

MiniReview

Thiol-activated cytolysins: structure, function and role in pathogenesis

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Abstract

Members of the thiol-activated family of cytolysins are involved in the mechanism of pathogenesis of a number of Gram-positive species. While they are pore-forming toxins, their major pathogenic effects may be more subtle than simple lysis of host cells, and may include interference with immune cell function and cytokine induction. Crystal structure, electron microscopy, mutagenesis and antibody binding studies have led to the modeling of a novel mechanism of pore formation, encompassing membrane-binding, membrane insertion and oligomerization. Despite their designation as thiol-activated cytolysins, it is now clear that thiol activation is not an important property of this group of toxins. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The thiol-activated cytolysin (TACY) family is a prominent group of bacterial toxins, of which streptolysin O (SLO) is the prototype. Members of this group are elaborated by more than 20 species of Gram-positive bacteria [1] (Table 1) and are intimately involved in the pathogenesis of infections by species such as *Arcanobacterium pyogenes*, *Clostridium perfringens*, *Listeria monocytogenes* and *Streptococcus pneumoniae*. These toxins have a number of common characteristics which classify them as a group [2]. They are synthesized as water-soluble, single-chain polypeptides, with molecular masses ranging from 47 to 60 kDa and are lytic for eukaryotic cells by the formation of pores in the cell membrane. The group is named for the sensitivity of their cytolytic activity to oxygen and their activation by reducing compounds (thiol activation). Cholesterol is the major target cell receptor for these toxins and small amounts of free cholesterol can inhibit lytic activity [2]. Members of the group show 30–60% similarity in primary amino acid sequence, and contain an almost

invariant undecapeptide sequence (ECTGLAWEWWR) located near the C-terminus of the protein (Fig. 1).

2. Role of TACYS in bacterial pathogenesis

2.1. TACYS are important virulence factors

TACYS have been implicated in the pathogenesis of infections by a number of Gram-positive pathogens. However, definitive gene knockout experiments to prove the role of TACYS in infection have been performed on only a few Gram-positive pathogens. Insertional disruption of the *ply* gene, encoding pneumolysin (PLY) in *S. pneumoniae*, results in reduced virulence in several mouse models [3]. These mutants are less able to damage the alveolar capillary barrier, multiply in the lungs and escape into the bloodstream [3]. *L. monocytogenes* is the only true intracellular pathogen that produces a TACY. Tn916 insertion mutants of the *hly* gene, which encodes listeriolysin O (LLO), are avirulent, as LLO is required for bacterial escape from the phagolysosome [4]. A pyolysin (PLO)-deficient mutant of the opportunistic pathogen *A. pyogenes* is significantly reduced for virulence in an intraperitoneal mouse model [5]. Although *C. perfringens* phospholipase C is the prominent factor in the pathogenesis of myonecrosis, insertional inactivation of *pfoA*, the gene encoding perfringolysin O (PFO), results in a more moderate infec-

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Table 1
List of known members of the thiol-activated cytolysin family

Organism	Toxin	Abbreviation	GenBank accession number
<i>Arcanobacterium pyogenes</i>	Pyolysin	PLO	U84782
<i>Bacillus cereus</i>	Cereolysin	CLY	D21270
<i>B. thuringiensis</i>	Thuringiolysin O		
<i>Brevibacillus laterosporus</i>	Laterosporolysin		
<i>Clostridium bifermentans</i>	Bifermentolysin		
<i>C. botulinum</i>	Botulinolysin		
<i>C. chauvoei</i>	Chauveolysin		
<i>C. histolyticum</i>	Histolyticolysin		
<i>C. novyi</i> type A	Oedematolysin		
<i>C. perfringens</i>	Perfringolysin O	PFO	M36704
<i>C. septicum</i>	Septicolysin O		
<i>C. tetani</i>	Tetanolysin		
<i>Listeria ivanovi</i>	Ivanolysin O	ILO	X60461
<i>L. monocytogenes</i>	Listeriolysin O	LLO	M29030
<i>L. seeligeri</i>	Seeligerilysin O	LSO	X60462
<i>Paenibacillus alvei</i>	Alveolysin	ALY	M62709
<i>Streptococcus canis</i>	Streptolysin O	SLO	D16825
<i>S. equisimilis</i>	Streptolysin O	SLO	D16824
<i>S. intermedius</i>	Intermedilysin	ILY	
<i>S. pneumoniae</i>	Pneumolysin	PLY or PLN	X52474
<i>S. pyogenes</i>	Streptolysin O	SLO	M18638
<i>S. suis</i>	Suilysin	SLY	Z36908

tion in a mouse model, with decreased muscle necrosis [6] and reduced vascular leukostasis [7].

