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MINIREVIEW

Cholesterol-Dependent Cytolysins, a Family of Versatile Pore-Forming Toxins

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The cholesterol-dependent cytolysins (CDCs) are a large family of pore-forming toxins that are produced by more than 20 species from the genera *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, and *Arcanobacterium*. The pore-forming mechanism of these toxins exhibits two hallmark characteristics: an absolute dependence on the presence of membrane cholesterol and the formation of an extraordinarily large pore. Each CDC is produced as a soluble monomeric protein that, with the exception of one member, is secreted by a type II secretion system. Upon encountering a eukaryotic cell, the CDCs undergo a transformation from a soluble monomeric protein to a membrane-embedded supramolecular pore complex. The conversion of the monomers to an oligomeric, membrane-inserted pore complex requires some extraordinary changes in the structure of the monomer, yet the mechanism of pore formation may be only the beginning of the CDC story.

Although the CDCs are well known as beta-hemolytic proteins, it has become increasingly apparent that bacterial pathogens use these proteins in much more sophisticated ways than as simple hemolysins or general cell-lytic agents. The CDC structure also exhibits a plasticity that has allowed the evolution of unique features for some CDCs, without compromising the fundamental pore-forming mechanism. Some of these features are reflected in CDCs that activate complement, that utilize a nonsterol receptor, that exhibit a pH-sensitive, pore-forming mechanism, or that can function as a protein translocation channel. As will be apparent in this review, our knowledge of how the CDCs are used by pathogens lags behind our understanding of the CDC pore-forming mechanism, although some studies have provided some provocative glimpses into how the bacterial cells use CDCs during an infection.

This review will focus on the advancements in our understanding of the CDC pore-forming mechanism, the unique structural and mechanistic features that are exhibited by many of the CDCs, and how these features might contribute to pathogenesis.

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OVERVIEW OF THE CDC PORE-FORMING MECHANISM

The structures of the soluble monomers of perfringolysin O (PFO) (80) and intermedilysin (ILY) (72) are shown in Fig. 1, and the general features of the CDC pore-forming mechanism are shown in Fig. 2. Both CDCs are β-sheet-rich, four-domain proteins. The highly conserved tryptophan-rich undecapeptide is present in domain 4. The undecapeptide appears to mediate the binding of some CDCs to cholesterol-rich membranes, and it has been thought that the undecapeptide may interact directly with cholesterol. However, it has also been shown that at least one CDC from *Streptococcus intermedius*, ILY, specifically uses human CD59 as its receptor. The undecapeptide has also been shown to insert into the membrane (28, 58), though not to a significant depth (28). In addition, three other short hydrophobic loops juxtaposed to the undecapeptide at the tip of domain 4 have been shown to also insert into the membrane surface and anchor the CDC to the membrane in a perpendicular orientation (12, 75, 76).

After membrane binding, the CDC monomers diffuse laterally to initiate formation of the membrane oligomer. The interaction of the domain 4 loops with the membrane may be involved in triggering a rearrangement of a small loop within domain 3 comprised of a short β-strand and an α-helix (β5 and α1) (Fig. 1) that frees up the edge of β-strand 4 of the domain 3 core β-sheet. The edge-on hydrogen bond interaction of β-strand 4 with β-strand 1 of another monomer initiates the oligomerization of the monomers into the membrane-bound prepore complex. The prepore complex is an oligomeric complex in which the transmembrane β-barrel has not inserted to create the pore. Once the prepore complex reaches a large size, presumably a complete ring structure, it then makes the transition to the pore complex. The transmembrane pore is formed when two α-helical bundles in domain 3 of each monomer within the prepore complex are converted to two extended amphipathic transmembrane β-hairpins (TMHs). Upon the conversion of the prepore to the pore, the height of the prepore structure undergoes a vertical collapse of about 40Å. The collapse of the prepore structure brings the domain 3 TMHs within striking distance of the membrane surface, at which point they undergo a concerted insertion into the membrane that results in the formation of the large transmembrane β-barrel pore. The CDC pore is large: it is comprised of 35 to 50 monomers and exhibits a diameter of 250 to 300 Å. As described in detail below, the CDCs undergo a series of remark-

