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Microbial Protein Toxins

With 41 Figures, 6 of Them in Color; and 3 Tables

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The cover illustration depicts pseudohyphal filaments of the ascomycete *Saccharomyces cerevisiae* that enable this organism to forage for nutrients. Pseudohyphal filaments were induced here in a wild-type haploid MATa Σ1278b strain by an unknown readily diffusible factor provided by growth in confrontation with an isogenic petite yeast strain in a sealed petri dish for two weeks and photographed at 100X magnification (provided by Xuewen Pan and Joseph Heitman).

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Diphtheria toxin, diphtheria-related fusion protein toxins, and the molecular mechanism of their action against eukaryotic cells

Ryan Ratts and John R. Murphy

Abstract

Diphtheria toxin remains one of the most successfully studied of the bacterial protein toxins. A detailed understanding of the structure function relationships of the toxin and the role of each domain in the intoxication process is well understood. This understanding has led to the development of diphtheria toxin-related fusion protein toxins which are targeted toward specific cell surface receptors. The first of these targeted toxins are now approved for human clinical use in the treatment of refractory hematologic malignancies and graft-versus-host disease. In recent years, studies on the molecular mechanism by which the diphtheria toxin catalytic domain is translocated across the early endosomal membrane has revealed that a host cell cytosolic translocation factor complex facilitates the entry process. A detailed understanding of this process will further stimulate the development of new approaches toward the delivery of cargo, ranging from protein to nucleic acid and/or protein nucleic acids, to the eukaryotic cell cytosol.

1 Diphtheria toxin

Diphtheria toxin was first described by (Roux and Yersin 1888) in the culture medium of *Corynebacterium diphtheriae*. Of all the bacterial A/B toxins which exert their toxicity by the translocation of their enzymatically active domains into the cytosol of eukaryotic cells, diphtheria toxin (DT) and the DT-based fusion protein toxins have been among the most extensively and successfully studied. Diphtheria toxin is naturally expressed by strains of *C. diphtheriae* which are lysogenic for one of a family of closely related corynebacteriophages whose genome carries the structural gene encoding the toxin, *tox.* Diphtheria toxin is produced in precursor form with a 25 amino acid signal peptide and is co-translationally secreted as a single polypeptide chain. Upon cleavage of the signal sequence the toxin is released into the culture medium in its mature form as a 535 amino acid residue protein with a molecular weight of 58 kDa (Smith 1980; Smith et al. 1980; Kaczorek et al. 1983; Greenfield et al. 1983).

Early biochemical analysis of diphtheria toxin revealed that the protein is exquisitely sensitive to serine protease (e.g. trypsin) attack which results in 'nicking'

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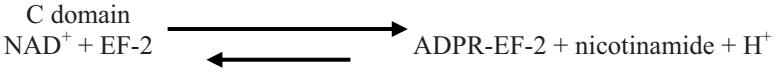
in vitro and the formation of two fragments, A and B, which remain covalently attached by an interchain disulfide bond (Gill and Pappenheimer 1971; Drazin et al. 1971). Characterization of non-toxic serologically related mutants (Uchida et al. 1971) demonstrated that fragment A, the N-terminal 21.1 kDa peptide, contains the enzymatic activity (Collier and Kandel 1971; Gill and Dinius 1971); whereas, fragment B, the C-terminal 37.1 kDa peptide, mediates binding of the toxin to its cell surface receptors and facilitates the delivery of fragment A to the cytosol (Gill and Dinius 1971; Drazin et al. 1971; Uchida et al. 1971). X-ray crystallographic analysis, at a resolution of 2.5 Å, demonstrated that diphtheria toxin is composed of three structural domains: the amino terminal catalytic (C) domain corresponds to fragment A; whereas, the transmembrane (T) and carboxy-terminal receptor binding (R) domains comprise fragment B (Choe et al. 1992; Bennett et al. 1994).

A disulfide bond between Cys186 and Cys201 of the toxin subtends a 14 amino acid protease sensitive loop and connects fragment A with fragment B (Gill and Pappenheimer 1971). Furin mediated cleavage within this loop and retention of the disulfide bond are prerequisites for both the DT and DT-related fusion protein toxins action against eukaryotic cells (Williams et al. 1990; Ariansen et al. 1993; Tsuneoka et al. 1993). While the mechanisms of enzymatic activity, receptor binding, and receptor mediated endocytosis are known in great detail, relatively little is known about the precise molecular mechanisms which mediate C domain translocation across the endosomal membrane and the release of an active appropriately folded enzyme into the cytosol.

2 Catalytic domain delivery into the eukaryotic cytosol results in an inhibition of protein synthesis

The intoxication of eukaryotic cells by either DT or the DT-related fusion protein toxins requires the productive delivery of the C domain from the lumen of an early endosomal compartment to the cytosol. It is well known that the C domain (amino acid residues 1 – 193, fragment A) catalyzes the NAD^+-dependent ADP-ribosylation of elongation factor 2 (EF-2). Elongation factor 2 is a soluble translocase involved in protein synthesis, and is the only known substrate for the DT C domain in eukaryotic cells (Pappenheimer 1977). The enzymatic transfer of the ADP-ribosyl (ADPR) moiety from NAD^+ to a modified histidine residue in EF-2 (diphthamide), as shown below, results in the irreversible arrest of polypeptide chain elongation, which ultimately leads to cell death (Collier et al. 1969).



The DT C domain has been shown to ADP-ribosylate EF-2 from archea to man (Pappenheimer et al. 1972); whereas, the homologous elongation factors from both prokaryotic cells and mitochondria are not recognizable substrates (Richter et al. 1970). The crystal structure of DT indicates that the C domain is composed of

eight β-strands and seven α-helices. Binding of NAD^+ is mediated by residues His21 and Tyr65 (Papini et al. 1989, 1990, 1991), and Glu148 also plays a key role in catalysis (Carroll et al. 1984). The requirement for NAD^+ is highly specific and it cannot be replaced by $NADP^+$ or NADH (Goor et al. 1967).

The role that the C domain plays in its own delivery to the cytosol of targeted cells is, as yet, unknown. However, the complete unfolding of the C domain was postulated more than twenty years ago as a prerequisite for translocation (Donovan et al. 1981; Kagan et al. 1981). The necessity for complete unfolding of the C domain prior to translocation was then indirectly demonstrated by Wiedlocha et al. (1992) and by Falnes et al. (1994). Wiedlocha et al. (1992) genetically fused acidic fibroblast growth factor (aFGF) to the amino terminus of the C domain. The aFGF-DT fusion protein was found to be cytotoxic for eukaryotic cells, confirming the observation that polypeptides fused to fragment A may be delivered into the cytosol of targeted cells (Stenmark et al. 1991). In the presence of heparin, however, aFGF is known to retain a rigid tertiary structure and the aFGF-DT fusion protein was no longer cytotoxic. These results strongly implied that unfolding is a requirement for delivery through the nascent channel formed by the T domain.

Based upon the X-ray crystal structure of the toxin, Falsnes et al. (1994) constructed a double cysteine insertion mutant which formed an internal disulfide bond within the C domain. Under oxidizing conditions, with the disulfide bond intact, unfolding of the C domain is prevented. While this mutant toxin retained ADP-ribosyltransferase activity *in vitro*, it was not cytotoxic toward cells in culture. These results provided additional indirect evidence that complete unfolding of the C domain was prerequisite for intoxication of eukaryotic cells. In the case of the DT-related fusion protein toxins, tracking of internalized epitope tagged toxin has also been used to demonstrate that the C domain is denatured and loses its enzymatic activity in lumen of acidified early endosomes (Zeng 1998). Taken together, these studies indicate that the cytosolic delivery of the C domain occurs in at least a partially unfolded enzymatically inactive state.

3 Receptor binding and substitution of the native diphtheria toxin receptor binding domain with surrogate ligands

As with all of the A/B bacterial protein toxins, the intoxication of target cells by DT and DT-related fusion protein toxins begins with receptor binding, followed by receptor mediated endocytosis. The cell surface receptor for DT, DTR, has been shown to be the heparin binding epidermal growth factor-like precursor (Naglich et al. 1992a, 1992b). The relative sensitivity of a given cell line to intoxication by DT is related to the number of receptors present on the cell surface (Middlebrook et al. 1978; Dorland et al. 1979). Analysis of diphtheria toxin binding has also suggested that the sensitivity of targeted cells is also dependent upon the presence of DTR modulators, such as the human CD9 antigen (Mitamura et al.

1992). Human CD9 antigen, which is associated with the DTR (Mitamura et al. 1992; Iwamoto et al. 1994), enhances sensitivity to DT through an as yet unknown mechanism.

Analysis of the X-ray crystal structures indicates that the receptor binding (R) domain of native DT is composed of residues 386-535 (Choe et al. 1992; Bennett et al. 1992). Substitution of the native R domain with a surrogate cell surface receptor specific ligand has been shown to result in the formation of a fusion protein toxin whose action is targeted to only those cells which express that cell surface receptor. The first genetically engineered fusion protein toxin, $DAB_{486}\alpha MSH$, consisted of the diphtheria toxin fragment A and a portion of fragment B fused to α-melanocyte stimulating hormone (α-MSH) (Murphy et al. 1986). While $DAB_{486}\alpha MSH$ was selectively toxic toward cells that displayed the MSH receptor, it was prone to extensive proteolytic degradation when expressed in recombinant *E. coli.* Human interleukin-2 (hIL-2), 133 amino acid peptide versus the 13 amino acid aMSH, was used as the next surrogate receptor binding domain (Williams et al. 1987). Remarkably, DAB_{486}IL-2 was more stable when expressed in *E. coli* and was found to be extremely potent (IC_{50} of 2-4 x 10^{-11} M) against only those cells that displayed the high affinity receptor for IL-2 (Bacha et al. 1980; Williams et al. 1990a, 1990b). Deletion analysis of the carboxy-terminal residues in the diphtheria toxin portion of DAB_{486}IL-2 genetically mapped the junction between the transmembrane (T) domain of the toxin and the receptor binding domain (Williams et al. 1990a, 1990b). The internal in-frame deletion mutant, DAB_{389}IL-2, was shown to be at least a log more potent (*e.g.* IC_{50} = 2 -5 x 10^{-12} M) toward high affinity IL-2 receptor bearing cell *in vitro*.

In the case of DAB_{389}IL-2, only the high affinity and intermediate affinity IL-2 receptor:: fusion protein toxin complexes are internalized (Re et al. 1996). The IL-2 receptor is composed of three subunits α, β, and γ_c (Takeshita et al. 1992). The α subunit alone forms the low affinity receptor (Kd ~10 nM), while the β and γ_c subunits form a heterodimeric intermediate affinity receptor (Kd ~5 nM). All three subunits together form a heterotrimeric high affinity receptor (Kd ~60 pM). IL-2 is a hematopoietic cytokine, and binding to the high affinity IL-2 receptor stimulates the activation of genes promoting DNA synthesis and the proliferation of IL-2 dependent cells (Rayhel et al. 1988). Signaling pathways mediated by the IL-2 receptor are a requirement for T-cell activation and proliferation, and IL-2 receptor expression is frequently upregulated in leukemia and lymphoma tumors of T-cell origin (Waldmann 1989). Expression of the high affinity IL-2 receptor is also an obligatory event in the development of T-cell mediated immune response, and upregulation of the receptor on auto-aggressive T-cells marks an early common event in the pathogenesis of essentially all autoimmune diseases (Ratts and vanderSpek 2002). The specific expression of the high affinity IL-2 receptor on only activated and proliferating T-cells suggested that intervention with DAB_{389}IL-2 could result in a beneficial outcome for patients presenting with CD25 positive (α-chain of the IL-2 receptor) malignancies and autoimmune diseases. Indeed in 1998, DAB_{389}IL-2 (Ontak®) was approved by the U.S. Food and Drug Administration for the treatment of refractory cutaneous T cell lymphoma,

and current studies have shown this fusion protein toxin to be an effective therapeutic in the treatment of steroid resistant graft vs. host disease (Ho et al. 2004). Based on the success of DAB_{389}IL-2 in the clinic, a growing number of diphtheria toxin based fusion protein toxins have been constructed as potential therapeutics for a variety of malignant oncological disorders (Ratts and vanderSpek 2002).

4 Intoxication of target cells requires the toxin to pass through a low pH endosomal compartment

Ammonium salts (e.g. NH_4Cl), glutamine, certain other amines, and choloroquine were the first compounds found to block the action of diphtheria toxin by inhibiting the cytosolic delivery of the C domain (Kim et al. 1965). It is well known that choloroquine and ammonium salts are ionophores, and raise the luminal pH of endocytidic vesicles and lysosomes (Wibo et al. 1974). These early results led to the hypothesis that passage of diphtheria toxin through an acidic compartment was an integral step in the intoxication process. Umata et al. (1990) confirmed the hypothesis by demonstrating that acidification of the endosomal lumen by membrane associated vesicular (v)-ATPase was, in fact, a required step in DT intoxication. The v-ATPase inhibitor bafilomycin A1 (Bowman et al. 1988) blocked acidification of the endosomal lumen and protected cells against DT intoxication (Umata et al. 1990).

In contrast to the internalization and endosomal route of entry, brief exposure of toxin bound to the surface of cells to low pH (*e.g.* 5.0) results in the delivery of the C domain to the cytosol and a decrease protein synthesis even in the presence of choloroquine and ammonium ions (Sandvig et al. 1980). This study also demonstrated that the entry of pre-nicked diphtheria toxin through the cell membrane in the low pH environment was time and temperature dependent. Using the same system, Sandvig and Olsnses (1981b) also demonstrated that the cytosolic entry of the C domain could be blocked by the metabolic inhibitors 2-deoxyglucose and sodium azide, implying that ATP was required for the membrane translocation (Sandvig et al. 1981b). Proteinase protection assays were used to examine which portions of diphtheria toxin inserted into the plasma membrane when toxin bound cells were exposed to low pH (Moskaug et al. 1988). These investigators described the translocation of fragment A (20 kD) into the cytosol and a plasma membrane associated 25 kDa peptide derived from fragment B. Furthermore, an inwardly directed proton gradient was required for the translocation of fragment A, but not for membrane insertion of fragment B (Sandvig et al. 1988). In addition, it has been shown that translocation of fragment A into the cytosol requires a lower pH then the membrane insertion of fragment B (Stenmark et al. 1989; Falnes et al. 1992).

5 Internalization of the toxin by receptor mediated endocytosis

Diphtheria toxin reaches an endosomal compartment following internalization of a toxin-receptor complex into clathrin coated pits. Clathrin is recruited to the plasma membrane by the tetrameric adapter protein (AP) complex AP2 (Chang et al. 1993). The membrane association of AP2 is dependent upon interactions with membrane phospholipids (*e.g.* phosphoinositides) (Jost et al. 1998), membrane proteins (Chang et al. 1993), and specific sorting signals in the cytoplasmic tails of receptors and other cargo materials (Fingerhut et al. 2001). Coat assembly results in the formation of a deeply invaginated constricted pit containing clustered ligand-receptor complexes (Keen et al. 1982; Hanover et al. 1984). Assembly of the clathrin coat may be inhibited by depletion of intercellular potassium stores, and cells are protected against diphtherial intoxication under such conditions (Moya et al. 1985; Sandvig et al. 1985). Sequestration of the coated pit from the plasma membrane requires additional proteins, including the GTPase dynamin (Damke et al. 1995). Overexpression of dominant negative dynamin blocks clathrin dependent endocytosis and has also been found to protect cells against the action of diphtheria toxin (Simpson et al. 1998).

After entering the cytoplasm, these vesicles lose their clathrin coat and a rapid homotypic fusion of uncoated vesicles then results in the formation of an early endosomal compartment (Luzio et al. 2001). Each vesicle has a pair of SNARE proteins (soluble N-ethylmaleimide sensitive factor attachment protein receptor), a v-SNARE and t-SNARE, which are arranged in stable heterotetrameric structures of 'coiled-coils' called *cis*-SNARE complexes, *i.e.* complexes of SNAREs residing on the same membrane (Littleton et al. 2001). Priming of the vesicles for membrane fusion requires the untangling of the *cis*-SNARE pairs by an n-ethylmaleimide-sensitive factor (NSF) (Littleton et al. 2001). The untangled t-SNAREs are stabilized by LMA1 (low molecular weight activator), which is a heterodimer composed of thioredoxin (Trx) and an inhibitor of vacuolar proteinase B (IB-2) (Xu et al. 1997, 1998). LMA1 binds to vesicles in the presence of NSF, is transferred upon ATP hydrolysis to t-SNAREs, and is released during fusion in a phosphatase regulated reaction (Xu et al. 1997, 1998).

6 The early endosomal compartment

The acidification of endosomes is mediated by vesicular (v)-ATPase in a strictly ATP dependent process; whereas the acidification of lysosomes is stimulated by both ATP and GTP (Merion et al. 1983; Bowman et al. 1988). Merion et al. (1983) showed that endosomes isolated from DT resistant mutants of Chinese hamster ovary (CHO)-K1 cells were defective in acidification. In contrast, lysosomes isolated from the same mutants were not defective in acidification, suggesting that the endosomal compartment was the site of fragment A translocation (Merion et al. 1983).

Several studies have confirmed the early endosomal compartment as the site of fragment A translocation. The v-ATPase inhibitor bafilomycin A1 blocked acidification of the endosomal lumen and protected cells against DT intoxication (Bowman et al. 1988; Umata et al. 1990). Cell fractionation experiments also indicated that translocation of the DT C domain occurs from early endosomes (Papini et al. 1993a, 1993b; Lemichez et al. 1997). Nocodazole, an inhibitor of microtubule formation, blocks transport from early to late endosomes (Bomsel et al. 1990; Aniento et al. 1993), but does not alter DT cytotoxicity (Lemichez et al. 1997).

Importantly, Lemichez et al. (1997) showed that membrane translocation of the DT C domain exploits the early to late trafficking machinery. Acidification of the endosomal lumen is a critical step in the sorting of proteins within the endosomal pathway. Acidification results in the dissociation of ligand-receptor complexes, allowing receptors to be recycled back to the plasma membrane. Acidification is also required for the formation of endosomal carrier vesicles (ECVs) which carry ligands and non-dissociated ligand-receptor complexes from early to late endosomes. Bafilomycin A1 inhibits the formation of these ECVs, resulting in the accumulation of early endosomes which are highly tubular in structure (Clague et al. 1994). The formation of ECVs requires the binding of both β'COP (Sec 27) and ADP-ribosylation factor 1 (ARF1) to the cytoplasmic surface of the endosomal membrane, and the binding of both factors is dependent upon a low endosomal lumen pH (Aniento et al. 1996; Gu et al. 1997). The formation of ECVs is also regulated by GTPases, *e.g.* Rab 6 (Zerial and Stenmark 1993), and the non-hydrolysable GTP analogue GTPγS inhibits ECV formation from early endosomes (Aniento et al. 1996). Once formed, the ECVs become fusogenic with late endosomes (Zerial and Stenmark 1993). Inward invaginations of the endosomal membrane also occurs during ECV formation, resulting in the production of multivesicular bodies (Hopkins et al. 1990; Futter et al. 1996).

Confocal microscopy studies have shown that when diphtheria toxin is loaded into early endosomes, and fixed at the point of half-life of maximal translocation, that the toxin colocalizes with β'COP in predominantly tubular structures (Lemichez et al. 1997). Furthermore, antibodies to β'COP inhibited the *in vitro* translocation of the DT C domain from the lumen of purified early endosomes (Lemichez et al. 1997). Interestingly, Lemichez et al. (1997) also noticed that GTPγS had no effect on the *in vitro* translocation of the DT C domain from early endosomes. Since GTPγS increases the amount of β'COP bound to endosomal membranes, it has been suggested that the binding of β'COP precedes GTP hydrolysis in the formation of ECVs (Aniento et al. 1996). Most importantly, the work of Lemichez et al. (1997) suggested that the binding of β'COP, and possibly other cytosolic proteins, that preceded GTP hydrolysis in ECV formation played an important role in facilitating the translocation of the DT C domain from the lumen of early endosomes to the cytosol.

While fragment A is most efficiently translocated out of early endosomes, the bulk of endocytosed fragment A ends up sorted into late endosomes where translocation of fragment A into the cytosol is marginal (Lemichez et al. 1997). The

presence of Rab 7 distinguishes ECVs and late endosomes from early endosomes, and the contents of these vesicles are targeted for the lysosomal pathway and eventually degraded. Zeng (1998) demonstrated that the internalized C-domain is unfolded and catalytically inactive within the lumen of early endosomes. Furthermore, Zeng (1998) demonstrated that ATP alone does not support translocation from the lumen of early endosomes to the external milieu. The addition of cytosolic factors was required, and cytosol mediated translocation was not only ATP, but also time and temperature dependent.

7 The transmembrane domain and channel formation

The T domain, residues 195-389, is composed of nine α-helices (TH1-9) and their connecting loops. The crystal structure of DT shows that the helices are arranged in three layers (Choe et al. 1992). The first three helices (TH1-3) comprise the first layer and are amphipathic in nature. Helices TH5, 6, and 7 compose a second hydrophobic layer. The third, central core layer is composed of the hydrophobic helices TH8 and 9, connected by transmembrane loop 5 (TL5).

Exposure of DT to planar lipid bilayers at low pH results in spontaneous membrane insertion of the T domain and the formation of voltage dependent and cation selective channels (Boquet et al. 1976; Donovan et al. 1981). A channel diameter of approximately 18 Å was observed, which is theoretically large enough to accommodate the passage of a fully denatured and extended fragment A (Kagan et al. 1981). Upon acidification of the endosomal lumen, residues Glu 349 and Asp 352 located at the tip of loop connecting TH5 and TH6 are protonated, and the third helical layer spontaneously inserts into the membrane forming cation selective channels (Mindell et al. 1992, 1994a, 1994b; O'Keefe et al. 1992). Deletion or disruption of these helices by introducing proline residues ablates channel formation and results in non-cytotoxic mutants (vanderSpek et al. 1994a, 1994b; Hu et al. 1998), suggesting that the full length helices arranged in a specific conformation is required for channel formation. While helices TH8 and 9 alone are necessary for channel formation (Silverman et al. 1994), mere channel formation alone is not sufficient for effective delivery of the C domain (vanderSpek et al. 1994a).

The role that the Pro 345 residue, located at the end of TH8, plays in channel formation remains unclear. Mutation of Pro 345 to either a Glu residue, an α-helix former, or to a Gln residue, an α-helix breaker, resulted in a marked decrease in DT toxicity (Johnson et al. 1993). Although the *cis-trans* isomerization of proline by membrane associated peptidylprolyl *cis-trans* isomerases (PPIases) has been shown to be important in the gating mechanisms of other cation selective channels (Brandl et al. 1986; Deber et al. 1990; Woolfson et al. 1991), roles for PPIases in DT channel formation or translocation of the DT C domain have not been demonstrated.

Following membrane insertion of the third helical layer, the second helical layer is subsequently inserted and is thought to stabilize the channel formed by helices TH8 and TH9 (Cabiaux et al. 1994). Insertion of proline residues into the

second helical layer of DAB_{389}IL-2 resulted in non-cytotoxic mutants with abnormal channel formation (Hu et al. 1998). Although this layer is not required for channel formation, it appears that the second helical layer is required for the formation of productive channels capable of supporting C domain translocation across the early endosomal membrane.

Deletion of the first three helices of DAB_{389}IL-2 resulted in a non-cytotoxic mutant that still formed characteristic channels and retained enzymatic activity (vanderSpek et al. 1993). Several studies have suggested that the amino terminal residues of TH1 are translocated across the membrane and presented to the cytosol (Madshus et al. 1994a; Senzel et al. 1998; Umata and Mekada 1998). Replacement of the charged residues in TH1 with uncharged residues strongly inhibits translocation of TH1 (Madshus et al. 1994a). Replacement of TH1 with a helix in which the charge distribution and hydrophobicity was maintained, resulted in mutant fusion protein toxins that retained cytotoxicity (vanderSpek et al. 1994b). In contrast, when the charge distribution and hydrophobicity were not maintained, the resulting fusion protein toxin mutants were non-cytotoxic (vanderSpek et al. 1994b). While none of the mutants in this study showed defects in receptor binding or enzymatic activity, it is not known whether or not any of these mutants form channels. Finally, the insertion of proline residues into the first helical layer also resulted in non-cytotoxic mutants that formed characteristic channels and retained enzymatic activity (Hu et al. 1998). Taken together, these results suggest that the first helical layer facilitates the orientation and insertion of the C domain through the nascent channel formed by the T domain. Assuming that the disulfide bond connecting fragments A and fragment B remains intact, translocation of amino terminal residues of TH1 across the endosomal membrane would be anticipated to effectively thread the carboxy-terminal residues of the C domain through the nascent channel and present them to the cytosol (vanderSpek et al. 1994b).

8 The interchain disulfide bond

The C domain is separated from the T and R domains of diphtheria toxin by a protease sensitive loop that is created by the disulfide bond between residues Cys 186 and Cys 201. Upon binding to cell surface receptors and/or internalization of toxin-receptor complex, the loop is nicked by the enzyme furin (Tsuneoka et al. 1993). Although furin, involved in the maturation of polypeptide precursors during secretion, is predominantly located in the ER and Golgi, it is also found in endosomes and on the plasma membrane (Bosshart et al. 1994; Mayer et al. 2004). Retention of the DT interchain disulfide bond is a prerequisite for intoxication (Falsnes et al. 1992; Ariansen et al. 1993), and it presumably mediates threading the DT C domain through the channel formed by the T domain, or serves to tether the C domain to the isomerizing T domain during pore formation. However, the precise location where reduction of the interchain disulfide bond occurs has remained controversial.

Moskaug et al. (1988) showed that only membrane permeate sulfhydryl blockers were able to prevent the release of the C domain into cytosol (Moskaug et al. 1988). Observations by Papini et al. (1993b) indicated that reduction of the interchain disulfide bond occurs after the low pH induced membrane insertion of the T domain within the early endosomal compartment (Papini et al. 1993b). Since unreduced DT C domain and membrane inserted DT fragment B are both targeted for proteolytic degradation (Madshus et al. 1994b), it has been suggested that reduction of the interchain disulfide bond occurs post-translocation and is required for the cytosolic release of the DT C domain. Alternatively, it has also been proposed that reduction of the interchain disulfide bond occurs prior to translocation, presumably on the cell surface or within the endosomal lumen (Ryser et al. 1991; Mandel et al. 1993). In contrast to the results obtained by Moskaug et al. (1987), Ryser et al. (1991) found that membrane permeates sulfhydryl blockers prevented intoxication by DT. Nonetheless, the pivotal role of interchain disulfide bond reduction during DT intoxication is underscored by the observation that reduction and release of the C domain appears to be the rate limiting step for the entire intoxication process (Papini et al. 1993b).

In vitro studies have shown that thioredoxin 1 (Trx-1) (12 kDa) is capable of reducing the DT interchain disulfide bond under acidic conditions (Moskaug et al. 1987). This result is consistent with observations that exposure of the DT interchain disulfide bond on the protein surface occurs upon denaturation (Blewitt et al. 1985). Trx-1 is predominately cytosolic, but a shorter form (10 kDa) is actively secreted by a non-classical ER-Golgi independent pathway (Rubartelli et al. 1991; Rosen et al. 1995), presumably following proteolytic cleavage of 16-24 residues from the C-terminal end. As such, the observation that the DT interchain disulfide bond is exposed only under denaturing conditions, such as those found in the endosomal lumen, might be significant since premature reduction of the interchain disulfide results in the loss of toxicity. Although Trx-1 is presumably present on the luminal side of the endosomal membrane (Rosen et al. 1995), it is not known whether or not Trx interacts directly with DT *in vivo* during intoxication.

Protein disulfide isomerase (PDI) has also been implicated in mediating reduction of the DT interchain disulfide bond (Mandel et al. 1993). PDI is expressed predominantly in the ER and Golgi lumen, but it is also found on the cell surface (Freedman et al. 1989). Mandel et al. (1993) showed that bacitracin, a specific inhibitor of PDI, protected cells against diphtheria toxin intoxication, and therefore hypothesized that reduction of the interchain disulfide bond occurs prior to translocation across the endosomal membrane. This hypothesis seems unlikely since reduction by PDI within the endosomal lumen would most likely result in a release of the C-domain that may longer be threaded into and through the channel formed by the T-domain. Furthermore, the redox state of the endosomal lumen most likely mimics the extracellular environment. In such an environment, PDI is predominately involved in the isomerization and the formation of disulfide bonds rather than production of free thiols (Freedman et al. 1989).

9 Proposed mechanisms of diphtheria toxin C domain translocation

There are presently two hypotheses for translocation of denatured DT C-domain across the early endosomal membrane. The first hypothesis argues that diphtheria toxin is capable of facilitating the delivery of its C domain without the participation of other factors. In contrast, the second hypothesis maintains that a cytosolic translocation factor (CTF) complex is required to facilitate the translocation of the C domain across the early endosomal membrane and release it into the cytosol.

9.1 Unassisted spontaneous translocation of the diphtheria toxin C domain

Oh et al. (1999) labeled diphtheria toxin with an N-terminal histidine tag (6xHis) and demonstrated that the addition of Ni^{2+} to the *trans* compartment prevented rapid channel closure. These investigators argue that the 6xHis-tag, and presumably the amino terminal end of the C domain are translocated from the *cis* to the *trans* side of the lipid bilayer upon channel formation by the T domain. These investigators also used biotin to label cysteine site-directed mutants at either position 58 or 148. The addition of streptavidin to the *trans* side of the planar lipid membrane also interfered with the channel closure. Again, Oh et al. (1999) argue that these results demonstrate that Cys58 and Cys148 are on the *trans* side of the membrane following channel formation. Importantly, Oh et al. (1999) did not rule out the possibility that both Ni^{2+} ions, as well as the putative membrane-impermeable TCEP and streptavidin, pass through the nascent channel formed by the insertion of the T domain.

Ren et al. (1999) demonstrated that at low pH, the addition of proteins in a partly unfolded, molten globule-like conformation were able to convert the T domain from a shallow membrane-inserted form to its fully membrane inserted form. In these studies, the membrane inserted T domain was shown to behave in a chaperone-like fashion, and as a result, the authors argued that the membrane inserted T domain may function as a chaperone in the delivery of its own C domain across the planar lipid membrane.

There are several important caveats that have not been explained in the studies by Oh et al. (1999) and Ren et al. (1999). Since protease digestion patterns of DT inserted into planar lipid bilayers differ from those of DT inserted into the plasma membrane (Moskaug et al. 1991; Cabiaux et al. 1994), it seems likely that interaction(s) between the toxin and proteins associated with the endosomal membrane (*e.g.* receptor, CD9, etc.) influence the orientation and/or stoichiometry of insertion of the T-domain and translocation of the C domain. In addition, Ren et al. (1999) and Hammond et al. (2002) have shown that although the DT T-domain has chaperonin-like properties, it has a significantly greater affinity for other molten globule-like polypeptides compared to its own C-domain (Hammond et al. 2002). Furthermore, if the DT self-mediated C-domain translocation hypothesis of

Oh et al. (1999) and Ren et al. (1999) were correct, then C-domain translocation *in vitro* from the lumen of purified early endosomes charged with toxin would be expected to occur following the addition of only ATP. The observation by Zeng (1998) that C domain translocation from the lumen of purified early endosomes requires the addition of both ATP and cytosol suggests that the DT self mediated C domain translocation seen using artificial planar lipid bilayers may not be physiologically relevant.

9.2 A cytosolic translocation factor facilitates the membrane translocation of the diphtheria toxin C domain

As previously described, studies using partially purified early endosomes preloaded with toxin established the hypothesis that C-domain translocation across the vesicle membrane is dependent upon ATP and the presence of cytosolic components which include β'COP (Lemichez et al. 1997). This hypothesis is consistent with the analogous mechanisms of unfolded polypeptide translocation across membranes during mitochondrial import (for review see Neupert and Brunner 2002), protein synthesis into the ER, and the retro-translocation of the ER-association degradation (ERAD) pathway (for review see Tsai et al. 2002). In these systems, translocation is facilitated by the sequential binding and refolding of denatured proteins by chaperonins as they emerge through the membrane.

More recently, Ratts et al. (2003) have confirmed and extended the observations of Lemichez et al. (1997). In this study, Ratts et al. employed purified early endosomes that were charged with the diphtheria toxin-related fusion protein DAB_{389}IL-2 and used the *in vitro* translocation of the C domain ADP-ribosyltransferease activity as an assay for the partial purification of cytosolic factors that were required for C domain translocation. Following partial purification, selected proteins were identified by mass spectrometry sequencing and their potential role in facilitating translocation was probed by the use of specific inhibitors and/or neutralizing antibodies. Interestingly, crude cytosol from both human cells and yeast were found to support C domain translocation from purified early endocytic vesicles. Moreover, following partial purification, the chaperonin heat shock protein Hsp 90 and thioredoxin reductase were both identified by mass spectrometry sequencing in CTF complexes from both human T cell and yeast. A functional role for these two proteins in translocation and/or release of the C domain was then established through immuno-precipitation and the use of specific inhibitors.

Immuno-depletion of either Hsp 90 or thioredoxin reductase from either human or yeast CTF complexes, destroyed the ability to support *in vitro* translocation. Reintroduction of recombinant Hsp 90 and/or thioredoxin reductase to the depleted CTF complex mixture resulted in the restoration of C domain translocation *in vitro*. In addition, in the case of Hsp 90 the addition of the inhibitors geldanamycin and radicicol was found to block C domain translocation. Further, the addition of the thioredoxin reductase stereo-specific inhibitor *cis*-13-retinoic acid to the reaction mixture also blocked C domain translocation from purified early

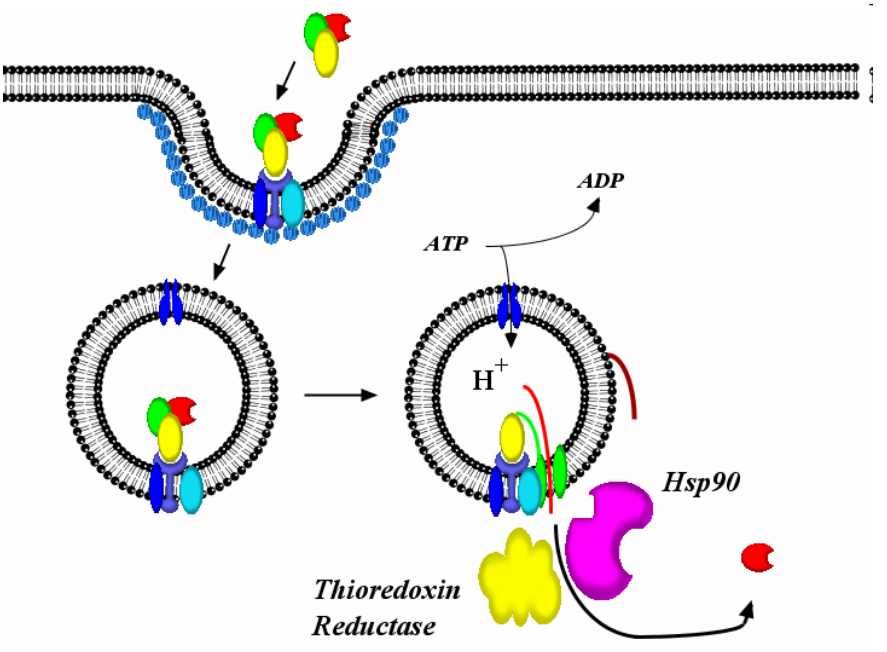


Fig. 1. Schematic diagram of the molecular mechanism of diphtheria toxin action. ***Step 1.*** Diphtheria toxin (C domain, red; T domain, green; R domain, yellow) binds to it cell surface receptor (heparin binding epidermal growth factor like precursor, HB-EGFLP; CD9) complex. ***Step 2***. The toxin::receptor complex is internalized by receptor-mediated endocytosis in clathrin coated pits. The endoproteinase furin specifically cleaves the toxin in the protease sensitive loop connecting the C- and T domains. ***Step 3.*** Upon un-coating, the lumen of early endosomes becomes acidified through the action of vATPase. Under conditions of low pH the T domain becomes partially denatured and spontaneously inserts into the vesicle membrane forming a channel. A fully denatured C domain is threaded into the T domain channel with T domain helix 1. ***Step 4.*** The membrane translocation of the C domain across the vesicle membrane is facilitated by a cytosolic translocation factor complex which is, in part, composed of Hsp 90 and thioredoxin reductase. Thioredoxin reductase functions to reduce the disulfide bond between the C- and T domains. The function of Hsp 90 is most likely to facilitate the refolding of the C domain to a fully functional ADP-ribosyltransferase.

endosomes. Taken together, these results demonstrate that both Hsp 90 and thioredoxin reductase are most likely required for productive translocation out of the lumen of the early endosome and release into the external milieu.

A current molecular model of the process by which diphtheria toxin C domain is translocated across the membrane of early endosomes is shown in Figure 1. The intoxication process begins with the binding of diphtheria toxin to its cell surface receptor. The toxin is then internalized into the cell in clathrin coated pits. The

endoproteinase furin “nicks” the a-carbon peptide chain after Arg194 in the sensitive loop subtended by Cys186 and Cys201. Upon maturation of the early endosomal compartment, and acidification of the lumen the diphtheria toxin transmembrane domain undergoes a low pH induced conformational change which results in the spontaneous insertion of the T domain into the vesicle membrane and subsequent formation of a pore or channel. Transmembrane helix 1, linked by the disulfide bond with the carboxy-terminal end of the C domain, is then threaded into the channel and the translocation process is initiated. As the amino terminal end of the T domain with the disulfide linked carboxy-terminal end of the C domain emerges through the channel formed by the T domain, it is recognized by and translocation is facilitated by a cytosolic translocation factor complex containing Hsp 90 and thioredoxin reductase. The disulfide bond between the C- and T domains is reduced most likely by thioredoxin reductase and released into the cytosol. The C domain is then refolded into an enzymatically active conformation. Once enzymatic activity is regained, the C domain then catalyzes the NAD^+-dependent ADP-ribosylation of elongation factor 2 and cellular protein synthesis is inhibited leading to the subsequent death of the cell.

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Anthrax toxin and genetic aspects regulating its expression

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Abstract

Anthrax toxin is a unique, tripartite bacterial toxin, capable of causing edema and lethality through binary combinations of protective antigen (PA) with either edema factor (EF) or lethal factor (LF). The toxin-encoding genes (*pagA*, *lef*, *cya*) are maintained on a ~44 kb pathogenicity island (PAI), found on the large plasmid, pXO1. The toxin encoding genes are subject to inducible expression in response to CO_2 and temperature through regulation by AtxA, which is also encoded by the pXO1 PAI. AtxA activity is not limited to toxin expression, as it influences the expression of chromosomal genes and genes involved in capsule synthesis found on a second large plasmid, pXO2. Thus, AtxA appears to be a major global regulator of inducible gene expression in *B. anthracis*. In response to AtxA induction, *pagA* is expressed as the first gene of the bicistronic operon, which also encodes *pagR*. PagR functions as an autogenous negative regulator of *pagA* expression and also modulates expression of two proteins involved in S-layer synthesis, thereby linking cell wall synthesis with toxin production. Therefore, expression of PA is subject to both positive and negative regulation. The review chapter presented here highlights the activity of anthrax toxin and the intertwining regulatory network that leads to AtxA-regulated, growth-dependent expression of PA, LF, and EF.

1 Introduction

Bacillus anthracis is a Gram-positive, aerobic, spore-forming pathogen (Mock and Fouet 2001). As a soil-borne microorganism, *B. anthracis* exists in the metabolically inactive spore form and is highly resistant to harsh conditions including heat, UV, and various chemicals (Mock and Fouet 2001). Following exposure to the host either by skin abrasion, ingestion, or inhalation, *B. anthracis* germinates within macrophages, escapes these cells, and grows as a rapidly dividing vegetative bacterium (Dixon et al. 2000). Upon death of the host, sporulation occurs, spores return to the soil, and the cycle is initiated once again when spores encounter an appropriate host. In this regard, the natural cycle for *B. anthracis* occurs when soil-borne spores are engulfed by herbivores, which subsequently succumb to anthrax disease. The success of *B. anthracis* in causing disease and maintaining

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this cycle is due to this organism's hardy nature and unique virulence factors (Mock and Fouet 2001).

Anthrax disease can be manifested in cutaneous, gastrointestinal, and inhalational forms depending on the route of infection. All three forms of anthrax disease have the potential to cause a fatal, systemic infection in the host with up to 10^7-10^9 organisms/ml of blood unless treated within the early stages (Duesbery and Vande Woude 1999b). Progression to the later, life-threatening stages of disease is dependent upon synthesis of an anti-phagocytic capsule and secretion of anthrax toxin (AT).

AT, the focus of this chapter, is composed of protective antigen (PA), edema factor (EF), and lethal factor (LF). As described within this chapter, expression of AT is regulated by precise host signals, including CO_2 and temperature, through a combination of positive and negative regulators. In this manner, *B. anthracis* has evolved to assure that toxin production occurs at the appropriate time and place within the host in order to provide an advantage to the organism during disease. Defining the exact role of AT in disease will require continued investigation and the current state of understanding has been the subject of several excellent reviews to which the reader is referred, and will be briefly described herein. For this reason, the current chapter is largely dedicated to describing the knowledge of genetic make-up and regulation of AT expression.

2 Overview of anthrax toxin

The three protein components of AT act in binary combination, whereby the binding domain (PA) facilitates the uptake and entry of the enzymatic domain (LF or EF) into the cytosol of host cells (Brossier and Mock 2001). In the first step of cell entry, PA binds to the cell surface receptors, tumor endothelial marker-8 and capillary morphogenesis-2 proteins (Bradley et al. 2001; Scobie et al. 2003), using an exposed carboxy-terminal region of domain IV. Following receptor binding, PA is cleaved by furin-like proteases (Klimpel et al. 1992) releasing ~20 kDa from the amino-terminus, thereby exposing sites for LF and EF binding and yielding a form of PA termed PA_{63}. In the next stage of intoxication, PA_{63} oligomerizes into a heptamer (Milne et al. 1994), forms a pre-pore complex (Miller et al. 1999), and binds 3 molecules of EF and/or LF (Mogridge et al. 2002). Utilizing a highly conserved amino terminal region (Arora and Leppla 1993), EF and LF bind the PA_{63} heptamer and the complex is endocytosed via lipid rafts (Abrami et al. 2003). Following acidification of the endocytic vesicle, the heptamer fully inserts into the membrane by formation of a 14-strand beta-barrel that serves as an acid-pH dependent channel (Collier and Young 2003). This insertion and channel formation then leads to exposure of LF and EF to the cytosol, although this process remains poorly defined. Once exposed to the cytosol, both EF and LF can have a dramatic impact on cell physiology.

EF functions as a calmodulin and Ca^{2+}-dependent adenylate cyclase to generate high levels of cyclic AMP within the cell (Leppla 1982). Cyclic AMP can act as a

second messenger in a variety of signaling pathways, yet those pathways directly affected by EF activity have not been identified. The adenylate cyclase activity and increases in cyclic AMP invoked by EF also correspond with an influx of Ca^{2+} into the cell (Kumar et al. 2002). This too, may have an impact on cell physiology. At the cellular level, ET inhibits neutrophil function, induces IL-6 production, and blocks LPS-induced expression of TNFα (Brossier and Mock 2001).

LF targets substrates that regulate well-studied signaling pathways and this has also provided a basis for more focused experiments. Within the cytosol, LF acts as a Zn^{2+} metalloprotease and cleaves mitogen-activated protein kinase kinases (MAPKKs) (Duesbery and Vande Woude 1999a; Vitale et al. 2000), including MEK1,2, and MKK3,4,6,7. LF proteolysis occurs at the amino-terminus of MAPKKs, removing between 8 to 47 residues depending on the particular target (Vitale et al. 1998). Extracellular signal-regulated kinase (ERK), p38, and c-jun n-terminal kinase (JNK) signaling are disrupted due to LF's ability to cleave the corresponding MAPKKs (Park et al. 2002). Unfortunately, a direct link has not been made between disruption of these pathways and cytotoxicity in the absence of inflammatory stimuli. Indeed, we recently reported that chemical inactivation of MEK1,2 in LT-sensitive cells did not lead to detectable changes in physiology, nor to effects similar to LT intoxication (Tucker et al. 2003). However, this does not dismiss the possibility that inactivation of MAPKKs works in synergy with other LF activities. Thus, while the targets of LF have been defined, the overall contribution of MAPKK inactivation to disease is still under investigation.

Recent studies suggest LT also alters cellular events outside of the MAPKK signaling pathways. We have reported that LT intoxication of macrophages leads to a noticeable decline in glycogen synthase kinase–3β (GSK-3β) levels and related changes in mRNA levels from genes regulated via this kinase (Tucker et al. 2003). Furthermore, studies by Dietrich and colleagues indicate that a kinesin motor protein (Kif1C) may be involved in resistance to LT, as single nucleotide polymorphisms correlate with sensitivity/resistance between various in-bred strains of mice (Watters et al. 2001). Overall, these studies indicate that LT intoxication is a multi-factorial process that inactivates MAPKK signaling, alters GSK-3β levels, and involves kinesin motor proteins.

3 pXO1, the plasmid encoding AT

B. anthracis maintains two large plasmids that encode capsule (pXO2) and AT (pXO1) (Green et al. 1985; Mikesell et al. 1983). Although the focus of this chapter is on the genetics of anthrax toxin, which is encoded on pXO1, it is worth briefly mentioning pXO2 since expression of genes on this plasmid is influenced by pXO1. The plasmid pXO2 consists of 96,231 nucleotides with 85 open reading frames (Mock and Fouet 2001). This plasmid has been shown to contain the genes necessary for synthesis of the anti-phagocytic capsule, which are encoded on the capBCAD operon (Bourgogne et al. 2003). In 1995, Vietri and colleagues identified a pXO2-encoded regulatory element referred to as acpA (Vietri et al. 1995).

AcpA is essential for capsule synthesis in *B. anthracis* strains possessing pXO2 (Bourgogne et al. 2003). In addition to AcpA, as discussed below, capsule synthesis is positively regulated by AtxA. pXO1 clearly influences capsule production, as strains possessing both pXO1 and pXO2, compared to strains harboring pXO2 alone, express higher levels of capsule (Guignot et al. 1997; Uchida et al. 1997). Furthermore, strains defective in the pXO1-encoded AtxA are attenuated in capsule production (Guignot et al. 1997; Uchida et al. 1997). Although pXO1 modulates pXO2 genes, a reciprocal regulation from pXO2 to pXO1 has not been identified.

Anthrax toxin was first reported to be encoded by a large plasmid that conferred toxigenic characteristics by Mikesell et al. in 1983. Preliminary analysis indicated strains lacking this extrachromosomal element did not produce EF, LF, or PA. Incubation at 42.5ºC cured toxigenic strains of the plasmid and completely eliminated toxin production. In addition to discovery of the toxin-encoding plasmid, this work also explained Pasteur's early observations regarding the loss of virulence when *B. anthracis* was incubated at 43ºC. Subsequent work found that the toxin structural genes, *cya*, *lef,* and *pag*, are all encoded on this plasmid, thereby making a direct link between toxin production and this extrachromosomal element. pXO1 has now been found in all toxigenic strains of *B. anthracis* and there is currently no evidence of strains harboring toxin genes within the chromosome.

Following discovery of pXO1, Leppla and colleagues subsequently cloned *pag* with cloning of *lef* and *cya* following soon thereafter. Each of these genes were confirmed to be encoded by pXO1 as this plasmid was used for cloning each of these elements (Mock et al. 1988; Robertson and Leppla 1986; Tippetts and Robertson 1988; Vodkin and Leppla 1983). Sequence comparisons revealed homology within the 5'end of *lef* and *cya*, suggesting this could be a common region of PA binding between these two enzymatic components (Bragg and Robertson 1989). This possibility was subsequently confirmed by numerous functional and biochemical experiments performed over the next decade. Each of these genes was found to share G + C base composition similar to the genome of *B. anthracis*, indicating co-evolution of these genes with this organism or one closely related.

Okinaka and colleagues reported the complete sequence of pXO1 in 1999 and the plasmid was found to contain 181,654 bp of DNA encoding 143 ORFs (Okinaka et al. 1999). The genes of *cya*, *lef*, and *pag* are located within a pathogenicity island, approximately 44.8 kb in size, and flanked by exact inverted repeats of IS*1627* elements (Okinaka et al. 1999). This pathogenicity island also contains the regulators *pagR* and *atxR*, which are described in the following sections; refer to Figure 1 for a diagram of the pathogenicity island. Of the 31 ORFs within the pathogenicity island, Okinaka and colleagues reported that 15 have no assignable function. Interestingly, three spore germination responsive elements, *gerX-A*,*B*,*C*, are present on the pathogenicity island of pXO1. Additionally, pXO1 encodes a type 1 topoisomerase, *topA*; a resolvase, and a transposase (Okinaka et al. 1999). The G + C content of pXO1 is similar to that of the *B. anthracis* chromosome, both of which are approximately 33% G + C (Mock and Fouet 2001).

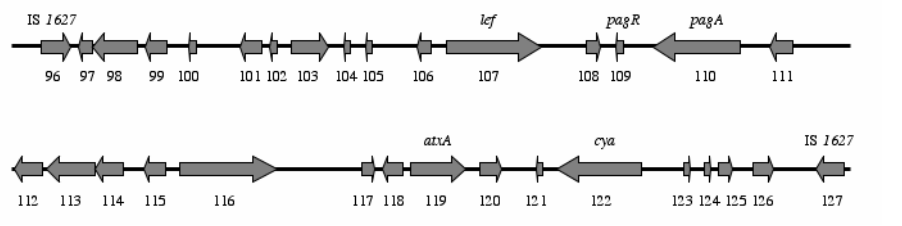

Fig. 1. Pathogenicity island (PAI) encoding PA, LF, and EF. The toxin encoding plasmid, pXO1, contains a 44.8 kb region flanked by inverted repeats of two IS*1627* elements. The toxin-encoding genes, *pagA*, *lef*, and *cya* are distributed throughout the PAI and are labeled in the figure. PAI also encodes *pagR*, a negative regulator, and *atxA,* a global regulator of toxin and capsule synthesis. Overall, the pXO1 PAI contains 32 ORFs encoding various physiological activities in addition to toxin production. ORFs are labeled within the figure according to their appearance within the completed sequence of pXO1. Figure adapted from Okinaka et al. 1999.

pXO1 is clearly necessary for virulence as it encodes the genes comprising anthrax toxin. Yet, genes outside of the pathogenicity island encode proteins involved in normal housekeeping activity. Additionally, a recent study examining 106 open reading frames from pXO1 revealed that over half of these genes were identified in *B. cereus* and *B. thuringensis*, suggestive of the occurrence of significant genetic exchange between these organisms (Pannucci et al. 2002). Direct horizontal gene transfer has not been shown definitively for pXO1; but, as pointed out by Pannucci and colleagues, the sequence of this plasmid is highly suggestive of exchange from this plasmid. pXO1 contains several insertion elements, as well as tranposons and integration sites, indicating a great deal of genetic exchange involving this plasmid may have occurred. A large plasmid, similar to pXO1 but lacking the pathogenicity island, was also recently identified in a strain of *B. cereus* (Rasko et al. 2004). Until recently, pXO1-like plasmids containing the pathogenicity island have not been found outside of strains of *B. anthracis*. However, a newly reported plasmid termed pBCXO1 was isolated from a unique strain of *B. cereus* (strain G9241), which was the cause of an anthrax-like disease (Hoffmaster et al. 2004). Plasmid pBCXO1 is highly homologous (99.6%) to pXO1 and contains the pathogenicity island encoding AT. Interestingly, pXO2 is not present in *B. cereus* G9241, but the genes encoding a polysaccharide capsule are maintained on a previously unidentified plasmid which does not share similarity with genes encoding capsule in *B. anthracis*. Thus, the occurrence of pXO1-derived genes has been found in at least one species of *Bacillus* other than *B. anthracis.*

4 The genes of anthrax toxin: *pagA*, *lef*, and *cya*

pagA. The gene encoding PA (*pagA*: accession number M22589) is found at region 133161 to 135455 on pXO1 and is encoded on the negative strand of DNA (Okinaka et al. 1999). Early literature refers to *pagA* as *pag*; but, as described be-

low, *pag* is encoded as a part of a bicistronic message with a regulatory element *pagR*. Thus, the more current literature correctly refers to the gene encoding PA as *pagA*. The DNA sequence of *pagA* encodes a protein 764 amino acids in size with a molecular weight of 83 kDa (Okinaka et al. 1999). The overall G + C content of PA is 31%. *pagA* shares sequence homology with other known binary toxins, such as *Clostridium perfringens* iota toxin Ib and *Clostridium spiroforme* Sb (Lacy and Collier 2002). These homologous proteins function in a similar fashion to PA, acting as cell entry components of binary toxins. PagA has been cloned and expressed in a variety of recombinant systems, and like the other two components of AT, the three dimensional structure of this protein has been solved.

lef. The gene encoding LF (*lef*: accession number M29081 and M30210) is located upstream of *pagA* with the intervening sequence containing the *pagR* region. LF is encoded on the positive strand of pXO1 in the opposite direction from the *pagA*/*pagR* bicistronic element. The open reading frame of *lef* lies at regions 127442 to 129871 on pXO1 and encodes a protein 809 amino acids in size (Okinaka et al. 1999). The mature protein consists of 776 residues following the cleavage of a 33-residue signal peptide (Duesbery and Vande Woude 1999b). Based on this sequence, the predicted size of LF is 90.2 kDa (Duesbery and Vande Woude 1999b). LF shares sequence homology with EF across the first 255 residues which reflects a common region of PA-binding (Collier and Young 2003). With the exception of homology with EF, LF does not have any known protein neighbors.

cya. The gene encoding EF (*cya*: accession number M23179 and M24074) is located downstream of both *lef* and *pagA* at region 154224 to 156626 and is encoded on the negative strand of pXO1 (Okinaka et al. 1999). *cya* encodes an 800 amino acid protein, with a 767 amino acid mature protein following cleavage of the secretion signal peptide (Duesbery and Vande Woude 1999b). The final predicted molecular weight of the mature protein is 88.8 kDa (Duesbery and Vande Woude 1999b). EF shares significant sequence homology with the adenylate cyclase produced by *Bordetella pertussis* and with the amino-terminal region of LF (Escuyer et al. 1988).

5 Environmental signals regulating AT expression

Anthrax toxin expression is inducible and responsive to environmental signals such as CO_2 and temperature (Leppla 1988; Sirard et al. 1994). Furthermore, growth of *B. anthracis* in defined media leads to increased expression of PA, LF, and EF (Leppla 1988). Expression of *pagA*, *cya*, and *lef* is coordinated in response to these environmental signals, yet the genes are not organized within an operon.

Growth of *B. anthracis* in the presence of bicarbonate (0.8%) provides CO_2 levels adequate for the induction of toxin expression (Sirard et al. 1994). In conjunction with CO_2 levels, toxin expression is impacted by medium composition. Bartkus and Leppla observed that toxin production was enhanced when *B. anthracis* was grown at 37° C in R minimal medium containing 0.1M Tris hydro-

chloride (pH 8.0) supplemented with uracil (40 μg/ml), sodium bicarbonate (0.8%), horse serum (5%), and streptomycin (500 μg/ml) compared to *B. anthracis* grown in brain heart infusion broth (Bartkus and Leppla 1989). Finally, under these growth conditions, toxin expression is also influenced by temperature (Sirard et al. 1994). Using a *lacZ* reporter system, Sirard and colleagues found a four to six-fold higher expression level (based on β-galactosidase activity) when *B. anthracis* was grown at 37° C compared with 28° C.

It is important to note that, despite the coordinated expression of *pagA*, *cya*, and *lef* in response to the environmental signals, the corresponding proteins do not accumulate at similar levels. Results from *lacZ* fusion/reporter assays indicate *pagA* is transcribed at higher levels than *lef* and *cya*, with a ratio of about 5:1 for *pagA*:*lef* and 10:1 for *pagA*:*cya* (Sirard et al. 1994). This corresponds with and explains the fact that protein levels of PA exceed LF and LF exceeds that of EF. Reportedly, PA, LF, and EF are produced in relative levels of 20 μg PA, 5 μg LF, and 1 μg EF (Leppla 1988).

6 AtxA, a global regulator of AT expression

Expanding on the initial observations of regulation of toxin production, Koehler and colleagues further demonstrated that regulation of *pagA* was modulated by CO_2 through a specific trans-activating element (Koehler et al. 1994). Screening a transposon mutant library for the absence of toxin production led to identification of a site 13 kb upstream of *pagA,* proximal to the 3' end of *cya*, which was important for toxin expression (Koehler et al. 1994). This region corresponded to a trans-activator element termed AtxA (~55.6 kDa), which had recently been reported by Uchida and colleagues (Uchida et al. 1993).

AtxA is now known to control expression of both the pXO1-encoded toxin genes as well as the genes involved in capsule synthesis that are located on pXO2 (Dai et al. 1995; Guignot et al. 1997; Uchida et al. 1997). Consequently, *atxA*-null mutant strains have been shown to be avirulent in a mouse model (Dai et al. 1995). Studies conducted with *B. anthracis* strain variants, differing in plasmid components, suggest that *atxA*-mediated expression is required to direct the release of vegetative bacilli from macrophages during disease establishment (Dixon et al. 2000). This is based on observations with an *atxA*-null mutant strain that was incapable of escaping from the infected macrophages.

Recent studies demonstrated that, in addition to controlling synthesis of the toxin and capsule genes, AtxA controls the expression of numerous other genes on both plasmids and the chromosome (Bourgogne et al. 2003). For this reason, AtxA is now believed to be a major global regulator of virulence, controlling the expression of capsule, S-layer, and toxin.

Once the AtxA-responsive region of *pagA* had been defined, it was also found that initiation of *pag* transcription occurred at 2 promoter binding sites termed P1 and P2 (Koehler et al. 1994). Koehler and colleagues reported that P1, which lies 58 bp upstream of the *pag* start codon, is modulated by AtxA. Initiation from P2 is

minimal but does not require CO_2 or AtxA (Koehler et al. 1994). As expected, further studies found that in addition to *pag*, *lef* and *cya* expression are also modulated by AtxA (Dai et al. 1995).

Most telling about AtxA as a major global regulator are the recent experiments of Bourgogne and colleagues which used transcriptional profiling to identify genes subject to regulation by AtxA (Bourgogne et al. 2003). Comparisons of mRNA profiles between wild type organism and *atxA*-null mutants revealed several chromosomal genes regulated by AtxA. Furthermore, this group reported that of the 38 plasmid-encoded genes expressed under specific growth conditions, 18 were subject to regulation by AtxA (Bourgogne et al. 2003).