2.2. Cytotoxic effects of TACyS

TACyS were originally described as hemolysins since they show lytic activity against erythrocytes from a number of animal species. However, the primary targets for TACyS in pathogenesis may be cells involved in defending the host from infection. PLY interferes with the phagocytic and ciliary clearance of pneumococci, probably due to its cytotoxicity for human immune cells, and respiratory endothelial and epithelial cells [8]. PFO causes vascular injury through a direct cytotoxic effect on host inflamma-

tory cells and tissues [9]. PLO is cytotoxic for murine macrophages, and bovine and ovine polymorphonuclear leukocytes (PMNs) [5], while SLO of *S. pyogenes* is cytotoxic for human PMNs [10]. TACyS can also be cytotoxic for different cell types by different mechanisms. Invasion of dendritic cells or hepatocytes by *L. monocytogenes* results in cell apoptosis, promoted by LLO [11]. In contrast, *L. monocytogenes* apparently kills bone marrow-derived macrophages by delayed necrosis following multiplication in the cytoplasm, a process also reliant on LLO [12].

2.3. Other effects of TACyS

In addition to direct cytotoxic effects, TACyS are also capable of interfering with host defense and immune cell function including the migration, respiratory burst, phagocytic ingestion and bacteriocidal activity of phagocytes and the proliferative responses of lymphocytes (Table 2). These effects can be observed even at sub-lytic concentrations of TACyS, which may be more representative of toxin concentrations in vivo, but it is likely they still require the formation of pores in immune cell membranes, thus disrupting normal immune cell function. Therefore, the major effects of TACyS in pathogenicity may be more subtle than host cell destruction by lysis. Interference with immune cell functions, especially in the early stages of infection, prevents clearance of the pathogen and allows the invading organism to home to a preferred niche.

TACyS can also modulate the inflammatory response by inducing the expression of cytokines and other inflammatory mediators (Table 2). PLY can trigger expression and release of cytokines at femtomolar concentrations,

SLO	529	E	C	T	G	L	A	W	E	-	W	W	R
PLY	427	E	C	T	G	L	A	W	E	-	W	W	R
SLY	455	E	C	T	G	L	A	W	E	-	W	W	R
LLO	483	E	C	T	G	L	A	W	E	-	W	W	R
LSO	484	E	C	T	G	L	F	W	E	-	W	W	R
ILO	458	E	C	T	G	L	A	W	E	-	W	W	R
PFO	458	E	C	T	G	L	A	W	E	-	W	W	R
ALY	460	E	C	T	G	L	A	W	E	-	W	W	R
CLY	465	E	C	T	G	L	A	W	E	-	W	W	R
PLO	491	E	A	T	G	L	A	W	D	P	W	W	-
ILY	485	G	A	T	G	L	A	W	E	P	W	-	R

Fig. 1. The conserved undecapeptide sequence of TACyS. Alignment of the undecapeptide sequence of TACyS for which sequences are available. Amino acids which match the consensus undecapeptide sequence are boxed. Amino acid numbers are given for the first amino acid in the sequence for each protein. Toxin abbreviations and GenBank accession numbers are shown in Table 1. The ILY undecapeptide sequence was obtained from Nagamune [48]. Amino acid sequences of SLO molecules from *S. canis*, *S. equisimilis* and *S. pyogenes* are identical over the region shown.

and is one of the most potent cytokine-inducing bacterial molecules, even more so than bacterial LPS [13]. Both PLY and SLO activate components of the arachidonic acid cascade in host defense cells [3,14] (Table 2), which contributes to the inflammatory response by the dilation of blood vessels at the site of infection. The formation of pores in cell membranes results in Ca^{2+} influx, which may stimulate intracellular signal transduction pathways, leading to the expression of cytokines and other inflammatory mediators, possibly through the induction of stress-sensing transcriptional factors such as NF- κ B [15,16]. Inhibition of pore formation by preincubation of LLO with cholesterol does not affect the ability of LLO to induce cytokine expression [17]. However, cholesterol-inactivated LLO is still able to partially insert into membranes [18], and thus may perturb the integrity of the membrane.