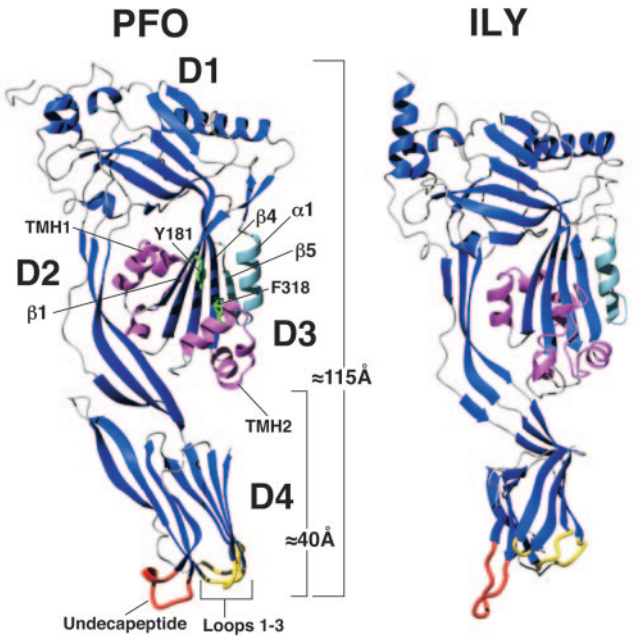

FIG. 1. Crystal structures of PFO and ILY. Shown are the ribbon representations of the α-carbon backbone structures of PFO and ILY (72, 80). The locations of TMH1 and TMH2 (magenta), the three domain 4 loops of Ramachandran et al. (Loops 1 through 3) (yellow), the undecapeptide (red), residues Y181 and F318 (green), the β5-α1 loop (light blue), and β-strands β1 and β4 of the domain 3 core β-sheet are shown for PFO. Most of the corresponding regions are shown for ILY on the right and are color coded as for PFO. D1 through D4, domains 1 through 4, respectively.

able conformation changes as they make the transition from a soluble monomer to a membrane-embedded pore complex.

CELLULAR RECOGNITION AND BINDING

Cholesterol and the CDC mechanism. The CDC cellular receptor has traditionally been accepted to be cholesterol. This paradigm was established as a result of two general observations: hemolysis by the CDCs is inhibited by the addition of free cholesterol to the toxin prior to incubating it with the erythrocytes (3, 11, 14, 17, 34, 50, 51, 74, 85, 86, 98), and some CDCs have been shown to bind directly to cholesterol (36, 38, 63). The inhibitory effect of free cholesterol was thought to result from the occupation of the receptor-binding site of the CDC, thus preventing it from binding to the membrane cholesterol in the erythrocyte membrane. Much of the direct evidence that the CDCs use cholesterol as a receptor has come from several studies of the CDC from *Clostridium perfringens*, PFO (35, 36, 58–63). Because of its ability to directly bind to cholesterol-containing membranes, PFO has been used as a probe for membrane cholesterol (26, 52, 57, 64, 92, 96). The contribution of membrane cholesterol to the mechanism of the CDCs, however, appears to be more complicated than just functioning as a CDC membrane receptor. Furthermore, it is not entirely clear that cholesterol functions as a receptor in the classical sense in which there is a 1:1 stoichiometry of CDC to cholesterol.

Studies with liposomal systems have shown that the total concentration of cholesterol in liposome membranes required for successful binding of PFO and other CDCs is quite high, on the order of 50 mol% of the total membrane lipid (3, 27).