As one might expect, regulation of toxin production does not occur independently of other factors within the cell. Coordinated regulation of multiple factors important to virulence works to the advantage of *B. anthracis*. Thus, it is important to briefly mention the influence toxin regulation has on other expression events within *B. anthracis*. AtxA regulates the expression of capsule through two regulators encoded by *acpA* and *acpB* (Drysdale et al. 2004). Thus, pXO1$^-$ strains and *atxA*-null mutants are defective in capsule production. Since the capsule encoding operon *capBCAD* is located on pXO2, it is evident that factors from pXO1 can influence pXO2. To date, there are no reports of pXO2-dependent regulation by pXO1. Finally, it is worth noting that the attenuation of pXO1$^-$ mutants is often attributed to loss of toxin production; however, given the influence of this plasmid and AtxA on expression of numerous genes, the possible involvement of other pXO1-regulated factors in virulence cannot be excluded.

7 PagR, a negative regulator of PA expression

Experiments by Hoffmaster and Koehler found that *pag* transcription resulted in two distinct mRNA transcripts, 2.7 kb and 4.2 kb in length (Hoffmaster and Koehler 1999). The smaller transcript encoded PA alone while the second, larger transcript produced a bicistronic message containing an additional region termed *pagR*. Hoffmaster and Koehler reported *pagR* to be encoded on a 300 bp ORF, which is preceded by a putative ribosomal binding site. Inactivation of *pagR* resulted in increased levels of PA, thereby suggesting this element might function as a negative regulator of *pag* expression. The differential expression of *pag* and *pagA*/*pagR* occurs through inverted repeated sequences that act as an attenuator to modulate levels of bicistronic and monocistronic mRNA. PagR appears to be subject to *pagA*'s transcriptional regulation, as there are no intervening regions for regulation of *pagR*. As levels of PagR increase, this protein serves as a negative regulator of *pagA* expression by modulating AtxA induction of this gene. However, it has also been found that overexpression of AtxA yields reduced levels of PA (Dai and Koehler 1997), indicating that PagR may repress PA synthesis independently of AtxA; or, other, yet unidentified factors may play a role in this regulation. Indeed, PagR has also been shown to bind directly to the upstream promoter region of *pag* in order to block expression of PA (Mignot et al. 2003). This

modulation appears to cover both the P1 and P2 promoter binding regions, indicating that PagR can repress both CO_2-dependent and CO_2-independent expression.

PagR has also been shown to regulate the expression of two genes, *sap* and *eag*, which encode protein components of the cell surface S-layer (Mignot et al. 2004). As AtxA increases expression of the *pag* operon, PagR accumulates and represses *sap* expression. Strains defective in AtxA expression demonstrate unregulated expression of Sap, and a similar phenotype is observed when *pagR* deletion strains are examined. As shown with *pagA* regulation, *pagR* binds upstream regulator regions of both *sap* and *eag*. Thus, *pagR* provides a direct connection between toxin production and cell surface structures.

8 AbrB, a growth phase-dependent regulator of AT production

Expression of *pagA*, *lef*, and *cya* and corresponding toxin production reaches maximal levels during late log phase when *B. anthracis* is grown in laboratory medium, and is retained at steady state into stationary phase of growth (Koehler et al. 1994; Leppla 1988; Sirard et al. 1994). There is limited production of AT during early exponential phase of growth. This growth phase-dependent expression of AT is modulated by a negative regulator, AbrB (Saile and Koehler 2002). Studies showed that AbrB is a growth phase-dependent regulator of anthrax toxin synthesis with a predominant impact on *pag* activation. Orthologues of AbrB are encoded on the chromosome and pXO1; however, the plasmid-encoded element does not encode the first 27 residues of *abrB* and isogenic strains lacking this gene are not altered in toxin production (Saile and Koehler 2002). Conversely, chromosomal *abrB* encodes full-length protein and disruption of this gene leads to altered toxin production. In the absence of chromosomal AbrB, toxin production is substantially increased during early and mid-exponential phase growth (Saile and Koehler 2002). AbrB apparently modulates toxin expression, at least in part, by the repression of AtxA. Interestingly, AbrB has a predominant impact on *pagA* expression with limited influence on *cya* and *lef*.

AbrB is not responsive to AtxA, but appears to be regulated by Spo0A such that increases in phosphorylated Spo0A result in a decline in AbrB levels (Saile and Koehler 2002). Thus, the growth phase effect may occur as phosphorylated Spo0A accumulates in late exponential to stationary phase growth.

9 Conclusions and outlook

The genes encoding anthrax toxin are maintained within a pathogenicity island on pXO1. Despite their coordinated regulation, *pagA*, *cya*, and *lef* are not organized within an operon but are subject to global regulation through a regulon guided primarily by AtxA. Regulation involves the coordinated effects of a negative regu-

lator, PagR, and a growth phase-dependent regulator, AbrB. A diagram summarizing essential components of this regulatory system is shown in Figure 2. The model, as it now stands, has AtxA responding to environmental cues, such as CO_2 and temperature. In response to environmental signals, AtxA activates the expression of *pagA*, *cya*, and *lef*. Read-through from *pagA* leads to a bicistronic message that encodes PagR, which subsequently represses the activity of AtxA and causes a decline in expression of PA. AbrB provides a link between toxin production and growth phase as *B. anthracis* moves into stages of sporulation.

Regulation of AT production allows *B. anthracis* to commit energy to the expression of this virulence factor at the most appropriate time. Signals from within the host, such as temperature and CO_2, permit the organism to sense when toxin production will be necessary. Additionally, linking expression to growth phase allows the bacterium to shift efforts away from toxin production when entering sporulation.

It is important to note that regulation of toxin production is only part of the coordinated response to host conditions. Furthermore, feedback mechanisms exist that allow expression of toxin to influence other physiological events in *B. anthracis*. PagR is an excellent paradigm for such a coordinated event.

Despite the advances made over the past two decades, several important questions remain unanswered. First, the mechanism by which CO_2 and temperature influences AtxA activity is not known and a better understanding of this process could indicate whether other factors (e.g. cell surface sensory molecules) are involved. It may be reasonable to assume that AtxA is directly impacted by these conditions, since screens of transposon mutants have not identified other factors which might work upstream of AtxA. Second, the explanation for differential levels of *cya*, *lef*, and *pagA* expression is not clear. Each gene seems to be subject to similar regulation, but the accumulated amount of protein is significantly different for each of these toxin components. Further studies on AbrB may provide part of the explanation since *pagA* seems to be more sensitive to this regulator than the genes encoding LF and EF. Detailed studies on mRNA stability for each of these genes could highlight important differences in overall protein production for PA, LF, and EF. Third, little is known about the time of infection at which toxin expression is most pronounced within the host. During the initial stages of disease, *B. anthracis* germinates within macrophages prior to becoming systemic. Previous reports indicate that toxin production is important for survival within the macrophage following germination (Dixon et al. 2000; Guidi-Rontani et al. 2001). Temperature would obviously serve as a signal for activation of AtxA, but whether CO_2 levels are sufficient to support toxin production within the macrophage is unclear.

B. anthracis continues to be a major threat as a bioterrorist weapon, and this possibility came to fruition during the fall of 2001 with the intentional release of anthrax spores through the United States postal system. Effective therapies targeting this bacterium's toxin will necessarily need to be part of a robust treatment regimen because, in many cases, the disease will have progressed beyond the early

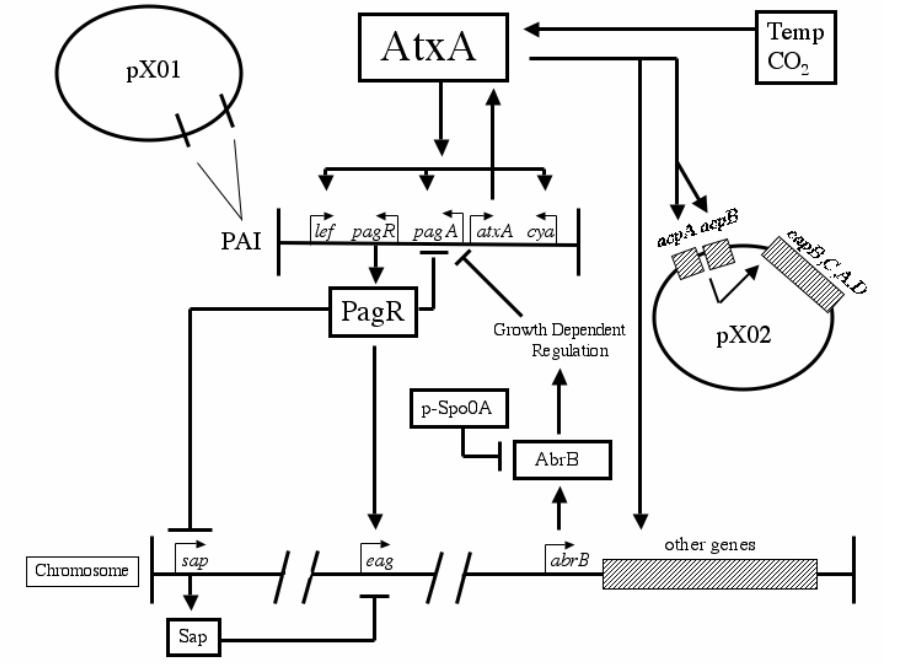

Fig. 2. Diagram of regulatory network modulating anthrax toxin expression. In response to environmental signals, AtxA induces expression of *cya*, *pag*, and *lef*. A bicistronic operon of *pagApagR* encodes the negative regulator *pagR*, which represses *pagA* expression and modulates levels of S-layer components Sap and Eag. In addition to regulation of toxin production, AtxA modulates capsule synthesis genes on pXO2 as well as several genes on the chromosome. AbrB functions as a growth-phase regulator of toxin production by repressing expression in lag and early exponential phase growth. AbrB is subject to repression by phosphorylated Spo0A, which accumulates near stationary phase and allows increased toxin production and steady state levels during late stages of growth.

stages of disease at which point antibiotic treatment is ineffective. Understanding the mechanisms of toxin gene regulation will not only improve our appreciation of the basic biology of anthrax, but may also suggest novel approaches for treatment of this disease.

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Shiga toxins and their mechanisms of cell entry

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Abstract

The Shiga toxins (Stxs) are related toxins secreted by *Shigella dysenteriae, Escherichia coli* in addition to other bacteria. They all act by entering cells and inhibiting protein synthesis enzymatically, thereby, killing the cells. Toxicity can also be mediated by induction of apoptosis. Furthermore, the Shiga toxins can in some cells induce secretion of a number of cytokines, some of which may induce synthesis of the toxin receptor Gb3 in other cell types. The toxins are important tools in studies of intracellular pathways, and knowledge about interactions of the toxins with cells are important to prevent and cure diseases caused by these highly potent agents. Knowledge about the toxins and their entry process can be exploited in attempts to use Stxs or their subunits also for therapeutic purposes. This article will focus on the entry process of Stxs.

1 Introduction

Shiga toxins, which all have one A-fragment and five B-fragments (Fig. 1), belong to a group of bacterial and plant toxins that all enter the cytosol of cells and act enzymatically on a cytosolic target. This group includes the bacterial toxins diphtheria toxin, anthrax toxin, cholera toxin, *Pseudomonas* exotoxin A, and plant toxins such as ricin, abrin, and viscumin (for review, see Schiavo and van der Goot 2001; Sandvig and van Deurs 2002; Sandvig 2003). After binding to the cell surface, these toxins are taken in by different types of endocytosis, and they fall into two groups when it comes to the destination in the cell from where they enter the cytosol. One group of toxins, including diphtheria toxin and anthrax toxin, enters from endosomes. The low endosomal pH triggers a conformational change in the toxin molecules followed by membrane insertion and translocation of the toxin to the cytosolic side. The other group of toxins, including Stxs, cholera toxin, *Pseudomonas* exotoxin A, and the plant toxins ricin, abrin, and viscumin have to undergo a much longer intracellular journey before being translocated to the cytosol. These toxins are transported from the endosomes to the Golgi apparatus and retrogradely to the ER before translocation to the cytosol (for review, see Sandvig and van Deurs 2002; Lencer and Tsai 2003). The pathway used by Stx on its way to the cytosol is illustrated in Figure 2.

There are a number of reasons why studies of protein toxins and their action should be performed. Several of the bacterial toxins are still a threat in connection

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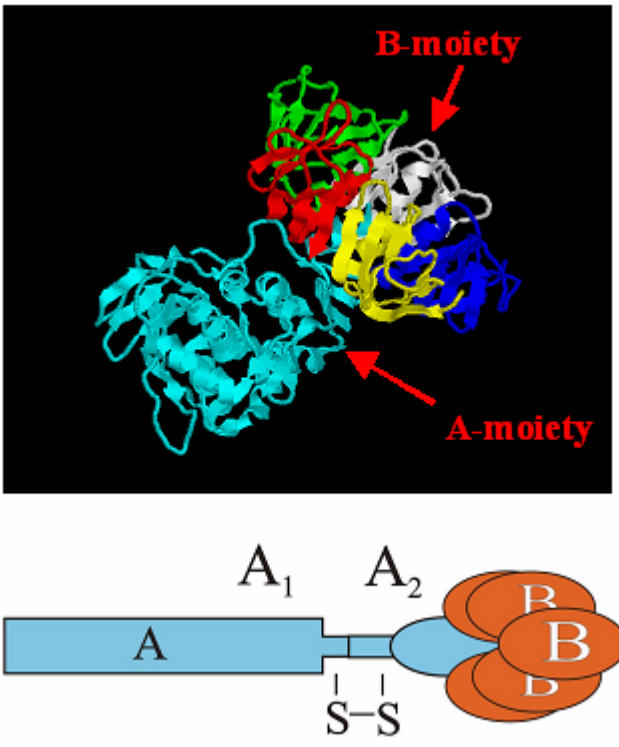

Fig. 1. Crystallographic and schematic structures of Shiga toxin (PDB protein data bank 1DMO). As indicated the A fragment of the toxin is cleaved into the A1 and A2 fragments, and the A1 fragment can then inactivate ribosomes. The five small B fragments are responsible for binding to Gb3.

with infectious diseases, and increased knowledge about their structure, interaction with cells, and intracellular trafficking is warranted to prevent and cure diseases. *E. coli* secreting Stxs seems to be an increasing problem and causes not only diarrhea, but also haemolytic uremic syndrome (Paton and Paton 1998; Andreoli et al. 2002; Siegler 2003). Importantly, the toxins can also be exploited for therapeutic purposes. For instance, as described below, toxins can be used as components of immunotoxins or other toxin conjugates designed to kill certain cell types (Frankel et al. 2000; Gura 2002; Sinha 2003; Weaver and Laske 2003; Kreitman 2003a; Kreitman 2003b). In the case of Stx, it has even been proposed that one of the toxin variants, Stx1 (see below), can be used as an antineoplastic agent as such (Arab et al. 1999; Salhia et al. 2002; Heath-Engel and Lingwood 2003). Furthermore, since the toxin targets are in the cytosol, the idea arose that the toxins can be used as vectors to bring in molecules with biological activity or to bring in epitopes for the purpose of vaccination (Smith et al. 2002b). These approaches are facilitated by increased knowledge about the toxin molecules and their behaviour in cells. Importantly, toxins are now well-established tools in stud-

ies of basic cell-biological processes such as in intracellular transport (Schiavo and van der Goot 2001; Sandvig and van Deurs 2002). They have proven valuable in studies of endocytosis, fusion processes, and in characterization of previously unknown pathways, For instance, Shiga toxin was the first toxin demonstrated to undergo transport all the way from the cell surface to the Golgi apparatus and the ER (Sandvig et al. 1992), a pathway which later on was shown to be exploited also by other toxins.

2 Structure of Shiga toxins

The Shiga toxins (Shiga toxin and Shiga-like toxins (Stxs), also called verotoxins) all have, in principle, the following structure (Fig. 1): there is one moiety of the molecule (the B-moiety) that is responsible for binding of the toxin to the cell surface receptors, and one moiety (the A-moiety) that enters the cytosol and inhibits the protein synthesis enzymatically by removing one single adenine from the 28 S RNA (Endo et al. 1988). The binding moiety (35 kD), which consists of five B subunits, binds to a cell surface receptor, the neutral glycosphingolipid Gb3 (see below). The A-moiety can be cleaved to an A1 and an A2 part that are linked by a disulfide bond, and this cleavage is necessary for activation of the toxin. The proteolytic cleavage can occur if the toxin is exposed to trypsin. Nevertheless, it seems to be the enzyme furin that is responsible for cleavage when the toxin is entering cells. The A fragment has a typical furin recognition site (Garred et al. 1995b), and *in vitro* studies demonstrate that the toxin is cleaved by furin most efficiently at a slightly low pH, suggesting that Shiga toxin is processed and activated in endosomes. The toxin acts on ribosomes at a maximal rate only after reduction of the disulfide bond connecting the A1 and A2 subunit. Reduction of the disulfide bond may, in analogy with cholera toxin, occur in the endoplasmic reticulum (ER) (Lencer and Tsai 2003). However, at the present stage, we cannot exclude that this also occurs in the cytosol (for further discussion of this point, see the paragraph “Transport from the ER to the cytosol”). There are a number of different Stxs. The toxin secreted by *Shigella dysenteriae*, Shiga toxin (Stx), is almost identical to Stx1 (Shiga-like toxin 1) secreted by *E. coli*. There is only one amino acid that differs (Calderwood et al. 1987; Strockbine et al. 1988). Related toxins (Paton and Paton 1998; Schmidt 2001; O'Loughlin and Robins-Browne 2001; Binnington et al. 2002; Leung et al. 2003) comprise Stx2 (60% homology) and Stx2c, which is quite similar to Stx2 (95 % homology). Bacteria infecting humans secrete all these toxins. The toxin called Stx2e is produced by an *E. coli* that infect pigs. Stxs produced by *E. coli* are believed to be encoded by the genome of prophages, and Stx phages can be transduced into other bacteria as well. Thus, toxin production by *Aeromonas hydrophila, A. cavia, Citrobacter freundii* and *Enterobacter cloacae* has also been reported (for review, see (Paton and Paton 1998; Schmidt 2001).

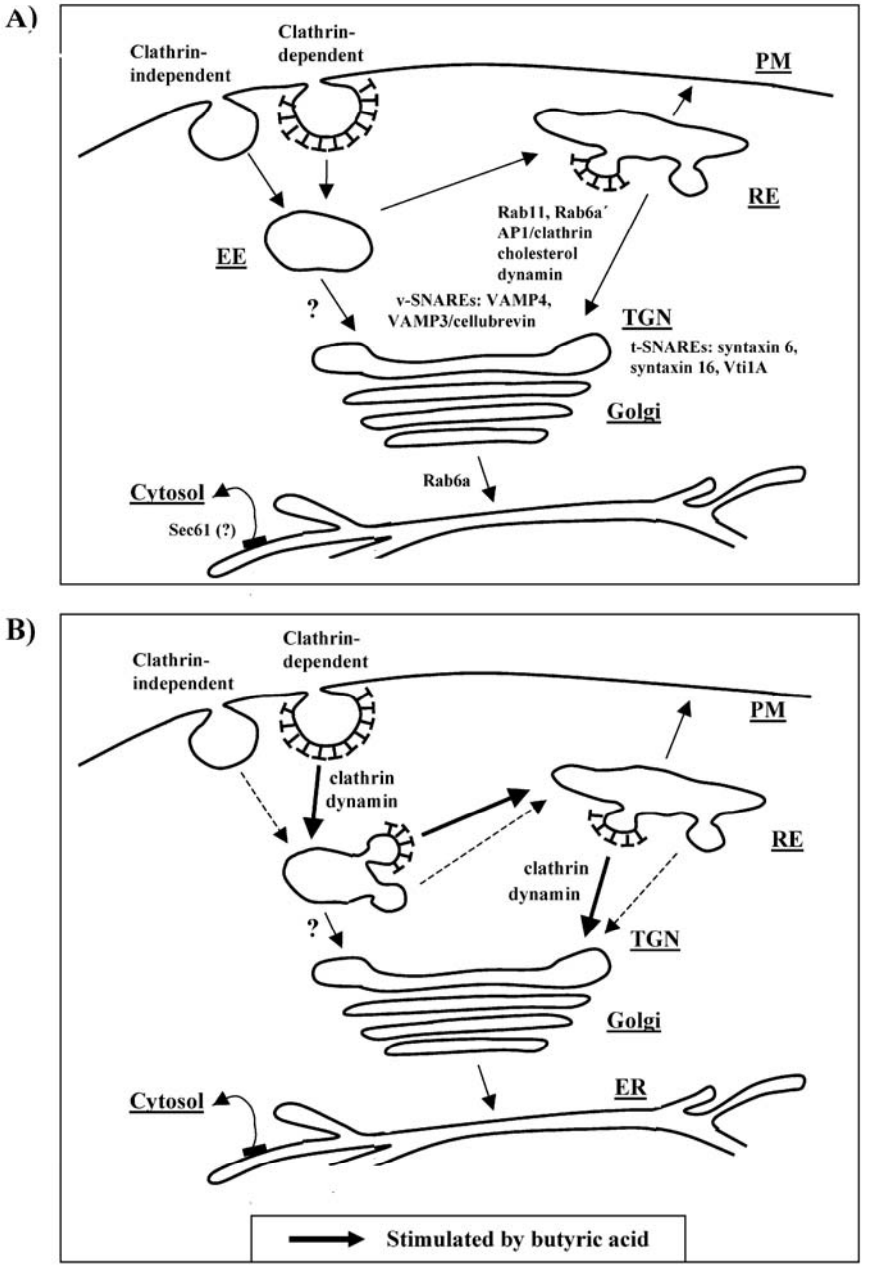
A)
Clathrin-independent
Clathrin-dependent
PM
RE
Rab11, Rab6a´
AP1/clathrin
cholesterol
dynamin
EE
?
v-SNAREs: VAMP4,
VAMP3/cellubrevin
TGN
t-SNAREs: syntaxin 6,
syntaxin 16, Vti1A
Golgi
Rab6a
Cytosol
Sec61 (?)
B)
Clathrin-independent
Clathrin-dependent
PM
clathrin
dynamin
RE
clathrin
dynamin
?
TGN
Golgi
ER
Cytosol
Stimulated by butyric acid

Fig. 2. (overleaf) Model of Shiga toxin entry. In **A**, the pathway and some of the molecules involved in Stx entry are indicated. Stx is able to enter cells by both clathrin-dependent and -independent mechanisms. After endocytosis the toxin can be transported retrogradely to the Golgi apparatus and the ER, and then to the cytosol, where it exerts its toxic effect by inactivating ribosomes. Endosome-to-Golgi transport of Shiga toxin does not seem to involve the late endocytic pathway; the toxin seems to move from early endosomes (EE) to the TGN, either by a direct pathway (indicated by a question mark), or through the recycling compartment (RE), since the transport is dependent on Rab11. **B** shows a model of the involvement of dynamin and clathrin in endocytosis and endosome-to-Golgi transport of Shiga toxin – and the effect of butyric acid on these steps. Endosome-to-Golgi transport of Shiga toxin is dependent on dynamin. Clathrin is also involved in this step, however, the clathrin-dependency differs depending on the transport efficiency. Toxin transport in cells that are sensitised by butyric acid is more dependent on a pathway that involves clathrin than the transport in non-treated cells that are less sensitive to the toxin (Lauvrak et al. 2004). Confocal microscopy experiments revealed a higher extent of colocalization between Shiga toxin and clathrin after butyric acid-treatment. A similar effect is observed at the level of endocytosis – butyric acid increases the fraction of toxin uptake that is dynamin- and clathrin-dependent (Lauvrak et al. 2004).

3 Toxin interaction with cell surface receptors

The binding of most Stxs occurs to Gb3 at the cell surface, the only exception being Stx2e, which binds to Gb4. Nevertheless, the interaction of these toxins with Gb3 is quite complex and shows unusual properties (for review, see (Binnington et al. 2002; Lingwood and Mylvaganam 2003). The carbohydrate structure of Gb3 interacts with the Stxs in a toxin-specific manner. Studies with deoxy-analogs of Gb3 revealed differences in the requirements for hydroxyl groups in the carbohydrate moiety. Interestingly, one single Stx1 B-subunit is able to interact with three Gb3 analogs (Ling et al. 1998), and mutational analysis of the Gb3-binding sites indicates that all three sites are important for interaction of the toxin with membrane-associated Gb3 (Soltyk et al. 2002). However, studies of the interaction of Stx1 B with soluble aminodeoxy analogs of Gb3 suggest that the interaction of site 3 is weak and that its function might be to orient the molecule to facilitate binding to the two other binding sites (Mylvaganam et al. 2002). Importantly, it is not only the carbohydrate part of Gb3 that is important for the binding (for review, see (Binnington et al. 2002), and the references herein). The ability of Gb3 to bind the various toxins is also dependent on the type of fatty acid in Gb3. Fatty acids with C22 and C18 were found to have the highest binding capacity for Stx1 and Stx2c, and also the degree of unsaturation of the lipid may influence the binding. Furthermore, hydroxylation of C22 and C18 fatty acids increased the toxin binding, both the affinity and the capacity were increased (Binnington et al. 2002). However, the binding of the different toxins are not affected to the same extent by the different parameters, making it likely that some of the differences observed with the various Stxs might arise already at the stage of the toxin-receptor interac-

tion. Remarkably, the phospholipids in the surrounding membrane can also play a role for the interaction (Arab and Lingwood 1996; Arab and Lingwood 1998), a finding that can explain the fact that *in vitro* binding studies do not necessarily reflect binding properties seen with intact cells. Toxin-lipid interactions measured *in vitro* generally show a lower affinity between the ligand and the receptor than what is actually observed in intact cells. Even results obtained from different *in vitro* systems such as microtitre plate assays and liposomes may give different results (Arab and Lingwood 1998). The structural properties of the receptors (Gb3) are probably also important for the ability of the toxin-receptor complex to localize to lipid rafts, a property which is important for the destiny of the toxin-receptor complex in the cells (Falguieres et al. 2001; Kovbasnjuk et al. 2001). If the toxin is bound to receptors with fatty acids with intermediary chain length (Sandvig et al. 1994; Sandvig et al. 1996; Lingwood 1999), and the toxin-receptor complex is associated with lipid rafts, then the cell is likely to become intoxicated. Thus, one has to be careful with extrapolation from *in vitro* studies, such as microtitre plate binding assays and even liposomes, to what might happen when the toxin is interacting with the whole cell and its variety of components. The importance of studies of toxin-interactions at the intact cell surface also becomes evident from recent results suggesting that receptor-bound toxin may interact with other proteins at the surface (Shimizu et al. 2003). Interestingly, also these studies revealed differences between Stx1 and Stx2. Interactions of this type might be relevant for the old observation that Stx, when bound to its receptor, can induce its own transport to clathrin-coated pits at the cell surface (Sandvig et al. 1989). However, not all differences between the various Stxs can be explained by individual receptor interactions. Although Stx2 was reported to be more toxic to human renal endothelial cells than Stx1 (Louise and Obrig 1995), this was not reflected by the binding to the cell surface as the cells bound more Stx1 than Stx2 (Louise and Obrig 1995).

A number of different cell types are able to bind the Stxs, and in most cases glycolipids have been found to be responsible for the binding. However, in some cases low-affinity interactions with proteins could play a role (te Loo et al. 2000). Different cells have been suggested as carriers of Stxs in blood, including erythrocytes and platelets (Binnington et al. 2002). Nevertheless, when Stx was added to intact blood, polymorphonuclear lymphocytes (PMNs) were found to bind toxin in a rapid and reversible manner (te Loo et al. 2000). These cells might be responsible for transfer of the toxin from the infected gastrointestinal tract and to the kidney where the toxin could be transferred to kidney epithelial cells and exert its effect locally. Interestingly, the interaction of Stx with PMNs might be through a protein, and the affinity ($K_d = 10^{-8}$mol/l) is lower than the normal toxin-receptor interaction, facilitating transfer to the kidney cells.

Hemolytic-uremic syndrome, the leading cause of acute renal failure in children, was several years ago suggested to be due to age-related differences (Lingwood 1994). Although this view was recently challenged (Ergonul et al. 2003), the lack of differences as a function of age was apparently due to the methods used (Stoddart et al. 2002). A recent report indicates that the presentation of Gb3 and its ability to bind Stx is a function of age (Chark et al. 2004). Binding of toxin and B subunits could be changed both by extraction of cholesterol and by

lowering the temperature to 4 °C, i.e. unphysiological conditions (Chark et al. 2004).

Important, both for studies of Stx entry into cells and for progression of disease, is the fact that the presence of Stx-receptors on cells can be regulated by a number of factors. Bacterial lipopolysaccharide (LPS), TNFα, and interleukin 1 have all been reported to increase synthesis of Gb3 and exposure of binding sites at the cell surface, and the same is the case with exposure of some cell types to butyric acid (for review, see (Lingwood 1999; Sandvig 2001). Under these conditions, it takes a long time before the receptor-content at the cell surface is changed. A more rapid change in Shiga toxin binding was recently reported to occur after incubation of endothelial cells with sphingomyelinase (Obrig et al. 2003).

4 Endocytosis of Shiga toxins

Stx can, after binding to Gb3, be rapidly internalised from clathrin-coated pits (Sandvig et al. 1989; Sandvig et al. 1991) (Fig. 2). Remarkably, this is a toxin-driven process (Sandvig et al. 1989). When the toxin is bound to the cell surface receptors on ice, there is an even distribution of the toxin. However, after a short incubation at 37 °C the toxin-receptor complex moves to clathrin-coated pits (Sandvig et al. 1989). The mechanism behind this phenomenon is not known, but the toxin may interact with a transmembrane protein that can interact with AP-2 in the clathrin-coated structures. The fraction of Stx that enters by clathrin-dependent endocytosis is cell type-dependent; Stx can enter also by clathrin-independent endocytosis (Lingwood 1999; Sandvig et al. 2002; Nichols 2002; Lauvrak et al. 2004). Interestingly, we have recently found that the importance of clathrin both for endocytosis of the toxin and for its intracellular transport can be changed even in one and the same cell type. A factor such as butyric acid that sensitizes cells to the toxin also seems to increase the importance of clathrin (Fig. 2B). When BHK cells are treated with butyric acid they are sensitised to Shiga toxin, and a larger fraction of the toxin is endocytosed from clathrin-coated pits (Lauvrak et al. 2004). Thus, efficient internalisation and intoxication is clathrin-dependent (Lauvrak et al. 2004). Not only can Stx-endocytosis occur in a clathrin-independent manner, it can also occur independently of other endocytic processes that are dependent on functional dynamin (such as uptake from caveolae) (Lauvrak et al. 2004). HeLa cells expressing a dominant negative mutant of dynamin (K44A) still endocytose Stx (Lauvrak et al. 2004). In this case, the exact mechanisms used are not known. Nevertheless, it is now accepted that there are several types of endocytosis (for a recent review, see (Conner and Schmid 2003). Evidence for different pathways has actually been accumulating over a number of years (Moya et al. 1985; Sandvig et al. 1987; Sandvig and van Deurs 1991), but so far clathrin-dependent endocytosis has been investigated in most detail. In HeLa cells the B-fragment of Stx was found to be associated with detergent-resistant microdomains (Falguieres et al. 2001). To which extent such an association is important for the endocytic pathway used is not known. However, as described be-

low, lipid rafts and cholesterol are important for endosome to Golgi transport, and treatment of HEp-2 cells with methyl-β-cyclodextrin to extract cholesterol protects the cells against Stx (Sandvig et al. 2002). Nevertheless, this does not necessarily mean that the presence of Stx in lipid rafts is essential since an important process such as clathrin-dependent endocytosis is also inhibited upon removal of cholesterol (Rodal et al. 1999; Subtil et al. 1999).

After endocytosis, the toxin can be recycled, transported to lysosomes, to the Golgi apparatus (see below) and even transcytosed (Hurley et al. 1999). Again, when it comes to transcytosis across polarized intestinal cells (T84 and CaCo2A), differences between Stx1 and Stx2 were reported. However, for both toxins the ionophore monensin was found to reduce the transcytosis, whereas the drug brefeldin A, which disrupts the Golgi apparatus and which might release coat components also in other locations, did not affect the trancytosis (Hurley et al. 1999).

5 Endosome to Golgi transport of Shiga toxins

An important step in Stx-intoxication is transport of the toxin from endosomes to the trans-Golgi network. A well-studied pathway between endosomes and the Golgi-apparatus goes via late endosomes and is Rab9-dependent (Lombardi et al. 1993; Riederer et al. 1994). Nonetheless, Stx seems to use a more direct pathway from the early endosomes (Mallard et al. 1998), and trafficking along this pathway occurs, as expected, independently of Rab9 (Sandvig et al. 2002). It is not clear whether endosome to Golgi transport of Stx has to go via the perinuclear recycling compartment or whether the toxin can be transported more directly from sorting endosomes to the trans-Golgi network. Stx has been visualized in clathrin-coated structures on endosomes, and this structure was suggested to be involved in Golgi transport (Johannes and Goud 2000). Recent results reveal that clathrin is essential in endosome to Golgi transport under conditions where there is an efficient transport of the toxin to the Golgi apparatus (Lauvrak et al. 2004; Saint-Pol et al. 2004) (Fig. 2B). Also, dynamin seems to be essential for endosome to Golgi transport of Stx (Lauvrak et al. 2004). In addition, Rab11 and Rab6a′ as well as the v-SNARES VAMP3 and VAMP4 and the t-SNARES syntaxin 6, syntaxin 16 and Vti1A, have been reported to be involved in this transport step (for review, see (Johannes and Goud 2000; Sannerud et al. 2003)). Interestingly, transport of StxB from endosomes to the Golgi apparatus is, as first shown for the plant toxin ricin (Grimmer et al. 2000), dependent on cholesterol (Falguieres et al. 2001). However, the reason for this is not known. Cholesterol might be required to obtain the right membrane curvature (Farsad and De Camilli 2003) and to allow vesicle formation to occur (Grimmer et al. 2000). Furthermore, cholesterol could be important for sorting of receptors into rafts as a way to up-concentrate the toxin-receptor complex, or for recruiting cytosolic proteins that are important for transport or fusion.

6 Toxin transport from the trans-Golgi network to the ER

How do protein toxins move retrogradely from the trans-Golgi network to the ER? Interestingly, the different toxins may exploit more than one pathway leading from the TGN to the ER (for review, see (Sandvig and van Deurs 2002; Sannerud et al. 2003). For some of the protein toxins which have a KDEL or KDEL-like sequence, transport via the KDEL-receptor in COP I-coated vesicles may play a role for the retrograde transport or for keeping the toxins in the ER (Jackson et al. 1999; Lencer and Tsai 2003). However, in the case of Stx, which does not have a KDEL sequence, transport to the ER seems to occur by a COP I–independent but Rab6a-dependent route (White et al. 1999; Girod et al. 1999) (Fig. 2). Thus, transport of Stx differs from that of *Pseudomonas* exotoxin A in that overexpression of lysozyme-KDEL, which saturates the KDEL-receptors protects against *Pseudomonas* exotoxin A, but not against Stx1 (Jackson et al. 1999). To which extent transport from the TGN to the ER bypasses some or all of the Golgi cisterns is currently not known, but Stx can be seen in the various Golgi cisterns in some cells (Sandvig et al. 1992). The retrograde transport of Stx is not due to a signal in the A-fragment since also the B-fragments can be transported retrogradely (Sandvig et al. 1994). It should be noted that protein toxins are not the only molecules that use a retrograde pathway in cells. CD19, which has sequence similarities with the Shiga B-fragment, is also able to bind Gb3, and interestingly retrograde transport of this molecule seems to be important for induction of apoptosis in B cells (Khine et al. 1998).

7 Furin-induced cleavage and activation of toxins

Cleavage of the A-fragment of Stx is essential for maximal activity of the toxin on cells (Garred et al. 1995b). The A-fragment of Stx is easily cleaved by trypsin to form the two fragments, A1 and A2, connected by a disulfide bond. However, when the intact toxin is added to cells it is furin, which cycles between the cell surface and the Golgi apparatus, which is normally responsible for cleavage and activation of the A-fragment (Garred et al. 1995b). A loop formed by the disulfide bond in the A-fragment contains the sequence Arg-Val-Ala-Arg, a sequence recognized by furin (which is able to interact with Arg-X-X-Arg). Interestingly, the cleavage occurs at an optimal rate at slightly low pH, suggesting that furin acts on the toxin in endosomes. This is in agreement with data showing that cells cleave Shiga toxin even when the Golgi apparatus is disrupted by brefeldin A (Garred et al. 1995b). If toxin with mutations in the furin recognition site, or deletion of this site, is given to cells, there is a much slower processing of the toxin. This processing seems to occur after retrograde transport of Stx to the ER or the cytosol. Evidence suggests that the cytosolic enzyme calpain is able to cleave such mutant toxins (Garred et al. 1995a). It should be noted that Stx is not the only toxin that is cleaved and activated by furin. Also diphtheria toxin, anthrax toxin, and *Pseudomonas* exotoxin A are processed by this enzyme (Gordon and Leppla 1994).

8 Transport from ER to the cytosol

Little is actually known about the translocation of Stx from the ER to the cytosol. For some of the other protein toxins that are transported retrogradely to the ER, there is evidence that Sec61 is the channel involved in translocation to the cytosol (Wesche et al. 1999; Simpson et al. 1999; Schmitz et al. 2000). This might also be the case for Stx. Interactions of the channel Sec61 with ricin (Wesche et al. 1999; Simpson et al. 1999) and cholera toxin (Schmitz et al. 2000) have been demonstrated. It is not known whether only the A1-fragment of Stx or also other parts of the toxin molecule can be transported into the cytosol. The fact that toxin mutants where the furin cleavage site has been removed are activated by a calpain-like enzyme (Garred et al. 1995a), suggests that the whole A-fragment can be translocated. However, this remains to be demonstrated. It has been reported that epitopes linked to the B-chain of Stx can be presented by MHC class I (Smith et al. 2002b), a result which might suggest that the whole construct is transported to the cytosol where it is cleaved and that the epitope is then transported back into the ER by the TAP-transporter before becoming bound to the MHC class I molecules. However, the possibility exists that cleavage and release of the epitope occur in the ER. So far transport of the B-fragment to the cytosol has not been demonstrated.

9 Shiga toxin stimulates secretion of cytokines/chemokines

An important function of Stxs in relation to disease is the ability of these toxins to induce synthesis and release of cytokines such as interleukins and other active peptides from different cell types (Thorpe et al. 2001; Lee et al. 2002; Zoja et al. 2002; Smith et al. 2003; Cameron et al. 2003). Thorpe et al. reported that ribotoxic stress caused by Stxs seems to be responsible for induction and stabilization of different chemokine mRNAs in intestinal cells (Thorpe et al. 2001). Stx was found to induce synthesis and secretion of interleukin-8 from the intestinal epithelial cell HCT-8 (Thorpe et al. 1999) and from Caco-2 cells (Yamasaki et al. 1999). Interestingly, the protein toxin ricin had a similar effect as Stx on HCT-8 cells. Also the studies by others (Yamasaki et al. 1999) indicated that a toxic effect on ribosomes was involved in cytokine production since a non-toxic mutant of Stx1 was unable to induce such a response. Furthermore, inhibition of the MAP kinase (mitogen activated protein kinase) p38 by addition of a specific inhibitor decreased the IL-8 release (Thorpe et al. 1999). More recent studies have revealed that Stxs can induce both *c-jun* and *c-fos* and activation of the stress-activated protein kinases (SAPK), JNK and p38 in intestinal cells (Smith et al. 2003). As a result of the chemokines synthesized in response to Shiga toxin, neutrophiles may be recruited to the site of infection, the gut, and the intestinal barrier may become compromised (Thorpe et al. 2001). Also in monocytes, p38 MAP kinase and also

ERK are involved in cytokine generation (tumour necrosis factor alpha (TNFα) and granulocyte-macrophage colony-stimulated factor (GM-CSF)) in response to Stxs (Foster and Tesh 2002; Cameron et al. 2003). The authors suggest that the slow kinetics involved could be due to a requirement for uptake of the toxin. Use of cDNA array has revealed that in renal tubular cells one third of the cytokine genes were upregulated by Stx2 produced by *E. coli* O157:7 isolated from a HUS patient. These changes are probably important for renal tubular injury in HUS (Lee et al. 2002). *E. coli* producing Stx2 is more likely to cause HUS than bacteria producing Stx1, and also when it comes to cytokine production in animal models after Stx injection, different responses are observed after injection of Stx1 and Stx2. It was recently reported that in a baboon model of HUS, Stx2 and not Stx1 induced a cytokine response and also other changes associated with HUS (Siegler et al. 2003).

10 Toxin-induced apoptosis

Stx itself and the related toxins are known to induce apoptosis in a number of different cell types (for review, see (Cherla et al. 2003). It is possible that the mechanism by which this occurs differs. In some cells the apoptotic process seems to depend on entry of the A-fragment into the cytosol since it can be inhibited by brefeldin A, a drug known to block toxin transport to the cytosol and intoxication (Kojio et al. 2000; Yoshida et al. 2002; Fujii et al. 2003). Also, apoptosis has been linked to the Stx1-induced activation of the JNK and p38 MAP kinase pathways. These pathways are apparently activated by the Stx1-induced ribotoxic stress. Inhibitors of this pathway not only inhibited chemokine secretion (see above), but also inhibited apoptosis (Smith et al. 2003). Different studies have concluded that caspases are involved in Stx1-induced apoptosis (Tetaud et al. 2003; Smith et al. 2003; Cherla et al. 2003) and references therein), although also caspase-independent apoptosis may occur (Cherla et al. 2003). The link between the effect of the toxins on ribosomes, and the activation of kinases and caspases is not known. However, the Bcl-2 family of proteins can be involved in the apoptotic process (Jones et al. 2000). In HEp-2 cells, Bcl-2 overexpression was shown to protect against Stx1 and Stx2-induced apoptosis (Jones et al. 2000), and in human dermal microvascular endothelial cells Stx1 and Stx2 inhibited expression of the anti-apoptotic protein Mcl-1 that is a Bcl-2 family member (Erwert et al. 2003). Interestingly, inhibition of protein degradation by addition of the proteasome inhibitor lactacystin inhibited apoptosis (Erwert et al. 2003). In some cases, even the B fragment in itself has been reported to cause apoptosis (Mori et al. 2000; Tetaud et al. 2003). In Ramos cells Stx caused a rapid activation of Syk and the Src family kinase Lyn (Mori et al. 2000). Thus, the kinetics of kinase activation seem to be different from the slower activation of other kinases observed after entry of the A-fragment into the cytosol. In Burkitt's lymphoma cells, both antibodies to Gb3 and Stx-1 induce apoptosis, but by different mechanisms (Tetaud et al. 2003). The antibody induced a caspase-independent apoptosis, whereas the toxin-induced

process was caspase-dependent and involved mitochondria. Interestingly, in the human renal tubular cell line, ACHN, addition of Stx caused a rapid activation of the Src family kinase Yes (Katagiri et al. 1999). Nevertheless, the possible consequences of this signalling are not yet clarified (Katagiri et al. 1999). Importantly, exposure of a cell to multiple signals, such as Stx, LPS and cytokines, can increase an apoptotic response (Pijpers et al. 2001; Cherla et al. 2003).

11 Protection against Shiga toxin-induced disease

Detailed knowledge about the Stx-receptor and the binding of toxin has allowed construction of soluble receptor-based compounds that are designed to bind Stx1 and Stx2 either in the gut or after passage of the toxin into circulation (Mulvey et al. 2003; Karmali 2004; Watanabe et al. 2004) and references herein). Importantly, slight modification of such compounds may facilitate interaction of one or the other toxin (Mulvey et al. 2003). An interesting approach for protection against Stx-producing bacteria is the production of bacteria with a receptor mimic at the cell surface, again with the idea of binding Stx in the gut, thereby, preventing toxin transport into the circulation (Paton et al. 2000). Also Stx-liposome conjugates used for vaccination are being investigated (Uchida 2003). Increased knowledge about the toxins and their interactions with receptors and cells facilitate use of different strategies to protect against the toxins.

12 Therapeutic use of Shiga toxins

Epitopes presented by MHC class I can arise from proteins that are processed by proteasomes or other proteolytic systems in the cytosol. These peptides are transported into the ER by the TAP-transporter, and they then become bound to MHC class I molecules. The idea, therefore, arose that toxins entering the cytosol could transport epitopes into the cell for presentation by MHC class I. There are now many examples that this principle works (Noakes et al. 1999; Lippolis et al. 2000; Smith et al. 2002b), and that different toxins or parts of toxins can mediate such a process. In the case of Stx, even the B-subunit can be used. However, this does not mean that the B-fragment actually enters the cytosol with the epitope. Epitopes might also be released in the ER, and in the case of an epitope added to the plant toxin ricin, this has actually been shown to occur (Smith et al. 2002a). Inhibition of presentation by MHC class I by the drug lactacystin does not necessarily mean that the epitope is released in the cytosol. In this context, it is important to note that lactacystin is able to inhibit sorting along the endosomal pathway in the direction of lysosomes (van Kerkhof et al. 2001).

Several protein toxins are used to selectively kill certain cell types. The toxins are then used as components of molecules that are directed to certain cell types, for instance to cancer cells, by monoclonal antibodies or other ligands that selec-

tively bind the cell one wants to kill (Frankel et al. 2000; Gura 2002; Sinha 2003; Weaver and Laske 2003; Kreitman 2003a; Kreitman 2003b). In the case of Stx, the toxin might be used against certain types of cancer without any further modification (Arab et al. 1999; Salhia et al. 2002; Heath-Engel and Lingwood 2003). However, future studies are required to prove the usefulness of these approaches.

13 Conclusions

Knowledge about the complex interaction of Stxs with cells and the regulation of cellular sensitivity to these toxins is accumulating, thereby, facilitating development of new approaches to protect from disease and to cure patients affected by the toxins. Furthermore, the ongoing use of Stxs or parts of the toxins for therapeutic purposes and in cell biological studies are likely to provide us with information about basic processes in cell biology as well as with compounds that are useful in practical medicine.

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Cholera toxin: mechanisms of entry into host cells

David E. Saslowsky, Michael Kothe, and Wayne I. Lencer

Abstract

Cholera toxin moves from the plasma membrane to the ER of host cells to cause disease. Trafficking in this pathway depends on toxin binding to specific ceramide-based glycolipids that associate with lipid rafts at the cell surface. In the ER, a portion of the toxin is unfolded, dissociated from the rest of the toxin and retro-translocated to the cytosol where it activates adenylyl cyclase to initiate the severe secretory diarrhea seen in cholera.

1 Introduction

Cholera toxin (CT) is an AB_5 subunit protein complex responsible for the massive secretory diarrhea caused by *Vibrio cholerae* infection. After colonization of the intestine, the toxin is secreted from the bacterium into the intestinal lumen without damage to the epithelium. It enters host intestinal cells as a fully folded protein complex by co-opting a membrane glycolipid to move from the plasma membrane (PM) to the endoplasmic reticulum (ER) (Fig. 1). In the ER, the toxin hijacks the cellular machinery that allows misfolded proteins to cross the membrane for degradation in the cytosol, a process termed retro-translocation (Hazes and Read 1997; Tsai and Rapoport 2002a). Here the enzymatic portion of the toxin, the A1 chain, is unfolded, dissociated from the B subunit pentamer and retro-translocated to the cytosol where it acts to induce disease. This pathway followed by CT into the cytosol of host cells is almost the total reversal of the biosynthetic pathway for secretory and membrane proteins.

The A1 chain of CT is an ADP-ribosyltransferase that modifies the heterotrimeric G protein $G_{s\alpha}$ to activate adenylyl cyclase. The rapid production of cAMP by adenylyl cyclase induces intestinal chloride secretion, which, due to the accompanied movement of water, is responsible for the massive fluid loss characteristic of cholera. Previous studies on the biochemistry of CT action as an ADP-ribosyltransferase have contributed fundamentally to our understanding of heterotrimeric G-protein function in eukaryotic cells and to the identification of the ADP-Ribosylation Factor (ARF) family of small GTPases (Spangler 1992; Kahn et al. 2002).

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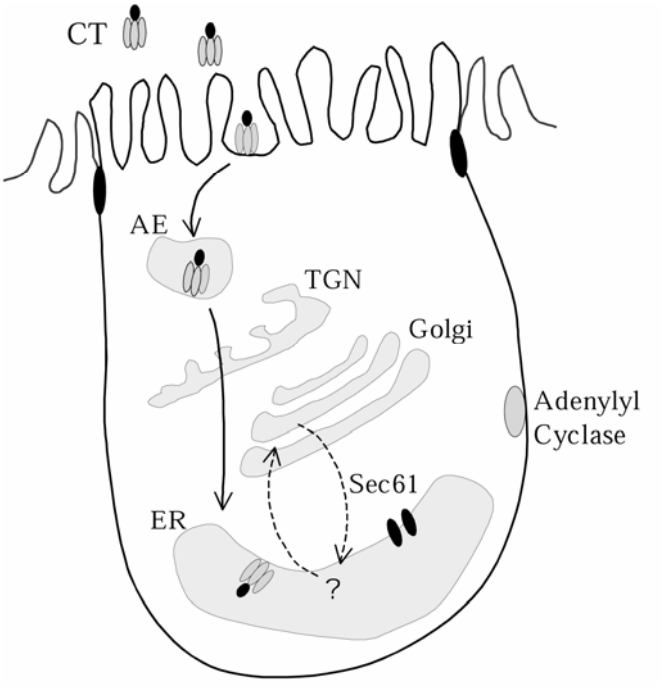

Fig. 1. Transport of CT from the Plasma Membrane to the ER by association with lipid raft glycolipids. CT enters the cell by binding ganglioside G_{M1} in lipid rafts and travels with the ganglioside to the Golgi and then retrograde to the ER. The toxin enters the ER as a fully assembled protein. AE, apical endosome; TGN, trans-Golgi network.

Studies on CT and other related AB_5 toxins have also elucidated critical aspects of epithelial cell biology that affect intestinal physiology in both health and disease. Here we discuss recent advances in our understanding of the mechanisms of toxin transport from the PM to the ER and of the reactions that unfold and retrotranslocate the A1 portion of the toxin to the cytosol.

2 Toxin structure

CT is a member of the AB family of toxins, where the A component is enzymatically active and causes toxicity and the B component is responsible for membrane-binding and mediates toxin entry into the cell. While several members of this family consist of single polypeptides with distinct A and B domains (e.g. ricin toxin, diphtheria toxin), CT belongs to the subfamily of AB_5 toxins, where the A and B components are expressed as separate polypeptides and assemble with a stoichiometry of 1:5 to form the holotoxin (Fig. 2). Other members of the AB_5 subfamily of toxins include the closely related *E. coli* heat-labile enterotoxins LTI, LTIIa and LTIIb, as well as shiga toxin, Shiga-like toxin and pertussis toxin.

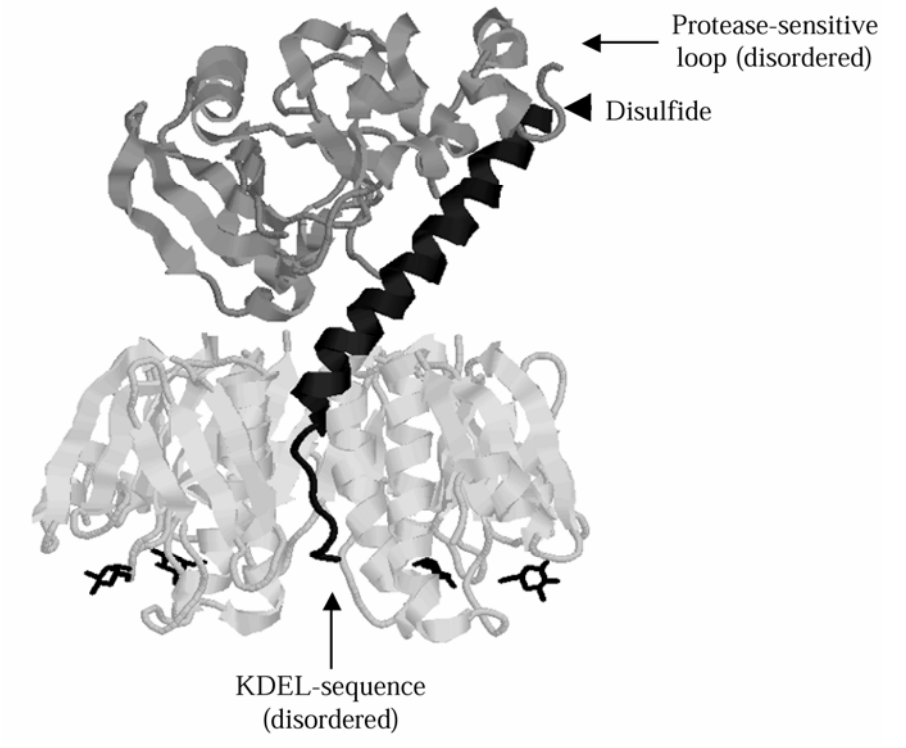

Fig. 2. Structure of cholera toxin (PDB file 1S5F). Three shades of grey are used to highlight the A1 and A2 domains of the A subunit (medium and dark grey, respectively) and the B subunits (light grey). Galactose molecules, which are bound to the G_{M1}-binding sites, are shown as black stick representations. One subunit of the B pentamer has been removed to aid in visualization of the A subunit C-terminus entering the B pentamer pore. The locations of the protease-sensitive loop connecting the A1 and A2 domains and of the KDEL sequence at the extreme C-terminus of A2 are indicated by arrows (both features are disordered in the crystal structure). The location of the disulfide bond linking Cys187 and Cys199 in the A subunit is indicated by an arrowhead.

The five copies of the B subunit form a highly stable ring-like assembly. The CT B pentamer contains five binding sites for G_{M1}, a ganglioside located in the epithelial cell apical membrane. Residues from two adjacent B subunits are involved in each of these binding sites. Binding of G_{M1} tethers the toxin to the membrane and results in the association of CT with specialized microdomains termed lipid rafts (Fig. 3). G_{M1}-mediated localization of CT in lipid rafts is required for toxin function (Orlandi and Fishman 1998; Wolf et al. 1998, 2002).

The A subunit assembles non-covalently with the B pentamer. It is divided into a compact N-terminal domain (A1) that contains the enzymatic activity of the toxin and an extended C-terminal domain (A2) that is responsible for tethering the A and B subunits together by protruding with its C-terminus through the central

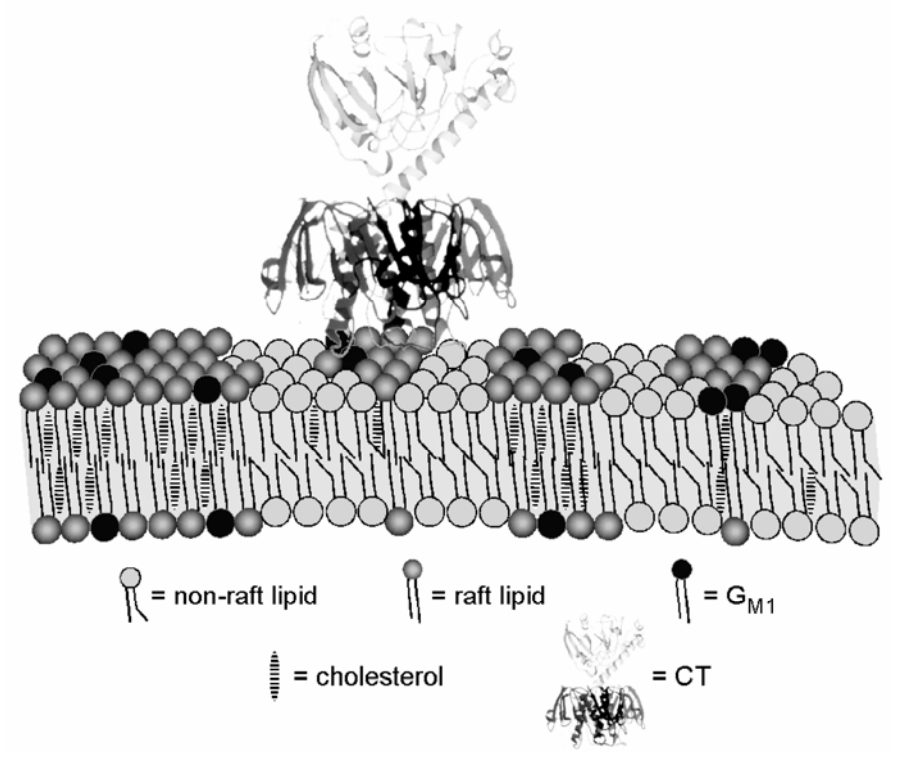

Fig. 3. Binding of CT to raft-associated G_{M1}. G_{M1} is shown to be preferentially associated with raft domains enriched in sphingolipids (e.g. sphingomyelin) and cholesterol. Association of CT with such domains is important for efficient endocytosis and toxin trafficking.

pore in the B pentamer (Fig. 2). The extreme C-terminus of the A2 chain has an ER-retention KDEL motif that faces the membrane when the toxin binds to G_{M1}. The A1 and A2 domains are linked by a flexible protease-sensitive loop. Cleavage of the loop occurs in the intestinal lumen after secretion of the toxin from *V. cholerae* and is mediated by proteases either endogenous to the microbe or the host. After cleavage, the A1 and A2 chains remain stably linked by a disulfide bond and extensive non-covalent interactions (Sixma et al. 1993) until arrival of the toxin in the ER, where reduction of the disulfide and PDI-mediated unfolding separate the A1 chain from the A2-B_5 complex.

3 Endocytosis and lipid rafts

Both clathrin-dependent and -independent mechanisms of endocytosis are involved in the initial uptake of CT into the cell (Parton 1994; Nichols et al. 2001; Shogomori and Futerman 2001a; Sandvig and van Deurs 2002; Singh et al. 2003). While all forms of endocytosis may allow CT access to the Golgi and ER, recent studies show that neither endocytosis by clathrin-, caveolin-, or Arf6-dependent

mechanisms are required for the induction of toxicity (Massol et al. 2004). These results are consistent with the existence of a novel alternative mechanism of endocytosis that allows CT to move from the PM to the Golgi en route to the ER. The molecular identity of this pathway remains to be identified but likely involves cholesterol homeostatic machinery as well as lipid rafts.