The pathogenic advantage of cytokine induction and stimulation of the inflammatory response is not immediately obvious, although inflammation is often a key process in diseases caused by, for example, *S. pneumoniae*, *S. pyogenes*, *C. perfringens* and *A. pyogenes*. Since the cytokine network effectively controls the host inflammatory response, TACYs may act as regulators of bacterial invasion [13]. Cytokines may promote bacterial infection, as tumor necrosis factor- α (TNF- α) and interleukin-1 α (IL-1 α) enhance the adherence of *S. pneumoniae* to respiratory epithelium, which may contribute to diseases such as otitis media [19]. There is also evidence that cytokine induction may promote the intracellular survival and growth of ingested bacteria, which would normally be killed by phagocytic cells [20].

PLY can directly activate the classical complement pathway, in the absence of specific antibody, by binding directly to the Fc region of human IgG [21]. Complement

activation may lead to direct complement-mediated attack on host cells [3]. The complement-activating ability of PLY is associated with increased persistence of pneumococci during the later stages of infection of the mouse lung and contributes to pneumococcal virulence in a rabbit intracorneal model [3]. While direct activation of the complement pathway has only been demonstrated for PLY, SLO polymers acted as foci for specific antibody-dependent complement activation on host cell membranes, leading to decompensation and self-attack [22].

2.4. Role of TACYs as host protective antigens

Antibodies to TACYs provide an effective host defense. Passive and active immunization experiments have demonstrated that antibodies which neutralize the effects of PLO can protect mice against *A. pyogenes* infection [5,23]. Mice immunized with genetically toxoided PLY were protected against challenge with multiple capsular serotypes of *S. pneumoniae* [8], and recent vaccine studies indicated that *Salmonella typhimurium aroA* strains expressing LLO confer excellent protection against listeriosis in mice [24]. Passive immunization using a neutralizing monoclonal antibody to PFO significantly delayed mortality in fulminant, wild-type *C. perfringens* infection in mice [9]. While less well studied than the more prominent members of this family, vaccination with purified sulysin, the TACY of *S. suis*, completely protected both mice and pigs from experimental infection [25].

3. Structure-function analysis of TACYs

Complete nucleotide sequence data are now available

Table 2
Sub-lytic effects of TACYs on host immune and defense cells

Toxin	Property	Reference
SLO	Inhibition of chemotaxis and mobility of human PMNs	[51]
	Induction of expression of IL-1 β , IL6 and IL8 from human keratinocytes, and IL-1 β and TNF α from monocytes	[14,52]
	Induction of the release of prostaglandin E ₂ from human keratinocytes	[14]
	Antibody-mediated induction of the complement pathway	[22]
PLY	Inhibition of migration, respiratory burst and bacteriocidal activity of human PMNs and monocytes	[3]
	Inhibition of human lymphocyte proliferative responses and immunoglobulin production	[3]
	Induction of IL-1 β and TNF α expression from human monocytes	[53]
	Activation of phospholipase A ₂	[3]
	Direct activation of the complement cascade	[21]
LLO	Induction of nitric oxide production from murine macrophages	[54]
	Suppression of phagocytosis by murine macrophages	[55]
	Induction of expression of IL-1 α , IL12 (murine macrophages), IFN- γ (murine NK cells) and IL-8 and macrophage chemotaxis protein 1 (human epithelial cells)	[16,17]
	Induction of expression of adhesion molecules on the surface human epithelial cells	[16]
PFO	Activation of transcriptional factor NF- κ B	[16]
	Promotion of dysfunctional human PMN/endothelial cell adhesive interactions	[9]
	Inhibition of human PMN chemotaxis	[9]
	Priming of human leukocytes for increased respiratory burst	[9]
ALY	Promotion of vascular leukostasis	[7]
	Induction of IL8 expression in human PMNs and lymphocyte-monocyte-basophil cell populations	[56]