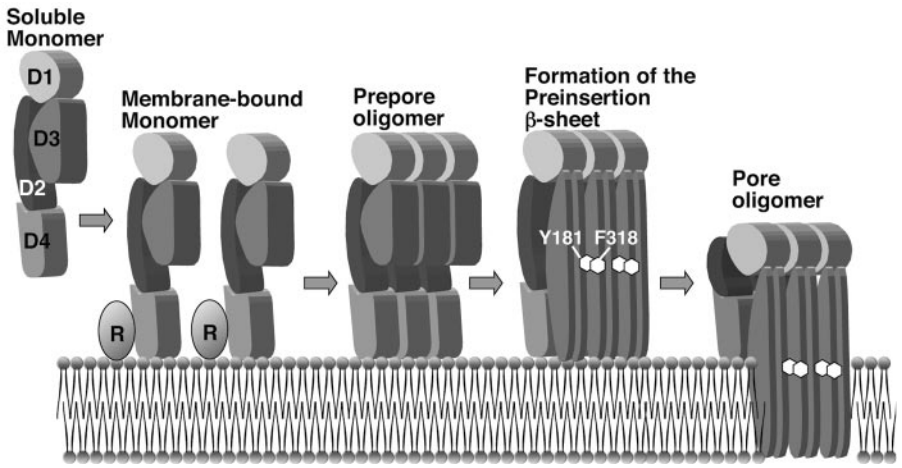

FIG. 2. The CDC pore-forming mechanism. The first step in the CDC mechanism is the interaction of the soluble CDC monomers with the host membrane. Binding to cholesterol-rich membranes, presumably via lipid rafts, appears to be mediated by the undecapeptide of many CDCs; however, the CDC from *Streptococcus intermedius*, ILY, has been shown to have a modified undecapeptide that has lost this function. Instead, ILY binds to human CD59 (R) and the CD59 binding site is contained elsewhere in the domain 4 structure. Upon membrane binding (either by direct binding to cholesterol-rich membranes or via a specific receptor), structural changes within domain 3 are triggered that initiate oligomerization of the membrane-bound monomers into the prepore structure (29, 33, 75–77, 89). The insertion of the undecapeptide tryptophans also appears to control the next stage of the CDC mechanism, prepore-to-pore conversion (19), in which the domain 3 α-helical bundles unravel to form TMH1 and TMH2 and each monomer of the prepore contributes these two β-hairpins to the ultimate formation of the transmembrane β-barrel. The formation of a preinsertion β-barrel is hypothesized, but as described in the text, its formation would be the most energetically favorable structure for the TMHs when they are inserted into the membrane to form the β-barrel pore. Cooperation of the monomers appears necessary for the membrane insertion of the TMHs (32). Upon conversion of the prepore to pore, the domain 2 structure appears to collapse, which brings domains 1 and 3 35 to 40 Å closer to the membrane and allows the transmembrane β-barrel to insert across the bilayer (12, 76, 87, 88). Depletion of membrane cholesterol blocks the prepore-to-pore transition.

Interestingly, it has been shown that the transition from little or no binding to full binding to lipid-cholesterol liposomes occurs in a narrow range of cholesterol concentration. At 40 mol%, both tetanolysin, a CDC from *Clostridium tetani*, and PFO exhibit little or no binding, whereas at approximately 50 to 55 mol%, they both exhibit maximal binding (27, 81). These observations suggest that the interaction between the CDC and cholesterol is not simply the result of one toxin molecule binding to one cholesterol molecule since cholesterol was not limiting under any of the conditions described in the above studies.

It appears that the distribution of the cholesterol in the membrane may be important for CDC binding. The fact that these toxins can go from virtually no binding to maximal binding in such a narrow range of cholesterol suggests that they may be more likely to interact with eukaryotic cells via cholesterol-rich lipid rafts where their cholesterol concentration is high. It has been shown that PFO is preferentially associated with lipid rafts in cells (90, 96). The ability to target rafts with a CDC may enable the bacterial pathogen to disrupt essential functions of the cell by disrupting the structure of the lipid rafts, a cellular feature where it is increasingly evident that a significant number of essential cellular processes, such as signaling and endocytosis, occur. Why high cholesterol concentrations are necessary for binding is not understood, although it is likely to be related to the structure and accessibility of cholesterol in cholesterol-rich domains.

The role of cholesterol as the CDC receptor has been challenged to some extent in the last decade by studies that suggest that its role may be downstream of the membrane recognition step of the CDC mechanism (8, 37). The complex role of cholesterol in the CDC mechanism was recently reinvestigated, primarily because a CDC was discovered that specifically recognized human cells, yet remained sensitive to membrane cholesterol concentration.

The CDC ILY from *Streptococcus intermedius* posed a special problem to the dogma that cholesterol was the CDC receptor since ILY is specific for human erythrocytes (54, 55). The human cell specificity of ILY was difficult to reconcile with the notion that cholesterol served as the common receptor for the CDCs. Ultimately, the study of how ILY could specifically target human cells provided a deeper understanding of the role of cholesterol in the CDC mechanism and also revealed that cholesterol was not necessarily the receptor for all CDCs. We (19) reinvestigated the role of cholesterol in the cytolytic mechanisms of three CDCs: ILY, *Streptococcus pyogenes* streptolysin O (SLO), and PFO. It was determined that depletion of approximately 90% of the cholesterol from the membrane of human erythrocytes essentially abolished the ability of these CDCs to lyse the erythrocytes, yet binding of SLO and ILY to the cholesterol-depleted cells remained essentially unchanged. PFO binding decreased approximately 10-fold, but its activity decreased more than 11,000-fold. In all three cases, the nearly complete loss of cytolytic activity did not correlate with changes in binding.