It has long been appreciated by chemists and biophysicists that the lipid species present in cell membranes are capable of phase-partitioning into distinct domains in artificial membrane systems (Anderson and Jacobson 2002; Simons and Ehehalt 2002; Edidin 2003). Studies investigating GPI-anchored proteins involved in signal transduction as well as the finding that membrane fragments enriched in such proteins persist after detergent extraction of intact cells form the basis of the raft hypothesis. In this model, phase-partitioned microdomains (rafts) concentrate or exclude protein species in cells based on the thermodynamic properties of the membrane-associated portion(s) of the protein or by direct interaction with the head groups of raft-associated lipids. This preferential lateral sorting of membrane proteins may also serve either to activate or inhibit protein function, a phenomenon that is typically determined empirically on a case-by-case basis. Such interactions with rafts are probably dynamic and are likely influenced by protein conformation and state of post-translational modification, as well as membrane lipid composition. This model predicts a powerful mechanism of control over membrane physiology and has been given credence with respect to living cells through recent studies attempting to visualize raft domains non-invasively at the molecular level (Nichols 2002; Gaus et al. 2003; Sharma et al. 2004). Rafts are enriched in sphingomyelin and cholesterol, species that posses a mutual affinity and that are synthesized and transported from the ER to other sites in the cell such as the PM. The biosynthesis, trafficking, and homeostasis of these two raft components are tightly coupled and may provide the basis for the retrograde trafficking observed for CT via interactions with its raft-associated receptor G_{M1}.

Several studies suggest that CT enters host cells by binding G_{M1} in lipid rafts and that this process is dependent on membrane cholesterol (Orlandi and Fishman 1998; Wolf et al. 1998, 2002; Fujinaga et al. 2003). The inhibition of toxin function after cholesterol depletion correlates with a change in raft structure characterized by the loss of proteins normally associated with the lipid raft fraction. The CT-G_{M1} complex, however, is not displaced from the raft fraction by cholesterol depletion. Thus, toxin trafficking and function in intestinal cells depends on membrane cholesterol, but toxin localization in apical PM rafts does not. It is possible that cholesterol depletion changes the lipid composition of rafts in a way that alters raft function regarding processes such as endocytosis. Together, these results raise the possibility that cholesterol functions to couple the CT-G_{M1} complex with membrane components that are involved in raft dynamics in a way that actuates toxicity in host cells.

One of the proteins displaced from the raft fraction in intestinal cells by cholesterol depletion is actin (our unpublished results, Badezadigan and Lencer). An intact actin cytoskeleton is necessary for the efficient transport of CT to the Golgi and for the subsequent activation of adenylyl cyclase. We have found that CT fractionates with heterogeneous populations of lipid rafts, a portion of which are

enriched in actin and other cytoskeletal proteins. CT and actin co-immunoprecipitate, suggesting that they are present on the same membrane microdomain. When tested *in vivo*, depolymerization or stabilization of actin filaments inhibits transport of CT from the PM to the TGN, and reduces the levels of cAMP generated in the cytosol. Thus, the CT-G_{M1} complex might be associated with the actin cytoskeleton via lipid rafts, and this appears to be required for trafficking of CT from the PM to the ER. This phenomenon is similar to that found for SV40 virus, which also binds G_{M1} and enters host cells via caveolae (Pelkmans et al. 2001, 2002; Tsai et al. 2003).

4 Retrograde transport into the Golgi and ER

Subsequent to endocytosis, CT can be found in early and recycling endosomes, the Golgi apparatus, and the ER (Majoul et al. 1996, 1998; Richards et al. 2002). Movement within the Golgi apparatus can be inhibited by blockade of COPI- and COPII-mediated vesicular transport, and this affects toxin function, implicating trafficking through the Golgi as a necessary step in toxin action (Richards et al. 2002). It is proposed that the A and B subunits of CT dissociate in the Golgi apparatus, and that the A subunit, which contains an ER-targeting KDEL-motif at its C-terminus, may then traffic retrograde into the ER by binding the KDEL-receptor Erd2 (Bastiaens et al. 1996; Majoul et al. 1998, 2001). Two lines of evidence, however, show that this is not likely to be the case. First, the KDEL-motif is not required for toxin function, though it improves the efficiency of intoxication (Lencer et al. 1995). Second, recent studies using a mutant CT B subunit containing sulfation and N-glycosylation motifs for direct assay of toxin entry into the Golgi and ER show that the lipid raft ganglioside G_{M1} is the vehicle for transport into these compartments (Fujinaga et al. 2003). The A subunit is completely dispensable for retrograde trafficking to the ER. Toxin binding to any membrane glycolipid, however, is not sufficient for transport in this pathway. The glycolipid must also associate with lipid rafts. In intestinal cells, for example, the *E. coli* toxin LTIIb that binds to the non-raft glycolipid G_{D1a} does not move backwards to the Golgi or ER. Thus, CT is transported from the PM to the ER by a specific lipid sorting pathway and not a pathway that depends on direct sorting of proteins.

4.1 Possible mechanisms of ganglioside trafficking

We believe the ability of G_{M1} but not G_{D1a} to sort CT retrograde into the Golgi and ER in intestinal cells is based on differences in the structures of their lipid moieties. The ceramide domain of G_{M1} likely defines the affinity of the molecule for association with lipid rafts (Brown and London 2000). Raft association, however, may not be sufficient for retrograde transport all the way into the secretory pathway. GPI-anchored proteins, for example, also fractionate with lipid rafts, but there are conflicting reports whether these molecules traffic retrograde from the

PM to the Golgi or into the endosome (Fivaz et al. 2002; Nichols 2002; Sabharanjak et al. 2002). Thus, it is possible that only ceramide-based raft glycolipids move retrograde from the PM to the ER.

Sorting of raft from non-raft glycolipids, and delivery of raft glycolipids into the retrograde pathway probably depend on biophysical features of the lipid moiety that can influence its incorporation into certain membrane domains, vesicle buds, and tubules (Mukherjee et al. 1999). The ability of the AB_5 toxins to cross-link up to five glycolipids at once may further enhance these properties. It is also possible that specific components of the raft microdomain may function to couple the toxin-ganglioside complex with sorting machinery inside the cell necessary for retrograde transport as discussed above. Such lipid-based sorting may occur during endocytosis at the level of the PM, after arrival in the endosomal compartment, or both (Parton 1994; Nichols et al. 2001; Shogomori and Futerman 2001b).

4.2 A direct pathway from TGN to ER

Shiga toxin is a structurally related AB_5 toxin that also enters the ER of host cells by binding a membrane glycolipid (Sandvig and van Deurs 2002). There is evidence that the lipid-dependent trafficking pathways from the PM to the ER for CT and Shiga toxin are independent of sorting by the KDEL receptor and COPI-coated transport vesicles (Girod et al. 1999; Fujinaga et al. 2003).

An explanation for this independence was recently provided by the results of studies on the novel small molecule Exo2 (Feng et al. 2004). Exo2 collapses the Golgi apparatus but leaves the TGN intact. CT remains fully functional in cells treated with Exo2, even though the Golgi apparatus is completely dispersed. Thus, it is possible that CT can bypass the Golgi apparatus and move directly from the TGN to the ER, at least in cells treated with Exo2. Such a direct pathway for transport from the PM to the ER has been suggested for the membrane protein caveolin and cholesterol (Smart et al. 1996) and for the G_{M1}-binding virus SV40 (Pelkmans et al. 2001, 2002; Fujinaga et al. 2003). Why then does inhibition of COPI affect toxin function? Here, we propose that the KDEL-motif on CT and COPI-dependent transport, while not required for trafficking into the ER, play a role in retaining CT in the ER (Fujinaga et al. 2003) (Fig. 1). Interference with the KDEL-dependent retrieval pathway from Golgi to ER affects toxin function by reducing the availability of the A1 chain for retro-translocation to the cytosol.

5 Mechanisms of retro-translocation across the ER membrane

Our studies on B subunit trafficking into the ER show that CT enters the ER as a fully folded protein. In the ER, the luminal chaperone protein disulfide isomerase (PDI) recognizes the A1 chain (Fig. 4). In its reduced form, PDI binds to the A1

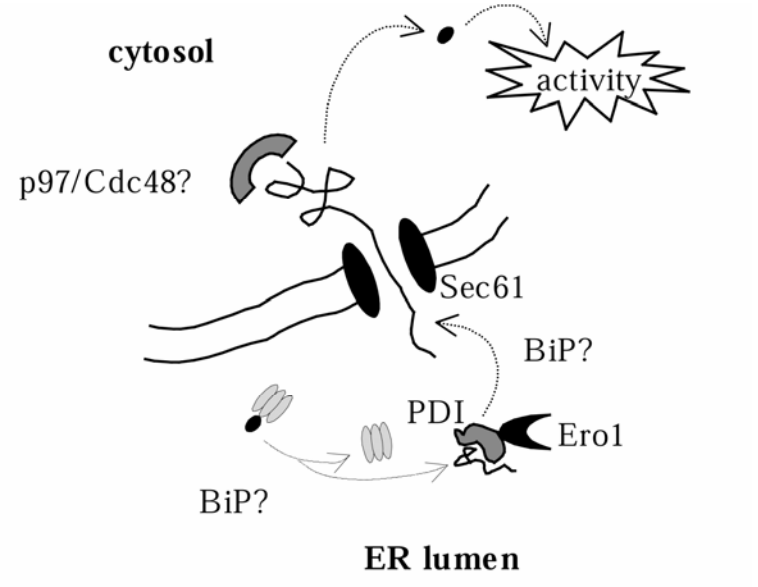

Fig. 4. Unfolding and retro-translocation of the CT A1 chain. In the ER, protein disulfide isomerase (PDI) in its reduced form recognizes the proteolytically nicked form of the CT A subunit and unfolds and dissociates the A1 chain from the B subunit. The PDI-A1chain complex may be targeted to the ER luminal membrane where the oxidoreductase Ero1 catalyzes the oxidation of PDI, causing the release of the unfolded A1 chain. Retro-translocation may occur by passage of the A1 chain through the Sec61 channel. The driving force for movement of the A1 chain out of the ER into the cytosol does not depend on polyubiquitination of the A1 chain but might involve interaction with the AAA ATPase p97/Cdc48. Involvement of BiP in retro-translocation has also been suggested.

chain, dissociates it from the B subunit, and unfolds it (Tsai et al. 2001). PDI will recognize the A1 chain only when the peptide loop that connects the A1 and A2 chains is proteolytically cleaved. This site of cleavage has long been recognized to represent a critical post-translational modification required for toxicity. Thus, the peptide loop between the A1 and A2 chains acts as a molecular switch, allowing for proper folding of CT in the periplasm of *V. cholerae* when the loop is intact and for unfolding in the ER of host cells when the loop is cleaved by proteolysis. Both the periplasm and ER are similar oxidizing environments containing chaperones to assist in protein folding and unfolding.

The molecular mechanism that explains why PDI recognizes the nicked toxin as a substrate is not known. It may recognize a hydrophobic domain in the A1 chain that is exposed by cleavage and reduction of the A subunit (Hazes and Read 1997). After unfolding and dissociating the A1 chain from the B subunit, the A1 chain-PDI complex appears to be targeted to the luminal surface of the ER membrane where it is then oxidized by Ero1 (Tsai and Rapoport 2002b). The oxidized form of PDI releases the A1 chain, presumably into the protein-conducting chan-

nel that transports the A1 chain to the cytosol. *In vitro*, the A1 chain rapidly and spontaneously refolds after release from PDI. Thus, PDI acts as a chaperone that is driven by a redox- rather than an ATP-cycle. There is good evidence that the protein translocation channel that transports the A1 chain to the cytosol may be Sec61 (Schmitz et al. 2000). There are also recent studies that suggest the ER chaperone BiP may play a role in the retro-translocation of the A1 chain (Winkeler et al. 2003).

Since most proteins that are dislocated from the ER are polyubiquitinated and targeted for degradation by the proteosome, we recently tested whether polyubiquitination is required for the retro-translocation reaction. Here, we prepared mutant toxin lacking all lysines in the A1 chain and with the N-terminus blocked by carbamylation. When applied to human intestinal cells, this mutant toxin lacking all sites for polyubiquination still induced a robust Cl^- secretory response (Rodighiero et al. 2002). Thus, polyubiquitination is not required for retro-translocation of the A1 chain to the cytosol.

Why are the two lysines in the wild type toxin not ubiquitinated after retro-translocation to the cytosol? Since the A1 chain must be unfolded initially after entry into the cytosol, we hypothesized that the toxin may refold rapidly after emerging from the translocation channel. To test this idea, we modeled the refolding reaction *in vitro*, and found that the A1 chain refolds in as little as 5 seconds after release from PDI (Rodighiero et al. 2002). Such rapid refolding renders the A1 chain resistant to polyubiquitination and may explain the driving force for the retro-translocation reaction itself.

Retro-translocation of the A1 chain might also be assisted by the AAA ATPase p97, a cytosolic protein that is involved in extracting misfolded proteins from the ER (Meyer et al. 2000, 2002; Ye et al. 2001, 2003). p97 interacts with the ER membrane and translocates proteins by alternating ATP hydrolysis on its two ATPase domains. While p97 seems to be mainly involved in retro-translocation of polyubiquitinated substrates as a complex with its cofactors Ufd1 and Npl4, it has recently been shown to be able to also interact with non-ubiquitinated substrates (Ye et al. 2003). It is, therefore, possible that the initial phase of A1 chain retro-translocation is assisted by p97, with spontaneous refolding taking over as the driving force once a portion of the chain has been translocated.

6 Conclusions

CT moves from the PM to the ER by binding to G_{M1} gangliosides intrinsic to lipid rafts. Raft structure probably dictates this function and depends on membrane cholesterol and likely on association with the actin cytoskeleton. Because many of the bacterial toxins and some viruses use gangliosides or neutral ceramide glycolipids to enter the ER of host cells, the raft glycolipids, and in particular the ceramide-based lipids represent a general vehicle for transport in this pathway. The lipid-dependent sorting pathway from the TGN to the ER may bypass the Golgi apparatus entirely. The toxin enters the ER as a fully folded protein and it is unfolded in

the ER by the ER-luminal chaperone PDI, and possibly BiP. The retro-translocation reaction that moves the A1 chain to the cytosol likely involves protein transport across Sec61 but does not depend on polyubiquitination. The driving force may be provided instead by the intrinsic ability of the A1 chain to refold spontaneously or by the cytosolic AAA ATPase p97.

There are still numerous gaps in our understanding of the mechanisms by which CT and G_{M1} traffic through the aforementioned pathways. As clathrin and caveolin are not requisite for endocytosis of the CT-G_{M1} complex, are there any other as yet undiscovered proteins involved in this process, and if so, are they specific for the non-caveolae raft pathway? To what degree does the trafficking of CT overlap with that of cholesterol and sphingolipids such as sphingomyelin between the PM and ER, and does this explain why G_{M1} carries CT from the PM to the ER? Questions also remain with respect to the mechanism of retro-transocation. What are the exact sequence of events and identity of molecular components required to unfold and retro-translocate the A1 chain to the cytosol, and what is the driving force for this reaction? Future studies on CT and the related AB_5 toxins will continue to advance our understanding of these fundamental pathways essential to eukaryotic cell biology that are co-opted by certain toxins and viruses to induce disease.

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ExoU: A cytotoxin delivered by the type III secretion system of *Pseudomonas aeruginosa*

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Abstract

ExoU is a potent cytotoxin that is injected into host cells by the type III secretion system of *Pseudomonas aeruginosa.* It has recently been shown to possess phospholipase activity, which is essential for its ability to rapidly kill a broad variety of eukaryotic cell types. As part of the type III secretion regulon of *P. aeruginosa,* ExoU is regulated at the level of both transcription and translation by environmental stimuli that include contact with eukaryotic cells, low calcium concentrations, and a temperature of 37°C. The *exoU* gene itself is a variable trait and is found in only a third of *P. aeruginosa* strains isolated from patients with acute infections. Although a minority of strains secrete ExoU, these strains are in general more virulent. Likewise, disruption of the ExoU-encoding gene in strains that normally secrete this protein results in decreased virulence. Thus, ExoU is a potent toxin that augments the ability of *P. aeruginosa* to cause disease.

1 Introduction

Microbial pathogens have evolved diverse and ingenious mechanisms to manipulate, injure, or kill the host cells they encounter while causing disease. In this regard, *Pseudomonas aeruginosa* is no exception. This gram-negative bacterium is called an opportunistic pathogen because of its predilection to cause disease in compromised hosts. For example, it is a leading cause of hospital-acquired infections (Stryjewski and Sexton 2003) as well as chronic respiratory infections in individuals with cystic fibrosis (Davidson et al. 2003). *P. aeruginosa* produces a large number of factors that contribute to its ability to colonize, persist, and disseminate within the human body (Engel 2003). These virulence determinants allow *P. aeruginosa* to infect many different anatomical sites, including the respiratory tract, bloodstream, urinary tract, eye, and skin (Stryjewski and Sexton 2003).

Recently one particular virulence determinant of *P. aeruginosa*—ExoU—has attracted considerable attention. ExoU is a type III-secreted phospholipase with a predicted molecular mass of 73.9 kDa that when injected into cells of infected tissues results in rapid death. As the result of recent advances, a clearer understanding is emerging of ExoU's molecular activity, how this activity leads to its cytotoxic effect on host cells, and how this in turn results in severe disease. In this

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chapter, we will review what is known about the molecular, cell biological, and clinical aspects of ExoU intoxication.

2 Discovery

A pivotal study in the discovery of ExoU was the careful examination of *P. aeruginosa*-infected epithelial cell monolayers by Apodaca and colleagues (Apodaca et al. 1995). These investigators noted that certain strains of *P. aeruginosa* induced epithelial cell death during co-incubation in cell culture. This killing—hereafter referred to as cytotoxicity—was rapid, resulted in the loss of plasma membrane integrity, and required direct contact between viable bacteria and the affected cells. Studies using isogenic mutants demonstrated that this cytotoxicity did not require the well-characterized virulence factor exotoxin A, but rather the AraC-like transcriptional regulator ExsA (Frank and Iglewski 1991). ExsA had previously been shown to be essential for expression of the putative toxin ExoS and several other uncharacterized proteins (Frank et al. 1994). Interestingly, not all strains of *P. aeruginosa* possessed cytotoxic activity but those that did were more virulent in animal models of infection than non-cytotoxic strains or mutants (Wiener-Kronish et al. 1993; Kudoh et al. 1994; McElroy et al. 1995; Sawa et al. 1998). Thus, there was significant interest in identifying the bacterial factor responsible for cytotoxicity.

The development of an *in vitro* cytotoxicity assay laid the foundation for the identification of ExoU. This assay was used by two groups of researchers who independently discovered ExoU using different approaches. Fleiszig, Frank, Weiner-Kronish, and colleagues demonstrated that cytotoxic strains of *P. aeruginosa* secreted an approximately 70 kDa protein that was absent in the culture supernatants of non-cytotoxic strains (Fleiszig et al. 1997). Furthermore, production of this protein required the transcriptional activator ExsA (Frank et al. 1994), which had recently been shown to regulate expression of a previously unidentified type III secretion system in *P. aeruginosa* (Yahr et al. 1996). Together, these results suggested that the 70 kDa protein was responsible for *P. aeruginosa*-associated cytotoxicity and was secreted by this type III pathway. Random transposon mutagenesis of a cytotoxic strain of *P. aeruginosa* was used to generate a library of mutants, which was then screened for defects in secretion of the 70 kDa protein (Finck-Barbançon et al. 1997). Using this approach, the gene that encoded the 70 kDa protein was identified and named ExoU (Finck-Barbançon et al. 1997). The hypothesis that ExoU was essential for cytotoxicity was confirmed when the mutant containing the disrupted *exoU* gene was shown to be non-cytotoxic and complementation with an intact copy of the *exoU* gene restored cytotoxicity (Finck-Barbançon et al. 1997).

Kang, Engel, and colleagues independently identified ExoU using a different approach (Kang et al. 1997). They also used random transposon mutagenesis of a cytotoxic strain of *P. aeruginosa* to generate a library of *P. aeruginosa* mutants. However, this library was then directly screened for loss of cytotoxicity by co-

incubating each mutant with epithelial cells in an assay for cell death. A subset of the identified mutants had defects in protein secretion. Most of the disrupted genes in these mutants were similar to the type III secretion genes of *Yersinia* spp. Two of the mutants, however, had transposons inserted in a gene that encoded an approximately 70 kDa secreted protein, which was designated PepA (Hauser et al. 1998a). The other type III secretion-like genes appeared to be required for the secretion or translocation of PepA (Kang et al. 1997; Hauser et al. 1998b) while PepA itself was required for cytotoxicity (Hauser et al. 1998a). Comparison of the sequences of PepA and ExoU indicated that they were the same protein; ExoU is now the accepted name of this protein.

It is now clear that ExoU is part of the *P. aeruginosa* type III secretion system (Yahr et al. 1996; Finck-Barbançon et al. 1997; Kang et al. 1997; Hauser et al. 1998a). Type III secretion systems are present in a large number of gram-negative bacteria and function to inject bacterial proteins, called effector proteins, directly into host cells (reviewed in Hueck 1998). This occurs through the action of a complex secretion and translocation apparatus that in some bacterial species has been visualized as a needle-like structure extending from the bacterial surface (Kubori et al. 2000; Blocker et al. 2001). In the *P. aeruginosa* type III system, ExoU as well as ExoS, ExoT, and ExoY are the effector proteins that are injected into host cells (reviewed in Frank 1997). The *P. aeruginosa* type III system is most similar to the type III system of *Yersinia* (Yahr et al. 1996), although no homologous cytolytic effector proteins have been found in the *Yersinia* system.

3 Activity

3.1 Cell biological activity

As described in the preceding section, ExoU was identified based upon its cytotoxicity towards mammalian cells. Isogenic mutants with disrupted *exoU* genes were significantly less cytotoxic than their wild type parent strains (Finck-Barbançon et al. 1997; Hauser et al. 1998a), clearly showing that ExoU is necessary for high levels of cytotoxicity. Furthermore, transfection of Chinese hamster ovary (CHO) cells with the *exoU* gene resulted in increased cell permeability and decreased detection of a luciferase reporter from a cotransfected construct, indicating that ExoU is not only necessary but sufficient to kill mammalian cells in the absence of other bacterial products (Finck-Barbançon and Frank 2001). The onset of ExoU-mediated cell death is rapid, within 1 1/2 to 3 hours of infection (Apodaca et al. 1995). The membranes of intoxicated cells become permeable, and features of apoptosis, such as DNA fragmentation, are not observed, indicating that killing occurs by necrosis (Apodaca et al. 1995; Finck-Barbançon et al. 1997; Hauser et al. 1998a; Hauser and Engel 1999; Finck-Barbançon and Frank 2001).

In cell culture assays, ExoU's cytotoxic activity is not limited to one or a few cell types. ExoU-mediated killing has been observed in cell lines derived from the following cell types: fibroblasts (Evans et al. 2002), macrophages (Hauser and

Engel 1999; Sawa et al. 1999), and many types of epithelial cells, including Madin-Darby canine kidney cells (Apodaca et al. 1995; Finck-Barbancon et al. 1997; Kang et al. 1997), primary corneal cells (Fleiszig et al. 1996), T84 human colon carcinoma cells (Allewelt et al. 2000), BEAS-2B human bronchial epithelial cells (Kurahashi et al. 1999), and CHO cells (Vallis et al. 1999a; Finck-Barbancon and Frank 2001). This suggests that delivery of ExoU and its mechanism of cytotoxicity utilize factors that are common to many cell types.

3.2 Enzymatic activity

Initially, the molecular mechanism by which ExoU caused cell death was a mystery. ExoU was not homologous to any characterized proteins and did not possess known motifs that suggested a specific enzymatic activity. Results, however, indicated that large portions of this protein were essential for killing. For example, a random transposon insertion in the 3' end of the *exoU* gene resulted in the production of a C-terminal truncated variant of ExoU that was non-cytotoxic, indicating that the last 30 amino acids were necessary for killing (Hauser et al. 1998a). Subsequent structure-function studies were performed to more carefully define the regions of ExoU necessary to cause cell death. Transfection of CHO cells with plasmids expressing wild type ExoU resulted in cell death, whereas transfections with plasmids encoding ExoU variants containing N-terminal, C-terminal, and internal deletions resulted in viable CHO cells (Fig. 1) (Finck-Barbançon and Frank 2001). These data indicated that all three regions of ExoU were essential for cytotoxicity in mammalian cells, conclusions that were confirmed and refined in a yeast model system (Rabin and Hauser 2003; Sato et al. 2003). Specifically, whereas yeast were rapidly killed by the expression of wild type ExoU, *S. cerevisiae* remained viable upon expression of ExoU variants containing deletions of amino acids 83-119, 301-342, or 624-687, indicating that at least portions of each of these three regions were essential for cytotoxicity (Fig. 1). Subsequent studies using CHO cells demonstrated that deletion of as few as 20 amino acids from the C-terminus of ExoU was sufficient to abolish cytotoxic activity (Phillips et al. 2003). Conversely, an ExoU variant lacking amino acids 1-82 remained cytotoxic to yeast, indicating that this portion of the protein was dispensable for killing (Rabin and Hauser 2003). This is not surprising, since the extreme N-termini of type III effector proteins carry information essential for secretion and translocation through the type III needle but not for biological activities (Hueck 1998). Together, the results of these deletion studies indicated that portions of the N-terminal, C-terminal, and internal regions of ExoU are necessary for cytotoxicity.

In the absence of a known activity, little else could be inferred about the mechanism of ExoU-mediated killing. It did appear that ExoU was quite potent. Expression of even small amounts of ExoU were sufficient to rapidly lyse CHO cells, preventing visualization of green fluorescent protein (GFP)-tagged ExoU (Finck-Barbançon and Frank 2001). In fact, half-maximal killing of CHO cells required as few as 300-600 molecules per cell (Phillips et al. 2003). Thus, although

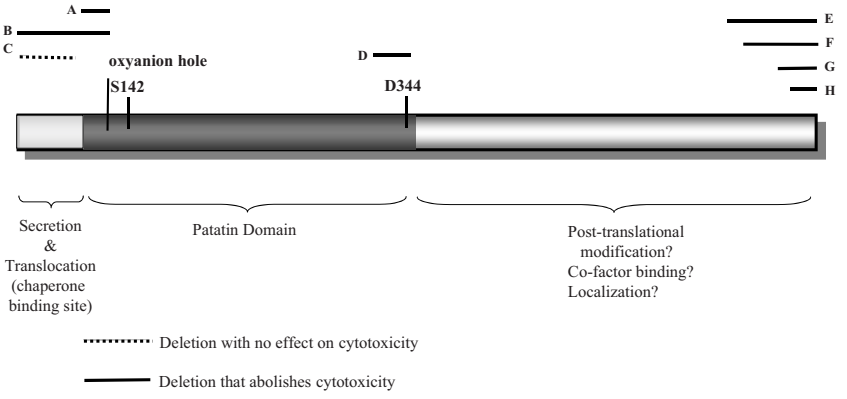

Fig. 1. Regions of ExoU essential for cytotoxicity. Currently, three regions of ExoU have been defined. First, the N-terminal portion is thought to contain information necessary for secretion, translocation, and chaperone binding. This is adjacent to a patatin-like domain, which contains the putative oxyanion hole, catalytic serine (S142), and catalytic aspartate (D344) residues and is essential for lipase activity. Finally, the C-terminal half of the protein has unknown function but is required for cytotoxicity. The bars above the ExoU molecule summarize the results of deletion studies. The deletions are comprised of the following amino acids: A: 83-119, B: 1-119, C: 1-82, D: 301-342, E: 624-687, F: 633-687, G: 657-687, H: 667-687. Data in this figure are taken from the following: (Hauser et al. 1998a; Finck-Barbançon and Frank 2001; Phillips et al. 2003; Rabin and Hauser 2003).

ExoU appeared to be an extremely potent cytotoxin, its mechanism of action remained a mystery.

A clue to ExoU's activity became available when continued expansion of the protein domain databases led to the identification of a patatin-like domain within its N-terminus (Sato et al. 2003) (Fig. 1). Patatin is the major storage protein found in potatoes (Vancanneyt et al. 1989; Ganal et al. 1991) and has lipid acyl hydrolase activity used for protection under conditions of stress or infection (Strickland et al. 1995). Patatin possesses a Ser-Asp catalytic dyad similar to that of human calcium-independent phospholipase A_2 ($iPLA_2$) and human cytosolic phospholipase A_2 ($cPLA_2$), and has itself been associated with phospholipase A_2 activity (Hirschberg et al. 2001; Rydel et al. 2003) (Fig. 2). These proteins also share an oxyanion hole motif, whose role is to stabilize the tetrahydral intermediate of the enzyme during catalysis (Hirschberg et al. 2001). Sequence alignments demonstrated that ExoU also contains a putative oxyanion hole (residues 111-116), a catalytic serine motif (residues 140-144 containing S142), and a catalytic aspartate motif (residues 344-346 containing D344) within its amino terminus (Figs. 1 and 2) (Phillips et al. 2003; Sato et al. 2003). The identification of a patatin-like domain in ExoU suggested that this toxin was a phospholipase, a hypothesis confirmed by direct experimental evidence. Expression of ExoU in yeast resulted in increased amounts of free palmitic acid and decreased amounts of neutral lipids (Sato et al. 2003). Likewise, syringe loading of ExoU into CHO cells resulted in release of arachidonic and palmitic acids (Phillips et al. 2003). ExoU re-

quires a eukaryotic cofactor to exhibit lipase activity (Sato et al. 2003), but it is unlikely that ExoU is merely activating an endogenous lipase. Addition of purified recombinant ExoU along with yeast extracts to liposomes released free fatty acids but treated yeast extracts subsequently depleted of exogenously added recombinant ExoU did not (Sato et al. 2003). As expected, mutation of the putative active site residues to S142A or D344A resulted in a loss of *in vitro* and *in vivo* lipase activity (Phillips et al. 2003; Sato et al. 2003).

The evidence is quite strong that ExoU's phospholipase activity is an integral part of its ability to kill eukaryotic cells. Inhibitors of $iPLA_2$ and $cPLA_2$ blocked ExoU-mediated killing in both the yeast model system and in co-culture experiments with *P. aeruginosa* and BEAS-2B or CHO cells (Phillips et al. 2003; Sato et al. 2003). Inhibitors specific for human phospholipase A_2's did not prevent killing, indicating that this protection was not an indirect effect of inhibiting host cell phospholipases (Phillips et al. 2003). Furthermore, a point mutation in the catalytic serine residue (S142A) or aspartate residue (D344A) resulted in non-cytotoxic variants of ExoU when tested with CHO cells or BEAS-2B cells (Phillips et al. 2003; Sato et al. 2003).

The exact substrate specificity of ExoU has not been fully defined, but as is the case for patatin, it appears to be broad. Patatin catalyzes the hydrolysis of phospholipids, glycolipids, sulfolipids, monoacylglycerols, perhaps diacylglycerols, but not triacylglycerols (Galliard 1970, 1971; Hirayama et al. 1975; Wardale 1980; Senda et al. 1996). Likewise, ExoU appears to hydrolyze neutral lipids as well as phospholipids (Sato et al. 2003). Inhibitor studies were consistent with the presence of phospholipase A_2 activity (Phillips et al. 2003; Sato et al. 2003), and the release of fatty acids when activated recombinant ExoU was incubated with lysophospholipid indicated lysophospholipase activity (Tamura et al. 2004). Interestingly, this second activity was much stronger than the PLA_2 activity (Tamura et al. 2004). Whether ExoU has additional enzymatic activities has not yet been examined.

The requirement of the patatin-like domain for cytotoxicity presents an explanation for the results of earlier deletion studies showing that a region adjacent to the N-terminus as well as an internal region of ExoU were essential for cell killing (Finck-Barbançon and Frank 2001; Rabin and Hauser 2003) (Fig. 1). Both these regions lie within the patatin-like domain. However, the mechanism by which the C-terminus of ExoU contributes to cytotoxicity has not yet been determined. Multiple studies found that the C-terminus was required for cytotoxicity, yet this portion of the protein is well removed from the patatin-like domain (Kang et al. 1997; Finck-Barbançon and Frank 2001; Rabin and Hauser 2003) (Fig. 1). It is conceivable that the C-terminus modifies the lipase activity of the N-terminus, perhaps by providing a binding site for the eukaryotic cofactor or a site for post-translational modification. Alternatively, lipase activity alone may not be sufficient for cell death. The C-terminus may play an essential role in localizing the patatin-like domain to a particular cellular compartment or may itself have a second activity required for killing.

	oxyanion hole	active site serine	active site aspartic acid
ExoU:	110-S**GG**GA**KG**A-117	140-**GS**S**AG**GI-146	343-Q**DG**GVM-348
PatatinB2:	12-D**GG**GI**KG**I-19	52-**GT**S**TG**GL-58	191-V**DG**AVA-196
$iPLA_2$:	430-D**GG**GV**KG**L-437	463-**GT**S**TG**GI-469	597-L**DG**GLL-602
$cPLA_2$:	195-S**GG**GF**R**AM-202	226-**GL**S**S**GSTW-231	548-V**D**SGLT-553

Fig. 2. Alignments of portions of the amino acid sequences of patatin, ExoU, $iPLA_2$, and $cPLA_2$ showing similarities between the oxyanion hole, active site serine, and active site aspartate motifs of these proteins. Letters in bold represent residues conserved in bacterial patatin-like proteins. Adapted from the following: (Banerji and Flieger 2004; Hirschberg et al. 2001; Sato et al. 2003).

Together, these findings support a model in which ExoU kills eukaryotic cells by acting as a phospholipase. This toxin is the first identified lipase delivered by a type III secretion system and the first bacterial patatin-like protein shown to be important in virulence. Interestingly, patatin-like domains have recently been identified in many bacterial pathogens and symbionts, suggesting that ExoU may be the first characterized member of a large family of bacterial proteins (Banerji and Flieger 2004).

3.3 Mechanism of cytotoxicity

At the cellular level, the net result of intoxication with ExoU is clear; cell death occurs rapidly and is characterized by loss of integrity of the affected cell's plasma membrane (Finck-Barbançon et al. 1997; Hauser et al. 1998a; Hauser and Engel 1999; Finck-Barbançon and Frank 2001). The mechanism by which this occurs is less well understood. Pore formation by ExoU is not involved, since this toxin does not oligomerize or form pores in liposomes (Finck-Barbançon and Frank 2001). Rather, ExoU's phospholipase activity is essential for killing. Very little is known about the mechanism by which this activity leads to cell death, in part because the rapidity of ExoU-induced cytotoxicity makes it difficult to study. However, several recent reports have begun to elucidate certain aspects of this process.

Following injection of ExoU into the host cell, activation by a eukaryotic cofactor is required (Sato et al. 2003; Tamura et al. 2004). For this reason, ExoU's phospholipase activity was initially difficult to detect with *in vitro* assays using recombinant ExoU (Phillips et al. 2003; Sato et al. 2003). Lipase activity was only noted in the presence of yeast or epithelial cell extracts, indicating the requirement for such a eukaryotic factor (Phillips et al. 2003; Sato et al. 2003; Tamura et al. 2004). In this regard, ExoU is similar to ExoS and ExoY, two other *P. aeruginosa* type III effector proteins that also require eukaryotic factors for enzymatic activity. The identity of ExoU's eukaryotic cofactor is not known.

Whether ExoU's phospholipase activity directly or indirectly leads to cell death is unclear. ExoU may act directly on the phospholipids in the host cell plasma or

organellar membranes, resulting in cellular dissolution and lysis. Alternatively, ExoU may activate or inhibit endogenous host cell processes that in turn result in rapid cell death. Inhibitors of cyclooxygenase and lipoxygenase, downstream signaling molecules of the arachidonic acid pathway activated by $cPLA_2$, did not protect cells from ExoU, suggesting that the cytotoxic effect of ExoU does not require this pathway (Phillips et al. 2003). Others have shown that protein tyrosine kinases such as Src-family kinases may be involved in ExoU-mediated cell death (Evans et al. 1998, 2002). Calcium is an important modulator of many rapid signaling pathways within the mammalian cell and, therefore, could play an important role in the downstream effects of ExoU. When *P. aeruginosa* was added to the basolateral side of Calu-3 cell monolayers, a relatively slow but sustained increase in intracellular calcium was observed and was followed by loss of integrity of Calu-3 cell membranes (Jacob et al. 2002). This phenomenon required the presence of calcium-supplemented media and was prevented by the addition of the calcium channel blocker La^{3+} (Jacob et al. 2002). From these results, it was hypothesized that ExoU led to the depletion of intracellular calcium stores that in turn resulted in the opening of basolateral calcium channels. Thapsigargin, an inhibitor of an endoplasmic reticulum calcium pump, mimicked the increased intracellular calcium levels observed with ExoU intoxication but did not cause cell death. Thus, increased intracellular calcium levels alone are not sufficient to cause cytotoxicity, indicating that ExoU affects host cells in additional ways.

In a separate approach to characterize the effects of ExoU on host cells, McMorran and colleagues used microarray analysis to show that 14 genes were upregulated in lung epithelial cells following infection with a wild type ExoU-secreting strain but not with an isogenic ExoU mutant (McMorran et al. 2003). Their analysis suggested that injection of ExoU resulted in the activation of AP1, which functions as a transcriptional activator of genes involved in cell protection and survival. Whether these changes represent the manipulation of host cell signal transduction pathways by ExoU to cause cell death or are merely generic cell responses to injury remains to be determined.

4 Regulation

Much of what is known about the regulation of ExoU has been inferred from studies of other genes of the *P. aeruginosa* type III regulon. Recent advances indicate that the regulation of this system is quite complex, but certain aspects are now clear. For example, at least five environmental stimuli are known to induce production of *P. aeruginosa* type-III-secreted products *in vitro*: contact with host cells (Vallis et al. 1999b), a NaCl concentration of 200 mM (Hornef et al. 2000), the presence of serum (Vallis et al. 1999b), a temperature of 37°C (Hornef et al. 2000), and the presence of calcium chelators, presumably through the establishment of a low-calcium environment (Iglewski et al. 1978; Thompson et al. 1980; Finck-Barbançon et al. 1997). The relevance of these signals to type III secretion *in vivo* is less well understood.

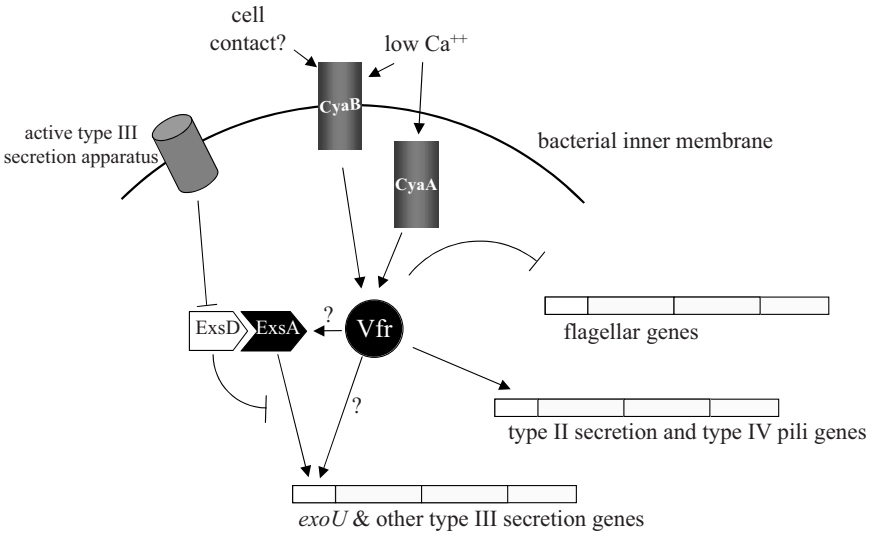

Fig. 3. Model of regulation of the *P. aeruginosa* type III secretion regulon. See text for details. Adapted from the following: (McCaw et al. 2002; Wolfgang et al. 2003b).

The details of how environmental signals lead to increased expression of type III secretion genes are beginning to emerge and have led to the following model (Fig. 3). Two *P. aeruginosa* adenylate cyclases, CyaA and CyaB, mediate an increase in intracellular 3',5'-cyclic AMP (cAMP) levels in response to low environmental calcium levels (Wolfgang et al. 2003b). CyaB, which contains multiple putative transmembrane domains, may also be able to sense contact with host cells. cAMP then binds and activates Vfr, a CRP family transcriptional factor. Vfr in turn activates the expression of a large number of genes, including those encoding components of the type III secretion, type IV pili, and type II secretion systems. In contrast, flagellar genes are downregulated by this signaling pathway. The net effect is that the bacterium is prepared for intimate attachment to the host cell and secretion of multiple toxins, including ExoU. However, Vfr alone is not sufficient to activate expression of type III secretion genes. ExsA, an AraC-like transcriptional activator encoded by a gene within the type III secretion locus of *P. aeruginosa,* is also required and functions as a central regulator of the type III system (Yahr and Frank 1994; Hovey and Frank 1995; Yahr et al. 1995). Under appropriate conditions, ExsA binds to a consensus sequence (TXAAAAXA) located upstream of the operons that encode the secretion machinery, translocation apparatus, and effector proteins (including ExoU) of the type III system (Hovey and Frank 1995; Yahr et al. 1996; Finck-Barbançon et al. 1997, 1998; Hauser et al. 1998a). It is unclear whether Vfr activates ExsA, which then binds to type III promoters or whether the two proteins together act upon type III promoters. Interestingly, it appears that overexpression of ExsA may obviate the need for activated Vfr (Dacheux et al. 2001; Wolfgang et al. 2003b).

As has been noted with other type III secretion systems, expression of type III genes and secretion of type III proteins in *P. aeruginosa* are linked, such that in the absence of secretion, type III genes are repressed (Yahr and Frank 1994; Hornef et al. 2000; McCaw et al. 2002). The type III secretion repressor ExsD plays an important role in this linkage (Fig. 3). Under conditions that prevent secretion, ExsD appears to bind ExsA, inhibiting ExsA-mediated transcriptional activation (McCaw et al. 2002). When type III secretion is active, ExsD is thought to release ExsA (McCaw et al. 2002). ExsA is then free to bind type III secretion promoters and cause expression of these genes.

Regulation in *P. aeruginosa* is not solely at the level of transcription, but also occurs at the level of secretion. The details of this regulation have not been worked out, but the type III protein PcrV is involved. A mutation in the gene encoding this protein resulted in a "calcium-blind" phenotype, meaning that the presence of calcium was no longer sufficient to prevent secretion of type III proteins (Sawa et al. 1999). Interestingly, although effector proteins were secreted by this mutant, they were not injected into host cells, suggesting a role for PcrV in the assembly or function of the translocation complex (Sawa et al. 1999). Like many type III effector proteins, ExoU has an associated chaperone, designated SpcU (specific *Pseudomonas* chaperone for ExoU), which was required for maximal secretion (Finck-Barbançon et al. 1998). SpcU binding to ExoU required amino acids 3-123 of ExoU (Fig. 1) and may function to maintain ExoU in an appropriate cytosolic conformation or to target ExoU to the type III secretion-translocation machinery (Finck-Barbançon et al. 1998).

Several other bacterial factors have been implicated in either the direct or indirect regulation of type III secretion in *P. aeruginosa*. These include DsbA, a periplasmic thiol:disulfide oxidoreductase that contributes to proper protein folding by catalyzing the formation of disulfide bonds (Ha et al. 2003); TruA, a tRNA pseudouridine synthase homologue (Ahn et al. 2004); the PDH-E1 and -E2 subunits of pyruvate dehydrogenase (Dacheux et al. 2002); HutT, a histidine transporter (Rietsch et al. 2004); the RhlR/RhlI quorum sensing system (Hogardt et al. 2004); and the stationary phase sigma factor RpoS (Hogardt et al. 2004). The roles these factors play in regulating type III secretion are less well understood.

Most of the preceding discussion applies to the regulation of the *P. aeruginosa* type III secretion system as a whole. In the type III secretion systems of other bacteria, the transcription, secretion, or stability of individual effector proteins is sometimes differentially regulated (Kubori and Galan 2003; Thomas and Brett Finlay 2003). Besides SpcU, it is unclear whether any mechanisms of regulation are directed specifically at ExoU.

5 ExoU secretion as a variable trait

The initial observation that mammalian cells were rapidly killed by some strains of *P. aeruginosa* but not by others led to the discovery of ExoU. Subsequent studies have shown that the reason for this difference in cytotoxicity is that ExoU se-

cretion is a variable trait (Hirakata et al. 2000; Roy-Burman et al. 2001; Hauser et al. 2002; Berthelot et al. 2003; Schulert et al. 2003). Among clinical isolates, heterogeneity of ExoU production exists on two levels: genotypic and phenotypic.

Although all strains of *P. aeruginosa* contain genes encoding the type III secretion apparatus, they tend not to harbor a complete complement of effector-encoding genes (Feltman et al. 2001). For example, only 28-34% of strains causing acute infections contained a chromosomal copy of the *exoU* gene (Feltman et al. 2001; Lomholt et al. 2001; Berthelot et al. 2003). In contrast, the *exoS* gene was found in 65-72% of these strains (Feltman et al. 2001; Lomholt et al. 2001). Interestingly, very few isolates contained both the *exoU* and the *exoS* genes or neither of these genes (Feltman et al. 2001; Berthelot et al. 2003). The reason for the inverse relationship between the *exoU* and *exoS* genes is unclear but does not reflect an obvious redundancy in function at a molecular level--ExoU is a phospholipase, whereas ExoS is a GTPase-activating protein (GAP) for Rho GTPases and an ADP-ribosyltransferase (reviewed in Barbieri 2000). In addition, these two genes occupy distinct chromosomal loci, so the presence of one gene would not be expected to preclude acquisition of the second (Wolfgang et al. 2003a). In strains that do contain the *exoU* gene, it appears to be under strong selective pressure or recently acquired. Sequencing of the 2,064 bp *exoU* genes from six strains identified only 14 total single nucleotide polymorphisms and only five nonsynonymous single nucleotide polymorphisms, indicating that ExoU is highly conserved from strain to strain (Ajayi et al. 2003).

One explanation for the variable presence of the *exoU* gene in *P. aeruginosa* strains is that the gene was exogenously acquired. In support of this hypothesis, the G+C content of the *exoU* gene is 59%, significantly lower than the 67% G+C content of the *P. aeruginosa* genome as a whole (Palleroni 1984; Finck-Barbançon et al. 1997; Hauser et al. 1998a). In addition, an insertion sequence-like element is located immediately upstream of the *exoU* gene. Genomic analysis of several strains has shown that the *exoU* gene is indeed located in a hypervariable portion of the *P. aeruginosa* chromosome, adjacent to a tRNA-Lys gene (Wolfgang et al. 2003a; He et al. 2004). As is common with tRNA genes, this tRNA-Lys gene acts as a hotspot for integration of genetic elements, including several small and large islands (Kiewitz et al. 2000; Wolfgang et al. 2003a). In this locus, the chromosome of strain PA14 harbors a 10.7 kb pathogenicity island, named PAPI-2 (He et al. 2004). PAPI-2 has multiple features of a genomic island, including an integrase gene, four transposase genes and an insertion sequence-like element. The genes encoding ExoU and its chaperone SpcU are located at the right border of this element (He et al. 2004) (Fig. 4). The G+C content of this island is similar to that of the *exoU* gene itself (57% vs. 59%, respectively), supporting the hypothesis that the *exoU* gene is carried by PAPI-2. However, a related 8.9 kb genomic island containing some of the same genes as PAPI-2 but lacking the *exoU* and *spcU* genes is present at the same locus in strain PAO1 (He et al. 2004). Thus, PAPI-2 may be a mosaic island, of which a smaller independent *exoU*-containing genetic element is one part. The genomic sequencing of additional *exoU*-containing strains should clarify this issue.

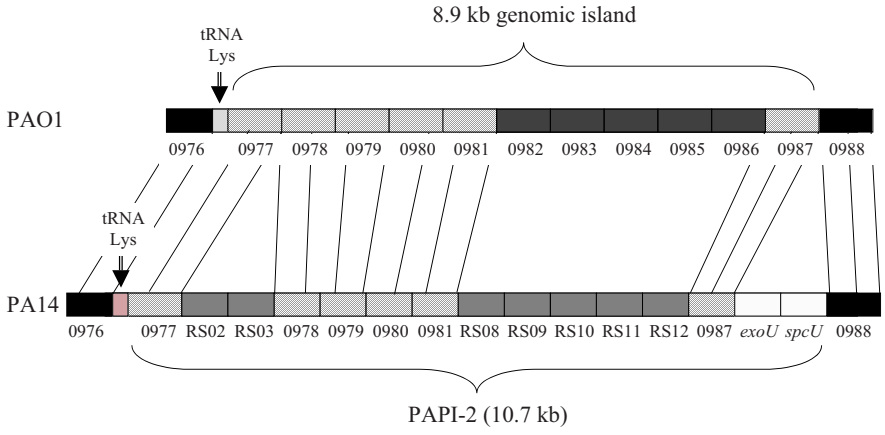

Fig. 4. Corresponding genetic elements inserted at the tRNA-Lys locus of *P. aeruginosa* strains PAO1 and PA14. Alphanumeric designations refer to individual open reading frames (Stover et al. 2000; He et al. 2004). The diagram is not drawn to scale. Adapted from the following: (Wolfgang et al. 2003a; He et al. 2004).

Although the presence of the *exoU* gene is variable, its distribution among *P. aeruginosa* strains does not appear to be random. Strains harboring this gene often have other unlinked genetic elements in common, suggesting that they are phylogenetically related (Wolfgang et al. 2003a). Consistent with this interpretation, multilocus enzyme electrophoresis analysis indicated that strains containing the *exoU* gene clustered in a limited number of electrophoretic types (Lomholt et al. 2001). Also, ExoU secretion was associated with specific serotypes; serotype O10 and O11 strains secreted ExoU but serotype O3, O4, O6, O12, and O16 strains did not (Berthelot et al. 2003; Faure et al. 2003). Together, these findings suggest that *exoU*-containing strains comprise a limited number of phylogenetically related groups.

Even among strains that harbor the *exoU* gene, phenotypic differences exist in the secretion of ExoU because not all strains that harbor type III secretion genes secrete these proteins. From 23% to 28% of isolates causing acute infections harbored type III genes but did not secrete type III proteins under secretion-inducing conditions *in vitro* (Roy-Burman et al. 2001; Hauser et al. 2002). This proportion was much higher in isolates from chronically infected patients with cystic fibrosis (59%) (Roy-Burman et al. 2001). The reason for this disparity between genotype and phenotype is unclear but does not appear to be due to the accumulation of mutations in the type III secretion apparatus itself since overexpression of the transcriptional regulator ExsA restored secretion in some strains (Dacheux et al. 2001).

Thus, there are both genotypic and phenotypic differences in ExoU production among strains of *P. aeruginosa*. As discussed in the following section, this heterogeneity has important implications with regard to the virulence potential of different strains.

6 Role in virulence

ExoU's ability to rapidly kill many mammalian cell types suggests that it plays an important role in the development and progression of disease. The use of animal models of disease indicated that this is indeed the case. Pulmonary inoculation of mice with a mutant strain of *P. aeruginosa* containing a disrupted *exoU* gene resulted in decreased destruction of the pulmonary epithelial barrier (Finck-Barbançon et al. 1997), decreased wet/dry ratios of the infected lungs (Finck-Barbançon et al. 1997), and decreased mortality (Hauser et al. 1998a) relative to the wild type strain. Likewise, transformation of an *exoU*-containing plasmid into *P. aeruginosa* strains that did not naturally harbor this gene resulted in increased pulmonary bacterial loads and increased mortality in a mouse pneumonia model (Allewelt et al. 2000). ExoU also contributed to bacterial colonization and disease severity in a corneal model of infection (Lee et al. 2003). The involvement of ExoU in other *P. aeruginosa* infections, such as urinary tract infections, bacteremia, burn infections, and wound infections, has not yet been investigated.

Although ExoU clearly plays an important role in pathogenesis, it is not required for *P. aeruginosa* to cause disease. In mice infected with a large inoculum of a *P. aeruginosa* strain containing a disrupted *exoU* gene, disease and mortality still occurred (Garrity-Ryan et al. 2000). Likewise, strains that naturally lacked the *exoU* gene were nonetheless capable of causing disease in animal models (Roy-Burman et al. 2001; Schulert et al. 2003). Together, these observations indicate that ExoU augments the ability of *P. aeruginosa* to cause disease but is not an essential part of this organism's virulence arsenal.

The emerging model, therefore, is that any given *P. aeruginosa* strain is associated with a basal level of virulence dependent upon the collection of virulence determinants it produces, and that in certain strains this level is augmented by the production of ExoU. In support of this model, strains of *P. aeruginosa* that naturally secreted ExoU were noted to be more virulent in a mouse model of pneumonia than isolates that did not secrete this toxin (Roy-Burman et al. 2001; Schulert et al. 2003). The extent of this augmentation likely varies from strain to strain, but transformation of a strain lacking the *exoU* gene with a plasmid encoding ExoU was associated with a tenfold reduction in the 50% lethal dose (and, therefore, an increase in virulence) in a mouse model of acute pneumonia (Allewelt et al. 2000).

In contrast to the abundance of data regarding the molecular and cellular effects of intoxication with ExoU, relatively little is known about how these effects lead to the pathophysiological consequences characteristic of disease. ExoU-mediated death in an animal model occurred relatively rapidly, within three to four days (Hauser et al. 1998a), suggesting a largely immediate effect of this toxin. Kurahashi and colleagues showed that both a wild type strain of *P. aeruginosa* that secreted both ExoU and ExoT and an isogenic mutant that secreted neither ExoU nor ExoT caused increased pulmonary levels of the proinflammatory cytokines tumor necrosis factor-α (TNF-α), IL-8, GRO, and MCP-1 (Kurahashi et al. 1999). Importantly, TNF-α leakage from the lungs into the circulation occurred only with the wild type strain. Also, infusion of the wild type strain directly into the blood-

stream did not result in high levels of plasma TNF-α. Based upon these findings, the authors hypothesized that, although significant amounts of TNF-α accumulated in lungs infected with either ExoU$^+$ or ExoU$^-$ strains of *P. aeruginosa,* only ExoU$^+$ strains caused disruption of the epithelial cell barrier in the lungs and allowed intrapulmonary TNF-α to escape into the circulation, leading to sepsis. Additional experiments will be necessary to determine whether ExoT also plays a role in this process.

Interestingly, ExoU's toxic effects are not limited to mammalian hosts. ExoU-dependent disease has been observed using such diverse model systems as caterpillars (*Galleria mellonella* (Miyata et al. 2003)), amoebae (*Dictyostelium discoideum,* (Pukatzki et al. 2002)), and yeast (*Saccharomyces cerevisiae* (Rabin and Hauser 2003; Sato et al. 2003)). Thus, a variety of model systems, each with its own advantages, are available to further decipher the mechanism of action of this toxin. This broad host range suggests that the molecular target of ExoU is evolutionarily conserved across distantly related eukaryotic organisms. It may also provide an answer to the puzzling question of how ExoU evolved. *P. aeruginosa* is a bacterium that normally inhabits moist soil, lakes, and streams (Rhame 1979). Infections in humans are by and large the unintended consequence of modern medical technology. Most commonly, *P. aeruginosa* infects people requiring mechanical ventilation, receiving chemotherapy for malignancies, or implanted with prosthetic devices, such as intravenous catheters (Stryjewski and Sexton 2003). Even individuals with cystic fibrosis rarely lived long enough to acquire *P. aeruginosa* infections prior to the advent of modern antimicrobial therapies (Govan and Deretic 1996). In a sense, then, humans are accidental hosts for this bacterium, becoming infected only when receiving medical therapies or harboring genetic defects that disrupt normal innate immune defenses. Thus, the selective pressures that led to the evolution of ExoU must lie elsewhere. Given the diverse spectrum of eukaryotic organisms susceptible to ExoU's mechanism of killing, it is likely that this toxin evolved to protect *P. aeruginosa* from or allow *P. aeruginosa* to attack environmental organisms. Further studies of the ecology of *P. aeruginosa* in the environments will be necessary to more accurately define ExoU's natural targets.

7 Role in human disease

Recent studies suggest that ExoU is important to the pathogenesis of *P. aeruginosa* infections in humans, indicating that the animal models used to study this toxin accurately mimic human disease. For example, as was the case in mouse models of acute pneumonia, ExoU was not required for the development of pneumonia in humans. The majority (71%) of *P. aeruginosa* strains bronchoscopically isolated from patients meeting strict criteria for ventilator-associated pneumonia did not secrete ExoU (Schulert et al. 2003). Nonetheless, ExoU secretion did appear to be associated with increased disease severity. In a study of 108 patients with *P. aeruginosa* cultured from their respiratory tracts or blood, a functional

type III secretion system was associated with a six-fold increase in mortality (21% vs. 3%) (Roy-Burman et al. 2001). Of these secreting strains, 32% secreted ExoU. In a study of patients with ventilator-associated pneumonia, nine (90%) of ten patients infected with ExoU-secreting strains had severe disease, whereas only three (38%) of eight patients infected with strains that did not secrete type III proteins had severe disease (Hauser et al. 2002).

Are ExoU-secreting strains over-represented in certain illnesses? Although the *exoU* gene was found in the same proportion (25-40%) of blood, urine, acute respiratory tract, and wound isolates, fewer isolates from chronically infected cystic fibrosis patients contained this gene (10%) (Feltman et al. 2001; Berthelot et al. 2003). Additional studies have verified that secretion of ExoU is extremely rare among isolates from cystic fibrosis patients (M. Jain, A. Hauser, unpublished data). It may be that secretion of such a destructive toxin is not compatible with establishment of a chronic infection. In contrast to cystic fibrosis isolates, strains cultured from patients with acute keratitis, an infection involving the cornea of the eye, frequently (52%) contained the *exoU* gene (Lomholt et al. 2001; Cowell et al. 2003). This may indicate a role for ExoU or other factors present in *exoU*-containing strains in the pathogenesis of keratitis.

8 Conclusion

Although many gaps remain in our understanding of the mechanisms by which ExoU causes disease, significant advances are being made at a rapid pace. Currently, it is clear that ExoU is expressed and transported by the *P. aeruginosa* type III secretion system. Once injected, this toxin acts as a phospholipase that rapidly kills many types of eukaryotic cells. Although only a minority of clinical isolates of *P. aeruginosa* secrete ExoU, these isolates are associated with particularly severe disease. Remaining areas of uncertainty include the substrate specificity of ExoU, the mechanism by which phospholipase activity leads to cell death, and the role of cytotoxicity in the development of the pathophysiological consequences of severe disease. A better understanding of these fundamental processes will identify opportunities for novel therapeutic interventions that may limit the morbidity and mortality currently associated with infection by ExoU-secreting strains of *P. aeruginosa*.