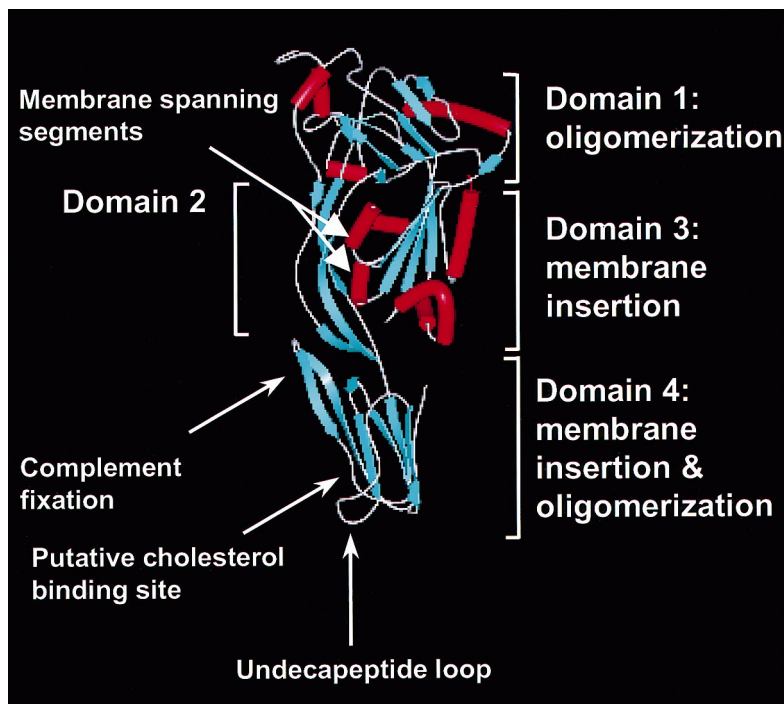


Fig. 2. 3D structure of a TACY. Representative structure of the PFO monomer [26] displayed by WebLab viewer (Molecular Simulations Inc.). The four domains are indicated, as well as the functions of various regions described in the text.

for the genes of 11 TACYS, including SLO molecules from *S. pyogenes*, *S. equisimilis* and *S. canis* (Table 1). In addition, incomplete sequences have been determined for cereolysin from *Bacillus cereus* and intermedilysin (ILY) from *S. intermedius*. In primary amino acid structure, the TACYS are defined by an almost invariant undecapeptide sequence (Fig. 1), which is important for cytolytic activity. However, the mechanism by which these toxins form pores in eukaryotic cell membranes is not easily deduced from the primary amino acid structure, since there are no sustained regions of hydrophobicity which may constitute membrane-spanning domains.

3.1. Three-dimensional structure of TACYS

The 3D crystal structure of the PFO monomer has recently been described, using X-ray crystallography, as an elongated molecule comprised of four β -sheet-rich domains (Fig. 2), only one of which, the C-terminal domain 4, is contiguous within the primary amino acid sequence [26]. A proposed structure for PLY based on a homology model with PFO suggests that PLY takes on a very similar conformation [27] and it appears likely, given the conservation of residues that map to the hydrophobic core of PFO, that all TACYS share a similar 3D structure [26]. The major primary sequence motif, the undecapeptide sequence, occupies a prominent position as a loop within domain 4 at one end of the elongated structure (Fig. 2).

3.2. Membrane insertion and pore formation

Proteins of the TACY family bind to cholesterol-containing membranes and oligomerize to form transmembrane pores up to 30 nm in diameter and composed of 40–80 monomer subunits. These pores are particularly large and allow the passage of both ions and macromolecules. The description of the crystal structure of the PFO monomer has spawned a number of models of pore formation [15,26,28], all of which include a basic multistep process encompassing membrane binding, membrane insertion, and oligomerization (Fig. 3).