Additional analyses showed that cholesterol depletion blocked prepore-to-pore conversion (Fig. 2) of the CDCs (19). The loss of cholesterol from the membrane prevented the insertion of the transmembrane β-barrel of the prepore complex, thus trapping the oligomer in the prepore state. Why the loss of cholesterol from the membrane prevents the insertion of the β-barrel remains unknown, but it seems reasonable that it is linked to the changes induced on the membrane structure by the presence of specific levels of cholesterol (i.e., lipid raft formation). These studies showed that the CDC pore-forming mechanism was far more sensitive to membrane cholesterol levels at the prepore-to-pore step rather than at the membrane-binding stage. This discovery also provided an explanation for the general enhancement of pore formation that cholesterol has on other, unrelated membrane-penetrating toxins (19).

A novel CDC receptor. The fact that cholesterol depletion had no effect on the binding of ILY suggested that ILY might use a nonsterol receptor for cellular recognition. Giddings et al. (20) subsequently showed that the human cell specificity of ILY was based on its ability to specifically bind to the human form of the glycosylphosphatidylinositol-anchored protein CD59. CD59 is a late-stage complement inhibitor present on mammalian cells (79). CD59 specifically binds to complement proteins C8α and C9 after they have assembled into the complement membrane attack complex (MAC), thus blocking pore formation by the MAC on host cells. Residues 42 through 58 of human CD59 mediate its interaction with C8α and C9 (102), and this same peptide region is recognized by ILY (20). This region is responsible for the species-selective nature of the CD59 interaction with complement. In many cases, one species, CD59, cannot effectively inhibit the formation of the MAC from another species (79, 101, 102). These residues are the most variable among all CD59s, particularly when the primary structures of higher-primate CD59 molecules are compared with human CD59, consistent with the observation that erythrocytes from higher primates are also relatively insensitive to ILY (54). Therefore, the variation within these residues is apparently responsible for the highly restricted human cell specificity of ILY. It should be noted that although ILY uses a nonsterol receptor, as described above, it still remains sensitive to membrane cholesterol concentrations (19). Therefore, the nomenclature of a CDC is the most appropriate name for these toxins since it does not necessarily imply that a specific feature of the CDC mechanism is the sole basis for the cholesterol dependence of these toxins.

Domain 4 and the undecapeptide. During the process of the CDC monomer interaction with the membrane, the undecapeptide and three other short loops at the tip of the domain 4 β-sandwich (Fig. 1), insert into the membrane upon the interaction of the CDC monomers with the membrane surface (27, 75). These loops do not penetrate deeply into the membrane and so do not directly participate in the structure of the transmembrane pore. One function of the loops is to anchor the monomers to the membrane in an upright position. We have shown that domain 4 exists in a perpendicular orientation to the membrane and is surrounded by the aqueous milieu, even in the oligomeric state (75). Interestingly, domain 4, once thought to form part of the interface with neighboring monomers in the oligomeric complex (35, 94), does not form a permanent interaction with its neighbors. It appears that if there is an interaction between monomers of the oligomer via domain 4, it is only transient and is not present in the oligomer (75, 99).

Domain 4 of the CDCs mediates membrane recognition,

whether it is via cholesterol or another receptor, as in the case of ILY (20, 53, 72, 90, 99) (Fig. 1). The three-dimensional α-carbon backbone structure of domain 4 of ILY is not significantly different from that of PFO, and so the ability of ILY to bind CD59 must be based on differences in the primary structure of domain 4. The most obvious difference in the domain 4 structures of ILY and PFO is that the conserved undecapeptide region of the CDCs is altered from ECTGLAWEWWR in PFO to GATGLAWEPWR in ILY. The study of the ILY undecapeptide structure has provided additional support to previous studies that suggested that the undecapeptide contributes to CDC binding to cholesterol-rich membranes (36, 58). Interestingly, the altered structure of the ILY undecapeptide contributes to the ability of ILY to specifically bind human CD59, but in a completely unexpected way. The structure of the ILY undecapeptide is not involved in ILY receptor binding (72), but its alteration appears to be necessary to prevent ILY from binding directly to cholesterol-containing membranes. Nagamune et al. (53) showed that the substitution of the SLO undecapeptide sequence for the ILY undecapeptide converted ILY into a nonspecific CDC that could lyse both human and animal erythrocytes. These results suggested that the substitution of the consensus undecapeptide from SLO into the ILY enabled the ILY mutant to bind to cholesterol-rich membranes, as evidenced by its ability to lyse both human and rabbit erythrocytes.