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Staphylococcal alpha-toxin

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Abstract

Staphylococcal alpha-toxin, archetype of an oligomerizing pore-forming toxin, is produced and secreted as a water-soluble 34 kD monomer. High affinity binding possibly occurs to clustered phosphocholine head groups in cholesterol/sphingomyelin-rich membrane microdomains. Toxin heptamers can rapidly assemble at such sites, and oligomerization triggers conformational changes leading to insertion of an amphipathic hairpin β-barrel sequence into the lipid bilayer, so that a protein-lined pore of approximately 1 nm diameter is formed. Many cellular reactions are triggered by the uncontrolled transmembrane flux of ions, and these can account for short- and long-range effects of the toxin. Moreover, nucleated cells can repair a limited number of membrane lesions, and transcriptional responses are then triggered that further contribute to long-term effects of the toxin. The biological relevance of the toxin is well established, and novel prophylactic and therapeutic strategies targeting this major virulence factor of *S. aureus* are currently being developed.

1 Occurrence and biological significance

Staphylococcal alpha-toxin is a major pathogenicity determinant of *S. aureus* (Bhakdi and Tranum-Jensen 1991). It is not produced by coagulase-negative staphylococci, and this is probably one reason for their reduced virulence compared with *S. aureus.* The toxin is secreted as a 293-residue water-soluble monomer of Mr 33000 (Gray and Kehoe 1984). Gene expression is subject to complex regulation (Recsei et al. 1986). Antibodies against alpha-toxin become detectable in sera of all young adults, indicating that toxin production occurs in humans even in the absence of clinical disease. Similarly, antibodies can be found in sera of domestic animals, reflecting the fact that *S. aureus* is a most versatile pathogen that infects a broad spectrum of hosts. *S. aureus* infections range from self-limiting skin afflictions to life-threatening systemic diseases, and *S. aureus* is a major causative agent of hospital infections. Inasmuch as the pathogenesis of staphylococcal disease is multifactorial, alpha-toxin probably assumes a dominant role in most cases. In animal models, purified toxin has been shown to be hemolytic, dermonecrotic, and lethal (McCartney and Arbuthnott 1978). The biological importance of toxin production has formally been shown by two approaches. First, the use of genetically deficient bacterial mutant strains has proven alpha-toxin to

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be a major cause of tissue destruction and abscess formation (Bramley et al. 1989; Menzies and Kernodle 1994; Patel et al. 1987; Jonsson et al. 1985). siRNA-mediated suppression of alpha-toxin production has been shown to eliminate lethality of *S. aureus* infections in a rabbit model (Yinduo et al. 1999) Second, the application of mono- and polyclonal antibodies against alpha-toxin has been found to protect against development of staphylococcal lesions. Alpha-toxin was, thus, not only the first channel-forming bacterial toxin to be discovered (Füssle et al. 1981), it also constitutes one of the best-studied examples of how a pore-forming toxin can contribute to microbial virulence (Bhakdi and Tranum-Jensen 1991; Bhakdi et al. 1996).

2 Properties of native toxin

Alpha-toxin can be recovered in culture supernatants of *S. aureus* strains. The classic high-producing strain Wood 46 is generally used for purification purposes. Naturally occurring toxin mutants have not been detected to date, so the toxin gene is highly conserved. Somewhat surprisingly, alpha-toxin represents a unique molecular entity, and no other molecule displaying extensive homology has yet been discovered. Restricted homologies do exist in the pore-forming domain, however; these have been noted in a family of bi-component, pore-forming leukocidins, also produced by *S. aureus* (Supersac et al. 1993; Gouaux et al. 1997)

Under optimal culture conditions, alpha-toxin comprises the major protein component in overnight bacterial supernatants. The bulk of the toxin is present in monomeric form. Purification is easy and can be achieved by single-step ion exchange chromatography (Valeva et al. 1996) or by fast protein liquid chromatography (Lind et al. 1987). Toxin preparations can be transferred to ammonium acetate and lyophilized. In this form, the protein is stable for years at -20°C and for weeks at room temperature. The A_{280} of a 1 mg/ml solution is approximately 2.0. The toxin is soluble in water or any aqueous buffer at pH 5-9. Native toxin migrates as a single polypeptide band of molecular weight 33000 in SDS-PAGE. The pI is approximately 8.6 (Bhakdi and Tranum-Jensen 1991).

The three-dimensional structure of monomeric alpha-toxin has not yet been solved. However, the crystal structure of the related leukocidin F monomer is available and the salient features are transferable to alpha-toxin. This and analyses of secondary structure have shown that native toxin is an ellipsoid molecule harboring an abundance of β-sheet structure (approximately 60%), approximately 10% is α-helical, and the rest consists of non-α/non-β elements (Tobkes et al. 1985). The membrane-inserting domain, which is located near the center of the polypeptide chain, is totally exposed to the hydrophilic environment (Valeva et al. 1995, 1996, 1997a, 1997b). Both alpha-toxin and leukocidin F contain a binding cleft for phosphorylcholine (Trp179-Arg 200 in alpha-toxin, Trp177-Arg198 in leukocidin F) (Olson et al. 1999).

Monomeric alpha-toxin is destroyed by proteases. Under controlled conditions, proteolytic cleavage first occurs in the pore-forming domain, generating a nicked

molecule that still binds to form smaller pores in membranes of erythrocytes (Palmer et al. 1993b).

Native alpha-toxin can be radio-iodinated without substantial loss of function (Hildebrand et al. 1991), and cysteine substitution mutants can be site-specifically labeled with fluorescent molecules, or with biotin, or with radiolabeled fluorescein (Palmer et al. 1993a; Valeva et al. 1996, 1997a, 1997b). It is, therefore, possible to produce various probes for *in vitro* and *in vivo* studies.

3 Mechanism of action

3.1 Binding

The toxin binds as a monomer in the absence of any molecular modification (Bhakdi and Tranum-Jensen 1991; Hildebrand et al. 1991). Binding studies have revealed that interaction with red blood cells can occur via two distinct mechanisms (Hildebrand et al. 1991). Highly susceptible cells, e.g. rabbit erythrocytes, express a relatively small number (approximately 2000 per cell) of high affinity binding sites that are responsible for the interaction of toxin with membranes at low concentrations (≤ 2 nM; 60 ng/ml). Binding is optimal at ambient temperature (22-25°C). Both rabbit erythrocytes and human erythrocytes additionally display an unsaturable number of low affinity bindings sites, which account for the hemolytic properties of alpha-toxin on less susceptible red blood cells (human erythrocytes). Neither the low nor the high affinity binding sites have been definitively identified. They are not destroyable by the action of proteinase K, pronase, phospholipase A_2, or phospholipase D. Binding to erythrocytes can be inhibited by millimolar concentrations of phosphocholine (Tomita and Kamio 1977). Because a putative binding site for phosphorylcholine is present on each toxin monomer, it is most likely that this interaction bears biological relevance.

High affinity binding sites are believed to be present on highly susceptible nucleated cells (e.g. keratinocytes, endothelial cells, lymphocytes, and monocytes) and on platelets, although this contention has not been proven experimentally.

3.2 Oligomerization

Toxin oligomers form when membrane-bound monomers collide with each other in the bilayer. Our working hypothesis is that clustered phosphocholine groups predominantly contained in sphingomyelin/cholesterol-rich domains serve as binding sites where collision and oligomerization preferentially occur (Valeva et al. unpublished data). Oligomerization takes place at 0°C (Valeva et al. 1997a), and so membrane fluidity is of negligible significance. Oligomerization also occurs spontaneously in solution (Bhakdi et al. 1981, 1996). We have postulated that the main effect of membrane binding is to uniformly orient clustered toxin monomers so their contact surfaces are correctly positioned when collision occurs

(Bhakdi and Tranum-Jensen 1991). Non-circularized structures probably representing oligomeric intermediate stages have been detected by atomic force microscopy on liposomes (Czajkowsky et al. 1998), but they have eluded biochemical characterization, possibly because they dissociate in detergents. Two different oligomers have been clearly defined that successively assemble to form pre-pores before membrane-insertion occurs. The first is an oligomer that is stable in non-denaturing detergent such as deoxycholate, but that dissociates in SDS. This early pre-pore complex forms on erythrocytes at 4°C. Pre-pores with similar properties can be generated at ambient temperature through the use of a substitution mutant in which His-35 is replaced by arginine. This substitution causes oligomer assembly to be blocked at this pre-pore stage. Application of H35R-alpha-toxin to cells consequently leads to blockade of high affinity binding sites and to the generation of SDS-labile, non-functional oligomers (Jursch et al. 1994).

The first pre-pore stage is succeeded by a second stage resulting from cooperative effects within the oligomer complexes. Hydrophobic protein-protein interactions gain dominance and an SDS-stable oligomer is generated whose pore-forming domain is still located outside the bilayer (Walker et al. 1992; Valeva et al. 1997a). Such late pre-pore complexes can form on certain cells that display a natural resistance towards toxin attack, for example, on human granulocytes. These cells then carry large numbers of SDS-stable oligomers without becoming permeabilized (Valeva et al. 1997b).

3.3 Pore formation

In a final step, assembled oligomers insert their pore-forming sequences into the bilayer and a channel is formed (Fig. 1). There is evidence that this step is governed both by cooperative effects and by conformational changes occurring within each of the protomers (Valeva et al. 1997, 2001).

The diameter of channels created by alpha-toxin has been sized to approximately 1 nm in planar lipid bilayers (Menestrina 1986; Belmonte et al. 1987) and in cell membranes (Kasianowicz and Bezrukov 1995). However, it seems that pore diameters are slightly smaller when susceptible cells (e.g. keratinocytes) are exposed to low toxin concentrations, because the cells then leak K^+ without permitting influx of Ca^{2+} (Walev et al. 1993). At higher toxin concentrations, slightly larger pores appear to be created and rapid Ca^{2+} influx is noted (Walev et al. 1993; Jonas et al. 1994). The cause of this small heterogeneity in pore size has remained enigmatic. Initially, cytoplasmic proteins do not leak out of toxin-damaged cells.

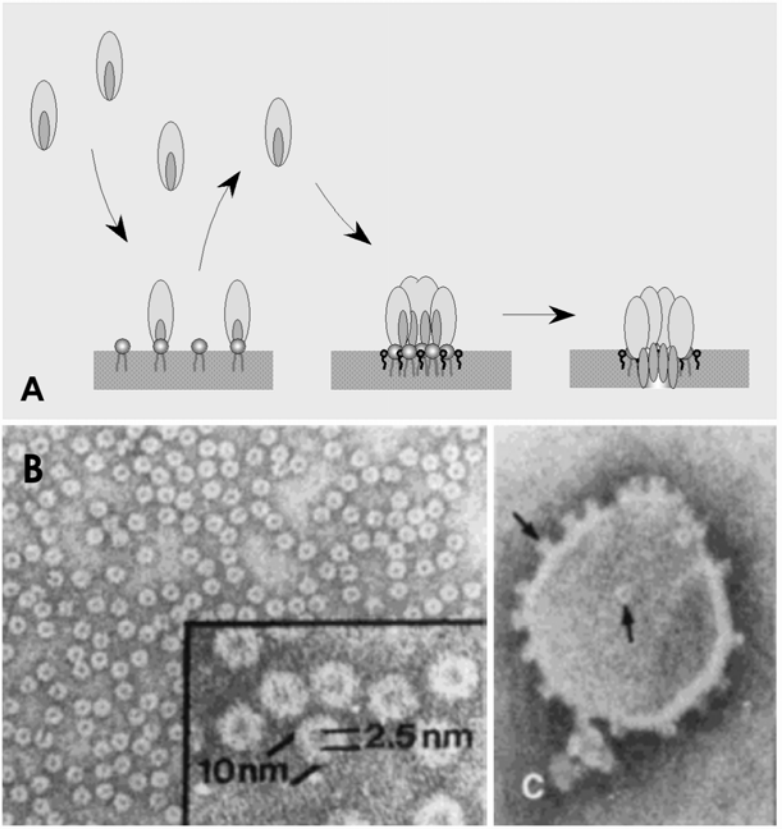
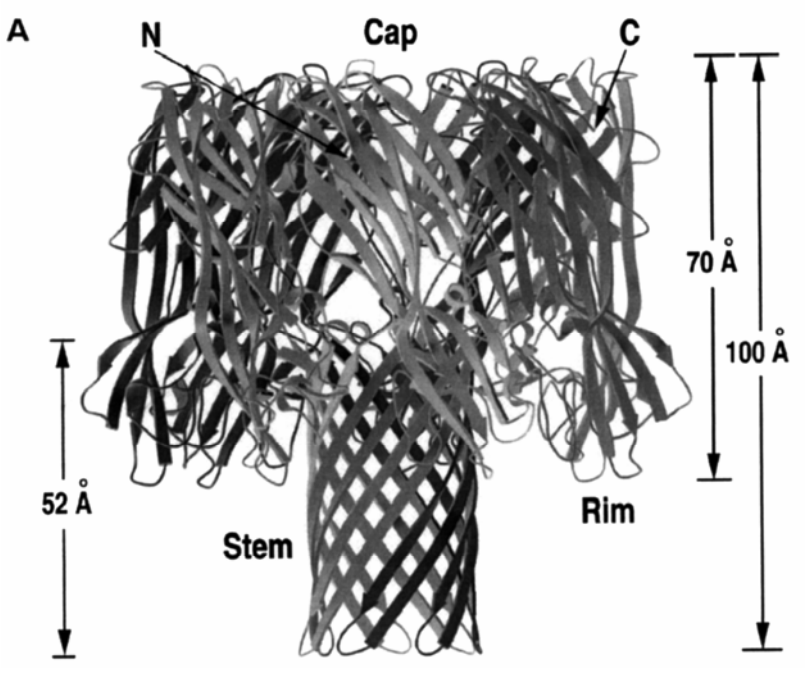


Fig. 1. Assembly model for alpha-toxin in lipid bilayers. Water-soluble native monomers bind to phosphocholine groups on cell surfaces. Binding is of low affinity and transient unless several molecules chance to contact each other on membrane domains where phosphocholine groups are exposed in clusters. Oligomerization can then occur that provide for firm binding to membranes. Oligomerization provides the driving force for insertion of the central molecular domain into the bilayer. A hydrophilic transmembrane pore traverses the center of the circularized, heptameric protein complex. **B** Isolated toxin heptamers in detergent solution. **C** Lecithin liposomes carrying reincorporated alpha-toxin heptamers. The heptamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (arrows).

4 Structure

4.1 Structure of the heptameric pore formed in detergent solution

The key finding that ultimately led to crystallographic studies of the alpha-toxin oligomer was made in 1981, when it was found that oligomerization of alpha-


A
N
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70 Å
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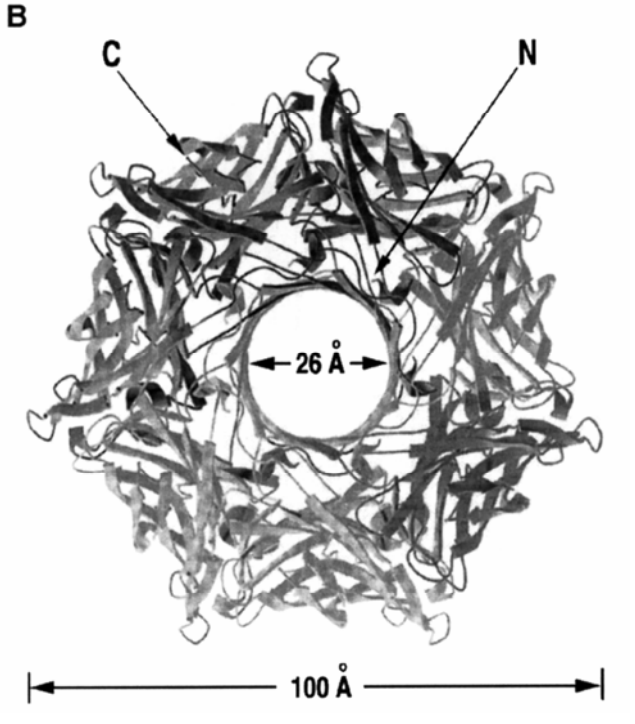
52 Å
Rim
Stem


B
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100 Å

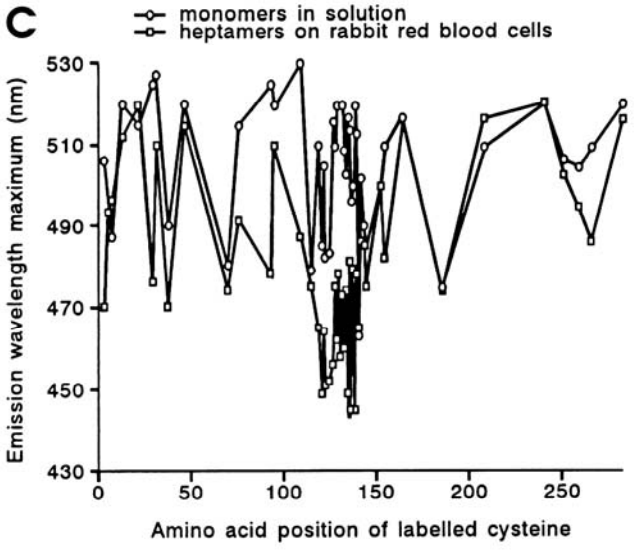

Fig. 2. Ribbon representations of the alpha-hemolysin heptamer with each protomer in a different color. **A** (overleaf) View perpendicular to the seven-fold axis and approximately parallel to the putative membrane plane. The mushroom-shaped complex is approximately 100 Å tall and up to 100 Å in diameter, and the stem domain measures about 52 Å in height and 26 Å in diameter from C_α to C_α. Approximate locations of the cap, rim and stem domain are shown. Thr^{129} is located at the base of the stem domain. **B** (overleaf) View from the top of the structure and parallel to the sevenfold axis. The amino latch of one protomer makes extensive interactions with its clockwise-related immediate neighbor and residues in each glycine-rich region wrap around the sevenfold axis approximately 180°. Protomer-protomer contacts consist almost exclusively of side chain-side chain interactions in the cap domain while in the stem domain main chain-main chain contacts predominate as the β strands from a continuous β sheet. Reproduced from Song et al. (1996) with permission from Science. **C** Fluorescence emission maxima of acrylodan-labeled alpha-toxin cysteine mutants in solution and after heptamer assembly on rabbit erythrocytes. An emission blue shift indicates movement of the fluorescence label to a more hydrophobic environment. Significant blue shifts were observed exclusively in the central region spanning residues 118-140.

toxin could be induced simply through the addition of certain detergents such as deoxycholate (Bhakdi et al. 1981). Until today, the reason for this phenomenon has not been clarified.

Crystallization of the detergent-induced oligomer was achieved a decade later (Gouaux et al. 1994), and its three-dimensional structure was solved at 1.9 Å resolution (Song et al. 1996). The oligomer measures 10 nm in height and 10 nm in diameter and has the shape of a mushroom, with a 1 nm pore running through its center (Fig. 2A, 2B). Each oligomer is composed of 7 alpha-toxin molecules. The heptamer has a cap, rim, and stem domain. The latter is a 14 strand anti-parallel β-

barrel that forms the wall of the putative transmembrane pore. The cap domain represents approximately 70% of the total mass of the oligomer and resides outside the bilayer. The rim domain underlies the cap and is probably in close contact with the outer membrane leaflet.

The putative membrane spanning stem domain measures 52 Å in height and 26 Å in diameter. Thereby, each protomer contributes two anti-parallel beta-strands formed from the amino acid sequence 118-140. The side chains of the even residues in this sequence contact lipids, whereas the side chains of the uneven residues are located in the pore lumen. Residue 129 represents the turning point of each beta-sheet at the cytoplasmic face of the bilayer (Song et al. 1996).

4.2 Structure of the membrane-bound oligomer

Spectroscopic studies have provided data that are in beautiful accord with the crystallographic analyses, and there can be no doubt that the pore is indeed formed through insertion of a single amino acid sequence encompassing residues 118-140. The novel experimental strategy leading to *in situ* identification of this domain involved production of single cysteine substitution mutants that were derivatized with acrylodan, a sulfhydryl-specific, polarity-sensitive fluorescent dye. The emission spectrum of acrylodan depends on the polarity of its environment: the probe fluoresces green in water and blue in a hydrophobic environment (Prendergast et al. 1983). Analyses of 50 labeled toxin mutants revealed that only acrylodan attached to residues 118-140 exhibited a marked blue shift in emission spectrum coincident with oligomer formation in lipid bilayers. Already in 1994, fluorescence energy transfer experiments showed that membrane-inserted amino acid residue 130 must be located very close to the cell cytoplasm (Ward et al. 1994). By employing mutant toxins exhibiting defects at defined stages of assembly, insertion of the pore-forming sequence could be shown to correlate with function. When susceptible cells were examined, periodicity in side-chain environmental polarity was detected: thus, every even residue in the sequence entered a nonpolar environment in a striking display of an amphipathic transmembrane beta-barrel (Fig. 2C). These data have resolved the molecular organization of the alpha-toxin channel in living cells and they are in superb accord with the crystallographic data (Song et al. 1996; Valeva et al. 1997a, 1997b).

4.3 Structurally related pore-forming toxins

Early analyses of amino acid sequences revealed that alpha-toxin stood alone on the large list of pore-forming toxins and to date, no second protein with high overall sequence homology has been discovered. Thus, the realization that alpha-toxin represents the prototype of a toxin family with members from both the gram-positive and gram-negative bacterial realm came as a recent surprise. It is now evident that close structural and functional homology exists with the staphylococcal leukocidins (Gouaux et al. 1997; Tomita and Kamio 1997; Olson et al. 1999).

Furthermore, the mechanism of pore-formation is shared by anthrax-protective antigen (Collier and Young 2003), by *Vibrio cholerae* cytolysin (VCC), and probably by aerolysin (Olson and Gouaux 2003). In all cases, pores are formed by insertion of two antiparallel β-strands each derived from one monomer in the assembled oligomer, so that circular transmembrane β-barrels are created.

Leukocidins are two-component toxins that assemble into pore-forming heteroheptamers (Sugawara-Tomita et al. 2002) or heterooctomers (Miles et al. 2002). *Vibrio cholerae* cytolysin (VCC) is produced as a monomeric protoxin precursor that is activated by proteases through removal of the 15 kD protoxin domain at the N-terminus (Valeva et al. 2004). A cytolytic domain was identified based on PSI-blast similarity with members of the hemolysin/leukocidin/aerolysin-family. The predicted cytolytic domain of 250 amino acids contains a putative antiparallel β-barrel that is proposed to represent the pore-forming domain (Olson and Gouaux 2003). Unpublished experimental data from this laboratory support this prediction.

The similarity of anthrax-protective antigen and alpha-toxin surfaced when the three-dimensional structure of the PA-oligomer was solved in 1997. PA-heptamers were found to display structural homology with alpha-toxin, and the β-barrel serves as a translocation pore for enzymatically active anthrax toxin components to the cytosol (Petosa et al. 1997).

5 Cellular resistance and repair mechanisms

Resistance towards toxin attack may be due to various causes. The first is the absence of high affinity binding sites. This is exemplified by human erythrocytes, which escape attack when alpha-toxin is present even at comparatively high concentrations (10 μg/ml). This type of resistance can be overcome by increasing toxin doses to 30 - 100 μg/ml, since non-specific, absorptive binding occurs (Hildebrand et al. 1991). It is notable that no marked differences had yet been observed between pores formed at low and high toxin concentrations, although, as alluded to above, it is possible that small variations do exist regarding stochiometry and pore size.

A second cause for resistance is that insertion of the pore-forming sequence may be inhibited despite assembly of oligomers. This is the case with human granulocytes. Thus, alpha-toxin binds to these cells as efficiently as it does to highly susceptible lymphocytes. Granulocyte-bound toxin oligomers form SDS-stable pre-pores that remain associated at the cell surface for surprisingly long periods (≥ 1 hour). It is intriguing that very little endocytosis or shedding occurs. However, membrane permeabilization does not take place (Fig. 3A), and this has been related by spectroscopic studies to the inability of the oligomers to insert their pore-forming sequences into the membrane (Fig. 3B; Valeva et al. 1997a). The mechanisms underlying inhibition of membrane insertion have not been clarified.

A third reason for relative resistance of cells towards the lethal action of alpha-toxin is their capacity to repair the lesions. This is exemplified by the reaction of

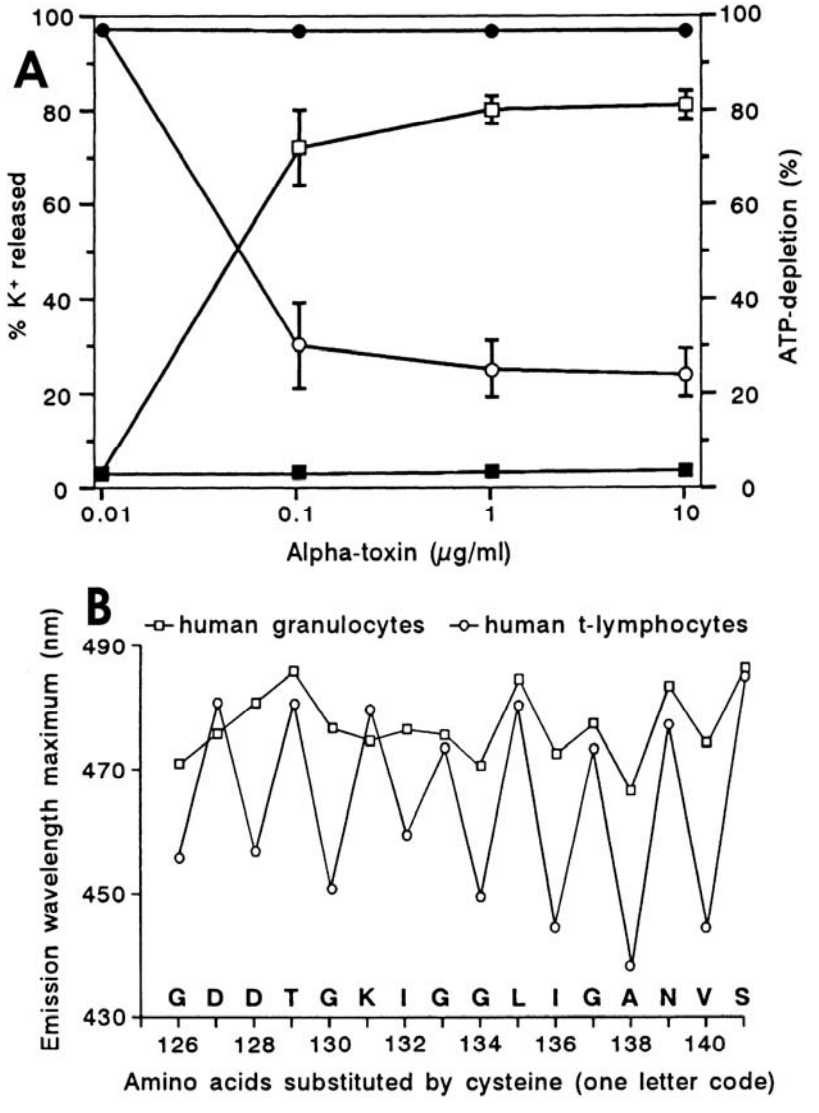

Fig. 3. Granulocytes are resistant towards the permeabilizing effects of alpha-toxin. **A** Measurement of cellular ATP-depletion and K^+ -efflux in alpha-toxin-treated cells. Lymphocytes (open symbols) or granulocytes (filled symbols) were treated with alpha-toxin for 60 min at 37°C. K^+ concentration (squares) in supernatants were determined. Cells were lysed with Triton- X-100 and ATP concentrations in the lysates (circles) were determined. Data represent means ± SD (error bars) for three independent experiments. **B** Emission maxima of acrylodan-labeled alpha-toxin cysteine mutants measured on human granulocytes and human T-lymphocytes, showing lack of lipid contact of the pore-forming domain in the toxin-resistant granulocytes.

fibroblasts. Here, alpha-toxin binds efficiently to and permeabilizes the cells, provoking initial K^+-efflux and depletion of cellular ATP (Walev et al. 1994) The latter may be due to consumption rather than to direct egress of the nucleotide through the toxin pores. After approximately 2 hours, the cells start to recuperate and replenish their ATP content. After overnight culture, no residual signs of toxin attack remain, although approximately 50% of the toxin oligomers remain detectable at the cell surface. Utilization of labeled cysteine substitution alpha-toxin mutants has generated information on the conformation of the alpha-toxin oligomers in the repaired lesions. After repair, the amino acid side chains originally in contact with lipid continue to display blue fluorescence, indicative of their retention within the membrane. Remarkably, fluorescence of acrylodan attached to side chains in the pore lumen shift from a green to blue fluorescence (Valeva et al. 2000). This would best be explained by exclusion of water from the pore, and a speculative model emerges in which the channel is closed through constriction. Pore closure is likely to be a process of widespread relevance in the context of attack by and defense against pore-forming proteins. The underlying molecular mechanism remains to be elucidated.

6 Biological effects

6.1 Cytocidal action

Cell death is the most obvious and inevitable consequence of pore formation if a lesion is not removed or repaired. Cell death can have immediate detrimental consequences. Tissue necrosis generates niches for bacterial survival and multiplication. Death of phagocytes and lymphocytes fosters microbial persistence and invasion. Monocytes (Bhakdi et al. 1989) and lymphocytes (Jonas et al. 1994) are indeed prime targets for alpha-toxin attack. Endothelial cells are also highly susceptible (Seeger et al. 1990; Suttorp et al. 1985) and their death can lead to major perturbations in the microcirculation. Further, alpha-toxin exerts potent cytocidal action on keratinocytes (Walev 1993), which would be in line with the known relevance of *S. aureus* as a major cause of skin infections.

6.2 Secondary cellular reactions

Secondary reactions mounted by cells under attack by any pore-forming toxin are of prime importance, and they can explain the long-range effects of these toxins. The reactions provoked by alpha-toxin may conveniently be grouped into three major categories.

6.2.1 Reactions provoked by transmembrane flux of monovalent ions

Pores that allow passage of K^+ and Na^+, but that restrict passage of Ca^{2+} and larger molecules elicit unexpected effects. When alpha-toxin is applied to activated T lymphocytes at low concentrations such that Ca^{2+} flux is restricted, programmed cell death is triggered and DNA-fragmentation occurs after 2-4 hours (Jonas et al. 1994). When applied at similarly low concentrations to monocytes, alpha-toxin provokes the processing and transport of IL-1β (Bhakdi et al. 1989; Walev et al. 1995). The latter phenomenon is due to rapid cleavage of intracellular pro-IL-1β precursor by the membrane-bound interleukin-converting enzyme ICE alias caspase 1. Since ICE-related proteases are also involved in the apoptosis pathway, these two findings may be linked to one another.

How monovalent ion flux is linked to caspase activity is not yet known. Recent data suggest that K^+ efflux enhances the autocatalytic activation of pro-caspase 1 (Cheneval et al. 1998). The intriguing concept emerging from these studies is that K^+ directly or indirectly controls the function of vitally important proteolytic enzymes in the cell. Both programmed cell and interleukin 1β conversion were inhibited by high extracellular concentrations of K^+ in alpha-toxin permeabilized cells, and recent work emphasizes that K^+ efflux is an early event occurring in apoptotic cells (Hughes et al. 1997; Bortner et al. 1997).

A second possibility is that intracellular K^+ levels influence pro-IL-1β processing more indirectly. Recent data have shown that processing is enhanced upon activation of Ca-independent phospholipase A_2 ($iPLA_2$), and that the latter can be invoked by K^+-depletion. $iPLA_2$ did not directly affect the activity of the IL-1β transporter or the activity of the IL-1β converting enzyme (caspase 1). Therefore, a working hypothesis is that cleavage of phosphorylcholine in the inner membrane leaflet by $iPLA_2$ facilitates trafficking of pro-IL-1β to the "inflammasome" (Martinon et al. 2002) containing caspase 1 (Walev et al. 2000).

6.2.2 Ca^{2+}-dependent reactions

Alpha-toxin generates Ca^{2+}-permissive pores when applied to certain cells such as endothelial cells (Suttorp et al. 1985) and platelets (Bhakdi et al. 1988). In other cells such as monocytes and keratinocytes, low toxin concentrations generate smaller pores that are not Ca^{2+}-permissive. At higher doses, however, Ca^{2+}-influx also occurs. In no instance are large membrane defects created such that cytoplasmic proteins egress from the cells. Therefore, Ca^{2+}-dependent machineries remain intact and are vulnerable to flooding by extracellular Ca^{2+} with various consequences including secretion, activation of phospholipases (Seeger et al. 1984; Suttorp et al. 1985), contraction of cytoskeletal elements Suttorp et al. 1988), and the stimulation of constitutive NO-synthase (Suttorp et al. 1993). By stimulating secretion of procoagulatory substances from platelets, alpha-toxin exerts potent procoagulatory effects (Bhakdi et al. 1988). By activating the calcium-dependent phospholipase A_2, alpha-toxin provokes generation of biologically active lipid mediators (Fig. 4) (Seeger et al. 1984; Suttorp et al. 1985). Calcium-influx into endothelial cells provokes cytoskeletal contraction, and this leads to rounding up

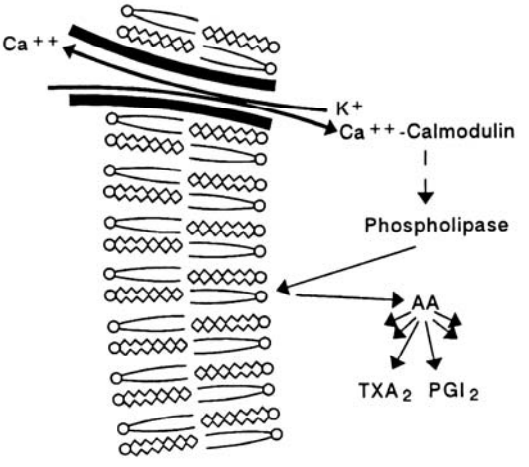

Fig. 4. A staphylococcal alpha-toxin-created transmembrane pore serves as a nonphysiological calcium channel. Incoming calcium, possibly after binding to calmodulin, activates phospholipases with subsequent cleavage of arachidonic acid and formation of tissue specific arachidonic acid metabolites. TXA_2, thromboxane A_2; PGI_2, prostacyclin.

of the cells and formation of intracellular gaps that permit rapid leakage of macromolecules across a confluent monolayer (Suttorp et al. 1988). All these events could contribute to the development of multi-organ dysfunction during staphylococcal infections.

Further to these secondary effects, liberation of biologically active substances from dead or dying cells may also have long-term effects. For example, keratinocytes killed by alpha-toxin would release their content of intracellularly stored IL-1α, which would trigger local inflammatory reactions.

6.2.3 Transcriptional effects accompanying recovery

Because cells attacked by pore-forming toxins are not inevitably killed, they retain the capacity to mount active responses following membrane damage. Recently, it was found that transcriptional processes are activated as a response of cells to low doses of alpha-toxin (Dragneva et al. 2001). The transient drop in cellular ATP levels was followed by secretion of interleukin 8 from monocytic and epithelial cells. Electrophoretic mobility shift and immunofluorescence assays demonstrated that IL-8 secretion was preceded by activation of NF-κB. Transfection experiments with NF-κB-deficient cells showed that activation of the IL-8 promoter/reporter by alpha-toxin was dependent on NF-κB. By contrast, the transcription factor was not required for lesion repair. Thus, attack of cells by low doses of a pore-forming toxin can lead to transcriptional gene activation, which is followed by production of mediators that may be relevant to the initiation and propagation of inflammatory lesions. A systematic investigation on other tran-

scriptional effects accompanying recovery of cells after toxin attack is currently underway, and they are beginning to broaden our understanding of fundamental processes underlying long-term effects of the toxin on cells and tissues.

6.3 Long-range effects

Alpha-toxin can provoke acute organ dysfunction and is lethal in experimental animals (McCartney and Arbuthnott 1978). In this regard, alpha-toxin is no less potent than endotoxins. When applied intravascularly, alpha-toxin provokes profound pathophysiological alterations in the pulmonary microvasculature, subnanomolar concentrations leading to the development of lethal pulmonary edema (Seeger et al. 1984, 1990). The underlying mechanisms are complex, and encompass the direct toxic action on endothelial cells and the production of mediators that in turn promote pulmonary arterial hypertension. Systemic application of alpha-toxin in monkeys causes the animal to succumb with symptoms that may be explained from the *in vitro* observations. Further to development of pulmonary edema, a profound thrombocytopenia is observed, caused by the action of alpha-toxin on platelets (Bhakdi et al. 1989a). Human patients suffering from severe staphylococcal infections (e.g. septicemia, burn wound infections, pneumonia) all present with symptoms that can be partially explained by the local and systemic action of alpha-toxin.

6.4 Synergism between alpha-toxin and other toxins

An important recognition emerging in recent years is that different toxins can synergize with each other to enhance the detrimental effects in the host macroorganism. For example, priming of pulmonary cells in isolated lungs with endotoxin potentiates the vascular abnormalities in response to alpha-toxin (Walmrath et al. 1993). Such synergism and the resulting vascular abnormalities are probably relevant to the pathogenesis of organ failure in systemic infections.

7 Use of alpha-toxin in cell biology

Alpha-toxin was the first pore-forming cytolysin to be exploited as a tool for selective permeabilization of plasma cell membranes (Ahnert-Hilger et al. 1985). Permeabilized chromafine cells were shown to retain the capacity to secrete granular constituents upon stimulation with low concentrations of Ca^{2+}. Since then, an ever-increasing number of investigators are confirming that pore-forming toxins are superior tools for membrane permeabilization than conventional agents such as digitonin or saponin. The assets of pore-forming toxins are multiple (Bhakdi et al. 1993). The actions of these agents are well characterized and easy to control. Since alpha-toxin pores do not permit passage of proteins, guarantee is

given that channels are exclusively formed in the plasma membrane. Alpha-toxin pores are quite well defined in size, so that stable membrane lesions of approximately 1 nm diameter can be produced. It is possible to manipulate the intracellular ionic milieu, and to introduce Ca^{2+} and small molecules such as nucleotides into the cells. Since pure preparations of alpha-toxin contain no enzymatic activity, there will be no proteolytic alterations of cell constituents.

Native alpha-toxin contains no cysteines, and single cysteine substitution mutants can be produced that usually remain functional. These substitution mutants have played a major role as tools for the elucidation of structure-function relationships during pore assembly (Valeva et al. 1995, 1996, 1997a, 1997b). Mutants containing histidine replacements (Jursch et al. 1994; Menzies and Kernodle 1994), or with nicks and overlaps in the pore-forming domain (Walker and Bayley 1994; Walker et al. 1993, 1994, 1995), have also proved valuable and harbor the potential of becoming useful tools in cell biology (Bhakdi et al. 1993; Bayley 1994). The utility of alpha-toxin may be broadened through the availability of manipulatable toxin derivatives. Thus, it is now possible to assemble oligomers that can be opened and closed (Bayley 1994; Walker et al. 1994, 1995; Chang et al. 1995; Valeva et al. 1996; Russo et al. 1997).

Further to its application as a permeabilizing agent, alpha-toxin may also become useful for the study of turnover and dynamics of membrane microdomains, since it can be derivatized and stably labeled for use as a non-toxic membrane marker.

8 Medical relevance

Although it is presently not possible to unequivocally prove that alpha-toxin represents a microbial virulence factor in humans, all available data do clearly support this contention. The major arguments are: (1) many human cells including monocytes, lymphocytes, endothelial cells, keratinocytes, and platelets are effectively attacked by the toxin under physiological conditions. (2) Damage to these cells triggers pathological sequelae. These processes have been studied in detail *in vitro*, and are mirrored by characteristic clinical presentations. Thus, homeostasis disturbances, thrombocytopenia, and pulmonary lesions are frequently encountered in patients during severe staphylococcal infections. The clinical presentation of patients with *S. aureus* skin infections can also be easily explained by the action of alpha-toxin on keratinocytes, macrophages, and fibroblasts. In all instances, the possibility that alpha-toxin synergizes with other virulence factors is imminent.

A theoretical approach to relate toxin production with clinical relevance is to quantify the level of alpha-toxin in tissues or body fluids and attempt to make a correlation with disease. Unfortunately, alpha-toxin avidly binds to target cells, antibodies and lipoproteins, and quantification methods must, therefore, first solve the task of liberating the toxin from these binders. A sensitive immunoassay for quantifying alpha-toxin in tissues and body fluids is consequently not yet available.

Indirect demonstration of a significant role for alpha-toxin as a virulence factor would be possible if application of specific high titered antibodies were found to be therapeutically effective. Polyclonal and monoclonal human hyperimmune globulins against alpha-toxin have been prepared, and they are able to suppress all experimental toxic effects *in vitro* and *in vivo* (Bhakdi et al. 1989). Patients with early diagnosis of severe *S. aureus* infections are candidates for therapy with such hyperimmune globulins, but clinical trials with these antibodies have not yet been conducted. Construction and production of alpha-toxin vaccines are very simple. A single substitution mutant has been found to be devoid of all toxic effects in experimental animals, and to induce high protective antibody levels (Bhakdi et al. 1994). The feasibility of using such vaccines in veterinary medicine is presently being explored.

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S. cerevisiae K28 toxin - a secreted virus toxin of the A/B family of protein toxins

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Abstract

Since the initial discovery of toxin-secreting killer strains in the yeast *Saccharomyces cerevisiae* more than 40 years ago, continuous research on this wide-spread phenomenon substantially strengthened our knowledge in different areas of modern biology, providing deeper insights into basic aspects of eukaryotic cell biology and virus-host cell interactions. This chapter will focus on the K28 killer system in *S. cerevisiae*, in particular the toxin-encoding K28 killer virus *Sc*V-M28 and the intracellular processing, maturation, and toxicity of its viral protein toxin. The toxin's receptor-mediated mode of action, its strategy to penetrate and to finally kill a eukaryotic target cell will be discussed, and the mechanism of toxin immunity, i.e. the question of how a virus-infected killer yeast cell resists its own toxin, will be addressed.

1 Introduction

The initial discovery of toxin secreting 'killer' strains in the yeast *Saccharomyces cerevisiae* and its phenotypic association with the presence of cytoplasmically inherited double-stranded RNA (dsRNA) viruses initiated yeast virology in the late sixties and early seventies of the past century (Bevan and Makower 1963; Wickner 1996). At that time it was shown that certain strains of baker's yeast secrete protein or glycoprotein toxins that are lethal to sensitive strains of different species and genera. Because of this easily recognizable phenotype, these yeast strains have been designated 'killer' yeasts, and the frightening term 'killer toxins' has been created to describe the lethal proteins secreted by these strains. Shortly after this discovery it became evident that toxin producing killer strains exhibit a remarkable antimycotic potential, and that toxin-producing killer strains are not only restricted to *S. cerevisiae* but can also be found among many different yeast genera (Magliani et al. 1997; Schaffrath and Breunig 2000; Schmitt and Breinig 2002; Bruenn 2002).

In *S. cerevisiae* the killer phenotype is caused by an infection with cytoplasmic persisting double-stranded RNA viruses that belong to the constantly growing class of mycoviruses, which are widely distributed among yeast and higher fungi

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(Buck 1986). Since the majority of fungal mycoviruses is non-infectious and symptomless in the corresponding host, they often are being referred to as cryptic viruses or 'virus-like particles' (VLP). All known fungal viruses spread horizontally by cell-cell mating and/or heterokaryon formation. In addition to the rigid yeast and fungal cell wall barrier to extracellular spread, it has been suggested that fungal viruses adopt this strategy, because mating and hyphal fusion is frequently occurring in nature; thus, making the extracellular route of spread dispensable (El-Sherbeini and Bostian 1987; Tipper and Schmitt 1991; Wickner 1996). While some of these viruses can be associated with adverse phenotypic effects on the fungus (like La France disease in *Agaricus bisporus*, plaque formation in *Penicillium*, and hypovirulence in *Endothia*), dsRNA viruses in *S. cerevisiae*, *Ustilago maydis*, *Hansenisaspora uvarum*, and *Zygosaccharomyces bailii* were shown to be responsible for a killer phenotype that is based on the secretion of polypeptide toxins (killer toxins), which are lethal to sensitive yeast strains of different species and genera (Schmitt and Neuhausen 1994; Schmitt et al. 1997; Radler et al. 1993; Zorg et al. 1988; Park et al. 1996; Dawe and Nuss 2001). In the few cases where killer determinants have been identified, they are either cytoplasmically inherited by encapsidated dsRNA viruses or genetically encoded by linear dsDNA plasmids or chromosomal yeast genes. In all cases studied so far, it became clear that yeast killer toxin production is always associated with specific toxin immunity, just as is true for bacteriocin production in bacteria (reviewed in Bussey 1991; Magliani et al. 1997). More recent studies on the virally encoded killer system in *S. cerevisiae* has shed light on some aspects of yeast virology (such as host-cell interactions and viral RNA transcription, replication and packaging), and also provided important insights into basic and more general aspects of eukaryotic cell biology (Wickner 1996; Schmitt and Breinig 2002). This chapter will focus on the K28 killer system in *S. cerevisiae*, in particular on the toxin-encoding K28 killer virus *Sc*V-M28 and the intracellular processing, maturation, and toxicity of its viral protein toxin.

2 dsRNA viruses cause a killer phenotype in *S. cerevisiae*

The genetic basis for killer phenotype expression in yeast is rather variable, and in the few cases where killer determinants have clearly been identified, they are either cytoplasmically inherited encapsidated dsRNA viruses, linear dsDNA plasmids or chromosomal yeast genes (the latter two are dealt with in more detail in accompanying chapters of this book). So far, in *S. cerevisiae* three major killer classes (K1, K2, K28) have been classified on the basis of killing profiles and missing cross-immunity, each of them producing a specific killer toxin as well as an unidentified immunity component that renders killer cells immune to their own toxin (Hanes et al. 1986; Dignard et al. 1991; Schmitt and Tipper 1990; Schmitt 1995; Wickner 1992). In each case, the killer phenotype is associated with the presence of two types of double-stranded RNA viruses: an L-A helper virus and one type of a toxin-coding M satellite ('killer') virus. *In vivo*, both dsRNA genomes are separately encapsidated into virus-like particles (VLP) that stably

Table 1. Viral double-stranded (ds)RNA genomes involved in killer phenotype expression in *S. cerevisiae*

Virus	Function of virus	dsRNA genome (kb)	Encoded protein(s)	Reference
*Sc*V-LA	helper virus	4.6	Gag, major capsid protein; Pol, RNA dependent RNA polymerase (Pol is *in vivo* expressed as Gag-Pol fusion protein)	Icho and Wickner (1989)
*Sc*V-M1	satellite virus ('killer' virus)	1.6	K1 preprotoxin (unprocessed K1 toxin precursor and immunity determinant)	Hanes et al. (1986)
*Sc*V-M2	satellite virus ('killer' virus)	1.5	K2 preprotoxin (unprocessed K2 toxin precursor and immunity determinant)	Dignard et al. (1991)
*Sc*V-M28	satellite virus ('killer' virus)	1.8	K28 preprotoxin (unprocessed K28 toxin precursor and immunity determinant)	Schmitt and Tipper (1995)

persist within the cytoplasm of the infected host (yeast) cell. L-A itself does not confer any (recognizable) phenotype upon a yeast cell, nor does it lead to host cell lysis or cell growth slowing. As summarized in Table 1, the linear dsRNA genome of L-A (4.6 kb) contains two open reading frames (ORF) on its (+) strand: ORF1 encodes the major capsid protein Gag necessary for encapsidation and viral particle structure; the second gene (ORF2) represents the RNA-dependent RNA polymerase Pol, which *in vivo* is expressed as a Gag/Pol fusion protein by a -1 ribosomal frameshift event (Icho and Wickner 1989; Dinman et al. 1991; Fujimura et al. 1992; Dinman and Wickner 1994). In contrast to L-A, which itself does not confer a phenotype upon the infected yeast cell, each of the three known M-dsRNA genomes (M1, M2, M28) contains a single open reading frame coding for a preprotoxin (pptox) that represents the unprocessed precursor of the mature and secreted killer toxin that also gives functional toxin immunity (Table 1). Since each toxin-coding M virus depends on the coexistence of L-A in order to be stably maintained and replicated within the yeast cell, the killer viruses resemble classical satellites of L-A, which itself functions as helper virus. The presence of all three killer virions in a single yeast strain does not occur *in vivo* since the toxin-coding M-dsRNA genomes exclude each other at the replicative level (Schmitt and Tipper 1992). However, this limitation can be by-passed by introducing cDNA copies of the K2 and K28 pptox genes into a natural K1 killer, resulting in stable triple killers that simultaneously produce three different killer toxins and express the corresponding toxin immunity functions (Bussey et al. 1988; Schmitt and Schernikau 1997).

2.1 Viral replication cycle

Intensive studies mainly in the labs of Reed Wickner and Jeremy Bruenn have shown that L-A virions represent non-infectious, icosahedral particles 39 nm in diameter that in many aspects show striking similarities to mammalian reoviruses and rotaviruses (Bruenn 1991; Cheng et al. 1994; Naitow et al. 2002). Each L-A virus contains a single copy of the 4.6 kb L-A dsRNA genome, which is encapsidated by 60 asymmetric dimers of the 76 kDa coat protein Gag and two copies of the 171 kDa Gag/Pol fusion protein. During the conservative replication cycle of L-A, a single-stranded plus-strand RNA [(+)ssRNA] is transcribed within the viral particle (*in viro*), which is subsequently extruded from the virions into the yeast cell cytoplasm (reviewed in Wickner et al. 1995; Wickner 1996; Caston et al. 1997). On one hand, this (+)ssRNA serves as messenger RNA to be translated into the viral proteins Gag and Gag/Pol, on the other hand, the plus-strand serves as RNA template, which is packaged into new viral particles. Once this viral coat assembly has been completed, Gag/Pol functions as replicase, synthesizes a new minus-strand and, thereby, generating the double-stranded RNA genome of the mature virus and finally closing in this viral replication cycle. The replication cycle of the toxin-coding M 'killer' viruses resembles that of L-A with the only exception that each M virion can accept two copies of the smaller M-dsRNA genomes before the toxin-coding transcripts are extruded into the cytoplasm of the infected yeast cell (Esteban and Wickner 1986); a phenomenon also found in some DNA bacteriophages, which has been described as 'headful packaging' (Wickner 1996). Since toxin-coding M virions parasitize Gag and Gag/Pol, they resemble classical satellites of L-A (Bostian et al. 1980; Schmitt and Tipper 1992).

As in many dsRNA viruses, the entire replication cycle of L-A and M virions depend on the presence of specific packaging signals within the 3'-end of the viral ssRNA transcript (Fujimura et al. 1990, 1992). These RNA regions, also known as viral binding sites (VBS), consist of a stem-loop structure whose stem is interrupted by an unpaired, protruding A residue (Ribas and Wickner 1992; Ribas et al. 1994). For viral replication of L-A, two sequence elements were found to be essential: an internal replication enhancer (IRE), which is essentially indistinguishable from the VBS, and a small stem-loop structure 5 bp from the 3'-terminus (3'-TRE). While L-A just contains a single VBS element, the toxin-coding transcripts of M1 and M28 each have two such VBS domains (Schmitt and Tipper 1995; Huan et al. 1991). *In vivo*, these VBS elements are *cis*-acting sequences that are recognized by Gag/Pol; subsequently, used to initiate (+)ssRNA binding and packaging into new viral particles. The replicase reaction (i.e. *in viro* minus-strand synthesis on the (+)ssRNA template) requires a correct 3'-end sequence and structure, but either L-A, M1 or M28 3'-end are substantially different. Within the intact (mature) virion, conservative transcription of the plus-strand from the dsRNA template requires recognition of its very 5'-terminal sequence by Gag/Pol.

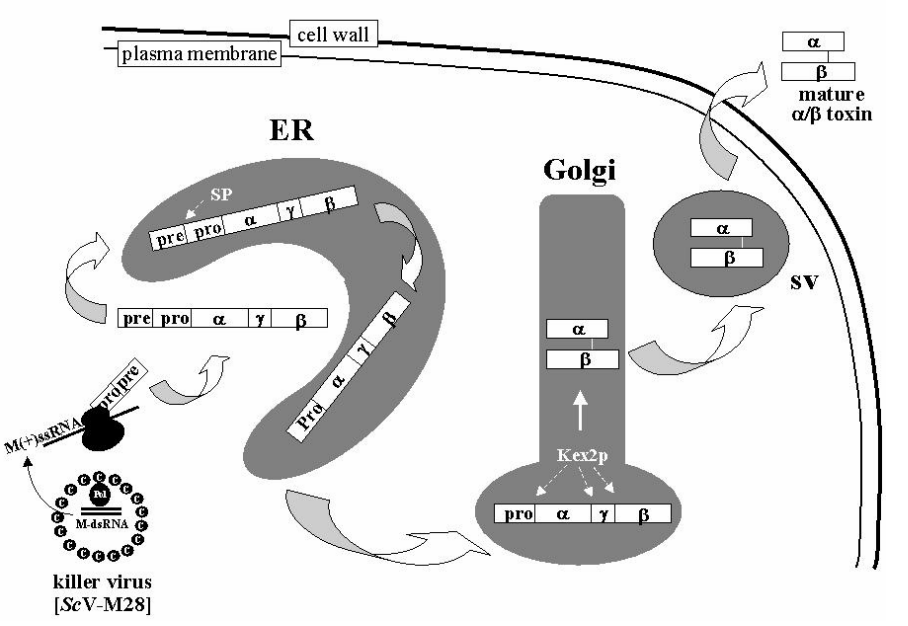

Fig. 1. Expression and processing of the K28 virus toxin within a killer virus (*Sc*V-M28) infected yeast cell. After *in vivo* translation of the preprotoxin (pptox) coding viral M28 transcript, pptox is posttranslationally imported into the lumen of the endoplasmic reticulum (ER) and signal peptidase (SP) cleavage removes the toxin's N-terminal secretion signal (pre-region). In a late Golgi compartment, the Kex2p endopeptidase cleaves the pro-region, removes the intramolecular γ-sequence and finally leads to the secretion of the mature α/β heterodimeric protein toxin (sv, secretory vesicle; modified after Schmitt and Breinig 2002).

In M28 (as in other dsRNAs), the plus-strand initiates with 5'-GAAAAA. Since there is little if any additional homology immediately downstream, this terminal recognition element (5'-TRE) may be all that is necessary for initiation of transcription (Schmitt and Tipper 1995).

3 Preprotoxin processing and toxin secretion

In a *Sc*V-M28 infected killer yeast, the K28 toxin encoding plus-strand RNA transcript is translated on free cytosolic ribosomes into a 38 kDa preprotoxin (pptox), which is posttranslationally imported into the secretory pathway, where it is further processed to the biologically active, α/β heterodimeric protein toxin (Fig. 1). Sequencing of a full-length cDNA copy of the viral M28-dsRNA genome identified a single 345-codon gene encoding the unprocessed toxin precursor (pptox) whose predicted size agrees very well with the 38 kDa protein species observed for the *in vitro* translation product of M28(+)ssRNA (Schmitt 1995). Expression

of the pptox gene in a sensitive non-killer yeast confers a complete killer phenotype, ensuring K28 toxin production and functional (i.e. protecting) immunity (Schmitt and Tipper 1995). The unprocessed toxin precursor consists of a hydrophobic secretion signal necessary for posttranslational toxin import into the lumen of the endoplasmic reticulum (ER), followed by the two subunits α (10.5 kDa) and β (11.0 kDa) of the mature toxin, which are separated from each other by a potentially N-glycosylated γ-sequence. During passage through the yeast secretory pathway, the K28 toxin precursor is enzymatically processed to the biologically active α/β heterodimer in a way that is highly homologous to the prohormon conversion in mammalian cells (Fuller et al. 1989; Rockwell and Thorner 2004); in a late Golgi compartment, the N-glycosylated γ-sequence is removed by the action of the furin-like endopeptidase Kex2p, the toxin's β-C-terminus is trimmed by carboxypeptidase Kex1p cleavage, and the biologically active protein is finally secreted as a 21 kDa α/β heterodimeric protein toxin whose two subunits are covalently linked by a single disulfide bond between amino acids α-Cys^{56} and β-Cys^{340} (Fig. 2). Most importantly, the C-terminus of β contains a four amino acid epitope, which represents a classical ER retention signal (HDELR). Since this signal is initially masked by a carboxyterminal arginine residue, ER retention of the toxin precursor is effectively prevented until the protoxin enters a late Golgi compartment in which carboxypeptidase Kex1p cleavage uncovers the toxin's intracellular targeting signal (see below; Riffer et al. 2002).

Extensive mutational analysis of the preprotoxin gene identified distinct domains within the toxin precursor that fulfil different biological functions within the overall killing process. While the toxin's β-subunit is mainly responsible for toxin binding to the outer yeast cell surface (i.e. to the α-1,3-linked mannotriose side-chains of a high molecular weight cell wall mannoprotein) and for retrograde toxin transport *via* Golgi and ER, the hydrophobic α-subunit represents the actual toxic component that - after nuclear import - interacts with essential host cell proteins that normally function in cell cycle progression, eukaryotic cell cycle control, and/or initiation of DNA synthesis in early S phase (see below; Schmitt and Breinig 2002).