The initial interaction of the toxin with the membrane probably occurs through the binding of membrane cholesterol. This serves the purpose of concentrating toxin subunits at cholesterol-rich regions of the lipid bilayer and promoting oligomerization. TACYS bind to membrane cholesterol with high affinity [29] and cholesterol likely represents the initial and only receptor on the host cell for these toxins. Arguments have been made for a C-terminal cell binding domain independent of the cholesterol binding site [30] from deletion analysis of C-terminal amino acids of PLY and PFO, which significantly reduces the ability of these toxins to bind to erythrocyte membranes [30,31]. However, at least in the case of the PFO deletions, there appears to be considerable structural disruption of domain 4, and interference with cholesterol binding [31]. The cholesterol binding site within TACYS has not yet been defined experimentally, although there is ample evi-

dence to suggest that it lies within domain 4 (Fig. 2). A C-terminal tryptic fragment of PFO, containing predominantly domain 4, is able to bind to both cholesterol and cholesterol-containing membranes [32], and the binding of cholesterol triggers conformational changes around the undecapeptide tryptophan residues at the tip of domain 4 [33,34]. Rossjohn et al. [26] have modeled a binding site on the 3D structure of the PFO monomer in which cholesterol binds to the flat sheet of domain 4 (Fig. 2), displacing the conserved undecapeptide loop which may be a key in membrane insertion.

It has been suggested that oligomerization of toxin molecules to form pores takes place on the membrane prior to insertion [26]. A similar mechanism is employed for other pore-forming toxins, but due to the large size of pores formed by TACyS, the energy requirements to fully insert a transmembrane channel of that size would be astronomical [35]. In addition, small oligomers can cause cytoplasmic leakage suggesting that membrane insertion is an early event in pore formation [15]. This mechanism of pore formation also does not account for the arc-shaped oligomers observed by electron microscopy. Capping experiments using mixtures of wild-type SLO and a mutant SLO molecule which polymerizes poorly suggest that arcs are intermediates in pore formation and actually breach the membrane to allow the passage of solutes [35]. Thus, a more likely scenario is that toxin monomers insert at least partially into the membrane and that new monomers are added to the growing oligomer as they insert.

The binding of membrane cholesterol induces a conformational change in the toxin monomer driving the subsequent events in pore formation, such as membrane insertion and oligomerization. TACyS cytolytic activity is inhibited by low levels of free cholesterol. However, cholesterol-inactivated LLO is still able to bind to host cell membranes, and apparently partially insert, although the

inactivated toxin is not able to oligomerize and form membrane pores [18]. These results suggest that conformational alterations caused by the binding of cholesterol transform TACyS from soluble molecules to hydrophobic molecules capable of at least partial membrane insertion. However, it would also appear that binding of cholesterol in the membrane is required for assembly of the pore [18].

Rossjohn et al. [26] predict that domain 4 of TACyS forms the major membrane insertion domain and hypothesize that the binding of membrane cholesterol to the flat sheet of domain 4 displaces the loop structure composed primarily of the undecapeptide to form a hydrophobic dagger that initiates membrane insertion of domain 4 of the toxin. The prediction of domain 4 as the membrane insertion domain is consistent with a mirroring of the four domain structure of PFO to the mushroom-shaped side views of oligomers within membranes [28,36] and the importance of domain 4 residues in cytolytic activity [37–41]. PFO activity can be inhibited by an isolated fragment of PFO containing domain 4 [32], or by antibodies that bind to domain 4 [42,43], although this latter evidence may reflect cell binding rather than actual membrane insertion. There is also evidence that parts of domain 3 are also inserted into the membrane [44,45]. Fluorescence microscopy has indicated that a 30-amino acid segment, predicted to form three short α -helices at the tip of domain 3 of monomer PFO (Fig. 2), forms a two-stranded, amphipathic β -sheet in membrane-bound PFO which creates a stable interface between the pore and the membrane [45]. Therefore, it is clear that membrane-bound TACyS take on a different structure to soluble monomer TACyS. A cryo-electron microscopy (cryo-EM) study of a helical oligomerized form of PLY, which forms naturally at high concentrations in solution, has been used as a predictor of an intermediate structure in membrane insertion [28]. Unlike its position in the soluble monomer structure, do-