Nagamune et al. (53) further showed that the chemical modification of the cysteine sulfhydryl of the solitary cysteine of the SLO undecapeptide sequence, when it was present in the ILY structure, abolished the activity of the ILY mutant on rabbit erythrocytes, but not on human erythrocytes. This observation was consistent with the much earlier observation of Iwamoto et al. (36), who showed that chemical modification of the cysteine sulfhydryl of the PFO undecapeptide caused a significant decrease in membrane binding of PFO. Therefore, the region of the undecapeptide in and around the cysteine residue appears to be important in endowing the ILY undecapeptide mutant with the ability to bind to cholesterol-rich membranes. These data also suggest that the altered structure of the native ILY undecapeptide no longer facilitates its binding directly to cholesterol-containing membranes, whereas the consensus undecapeptide in other CDCs, such as SLO and PFO, does facilitate the direct binding of these CDCs to cholesterol-rich membranes. Hence, in order for ILY to specifically bind human CD59, two evolutionary changes had to occur: the structure of the undecapeptide had to change to prevent the direct binding of ILY to cholesterol-containing membranes, and one or more mutations within the primary structure of domain 4, yet to be identified, were required to establish the human CD59-binding site.

Is the role of the undecapeptide therefore to simply direct those CDCs with the consensus undecapeptide structure to bind to cholesterol-rich membranes, and is the altered undecapeptide of ILY now nonfunctional? Although the ILY undecapeptide is no longer capable of directing ILY binding to cholesterol-rich membranes, it is still important to the pore-forming mechanism of ILY. An alanine scan of the native ILY undecapeptide by Polekina et al. (72) showed that mutation of Trp-491 to alanine abolishes its pore-forming activity while having only a minimal effect on its ability to bind to cells. It appeared that this mutation prevented prepore-to-pore conversion (72). Although the structural basis of this mutation is not understood, the observation by Heuck et al. (27), who showed that domains 3 and 4 are conformationally coupled, suggests a possible explanation. They showed that mutations that alter the rate of the insertion of the domain 3 TMHs had the same effect on the insertion of the domain 4 undecapeptide. The insertion of domain 4 precedes the insertion of the domain 3 TMHs, and so this effect could be observed only if the two domains were conformationally coupled. It is, therefore, not unexpected that mutations within the undecapeptide could affect the insertion of the TMHs. These observations suggest that while the ILY undecapeptide has lost the ability to facilitate ILY binding to cholesterol-rich membranes, it still contributes to pore formation by modulating prepore-to-pore conversion.

ASSEMBLY OF THE MEMBRANE PREPORE COMPLEX

Several pore-forming toxins have been shown to assemble into a membrane-bound oligomeric complex termed the prepore. The prepore is defined as the oligomerized membrane complex that has not inserted its transmembrane β-barrel and formed a pore. Although the concept of the prepore intermediate for the much smaller heptameric pore-forming toxins such as aerolysin, *Staphylococcus aureus* α-hemolysin, anthrax-protective antigen, and *Clostridium septicum* α-hemolysin (47, 69, 83, 95, 97) had been well established, it was more difficult to envision a prepore intermediate for the large CDC oligomeric complex. Assembly of the CDCs into a prepore complex in which the coordinate insertion of at least 70 β-hairpins would occur after the assembly of such a large complex was conceptually more difficult to envision than the assembly of a heptameric prepore and the insertion of 7 β-hairpins by the smaller pore formers. Palmer et al. (66) therefore proposed that membrane insertion of the CDCs might be initiated with the insertion of the transmembrane domains of a CDC dimer and that monomers would add to the dimer, thus growing the inserted oligomer from a small pore to a large pore.