4 Toxin uptake and retrograde transport

After the mature α/β heterodimeric protein toxin K28 has bound to the surface of a sensitive cell, it is taken up by endocytosis in order to reach the yeast secretory pathway. Yeast *end3* and *end4* mutants that are blocked in early steps of both fluid-phase endocytosis as well as receptor-mediated endocytosis (Raths et al. 1993) are toxin resistant, since in these mutants the toxin can no longer enter the cell and reach its final target compartment. Although the K28 membrane receptor has not yet been identified, there is growing evidence that it might be the cellular HDEL receptor Erd2p that colocalizes, in low copy number, to the cytoplasmic

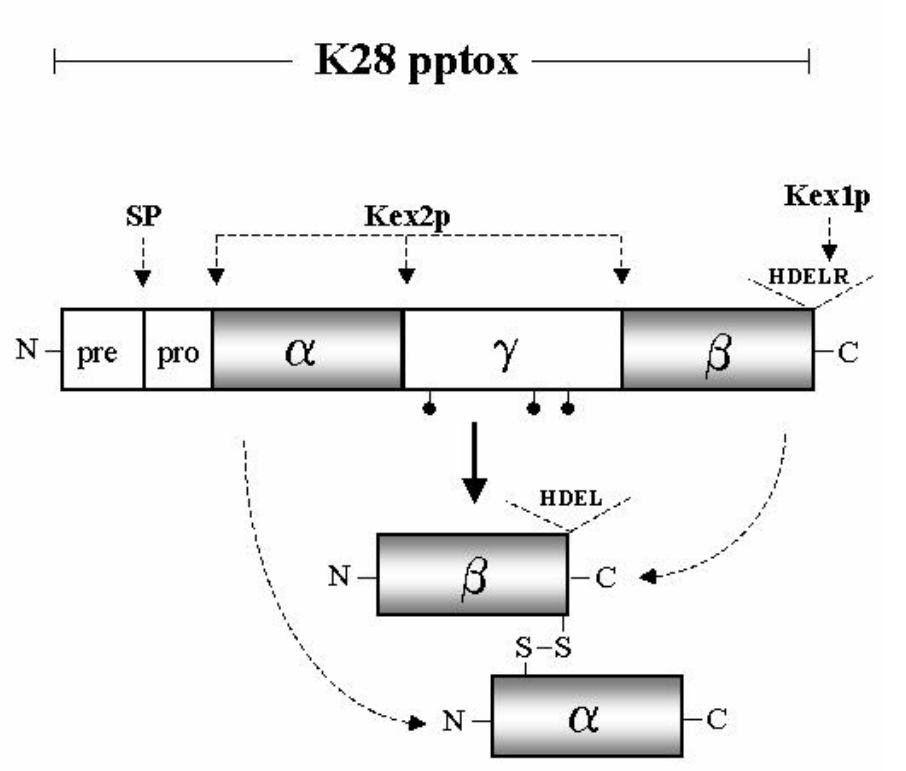

Fig. 2. K28 preprotoxin processing in a toxin-secreting killer strain of *S. cerevisiae*. Cleavage sites of the ER lumenal signal peptidase (SP) as well as cleavage sites of the late Golgi endopeptidase Kex2p and carboxypeptidase Kex1p at the toxin's β-C-terminus are indicated. Potential N-glycosylation sites within the intramolecular γ sequence of K28 pptox are indicated by filled circles and the carboxyterminal ER targeting sequence (HDELR) as well as the single disulfide bond between α and β (-S-S-) are shown.

membrane (Spindler, Leis, Schmitt, unpublished data). In accordance with this assumption, K28 toxin-treated yeast Δ*erd2* mutants that lack the HDEL receptor are completely K28 resistant, accumulate the toxin at the plasma membrane, and are incapable to internalize the α/β heterodimeric virus toxin (Eisfeld et al. 2000). Interestingly, this pronounced phenotype of a yeast Δ*erd2* mutant is exactly portrayed by a mutated K28 derivative lacking its β-C-terminal HDEL sequence; likewise, such a toxin variant is biologically inactive and has completely lost its ability to enter a cell (Riffer et al. 2002). Furthermore, since the same phenoptype can be observed in yeast mutants that are defective in the ubiquitin-conjugating enzyme (E2) Ubc4p and/or in the HECT domain ubiquitin ligase (E3) Rsp5p, we assume that both proteins, Ubc4p and Rsp5p, are involved in the *in vivo* ubiquitination and internalization of the receptor/ligand (i.e. Erd2p/K28) complex (Fig. 3).

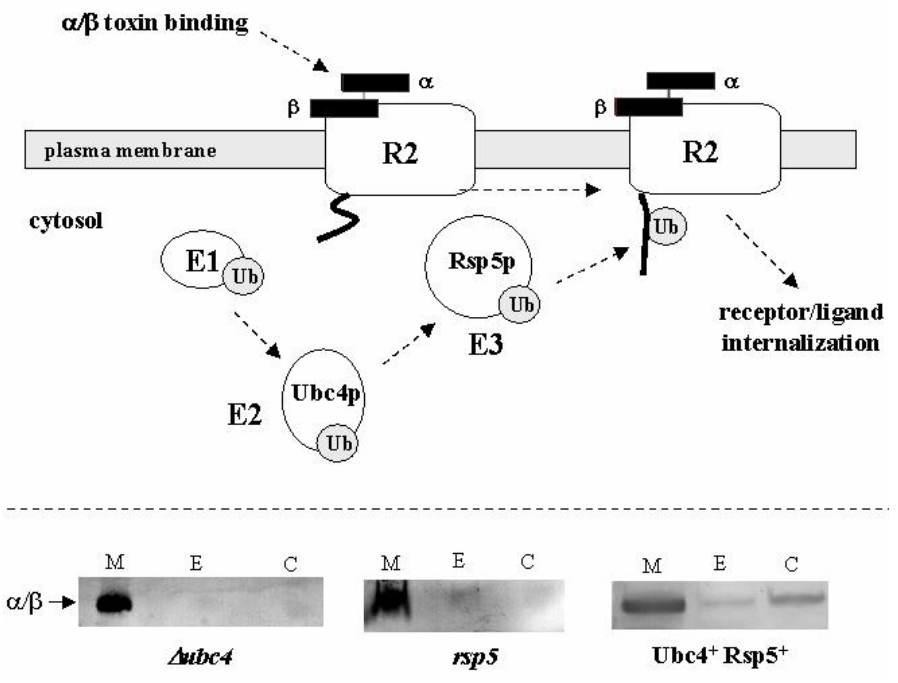

Fig. 3. K28 toxin binding to its plasma membrane receptor and Ubc4p/Rsp5p-mediated ubiquitination and internalization of the receptor/ligand complex – a working model (see text for details). The lower panel shows a western analysis of cell fractions derived from K28 toxin-treated cells of the indicated yeast mutants (Δ*ubc4*, *rsp5*) and a $Ubc4^{+}$ $Rsp5^{+}$ wild type strain. Spheroplasts of the indicated strains were treated with K28 for 2 hours, and after cell lysis and differential centrifugation the resulting cell fractions were separated by SDS-PAGE and probed with a polyclonal anti-β antibody (M, membrane fraction [plasma membrane, ER and Golgi membranes]; E, endosomal vesicle fraction; C, cytosol). Arrows indicate the position of the α/β heterodimeric K28 virus toxin.

Yeast Rsp5p is an essential peripheral membrane protein that regulates the internalization step of endocytosis and directs ubiquitination of endosomal cargo (Galan et al. 1996; Soetens et al. 2001; Dunn et al. 2004). It, therefore, represents a prime candidate for receptor-mediated endocytosis and subsequent sorting of the K28 virus toxin to the secretory pathway.

Independent of the question whether or not Erd2p resembles the long-sought K28 membrane receptor, after the toxin has been internalized and targeted to an early endosomal compartment, it travels the secretion pathway in reverse and enters the yeast cell cytoplasm. Responsible and essential for this unique, COPI-mediated retrograde toxin transport is a short amino acid motif at the carboxyterminal end of the toxins' β-subunit. In the unprocessed toxin precursor, the β-C-terminus carries an HDELR amino acid motif, which is converted into β-HDEL of the mature toxin after Kex1p cleavage occurred in a late Golgi compartment (see

Fig. 2). Thus, the mature and secreted toxin carries a C-terminal HDEL motif, which (in yeast) is known to function as classical ER retention signal and can, therefore, be found at the C-termini of the ER chaperones Kar2p (BiP) or the yeast protein disulfide isomerase Pdi1p (Pelham et al. 1988; Pelham 1990; Eisfeld et al. 2000). In yeast and in higher eukaryots, HDEL- and/or KDEL-carrying proteins resemble resident proteins of the ER lumen that are recognized and subsequently recycled from an early Golgi compartment back to the ER by a membrane-bound H/KDEL receptor (Semenza et al. 1990; Townsley et al. 1994). In case of the K28 virus toxin, this sequence allows retrograde toxin transport *via* Golgi and ER and finally ensures that the toxin can enter the yeast cell cytoplasm and subsequently transduce its lethal signal into the nucleus (Eisfeld et al. 2000; Riffer et al. 2002). The strategy of using endocytosis and retrograde toxin transport is a common phenomenon in many bacterial protein toxins. *Pseudomonas* exotoxin A for instance has also been shown to be internalized by receptor-mediated endocytosis, followed by reverse secretion *via* Golgi and ER. Since many other toxins like the heat-labile *E. coli* toxin or even the Shiga toxin contain putative ER retention signals at their C-termini, H/KDEL-dependent mechanisms seem to have a more general importance for toxin entry into eukaryotic target cells (Kreitman and Pastan 1995; Lord and Roberts 1998; Pelham et al. 1992; Lord et al. 2003; Lencer and Tsai 2003). In this respect, the major difference between the virally encoded killer toxin and the majority of all bacterial toxins is that K28 itself is produced and secreted by a eukaryotic cell; therefore, the ER-targeting signal at the toxins' β-C-terminus is initially masked by a terminal arginine residue. This 'intelligent' strategy ensures that the K28 virus toxin can successfully pass both, ER and early Golgi. Once it has reached a late Golgi compartment, the terminal arginine residue is no longer needed for intracellular toxin transport, and is being cleaved off by the action of the late Golgi carboxypeptidase Kex1p. Consequently, *in vivo* expression of a truncated K28 preprotoxin derivative in which the carboxterminal arginine residue had been deleted, results in a total loss of K28 toxicity since the toxin is completely retained within the ER lumen and can no longer be secreted (Riffer et al. 2002).

4.1 A single disulfide bond between α and β exposes the ER targeting signal of the toxin

In the mature heterodimeric protein toxin, α and β are covalently linked by a single disulfide bond (see Fig. 2). Since the α-subunit possesses only one cysteine residue close to its N-terminus, this cysteine residue (Cys^{56}) must be involved in disulfide bond formation. Correspondingly, a toxin derivative in which the cysteine residue in α had been destroyed ($Cys \rightarrow Trp^{56}$) is biologically inactive since intermolecular disulfide bond formation is prevented. In contrast to the cytotoxic α-subunit, β has four cysteine residues, each of them being possibly involved in disulfide bond formation. However, site-directed cysteine mutagenesis revealed that only Cys^{340} is responsible for correct disulfide bond formation in the K28 heterodimer: Cys^{340} is located right next to the toxin's β-C-terminal HDEL signal.

Thus, ensures (i) that the toxin's ER targeting signal is correctly exposed to the HDEL receptor Erd2p of the target cell, and (ii) that the K28 heterodimer can retrograde pass the cell (Eisfeld et al. 2000; Riffer et al. 2002). Once the toxin has entered a cell by endocytosis, it travels the secretion pathway in reverse in order to reach the yeast cell cytosol where the cytotoxic signal is transmitted into the nucleus (Schmitt and Eisfeld 1999). In this respect, the K28 virus toxin resembles certain bacterial and plant toxins that enter and kill a eukaryotic cell by modifying essential cellular components within the cytosol. For some of these heterodimeric protein toxins it has been shown that carboxyterminal K/HDEL-like sequences are responsible for intracellular targeting and retrograde transport of the toxins (Eisfeld et al. 2000; Yoshida et al. 1991). Correspondingly, mutations in the C-terminal ER targeting sequence dramatically reduce cytotoxicity, because interaction of the toxin's C-terminus with the K/HDEL receptor of the target cell is prevented (Chaudhary et al. 1990). In this respect, it is also interesting to note that some of these toxins contain disulfide bonds at or near their C-termini whose *in vivo* function is predicted to ensure access of the toxin's KDEL/HDEL-signal to the K/HDEL-receptor of the corresponding target cell (Pelham et al. 1992). This situation is also true for the K28 toxin since its cysteine residue right next to the β C-terminus is part of the disulfide bond that covalently joins α and β. Interestingly, yeast cells expressing a mutated toxin (Cys→Ser^{340}) show normal toxin secretion, i.e. they still secrete a α/β heterodimeric protein, but the mutated K28 derivative is completely inactive and incapable to enter a cell (Riffer et al. 2002). Thus, incorrect disulfide bonding in the mutated toxin does not prevent heterodimer formation, but it eliminates *in vivo* toxicity. Phenotypic analysis of additional toxin variants obtained after cysteine mutagenesis identified a Cys→Ser^{340} derivative of K28 as being completely inactive, incapable of killing a sensitive yeast. Cell fractionation studies on sensitive yeasts treated with the mutant toxin further indicated that the observed loss in toxicity is caused by its inability to retrograde pass a cell and to successfully reach its intracellular target. In this respect, the phenotype of a Cys→Ser^{340} toxin derivative exactly portrays the phenotype of a truncated toxin in which the β C-terminal HDEL sequence has been deleted; *in vivo* such a toxin (β-ΔHDEL) is, likewise, no longer capable to enter the secretion pathway of a sensitive cell (Eisfeld et al. 2000). In the wild type toxin, therefore, formation of a disulfide bond between α-Cys^{56} and β-Cys^{340} is of major importance to ensure accessibility of the β-C-terminal ER targeting signal to the HDEL-receptor of the target cell.

4.2 Toxin dislocation out of the ER

Once the toxin has reached the ER lumen, it is retrotranslocated into the cytosol from where it transduces the toxic signal into the nucleus. ER/cytosol export of K28 is mediated by the Sec61 complex/translocon in the ER membrane, which represents the major export channel in yeast and in higher eukaryotes (Eisfeld et al. 2000; Snapp et al. 2004). The yeast Sec61 complex itself, which consists of the

transmembrane ER protein Sec61p and the two smaller subunits Sbh1p and Sss1p (Beswick et al. 1998), has also been shown to be involved in the export and removal of misfolded ER proteins from the secretory pathway and their subsequent proteasomal degradation in the cytosol (Pilon et al. 1997; Plemper et al. 1997; Tsai et al. 2002; Hitt and Wolf 2004). More recently, Sec61 translocons have also been shown to be responsible for ER retrotranslocation of certain plant and microbial A/B toxins like ricin (Wesche et al. 1999), cholera toxin (Schmitz et al. 2000), *Pseudomonas* exotoxin A (Koopman et al. 2000), and the yeast K28 virus toxin (Eisfeld et al. 2000). However, in contrast to the majority of H/KDEL-carrying microbial toxins, ER-to-cytosol translocation of K28 does not depend on ERAD (ER-associated protein degradation; Ellgaard and Helenius 2003) - at least not on those ERAD components that have been identified so far - and yeast mutants defective in "classical" ERAD components such as Der3p/Hrd1p and/or Der1p show wild type sensitivity. Thus, K28 export from the ER into the cytosol might either be mechanistically different from ER-to-cytosol export of misfolded proteins, or dependent on potentially novel ERAD components that have not yet been identified.

Yeast cells carrying a mutated Sec61p translocon as well as yeast *sec63* mutants are completely K28 resistant, since in these mutants the toxin is no longer dislocated into the cytosol. Recently, we could show that this Sec61p/Sec63p-mediated ER/cytosol export of K28 greatly depends on the action of the luminal ER chaperones Kar2p [BiP], Pdi1p [protein disulfide isomerase], Jem1p and Scj1p, and that K28 toxin resistance in yeast *kar2*, *pdi1*, *jem1*, and/or *scj1* mutants is caused by the fact that the toxin sticks in the ER lumen and is no longer released into the cytosol of the corresponding yeast mutant. Interestingly, however, neither components of the ER degradation pathway (ERAD) such as Der3p/Hrd1p and/or Der1p (Sommer and Wolf 1997), which are known to be essential for retrograde transport and ER dislocation of misfolded and/or incorrectly assembled secretory proteins, nor components of the ubiquitin/proteasome degradation machinery such as Cue1p, Ubc6p and Ubc7p, are involved in K28 toxin translocation into the cytosol (Fig. 4). So far, it is not known what particular component within or near the ER membrane is responsible for toxin exit out of the ER, or if subsequent ubiquitination and proteasomal degradation of the toxin's β-subunit generates the driving force for retrograde toxin transport through the ER membrane. In this respect, it is also interesting to note that a K28 toxin variant that lacks all lysine residues in its β subunit is still ubiquitinated (*via* its N-terminus) and subsequently dislocated into the cytosol; therefore, it can be assumed that ubiquitination of the β-toxin, which might also be catalyzed by Ubc4p (E2) and Rsp5p (E3), is responsible for the *in vivo* export of the α/β heterodimeric protein toxin out of the ER lumen (Leis and Schmitt unpublished data). Once within the cytosol, the toxin dissociates into its two subunits α and β, most likely, because of the reducing environmental conditions of the yeast cell cytosol. As indicated in Fig. 4, β is subsequently ubiquitinated and targeted for proteasomal degradation, while the toxin's α-subunit enters the nucleus in order to exhibit its toxic effects (see below).

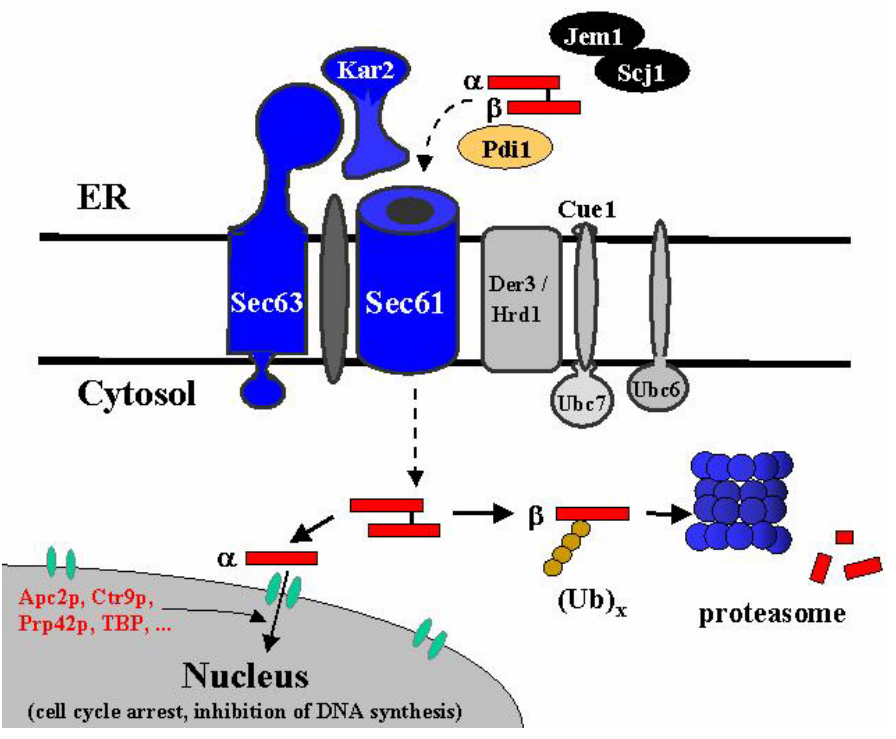

Fig. 4. Model of ER-to-cytosol dislocation of the K28 toxin in yeast. After endocytotic uptake and retrograde transport, K28 reaches the ER lumen from where it is retrotranslocated into the cytosol *via* the Sec61 complex of the ER membrane. Components of the ER-associated protein degradation pathway (ERAD) such as Der3p/Hrd1p, Ubc7p, Cue1p, and Ubc6p are not involved in K28 dislocation, while the lumenal ER chaperones Kar2p (BiP), Pdi1p, Jem1p, and Scj1p are essential for ER exit of the toxin (see text for details).

5 Mode of K28 toxin action

The viral K28 toxin kills sensitive yeast cells in a receptor-mediated process by interacting with receptors at the level of the yeast cell wall and the cytoplasmic membrane (Fig. 5). The initial step involves a fast and energy-independent binding to a primary K28 receptor, which has been identified as high molecular weight α-1,3-linked cell wall mannoprotein (Schmitt and Radler 1988). It was speculated, however, that toxin binding to the primary cell wall receptor either concentrates the toxin at the level of the cell wall or mediates close contact between the toxin and the target cell membrane (Schmitt and Radler 1987). Susceptible strains can become toxin resistant by chromosomal mutations in any of a set of genes that are involved in the structure and/or biosynthesis of yeast cell wall components; in

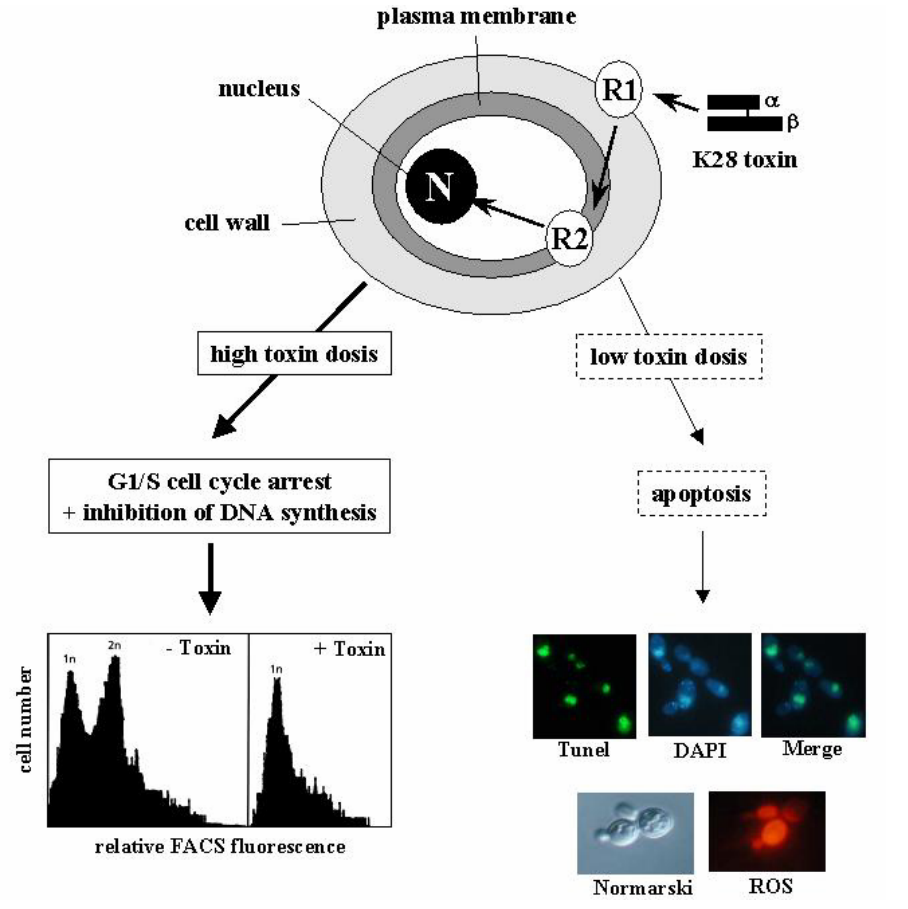

Fig. 5. Receptor-mediated mode of action of the K28 virus toxin. Killing of a sensitive yeast cell is envisaged in a two-step process involving initial toxin binding to receptors at the level of the cell wall (R1) and the cytoplasmic membrane (R2). After interaction with the plasma membrane, K28 enters the cell by endocytosis in order to reach its final target, the yeast cell nucleus. At high K28 toxin doses, sensitive yeast cells arrest at the G1/S boundary of the cell cycle with pre-replicated DNA (1n; left panel), while cells treated with K28 in sublethal concentrations respond with typical apoptotic markers such as chromosomal DNA fragmentation and accumulation of reactive oxygen species, ROS (right panel).

fact, a whole set of yeast *mnn* mutants (for mannoprotein) has been shown to be highly resistant to K28 at the cell wall level (Schmitt and Radler 1988; Tipper and Schmitt 1991).

The second, energy-dependent, step involves toxin translocation to the cytoplasmic membrane and interaction with a secondary membrane receptor. While the membrane receptor for K28 is still unknown, the K1 membrane receptor was recently identified as Kre1p, an O-glycosylated yeast cell surface protein, which is

initially GPI-anchored to the plasma membrane and is also involved in β-1,6-glucan biosynthesis and K1 cell wall receptor assembly (Breinig et al. 2002). After having reached the cytoplasmic membrane, K28 enters the cell by endocytosis, traverses the secretion pathway in reverse (*via* Golgi, ER and cytosol), and finally enters the nucleus, where the lethal events occur: DNA synthesis is rapidly inhibited, cell viability is lost more slowly, and cells arrest in early S phase of the cell cycle with a medium-sized bud, a single nucleus in the mother cell, and a pre-replicated 1n DNA content (Fig. 5; Schmitt et al. 1989, 1996). Since the cytotoxic α subunit of K28 resembles a 10.5 kDa low molecular weight polypeptide, it can enter the nucleus by simple passive diffusion without the need of an active nuclear import machinery. However, if α is additionally modified and extended by a classical nuclear localization sequence (NLS) at its N-terminus, *in vivo* toxicity of the K28 virus toxin is significantly enhanced due to a faster and more efficient nuclear import mediated by α/β importins of the host cell (Reiter and Schmitt, unpublished data). Once within the nucleus, K28-α specifically interacts with essential host cell proteins that are normally involved in eukaryotic cell cycle control and cell cycle progression as well as in initiating DNA synthesis in early S phase. Thus, because K28 is targeting essential and evolutionary conserved host proteins with basic cellular functions, resistance mechanisms based on mutations in essential chromosomal genes hardly occur *in vivo*, and so the K28 virus toxin has developed an amazing "intelligent" strategy to effectively penetrate and kill its target cell. Most recently, yeast two-hybrid screens identified a whole set of essential host cell proteins (like Apc2p, Ctr9p, Prp42p and TBP), which normally function in eukaryotic cell cycle control and/or initiation of DNA synthesis, and directly interact with K28-α, the cytotoxic component of the K28 virus toxin (Reiter and Schmitt, unpublished data). Within the nucleus, K28-α specifically blocks G1 cyclin transcription (*CLN1*, *CLN2*, *CLN3*) and inhibits Apc2p, which is an ubiquitin ligase (E3) of the anaphase promoting complex (APC) that is normally involved in the ubiquitination and proteasomal degradation of B-type cyclins. Thus, B-type cyclins are significantly stabilized in toxin-treated cells, explaining the *in vivo* observed cell cycle arrest of K28 treated cells. Because of the pleiotropic effect of K28, further analysis is needed to explain the still missing link between cell cycle arrest and inhibition of DNA synthesis.

5.1 Toxin-induced apoptotic host cell responses

In yeast, apoptotic markers such as DNA fragmentation, chromatin condensation, exposure of phosphatidylserine on the outer surface of the plasma membrane, accumulation of reactive oxygen species (ROS), and phenotypic changes can be induced by various factors such as H_2O_2, cell ageing, acetic acid, and α-factor pheromone treatment (Del Carratore et al. 2002; Laun et al. 2001; Ludovico et al. 2001; Madeo et al. 1999; Severin and Hyman 2002). Interestingly, active cell death can also be induced by treating yeast cells with low doses of the K28 virus toxin, while high toxin concentrations prevent apoptotic cell responses and cause

K28 specific, apoptosis-independent cell killing (Fig. 5). Phenotypic analysis of a glutathion-deficient yeast Δ*gsh1* mutant further confirmed that ROS accumulation represents the actual trigger of apoptosis in yeast (Severin and Hyman 2002) as well as in K28 toxin-treated cells. While apoptosis is clearly not the primary lethal effect of the toxin, a chromosomal deletion in the yeast caspase 1 gene *YCA1*, whose gene product is needed for apoptotic host cell responses (Madeo et al. 2002), results in a markedly reduced toxin sensitivity; thus, mutations in the caspase encoding yeast gene *YCA1* can rescue cells from K28-mediated toxicity. We, therefore, conclude that in the natural environment of a toxin-secreting killer yeast, where toxin concentration is usually low, induction of apoptosis plays a critical, if not an essential role in efficient toxin-mediated cell killing (Reiter and Schmitt, unpublished data).

6 Toxin immunity

Besides the fact that the primary toxin target has yet to be identified, the question of how immunity occurs *in vivo* has still not been answered. Although the precise molecular basis for toxin immunity is still unknown, it has been speculated that it might be conferred by the toxin precursor itself. In case of K1, which represents another virally encoded protein toxin in *S. cerevisiae*, the toxin precursor might act as competitive inhibitor of the mature toxin by saturating or eliminating the toxin's plasma membrane receptor that normally mediates toxicity (Bussey et al. 1982, 1983; Tipper and Schmitt 1991). It was also shown that the unprocessed toxin precursor is sufficient to confer immunity since *in vivo* expression of a toxin-coding cDNA in a Δ*kex2* null mutant lacking the ability of pptox processing; thus, incapable to release α and β from the intervening γ sequence results in immune non-killer yeast transformants (Sturley et al. 1986; Boone et al. 1986; Schmitt and Tipper 1992). Based on these observations, a model for K1 toxin immunity had been proposed in which either loss or modification of the toxin's secondary plasma membrane receptor would cause immunity. When it was demonstrated that expression of a cDNA copy of the preprotoxin was sufficient to confer normal immunity, it was postulated that the γ component of the toxin precursor might not only act as intramolecular chaperone ensuring proper pptox processing within the secretory pathway, but also by providing some sort of masking function by protecting membranes of toxin-producing killer cells against damage by the hydrophobic α subunit of K1 (Bostian et al. 1984). Later on, a more plausible model was proposed, in which it was speculated that interaction of the secreted protoxin with the receptor, through the same toxin domain involved in lethality, results in the diversion of the complex to the vacuole and its subsequent destruction (Sturley et al. 1986). This model was further strengthened by phenotypic analyses of various mutant pptox derivatives, which clearly indicated that the α toxin was the lethal component and that its secretion in the mature form caused a severe growth inhibition, while secretion of α fused to an N-terminal fragment of γ was sufficient to confer immunity (Zhu et al. 1993). Dependence of immunity on

diversion of the putative membrane receptor to the vacuole is consistent with the defect in immunity observed in many *vps* mutants. One class of killer resistant *kre* mutants, therefore, should show similar resistance in spheroplasts. More recently, a chromosomal *kre1-12* mutation was described that caused K1 resistance in yeast cell spheroplasts without affecting toxin binding to the cell wall, and the wild type gene product, Kre1p, was identified as being the long-sought membrane receptor for the K1 viral toxin (Schmitt and Compain 1995; Breinig et al. 2002).

Although the precise mechanism for K1 immunity is still obscure (Sesti et al. 2001; Schmitt and Breinig 2002), a completely different mechanism must be postulated for K28 killers, since it has been shown that K28 killer yeasts take up their own toxin after it had been secreted. Furthermore, the toxin subsequently re-enters the secretory pathway of a killer cell and reaches the cytosol, just as in a sensitive target cell. However, in contrast to the latter, the killer cell is not killed but rather protected against the lethal effect of the toxin. This means that K28 immunity is likely to affect some unknown step either within the yeast cell cytosol or eventually downstream of that, possibly within the nucleus. Most recently, we could show that the K28 toxin presursor (pptox) within the yeast cell cytosol complexes the mature and re-internalized α/β heterodimer and initiates its ubquitination and proteasomal degradation; thus, a K28 secreting killer yeast effectively resists its own viral α/β toxin (Breinig and Schmitt, unpublished data).

7 Concluding remarks

Over the years, much has been learned about eukaryotic cell biology by studying virus-infected killer strains of the yeast *S. cerevisiae* and their viral protein toxins. In the case of the yeast K28 virus toxin, a remarkable variety of important cellular processes can be studied in detail, such as receptor-mediated endocytosis, retrograde protein transport, ER-to-cytosol retrotranslocation and protein ubiquitination, nuclear signal transduction, and even toxin-induced apoptotic host cell responses. However, there is still a great number of open and unresolved questions, and future research is needed to adequately address each aspect. Based on the strategy of microbial A/B toxins to enter and to retrograde pass a cell, it might be possible in future to specifically design chimeric α/β toxin variants that function as novel vehicles for protein import and retrograde transport in yeast and possibly higher eukaryotic cells as well.

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Kluyveromyces lactis zymocin and other plasmid-encoded yeast killer toxins

Raffael Schaffrath and Friedhelm Meinhardt

Abstract

Supported by an endogenous killer plasmid DNA system that codes for an anti-proliferative zymocin toxin, the dairy yeast *Kluyveromyces lactis* has attracted research into inter-yeast pathogenesis. Molecularly, gene disruption and shuffle techniques have proven powerful means to study components essentially required for the maintenance of the killer plasmid system, namely, autonomously operating replication and transcription apparatuses. Moreover, by studying zymocin action towards sensitive *Saccharomyces cerevisiae* cells, a multi-step response has surfaced that involves chitin docking, import and communication of zymocin's γ-toxin with Elongator's toxin-target (TOT) function and eventually, a terminal G1 block. Though apparently different with respect to toxin-target strategies, novel yeast killers that are phylogenetically related to the *K. lactis* system have shown to establish cell surface contact by zymocin-like chitin binding. Hence, research into zymocin as a model may provide new insights into evolution of yeast pathosystems and proliferation control by toxins.

1 Introduction

Secretion of cytotoxic substances by microorganisms is one strategy to combat microbial competitors. In yeast, numerous such killer toxin systems, either chromosomally encoded or associated with dsRNA viruses or cytoplasmic dsDNA plasmids, have evolved (Wickner 1996; Walker 1998; Schaffrath and Breunig 2000). Biochemically, these toxins vary by receptor specificity, by being secreted as monomers or multi-subunit complexes and by their cytotoxic effects towards target cell proliferation (Meinhardt and Schaffrath 2001; Schmitt and Breinig 2002). The lethal interaction between *Kluyveromyces lactis* and *Saccharomyces cerevisiae* constitutes a model pathosystem that relies on zymocin, a trimeric protein toxin complex secreted by the dairy yeast (Stark et al. 1990). This killer phenomenon is encoded by a cytoplasmic pair of killer plasmids, pGKL1 (k1) and pGKL2 (k2), and ultimately causes a G1 block (Gunge at al. 1981; Butler et al. 1991a). Reviews describing yeast linear plasmids in general and the *K. lactis* killer system in particular have been written in the past (Stark et al. 1990; Gunge 1995; Fukuhara 1995; Meinhardt et al. 1997; Schaffrath et al. 1999; Meinhardt and

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Schaffrath 2001; Gunge and Tokunaga 2004). However, since the recent advances towards dissecting the zymocin response pathway in *S. cerevisiae* and identifying other zymocin-like killers, our knowledge about these yeast pathosystems has greatly increased. Consequently, the *K. lactis* zymocin has acquired considerable interest as a model system to study eukaryotic plasmid biology and control of cell proliferation in yeast.

2 The plasmid-encoded killer system from *Kluyveromyces lactis*

2.1 Plasmid structures, genome organizations, and gene functions

Linear extranuclear dsDNA elements are widespread among bacteria, plants, and fungi (Meinhardt et al.1997; Klassen et al. 2002). They are especially common in yeasts with the majority being cytoplasmic (Fukuhara 1995). Among yeasts, the most thoroughly studied are the killer plasmids pGKL1 and pGKL2 from *K. lactis*. pGKL1/2 are peculiar in many respects since both elements are (i) cytoplasmically present in multi-copy (50-100 per cell) and (ii) linear with (iii) distinct proteins covalently linked to (iv) terminal inverted repeats (TIRs). For adenoviruses and some phages, these structures are relevant for replication initiation (Meijer et al. 2001). The A/T bias of pGKL1 (73.2 %) and pGKL2 (74.7 %) is remarkably high compared to the *K. lactis* genome (Hishinuma et al. 1984; Tommasino et al. 1988). Both elements are tightly packed with genetic information and carry fifteen intron-less open reading frames (ORF) (Fig. 1). There are four on pGKL1 encoding a DNA polymerase (ORF1), the α, β (ORF2) and γ subunits (ORF4) of the zymocin toxin and toxin-immunity (ORF3) (Fig. 1; see below). Out of eleven pGKL2 genes, four (ORF1, 8, 9, and 11) are functionally unassigned. However, ORF1 is dispensable and ORF8-9 are essential for plasmid biology (Schaffrath et al. 1992, 1999; our unpublished data). The pGKL2-specific DNA-polymerase/terminal protein is encoded by ORF2 (Takeda et al. 1996). Viral-like mRNA capping and helicase enzymes are the predicted products of ORF3 and ORF4, respectively (Larsen et al. 1998; Tiggemann et al. 2001; Stark et al. 1990). ORF5 specifies a single-stranded DNA binding protein involved in pGKL1/2 replication (Schaffrath and Meacock 2001). Since cytoplasmic localization conditions autonomous replication and transcription machineries, it is not surprising that ORF6 codes for a pGKL1/2-specific RNA polymerase and ORF7 provides a putative subunit of the latter (Wilson and Meacock 1988; Schaffrath et al. 1995a, 1995b, 1997a). ORF10 codes for a replication factor that binds both pGKL-TIRs (McNeel and Tamanoi 1991; Tommasino 1991). Since the discovery of ORF11, a novel actively transcribed pGKL2 orphan gene (Fig. 1), ORF11-like loci have been located on pGKL2-related plasmids (Larsen and Meinhardt 2000; Klassen et al. 2001).

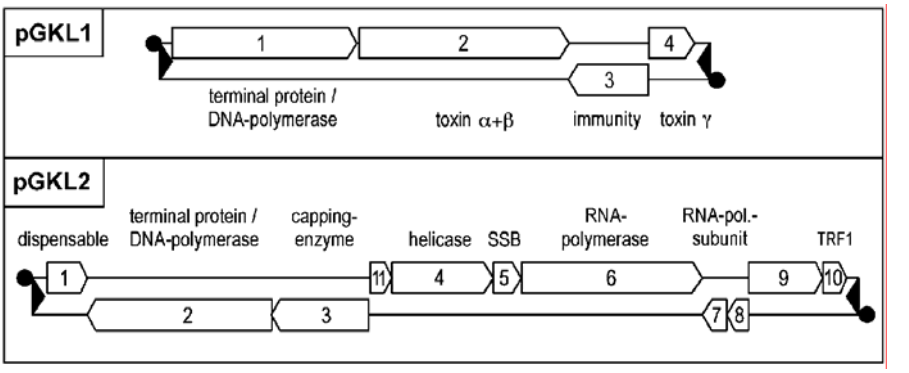

Fig. 1. Genetic organization of pGKL1 (k1) and pGKL2 (k2). Polarity of open reading frames and gene transcription are indicated by arrowheads (Hishinuma et al. 1984; Tommasino et al. 1988; Larsen and Meinhardt 2000). Known or assumed gene functions are depicted. Black circles correspond to terminal proteins; black triangles indicate TIRs. SSB: single-stranded DNA binding protein; TRF1: terminal region recognition factor 1.

2.2 Molecular genetic manipulation, gene shuffles, and applications

Plasmid gene activation probably occurs autonomously at an upstream conserved sequence (UCS) motif, a promoter preceding each of the pGKL1/2 ORFs (Romanos and Boyd 1988; Wilson and Meacock 1988; Kämper et al. 1991; Schickel et al. 1996; Schaffrath et al. 1996). Being cytoplasmic, pGKL1/2 hardly expressed genes governed by nuclear promoters and, likewise, pGKL1/2 genes are not expressed in the nucleus (Kämper et al. 1989b, 1991). Selection for integration of a gene governed by a nuclear promoter resulted in structurally altered circular or telomer-attached nuclear plasmids, most likely as a result of fortuitous autonomously replicating sequences (ARS) present in the pGKL-DNAs (Kämper et al. 1989b, 1991, Takata et al. 2000). However, by fusing UCSs in phase to selectable genes (Kämper et al. 1989a, 1991; Schaffrath et al. 1992) plasmid-specific expression of the markers was achieved from both pGKL1/2 hybrids that, like parental pGKL1/2, were cytoplasmic and had proteins covalently linked to their TIRs (Kämper et al. 1989a, 1991; Meinhardt et al. 1994; Schaffrath et al. 1992, 1995a, 1996, 1999, 2000; Tanguy-Rougeau et al. 1990). By applying such *in vivo* gene disruption strategies, all ORFs (except for ORF11) have been targeted (Kämper et al. 1989a, 1991; Schaffrath et al. 1999; Tiggemann et al. 2001; our unpublished results). Non-essential ORFs (pGKL2 ORF1 and pGKL1 ORF2) were identified by induced loss of non-marked parental plasmids. As for other ORFs, disruption never resulted in segregation of native plasmids indicating essential gene functions for the dyadic pGKL1/2 system (Schaffrath et al. 1999; Meinhardt and Schaffrath 2001). Gene functional analysis has been extended by a gene shuffle system that allows genes from pGKL2 to be transplaced onto pGKL1 (Schaffrath 1995, 2003). For this purpose, a wild type killer strain was sequentially transformed with two

markers. First, a recombinant plasmid was constructed by disruption of a target gene (ORF5) on pGKL2 using UCS-*LEU2* for selection. Next, the target gene (ORF5) was shuffled into the non-essential pGKL1 ORF2 using UCS-*TRP1* as second marker. As a consequence, recombinants harboured four plasmids, wild type pGKL1/2 and two hybrids (Schaffrath and Meacock 1995, 1996). Selection of both markers eventually led to segregation of the wild type plasmids, indicating the functionality of the transplaced ORF5 (Schaffrath and Meacock 1995, 1996). Similarly, the DNA helicase and RNA polymerase genes (Fig. 1) have been shuffled, reinforcing that pGKL genes are functionally interchangeable (Schaffrath et al. 1999, 2000). UCS-promoter function has also been addressed by UCS-driven reporter gene expression from pGKL1 (Schaffrath et al. 1996; Schickel et al. 1996). This defined the UCS motif as *cis*-active and sufficient for expression of native pGKL and heterologous genes (Meinhardt et al. 1994; Schründer and Meinhardt 1995; Schründer et al. 1996). Moreover, expression studies using various UCSs revealed a 12-fold variation in promoter strength with pGKL2 UCS6 most efficiently activating plasmid transcription (Schickel et al. 1996).

3 The *K. lactis* zymocin complex

3.1 Zymocin subunit composition and biogenesis

Zymocin is a trimeric complex with subunits of 99 (α), 30 (β), and 28 (γ) kDa (Stark and Boyd 1986). SDS-PAGE analysis of killer and non-killer filtrates has shown α, β, and γ to be pGKL1-dependent (Stark and Boyd 1986); furthermore, micro-sequencing revealed the mature N-termini of the α, β and γ polypeptides to be A_{30} (ORF2), G_{895} (ORF2), and A_{19} (ORF4), respectively. Hence, the larger subunits result from proteolysis of an αβ precursor (ORF2), while γ is processed from ORF4 (Fig. 1) (Stark and Boyd 1986). Synthesis of holo-zymocin is inhibited by tunicamycin suggesting post-translational glycosylation. Indeed, as judged from SDS-PAGE and lectin assays, an *N*-linked oligosaccharide chain is attached to the α subunit, a modification missing from β and γ (Sugisaki et al. 1984; Stark and Boyd 1986). The mature α and β subunits commence at positions preceded by Kex2 procession sites (α: KR_{29}; β: KR_{894}) and α/β, but not γ maturation requires an *S. cerevisiae* Kex2-like protease encoded by the *K. lactis KEX1* gene (Tanguy-Rougeau et al. 1988; Wesolowski-Louvel et al. 1988; Stark et al. 1990). While *S. cerevisiae* strains that maintain pGKL1/2 secrete authentic zymocin, a *kex2*Δ mutant fails to do so. Thus, zymocin biogenesis is Kex2-dependent and very similar in both yeasts. Although γ carries a leader peptide that efficiently drives protein secretion; γ itself is not secreted in the absence of αβ production. Secretion is, however, restored by introducing a heterologous pre-pro-sequence (Tokunaga et al. 1987b, 1988, 1989). If that foreign signal bypasses the requirement for αβ to

promote γ export, how then is αβ engaged in zymocin biogenesis? Obviously, subunit processing and zymocin secretion are coupled events. So, one role may involve assembly of γ into secretable holo-zymocin. Indeed, α-intra- and β/γ-intermolecular S=S bridges support zyomcin integrity and treatment with -SH reagents abolishes zymocicity (Stark et al. 1990).

3.2 Functional assignment of individual zymocin subunits

Toxicity of holo-zymocin appears to reside just within the γ subunit (γ-toxin): conditional expression of its gene (Fig. 1) from regulated promoters mimics holo-zymocin and is lethal to *S. cerevisiae* (Tokunaga et al. 1989, Butler et al. 1991b; Frohloff et al. 2001). While endogenous γ-toxin is cytotoxic, exogenously applied, it is inert. This implies, that γ-toxin has to enter the target cell in order to function from within and that holo-zymocin acts from the cell's exterior to promote γ-toxin access. Given that the α subunit has a chitin binding domain (CBD) and a chitinase motif, holo-zymocin is likely to recognize chitin (Butler et al. 1991c). Consistently, zymocin binds chitin *in vitro* and displays exo-chitinase activity (Butler et al. 1991c; Jablonowski et al. 2001a, 2003). The chitinase motif contains a key catalytic residue (E466) and zymocin is blocked by allosamidin, a competitive chitinase inhibitor (Terwisscha van Scheltinga et al. 1995). Intriguingly, the β subunit is hydrophobic and similar to *E. coli* tolQ, a factor required for cellular entry of colicins (Sun and Webster 1987). Concluding, chitin binding and chitin hydrolysis is likely to be crucial for zymocin docking. So, in addition to the G1 block, zymocin may cause cell wall damage. This may explain why exo-zymocin induces a terminal phenotype, whereas inhibition by intracellular γ-toxin can be reversed (Butler et al. 1991b). As for γ-toxin import, a poorly understood process, factors that ensure a β/γ disulphide exchange mechanism may promote the release of free, translocable γ-toxin from holo-zymocin once cell surface contact is established (Stark et al. 1990).

3.3 Autoimmunity of *K. lactis* zymocin producers

Not surprisingly, *K. lactis* zymocin producers are immune towards their own zymocin. This depends on killer plasmid pGKL1 and cured *K. lactis* isolates become zymocin sensitive (Niwa et al. 1981). In contrast, non-killer strains carrying orf2Δ plasmids remain immune but become killer-negative due to deletion of the αβ subunit gene, ORF2 (Fig. 1) (Stark and Boyd 1986). As strains carrying orf2-4Δ deletion plasmids lose immunity and zymocin secretion (Kitada and Gunge 1988), pGKL1 ORF3 has been evidenced as a candidate immunity gene. In fact, following cloning ORF3 into an ARS vector and transformation of a pGKL1-free host strain, immunity was partially restored as long as the recipient would maintain pGKL2 (Tokunaga et al. 1987a). Therefore, pGKL2 was postulated by Stark et al. (1990) to be essentially needed for ORF3 expression on the ARS vector or, alter-

natively, to encode an additional immunity factor. Despite sequence similarities between pGKL1 ORF3 and pGKL2 ORF1, a role of the latter in immunity has been ruled out: mutants carrying pGKL2 orf1Δ plasmids remain immune (Schaffrath et al. 1992). Collectively, though pGKL1/2-dependent, immunity is not understood. Possible mode of actions may include exclusion of zymocin from the cell or intracellular detoxification by trapping the γ-toxin or masking its target(s).

4 The zymocin response pathway of *S. cerevisiae*

4.1 Target yeast spectrum and anti-proliferative effects

Zymocin inhibits various yeast genera including *Saccharomyces*, *Kluyveromyces,* and *Candida* and anti-zymosis is readily visualized by a colony-colony interaction assay (Gunge et al. 1981; Kishida et al. 1996; Fichtner et al. 2003a). Biochemically distinct from *S. cerevisiae* toxins, zymocin does not cause cell shrinkage or ion-effluxes, nor does it interfere with progression through S-phase (Butler et al. 1991a; Schmitt et al. 1996; Ahmed et al. 1999; Schmitt and Breinig 2002). Instead, zymocin causes *S. cerevisiae* to accumulate as large, unbudded cells suggesting that cell division rather than growth is affected (Butler et al. 1991a). Indeed, FACS demonstrated a 1n pre-replicative DNA content indicative for a G1 cell cycle block (Butler et al. 1991a; Schaffrath and Breunig 2000). Earlier reports that zymocin inhibits Cdc35, adenylate cyclase, and hence abolishes the roles of cAMP for cell division (Sugisaki et al. 1983) have been disproven by Stark and co-workers (White et al. 1989). By knocking out *BCY1*, which encodes the regulatory subunit of cAMP-dependent protein kinase, resultant *bcy1*Δ cells no longer required cAMP for cell division in the absence of Cdc35 and were as vulnerable to zymocin as the *BCY1* parent (White et al. 1989). Reminiscent of G1 blocks induced by pheromone or associated with *cdc28*^{ts} cells (Lew et al. 1997), zymocin was speculated to antagonize G1 cyclin function. A sufficient amount of the latter is essential for the activity of cyclin-dependent kinase (CDK) Cdc28 in order to exit G1 (Lew et al. 1997). Intriguingly, however, dominant G1 cyclin alleles would not suppress the zymocin G1 block, yet, they efficiently bypassed the pheromone G1 block (Butler et al. 1994; our unpublished data). Collectively, it is unlikely that zymocin mimics pheromone or intoxicates CDK or Cdc35 function. Thus, zymocin action remains elusive, yet, its analysis may allow to reveal G1 cell cycle events specific for and sensitive towards proliferation control.

4.2 Zymocin sensitivity conferring genes and zymocin resistance

Based on their ability to grow in the presence of holo-zymocin, zymocin-resistant *skt* (**s**ensitivity to ***K***. *lactis* **t**oxin), *iki* (**i**nsensitive to **ki**ller), *kti* (***K***. *lactis* **t**oxin **i**nsensitive), and *tot* (**to**xin-**t**arget site) mutants, respectively, have been isolated over the last decade (Butler et al. 1991b, 1994; Kawamoto et al. 1993; Kishida et al.

1996; Frohloff et al. 2001). Sensitivity of these towards intracellular expression of γ-toxin from inducible promoters (see above) has distinguished zymocin binding/uptake (class I) from toxin-target site (class II) mutants (Butler et al. 1991b, 1994; Schaffrath et al. 1997b; Frohloff et al. 2001) (Table 1). While analysis of the latter has identified the toxin target (TOT) function of RNA polymerase II (pol II) Elongator and Elongator-relevant factors, mutations in class I genes affect chitin synthesis and plasma membrane components (Table 1).

4.3 Zymocin docking and early response events

Consistent with a role for chitin as primary zymocin receptor (see above) are studies on *chs* (**ch**itin **s**ynthesis deficient) *S. cerevisiae* mutants (Takita and Castilho-Valavicius, 1993; Jablonowski et al. 2001a). These behave as class I resistant responders: they survive exo-zymocin but display unaltered sensitivity to intracellular γ-toxin. Moreover, *KTI2* is allelic with *CHS3* and *SKT5* corresponds to *CHS4* (Table 1) (Kawamoto et al. 1993; Butler et al. 1994; Jablonowski et al. 2001a). *CHS4* encodes a post-translational activator of chitin synthase III (CSIII), the *CHS3* gene product (Kawamoto et al. 1993; Trilla et al. 1997). *In vivo*, CSIII accounts for >90% of a yeast cell's chitin resources. In line with class I resistance of single or combined mutations in *CHS4-7*, CSIII activity is lacking or significantly compromised in *chs4-7* mutants (Santos et al. 1997; Trilla et al. 1997, 1999; Ziman et al. 1998; Jablonowski et al. 2001a). Together with the findings that zymocin binds chitin *in vitro* and that chitin upregulation elicits hypersensitivity, holozymocin contacts the cell surface in a chitin-dependent manner. Thus, reduced chitin levels due to abolishing or deregulating CSIII activity render yeast cells class I protection (Jablonowski et al. 2001a, 2003).

Despite these advances, subsequent processes involved in entry or intracellular trafficking of γ-toxin are poorly understood. Consistent with a class I phenotype, *kti10* cells bear mutations in *PMA1* coding for major plasma membrane H^+ pump. It is needed for proton motive force (pmf) generation and membrane energization (ΔΨ) (Table 1) (McCusker et al. 1987; Mehlgarten and Schaffrath 2004). Hygromycin B, a ΔΨ-dependent antibiotic, no longer inhibits *kti10* cells and mutations in *PTK2*, coding for a Pma1 kinase, tolerate hygromycin B and zymocin (Perlin et al. 1989; Goossens et al. 2000; Mehlgarten and Schaffrath 2004). *kti10*/*pma1* cells phenocopy inviability at low pH and sublethal surplus of H^+ reinstates zymocin sensitivity suggesting zymocin is pmf-dependent. The H^+ effect, however, appears to follow chitin docking as it is not operating against zymocin resistant chitin mutants. Consistently, zymocin and γ-toxin dock onto and gain entry into *kti10* cells (Mehlgarten and Schaffrath 2004). Perhaps following uptake, γ-toxin is stuck unless mobilized by excess H^+. This suggests *KTI10* may promote γ-toxin activation or cytosolic release. Not knowing whether γ-toxin import requires the β subunit to be uncoupled and whether γ-toxin translocates by flip-flop insertion, voltage-gated import or endocytosis complicates a prediction as to when/where the β-S=S-γ linkage is split. Lack of ER-retention signals in β and γ and retrograde

Table 1. *S. cerevisiae* genes causing zymocin resistance when mutated or kept in multi-copy

Gene name/alias	Function of gene product	Zymocin-relevance	Reference(s)
I. class I resistance to zymocin			
CHS3/KTI2	chitin synthesis	docking	Butler et al. (1994); Jablonowski et al. (2001a)
CHS4/SKT5	chitin synthesis	docking	Kawamoto et al. (1993); Jablonowski et al. (2001a)
CHS5	chitin synthesis	docking	Jablonowski et al. (2001a)
CHS6	chitin synthesis	docking	Jablonowski et al. (2001a)
CHS7	chitin synthesis	docking	Jablonowski et al. (2001a)
PMA1/KTI10	H^+-ATPase	post-docking &	Mehlgarten and Schaffrath (2004)
PTK2	Pma1-activator	prior to TOT[a]	Mehlgarten and Schaffrath (2004)
KTI6	?	?	Butler et al. (1994)
GRX3[b]	glutaredoxin	post-docking ?	Jablonowski et al. (2001a)
II. class II resistance to zymocin			
KTI1	?	?	Butler et al. (1994)
IKI3/ELP1/ TOT1	Elongator	TOT	Yajima et al. (1997); Otero et al. (1999); Frohloff et al. (2001)
ELP2/TOT2	Elongator	TOT	Fellows et al. (2000); Frohloff et al. (2001, 2003)
ELP3/TOT3	Elongator	TOT	Wittschieben et al. (1999); Frohloff et al. (2001)
KTI12/ TOT4[b]	Elongator-AP[c]	TOT regulator ?	Butler et al. (1994); Frohloff et al. (2001); Fichtner et al. (2002a, b)
IKI1/ELP5/ TOT5/HAP2	Elongator	TOT	Yajima et al. (1997); Frohloff et al. (2001); Li et al. (2001); Winkler et al. (2001)
ELP6/TOT6/ HAP3	Elongator	TOT	Winkler et al. (2001); Jablonowski et al. (2001b); Krogan and Greenblatt (2001); Li et al. (2001)
ELP4/TOT7/ HAP1	Elongator	TOT	Winkler et al. (2001); Jablonowski et al. (2001b); Krogan and Greenblatt (2001); Li et al. (2001)
KTI11	Elongator-AP	TOT stability ?	Fichtner and Schaffrath (2002)
ATS1/KTI13	RCC1[d] homolog	Tot4-GEF[e] ?	Fichtner and Schaffrath (2002)
HRR25/ KTI14	casein kinase I	?	Mehlgarten and Schaffrath (2003)
SIT4	PPase[f]	TOT-DP[g]	Sutton et al. (1991); Jablonowski et al. (2001c, 2004)
SAP155[b]	Sit4-AP	?	Luke et al. (1996); Jablonowski et al. (2001c, 2004)

Table 1 continued. *S. cerevisiae* genes causing zymocin resistance when mutated or kept in multi-copy

Gene name/alias	Function of gene product	Zymocin-relevance	Reference(s)
SAP185	Sit4-AP	TOT-DP	Luke et al. (1996); Jablonowski et al. (2001c, 2004)
SAP190	Sit4-AP	TOT-DP	Luke et al. (1996); Jablonowski et al. (2001c, 2004)
URM1	urmylation[h]	TOT stability?	Furukawa et al. (2000); Fichtner et al. (2003b)
III. class of zymocin resistance/protection not assigned			
UBA4	urmylation	TOT stability?	Furukawa et al. (2000); Fichtner et al. (2003b)
KAP120	karyopherin	TOT comp.[i]?	Rout et al. (2000); Fichtner et al. (2003b)
DPH2	diphthamide	?	Mattheakis et al. (1993), Fichtner et al. (2003b)
YIL103w	diphthamide ?	?	Fichtner et al. (2003b)
MATa	mating-type	?	Butler et al. (1994)
$tRNA_3^{Glu}$ [b]	transfer-RNA	?	Butler et al. (1994)

[a]TOT: potential γ-toxin target; [b]multi-copy toxin suppressor; [c]AP: associated protein; [d]RCC1: regulator of chromatin condensation; [e]GEF: GTP exchange factor; [f]PPase: protein phosphatase; [g]DP: dephosphorylation; [h]urmylation: Urm1 (**u**biquitin-**r**elated **m**odifier) conjugation; [i]comp.: compartmentalization.

transport defects that do not protect against zymocin suggest this is not necessarily the route for γ-toxin entry (Jablonowski et al. 2001a). The fact, that class I mutant *kti6* is distinct from *chs*Δ cells points to another component required early in the zymocin response (Jablonowski et al. 2001a; Table 1). In analogy to K1 and KT28 killer toxins, it may represent a secondary plasma membrane receptor (Breinig et al. 2002). Finally, identification of *GRX3* as a multi-copy class I zymocin resistance factor (Table 1) implies that redox regulation affects zymocin, too. Here, higher-than-normal Grx3 glutaredoxin levels may impact β/γ uncoupling or γ-toxin import.

4.4 Toxin-target capacity of the RNA polymerase II Elongator complex

Ten class II target-site *kti* mutants suggest that a complex process transduces the zymocin G1 block (Butler et al. 1994). While some Kti proteins may be required for expression of target(s) inhibited by γ-toxin, others may participate in the process that is blocked by it. These may constitute a pathway or form a multi-subunit protein complex. In favour, analysis of *tot* (γ-toxin target) mutants identified the TOT function of Elongator, a histone acetylase (HAT) complex that purifies with pol II (Otero et al. 1999; Wittschieben et al. 1999; Fellows et al. 2000; Frohloff et al. 2001; Jablonowski et al. 2001b; Winkler et al. 2001; Li et al. 2001; Krogan and

Greenblatt 2001). Initially characterized as a trimer, Elongator was later found to be composed of two three-subunit sub-complexes (Otero et al. 1999; Krogan and Greenblatt 2001; Li et al. 2001; Winkler et al. 2001). Thus, holo-Elongator consists of six subunits encoded by *ELP1-6* (*TOT1-3* and *TOT5-7*) (Table 1). Elongator inactivation coincides with delayed gene activation and misadaptation to new environmental cues (Otero et al. 1999; Fellows et al. 2000). Other Elongator defects, known as Tot$^-$ phenotype, include G1 cell cycle delay, slow growth, thermosensitivity and hypersensitivity towards transcription and cell wall indicator drugs such as 6-azauracil and Calcofluor White (Frohloff et al. 2001; Jablonowski et al. 2001b; Krogan and Greenblatt 2001; Li et al. 2001; Winkler et al. 2001). Finally, defective Elongator conservatively phenocopies class II resistance against holozymocin and intracellular γ-toxin implying that in Tot$^-$ Elongator cells, the toxin target is altered or completely absent (Fichtner et al. 2002a, 2002b; Frohloff et al. 2001, 2003; Jablonowski et al. 2001b, 2001c, 2004; Li et al. 2001; Winkler et al. 2001). Elongator associates with elongating pol II and nascent mRNA (Otero et al. 1999; Gilbert et al. 2004). By virtue of its HAT subunit 3, Elongator may assist pol II during transcription through chromatin (Kim et al. 2002; Winkler et al. 2002). HAT activity reduction causes Elongator defects and survival in the presence of zymocin (Wittschieben et al. 2000; Frohloff et al. 2001). Mutagenesis of the HAT gene has revealed that TOT capacity is separatable from Elongator wild type properties (Jablonowski et al. 2001b). In these toxin resistant, but otherwise Elongator HAT-positive cells, γ-toxin is not lethal suggesting that, in wild type, it is subject to Elongator-dependent registration. The majority of the mutations locate outside the HAT-relevant domains indicating that while essential for zymocin, HAT activity *per se* is not sufficient (Jablonowski et al. 2001b). This argues against a downstream *bona fide* toxin target controlled by Elongator but instead reinforces Elongator's role as a veritable TOT.