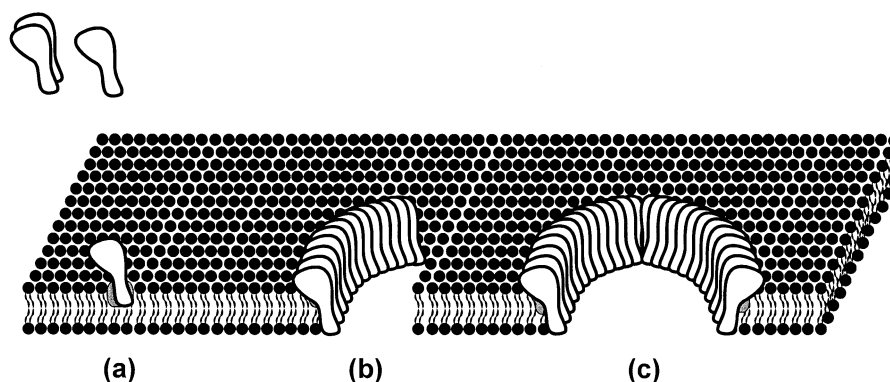


Fig. 3. Membrane insertion of the TACyS. A model for the insertion of TACyS into the host cell membrane which adopts aspects from several models [15,26,28]. a: Prior to binding to the host cell membrane, TACyS monomers may undergo some oligomerization in solution. The monomers descend onto the lipid bilayer and bind to cholesterol-rich regions (shown in gray). The binding of cholesterol displaces the undecapeptide allowing it to form a hydrophobic dagger which facilitates membrane partitioning of toxin into the membrane. b: Oligomerization stimulates conformational changes which allow domain 4 and domain 3 to assemble in the membrane. The formation of arcs allows leakage of solutes either by forming a pore with a free bilayer edge (as shown here) or by sufficiently perturbing the bilayer structure. c: Successive oligomerization of toxin subunits allows the arc to grow into a pore.

main 3 is displaced away from its interface with domain 2 and can be found in multiple positions or conformations in the helical oligomer [28]. This displacement may allow a refolding of domain 3 more consistent with its membrane-bound form and may explain the transition of α -helix to β -sheet at the tip of domain 3 [45].

The expulsion of domain 3 in the helical oligomer from its position in the monomer coincides with a 45° rotation of domain 4, with respect to domain 2, a position it apparently occupies in a membrane-bound state [28]. However, domain 3 in the pore state is more tightly packed against domains 2 and 4, presumably allowing the tip of domain 3 to extend towards, and insert into, the membrane [28].

As the isolated helical form in solution is not cholesterol-bound, the domain 4 rotation and domain 3 expulsion are likely stimulated by oligomerization, but it is only membrane-bound oligomers that can repack domain 3 and allow membrane insertion. There appear to be significant contact points within domain 1 [26,42,43] and domain 4 [46] between oligomer subunits. While the majority of oligomerization probably occurs in the membrane, there is evidence that oligomerization of TACYs, akin to that seen in pores, can also occur in solution [46]. However, membrane-driven oligomerization appears to proceed more rapidly, probably due to increased local concentration of toxin [46].

EM studies of TACY pores suggest a transmembrane pore [47], and the passage of ions and macromolecules is consistent with a large pore. In addition, disruptions of the membrane by arcs which are lined with a free edge of the lipid bilayer have been reported [35]. However, cryo-EM of sections of PLY pores in liposomes [28] suggests that the membrane may be continuous inside the pore and that the insertion of domains 3 and 4 may not even cross the bilayer [28]. Gilbert et al. [28] suggest that insertion of oligomers into the membrane may be enough to destabilize the bilayer structure leading to the formation of non-lamellar structures and a breakdown of the permeability barrier.

3.3. Conserved undecapeptide Trp residues

The conserved undecapeptide sequence is intimately involved in membrane insertion by TACYs [26]. It is the largest region of conservation among the primary amino acid sequences of TACYs. Variations from this conserved sequence have only been observed in seeligerilysin O of *L. seeligeri*, which has a simple Ala to Phe substitution, and PLO and ILY which have highly variant undecapeptide sequences [23,48] (Fig. 1).