In contrast, Shepard et al. (89) showed by planar bilayer analysis that PFO formed only large channels and that pore formation could be separated from oligomerization at low temperature. Their studies suggested that a large prepore complex was indeed formed prior to the formation of the CDC pore. Hotze et al. (33) subsequently showed that PFO could be trapped in the prepore complex by the introduction of a disulfide bond between serine 190 of the domain 3 TMH1 and glycine 57 of domain 2 of PFO. The disulfide bond prevented the unfurling of the TMH1 and so trapped PFO in the prepore. Upon reduction of the disulfide, the oligomerized prepore complex rapidly inserted into the membrane and formed a pore. In addition, we have shown that at limiting concentrations of PFO and SLO, which on average, only a single pore per liposome is formed (about 45 monomers per vesicle), the release of a small marker (~10 Å diameter) and a large marker (~100 Å diameter) was similar (28). These studies showed that only large pores are formed by PFO and SLO and suggest that their prepores must reach an appreciable size before they insert and form the pore. If small pores could be formed by the CDCs, then a difference in the rate of release of the small and

large markers should have been apparent as the CDCs approached limiting concentrations. Statistically, the preference would be for smaller pores to form at limiting concentrations. There are also reasons that the formation of a prepore complex would be the energetically preferred model for insertion, as is discussed below in the section on pore formation.

With the exception of pneumolysin (PLY) from *Streptococcus pneumoniae*, CDC oligomers normally do not form to any significant extent in aqueous solutions of CDC monomers, even at high protein concentrations. Only upon membrane binding does significant oligomerization occur. One common mechanism to control oligomerization is the proteolytic cleavage of a propeptide of the toxin by a membrane-restricted protease. The small pore-forming toxins such as aerolysin from *Aeromonas hydrophila*, *C. septicum* alpha-toxin, and anthrax-protective antigen all require the proteolytic cleavage of a propeptide to initiate oligomerization (2, 16, 24, 48, 84). Typically, a membrane-restricted protease, such as furin (25), cleaves at an amino- or carboxy-terminal site of the membrane-bound toxin that removes the covalent attachment of the propeptide from the main structure of the toxin. The propeptide presumably occludes, directly or indirectly, a site or sites on the toxin that are involved in the intermolecular interactions required for oligomer formation. The fact that the activating protease is restricted to the membrane surface ensures that the precursor toxin must bind before it is activated. The CDCs, however, do not undergo proteolytic processing at any stage of the assembly of the membrane pore.

Ramachandran et al. (77) determined that CDC oligomerization is controlled by a small loop in domain 3 that is comprised of a short β-strand (β5) and α-helix (α1) (Fig. 1). β5 is hydrogen bonded to β4 of the core β-sheet in domain 3. For oligomerization of the membrane-bound toxin monomers, β5 must break its interaction with β4 and the β5α1 loop must rotate away from β-strand 4 (β4) to allow the edge-on interaction of β4 with β1 of a second monomer. Therefore, the edges of β1 and β4 form part of the interface between monomers. The interaction of β4 and β5 is likely disrupted when the toxin is anchored to the cell surface, since only membrane-bound monomers oligomerize to any significant extent.

The mechanism by which the CDCs control oligomerization so that it primarily occurs at the membrane surface is thus far unique among pore-forming toxins, although it is likely that other pore-forming toxins that lack a propeptide activation scheme may use an analogous membrane-dependent mechanism to control oligomerization. The activation mechanism of the CDCs ensures that they do not prematurely oligomerize before they are bound to their intended target cell. However, in the case of pneumolysin, it appears that it may have evolved to oligomerize more easily in solution in order to facilitate its ability to activate complement via the classical pathway. This phenomenon is discussed in detail later in the review in the section "CDC Structural Diversity and Pathogenesis."

