
- B cell signaling assays
Akt phosphorylation, 134
B cell stimulation, harvesting, and lysis,
138–139
extracellular signal-regulated kinase
phosphorylation, 136–137
flow cytometry detection of
phosphoproteins
advantages and limitations, 148–150
Akt phosphorylation detection, 144–145
B cell inhibitor treatment, stimulation,
and harvesting, 144
data analysis and interpretation, 145, 147
extracellular signal-regulated kinase
phosphorylation detection, 144–145
overview, 142–143
S6 phosphorylation detection, 146
surface marker staining for cell type
discrimination, 143–144
inhibitor selection, 137–138
mammalian target of rapamycin activation,
135–136
overview, 133–134
Western blot and interpretation, 139–142
- class I signaling pathway, 118
- classes, 132
- inhibitors, 132
- knockout mice, 132
- product quantification, *see* Phosphatidylinositol
3,4,5-trisphosphate
- Phosphatidylinositol 4-phosphate 5-kinase
activators
 β 2 adaptin preparation, 161
ADP-ribosylating factor purification,
159–161
coimmunoprecipitation, 165–166
glutathione *S*-transferase pull-down assay,
164–165
overview, 157
stimulation assays, 161–163
talin head domain preparation, 161
functions, 156–157
isoforms, 156
purification of recombinant FLAG-tagged
protein, 158–159
- Phosphatidylinositol 3,4,5-trisphosphate
assays
comparison of assays, 126–128, 150
protein–lipid overlay assay
neomycin bead-based purification of
total phosphoinositides, 126
neutrophil stimulation and lipid
extraction, 125
overlay and analysis, 126
overview, 122–123
recombinant GRP1 PH domain
preparation, 123, 125
radioassay
deacylation of extracted lipids, 122
extraction of lipids, 121–122
high-performance liquid
chromatography of deacylated
lipids, 122
inorganic phosphate labeling of cells, 121
monoethylamine reagent preparation, 121
overview, 120–121
metabolism, 118
PH domain proteins in signaling, 118–119
- Phosphoinositides, *see also specific compounds*
chemical identification
acidified butanol, 214
periodate oxidation, 212–214
electrospray ionization–tandem mass
spectrometry
glycerophosphoinositol 4-phosphate versus
methylphosphoinositol 4-phosphate,
216–217, 219
glycerophosphoinositols, 215–216
overview, 214–215
standards
cyclic inositol phosphates, 224–225
glycerophosphoinositol 5-phosphate,
222–224
InsP(*n*-1), 219–221
lysophosphatidylinositol 4-phosphate,
221–222
methylphosphoinositol 4-phosphate,
225–226
extraction from cell samples
acidified Bligh and Dyer extraction,
194–196
neutral extraction, 196–197
overview, 191
high-performance liquid chromatography
deacylated phosphoinositides, 122
desalting, 202–203, 209–211
direct Econosphere NH2
HPLC, 201–202
Nucleodex β -OH HPLC, 206–207
Partisil 5-PAC HPLC, 209
Partisil 5 WAX HPLC, 207, 209
Partisil 10 SAX HPLC
formate gradient, 206

- Phosphoinositides, *see also specific compounds (cont.)*
indirect analysis of deacylated
phosphoinositides, 199–201
scintillant extraction, 211–212
metabolism, 188–191
neomycin bead-based purification, 126
thin-layer chromatography, 197–199
- Phospholipase A1
assays
mass spectrometry assay
incubation conditions and
analysis, 8, 10
intracellular enzyme activity, 10–11
materials, 6
recombinant enzyme preparation, 6–8
overview, 2
radioassay
incubation conditions and analysis, 5
materials, 3
radiolabeled phospholipid substrate
preparation, 3, 5
prospects for study, 11
types, 2–4
- Phospholipase A2
classification, 16
fluorescence real-time assay
applications, 22–23
calcium-dependent cytosolic enzyme
assay, 24–25
secretory enzyme assay, 23–24
substrates
design, 20–22
enzyme isoform substrate specificity, 18
fluorophore selection, 18–20
structures, 17
functions, 16
- Phospholipase C β
functions, 30
G protein interactions
activation, 30
activity assay
calcium solution preparation, 40
calculations, 41
incubation conditions and liquid
scintillation counting, 40–41
lipid vesicle preparation, 38–39
optimization, 41–42
overview, 37–38
protein solution preparation, 39–40
binding regions, 31
gel filtration chromatography, 45
isoform sensitivity, 30
modulator evaluation, 42–43
mutant studies, 45–46
pull-down assay, 44–45
purification of G $\beta\gamma$
cell harvesting and lysis, 35–36
chromatography, 36–37
expression in baculovirus–insect cell
system, 34–35
structure of complex, 30
isoforms, 30
purification of histidine-tagged protein
cell lysis, 32
expression in baculovirus–insect cell system,
31–32
heparin affinity chromatography, 33
nickel affinity chromatography, 32–33
- Phospholipase D
activator studies
assay, 61–62
kinetic analysis, 65–69
phosphatidylinositol 4,5-bisphosphate,
69, 71, 73, 74
purification of activators
Arf1, 60
Cdc42, 60–61
protein kinase C α , 60
Rac1, 60–61
RhoA, 60–61
synergistic effects, 62, 69
activity assays
fluorescence assay, 77–80
in vitro, 57, 59
in vivo assay using deuterated 1-butanol,
74–76
domains, 51
functions, 50–51
isoforms, 50–51
lysophospholipase D, *see* Autotaxin
phospholipid vesicle binding assays, 63–65
protein–protein interactions, 51
purification of phospholipase D1
amino-terminal truncated protein, 56–57
anion-exchange chromatography, 55–56
biophysical characteristics, 56
expression in baculovirus–insect cell
system, 52
gel filtration, 53, 55
nickel affinity chromatography, 52–53
PI3K, *see* Phosphatidylinositol 3-kinase
PIP2, *see* Phosphatidylinositol 4,5-bisphosphate
PIP3, *see* Phosphatidylinositol 3,4,
5-trisphosphate
PIP5K, *see* Phosphatidylinositol 4-phosphate
5-kinase
- Platelet-activating factor
bioassays, 106
liquid chromatography–mass
spectrometry assay
cleanup of samples, 112
lipid extraction, 109–112
materials, 107–108
overview, 106–107
quantitative analysis, 113, 115
running conditions, 113