4.5 Other factors linked to Elongator's TOT function

Class II mutations in genes coding for Elongator partners (*KTI11* and *KTI12/TOT4*) or Elongator-relevant factors (*KTI14/HRR25*, *KTI13/ATS1*, *URM1*, *UBA4*, *SIT4*, *SAP155*, *SAP185,* and *SAP190*) induce Tot$^-$ phenotypes (Fichtner et al. 2002a, 2002b, 2003b; Fichtner and Schaffrath 2002; Jablonowski et al. 2001c, 2004; Mehlgarten and Schaffrath 2003). This underscores the key role TOT/Elongator plays in the zymocin G1 block (Table 1). Tot4 is an Elongator-associator whose removal and overproduction protect from zymocin. This suggests that Tot4 impacts Elongator as a regulatory factor rather than a structural subunit (Frohloff et al. 2001; Fichtner et al. 2002a). Tot4 may mediate inter-complex communication between Elongator and pol II as it interacts both with Elongator and hyperphosphorylated pol II (Fichtner et al. 2002b). Consistent with this pol II modification being relevant for promoter escape, in coupled ChIP/PCR assays, Tot4 occupies promoter rather than actively transcribed DNA (Fichtner et al. 2002b). Tot4 contains calmodulin and ATP/GTP binding motifs, removal of which causes zymocin resistance (Fichtner et al. 2002a). Intriguingly, mutants of

Kti13/Ats1, a potential GTP exchange factor (GEF) homologue of mammalian RCC1, protect against zymocin in a class II manner (Fichtner and Schaffrath 2002). Whether it plays a role as a GEF for the candidate G-protein, Tot4, is elusive. However, DRL1, the *Arabidopsis thaliana* homologue of yeast Tot4 is a G-protein *in vitro* and phenotypic analyses of *drl1* mutants suggest that plant Elongator defects may connect to dysfunctional organogenesis (Nelissen et al. 2003). In line with transient communication between Elongator and Tot4, their contact is abolished when Elongator lacks or incorporates truncated versions of subunit 2 (Frohloff et al. 2003). The latter, a potential scaffold protein, carries eight WD40 protein-protein interaction domains and its mouse homologue, StIP1, shuttles nucleo-cytoplasmically (Fellows et al. 2000; Collum et al. 2000). Intriguingly, removal of subunit 2 results in partial Elongator assemblies that lack TOT capacity and Tot4-Elongator as well as Tot4-pol II interactions are not detected in such zymocin resistant scenarios (Frohloff et al. 2003).

Cells lacking the Sit4 phosphatase or its partners, Sap185 and Sap190, express class II resistance (Sutton et al. 1991; Luke et al. 1996; Jablonowski et al. 2001c). *SIT4* inactivation correlates with hyperphosphorylation of Elongator subunit 1, a scenario copied in *sap185*Δ*sap190*Δ mutants and in *SAP155* overexpressors (Jablonowski et al. 2001c, 2004). The latter accounts for out-competition of Sit4 binding to Sap185/Sap190 by Sap155. Consequently, multi-copy *SAP155* zymocin protection is suppressed by excess Sap185 and Sap190. Thus, among the Sit4 associating Sap proteins (Luke et al. 1996), Sap185 and Sap190 appear to be Elongator- and zymocin-relevant (Jablonowski et al. 2004). Similar to *sit4*Δ mutants, high-copy *TOT4* induces Elongator hyperphosphorylation. The *TOT4* dosage effect is suppressed by excess Sit4-Sap190, which restores Elongator wild type phosphorylation (Jablonowski et al. 2004). This antagonism requires presence of Tot4 suggesting competition for a common (Elongator) substrate or inhibition of Sit4-Sap190 by Tot4 (Jablonowski et al. 2004). In line with phosphorylation linking up to Elongator's TOT function, Hrr25/Kti14 casein kinase I defects elicit Tot$^-$ phenotypes (Mehlgarten and Schaffrath 2003). If Kti14 represents the Elongator-kinase is elusive. Optionally, resistance in *kti14* cells may correlate with seemingly Elongator-irrelevant factors such as Crz1, a transcription factor controlled by casein kinase I (Kafadar et al. 2003). Other processes linked to TOT include ubiquitin-like urmylation (*URM1-UBA4*: Table 1) and proteolysis (Furukawa et al. 2000; Fichtner et al. 2003b). As for the former, urmylation deficient *urm1*Δ and *uba4*Δ cells express zymocin resistance and can no longer sustain life without Elongator (Tong et al. 2001; Fichtner et al. 2003b; Goehring et al. 2003). Although there is no evidence that Elongator is urmylated, an *urm1*Δ null-allele appears to destabilize Elongator subunit 1 (Fichtner et al. 2003b). This coincides with partial N-terminal proteolysis and is counteracted by Kti11, a novel Elongator-associator (Fichtner et al. 2003b). In addition, Kti11 associates with translation elongation factor 2 (EF2) and Dph2, a diphthamide synthesis factor (Fichtner et al. 2003b). Diphthamide represents an exotic post-translational modification at EF2 that enables deadly ADP-ribosylation by *Corynebacterium diphtheriae* toxin (Mattheakis et al. 1993). Removal of Kti11 protects against zymocin and abrogates ADP-ribosylation of EF2 by diphtheria toxin (Fichtner and Schaffrath 2002;

Liu and Leppla 2003). Surprisingly, resistance to diphtheria toxin in *dph*Δ cells segregates with zymocin protection albeit not as pronounced as in Elongator mutants (Fichtner et al. 2003b). Thus, a functional overlap between lethal eubacterial and eukaryotic toxins may converge to Kti11, an Elongator partner protein.

4.6 Zymocin mode of action: a working model

In analysing a possible link between zymocin and pol II Elongator, pol II performance was found to be affected on exposure to zymocin: global polyA$^+$-mRNA levels and gene-specific pol II transcripts were downregulated, while rDNA transcription by pol I remained unaffected (Frohloff et al. 2001; Jablonowski et al. 2001b). Consistently, pol II under-production led to a hypersensitive read-out and, generally, transcriptionally compromised pol II mutants were found to be much more vulnerable to zymocin than wild type susceptible cells (Jablonowski et al. 2001b; Jablonowski and Schaffrath 2002; Kitamoto et al. 2002). The findings that Elongator associates with pol II hyperphosphorylated at its C-terminal domain (CTD), that overexpression of CTD phosphatase Fcp1 suppresses zymocin sensitivity, while a partially inactive *fcp1-100* allele elicits hypersensitivity suggest that zymocin action is modulatable by pol II CTD modification, and possibly requires nuclear pol II-Elongator contact (Otero et al. 1999; Jablonowski et al. 2001b; Kitamoto et al. 2002). The latter notion is supported by data that demonstrate nuclear pools of Elongator, that truncation of an Elongator NLS no longer supports TOT function, that NLS-GFP travels into the nucleus and that removal of Kap120, a karyopherin relevant for nucleo-cytoplasmic cargo transport, suppresses zymocin (Rout et al. 2000; Fichtner et al. 2002b, 2003b). Moreover, on studying CTD modification, which allows to distinguish initiation-compatible pol II form IIA from elongation-competent pol II form II0, zymocin was found to interfere with IIA/II0 ratios leading to an accumulation of hyperphosphorylated form II0 (Jablonowski and Schaffrath 2002). In line, using coupled ChIP/PCR assays, zymocin-treated cells were found to decrease pol IIA capable to occupy promoter-DNA while association of transcribed DNA with pol II0 was upregulated (Jablonowski and Schaffrath 2002). Based on these data, a model on the zymocin response emerges (Fig. 2). It assumes that following docking and translocation, the γ-toxin hijacks the TOT function of pol II Elongator (Fig. 2). Thus, cells may experience a negative effect on pol II performance and finally halt transcription of genes necessary for G1 exit (Fig. 2). In favour, both START execution and G1 exit are particularly sensitive towards compromised pol II function: temperature-sensitive mutations in eukaryotic pol II CTD subunits cause conditional G1 blocks (Drebot et al. 1993; Sugaya 2001). In future, some of the model's predictions should be tested by trapping protein-protein interaction between Elongator and γ-toxin and by transcriptomics to compare G1 blocks caused by zymocin and pheromone or as a result of CDK and pol II defects. This may reveal overlaps between pol II malfunction and cell cycle defects.

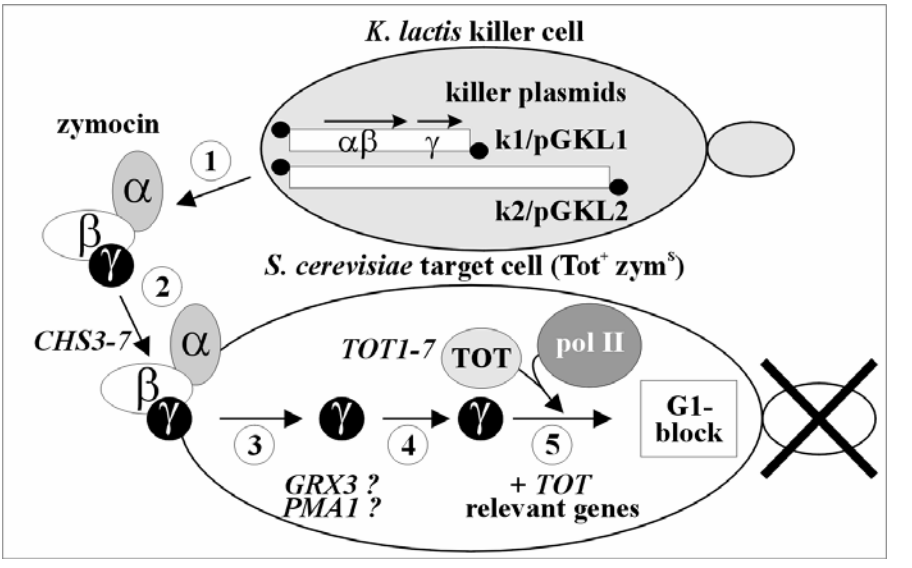

Fig. 2. Working model of the lethal interaction between *K. lactis* killer strains and sensitive *S. cerevisiae* cells. Lethal rivalry is a multi-step process that involves zymocin secretion by killer plasmid-carrying *K. lactis* cells (1), zymocin docking onto chitin of sensitive (zym^S) *S. cerevisiae* target (Tot$^+$) cells (2), import and activation of zymocin's γ-toxin subunit (3-4), lethal communication of the latter with pol II Elongator's TOT function (5) and a terminal G1 cell cycle block. The precise roles of a glutaredoxin (*GRX3*) and a proton pump (*PMA1*) in early, post-docking steps are not (?) yet clear. *CHS3-7* denote chitin synthesis genes. *TOT1-7* code for Elongator subunits and an Elongator-associator. TOT-relevant genes include *KTI11-14*, *SIT4*, *SAP185/190*, *UBA4*, and *URM1* (for details, see text and Table 1).

5 Zymocin-related yeast killer systems

Up to date, more than 20 linear dsDNA plasmid systems are known in yeast (Fukuhara 1995). Among these, elements isolated from *Pichia acaciae, Pichia inositovora,* and *Wingea robertsiae* do code for killer phenotypes (Worsham and Bolen 1990; Hayman and Bolen 1991; Klassen and Meinhardt 2002, 2003). As for *K. lactis,* in each of these killer systems, there is a smaller, non-autonomous plasmid coding for killer toxin subunits and a putative immunity factor (Fig. 3) (Klassen and Meinhardt 2002, 2003; Klassen et al. 2004). Among the four linear plasmid-encoded killer systems known so far, the pGKL1-based zymocin and the pPin1-3-encoded toxin from *P. inositovora* apparently belong to one corporate class (Klassen and Meinhardt 2003) (Fig. 3). Genetically very similar to pGKL1, pPin1-3 has four ORFs coding for immunity (ORF1), a DNA polymerase (ORF2) and a protein (ORF3) predicted to be similar to the α and β zymocin subunits of the *K. lac-*

tis system (Fig. 3). In line with a CBD and a chitinase signature of the latter, chitin affinity chromatography allowed isolation of a *P. inositovora* killer protein that cross-reacted with an anti-zymocin α subunit antibody. Adequately, inactivating CSIII rendered *S. cerevisiae* resistant to the *P. inositovora* toxin. ORF4 displays weak similarity to zymocin's γ-toxin, however, Elongator defects rescued from the *P. inositovora* killer effect. So, zymocin and the pPin1-3 encoded toxin may have overlapping modes of action (Klassen and Meinhardt 2003).

As with *K. lactis* and *P. inositovora*, the *P. acaciae* and *W. robertsiae* toxins bind chitin *in vitro* via CBDs (Klassen and Meinhardt 2002, 2003; Klassen et al. 2004). Further evidence for chitin as toxin receptor stems from analysis of chitin mutants that induce toxin resistance in a manner identical to class I zymocin protection (see above) (Klassen et al. 2004). Thus, comparable to zymocin, chitin constitutes a prerequisite for these novel killers and chitin binding is a common feature for zymocin-like toxins. As with zymocin, a characteristic hydrophobic profile was identified in pWR1A ORF2 and pPac1-2 ORF1 coding for zymocin β-like subunits of the *P. acaciae* and *W. robertsiae* systems (Fig. 3) (Klassen et al. 2004). For zymocin, it was assumed that the hydrophobic β subunit mediates uptake/delivery of the γ-toxin (see above). pWR1A ORF3 and pPac1-2 ORF2 (Fig. 3) are predicted to encode secreted proteins that substantially differ from the zymocin γ-toxin. However, similar to the latter, their intracellular expression is lethal towards a yeast reporter strain (Klassen et al. 2004). In both cases, however, lethality is Elongator-independent suggesting that these toxins differ mechanistically from the *K. lactis* zymocin. Unlike zymocin's G1 block, FACS analyses showed that the novel toxins affect S-phase progression (Klassen et al. 2004). In line, Rad53, an S-phase DNA damage-checkpoint kinase, is activated and *rad53* mutants behave hypersensitive to both toxins. Thus, damage checkpoint activation may contribute to coping with toxin stress, rather than being implemented in toxin action (Klassen et al. 2004). The picture emerging from these comparative studies reveals that following docking, a conserved complex consisting of hydrophobic and CBD/chitinase subunits mediates delivery of a protein toxin. It remains to be seen how the toxin enters the target cell and if such a protein complex can be designed as a delivery system for modified cargos. Also, criteria for such cargo to be incorporated into this delivery system are elusive at present.

6 Conclusions

Zymocin has been successfully exploited as an anti-zymotic additive in pilot studies; using lactose and bioengineered *K. lactis* killers unable to grow on lactic acid, growth of lactic acid-utilizing *Pichia* and *Candida* spoilage yeasts was counteracted (Kitamoto et al. 1999). Together with the emergence of zymocin as a tool to probe for Elongator's HAT proficiency, basic research that aims at the elucidation of its TOT function in proliferation control is a worthwhile option. Elongator regulation is a complex affair and subject to multi-level control including protein-protein interaction. In addition, post-translational phosphorylation, proteolysis and

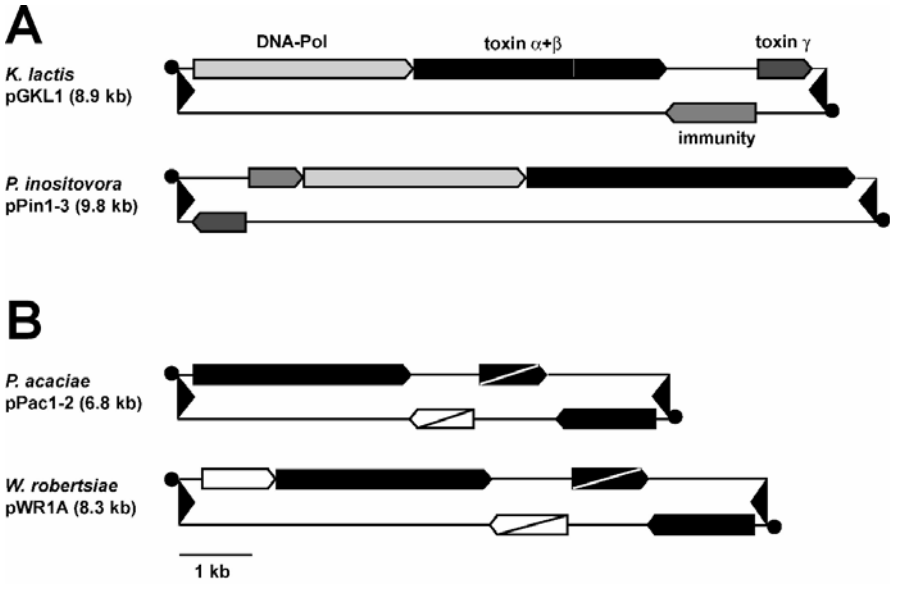

Fig. 3. Genetic basis of zymocin-like killer phenotypes in yeasts. A. Comparison of pGKL1 and pPin1-3 representing a corporate class of killer plasmids. Both elements encode their own element-specific DNA polymerase, an immunity function and zymocin-like subunits. Conserved functions between pGKL1 and pPin1-3 genes are represented in black or distinct shades of grey. B. Comparison of pPac1-2 and pWR1A, members of a second corporate killer plasmid class. In contrast to group 1 elements (A), there is no DNA polymerase gene, but zymocin-like α and β subunits. Toxin and immunity functions (slashed ORFs) are homologous between pPac1-2a and pWR1A but distinct from group 1 members.

diphthamide attachment collectively add to Elongator's plasticity. With zymocin-like yeast toxins surfacing, the zymocin response may serve as a model to further investigate, if and how Elongator is involved. Future efforts, therefore, will have to address whether zymocin communicates with and transforms Elongator in a dominant-negative fashion. Concluding, research into the *K. lactis*-*S. cerevisiae* pathosystem provides fundamentals of eukaryotic plasmid biology and facilitates studying inter-yeast rivalry and competition.

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The *Ustilago maydis* killer toxins

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Abstract

Killer toxins are small proteins secreted by a number of fungi that are lethal to susceptible cells (generally fungi of the same or related species). They bear some similarity to other families of protein toxins, which are ubiquitous in nature. The three known *Ustilago maydis* killer toxins typify, in many respects, the class of killer toxins. Two of them appear to exert their effects by affecting ion fluxes. Two are processed by a Golgi enzyme apparently extant in all eukaryotes. One of the three toxins has both structural and sequence homologs in other species. They have been useful in elucidating the mechanisms of ion channel function and processing of secreted proteins, and they may serve to confer resistance to fungi on a new generation of transgenic crop plants.

1 Introduction

Secreted protein toxins from fungi were first described in *Saccharomyces cerevisiae* (Bevan and Makower 1963; Somers and Bevan 1969; Bevan et al. 1973; Herring and Bevan 1974). They were subsequently discovered in *Ustilago maydis* (Puhalla 1968, 1969; Hankin and Puhalla 1971; Koltin and Day 1975; Koltin and Day 1976). There are now killer toxins known in a number of other fungi, including *Pichia* (Hayman and Bolen 1991; Suzuki and Nikkuni 1994; Kashiwagi et al. 1997; Klassen and Meinhardt 2003; Santos and Marquina 2004), *Zygosaccharomyces* (Schmitt and Neuhausen 1994), *Hansenula* (Kimura et al. 1993; Komiyama et al. 1995), *Kluyveromyces* (Stark et al. 1984, 1990; Stark and Boyd 1986), and possibly *Penicillium* (Olsen et al. 2004). There is also a putative killer toxin in *Gibberella zeae* (present work). In short, killer toxins are secreted by many fungi, including basidiomycetes and ascomycetes, yeasts, and mycelial fungi.

The origins of these toxins are various. Some are encoded by double-stranded RNA (dsRNA) viruses, some by DNA plasmids, and some by chromosomal genes. The structure of the *S. cerevisiae* k1 and k2 toxin genes suggests that they were adopted by their dsRNA viruses by adventitious packaging of cellular mRNAs, since they include, internally, but at the 3' ends of the toxin encoding sequences, long polyA stretches, which are absent from every other viral dsRNA. As we will see, there are also examples of dsRNA encoded toxins that are clearly homologous to toxins encoded by nuclear genomes.

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The structures and functions of these toxins are various. Some are composed of more than one polypeptide; some are single polypeptides. Some are single polypeptides that are homologous to toxins that are two polypeptides. Some multipeptide toxins are complexes held together by disulfide bonds, some by non-covalent bonds, while some are composed of more than one polypeptide acting independently. Their mechanisms of action are, in general, poorly understood. Some clearly are imported and act within the cell; others act at the cell surface. Some have cell wall receptors; some have cell membrane receptors. Several affect ion fluxes. Many of the killer toxins are processed from precursors (prepropolypeptides) by a Golgi subtilisin-like peptidase (KEX2p) now known to be ubiquitous in eukaryotes, but first discovered for its processing of the *S. cerevisiae* k1 killer toxin (Wickner 1974; Fuller et al. 1988, 1989; Mizuno et al. 1988). The three *Ustilago* toxins illustrate many features of the killer toxins taken as a class (Bruenn 1999, 2002).

There are three known protein toxins secreted by strains of *Ustilago maydis*: KP1, KP4, and KP6. These toxins were originally discovered by virtue of their inhibition of growth of some strains of *Ustilago* by others (Puhalla 1968) during studies on mating compatibility (Puhalla 1969). Heterokaryon transfer and mating experiments demonstrated that cytoplasmically inherited genetic elements are responsible for coding of the toxins (Puhalla 1968; Day and Anagnostakis 1973). These elements are now known to be resident dsRNA viruses. Nuclear genes control the sensitivity of cells to the three toxins (Puhalla 1968; Koltin and Day 1975, 1976). No single resistance allele confers immunity to all three toxins. There is some evidence for virally encoded resistance to the KP1 toxin (Peery et al. 1982), but not the KP6 toxin (Finkler et al. 1992). In general, immunity to the toxins does not seem to reside in the viral genome, a situation quite different from that for the k1 and k2 killer toxins of *Saccharomyces cerevisiae*, in which the preprotoxin confers immunity to the processed, secreted toxin (Bussey et al. 1982; Lolle et al. 1984; Boone et al. 1986; Hanes et al. 1986; Sturley et al. 1986; Douglas et al. 1988; Meskauskas and Citavicius 1992; Sesti et al. 2001).

The three *U. maydis* killer toxins are all encoded by M dsRNAs that appear to be satellite viruses, dependent on the packaging and replicase activities encoded by larger H dsRNAs (Tao et al. 1990; Park et al. 1994, 1996a; Kang et al. 2001). All are synthesized as prepropolypeptides with typical signal sequences cleaved during secretion. KP6 is composed of two polypeptides, not linked by intermolecular disulfides, derived from the same preprotoxin by KEX2p and KEX1p cleavages (Tao et al. 1990), while the KP1 and KP4 toxins are single polypeptides. However, the KP1 toxin is derived from a prepropolypeptide by KEX2p and KEX1p cleavage, while the KP4 toxin is processed from its precursor solely by signal sequence cleavage (Park et al. 1994, 1996a). All have multiple cysteine residues, which are known to be present in disulfides in KP4 and KP6 alpha, whose structures have been determined by x-ray crystallography (Gu et al. 1995; Li et al. 1999). These are two of only four killer toxin polypeptides whose structures have been determined, the others being the SMK (kk1) toxin from *Pichia* (Suzuki and Nikkuni 1994; Kashiwagi et al. 1997; Suzuki et al. 2000) and the

WmKT (HMK) toxin from Williopsis saturnus var. mrakii (*Hansenula mrakii*) (Antuch et al. 1996).

2 KP1 toxin

2.1 Synthesis

Of the three *U. maydis* killer toxins, least is known about KP1. A combination of genetics and biochemistry identified the toxin-encoding RNA as P1M2 (Koltin et al. 1980; Park et al. 1996a). The KP1 toxin is synthesized as a preprotoxin of 291 amino acids with a predicted signal sequence cleavage after Ala24 (von Heijne 1986, 1991; Park et al. 1996a). Further KEX2p cleavages are predicted at positions 142 and 173, yielding, after predicted KEX1p removal of terminal arginine residues (see KP6 below), predicted polypeptides of about 12.9 kDa (alpha, 117 amino acids) and 13.4 kDa (beta, 118 amino acids). The latter was shown to be the sole component of the toxin by purification using size exclusion chromatography in the presence of 7M urea (which does not denature the toxin) and sequence analysis of two internal peptides. Although it has two possible N-linked glycosylation sites, the KP1 toxin is not glycosylated, as judged by the estimated molecular weight of the purified protein. This would be consistent with the lack of glycosylation of KP6 alpha (as determined by mass spectrometry), which also has one possible site for N-linked glycosylation. The primary structure of the KP1 prepropolypeptide is similar to that of KP6 (Tao et al. 1990), or of *S. cerevisiae* k1 (Bostian et al. 1984; Skipper et al. 1984), or of the *Pichia* SMK toxin (Suzuki and Nikkuni 1994), with two polypeptides separated by a gamma spacer region removed by KEX2p processing (Park et al. 1996a). However, in KP1, in contrast to these three other killer toxins, only one of the resultant mature peptides is a component of the toxin.

2.2 Structure, mechanism of action, and heterologous expression

The KP1 toxin (P1M2 beta) has six cysteines, and its stability in 7M urea suggests that it has several disulfides, like those in KP6 beta or KP4 (see below). Unlike the KP4 and KP6 toxins, the KP1 toxin has not been successfully synthesized from cDNA expression vectors in heterologous (or homologous) cells. The lack of expression of KP1 from cDNA expression vectors is a result of degradation of the mRNA prior to export from the nucleus, and involves either the absence of an appropriate export signal in the message or the presence of multiple sequences that target the message for degradation (Park et al. 1996a). Since the viral message is normally produced in the cytoplasm, this is not entirely surprising. In addition, the toxin is made in small quantities and is susceptible to degradation in the growth medium prior to purification (like the *S. cerevisiae* k1 toxin), so it has not been ex-

tensively characterized. Consequently, nothing is known of its mode of action, cellular receptor, or structure.

3 KP4 toxin

3.1 Synthesis

The KP4 toxin, unlike all other known killer toxins, is the result of a single processing event during secretion, the removal of the signal sequence. It is synthesized as a prepropolypeptide of 127 amino acids and cleaved after Ser22 (Park et al. 1996a) as predicted (von Heijne 1986, 1991) and as verified by N-terminal sequence analysis (Ganesa et al. 1991). The KP4 prepropolypeptide does not have a predicted KEX2p site, and mass spectrometry and C-terminal protein sequencing of the purified toxin confirms that it does consist of the predicted 105 amino acids (Park et al. 1994). The toxin is not fully denatured by SDS-PAGE, running with an apparent molecular weight of 8.2 kDa. The toxin is fully denatured in SDS-urea-PAGE, running with an apparent size of 11.3 kDa, close to the size determined by mass spectrometry (11.045 kDa) and to that calculated from its known sequence (11.056 kDa). Some of the unusual stability of the protein may be due to its five intramolecular disulfide bonds, as demonstrated by x-ray crystallography (Gu et al. 1994, 1995).

The lack of KEX2p processing of the KP4 precursor was verified by expression in *S. cerevisiae*. Yeast does make and secrete the KP4 toxin in active form from a cDNA expression vector, and the toxin is made perfectly well in strains lacking the KEX2p enzyme (Park et al. 1994). The mature protein does not have any predicted N-glycosylation sites and has neither N- nor O-linked glycosylation (Park et al. 1994). Interestingly, cDNA expression vectors that successfully express the KP6 toxin do not express the KP4 (or KP1) toxin, even with extensive modification of non-coding 5' and 3' sequences, apparently because of rapid degradation of nuclear transcripts (C.M Park and J. Bruenn, unpublished.). This is not entirely inexplicable, since normally the viral RNA is transcribed and translated in the cytoplasm and never sees the nucleus, as it does in a cDNA expression vector.

3.2 Structure

The structure of the KP4 toxin was determined to 1.9 A resolution, refined to 1.4 A, using a single isomorphous replacement derivative, by x-ray crystallography (Gu et al. 1994, 1995). The toxin is an alpha/beta sandwich protein with a double split beta-alpha-beta motif. The toxin has seven beta strands and three alpha helices. The major structure consists of five antiparallel beta strands, with two antiparallel alpha helices crossing the strands at about a 45 degree angle. From one face of the molecule, the five beta strands are prominent, and from the opposite face the two alpha helices. Remarkably, the structure of KP4 is very similar to that

Fig. 1. Secondary structures (ss) from SMK and KP4 from the PDB files are compared by Clustal W, with a manual realignment of the first beta strand. The SMK peptides are strung together alpha C-terminus to beta N-terminus as though they were synthesized as a single polypeptide (without the intervening gamma segment removed by KEX2p). Secondary structures show about 80% identity, in contrast to less than 15% identity in amino acid sequences. L indicates loop, H alpha helix, and E beta strand. Asterisks indicate secondary structure identities.

of the *Pichia farinosa* kk1 (SMK) toxin, which is composed of two polypeptides held together by non-covalent bonds (Suzuki and Nikkuni 1994; Kashiwagi et al. 1997). The major features of the SMK toxin are the same as those of KP4 and are similarly situated in space. They also occur in the same order in the two polypeptides, if the two are considered a single polypeptide derived from the prepropolypeptide by excision of the gamma sequence between them (which appears to be removed by KEX2p). This is clear from looking at the structures and is verified by manually aligning the two sequences according to secondary structure (Kashiwagi et al. 1997). It is also possible to align the structures using Clustal W (Thompson et al. 1994), as shown in Figure 1. This automated alignment is essentially the same as that done manually, matching the five major beta strands and the two major alpha helices (beta 1 of KP4 with beta 1 of SMK; beta 3 of KP4 with beta 2 of SMK; beta 4 of KP4 with beta 3 of SMK; beta 6 of KP4 with beta 4 of SMK; beta 7 of KP4 with beta 5 of SMK; alpha 1 and alpha 2 of KP4 with alpha 1 of SMK; and alpha 3 of KP4 with alpha 2 of SMK). Alignments based on primary structure fail, because the two toxins have very little sequence similarity. In fact, the secondary structure alignment results in a primary sequence alignment in which fewer than 10% of the residues are identical. The level of significance for primary sequence alignments is usually around 23%.

One major difference between the two toxins is the lack of disulfides in SMK, which has four cysteines, none in disulfides. This may explain some of the chemical differences between the toxins; the KP4 toxin is relatively stable and effective at neutral pH and low salt (Gu et al. 1995, 2001, 2002) , while the SMK toxin is stable and effective only at low pH and high salt (Suzuki and Nikkuni 1994; Kashiwagi et al. 1997; Suzuki et al. 1997). The SMK toxin is active against *S. cerevisiae* (Suzuki et al. 2000), while the KP4 toxin is not (Park et al. 1994). The mechanism of action of the SMK toxin is poorly understood, while the KP4 toxin appears to be active against calcium channels.

3.3 Mechanism of action

There are several lines of evidence supporting the hypothesis that KP4 blocks calcium channels. First, calcium abrogates the effects of KP4 (Gage et al. 2001). This is also true of the SMK toxin (Suzuki and Shimma 1999). Second, KP4 blocks calcium uptake (Gage et al. 2001, 2002). Third, KP4 blocks the function of calcium channels in cultured mammalian cells as measured by electrophysiology (Gage et al. 2002). Fourth, KP4 eliminates the spike in calcium concentration that is the result of some perturbations of Neurospora cells (Nelson et al. in press).

The discovery of calcium channels in fungi has been facilitated by the sequencing of the *S. cerevisiae* genome, which has one copy of a gene (CCH1) coding for the alpha1 subunit of a typical calcium channel. Genetics has implicated this gene and an additional gene, MID1, in regulation of calcium import in yeast (Iida et al. 1994; Fischer et al. 1997; Paidhungat and Garrett 1997; Locke et al. 2000). MID1 appears to function alone as a stretch-activated cation channel (Kanzaki et al. 1999). Now that a number of fungal genomes are sequenced, it is clear that CCH1 occurs in every fungal genome (NP_011733.1in *Saccharomyces cerevisiae*, EAA67357.1 in *Gibberella zeae*, EAA34496.1 in *Neurospora crassa*, EAA54852.1 in *Magnaporthe grisea*, EAA66286.1 in *Aspergillus nidulans*, and NP_593894.1 in *Schizosaccharomyces pombe*). MID1 is also present in each fungal genome (NP_014108.1 in *Saccharomyces cerevisiae*, NP_592865.1 in *Schizosaccharomyces pombe*, EAA77435.1 in *Gibberella zeae*, EAA50242.1 in *Magnaporthe grisea*, EAA60130.1 in *Aspergillus nidulans*, and EAA31782.1 in *Neurospora crassa*).

Why do fungi need calcium channels? Calcium transport is important for growth regulation and mating in *S. cerevisiae* (Iida et al. 1990; Jackson and Heath 1993; Hartley et al. 1996; Paidhungat and Garrett 1997) and presumably has similar roles in other fungi. The phenotype caused by KP4, which is not really killing, but rather growth inhibition (which can be released by calcium washes after exposure to toxin) is consistent with blocking of a calcium channel, since calcium influx during certain periods of the cell cycle is necessary for progression through the cycle (Gage et al. 2001). Testing of a number of cations shows that only calcium alleviates the effect of KP4 toxin on *U. maydis*; KP4 also inhibits the uptake of radioactive calcium from the medium (Gage et al. 2001). KP4 is effective in blocking voltage-gated calcium channels in cultured mammalian cells, and the

block is not detectable in the presence of L-type channel inhibitors (nimodipine), implying that KP4 is specific for L-type calcium channels (Gu et al. 1995; Gage et al. 2001, 2002). L-type calcium channels are long-lasting voltage gated channels. These effects of KP4 on cultured cells occur at approximately the same concentration as that effective in inhibition of growth of *U. maydis*. Additionally, the blockage of calcium channels (usually measured in the presence of barium ions) is abolished by low concentrations of calcium, consistent with the abrogation of the effects of KP4 on *U. maydis* by extracellular calcium (Gage et al. 2002).

A comparison of the structure of KP4 with that of AaHII, a scorpion toxin with some structural similarities (Gu et al. 1995), lead to the prediction that KP4 residue K42 would be critical for function. Alteration of KP4 at K42 (but not at R68) nearly eliminates toxic activity toward *U. maydis* (Gage et al. 2001) and also eliminates the effect of KP4 on calcium channels in mammalian cells (Gage et al. 2002). A comparison of the structure of KP4 with that of certain plant defensins also predicts the importance in these small, secreted proteins of a similarly located lysine, which when present confers calcium channel blocking activity, but when absent obviates this activity (Tom Smith, personal comm.). Finally, KP4 blocks the spikes in internal calcium concentration resulting from high external calcium or hypo-osmotic shock in *Neurospora* (Nelson et al. in press). In summary, assuming that all fungi depend on calcium transport for progression through the cell cycle, that all fungi have similar calcium channels (which appears to be the case), and that these channels are similar to L-type mammalian calcium channels, the effects of KP4 are consistent with L-type calcium channel inhibition.

There are clearly subtleties in the mode of action of killer toxins that we do not understand. For instance, KP4 works on mammalian and *Neurospora* calcium channels, but not on *S. cerevisiae* channels. Even though the SMK and KP4 toxins are very similar in structure, the SMK toxin works on *S. cerevisiae*, but KP4 does not. They may actually have different targets, since the SMK toxin is also lacking a lysine (or any basic residue) in the position equivalent to K42 of KP4 (Fig. 1), and *S. cerevisiae* requires a P-type ATPase (SPF1p) for sensitivity to SMK (Suzuki and Shimma 1999). SMK does affect calcium levels in *S. cerevisiae*, but it is unclear what relationship SPF1p might have to calcium channels.

Unlike the KP1 and KP6 toxins, which currently have no known homologs, the KP4 toxin has both structural (SMK) and sequence homologs. A search of the databases shows that a predicted protein from *Gibberella zeae*, accession number XP_380236.1, has 41% sequence identity with KP4, much greater than would be expected by chance. This protein (GIZT for convenience) is encoded by a chromosomal gene (like SMK1). The amino acid sequence alignment generated by Clustal W is shown in Figure 2. GIZT has a predicted signal sequence, with a predicted cleavage site (A16) that creates an amino terminus with ten amino acids identical to those from the amino terminus of KP4 (von Heijne 1986, 1991). In addition, GIZT preserves five of the ten cysteines in KP4. Four of the conserved cysteines participate in disulfide bonds in KP4, so that GIZT probably preserves the disulfides equivalent to C5-C78 and C11-C81 of KP4. GIZT also has a match for C105, and a slight adjustment in the alignment would preserve C44, so that

GIZT may also have the equivalent of the C44-C105 disulfide. The boxed residues in Figure 2 show the conservation of cysteines.

An alignment of the predicted secondary structure of GIZT with KP4 is also shown in Figure 2, demonstrating an excellent match (as good as that of the known secondary structures of SMK and KP4; Fig. 1). Alignment by secondary structure is similar to that by amino acid sequence. A prediction from this alignment is that GIZT should recognize the same receptor as the KP4 toxin, although it might not have the same effects. For instance, the critical lysine residue (K42) of KP4 is missing in GIZT (Fig. 2). Since, as shown in Figure 1, SMK has no lysine homologous to K42 in KP4 (Kashiwagi et al. 1997), it will be interesting to see if GIZT does act as a killer toxin, and if so, whether its mode of action is more like that of SMK or of KP4.

In short, there are several good reasons for thinking that KP4 blocks calcium channels and that that is its method of inhibiting growth of *U. maydis*. What would be ultimately satisfying would be a demonstrated physical and electrochemical interaction between KP4 and a *U. maydis* calcium channel and a demonstration that sequence alterations in a calcium channel polypeptide(s) can confer KP4 resistance.

3.4 Heterologous expression

Ustilago species are pathogens on a number of important plants, including maize, wheat, oats, and barley (Koltin and Day 1975). Expression of the *Ustilago* killer toxins in crop plants could be a useful means of conferring resistance to infection. The killer toxins are not toxic to mammals when ingested and are highly specific for the ustilaginales. The vast majority of strains in the wild are sensitive to all three of the killer toxins (Day 1981). KP4, because of its ease of expression in heterologous systems, has become the poster child for this strategy.

KP4 is, as pointed out, readily expressed in *S. cerevisiae*, and it turns out, in plants. KP4 is expressed at a high level in tobacco plants transgenic for an appropriate cDNA expression vector, and the secreted KP4 is a toxin identical in size and properties to authentic KP4 (Park et al. 1996b). So much toxin is made that is easily detectable on the surface of leaves, which is where it would be desirable to confer resistance to *Ustilago* infection. In fact, transgenic wheat plants expressing the KP4 toxin exhibit increased resistance to *Ustilago* infection (Clausen et al. 2000). Similar experiments show some increased resistance to *Ustilago* infection in maize plants expressing KP4 (J. Duvick, S. Gold, J. Bruenn, unpublished).

Fig. 2. The known (KP4) and predicted (GIZT) mature polypeptides are aligned by Clustal W (Thompson et al. 1994), showing 41% identity in the upper panel. In the lower panel, the known (KP4) and predicted (GIZT) secondary structures (ss) are aligned by Clustal W. This alignment is slightly displaced from that of the upper panel. L indicates loop, H alpha helix, and E beta strand. The secondary structures are about 75% identical. In both panels, identities are indicated by asterisks, similarities by dots. The conserved cysteines are boxed and KP4 residue numbers indicated.

4 KP6 toxin

4.1 Synthesis

The KP6 toxin is encoded by P6M2 (1234 bp) and synthesized as a prepropolypeptide of 219 amino acids (Tao et al. 1990). Signal peptidase cleaves after alanine 19 and KEX2p cleaves after amino acids 27, 107, and 138. The C-terminal arginine is removed from KP6 alpha, leaving a KP6 alpha of 79 amino acids and a KP6 beta of 81 amino acids, as determined by N-terminal sequence analysis and by mass spectroscopy (Tao et al. 1990; Kinal et al. 1995) (J. Bruenn, unpublished). KP6 alpha has one possible N-linked glycosylation site, which is used in *S. cerevisiae*, but not in *Ustilago* or in tobacco (Tao et al. 1990; Kinal et al. 1995). In addition, in *S. cerevisiae*, KEX1p removes the terminal KR from KEX2p products (Toh-e et al. 1978; Cooper and Bussey 1989; Thomas et al. 1990; Jones 1991; Dmochowska et al. l987; Eisfeld et al. 2000), but processing in *Ustilago* removes only the terminal arginine, as confirmed by structural analysis (Li et al. 1999). The

structure of the prepropolypeptide and its processing is similar to that of the *S. cerevisiae* k1 preprotoxin, except that the k1 preprotoxin confers immunity to the mature toxin (Boone et al. 1986; Hanes et al. 1986; Sturley et al. 1986; Zhu et al. 1993), while the KP6 preprotoxin does not confer resistance to its toxin (Finkler et al. 1992). KP6 alpha has eight cysteines, all in intramolecular disulfides (Li et al. 1999), while KP6 beta has six cysteines. The two toxin components appear to exist independently in solution, since they can be purified as monomers (Peery et al. 1987; Steinlauf et al. 1988). This is considerably different from the *S. cerevisiae* k1 toxin in which alpha and beta are joined by disulfides (Palfree and Bussey 1979; Bussey et al. 1983)

The processing of the preprotoxin from two mutants, NK3 and NK13, demonstrates that KEX2p is responsive to structural cues (Tao et al. 1993), as well as to the KR or PR sequence at the processing site (Park et al. 1994). There did appear to be a requirement for a hydrophobic residue two amino acids prior to the KR or PR sequence (Park et al. 1994), but this generalization does not hold for SMK (Suzuki and Nikkuni 1994). Consequently, it is not surprising to find that residues outside the cleavage site influence KEX2p function. In NK3, a C78R change in the alpha sequence prevents maturation of alpha, but permits maturation of beta. In NK13, a T164P change in beta permits maturation of alpha, but precludes maturation of beta. Both of these changes are outside the KEX2p recognition sequences (27, 107, 138), but both would be likely to cause drastic changes in the structure of the preprotoxin; C78R removing one of the disulfides that stabilizes the structure, and T164P introducing an adventitious turn in a region that is predicted to be an alpha helix. Both KP6 alpha and KP6 beta are necessary for toxin function, although, as demonstrated by these mutants, they may be synthesized separately (Peery et al. 1987). It is unclear whether the two toxin polypeptides associate in solution or only at the site of action. However, the ability to express the preprotoxin from *Ustilago* expression vectors with P6M2 cDNA (Kinal et al. 1991, 1993) made it possible to show that sensitive cells (but not resistant cells) are killed by the expression of KP6 beta, but not by the expression of KP6 alpha (Finkler et al. 1992). It is not clear why the expression of KP6 beta in sensitive cells is lethal, while both toxin polypeptides are necessary to kill sensitive cells when applied externally. Expression of the SMK toxin in *S. cerevisiae* is also lethal, but only when overall structure and signal sequence are preserved (Suzuki et al. 2000). In general, the relationship between the lethality of killer toxins expressed in sensitive cells and supplied externally is unclear. The mechanisms may be somewhat different.

4.2 Structure

The structure of the KP6 alpha polypeptide has been determined by X-ray crystallography at 1.8 A resolution (Li et al. 1999). The structure is an alpha/beta sandwich motif consisting of four beta strands in an antiparallel beta sheet, a pair of antiparallel alpha helices that lie at an angle of about 20 degrees to the beta strands on one side of the sheet, a beta strand along one edge of the sheet, and a short N-

terminal alpha helix on the other side of the beta sheet. All eight cysteines are in disulfides, with one disulfide connecting the two longest beta strands. KP6 alpha does not have the same folding connections as SMK and KP4, although all have alpha/beta sandwich motifs.

Interestingly, KP6 alpha is present as a funnel-shaped trimer in crystals. The trimer is stabilized by intermolecular salt bridges and hydrogen bonds and two trimers associate to form a hexamer. The result is an hourglass-like structure in which the two trimers are related by a two-fold axis of symmetry and are joined by a narrow passage (4.2 A in diameter) filled with highly ordered water molecules. The distance between the two planes at the tops of the trimers is 34 A. This is suggestive of an ion channel, a model made more plausible by the presence of three phenyl rings (made by the GFG sequence in the amino terminal alpha helix of the monomer) at the mouth of the funnel. The resultant structure is quite similar to that of the bacterial potassium channel, which adopts a funnel shape with four monomers (Doyle et al. 1998). In this model, the role of KP6 beta would be to shield the hydrophilic surface of alpha and permit its insertion into cellular membranes (Li et al. 1999). There is little structural information on KP6 beta. However, KP6 beta appears to have a secondary structure dominated by beta sheet, and its tertiary structure is partially disrupted by an increase in temperature, but restored by interaction with lipids, consistent with insertion into membranes (Balasubramanian et al. 2000).

4.3 Mechanism of action

There is no direct evidence on the mechanism of action of the KP6 toxin. As described above, structural clues to its function hint at the formation of an adventitious ion channel, possibly causing the leakage of K^+ or $NH4^+$ from cells (Li et al. 1999). A similar mechanism has been proposed for the *S. cerevisiae* k1 toxin, which has been reported to form K^+ channels in sensitive cells (Martinac et al. 1990). It is suggestive that the k1 toxin can, like the KP6 toxin, form multimers (Martinac et al. 1990). However, there are also reports that k1 interacts with preexisting potassium channels (Sesti et al. 2001). These ambiguities and the lack of definitive evidence on the mechanism of action of the KP6 toxin (and even the KP4 toxin) are the result of the technological difficulties inherent in doing electrophysiology on fungi. However, there clearly are differences in the mode of action of the k1 and KP6 toxins, since the k1 toxin works on spheroplasts of otherwise insensitive cells (Zhu and Bussey 1989; Martinac et al. 1990), while the KP6 toxin does not work on spheroplasts at all (Steinlauf et al. 1988). The initial site of action of the k1 toxin is known to be a cell wall receptor (Bussey et al. 1979; Hutchins and Bussey 1983; Boone et al. 1990), so this could be a quantitative rather than qualitative difference. The KP6 toxin may need to be concentrated at the membrane target by prior interaction with cell wall components, while the k1 toxin need not. The KP6 toxin Interaction with the cell wall could also be necessary for KP6 alpha and beta interaction, something not necessary with the k1 toxin, in which the two polypeptides are joined by disulfide bonds. The resistance

of mutants of *S. cerevisiae* lacking normal (1-6)-β-D-glucan synthesis (Hutchins and Bussey 1983; Boone et al. 1990; Meaden et al. 1990) may reflect a requirement for interaction with the cell wall in order to gain access to the cell membrane. There are other killer toxins that also appear to affect potassium channels (Santos and Marquina 2004), while at least one other yeast killer toxin, k28, which is also an alpha/beta heterodimer, acts by an entirely different mechanism (Eisfeld et al. 2000).

4.4 Heterologous expression

Of the three *Ustilago* toxins, only KP6 has been expressed from cDNA clones in *Ustilago*, yeast, and plants. KP1 has not been expressed in any system, and KP4 has been expressed in yeast and plants, but not in *Ustilago*. The ability to express KP6 in heterologous systems is a little surprising, because the processing of the toxin varies in each system, and because a gene for KEX2p has not been demonstrated in plant systems. KP6 expression in yeast results in a toxin that is much less active, on a mass basis, than that synthesized in *Ustilago*. The resultant KP6 alpha is N-glycosylated, presumably at the one predicted site at residue 100, while KP6 alpha in *Ustilago* is not glycosylated (Tao et al. 1990). Although there is no direct evidence, it is assumed that KP6 alpha made in yeast is processed like other KEX2p products, so that it is missing the terminal KR, while mass spectroscopy and X-ray crystallography have demonstrated that KP6 alpha made in *Ustilago* has a C-terminal K and has had only the C-terminal R removed (Li et al. 1999) (J. Bruenn, unpublished). Either the adventitious glycosylation or the predicted incorrect C-terminus of KP6 alpha might explain the decreased activity of KP6 produced in yeast. In contrast, the KP6 toxin produced in tobacco has about the same activity per unit mass as the homologous toxin, and both alpha and beta are processed correctly (Kinal et al. 1995). This implies that plants have a KEX2p activity (and a KEX1p activity similar to that in *Ustilago*). However, none of the sequenced plant genomes shows any signs of a KEX2p homolog, although they have numerous other subtilisin-like proteases. Processing in tobacco is much less efficient than that in *Ustilago* as well, since despite an abundance of mRNA, the plants produce very little toxin. The KEX2p systems in both yeast and plants differ from that in *Ustilago*, since the NK3 and NK13 preprotoxins are processed in *Ustilago* (producing one of the mature peptides in each), but are not processed at all in plants or yeast (Kinal et al. 1995). The glycosylation enzymes also differ, since *Ustilago* and tobacco produce an unglycosylated alpha, but yeast produces a glycosylated species.

5 Summary

Small secreted protein toxins are ubiquitous in nature, and fungi produce their share of such proteins, which have the pleasant name killer toxins. *Ustilago* has

three known killer toxins, KP1, KP4, and KP6. These vary in mode of synthesis, sequence, structure, and probably mechanism of action. KP4 has both structural and sequence homologs among other fungal proteins, but so far neither KP1 nor KP6 has a homolog. The KP4 toxin appears to be a calcium channel blocker, and the KP6 toxin may affect ion channels as well. Interest in these toxins is motivated by a desire to understand the (apparently) universal proteinase processing of secreted proteins (including all serum proteins in mammals) and the various means of blocking existing ion channels and making new ones, and by the promise of designing transgenic crop plants resistant to fungal pathogens.

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Zygocin – a monomeric protein toxin secreted by virus-infected *Zygosaccharomyces bailii*

Frank Weiler and Manfred J. Schmitt

Abstract

Zygocin, a protein toxin secreted by virus-infected strains of the osmotolerant spoilage yeast *Zygosaccharomyces bailii* kills a broad spectrum of yeast and fungi of medical and agricultural relevance. Its mode of action requires initial binding to a toxin receptor in the fungal cell wall, but finally unfolds at the level of the cytoplasmic membrane. In the infected host, toxin expression is mediated by a 2.1 kb viral double-stranded (ds)RNA genome encoding a prepro-zygocin precursor, which is further processed to the biologically active toxin (zygocin) by endopeptidase Kex2p cleavage within the yeast secretory pathway. cDNA sequence of the toxin-coding dsRNA genome shows no significant homology to other protein toxins, or to any protein known so far. *In vivo* toxicity involves membrane permeabilization and critically depends on an energized susceptible host cell. Possible mechanisms of toxin resistance involve chromosomal host genes whose gene products affect plasma membrane lipid composition. Functional and structural properties of zygocin resemble those of antimicrobial peptides from higher eukaryotes and indicate a potential of this unique virus toxin as novel antimycotic drug.

1 Zygocin is genetically encoded by a dsRNA "killer" virus

Virus like particles (VLPs) are a common characteristic of virtually all yeast genera and can also be identified in many different fungi (Young and Yagiu 1978; Martinez-Espinoza et al. 2002). The majority of these viruses spread horizontally and contain linear double-stranded RNA (dsRNA) as viral genome. In yeast, one of the best-characterized dsRNA mycovirus is the *S. cerevisiae* helper virus *Sc*V-LA (Wickner 1996). Its L_A-dsRNA genome encodes a major capsid protein (Gag) and a RNA-dependent RNA polymerase (Pol) which is, *in vivo*, expressed as a Gag-Pol fusion by a –1 ribosomal frameshift during translation of the plus-strand RNA transcript (Fujimura et al. 1990; Wickner 1992; Cheng et al. 1994; Schmitt and Breinig 2002). Homologous to *Sc*V-LA in baker's yeast, occurrence of a *Z. bailii* helper virus (*Zb*V-L) likewise is not associated with a detectable phenotype, but necessary for proper propagation and replication of its toxin-coding satellite

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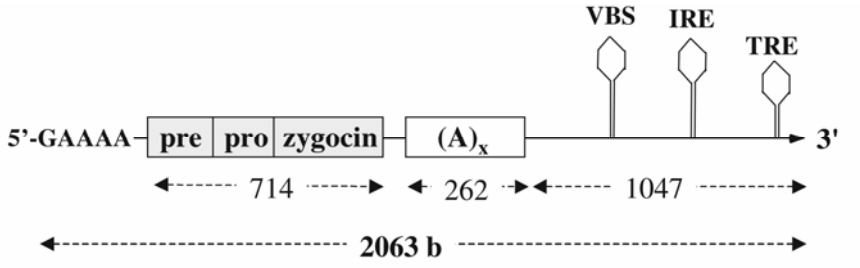


Fig. 1. Schematic illustration of the zygocin encoding ZbV-M(+)ssRNA. The preprozygocin open reading frame (ORF) is situated at the 5'-end, immediately downstream of the indicated 5'-GAAAA sequence that is highly conserved among yeast and fungal dsRNA viruses. The intramolecular poly A-rich stretch is shown [$A_{(X)}$] and the potential *cis*-acting 3'-sequences required for RNA packaging, transcription and replication are indicated (VBS, viral binding site; IRE, internal replication enhancer; TRE, 3'-terminal recognition element). Numbers indicate the size [bp] of the full length M_{Zb}(+)ssRNA virus transcript and its distinct elements.

"killer" virus *Zb*V-M whose 2.1 kb M-dsRNA genome encodes the preprozygocin precursor (Radler et al. 1993; Weiler et al. 2002). Besides the toxin-coding open reading frame, the viral M-dsRNA of *Z. bailii* comprises structural elements that are essential for viral RNA replication and packaging (Fig. 1). These elements are located near or at the 3'-terminus of the codogenic (+)ssRNA and include a viral binding site (VBS), an internal replication enhancer (IRE), and a terminal recognition element (TRE) that - according to their essential function within the viral life cycle - can be found in almost all yeast and fungal satellite viruses (Fig. 2). In addition to the 3'-sequences, a highly conserved 5'-GAAAA motif is present at the immediate 5' terminus of the viral plus-strand, which is presumed to be responsible for transcription initiation from the viral dsRNA template; besides that, other consensus sequence elements cannot be found in the 5'-region of M-dsRNA genomes (Ribas and Wickner 1992; Weiler et al. 2002). Similarity of these *cis*-active structural elements at the 5'- and 3'-flanking regions in mycoviral transcripts, however, does not extend to the level of the viral preprotoxins (pptox), although pptox processing to the biologically active protein toxin usually follows the same rules and principles in different killer yeasts, always being homologous and analogous to prohormone conversion in mammalian cells (Schmitt and Breinig 2002).

In addition to the *Zb*V-L helper virus and its toxin-coding *Zb*V-M satellite, killer strains of *Z. bailii* contain a third dsRNA genome, which is unique to *Zygosaccharomyces* and, therefore, has been designated *Zb*V-Z for *Z. bailii* virus Z (Schmitt and Neuhausen 1994). Although it has been speculated that the 2.8 kb Z-dsRNA genome of *Zb*V-Z might be involved in the expression of toxin immunity *in vivo*, i.e. in self-protection of the infected host against the virus toxin, transfection experiments with isolated *Z. bailii* VLPs resulted in a suicidal phenotype in

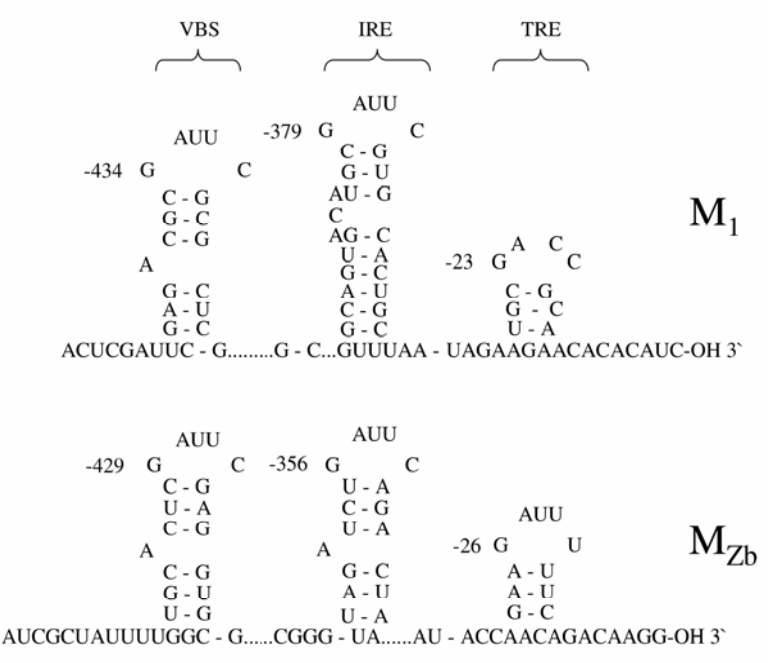


Fig. 2. Comparison of *cis*-active structural elements near the 3'-termini in the toxin-coding transcripts of yeast killer viruses *Zb*V-M and *Sc*V-M1. Data for the *S. cerevisiae* killer virus *Sc*V-M1 were taken from Hannig et al. (1984). [VBS, viral binding site; IRE, internal replication enhancer; TRE, 3'-terminal recognition element). Numbers are shown for distance from the 3'-terminus of each M(+)ssRNA.

S. cerevisiae transfectants (Neuhausen and Schmitt 1996). Thus, the precise role of *Zb*V-Z in killer phenotype expression and toxin immunity is still unclear.