The three undecapeptide Trp residues are conserved in all but ILY, which lacks the third Trp residue [48]. Each of these Trp residues contributes to the conformation, insertion and oligomerization of PFO molecules [34]. Mutations in the Trp residues of PLY [37,49], LLO [38], PFO

[39] and PLO (S.J. Billington, B.H. Jost and J.G. Songer, unpublished data) cause significant reductions in hemolytic activity. In all toxins, the greatest reduction in activity is observed with mutations in the first undecapeptide Trp residue. This residue lies at the very tip of the undecapeptide loop, as predicted by the 3D structure, and in Rossjohn's dagger hypothesis is likely to play a prominent role as the point of the dagger [26]. It is this residue which would be displaced from the hydrophobic pocket on the flat sheet of domain 4 by the binding of cholesterol, and presumably would be one of the first residues to penetrate the membrane. It is interesting to note that, despite the diverse nature of the undecapeptide sequences of PLO and ILY, with insertions and deletions with respect to the consensus sequence [23,48] (Fig. 1), the position of the first tryptophan within the undecapeptide is invariant. Results of conductance experiments suggest that not only is a greater amount of a PLY mutant in the first Trp residue required for hemolytic activity, but the very nature of the channels themselves is altered, being more highly conductive and less sensitive to cations and polysaccharide polymers [49].

3.4. Thiol activation

The TACY designation for this group of toxins is derived from the observation that crude toxins are only active in a reduced state. They reversibly lose activity upon oxidation, and activity can be regained by the addition of thiols or other reducing agents. This property arises from the presence of a Cys residue within the conserved undecapeptide sequence. Oxidation of this Cys residue results in reduced activity, probably through steric hindrance of important amino acids such as the three Trp residues, also located within the conserved undecapeptide [41].

Several lines of evidence imply that thiol activation is irrelevant, both to the function of this group as hemolysins/cytolysins and to their importance as virulence factors. In their pure form, TACYs are not susceptible to oxidation [1,37], suggesting that thiol activation is not an intrinsic property of the toxins. Site-directed mutagenesis of the Cys residue within the conserved undecapeptide indicates that, while some substitutions are deleterious, substitution with Ala at this position generally has little effect on the hemolytic activity [38,40,41], and renders the toxin insensitive to either oxidizing or reducing agents. Therefore, an essential role for the Cys residue, and thiol activation, in cytolytic activity in vitro can probably be ruled out. It has been argued that thiol activation may play a critical role in vivo by providing a post-translational means of regulating toxin activity, or that formation of intermolecular disulfide bonds may aid in toxin stabilization [38,40,41]. However, there is no evidence supporting these hypotheses and, in fact, a *L. monocytogenes* strain expressing a non-thiol-activated variant of LLO has only a slightly increased LD₅₀ for mice [38]. This difference in

LD₅₀ can probably be accounted for by a slightly reduced hemolytic activity [38].

Two recently induced members of the TACY family do not even exhibit thiol activation in their crude form. PLO, while clearly a member of this family on the basis of amino acid homology, is neither activated by reducing compounds, nor is its activity susceptible to thiol-blocking agents [23]. PLO is a divergent member of this family and contains a novel undecapeptide sequence (Fig. 1), which varies significantly from the consensus, including substitution of Ala for the conserved Cys [23]. While the results of initial experiments suggested that substituting the Cys residue into the PLO undecapeptide did not make PLO thiol-activated [23], more recent data indicate that substituting either the Cys alone or a consensus undecapeptide into PLO will render the molecule susceptible to thiol activation (S.J. Billington, B.H. Jost and J.G. Songer, unpublished data). It is probable that additional members of this family of toxins have been overlooked because they do not exhibit the property of thiol activation. Indeed, ILY, a *S. intermedius* cytotoxin specific for human cells, is also not thiol-activated [50]. ILY shows considerable amino acid sequence similarity with other TACYs [48]. However, its variant undecapeptide sequence includes an Ala substitution for the conserved Cys, similar to PLO, which explains its resistance to oxidation [48].

It is clear that the term 'thiol-activated cytotoxin' does not accurately reflect the true properties of this family of toxins. The most strongly conserved, and perhaps important, properties among this family are their abilities to bind cholesterol and to form pores in eukaryotic cell membranes. Therefore, we propose the use of the designation cholesterol binding, pore-forming (CHOP) toxins.