- standard curves, 108–109
- structure, 105–106
- Polymerase chain reaction, ceramide kinase
 - quantitative polymerase chain reaction of messenger RNA levels, 288–289
- Protein kinase C α , phospholipase D activation studies
 - assay, 61–62
 - purification of protein, 60
 - synergistic effects, 62

R

- Rac1, phospholipase D activation studies
 - assay, 61–62
 - kinetic analysis, 65–69
 - purification of protein, 60–61
 - synergistic effects, 62, 69
- RhoA, phospholipase D activation studies
 - assay, 61–62
 - kinetic analysis, 65–69
 - purification of protein, 60–61
 - synergistic effects, 62, 69
- RNA interference, ceramide kinase knockdown, 286–288

S

- S6, phosphatidylinositol 3-kinase signaling assay
 - in B cells, flow cytometry detection of phosphoprotein
 - advantages and limitations, 148–150
 - B cell inhibitor treatment, stimulation, and harvesting, 144
 - data analysis and interpretation, 145, 147
 - overview, 142–143
 - phosphorylation detection, 146
 - surface marker staining for cell type discrimination, 143–144
- Sphingolipids, *see also specific sphingolipids*
 - functions, 234, 244
 - liquid chromatography–mass spectrometry of C17 sphingoid bases
 - cell labeling with C17 sphingoid base, 239
 - ceramide synthase assay, 236–237
 - overview, 234–235
 - sphingosine kinase assay, 238–239
- Sphingosine kinase
 - functions, 237, 244
 - isoforms, 237–238
 - liquid chromatography–mass spectrometry assay using C17 sphingoid bases, 238–239
 - structure, 238
 - subcellular localization, 238
 - tissue distribution, 238
- Sphingosine-1-phosphate assay
 - adherent cell labeling, 260–261

- calculation, 261
 - differential extraction of sphingosine and sphingosine-1-phosphate, 260
 - materials, 259
 - nonadherent cell labeling, 261
 - principles, 259
 - tritium labeling, 260
 - functions, 258
 - receptor, 258
 - secretion, 268
 - sphingosine kinase effects of formation and secretion, 262–263
 - synthesis, 258
 - transporters, 259
 - Sphingosine-1-phosphate phosphohydrolase activity assays
 - cell lysate preparation, 249
 - incubation conditions and analysis, 251
 - live cell assay
 - incubation conditions, 252
 - sphingosine-1-phosphate uptake and hydrolysis in nonpermeabilized cells, 252–253
 - thin-layer chromatography of sphingoid base phosphates, 253
 - principles, 249
 - substrate radiolabeling, 250–251
 - function, 244
 - isoforms, 245–247
 - knockdown studies, 247–248
 - overexpression studies, 247–248
 - yeast mutant studies, 246, 248
- SPP, *see* Sphingosine-1-phosphate phosphohydrolase

T

- Talin, phosphatidylinositol 4-phosphate 5-kinase interactions
 - glutathione *S*-transferase pull-down assay, 164–165
 - head domain preparation, 161
 - stimulation assays, 161–163
- Tandem mass spectrometry, *see* Mass spectrometry
- Thin-layer chromatography
 - ceramide-1-phosphate quantification, 276–277, 283
 - diacylglycerol kinase assay, 302–303
 - inositol phosphates, 177–178
 - sphingoid base phosphates, 253
- TLC, *see* Thin-layer chromatography

W

- Western blot, phosphatidylinositol 3-kinase signaling assays in B cells
 - Akt phosphorylation, 134
 - B cell stimulation, harvesting, and lysis, 138–139

- Western blot, phosphatidylinositol 3-kinase
 signaling assays in B cells (*cont.*)
 extracellular signal-regulated kinase
 phosphorylation, 136–137
 inhibitor selection, 137–138
- mammalian target of rapamycin activation,
 135–136
overview, 133–134
Western blot and interpretation,
 139–142