2 Zygocin is synthesized as an inactive precursor

The ability of yeast to secrete antimycotic protein toxins is wide-spread among various genera (Gunge et al. 1981; Park et al. 1994; Hodgson et al. 1995; Schmitt et al. 1997; Schmitt and Breinig 2002). In most cases, the protein toxins are expressed as a pptox precursor and the corresponding toxin gene can either be located on chromosomal DNA, on linear dsDNA plasmids or can be associated with encapsidated viral double-stranded RNA. After receptor-mediated toxin binding to a sensitive cell, toxicity is mainly conferred by plasma membrane damage, G1 or S phase cell cycle arrest and/or by a rapid inhibition of nuclear DNA synthesis (Butler et al. 1991; Martinac et al. 1990; Schmitt et al. 1989, 1996). Zygocin as a virally encoded protein toxin produced and secreted by *Zb*V-M-infected strains of

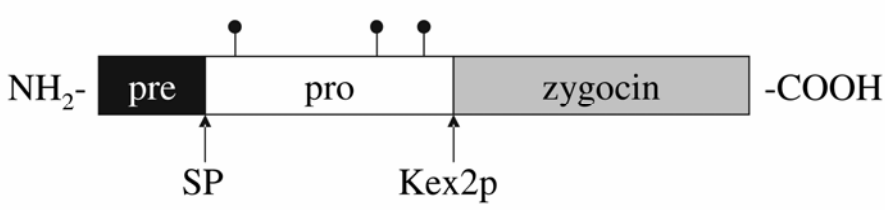


Fig. 3. Structure of the prepro-zygocin toxin precursor and its processing. Cleavage sites of the signal peptidase (SP) and Kex2p endopeptidase are indicated. Potential N-glycosyation sites are highlighted by black dots.

the osmotolerant spoilage yeast *Z. bailii* has originally been discovered in 1993 (Radler et al. 1993). The single open reading frame of the viral M_{Zb}-dsRNA genome encodes a 239 amino acid prepro-zygocin, representing the unprocessed zygocin precursor (Weiler et al. 2002). During secretion, the N-terminal signal peptide of the toxin precursor is removed by signal peptidase cleavage in the lumen of the endoplasmic reticulum (ER). The pro-region is N-glycosylated and in a late Golgi compartment, Kex2p endopeptidase cleavage removes the N-glycosylated pro-sequence, finally releasing the mature virus toxin (Fig. 3). MALDI-TOF analysis further indicated that the mature 99 amino acid protein toxin is free of any posttranslational modification (Weiler and Schmitt 2003). Synthesis as preprotoxin and subdivision of the toxin precursor into three distinct domains (pre-protoxin) is a common feature in many viral protein toxins, and in most cases Kex2p endopeptidase cleavage has been shown to be an essential step in toxin maturation and secretion (Schmitt and Breinig 2002). In contrast to the most intensively studied virus toxins of *S. cerevisiae* (K1, K28) and *Ustilago maydis* (KP6), the *Z. bailii* toxin is not a family member of microbial A/B toxins but rather resembles a monomeric, non-glycosylated protein toxin (Bostian et al. 1984; Schmitt and Tipper 1995; Tao et al. 1990; Weiler et al. 2002). Prepro-zygocin expression in the zygocin-resistant yeast *Schizosaccharomyces pombe* results in the secretion of a correctly processed and biologically active toxin, whereas transfection of zygocin encoding *Zb*V-M killer viruses into an *S. cerevisiae* non-killer results in a suicidal phenotype in which VLP transfectants are killed by their own toxin when cultivated under conditions of maximal zygocin activity (Schmitt and Neuhausen 1994). However, successful secretion of a correctly processed toxin demonstrated that the viral prepro-zygocin leader sequence is fully functional in fission yeast and, furthermore, suitable to drive efficient secretion of foreign proteins not only in baker's and fission yeast but also in *Pichia pastoris* and *Candida glabrata* (Eiden-Plach et al. 2004). Interestingly and in contrast to *S. cerevisiae* killer strains, there is no indication of a dsRNA encoded immunity component in *Z. bailii* killers, and it is not yet clear how *Zb*V-M infected cells protect themselves against their own toxin. In fact, toxin immunity in *Z. bailii* might not even be required since zygocin producing wild type killers are naturally toxin resistant, while the majority of all tested yeast and fungi, including *Zb*V-M transfectants of *S. cerevisiae*, are highly sensitive against the toxin (Weiler and Schmitt 2003). The reason for this intraspecific difference is not known, but it is not reflected by differ-

ences in toxin binding to the yeast cell surface since zygocin cell wall binding is not different in a toxin-secreting killer strain and a sensitive non-killer.

3 Zygocin toxicity involves disruption of cellular integrity

Susceptible yeast and fungal cells are killed in a two-step receptor-mediated fashion. In the initial step, the toxin binds to a primary receptor at the outer yeast cell surface, which has been identified as a high molecular weight cell wall mannoprotein (Radler et al. 1993). Natural binding affinity of zygocin to its cell wall mannoprotein receptor was also successfully used for receptor-mediated affinity purification of zygocin from cell-free culture supernatants of toxin-secreting killer strains (Weiler and Schmitt 2003). After toxin binding to the cell wall, zygocin interacts with the cytoplasmic membrane where the lethal effect takes place. An essential prerequisite for zygocin toxicity is an energized cell and, therefore, glucose-depleted cells as well as cells pretreated with an uncoupler such as NaN_3 become zygocin resistant, while control cells are rapidly killed within minutes. Furthermore, the fluorophoric compound propidium iodide (PI) penetrates yeast cells shortly after zygocin addition, indicating that zygocin is negatively affecting plasma membrane permeability. In comparison to K1, a well-known ionophoric α/β virus toxin of *S. cerevisiae*, kinetics of zygocin-mediated PI uptake indicate a much faster membrane permeabilizing effect of zygocin (Martinac et al. 1990; Weiler and Schmitt 2003). This is in good accordance with the observation that equivalent molar amounts of K1 require a significantly prolonged time span to achieve a comparable reduction in cell viability (Weiler and Schmitt 2003).

The ionophoric mode of action of zygocin is reinforced by *in silico* sequence analysis, comprising a stretch of potential α-helical conformation that forms an amphipathic structure, which is characteristic for many membrane-disturbing peptides such as alamethicin, melittin, and dermaseptin (Fig. 4A; La Rocca et al. 1999). In addition, this feature is accompanied by a highly predictable transmembrane helix at the C-terminus of zygocin, which would favour a membrane permeabilizing potential not by activating native ion channels but by establishing pores by itself after toxin oligomerization (Fig. 4B). So it might be postulated that the hydrophobic part of the amphipathic α-helix is responsible for toxin binding to the yeast cell surface. Initial toxin adsorption would only be limited by the toxin's ability to overcome the cell wall barrier and/or by additional physicochemical factors that affect zygocin's hydrophilic/hydrophobic transition from the aqueous medium to the cytoplasmic membrane; thus, the postulated model of zygocin action resembles the mechanism of toxicity in certain α-defensins from human origin (Fig. 5B; Tossi et al. 2000). Although unspecific toxin binding seems to be an essential step, it is not sufficient to initiate cell killing as zygocin producing cells are perfectly capable to bind toxin at the plasma membrane level without showing a zygocin-sensitive phenotype (Schmitt and Neuhausen 1994). In striking similarity and analogy to alamethicin, the toxic effect of zygocin would be mediated by

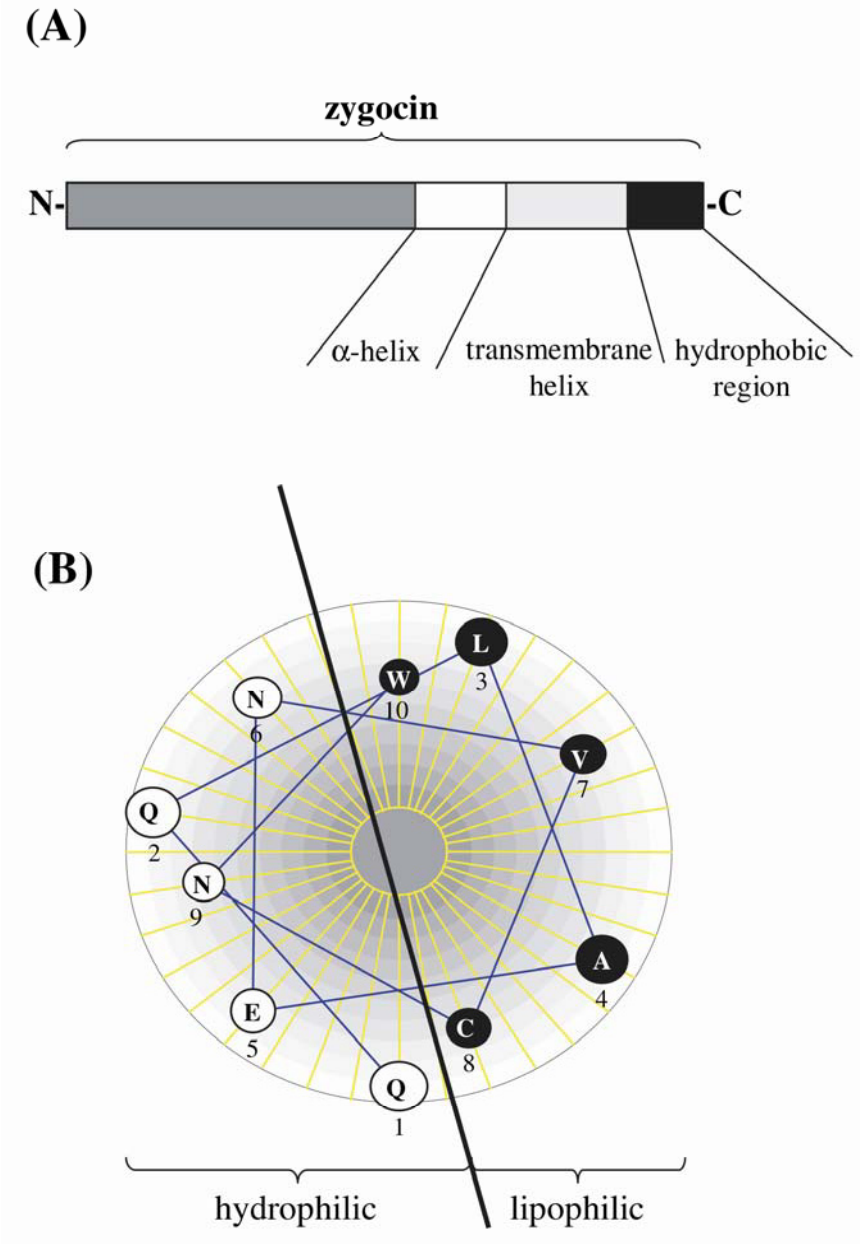


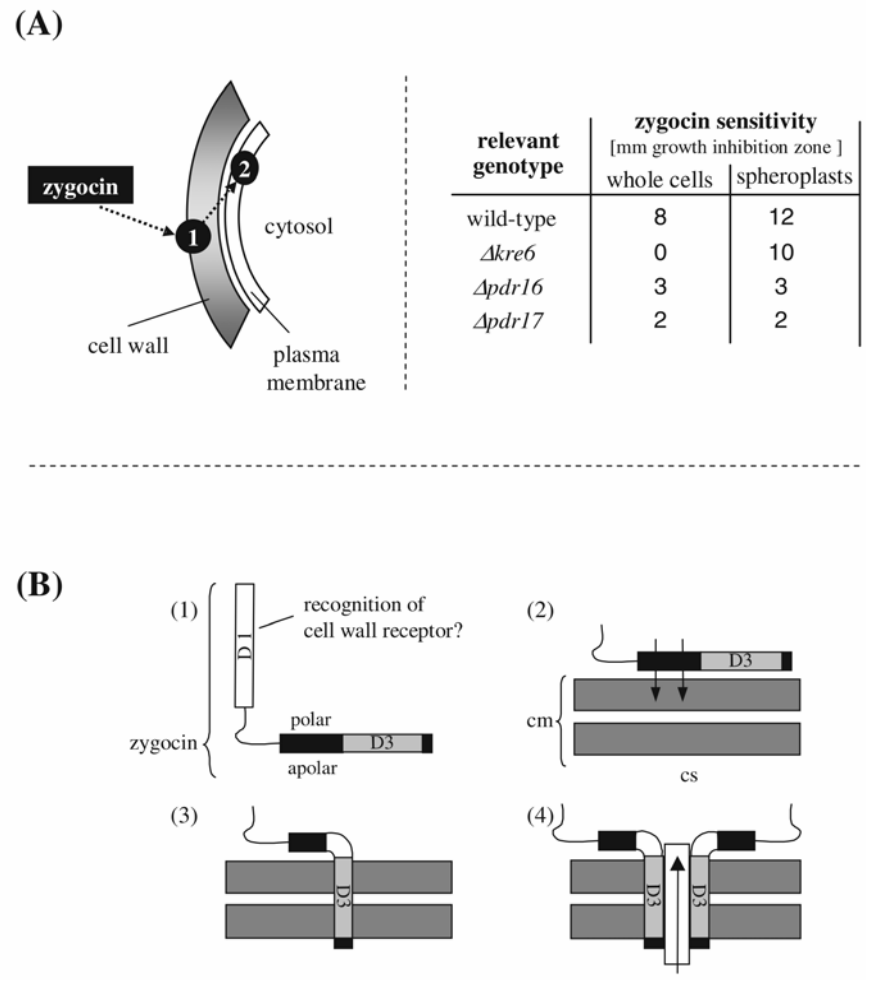
Fig. 4. Predicted secondary structures at the C-terminus of zygocin. **(A)** Potential α-helix has been envisaged employing a "hierarchical neural network" (Qian and Sejnowski 1988), whereas the transmembrane helix was predicted according to Hofmann and Stoffel (1993). **(B)** Helical wheel projection (Schiffer and Edmundson 1967) of the potential α-helix to demonstrate amphipathicity in zygocin.

incorporation of its transmembrane helix into the plasma membrane, a process which is solely driven by the natural transmembrane gradient that is only generated by energized cells (Sansom 1993; Cafiso 1994). A carpet-like mechanism of zygocin toxicity cannot be excluded, but seems unlikely since only minor molar amounts of zygocin are required for *in vivo* toxicity, contradicting cell killing *via* plasma membrane solubilization. Aim of future work, therefore, will be to gain deeper insight into zygocin conformation and its effect on toxicity.

4 Identification of host genes conferring zygocin resistance

More recently, we focused on the characterization of zygocin's mode of action. By Tn3 transposon mutagenesis (Burns et al. 1994), chromosomal yeast genes could be identified which - after disruption - render a former zygocin-sensitive yeast resistant. The phenotype of zygocin resistant Tn3 yeast disruptants was validated and further confirmed by phenotypically testing a knockout collection of yeast mutants defective in each single gene. As shown in Figure 5A, yeast strains containing chromosomal deletions in either *KRE6*, *PDR16*, or *PDR17* showed a significant change in zygocin sensitivity. While *kre6* mutants were completely toxin resistant at the cell wall level but highly sensitive as spheroplasts, *pdr16* and *pdr17* mutants were significantly less sensitive both, as intact cells as well as spheroplasts (Fig. 5A). Kre6p is a late Golgi glucan synthase involved in cell wall β-1,6 glucan synthesis. Consequently, mutations in *KRE6* affect cell wall composition and organization (Roemer and Bussey 1991). A Δ*kre6* null mutation causes a 50% decrease in the overall β-1,6 glucan content and a dramatic decline in cell wall mannoproteins, which constitute to the outer layer of the cell wall and are covalently linked to the inner β-1,3-glucan skeleton *via* β-1,6-glucan. Reduced cell wall mannoprotein content triggers an almost resistant phenotype of whole cells against zygocin, whereas after enzymatic removal of the cell wall, wild type sensitivity is fully restored (Fig. 5A). So the conversion of a sensitive yeast to a resistant mutant after loss of function of Kre6p reflects the decrease of primary toxin receptors and zygocin binding sites. Since toxin binding to the cell wall, however, can be completely by-passed in yeast cell spheroplasts (e.g. *kre6* mutant cells), it can be concluded that yeast and fungal cell walls represent an important natural barrier, which can be overcome and by-passed under conditions when cell wall structure is somehow altered; thus, enabling the toxin access to its plasma membrane target; an example for such a naturally occurring scenario would be mating yeast cells and fusion of fungal hyphae (El-Sherbeini and Bostian 1987; Schmitt and Tipper 1990).

Chromosomal mutations leading to zygocin resistance at the cell wall level give only limited insight into the actual mechanism of cell killing, since zygocin's toxicity manifests at the plasma membrane level by disrupting cytoplasmic membrane function in a sensitive target cell (Weiler and Schmitt 2003). This mode of action along with its rapid and energy-dependent toxicity shows striking similarities to



relevant genotype	zygocin sensitivity [mm growth inhibition zone]	
	whole cells	spheroplasts
wild-type	8	12
Δkre6	0	10
Δpdr16	3	3
Δpdr17	2	2

Fig. 5. Current model of zygocin action. **(A)** Receptor-mediated toxicity is envisaged in a two-step process in which the *Z. bailli* virus toxin interacts with a toxin receptor at the level of the cell wall (1) and subsequently binds to the cytoplasmic membrane (2). Whole cells as well as spheroplasts of yeast Δ*pdr16* and Δ*pdr17* mutants are significantly decreased in zygocin sensitivity, while yeast Δ*kre6* mutants are toxin resistant at the cell wall level but fully sensitive as spheroplasts. **(B)** Hypothetical model of zygocin membrane interaction based on experimental data, *in silico* structure prediction and analogy to antimicrobial peptides of higher eukaryotes [cm, cytoplasmic membrane; cs, cytosol; D1, 2, 3, functional domains of zygocin]. (1), domain structure of zygocin; (2), unspecific adsorption of the apolar side of the α-helix (D2) after overcoming the cell wall barrier by binding to a cell wall mannoprotein receptor (*via* domain D1) and recognition of a putative membrane receptor or docking protein; (3), membrane insertion of domain D3 driven by the transmembrane potential of the target cell and formation of a transmembrane helix; (4), toxin oligomerization by self assembly and subsequent plasma membrane permeabilization.

antimicrobial peptides that are produced by virtually all higher eukaryotes (Thevissen et al. 1996, 1997). Mechanisms of resistance against antimicrobial peptides are sparse and mostly limited to changes in the composition of the cytoplasmic membrane. Usually, the outer leaflet of microbial membranes is highly enriched in negatively charged lipids, which represents a major difference to the plasma membrane in higher eukaryotes. Due to the cationic net charge of antimicrobial peptides, including zygocin, an affinity to these lipids might exist, which facilitates toxin adsorption to its target membrane. So it was quite surprising when species of *Morganella* and *Serratia* were discovered, which showed a resistant phenotype to antimicrobial peptides that was triggered by a reduction of negatively charged lipids in the cytoplasmic membrane (Zasloff 2002). According to this observation, mutants of *Staphylococcus aureus*, which share a higher negatively charged membrane compared to the wild type, become much more susceptible to antimicrobial peptides, reflecting a significantly improved membrane binding of cationic antimicrobial peptides (Peschel et al. 2001). Thus, identification of Pdr16p and Pdr17p as gene products, which lead to a dramatic decrease in zygocin sensitivity at the plasma membrane level, is of high value with respect to further elucidate its molecular mode of action. Pdr16p and Pdr17p are known to affect plasma membrane lipid composition which, in turn, causes a significant decrease in the sensitivity of yeast *pdr16* and *pdr17* mutants against a variety of toxic substances (van den Hazel et al. 1999). Due to a dramatic reduction in membrane sterol concentration in yeast *pdr16* mutants, these mutant cells are significantly more susceptible to azole antimycotics, which interfere with ergosterol biosynthesis. In addition, the proportion of negatively charged lipids is decreased in yeast *pdr16* mutants, while *pdr17* mutants show the exact opposite effect. Previous studies indicate that Pdr16/17p activity is limited to the consistency of the cytoplasmic membrane. Consequently, a change of the cytoplasmic membrane is exclusively responsible for the reduced zygocin sensitivity in both mutants. Unfortunately, a zygocin-specific membrane receptor has not yet been identified, and the effect of a *pdr16/17* mutation cannot be attributed to the absence of a particular membrane lipid and/or membrane docking protein. However, membrane permeabilizing proteins do not necessarily require a specific secondary membrane receptor and/or docking protein, as has recently been shown for the antimicrobial polypeptide DmAMP1 produced by *Dahlia merckii*. The cytocidal effect of this antimicrobial protein, belonging to the diverse group of defensins, depends on sphingolipid containing membranes (Thevissen et al. 2000).

5 Zygocin as novel antimycotic

Because of its rapid *in vivo* killing kinetics, the *Z. bailii* protein toxin zygocin represents an attractive antimycotic in combating yeast and fungal pathogens. Its spectrum of activity encompasses phytopathogenic as well as human pathogenic yeast and fungi, including clinical isolates of *Candida albicans*, *C. glabrata*, *C. tropicalis*, *Sporothrix schenkii*, and the filamentous fungi *Fusarium oxysporum*

and *Colletotrichum graminicola* (Weiler and Schmitt 2003). When the molar activity of zygocin is compared to the antifungal activity of the common topic antimycotics clotrimazole and/or miconazole it becomes evident that only a fraction of the amount of zygocin is required to obtain the same inhibitory effect. As a remarkable advantage, zygocin combines a fast kinetic of cell killing with a high specificity to yeast and fungal cells. This displays an aspect which is neither shared by most antimycotics in use today nor by antimicrobial peptides of higher eukaryotes which all exert a dosis-dependent negative effect on the producing host. A drawback that interferes with a practical use of zygocin at least to medical application is determined by its temperature instability associated with an acidic pH optimum that limits zygocin application to topic purposes. So further work is aimed at elucidating the detailed mechanism of action and defining the basis for its high specificity.

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Acidophilic structure and killing mechanism of the *Pichia farinosa* killer toxin SMKT

Chise Suzuki

Abstract

SMKT, a killer toxin produced by a halotolerant yeast *Pichia farinosa* that consists of α and β subunits, is generated from a chromosomally encoded preprotoxin by enzymatic processing. SMKT is only stable under acidic conditions. Under neutral conditions, the subunits easily dissociate, consequently resulting in the aggregation of the α subunit and the concomitant loss of killer activity. The pH sensitive mechanism is discussed based on the crystal structure, circular dichroism, diffusion measurements by NMR, and mass spectrometry data. The *SPF1* (sensitivity to the *P. farinosa* killer toxin) gene encoding a novel P-type ATPase was isolated by complementation of SMKT-resistant mutants of *Saccharomyces cerevisiae*. It is possible that Spf1p and Pmr1p, the Golgi Ca^{2+} pump, collaborate to maintain ion homeostasis both in the ER and Golgi and contribute to glycosylation and protein trafficking. Possible mechanism of killing by SMKT is discussed in view of the function of Spf1p.

1 Introduction

Killer toxins are a general term of various proteins produced by yeasts or other fungal species that inhibit the growth or specifically kill sensitive strains of the same or related genera (Schmitt and Breinig 2002). In the "killer phenomenon", both the killer and the victim are unicellular.

It has long been known that certain yeast strains belonging to various species produce killer toxins, some of which are described in this issue. Most strains, both from natural habitats and existing laboratory strain collections, were identified in the absence of NaCl. Some halotolerant killer yeasts were isolated in the presence of 1 M NaCl from Japanese fermented food, which usually contain high concentrations of salt (Suzuki et al. 1989). One such killer yeast strain, *P. farinosa* NFRI 3621 was isolated from *koji*, a starter culture for *miso*. This strain produces a killer toxin which shows increasing killer activity with increasing concentration of salt or sorbitol on various strains of yeasts (Suzuki and Nikkuni 1989). For this means, the toxin termed SMKT (Salt-Mediated Killer Toxin) has a unique subunit structure that is stable only under acidic conditions (Suzuki and Nikkuni 1994). Mutants of *Saccharomyces cerevisiae* that lack the *SPF1* gene coding for a P-type

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ATPase are resistant both to exogenous SMKT and endogenous expression of the killer gene, *SMK1* (Suzuki and Shimma 1999; Suzuki et al. 2000).

Although killer toxins have offered attractive fields to many scientists for the past 40 years, the two common features, namely pH instability and the difficulty of heterologous expressions, have prevented a significant development in possible applications. This review will try to discuss these features in the terms of stable state and dissociation process of acidophilic subunit structure of SMKT and the sensitivity and resistance to SMKT of *S. cerevisiae* in which a novel P-type ATPase Spf1p is involved.

2 Properties of a halotolerant killer yeast, *Pichia farinosa*

The killer strain of *P. farinosa* (NFRI 3621) was isolated from a sample of *koji*, a starter culture for *miso* fermentation (Suzuki et al. 1989). The strain showed killer activity on some strains of *S. cerevisiae*, *Zygosaccharomyces rouxii*, and *Hansenula anomala* in the presence of 1 M NaCl. The killer spectrum of this strain against the standard killer strains (Young and Yagiu 1978) was examined in the presence or absence of 0.4 M NaCl. In the absence of NaCl, it killed only one killer strain of *Candida valida* (K7), whereas, in the presence of NaCl, it killed strains of *S. cerevisiae* (K2), *Candida glabrata* (K4), *Kluyveromyces marxianus* var. *marxianus* (K6), *H. anomala* (K8), *Kluyveromyces marxianus* var. *drosophilarum* (K10), in addition to the *C. valida* (K7). Further, the strain showed killer activity on some strains of *Schizosacchaomyces pombe* (Worsham and Bolen 1990; Facanha et al. 2002).

Killer strains of several *Pichia* species including *Pichia acaciae*, *Pichia inositovora*, *Pichia kluyvery*, *Pichia anomala*, and *P. farinosa* have been reported. Killer genes of *P. acaciae* and *P. inosito*vora are encoded by dsDNA plasmids similar to that of *Kluyveromyces lactis* (Worsham and Bolen 1990; Hayman and Bolen 1991). On the other hand, other killer genes are located on chromosomes.

A pioneering work using crude preparation of the killer toxin from *P. kluyvery* provides the only direct evidence for a killer toxin forming a protein channel in a lipid bilayer (Kagan 1983). Anti-ideotypic antibodies raised from a *P. anomala* killer toxin have been used in the development of novel antimycotics for the treatment of human and animal fungal infections (Polonelli et al. 1997). However, molecular structure of these toxins is still unknown. Such variety of killer toxins indicates that *Pichia* species include relatively wide range of strains. *P. farinosa* could be distinguished from other killer yeasts by its high resistance to osmotic stress, particularly to salt stress.

Pichia sorbitophila is considered as a synonym of *P. farinosa* according to Kurtzman (1998). In the Genolevure I project, 13 hemiacetomycetous yeasts including *P. sorbitophila* were analyzed (Souciet et al. 2000). The genomic DNA of *P. sorbitophila* was partially sequenced as 4829 random sequence tags of about 1 kb (de Montigny et al. 2000). The phylogenetic position of *P. sorbitophila* is close to the *Candida* species as suggested by cladograms established from 18S and 25S

rRNA sequence comparisons (Souciet et al. 2000). Non-universal decoding is widely distributed not only in *Candida* species but also in some hemiascomycetous yeasts. The CUG codon was shown to specify Ser instead of Leu in *P. sorbitophila* (Tekaia et al. 2000). The same is true of the CUG codon in *P. farinosa*. The *in vivo* evidence showing that positions specified by the CUG codon contain unmodified Ser in *P. farinosa* has been shown by mass spectrometry and Edman sequencing of peptides from the mature SMKT and secreted protoxin (Suzuki et al. 2002). However, comparative physiological studies together with PCR amplification of *P. farinose* DNA fragments homologous to known *P. sorbitophila* genes provided a strong indication that this strain should be classified as a separate species (Maresova and Sychrova 2003). Chromosome number and size of *P. sorbitophila* differ from those of *P. farinosa.* The genome of *P. sorbitophila* consists of 12 chromosomes, whereas that of *P. farinosa* consists of seven chromosomes. In addition, *P. sorbitophila* has two copies of *URA3* genes, termed *PsURA3* and *PsURA30*, on chromosome VIII and III, respectively. By contrast, P. *farinosa* has only one copy of *URA3* gene, termed *PfURA3*, on chromosome V (Suzuki et al. 2003).

3 SMKT with an acidophilic subunit structure

3.1 SMKT consists of two distinct subunits

The killer toxin produced by *P. farinosa* NFRI 3621 showed increasing killer activity on various strains of yeasts with increasing concentration of salt or sorbitol. In the light of this result, the killer toxin was, therefore, termed SMKT. The activity of SMKT, which is basically dependent on the pH of the medium, was completely retained in the pH range 2.5 to 4.0, but decreased to 50% at pH 5.0. No activity was observed at pH 6.0. When purifying SMKT from culture filtrate by ammonium sulfate precipitation and SP- and Butyl-Toyopearl chromatography, the buffer was, therefore, kept at pH 3.5 (Suzuki and Nikkuni 1994). Using a sensitive strain of *S. cerevisiae*, the killer activity was determined by a microtiter dilution method at pH 3.5 in the presence of 1.5 M sorbitol.

In the purified fraction of SMKT, Tricine-SDS-PAGE revealed two components (4-kDa and 8-kDa), which were not linked by any disulfide bonds. To confirm whether these components were genuinely associated with its biological activity or not, the purified fraction of SMKT was analyzed by electrophoresis in nondenaturing condition below pH 4. The toxin co-migrated with the killing activity as a single band that was found to contain the two components (4-kDa and 8-kDa) when analyzed by Tricine-SDS-PAGE. This result provides a strong evidence for the association of the two polypeptides with the killer activity. We termed the 4-kDa polypeptide as α-subunit and the 8-kDa polypeptide as β-subunit (Suzuki and Nikkuni 1994).

3.2 SMKT preprotoxin encoded by the chromosomal gene and the maturation process

The killer gene, *SMK1*, was cloned from the chromosomal DNA (Suzuki and Nikkuni 1994). Pulsed-field gel electrophoresis and subsequent Southern blot analysis showed that the genome of *P. farinosa* NFRI 3621 consisted of seven chromosomes, each approximately 1.1-2.2 Mb in size (11.8 Mb in total) (Suzuki et al. 2003), and that *SMK1* was located on chromosome VII (Suzuki, unpublished). *SMK1* encodes a preprotoxin consisting of 222 amino acid residues, comprising a typical signal sequence, a hydrophobic α-subunit (63 amino acids), an interstitial γ polypeptide with a putative glycosylation site (62 amino acids) and a hydrophilic β-subunit (77 amino acids) (Fig. 1).

On the basis of the amino acid sequences of the α and β subunits and those of the C-terminal peptides, the processing sites of the preprotoxin were determined (Suzuki and Nikkuni 1994). The sequence from Met1 to Ala18 has a typical feature for a signal peptide. The sequence from Trp19 corresponded to the N-terminal sequence of the α subunit. The C-terminal amino acid of the α subunit was a valine residue corresponding to Val81 of preprotoxin. In the preprotoxin sequence, Val81 is followed by two basic amino acid residues, Lys82-Arg83, suggesting that the preprotoxin is processed in a manner similar to that of the K1 toxin and α-factor precursor of *S. cerevisiae*, i.e. Kex1p-like carboxypeptidase trims C-terminal two basic residues generated by Kex2p-like endopeptidase cleavage (Julius et al. 1984; Dmochowska et al. 1987). The β subunit is generated by scission of the Arg145-Gly146 peptide bond of the preprotoxin. Consequently, the interstitial γ peptide is cleaved from the protoxin by processing enzymes resulting in the mature toxin consisting of the α and β subunits (Fig. 1). Although there is no amino acid sequence similarity between SMKT and the K1 toxin of *S. cerevisiae*, it is intriguing that the dsRNA-encoded K1 toxin and the chromosome-encoded SMKT are so similar in their overall structure, hydrophobicity profiles, and processing mechanism of preprotoxins. However, *kre1* and *kre5* mutants, which are resistant to the K1 toxin because of lack of cell wall (1->6) β-D-glucan (Boone et al. 1990; Meaden et al. 1990), are sensitive to SMKT, suggesting that (1→6) β-D-glucan is not involved in the recognition of the sensitive cells by SMKT.

Interestingly, *P. farinosa* NFRI 3621 secreted a protoxin of SMKT with the mature SMKT when cultured in a rich medium in the presence of high concentration of NaCl (Suzuki 1999). The secreted protoxin was generated by removal of the signal peptide from the preprotoxin and glycosylated presumably at the N-glycosylation site in the γ peptide region. It did not have any killer activity, suggesting that further processing is necessary for acquisition of the activity. Secretion of the protoxin increased with increasing concentration of NaCl in the medium and maximum secretion was observed at 2 M NaCl. Transcripts of *SMK1* and secretion of mature SMKT were observed in the presence or absence of NaCl, whereas secretion of the protoxin was only observed in the presence of NaCl. These results suggest that secretion of the protoxin is post-translationally controlled by NaCl.

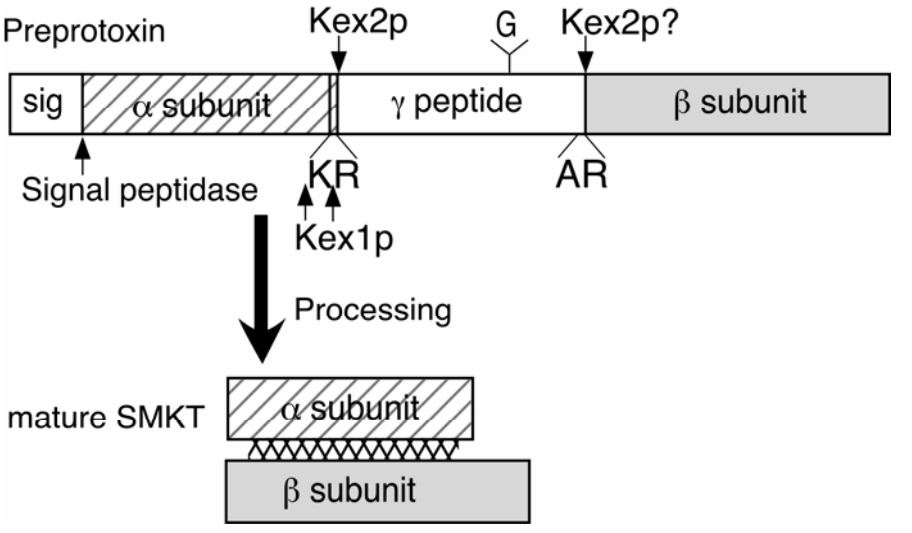

Fig. 1. Schematic drawing of the maturation process of SMKT. The structure of SMKT preprotoxin and mature SMKT consisting of the α and β subunits are indicated. The putative processing sites are indicated by arrows, and capital G represents the putative glycosylation site.

Further studies are necessary to elucidate the control mechanisms of the secretory pathway responding to salt stress that is unique in *P. fariniosa*.

3.3 Lethal effect of expression of *SMK1* in *S. cerevisiae*

Variant toxins in stability and activity are now being sought for further elucidation of the structure-function relationship and the molecular mode of action of killer toxins. In the case of SMKT, despite several attempts to establish an *SMK1* expression system, no successful result was obtained because expression of *SMK1* is lethal in various hosts, including *Escherichia coli*, *Pichia pastoris*, and *S. cerevisiae* (Suzuki, unpublished data). Therefore, instead of functional expression of SMKT, the lethal effect of expression of *SMK1* was used to elucidate the structure-function relationship of the *SMK1* product (Suzuki et al. 2000). When *SMK1* is expressed in *S. cerevisiae* under the control of a galactose-inducible promoter, the colonies of transformants showed a blue color in the presence of methylene blue, an oxidation-reduction indicator, employed to stain dead cells. Using the methylene blue plate assay, the *SMK1* regions required for the lethality in *S. cerevisiae* were examined.

Deletion of the interstitial γ peptide or the C-terminal loop from Ala208 to the C-terminal Asp222 had no effect on the lethality. On the other hand, deletion of the signal sequence resulted in complete loss of the lethality, suggesting that entering the secretory pathway is critical for the lethality of *SMK1* expression. Since expression of the γ subunit of the *K. lactis* toxin even without a signal sequence is

lethal for the host cells (Tokunaga et al. 1989), the mechanism responsible for the lethality of SMKT is different from that of the *K. lactis* toxin.

Deletion of the region from the C-terminus to Leu207 resulted in the loss of the lethal activity. In the crystal structure described in the next section, Leu207 is located at the C-terminus of the central strand of the β-sheet structure of SMKT and its side chain is thrust into a hydrophobic environment between the β-sheet and the α-helices (Fig. 2A). The result obtained upon substituting Ala, Ser, or Glu for Leu207 indicated that the nonpolar side chain of Ala or Ser fits the hydrophobic pocket, but the carboxyl group of Glu destabilizes the structure critical for the lethal effect of the *SMK1* product. Thus, it is possible that the side chain of Leu207 plays a key role in stabilizing the overall structure of the *SMK1* product.

In the case of K1 toxin of *S. cerevisiae*, association of the precursors with the cell membrane has been observed and this has been thought to be the immunity determinant and evidence of post-translational signal cleavage (Boone et al. 1986; Zhu and Bussey 1991). In the transformants expressing *SMK1*, most of the endogenous *SMK1* products corresponding to the preprotoxin were also observed in the membrane fractions but not in the soluble fractions, suggesting that stable association of the precursor occurred with the membrane.

In *P. farinosa*, positions specified by the CUG codon are translated to Ser (Suzuki et al. 2002). However, in *S. cerevisiae* which uses the universal genetic code, these CUG codons are translated to Leu. In order to express the authentic *SMK1* product in *S. cerevisiae*, the three CUG codons corresponding to Ser87, Ser137, and Ser206 in the *SMK1* gene were changed to universal Ser codons by site-directed mutagenesis (Suzuki et al. 2002). The expression not only of the modified *SMK1* gene with universal Ser codons but also of the unmodified *SMK1* gene with the CUG codons was found lethal in *S. cerevisiae*. The secretion of protoxin with the Ser codons was significantly increased. The presence or absence of CUG codon did not have much effect on the transcription level of *SMK1*. Therefore, the increased expression of protoxin with the Ser codons is considered as probably due to the stable conformation by the authentic amino acid residues and not due to the elevated level of transcription.

3.4 Crystal structure of SMKT

The crystal structure of SMKT has been determined at 1.8 Å resolution by our group (Kashiwagi et al. 1997). All 140 residues in each molecule of SMKT have been unambiguously assigned to the electron densities. The α and β subunits are jointly folded into an ellipsoidal, single domain structure, with overall dimensions of 41 Å×29 Å ×34 Å (Fig. 2A). Along the longest molecular principal axis, the N-terminal sides of the α and β subunits were designated as the molecular bottom and top, respectively. The α subunit possesses one α helix and two β strands, whereas the β subunit consists of one α helix and three β strands. These secondary structure elements are arranged so that a layer of two antiparallel α helices flanks a five-stranded antiparallel β sheet. The content of the α helix was estimated at

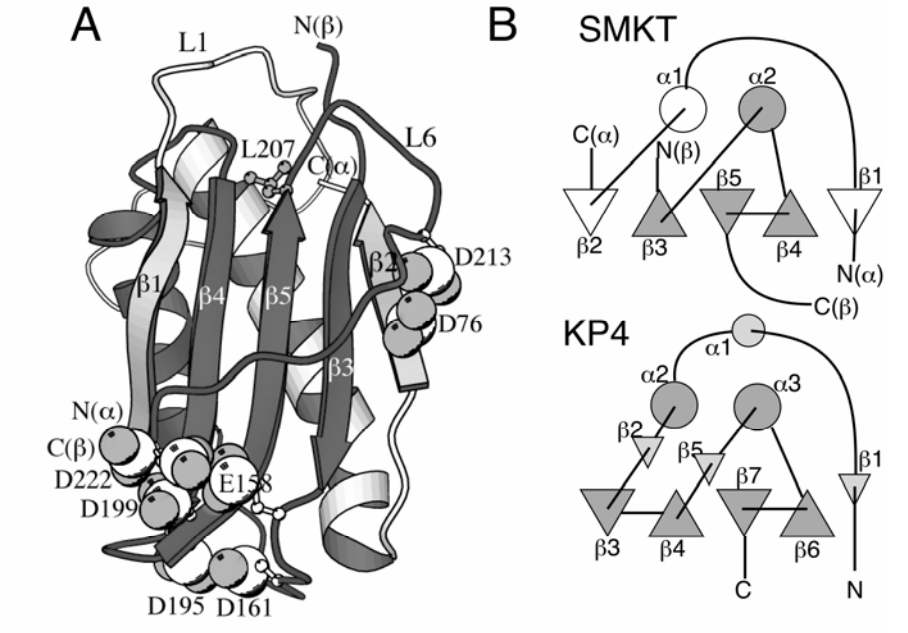

Fig. 2. (A) Crystal structure of SMKT. The α and β subunits are colored light and dark gray, respectively. Leu207, the critical residue for lethality, is shown in the ball-and-stick model. Carboxyl groups involved in the carboxyl-carboxyl interactions are shown in the space-filling model. The N- and C- termini of the α and β subunits are indicated as N(α), C(α), N(β), and C(β), respectively. (B) Topology diagrams of SMKT (upper) and KP4 (lower). The triangles and the circles represent β strands and α helices, respectively. The open and gray symbols in SMKT represents elements derived from the α and β subunits, respectively. The smaller symbols in KP4, shown in paler colors, represent short minor elements.

about 26%, consisting with the α helix content (20%) estimated by circular dichroism (CD) analysis using a mean residue molar ellipticity at 222 nm (Suzuki et al. 1997). There is no intersubunit disulfide bridge, instead two cysteine residues in each subunit form an intrasubunit disulfide bond. The C-terminus of the α subunit and the N-terminus of the β subunit are close to each other. This feature implies that a γ peptide may construct an independent domain attached to the top of the mature SMKT domain. It is reasonable to consider that the SMKT protoxin possesses the structure of the mature SMKT with the γ peptide domain that is ready for being cut off (Fig. 3). A long loop (L1 loop) of the α subunit locates at the top of SMKT. Reflecting the flexibility, moderately high B factors of the main chain atoms are observed in the L1 loop region. The L1 loop may provide room for the joining of the γ and SMKT domains in the protoxin structure and may cover the upper portions of SMKT including the C-terminus of the α subunit after the processing events.

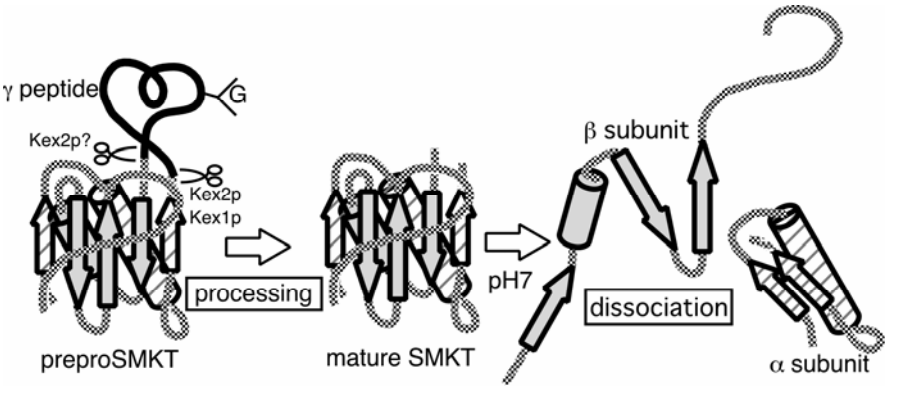

Fig. 3. Maturation and dissociation process of SMKT. The SMKT preprotoxin is processed to mature SMKT consisting of the α and β subunits. The subunit structure easily dissociates under neutral or basic condition (pH 7), resulting in the mutual aggregation of hydrophobic α subunit and loss of secondary structure of the β subunit.

Three–dimensional structures of several killer toxins have been determined. Structural studies on killer toxins have been focused on the analysis of native toxins secreted by killer yeasts, and not on recombinant proteins expressed in heterologous hosts. Successful expression of a functional killer toxin sufficient for structural analysis has not yet been achieved in a heterologous host. WmKT, a killer toxin produced by *Williopsis mrakii*, has been determined by NMR and shown to possess a four-stranded antiparallel β-sheet structure similar to that of βγ crystalline (Antuch et al. 1996). The crystal structure of KP4 (a single polypeptide chain of 105 amino acids) and the α-subunit (79 amino acids) of KP6 of *Ustilago maydis* have been determined (Gu et al. 1995; Li et al. 1999; Bruenn, this volume). The tertiary structure of KP4 consists of a central five-stranded anti-parallel β-sheet and two α-helices, which is essentially the same as that of SMKT (Fig. 2B). However, there are some differences between these two toxins. Whereas SMKT is a heterodimer encoded by a chromosome, KP4 is a monomer encoded by a double stranded RNA viral genome of *U. maydis*. The C-terminus of the α subunit and the N-terminus of the β subunit of SMKT are generated between the β2 and β3 strands, following the processing event. On the other hand, the β3 and β4 strands of KP4 corresponding to the β2 and β3 strands of SMKT are directly connected (Fig. 2B). SMKT has a slender ellipsoidal shape, in contrast with the globular shape of KP4. Furthermore, KP4 is a very stable protein with five intramolecular disulfide bonds, whereas SMKT is a pH-sensitive protein (as described in the next section). In addition to the overall folding topology, both toxins contain two left-handed split βαβ motifs. Therefore, it is of interest to discuss the common characteristics of their function. On the basis of the structural similarities between KP4 and scorpion neurotoxins and the experimental results showing that KP4 inhibited the Ca^{2+} channels of mammalian cells and that the killing activity of KP4 on the sensitive strain of *U. maydis* was inhibited by Ca^{2+}, it has been concluded that KP4 acts as a Ca^{2+} channel inhibitor. The killing activity of SMKT on

S. cerevisiae was also inhibited by 100 mM $CaCl_2$. However, circular dichroism (CD) analysis showed that the secondary structure of SMKT was not affected in the presence of $CaCl_2$, suggesting that $CaCl_2$ does not affect SMKT, but does affect the sensitivity of the cells (Suzuki et al. 1997).

3.5 Stable state and dissociation process of SMKT

Most killer toxins, including K1 toxin of *S. cerevisiae*, are active only in acidic solutions (Pfeiffer and Radler 1984). They lose their activities lost in neutral and basic solutions. Despite those common properties, the mechanism of the pH sensitivity of killer toxins has not yet elucidated. The results of biochemical and structural analysis of SMKT have provided for an example of the mechanism of their pH sensitivity.

The activity of SMKT was retained in a solution with pH 2.5-4.0 and was not observed at pH above 6 (Suzuki and Nikkuni 1989). Under acidic conditions, the α and β subunits of SMKT were tightly bound together even in the presence of 6 M urea or 1.5 M NaCl (Suzuki and Nikkuni 1994). The apparent molecular weight of SMKT in the presence of 6 M urea or 1.5 M NaCl was estimated to be about 13,000 by gel filtration analysis. As the killer assay is usually performed in the presence of 1.5 M NaCl, this result indicated that the active toxin exists as a single molecule composed of the α and β subunits. Consistent with the result, pulsed-gradient spin-echo NMR diffusion measurements showed that the tertiary structure of SMKT was stable at pH below 5.0 in the presence of 0.15 M or 2 M NaCl and that the native SMKT existed as an unaggregated heterodimer in the solution (Price et al. 1999). CD analysis also showed that the presence of 0.06, 0.15, and 1.5 M NaCl did not affect the secondary structure of SMKT. Furthermore, the crystal structures of SMKT analyzed at two different salt concentrations (3.3 M and 0.2 M ammonium sulfate) were almost identical to each other. These results indicate that SMKT stably exists as a single molecule regardless of the presence or absence of high concentration of salt under acidic conditions.

When the active SMKT solution was adjusted to pH 7, the solution became turbid. The turbidity was attributed to aggregation of the α subunit. After centrifugation of the turbid solution, the precipitate was found to consist of the α subunit and the supernatant, the β subunit. This result indicates that the inactivation of the toxin under neutral or basic conditions is caused by the dissociation of the α and β subunits (Suzuki and Nikkuni 1994). The dissociation of the subunits is irreversible. The dissociation and the aggregation of the α subunit occur at pH 5.3 at 4 ℃, as determined by dialysis (Fig. 3).

This dissociation process of SMKT was monitored by CD. The CD spectra in the pH range 3.4 to 5.1 did not change significantly. However, the solution of SMKT became turbid between pH 5.1 and pH 5.5. After removing the α subunit from the turbid solution by centrifugation, the soluble β subunit gave a CD spectrum which was exactly that of a peptide in a random coil. This result clearly indi-

cates that the β subunit cannot take any secondary structure in the absence of the α subunit (Suzuki et al. 1997).

The dissociation of the subunits could be explained by the feature of the interactions between the carboxyl groups. SMKT crystal was examined under acidic conditions (pH 3.5-4.0), wherein the carboxyl groups appear to be protonated (Kashiwagi et al. 1997). The protonated carboxyl groups are utilized as donors and are involved in the intramolecular hydrogen bond network of SMKT. SMKT is structurally distinguished from other toxins by the presence of four carboxy-carboxy hydrogen bonds. In each of four pairs of acidic residues, for instance, Asp76(β4)-Asp213(L6), Asp161(L4)-Asp195(β4), Glu158(L4)-Asp222(L6), and Asp199(β5)-Asp222(L6), the distance between carboxyl groups is sufficiently short to allow the formation of hydrogen bonds (Fig. 2A). These hydrogen bonds appear to be used for fixing loops to the β sheet. The long L6 loop is especially fixed by these hydrophobic interactions of acidic residues and by a disulfide bond so that the loop covers the upper half of the β sheet surface. These interactions may be the large stabilizing forces under acidic conditions.

Under neutral or basic conditions, the carboxyl groups bear negative charges and these interactions would presumably become repulsive and destabilize the structure, triggering the conformational changes that lead to the exposure of the buried hydrophobic residues of the β subunit (Kashiwagi et al. 1997). About 60% of the surface of the β subunit comprises hydrophobic residues, and almost all of hydrophobic residues of the β subunit are involved with the interactions with the hydrophobic α subunit. Therefore, it is unlikely that the α subunit stays in solution independent of the β subunit.

The pH-dependent denaturation mechanism of SMKT was also analyzed by pH titration utilizing electrospray-ionization mass spectrometry (ESI-MS) (Kashiwagi et al. 2000). The molecular ions of the heterodimer that possesses the highly ordered structure were mainly observed below pH 4.6. However, the two subunits immediately dissociated at this pH. The spectra measured at various settings of the mass spectrometer indirectly demonstrated that the pH-dependent dissociation occurs in the liquid phase. The result of ESI-MS analysis demonstrates the sudden dissociation of SMKT at pH 4.6, suggesting that the deprotonation of a specific carboxyl group triggers a cooperative dissociation process of SMKT.

Consistent with this result, mutational analysis of *SMK1* indicated that interaction between Asp195 and Asp161 is the candidate. As described in section 3.3, expression of *SMK1* is lethal in *S. cerevisiae* and amino acid substitutions that destabilize overall structure of the *SMK1* product caused the complete loss of the lethality. However, *SMK1* with substitutions of Ala for Asp213 and Asp222, and the deletion of the C-terminal Asp222 did not affect the lethality. The interactions between each pair of carboxyl groups, for instance, Asp213-Asp76, Asp222-Glu158, and Asp222-Asp199 are, therefore, not required for the stability of SMKT in solution. These interactions appear to stabilize the crystal structure by connecting the flexible C-terminal loop to the β-sheet structure. The remaining interaction between Asp195 and Asp161 may be involved in the maintenance of SMKT's stability under acidic conditions (Suzuki et al. 2000).

4 P-type ATPase *SPF1* required for sensitivity to SMKT

4.1 *SPF1* encoding a novel P-type ATPase

Analyses of resistant mutants against various killer toxins have provided clues not only for the killing mechanism itself, but also for physiological function of genes involved in the killing mechanism, such as cell wall synthesis, protein delivery (Bussey et al. 1990; Breinig et al. 2002; Schmitt and Breinig 2002).

SMKT-resistant mutants of *S. cerevisiae* were referred as *spf* (for sensitivity to *Pichia farinosa* killer toxin) (Suzuki and Shimma 1999). Genetic analysis showed that five mutants fall into the same complementation group, *spf1*. The wild type *SPF1* gene was isolated by complementation of the *spf1-1* mutation. *YEL031w*, the only open reading frame that could complement *spf1-1*, potentially encodes a protein of 1215 amino acids with a predicted molecular mass of 135 kDa. The sequence similarity suggested that the gene product is a P-type ATPase with 12 putative transmembrane domains.

SPF1 was also isolated by various genetic screens. The *cod1-1* mutants allowed the constitutive degradation of HMG-CoA reductase (Hmg2p) in the ER under normally stabilizing conditions of reduced feedback signals (Cronin et al. 2000). The *pio1* mutant failed to respond faithfully to a strong charge difference signal for protein insertion orientation (Tipper and Harley 2002). The *per9-1* mutation was defective in ER-associated degradation (ERAD) and showed synthetic lethality with *IRE1,* a sensor molecule of the ER stress (Vashist et al. 2002). Genes that complement those mutations were isolated and found to be identical to *SPF1*.

4.2 Feature of type V P-type ATPases

The P-type ATPases are members of a large superfamily of proteins involved in the transmembrane transport of charged substrates. Recently, 159 P-type ATPases were classified into five major types (Axelsen and Palmgren 1998). Type V, the ATPase family to which Spf1p belongs, is the only ATPase family, whose substrate specificities have not yet been identified. Among the 16 P-type ATPases in *S. cerevisiae*, two genes, *SPF1* and *YOR291w*, form this new family. Deletion of *YOR291w* showed no detectable effect on processes with which *SPF1* is involved including SMKT sensitivity, protein insertion orientation, and Hmg2p stability (Cronin et al. 2000; Tipper and Harley 2002). Although this result does not clarify the cellular role of*YOR291w*, it showed that it is not redundant with *SPF1*.

The type V ATPases are conserved from yeasts to human, suggesting that they play an important role in eukaryotic cells. In *S. pombe*, the *SPF1* homolog, *sev4*$^{+}$, was defined by genetic analysis of mutants defective in both proliferation and sporulation (Yoshida et al. 2003). Consistent with this, *spf1/spf1* homozygous diploid of *S. cerevisiae* also has defects in sporulation (Suzuki, unpublished data). *sev4*$^{+}$was also characterized as *cta4*$^{+}$and was found to be required for control of cell shape and microtubule dynamics (Facanha et al. 2002). Wild type cells of *S. pombe* showed sensitivity to SMKT, but not to other toxins like zygocin of *Zygo-*

saccharomyces bailii and KT28 of *S. cerevisiae* (Heintel et al. 2001; Weiler et al. 2002), whereas *cta4Δ* mutant cells acquire resistance to SMKT. Whether *SPF1* and *cta4*$^{+}$ genes are functionally interchangeable should be investigated. The *SPF1* homolog of *Drosophila melanogaster* restored the lovastatin (HMG-CoA inhibitor) sensitivity and regulation of Hmg2p degradation in *spf1/cod1* mutants (Cronin et al. 2002).

In P-type ATPases tested thus far, modification of the Asp residue in the highly conserved phosphorylation motif (CSDKTGTLT) resulted in complete loss of function (Harris et al. 1994; Sorin et al. 1997). This residue plays an important role as an acceptor of the terminal phosphate of ATP during ATP hydrolysis and energy transduction. To investigate the function of Spf1p, Asp487, the putative phosphorylation site of Spf1p, was replaced by Asn (referred to as Spf1D487Np) (Suzuki 2001). Expression of the Spf1D487Np did not suppress the SMKT-resistant phenotype and lovastatin sensitivity of *spf1* mutants (Cronin et al. 2002), suggesting that Asp487 at the putative phosphorylation site is necessary for the Spf1p function. The mutation point occurring in the *spf1-1* allele leads to a substitution of Ser for Gly815 (Suzuki and Shimma 1999). The Gly815 is conserved in almost all P-type ATPases and composes domain P according to the crystal structure of SERCA ATPase (Toyoshima et al. 2000). These results suggested that either Asp487 or Gly815 is necessary for the ATPase activity that is required for acquiring sensitivity to SMKT.

The type V ATPases differ from most other Ca^{2+} ATPases in that they lack two common motifs: an N-terminal metal-binding motif and a C-terminal domain involved in calmodulin binding and autoinhibition (Rudolph et al. 1989). However, the unique C-terminal structure of this group of proteins may be important because C-terminal truncation variants of Spf1p did not restore SMKT-sensitivity (Suzuki and Shimma 1999).

4.3 Localization of Spf1p

Many of the phenotypes of the *spf1* mutants suggested that Spf1p functions in the ER. Localization of Spf1p was determined by indirect immunofluorescence (Cronin et al. 2002; Vashist et al. 2002) and density gradient fractionation. Spf1p with the N-terminal 3myc-epitope tag (Cronin et al. 2002) and Spf1p with the C-terminal HA-epitope tag (Vashist et al. 2002) expressed from the native promoter were functional. Distribution of these epitope-tagged Spf1p was characteristic of the ER and overlapped with that of Kar2p, an ER marker. On the other hand, localization of 3myc-Spf1p was also determined by fractionation on sucrose density gradients in the presence of EDTA or $MgCl_2$ (Cronin et al. 2002). The distribution of 3myc-Spf1p overlapped that of the ER-localized Sec61p.

Differential centrifugation to give P13, P100, and S100 fractions was used to characterize the membranes where Spf1p is located (Suzuki 2001). P13 fraction typically contains large, dense membranes such as the ER, vacuole, and plasma membrane. Further resolution of P13 fraction on 1.2 M and 1.5 M sucrose layers gave a less dense membrane fraction within the upper sucrose layer (b-1) and a

dense membrane fraction at the interface between two sucrose layers (b-2). P100 fraction contains less dense membranes such as Golgi and transport vesicles (Gaynor et al. 1999). Bands corresponding to Spf1p were fractionated mainly in the P13, P100 and b-1 fractions and a little in the b-2 fraction. This fractionation pattern was distinct from that of an ER marker protein, Dpm1p, which was found mainly in the P13, b-1 and b-2 fractions. The b-2 fraction is abundant in Dpm1p. The same fractionation pattern was observed in another ER protein, Sec12p. On the other hand, a *cis*-Golgi marker, Och1p, although mainly recovered in P100, was also found in the P13 and b-1 fractions. The property of membrane where Spf1p is located is not always consistent with ER marker proteins, but is partially consistent with the *cis*-Golgi protein, suggesting the possibility that Spf1p is shuttling between the two organelles. When cell membranes from the *spf1* disruptants were analyzed, the ER marker proteins were fractionated in less dense membrane fractions. The b-1 fraction rather than b-2 is abundant in Sec12p and Dpm1p in the absence of Spf1p. Such altered distribution of ER membrane proteins may be due to multiple effects in consequence of the ER stress and the defect of glycosylation in the absence of Spf1p as described in section 4.5.