3.5. Complement activation

The ability of PLY to activate complement in the absence of specific antibodies is associated with a sequence, located within domain 4, involved in binding to the Fc portion of IgG (Fig. 4). Mutations in Tyr₃₈₄ and Asp₃₈₅ within this sequence significantly affected this function,

particularly an Asp₃₈₅ to Asn mutation, which reduced binding to IgG and abolished complement activation [21]. This mutation had no effect on the cytolytic activities of PLY. The homology model of PLY [27] predicts that domain 4 forms a β -sandwich structure similar to that of the Fc region of IgG, although no homology exists between them. As domain 4 appears to be involved in self-assembly of PLY molecules, and aggregated Fc promotes complement fixation, an association between PLY and Fc, similar to the self-association of PLY, may drive the direct activation of complement by PLY [27,46]. PLY is the only TACY demonstrated to activate complement. However, a similar structure for domain 4 is predicted for other members of this family [27,46] and residues required for direct binding of PLY to Fc are conserved amongst the family [23,27] (Fig. 4). Therefore, it appears likely that other TACYs may be capable of activating complement in the absence of specific antibodies.

4. Conclusions and future directions

As more information about the role of TACYs as important virulence factors becomes available, it becomes increasingly apparent that their effects may be much more subtle than simple destruction of host cells by pore formation. While specific effects may vary depending on the individual toxin, the apparent effects on immune cell function and induction of inflammatory pathways appear to be a constant, suggesting that the major role of TACYs may be as modulators of the immune response. However, there is little information on the mechanism of such effects. Are they purely the result of breakdown in the cellular permeability barrier due to pore formation, or are signal transduction pathways stimulated by the binding or insertion of these molecules?

Recent years have seen significant advances in our understanding of structure-function relationships within this group of toxins. However, while the growth of arcs into pores and the steps involved in oligomerization are now becoming clearer, there are still gaps in the pathways

PLY	368	S	G	A	Y	V	A	Q	Y	I	T	W	D	E	L	S	Y	D	H	Q	G	K	E	V	L	T	P	K	A	W		
SLY	396	S	G	A	Y	V	A	K	Y	N	I	T	W	E	E	V	S	Y	N	E	A	G	E	E	V	W	E	P	K	A	W	
PLO	432	G	G	G	Y	V	A	K	F	R	L	K	W	D	E	I	S	Y	D	P	Q	G	K	E	I	R	T	P	K	T	W	
LLO	424	S	G	G	Y	V	A	Q	F	N	I	S	W	D	E	V	N	Y	D	P	E	G	N	E	I	V	Q	H	K	N	W	
LSO	425	S	G	G	Y	V	A	Q	F	N	I	S	W	D	E	V	S	Y	D	E	N	G	N	E	I	K	V	H	K	K	W	
ILO	399	S	G	A	Y	V	A	R	F	N	V	T	W	D	E	V	S	Y	D	A	N	G	N	E	V	V	E	H	K	K	W	
ALY	401	S	G	A	Y	V	A	Q	F	E	V	Y	W	D	E	F	S	Y	D	A	D	G	Q	E	I	V	T	R	K	S	W	
CLY	406	Y	G	A	Y	V	A	Q	F	D	V	S	W	D	E	G	F	T	F	D	Q	N	G	K	E	I	L	T	H	K	T	W
PFO	399	S	G	A	Y	V	A	Q	F	E	V	A	W	D	E	V	S	Y	D	K	E	G	N	E	V	L	T	H	K	T	W	
SLO	470	Q	G	A	Y	V	A	Q	Y	E	I	L	W	D	E	I	N	Y	D	D	K	G	K	E	V	I	T	K	R	R	W	

Fig. 4. Conservation of complement activation domains among TACYs. Alignment of domain 4 sequences required for complement activation by PLY with similar sequences from other TACYs. Amino acids identical to the PLY sequence are boxed. Amino acid numbers are given for the first amino acid in the sequence for each protein. Toxin abbreviations and GenBank accession numbers are shown in Table 1. Amino acid sequences of SLO molecules from *S. canis*, *S. equisimilis* and *S. pyogenes* are identical over the region shown. Amino acids demonstrated to be important in complement activation by PLY (Tyr₃₈₄ and Asp₃₈₅) are indicated by the asterisks.

of membrane insertion. For example, the requirement for cholesterol in events subsequent to cell binding and perhaps displacement of the undecapeptide loop is unknown, as is the true binding site for cholesterol. The discovery of new members of this toxin family will create more questions in the area of mode of action. For example, if cholesterol is the only receptor for these toxins, why is ILY, a human-specific cytolysin, incapable of lysing erythrocytes from other species? Indeed, why do most TACYs show ranges of activities against cells from different species and cell types within a species? The answers to these questions and more await further study.

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