4.4 Phenotypes of disruptants of *SPF1*

Mutants lacking *SPF1* are highly resistant to exogenous SMKT. Using the complete set of deletion strains of *S. cerevisiae* (Giaever et al. 2002), SMKT resistant mutants were screened (Suzuki, unpublished data). Among approximately 5,000 strains, the deletion strain of *SPF1* showed the strongest resistance against SMKT.

Furthermore *spf1* disruptants showed resistance to endogenously expressed *SMK1*. When *SMK1* is expressed in the *spf1* disruptants as described in section 3.3, the colonies of transformants did not show a blue color on the methylene blue plate (Suzuki et al. 2000). This phenotype is specific to the *spf1* disruptants. Deletion strains, such as *gas1, erd1,* and *rer1*, which are partially resistant to exogenous SMKT, did not show such phenotype (Suzuki, unpublished data). The *spf1* disruptant concurrently acquired resistance to endogenously expressed *SMK1*, suggesting that the *spf1* disruptants have a modified toxin target site or have perturbed protein sorting so that SMKT cannot reach the target site.

Disruption of *SPF1* did not noticeably affect either mating or growth in the presence of high (600 mM) or low (10 μM) concentrations of $CaCl_2$ or 10 mM $MnCl_2$, respectively. The *spf1* cells grew more slowly than the wild type cells. The doubling time of the wild type cells in YPD medium was 1.9 hours, while that of the *spf1* cells was 2.3 hours. When cultured in rich media, the *spf1* cells aggregated in clusters, similar to cell wall mutant (Ballou et al. 1980). In addition, the *spf1* cells showed hypersensitivity to both calcofluor white (CFW) and hygromycin B and resistance to vanadate (Suzuki and Shimma 1999). CFW interferes with cell wall assembly (Ram et al. 1994) and inhibits the growth of cell wall mutants at lower concentrations compared to the wild type cells. Sensitivity to hygromycin B and resistance to vanadate are typical phenotypes of yeast mutants defective for N-glycosylation (Dean 1995; Shimma et al. 1997).

Disruption of *SPF1* is synthetic lethal with deletions of *HAC1* (Cronin et al. 2002) and *IRE1* (Vashist et al. 2002). *HAC1* is the unfolded protein response (UPR) regulator (Ogawa and Mori 2004) and *IRE1* is a protein kinase with a site-specific endoribonuclease activity responsible for the specific splicing of the *HAC1* transcript (Sidrauski and Walter 1997). The role of Spf1p in UPR is discussed in the next section. Tong et al. mapped a genetic interaction network containing approximately 1,000 genes and approximately 4,000 interactions (Tong et al. 2001; Tong et al. 2004), indicating several synthetic lethal or synthetic sick interactions of SPF1 with genes, such as RIC1, a guanyl-nucleotide exchange factor, SKT5, a gene encoding a positive regulator of chitin synthase III, and SMI1, a gene encoding a glucan synthesis regulator. SKT5 and SMI1 are isolated by complementation of mutants resistant to the killer toxins of *K. lactis* and *W. mrakii*, respectively.These results suggest that Spf1p affects cell wall assembly, possibly through protein transport.

4.5 Comparison of intracellular roles of Spf1p and Pmr1p

The existence of various phenotypes of *spf1* mutants and localization of Spf1p in the ER suggest that Spf1p sustains a variety of processes in the ER, in which Pmr1p is also involved (Deurr et al. 1998). Pmr1p is a yeast Ca^{2+}/Mn^{2+} pump belonging to type IIA P-type ATPase that resides in the *medial*-Golgi. *PMR1* is identical to *SSC1*, which was isolated by complementation of the sporulation defect of *ssc1-1* homozygous diploids. The *pmr1* mutants show hypersensitivity to both CFW and hygromycin B. Consistent with these phenotypes, proteins produced by *pmr1* mutants lack outer chain N-glycosylation, and this phenotype is restored by adding high concentration of $CaCl_2$ and $MnCl_2$ to the growth media (Rudolph et al. 1989; Antebi and Fink 1992; Deurr et al. 1998). These observations point to the fact that *spf1* mutant shares many phenotypic features with the *pmr1* mutant. However, overexpression of Pmr1p cannot substitute for Spf1p function (Suzuki and Shimma 1999). Similarly, overexpression of *SPF1/COD1* did not complement the sensitivity to EGTA of *pmr1* mutants (Cronin et al. 2002). To compare the two mutants, *pmr1spf1* double mutants were analyzed (Suzuki and Shimma 1999). The minimal inhibitory concentrations of SMKT for the wild type, *spf1*, *pmr1*, and *pmr1spf1* cells were 0.06, 10, 2.5, and 2.5 μM, respectively. This result demonstrated that the *pmr1* single mutant is partially resistant to SMKT. To compare outer chain N-glycosylation, activity staining of invertase was performed. Invertase isolated from the *spf1* cells migrates faster than that from the wild type cells, but slower than that from the *pmr1* cells. Although the outer chain N-glycosylation, which was lacking in *pmr1* invertase, was restored in part by the addition of 100mM CaCl2 to the medium, the size of the *spfl* invertase was not at all affected. The size of invertase from *pmr1spf1* double mutant cells, which was smaller than that of the *pmr1* cells, was partially restored by addition of $CaCl_2$. Since the *pmr1spf1* double mutant showed an additive effect on glycosylation-deficient phenotype, Spf1p and Pmr1p may be considered as participating in a different modification process of glycosylation.

Cronin et al. examined the level of a number of ions present in the wild type cells and *spf1* cells by inductively coupled plasma optical emission spectrometry (ICP-OES). Deletion of *SPF1* did not affect cellular calcium levels. However, deletion of both *SPF1* and *PMR1* showed a synergistic effect on the increase of intracellular calcium (Cronin et al. 2002). Although calcium-related phenotypes suggested direct involvement in calcium transport, the substrate of Spf1p has not yet been identified. Cronin et al. purified wild type histidine-tagged Spf1p and the nonfunctional Spf1D487Np using nickel affinity chromatography to identify the biochemical requirements for Spf1p. It was found that calcium failed to increase the ATPase activity of Spf1p and none of ions tested stimulated the ATPase activity (Cronin et al. 2002).

Phenotypes characteristic of perturbation of unfolded protein response (UPR), a stress-inducible pathway used to monitor and maintain ER homeostasis (Liu and Kaufman 2003), are also observed in *pmr1* cells and *spf1* cells (Deurr et al. 1998; Suzuki 2001; Cronin et al. 2002). Both mutants are hypersensitive to the glycosylation inhibitor tunicamycin and the reducing agent DTT, which alter protein folding in the ER and induce UPR. They also showed increased expression of Kar2p, an ER molecular chaperone regulated by the UPR. Under stress, *SPF1* mRNA and Spf1p synthesis are elevated 1.8-fold and 3.3-fold, respectively, following 60 min of tunicamycin treatment (Vashist et al. 2002). Loss of *SPF1* function leads to the constitutive activation of the UPR. By contrast, *PMR1* transcript levels do not change in response to ER stress. This is consistent with the data obtained by whole genome expression analysis. *SPF1* is one of 381 genes co-induced during the unfolded protein response, but *PMR1* is not (Travers et al. 2000). In contrast to the synthetic lethal interaction of *SPF1* with the UPR related genes, a *pmr1* null mutant does not show synthetic lethality with *IRE1* (Vashist et al. 2002).

Vashist et al. analyzed the processing of N-linked oligosaccharide (Vashist et al. 2002). Wild type cells showed mainly the trimmed $Man_8GlcNAc_2$ N-linked oligosaccharide. The *pmr1* and *spf1* single mutants contained $Man_8GlcNAc_2$ and $Man_9GlcNac_2$ oligosaccharide at equal quantities. The *pmr1spf1* double mutant accumulated most prominently the $Man_9GlcNac_2$ oligosaccharide, the N-glycan that does not support degradation when attached to CPY* (mutant carboxypeptidase Y) (Knop et al. 1996; Jakob et al. 1998). Oligosaccharide processing by the Ca^{2+}-dependent mannosidase I is most significantly impaired in the *pmr1spf1* double mutant. Since the untrimmed $Man_9GlcNac_2$ oligosaccharide is abundant in each mutant, a question arises whether the $Man_9GlcNac_2$ oligosaccharide is responsible for the killer resistance. For this question, a strain deleted of the *MNS1* gene coding for the ER mannosidase was examined for the killer sensitivity. This strain has no ability to trim $Man_9GlcNac_2$ to $Man_8GlcNAc_2$ oligosaccharide in the ER. However, the strain was found sensitive to SMKT (Suzuki, unpublished), suggesting that the $Man_9GlcNac_2$ oligosaccharide is not responsible for the SMKT resistance.

Protein trafficking from the ER to vacuole was monitored through the processing of pro-form to the mature vacuolar form of carboxypeptidase Y (CPY) (Vashist et al. 2002). The maturation of CPY was delayed in both the *spf1* and *pmr1* mutants and was most disrupted in the *pmr1spf1* double mutant, in which the

UPR is constitutively activated. This result apparently contradicts the other result that constitutive activation of the UPR by the overexpression of *IRE1* suppresses the *sec* mutants defective in vesicle budding from the ER but not others (Sato et al. 2002). Sato et al. suggested the possibility that induction of various target genes by the UPR increases the capacity of ER export, leading to alleviation of the defect of vesicle budding from the ER. It has also been reported that conditions that activate the UPR and elevate Kar2p expression lead to extracellular secretion of Kar2p (Belden and Barlowe 2001). It has been found that extracellular secretion of Kar2p was increased in *spf1* mutant, and that non-glycosylated CPY was secreted in the case of *spf1pmr1* double mutant (Suzuki, unpublished). These findings indicate that protein trafficking together with glycosylation is affected in the absence of the cooperate control of Spf1p and Pmr1p.

5 Killing and resistant mechanism of SMKT

5.1 SMKT interacts with the cell surface of resistant cells

In spite of the several attempts to find specific cell-surface molecules that might interact with SMKT, no binding affinity between SMKT and the cell walls of wild type cells have been detected, suggesting that the interaction between SMKT and its receptor is very weak or is different from those known of killer toxins and their cell wall receptors. To clarify this point, the interaction of SMKT with the wild type, *spf1*, *pmr1*, and *pmr1spf1*, cells was analyzed using fluorescence-activated cell sorting (FACS) analysis. The cells were incubated in the presence or absence of SMKT and fixed with formaldehyde to detect faint interaction. Fixed cells were incubated with rabbit anti-SMKT β subunit antibody, followed by FITC-conjugated goat anti-rabbit IgG. The SMKT that interacts with cell surface was then analyzed by FACS (Suzuki and Shimma 1999). No SMKT binding was observed in the wild type cells. In contrast, however, the FACS profile of the *spf1* cells showed significant SMKT binding. On the other hand, the *pmr1* cells showed a profile intermediate between the wild type and the *spf1* cells. The *pmr1spf1* cells showed the same FACS profile as the *spf1* cells. These results indicate that SMKT binds to the resistant cells, but not to the sensitive cells. Without fixation, no such interaction was observed, suggesting that the interaction is very weak.

Binding of SMKT was confirmed by fluorescence microscopy. Consistent with the FACS analysis, the FITC signal was observed only at the cell surface of the resistant cells and not on the wild type cells. Interestingly, no FITC signal was observed at the buds of resistant cells. Only the cell surface of the mother cells of resistant mutants was stained by indirect immunofluorescence, suggesting that mother-cell-specific molecules may interact with SMKT.

This is the first finding that provides clear evidence that the killer toxin interacts with the cell surface of the resistant cells, and that it is distinct from other toxins that interact with specific receptors of the cell wall of the sensitive cells. A possible interpretation for this interaction is that the cell wall components that

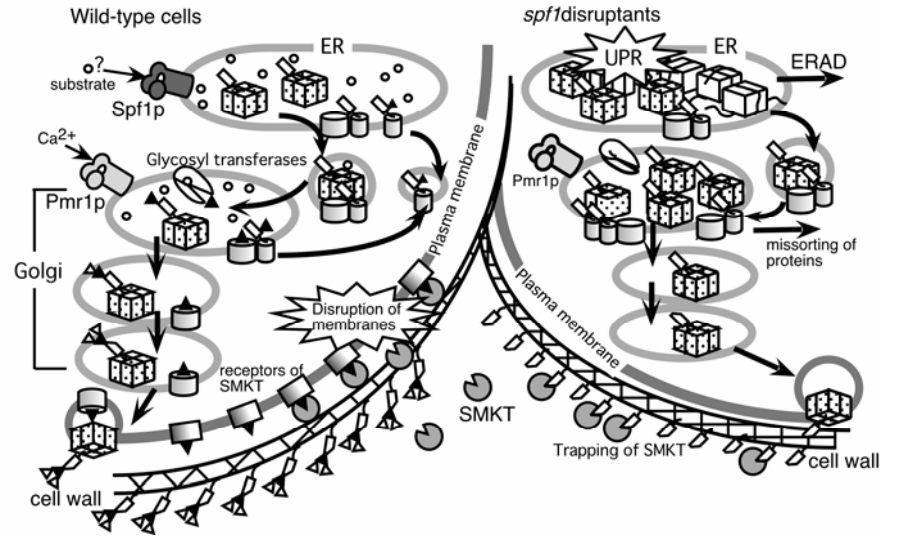

Fig. 4. What happens in the secretory pathway in the presence or absence of Spf1p? The port of entry for proteins destined for the secretory pathway is the ER, where Spf1p controls the homeostasis through an unidentified substrate. In the wild type cells, secretory proteins represented as parcels, and membrane proteins represented as cylinders including the putative SMKT receptor are folded and glycosylated in the ER. Sugar chains are represented as tags and triangles. Proteins are properly processed in the secretory pathway and transported to destination sites. In the absence of Spf1p, misfolded proteins accumulate in the ER, which consequently leads to perturbation of glycosylation and protein trafficking. Because of this, the putative SMKT receptor may not reach the plasma membrane and abnormal cell wall components may trap SMKT, conferring the cells with SMKT resistance.

mimic the true target molecule of SMKT may trap SMKT molecule at the surface and sequester it away from the target where it performs its toxic effect (Fig. 4). Although *spf1* cells accumulates the $Man_9GlcNac_2$ oligosaccharide, the oligosaccharide is not responsible for the resistance as mentioned above, suggesting that the loss of Spf1p affects other glycosylation enzymes and/or assembly of cell wall structure. It is very interesting to note that the binding of SMKT was observed only at the cell surface of the mother cells but not in the buds. Since loss of Spf1p also affects protein trafficking, it is possible that the molecule involved in the interaction with SMKT may have congested the mother cells.

5.2 Interaction of SMKT with cell membranes

SMKT that was trapped on the surface of the resistant cells was detected by means of fluorescent-labeled IgG, but SMKT that should be attacking the sensitive cells was not detected by the method. Under the conditions employed, no intracellular SMKT was detected because of the barrier function of the cell wall. Therefore, it is reasonable to conclude that SMKT that interacts with the cell membrane under

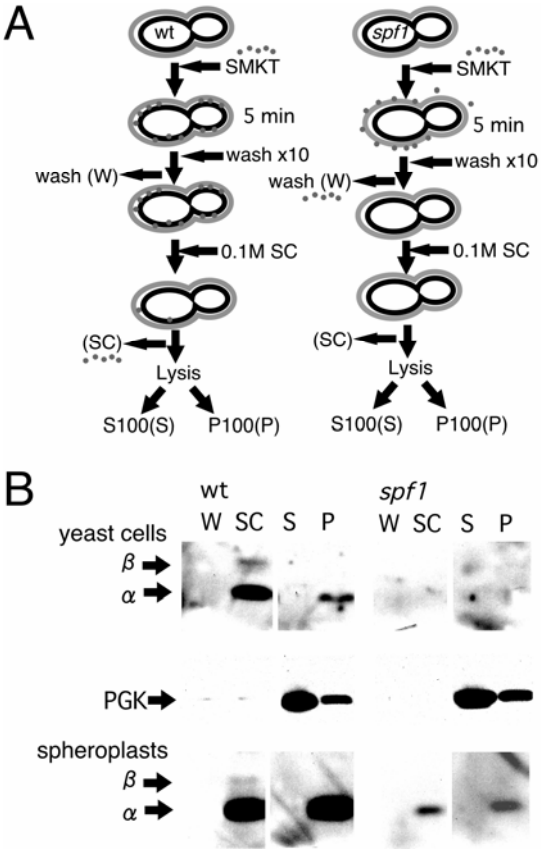

Fig. 5. Interaction of SMKT with sensitive wild type cells. (A) Schematic drawing of the flow of experiment and hypothesis of the phenomenon deduced from the result shown in B. Cells were treated with SMKT for 5 minutes, washed 10 times with a buffer. The final washing solution was kept for analysis (W). After extraction with 0.1 M sodium carbonate solution (SC), cells were lyzed and centrifuged at 100,000 x *g* to separate the S100 (S) and P100 fractions. The same experiment was performed using spheroplasts prepared from the wild type cells and *spf1* disruptants. (P). (B) Immunoblotting of each sample described in A. The α and β subunits of SMKT and 3-phosphoglycerate kinase (PGK) as a cytosol marker were analyzed by immunoblotting.

the cell wall could not be detected in the case of the sensitive cells, and that only SMKT superficially interacting with the resistant cells could be detected. Three approaches were taken to examine the interaction of SMKT with the membrane. First, direct interaction of SMKT with artificial liposomes was observed. Second, interaction of SMKT with yeast cell membrane was analyzed using sodium carbonate extraction. Then, the killing effect of SMKT on spheroplasts was examined to investigate the role of cell wall components in SMKT sensitivity.

SMKT has a pH sensitive structure and an optimum pH in the acidic range. Liposomes prepared using a buffer at pH 4.5 are a powerful tool to overcome the pH sensitivity and to analyze the interaction between SMKT and the cell membranes (Suzuki et al. 2001). Leakage of fluorescence-dye, calcein, from calcein-entrapped liposomes was observed in the presence of 0.1-5 μM SMKT. Leakage increased with increasing SMKT concentration. The effect of SMKT was less than half that of mastoparan-X, a tetradecapeptide toxin from hornet venom. No significant difference in calcein leakage was observed in various liposomes prepared from different composition of phospholipids, suggesting that SMKT nonspecifically destabilized liposomes. The process of destruction of liposomes in the presence of SMKT was directly observed under a dark-field microscope. When SMKT was added to the liposome suspension, a rapid morphological change was observed. The outer lamella of huge multi lamellar vesicles separated off and divided into small vesicles very rapidly, which then form aggregates of small vesicles. The effect of SMKT on membranes of yeast cells *in vivo* was also investigated by dark-field microscopy. In the absence of SMKT, each yeast cell appeared as a ring-like structure with a clear-cut outline, indicating that the yeast cells retained a spherical shape. However, SMKT-treated sensitive cells appeared hazy as turbid spheres, indicating that the spherical membrane structure was destroyed by SMKT. The number of cells with a turbid image increased with increasing concentrations of SMKT and prolonged incubation time. Although maximum leakage from LUV was less than 5% in the presence of 0.1 μM of SMKT, approximately 60-70% of the sensitive cells treated under same condition showed a turbid image. On the other hand, *spf1* disruptants showed a ring-like image and cells with a turbid image were not observed even in the presence of 1 μM SMKT. These results indicate that Spf1p is involved in the synthesis of the sensitivity determinants that are missing in the liposomes.

SMKT that interacts with the cell membrane under the cell wall was not detected by mean of fluorescent-labelled IgG. However, because disruption of cell membranes was observed by dark-field microscopy, it is believed that SMKT could have reached the membranes of the sensitive cells. Such SMKT that interacts with the sensitive cells was stripped off by sodium carbonate (SC) extraction (Suzuki et al. 2001) (Fig. 5). The sensitive wild type cells were treated with 1 μM SMKT for 5 min, washed with citrate-phosphate buffer (pH3.5) containing 1 M sorbitol until unbound SMKT was no longer detected in the washing solution. Then cells were treated with 0.1 M SC, which is typically used to extract proteins that are associated with membranes in a peripheral manner (Fujiki et al. 1982). Immunoblotting showed that both the α and β subunits of SMKT were extracted by SC-treatment from wild type cells. Since a cytosolic marker protein was not detected in SC-treated fractions, it could say that SC-treatment failed to cause cell lysis. This result suggests that SMKT is associated with the cell surface in a peripheral manner. Both the α and β subunits were extracted from the SMKT-treated wild type spheroplasts by SC-treatment as was observed in the intact cells, indicating that the cell walls are not involved in such interaction. These data provided a direct evidence for SMKT interaction with the membrane of sensitive cells.

On the other hand, neither the α nor β subunit was extracted by SC treatment from *spf1* cells nor detected in the cell lysates, suggesting that SMKT trapped on the cell walls was washed out during the washing process. A small amount of the α subunit was detected from spheroplasts of the *spf1* cells. Because *spf1* cells acquire resistance by trapping SMKT on the cell walls, SMKT may have interacted to some extent with the spheroplasts in the absence of the resistant cell walls. However, since more SMKT was extracted by SC-treatment in the case of wild type spheroplasts, it could be possible that SMKT preferably interacts with the membranes of sensitive cells.

To determine whether cell wall plays a role in the killing activity of SMKT, the killing effects of SMKT on intact cells and spheroplasts were examined. In the case of intact cells, the viability of the wild type cells was 3.6% after treatment with 5 μM SMKT for 1 hour, whereas that of *spf1* disruptants was 80%. The regeneration rate of the wild type spheroplasts was 28% in the absence of SMKT and was 1.4% after treatment of 5 μM SMKT for 1 hour, suggesting that wild type spheroplasts are sensitive to SMKT. On the other hand, the regeneration rate of *spf1* spheroplasts was in the range of 2-3% both in the presence and absence of SMKT, while less than 5% of intact cells remained in the spheroplasts. These results indicate that Spf1p is also involved in the regeneration of spheroplasts. However, it is difficult to conclude that *spf1* spheroplasts are resistant to SMKT when the regeneration rate is less than 5% because it is impossible to rule out the possibility that the regenerated colonies were derived from residual intact cells.

A receptor-mediated two-step model (Schmitt and Breinig 2002) is not likely to fit for that of SMKT. In the model, the toxin binds to a specific receptor and is then transferred to the plasma membrane where the toxin disrupts the membrane integrity. Binding to a specific cell wall component is thought to confer target specificity, although how a killer toxin moves from the cell wall to its target membrane remains unknown. In the case of SMKT, although spheroplasts are sensitive to SMKT, a specific receptor on the cell wall may not be involved in the killing. There are a few possibilities that may be considered regarding SMKT killing effect. A specific target molecule may be synthesized and transported to the membrane in the presence of Spf1p. Otherwise, it may not be synthesized and/or properly localized to the membrane in the absence of Spf1p. The loss of Spf1p may then perturb ER function that could consequently trigger the secretory pathway to accumulate abnormal cell wall components that mimic the true target molecule of SMKT (Fig. 4). Identification of SMKT binding molecule should give us a clue to understanding not only the killing mechanism of SMKT, but also the role of Spf1p on cell surface structure.

6 Concluding remarks

SMKT has a unique subunit structure that is sensitive to neutral pH. Biochemical approaches have shown the maturation process of SMKT from chromosomally encoded preprotoxin by enzymatic processing. An observation of turbidity in a ba-

sic solution of SMKT led various physicochemical approaches to elucidate the molecular dynamics of subunits dissociation under neutral and basic conditions. These studies have revealed the interaction between the hydrophobic α subunit and the hydrophilic β subunit to maintain a single domain structure where carboxyl groups play an important role.

Through the analysis of SMKT resistant mutants of *S. cerevisiae*, the *SPF1* gene encoding a novel type of P-type ATPase was identified. In cooperation with Pmr1p, Spf1p is involved in various indispensable functions in the ER by maintaining ER homeostasis. Loss of control of Spf1p results in perturbed glycosylation, accumulation of misfolded proteins and altered protein trafficking. ER is the port of entry for proteins destined for the secretory pathway where proteins are folded and glycosylated. Aggregation of misfolded proteins that escape the quality control mechanism of the ER is a common feature of 'protein-misfolding diseases', such as prion diseases, diabetes and cancer (Selkoe 2003). Identification of unknown substrate of Spf1p and SMKT binding molecule would elucidate the control mechanism of the ER homeostasis and the roles of the ER function on cell surface structure.

The study on SMKT and Spf1p may be a specific example. Proteins that lose their structure at neutral pH and as well as those that cannot be expressed in heterologous hosts are usually ruled out from various systematic analyses. However, as each killer yeast has selected a protein that interacts with a specific target and/or a specific receptor as an effective weapon, such specificity may be a clue to unknown biological processes, which are not yet uncovered by large-scale analyses.

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Ricin: structure, synthesis, and mode of action

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Abstract

Ricin is a lectin produced by the seeds of the *Ricinus communis* plant. It is potently toxic to mammalian cells, where it acts to inhibit the essential process of protein synthesis. Structurally, ricin is a heterodimer comprised of an enzymatic polypeptide (the A chain) disulphide bonded to a cell-binding lectin (the B chain). After surface binding, the holotoxin is internalised to endosomes from where a small fraction can be transported by a retrograde route to the endoplasmic reticulum (ER). After reduction in the ER lumen, the A chain is rendered competent for translocation to the cytosol and, whilst most is apparently degraded there, a proportion evades degradation to refold and inactivate ribosomes. In this review we present our current understanding of the biosynthesis and mode of action of this highly cytotoxic plant protein.

1 Discovery

It had been known for some time that extracts of the castor oil (*Ricinus communis*) seeds were poisonous to humans when, in the nineteenth century, Hermann Stillmark began his work to characterise the component(s) responsible for this toxicity. Stillmark mixed an extract of castor bean seeds with blood and observed a clumping of erythrocytes. He further showed that the causal agent for this was a protein, which he named 'ricin' (Stillmark 1888). Ricin was, therefore, the first described haemagglutinin, a discovery that opened the field of lectin research. The original lectins were termed phytohaemagglutinins, because they were initially found exclusively in plants. We now know that lectins are widespread throughout various phylogenetic kingdoms, and that Stillmark's ricin preparation contained both the potently cytotoxic, but weakly agglutinating dimeric protein ricin (*Ricinus communis* agglutinin II), and the closely related non-cytotoxic, but strongly agglutinating tetrameric protein *Ricinus communis* agglutinin I (Olsnes 1978). Stillmark's report describing ricin came to the attention of Paul Ehrlich who recognised the potential of a plant agglutinin as a model antigen. In a series of experiments that established some of the fundamental principles of immunology, Ehrlich demonstrated that mice became immune to a lethal dose of ricin after having received repeated non-toxic doses of the same preparation (Ehrlich 1891).

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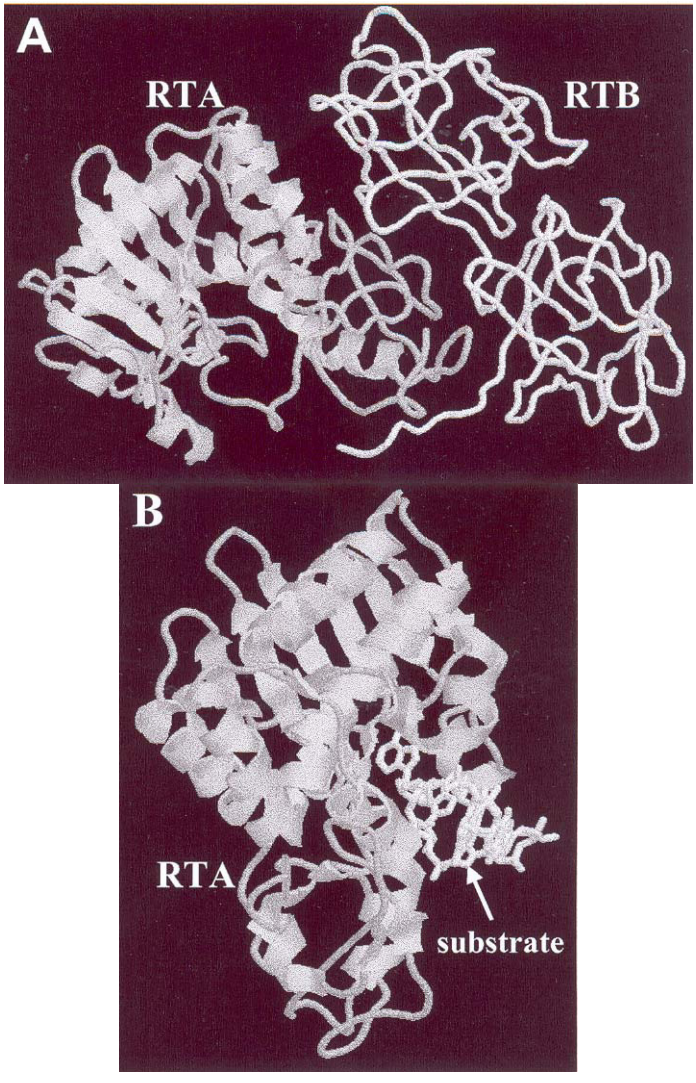

Fig. 1. Structure of ricin and substrate binding model. A: The α carbon backbones of ricin A chain (RTA) and ricin B chain (RTB) B: Model (as proposed in Monzingo and Robertus 1992) showing docking of the tetraloop into the active site of RTA.

2 Ricin structure

Ricin is a dimeric glycoprotein in which the 32 kDa A polypeptide/chain is covalently joined by a disulphide bond to the 34 kDa B polypeptide/chain. The 267

Table 1. Ricin A chain acts on a conserved loop in 23/25/28S ribosomal RNA

Source	Sequence
Rat 28S	4318 AGUACG**A**GAGGAAC 4331
Yeast 25S	3018 AGUACG**A**GAGGAAC 3031
E. coli 23S	2654 AGUACG**A**GAGGAAC 2667
Rice 25S	3011 AGUACG**A**GAGGAAC 3024
Tomato 25S	3018 AGUACG**A**GAGGAAC 3031

The target adenine removed by ricin A chain is shown in bold

residue **r**icin **t**oxin **A** chain (or RTA) and the 262 residue **r**icin **t**oxin **B** chain (RTB) each contain two potential *N*-glycosylation sites, and either no or four intrachain disulphide bonds, respectively. The primary sequence of each chain of various isoforms have been directly determined by protein sequencing (Funatsu et al. 1978, 1979) and deduced by cDNA- (Lamb et al. 1985) and genomic- (Halling et al. 1985; Tregear and Roberts 1992) cloning, and the X-ray crystallographic structure of ricin and ricin A chain have been determined (Montfort et al. 1987; Weston et al. 1994). While RTA has three structural domains with approximately 50% of the polypeptide arranged into α-helices or β-sheet (Katzin et al. 1991; Mlsna et al. 1993), RTB, a protein that probably arose after a gene duplication event, forms a bilobal structure lacking both α-helices and β-sheets (Villafranca and Robertus 1981) (Fig. 1A). Figure 1B shows a proposed model for the docking of the ribosomal RNA tetraloop into the active site of RTA.

3 Function of the ricin subunits

Ricin is a member of the so-called A-B toxin family in which a catalytic A subunit is associated with one or more cell-binding B subunits. RTA is a ribosomal RNA *N*-glycosidase that removes a specific adenine from 28S rRNA (A4324 in the case of rat liver 28S rRNA), and is, therefore, referred to as a ribosome-inactivating protein (RIP) (Endo and Tsurugi 1987) The target adenine lies in a tetraloop (G<u>A</u>GA) within a universally conserved stretch of 12 ribonucleotides known as the α-sarcin/ricin loop (SRL) (Szewczak et al. 1993) (Table 1) that is crucial for the binding of elongation factors (Moazed et al. 1988). Removal of this adenine leaves the phosphodiester backbone of the rRNA intact (Endo and Tsurugi 1987), but the ribosomes containing it are no longer able to synthesise protein. In this way, it is estimated that ~1,500 polysomes are inactivated per minute per RTA molecule (Endo and Tsurugi 1988).

RTA is not able to modify *all* ribosomes however, which highlights a possible role for ribosomal proteins. For instance, whilst RTA is active against naked 23S

prokaryotic rRNA, bacterial ribosomes are completely refractory (Nilsson and Nygard 1986), and plant ribosomes are ~5,000-fold less sensitive to this protein than mammalian ribosomes (Cawley et al. 1977; Harley and Beevers 1982; Taylor et al. 1994). The most plausible explanation for these substrate specificity differences is that particular ribosomal proteins may permit or hinder access of the toxin to its target SRL. It has also become clear that ricin can modify non-ribosomal nucleic acid substrates *in vitro* and in human endothelial cells, allowing it to be classified a polynucleotide:adenosine glycosidase (Barbieri et al. 1997; Brigotti et al. 2002). The physiological relevance of other enzyme activities currently ascribed to ribosome inactivating proteins like RTA is unclear (Morlon-Guyot et al. 2003).

RTB is a lectin that is responsible for binding the ricin holotoxin to appropriate complex carbohydrates containing terminal *N*-acetyl galactosamine or β-1,4-linked galactose residues exposed at the cell surface (Olsnes and Pihl 1982). The association constants for complex glycans are typically in the order of 1.10^7 M^{-1}. The relative contribution of glycoproteins and/or glycolipids as ricin binding sites on mammalian cells is unclear, although a role for glycosphingolipids as receptors has recently been ruled out (Spilsberg et al. 2003). Ricin can also bind some cells through its own mannose-rich N-glycans (Simmons et al. 1986; Magnusson et al. 1993).

4 Ricin biosynthesis

Ricin is located in the protein storage vacuoles of the endosperm cells of mature *Ricinus communis* (castor oil) seeds (Tulley and Beevers 1976; Youle and Huang 1976). In common with other storage protein components of the vacuole, ricin is synthesised during seed maturation and is hydrolytically degraded during the first few days of post-germinative growth (Roberts and Lord 1981). There are several isoforms of ricin including ricin D, ricin E, and the closely related lectin *Ricinus communis* agglutinin (RCA). Together they account for about 5% of the total particulate protein present in mature seeds. These proteins are encoded by a small multigene family composed of approximately eight members, some of which are non-functional (Tregear and Roberts 1992).

Although ricin is a heterodimer, its individual A and B subunits are initially synthesised together as part of a single precursor polypeptide (Fig. 2) (Butterworth and Lord 1983). This precursor contains 576 amino acids, the first 26 residues being a signal peptide to direct co-translational import of the nascent protein into the ER (Ferrini et al. 1995). This signal peptide is followed by a 9 residue propeptide, mature RTA, a short intramolecular propeptide and RTB (Lamb et al. 1985). Within the ER lumen, nascent proricin becomes core-glycosylated (Lord 1985a) and disulphide bonded (Roberts and Lord 1981) before being transported to the vacuoles via the Golgi (Lord 1985b). This intracellular transport is accompanied by several poorly characterised modifications, including trimming of oligosaccharides and the addition of fucose to RTA (Lord and Harley 1985). Upon reaching the vacuole, the mature disulphide-linked RTA-RTB dimer is generated by the

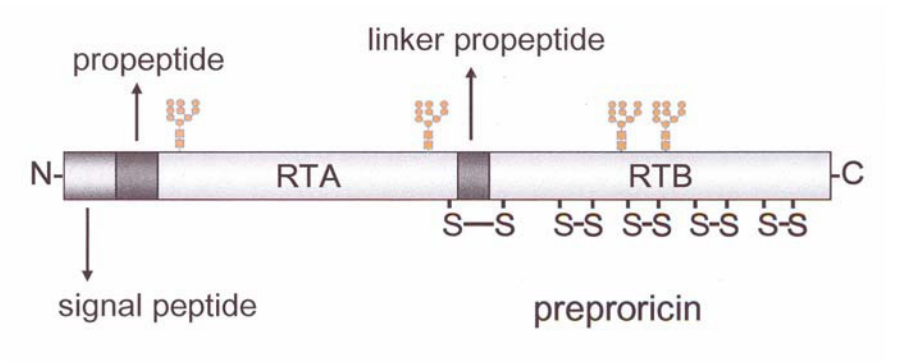

Fig. 2. The preproricin precursor. The signal peptide is removed upon co-translational segregation in the ER while the two propeptides are removed after delivery into storage vacuoles. Approximate positions of disulphide bonds and *N*-linked glycans are indicated.

endoproteolytic removal of the *N*-terminal and internal linker propeptides (Harley and Lord 1985).

While the role of the N-terminal propeptide in proricin remains unclear, the 12 residue linker peptide that lies between RTA and RTB has been shown to be both necessary and sufficient for targeting the proricin precursor to the storage vacuoles (Frigerio et al. 1998b, 2001). This vacuolar targeting signal is atypical in a number of respects. Firstly, it is intramolecular, whereas other defined vacuole targeting signals are located at the N- or C-terminus (Vitale and Raikhel 1999). Secondly, it contains LIRP (single letter amino acid code), a sequence more closely resembling the sorting signal (NPIR-like) used by proteins destined not to storage vacuoles but to the lytic vacuoles of vegetative plant tissues. The finding of a sequence-specific signal with a critical Ile (Frigerio et al. 2001) implicates the BP-80 family of vacuolar sorting receptors for the correct targeting of proricin to PSV, whereas hitherto these receptors were believed to be involved only in the targeting of NPIR-containing proteins to the lytic vacuoles, a process that involves the formation of clathrin coated vesicles. Thirdly, the position of the ricin targeting signal is functionally important, in that only when it is intramolecular or N-terminal does it function in a stringent sequence-specific manner (Jolliffe et al. 2003). The trafficking steps in the targeting of proricin to storage vacuole are summarised in Figure 3.

Recombinant proricin is a functional lectin able to bind galactose, but it lacks RNA *N*-glycosidase activity (Richardson et al. 1989). This is consistent with reports that in the mature ricin, the RTA subunit must be released from RTB to have catalytic activity (Lewis and Youle 1986; Wright and Robertus 1987). It appears that in both ricin holotoxin and proricin, RTB can sterically obstruct the active site of RTA. Biosynthesis in the form of an inactive precursor perhaps explains why *Ricinus* endosperm cells are able to synthesise large amounts of ricin even though endogenous ribosomes are susceptible to its action (Harley and Beevers 1982). However, the plant cell must also protect itself from errors in preproricin mRNA synthesis, processing, translation, and inefficient protein folding that may lead to

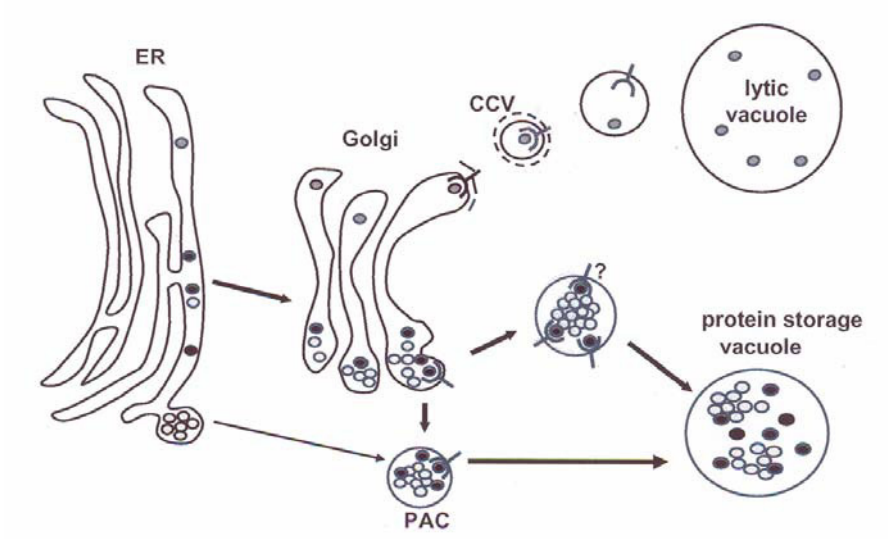

Fig. 3. Model for the targeting of proricin to plant vacuoles. Proricin (•) is transported from the ER through the Golgi for either delivery into precursor accumulating vesicles (PAC) that arise from the ER (Hara-Nishimura et al. 1998), or to be carried by other transport intermediates, to the protein storage vacuoles. The sequence-specific nature of the proricin targeting signal supports the idea of transport being receptor-mediated from the Golgi, although receptor interactions (denoted by ?) have not yet been demonstrated. Other castor bean storage proteins may follow a similar route or may aggregate within the ER to be transported by direct ER-to-vacuole pathways that are Golgi-independent. Proteins destined for lytic vacuoles are transported in a receptor-mediated manner from the Golgi via clathrin coated vesicles (CCV). In older cells, lytic and storage vacuoles may fuse (not shown).

the generation of truncated or mutated toxin. The danger lies in the propensity of free RTA to translocate the ER membrane (Di Cola et al. 2001). However, the presence of two lysine residues may ensure rapid ubiquitylation and proteasomal degradation of any nascent RTA or RTA fragment produced by premature termination of preproricin mRNA translation that could be accompanied by retrotranslocation or mislocalisation of the protein to the cytosol. If not rapidly destroyed, such active toxin would modify the sensitive *Ricinus* ribosomes leading to ablation of the producing cells.

Since *Ricinus* endosperm cells show no sign of ribosome damage whilst storing large amounts of ricin, it is clear that active RTA does not escape from the low pH vacuoles to reach the cytosol. Indeed, RTA and the interchain disulphide bond between RTA and RTB are structurally stable at low pH (Argent et al. 2000). The generation of catalytically-active ricin only within the confines of the storage vacuoles, therefore, enables *Ricinus* seeds to synthesise and store large quantities of this protein without compromising its own survival. The toxin is presumed to have a defensive role in the seed.

5 Ricin intoxication of mammalian cells

Cell entry by ricin involves the RTB-mediated binding to surface components containing exposed galactosides, and uptake by endocytosis. Following entry into the endosomal system, a proportion of ricin reaches the *trans*-Golgi network (TGN) from where retrograde Golgi to ER transport can occur. To reach the cytosol from the ER, the intrachain disulphide bond in ricin must be reduced to render RTA competent for membrane translocation. Other factors influencing this step are, at present, quite poorly understood, but preliminary findings indicate that RTA can exploit the machinery for perceiving and transporting misfolded ER proteins to the cytosol. Once in the cytosol, the toxin appears to evade degradation by minimising ubiquitylation that would otherwise target it to proteasomes, and by refolding into a protease-resistant, active conformation.

5.1 Endocytosis to the Golgi

When present at the cell surface, ricin binds to complex glycans containing terminal *N*-acetylgalactosamine or β 1,4-linked galactose residues (Olsnes and Pihl 1982). Most mammalian cells contain many different glycoproteins and glycolipids able to bind ricin. For example, HeLa cells possess around 3 x 10^7 potential ricin binding sites (Sandvig et al. 1976), although not all of these would be involved in a productive internalisation of toxin. The relative contribution of glycoproteins and glycolipids as toxin binding sites is unclear, although, as noted earlier, glycosphingolipids do not appear to act as ricin receptors (Spilsberg et al. 2003). Ricin is also able to bind to and enter some cell types by virtue of its own N-linked glycans. A limited number of cell lines express surface mannose receptors to which ricin, with high mannose glycans typical of plant glycoproteins, can bind. Mannose receptor-mediated uptake of ricin into both macrophages (Simmons et al. 1986) and rat liver endothelial cells (Magnusson et al. 1993) has been demonstrated.

Because of its opportunistic surface binding, ricin becomes localised to all types of plasma membrane invaginations (Sandvig and van Deurs 1996), and as such has been valuable in the study of endocytosis (Sandvig et al. 2002). The binding and internalisation of ricin by Vero cells was first visualised in clathrin-coated pits using a ricin-gold conjugate (van Deurs et al. 1985). Indeed, ricin uptake is reduced, though not abolished, in cells acidified to inhibit uptake from these structures (Sandvig et al. 1987) or in cells expressing antisense to clathrin heavy chain (Iversen et al. 2001). It is now well established that in addition to clathrin-mediated uptake, clathrin-independent mechanisms are also responsible for internalising this toxin (Moya et al. 1985). Clathrin-independent endocytosis (Nichols and Lippincott-Schwartz 2001) includes uptake by caveolae (specialised invaginations rich in lipid rafts and the protein caveolin) (Pelkmans et al. 2001) and macropinocytosis (Grimmer et al. 2002), and can depend on dynamin (Henley et al. 1998; Oh et al. 1998; Lamaze et al. 2001), or be independent of dynamin

(Damke et al. 1995). However, if the clathrin-independent mechanisms are perturbed by cholesterol extraction, the cells remain competent to take up ricin (Rodal et al. 1999). The picture that emerges is one in which ricin uses multiple entry pathways, some of which are rather poorly understood in terms of their interplay and regulation. The extent to which a particular internalisation mechanism is exploited by ricin will probably depend on cell type, though it is also recognised that interpretation of studies in which flux through specific entry pathways have been modulated can be complicated by the fact that disruption of one uptake mechanism might actually promote protein entry by a different mechanism (Damke et al. 1995).

Whatever the initial entry mechanism might be, the pathways appear to converge at endosomes (Fig. 4) from where most internalised ricin is either recycled to the cell surface or delivered via late endosomes to lysosomes, presumably for degradation (reviewed in Sandvig and van Deurs 1996). A small fraction of ricin (~5%) enters the Golgi of most cell types where it can be visualised by microscopy (van Deurs et al. 1988) or by biochemical means (Rapak et al. 1997). Indeed, disrupting the Golgi stack with drugs such as brefeldin A (Sandvig et al. 1991; Yoshida et al. 1991; Prydz et al. 1992) and ilimaquinone (Namibar and Wu 1995), or blocking endosome-to-Golgi transport by lowering the temperature (Sandvig and van Deurs 1996), protects cells against ricin showing that transport to or through the Golgi is important for a toxic effect.

So, how does ricin reach the Golgi apparatus? The well established Rab9-dependent pathway that transports the mannose-6-phosphate receptor from late endosomes to the TGN (Lombardi et al. 1993; Riederer et al. 1994) is one possibility. However, ricin transport to the Golgi can be independent of Rab9 (Simpson et al. 1995; Sandvig and van Deurs 2002). Whether this Rab9-independent transport occurs from late endosomes, or from early endosomes as reported for Shiga toxin (Mallard et al. 1998; Mallard and Johannes 2003) and TGN38 (Mallet and Maxfield 1999) is not entirely clear, although the reported Rab7-independence of ricin toxicity would indicate that the latter is more likely (Sandvig et al. 2002). The precise role of specific cellular proteins in the transport of ricin is unclear. Rab6' for example, has been implicated in the direct early endosome to TGN transport step used by Shiga toxin (Mallard et al. 2002), but the Rab6'-dependence of ricin uptake has not yet been determined. Dynamin, the mechanoenzyme that acts in both clathrin-dependent and –independent endocytosis, though not critical for ricin uptake from the cell surface, nevertheless influences intracellular transport of ricin to the Golgi with a concomitant reduction in toxicity (Llorente et al. 1998). Precisely how a dynamin-like protein affects the intracellular sorting of ricin requires further clarification.

5.2 Golgi to ER transport

The small amount of endocytosed ricin that undergoes retrograde transport to the early secretory pathway precludes its visualisation in Golgi cisternae or the ER lumen by conventional microscopy. Instead, this transport has been elucidated us-

ing biochemical assays that monitor protein synthesis inhibition to signal toxin arrival in the cytosol. Cells are protected against ricin when the Golgi is disrupted with chemical reagents (Sandvig et al. 1991; Yoshida et al. 1991; Prydz et al. 1992; Namibar and Wu 1995), or by expression of endogenous GTPase mutants that regulate intra-Golgi trafficking (Simpson et al. 1995). Conversely, the addition of an ER retrieval sequence (the tetrapeptide Lys-Asp-Glu-Lys or KDEL) to the C-terminus of RTA increased the potency of reconstituted ricin holotoxin (Wales et al. 1992; Wesche et al. 1999) and indeed of free RTA (Wales et al. 1993; Zhan et al. 1998) when taken up by fluid phase endocytosis. In both cases, the enhanced sensitivity of cells to ricin suggested an encounter of incoming toxin with KDEL receptors that cycle between the Golgi and ER, and that facilitate this late transport step.

To actually visualise ricin in the ER, it has been necessary to use a form of toxin that can be modified in this compartment and to employ gel analyses of the products. To this end, a recombinant RTA was engineered to contain an appendage carrying a tyrosine sulphation site and additional N-glycosylation sites. Internalisation of unlabelled holotoxin containing the modified RTA in cells supplied with [^{35}S]-sulphate led to a radiolabelling of the fraction passing through TGN where tyrosyl sulphotransferase is found. That Golgi to ER transport subsequently occurred was revealed by core glycosylation of the supplementary glycosylation sequons in the sulphated toxin (Rapak et al. 1997).

The classic pathway of Golgi to ER protein transport involves movement via coatomer protein 1 (COP1)-coated vesicles (Cosson and Letourneur 1994, 1997). A second pathway that is independent of COP1, regulated by Rab6A and exploited by Shiga toxin has also been described (Girod et al. 1999). When 1d1F CHO cells that carry a temperature-sensitive mutation in the ε-COP subunit of COP1 are challenged with ricin at the restrictive temperature, they remain sensitive to intoxication (Chen et al. 2002). Indeed, ricin was shown to intoxicate these cells when both the COP1-dependent and the COP1-independent pathways were simultaneously inhibited (Chen et al. 2002). In the 1d1F cells, ricin appears to use an alternative route from endosomes to the ER that bypasses the Golgi apparatus altogether (Llorente et al. 2003). Although the details of this Golgi-independent route to ER are lacking at present, it is thought not to involve caveosomes - non classical, caveolin-containing endosomes (Nichols et al. 2001).

In the absence of a KDEL sequence, it is not known how ricin accomplishes retrograde transport through the Golgi (Fig. 4). An intracellular role for RTB has been postulated (Newton et al. 1992) in which this subunit is invoked to interact with galactosylated KDEL-containing cellular proteins being salvaged by KDEL receptors, that are found throughout the Golgi (Griffiths et al. 1994). In this way, it is speculated that ricin may hitch a ride to reach the ER lumen. The RTB-dependent intracellular interaction of ricin with calreticulin is a possible example of this (Day et al. 2001), though how and where the toxin switches from its surface-bound receptor to this putative cycling ER chaperone or to other cycling proteins remains unclear.

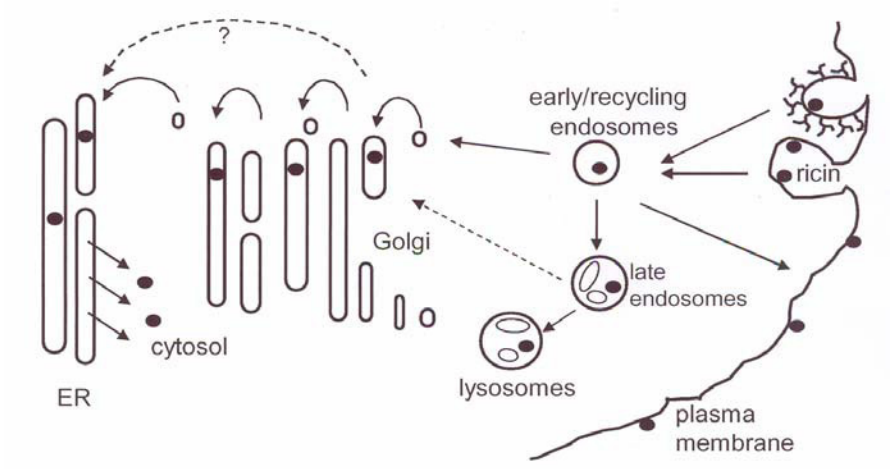

Fig. 4. Schematic of ricin uptake into sensitive mammalian cells. Ricin transport pathways are indicated by bold arrows and, where uncertainties remain, by dashed arrows.

6 Membrane translocation of ricin

To reach ribosomes, ricin holotoxin or at least its catalytic subunit, must cross an intracellular membrane. In 1992, we speculated that certain non-pore forming toxins, including ricin, might need to negotiate the entire endomembrane pathway from the cell surface to ER before entering the cytosol in order to exploit pre-existing, protein conducting machineries in otherwise non-permeable membranes (Pelham et al. 1992). Such machineries might conceivably be the abundant peptide transporters (TAP) (Russ et al. 1995) and/or the protein-transporting Sec61p complexes (Johnson and van Maes 1999) that are uniquely present in the ER membrane. However, TAP-independent cells have been shown to be sensitive to ricin (Sandvig and van Deurs 1996; Smith et al. 2002). The more plausible possibility that Sec61 channels could function in toxin export arose once it was recognised these conduits operate to transport misfolded ER proteins to the cytosol in a process termed retrotranslocation (Tsai and Rapoport 2002). Retrotranslocation is part of an ER quality control pathway known as ER-associated degradation (ERAD), by which terminally aberrant ER proteins are detected and dislocated to the cytosol for degradation by proteasomes (Sommer and Wolf 1997).

Sec61 translocons have now been implicated in the retrotranslocation of ricin (Simpson et al 1999, Wesche et al 1999) and other toxins that reach the ER such as *Peudomonas* exotoxin (JO et al. 2000; Koopman et al. 2000) and cholera toxin (Schmitz et al. 2000) and CHO cell lines with genetic defects in ERAD show an increased resistance to ricin intoxication (Teter and Holmes 2002). However, precisely how and in what form these toxins are targeted to the cytosol is not com-

pletely understood. For example, is ricin holotoxin dislocated prior to its reduction or are its subunits retrotranslocated separately after reduction? Although protein disulphide isomerase (PDI) has been shown to have a role in the reduction of cholera toxin (Majoul et al. 1997; Orlandi 1997; Tsai et al. 2001) and *Pseudomonas* exotoxin A (McKee and FitzGerald 1999) this has only recently been implicated for ricin (Bellisola et al. 2004). For ricin, it also appears that RTA can be retrotranslocated not only as a reduced polypeptide, but one that is physically separated from RTB. This was indicated after co-expression and separate targeting of ricin subunits to the ER lumen of transformed plant cells (Frigerio et al. 1998; Di Cola et al. 2001). In such cells, the pronounced toxic effect that is normally observed when free RTA is retrotranslocated from ER to cytosol can be mitigated when both RTA and RTB are simultaneously expressed. In this situation, heterodimers can be detected in the ER lumen that are incompetent for retrotranslocation (Frigerio et al. 1998). It remains possible that reductive cleavage and disruption of the non covalent interactions between the interface of the two ricin subunits, exposes region(s) within RTA normally masked in ricin that allow this subunit to interact with the membrane, or with other chaperones or the translocon itself. Significant structural changes have been shown to occur in RTA (but not in RTB or holotoxin) when added to artificial liposomes containing negatively charged lipids (Day et al. 2002). However, the physiological significance of this in relation to the endoplasmic reticulum membrane requires further study. It should be noted that the fate of reduced RTB is not yet known.

Studies addressing the folded state of toxins immediately before and during retrotranslocation have to date been largely limited to cholera toxin A1 chain and RTA. For both, it appears that an unfolding step is required. Cholera A1 requires a redox-dependent unfoldase activity of protein disulphide isomerase (PDI) (Tsai et al. 2001), whilst an RTA fusion with folded dihydrofolate reductase was poorly toxic compared with its unfolded counterpart (Beaumelle et al. 1997). In a similar vein, when a catalytically active RTA, engineered to contain an internal cleavage site, was added to cells as a cleaved RTA dimer reconstituted into holotoxin, it was not cytotoxic. However, the non-cleaved equivalent remained highly potent to cells (Marsden et al. 2004). These findings are consistent with the retrotranslocation of an unfolded RTA that cannot reform an active RIP from the unfolded RTA halfmers in the cytosol.

The ultimate fate of ERAD substrates is degradation by proteasomes. Treating cells with proteasomal inhibitors clearly sensitises them to ricin (typically by two to threefold) (Wesche et al. 1999; Smith et al. 2002), suggesting that a significant proportion of the exported toxin is normally degraded. However, since cells are quite clearly killed by RTA when proteasomes are active, cell death must be caused by some sort of uncoupling from the normally tightly linked sequence of steps leading to degradation. In this regard, it was noticed that the catalytic subunits of a number of toxins shown to reach the ER contained few, if any, lysine residues (Hazes and Read 1997). The 267 residue RTA for example, has just two lysines. Indeed, a comparison of the primary sequences of the catalytic subunits of seven cytotoxic type 2 (A-B) RIPs, shows them to contain an average of 2 lysyl residues per polypeptide. In contrast, a comparison of 36 type 1 (A chain only)

RIPs that do not normally enter mammalian cells by endocytosis to reach the ER, contain an average of 18 lysyl residues (Deeks et al. 2002). It was proposed that having such a low number of lysine residues allowed RTA (and other ER retrotranslocating toxins) to exploit the ERAD pathway whilst minimising the chances of lysine-directed ubiquitylation that would otherwise target these proteins to proteasomes. That this unusual uncoupling between transport to the cytosol and degradation was dependent on a low lysine content has been demonstrated by the introduction of additional lysyl residues into RTA and the related abrin A (Deeks et al. 2002) and into cholera A1 (Rodighiero et al. 2002). In all cases, the additional residues promoted enhanced proteasomal degradation, supporting the idea that a low lysine content confers a functional advantage by permitting an extended half-life in the cytosol, long enough for the toxins to damage their target substrates.

Native RTA is a glycoprotein, and from studies of this polypeptide in plant cells, we have shown that glycosylated RTA undergoes retrotranslocation across the ER membrane (Di Cola et al. 2001). Indeed, when proteasomes were inhibited, a deglycosylated RTA was recovered that had been generated by the action of a cytosolic peptide:*N*-glycanase (PNGase, reviewed in Suzuki et al. 2002). Neither yeast nor mammalian cytoplasmic PNGases act on folded proteins *in vitro* (Romisch and Ali 1997; Suzuki et al. 2000). Indeed, recent work has shown that such enzymes can specifically deglycosylate misfolded glycoprotein substrates (Hirsch et al. 1993, 2004), suggesting that the dislocating toxin must be an unfolded protein. Rapid refolding is, therefore, likely to be another means by which toxins can uncouple from the downstream steps of ERAD that lead to degradation. Rapid refolding has been experimentally demonstrated for cholera toxin A1 fragment *in vitro* (Deeks et al. 2002; Rodighiero et al. 2002) and there is some evidence that an encounter with substrate ribosomes can induce the refolding of RTA (Argent et al. 2000). The relative importance of evading ubiquitylation, refolding to a protease-resistant form and, in the case of ricin, of any preferential depurination of (and potential refolding on) membrane-bound ribosomes, remains to be established.

7 Concluding comments

Although much has been learned regarding the structure and mode of action of ricin, many aspects remain to be clarified. Prominent amongst these are the range and regulation of endocytic pathways used by ricin, the nature of any intramolecular carriers and transport intermediates, the interactions that lead to retrotranslocation and uncoupling from the degradation pathway, and an elucidation of the steps that can lead to toxin-induced apoptotic changes in some cell types. Ongoing research is attempting to address each of these issues.

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