PORE FORMATION

Structure of the membrane-spanning pore. The final stage in the CDC mechanism is the formation of the transmembrane β-barrel pore. We had originally suggested (80) that the membrane-spanning domain was formed by the β-sandwich structure of domain 4 of PFO (Fig. 1), based on early evidence that the undecapeptide of domain 4 entered the membrane (58). However, as described above, it has now been well established that domain 4 forms a superficial interaction only with the membrane and it does not contribute directly to the transmembrane β-barrel structure. Shortly before the elucidation of the PFO crystal structure, Palmer et al. (67) suggested that that a region within domain 3 might be associated with the membrane. Subsequently, Shepard et al. (87, 88) identified the membrane-spanning structures of domain 3. Using multiple fluorescent techniques, it was shown that each PFO monomer contributed two amphipathic transmembrane β-hairpins (TMH1 and TMH2) to the formation of a large transmembrane β-barrel. In combination with the crystal structure of monomeric PFO, these studies revealed a remarkable feature of the CDC transmembrane β-hairpins; each hairpin was packed in the soluble monomer as two sets of three α-helices in domain 3 (Fig. 1). Therefore, these α-helices undergo a relatively uncommon transition in secondary structure from α-helices to β-strands in order to form the two TMHs. Similar transitions are observed in the amyloid β-peptide, prion proteins, and elongation factor Tu (1, 68). Both PFO and ILY crystal structures (72, 80) reveal the same α-helical motif for the TMH sequences (Fig. 1) in the soluble monomers, so presumably the TMHs of all CDCs undergo the same structural transition.

The structural and mechanistic basis for the arrangement of the TMHs as α-helices in the soluble monomer is likely complex. One explanation for the α-helical packing motif may be that this packing in the soluble monomer minimizes the exposure of the hydrophobic residues of the amphipathic TMHs on the surface of the soluble monomer, thereby protecting the soluble nature of the monomer. The hydrophobic residues of the two TMHs of PFO are mainly packed against the core of the protein with the hydrophilic residues on the surface. The hydrophobic residues of TMH1 (Fig. 1) are mostly packed against domain 2, whereas those of TMH2 are packed against the core β-sheet of domain 3. Interestingly, the interface between TMH1 and domain 2 is not complementary (72, 80). It appears that the structure of this interface is such that it can be disrupted but stable enough to prevent the premature unraveling of the TMHs. Disruption of this interface is ultimately necessary for the extrusion of TMH1. In order to convert the α-helical bundle of TMH1 into an extended β-hairpin, the TMH1 α-helical bundle must extricate itself from its interface with domain 2, a process that would be more difficult if this interface was significantly more stable. At the same time that TMH1 breaks its interaction with domain 2, it is likely that the twist in the core β-sheet of domain 3 is relieved, resulting in the placement of each extended TMH on the opposite side from which the respective α-helical bundles reside in the monomer. As described below, the movement of the α-helices that form TMH1 away from domain 2 also likely removes a stabilizing influence from the structure of domain 2 that may lead to its ultimate collapse that brings the domain 3 TMHs into close proximity with the membrane surface during pore formation.

The use of amphipathic β-hairpins to form a membrane-spanning β-barrel by *S. aureus* α-hemolysin had previously been shown by Song et al. (91), who solved the crystal structure of the α-hemolysin heptameric membrane pore complex. How-

ever, several aspects of the PFO membrane-spanning domain were found to be unusual. First, each PFO oligomeric complex is comprised of about 35 to 50 monomers (12, 65) and each monomer contributes two β-hairpins to the β-barrel pore. Therefore, the coordinated insertion of up to 100 β-hairpins, or 200 β-strands, is necessary to form the CDC pore. In contrast, the monomers of other β-barrel toxins contribute a single amphipathic β-hairpin to the formation of the β-barrel. The oligomer of these other pore-forming toxins is generally heptameric, and hence seven β-hairpins form a 14-stranded β-barrel. By comparison, the CDCs must coordinate the insertion of 10 to 15 times as many β-strands.

As the TMHs begin their conversion from the α-helical structure in the monomer to their final β-hairpin structure as part of the transmembrane β-barrel, it is clear that they must proceed through a disordered state, but when and how they ultimately form the transmembrane β-barrel is not clear. The details of this process are not well understood, but there are some studies suggesting that the TMHs do not independently insert into the membrane. Hotze et al. (32) showed that a PFO mutant (PFO^{Y181A}) that was trapped in the prepore stage of the mechanism could be forced to insert its TMHs when mixed with sufficient native PFO. These studies suggested that the monomers cooperate to initiate the formation of the transmembrane β-barrel by the cooperative insertion of the TMHs.

The formation of a preinsertion β-barrel scenario is preferred to the individual insertion of the TMHs since the energetic cost of inserting a non-hydrogen-bonded peptide bond into the bilayer is high (reviewed in reference 100). It has been estimated that the free energy of partitioning an amino acid into a bilayer would be reduced 0.5 kcal mol^{-1} per residue if the hydrogen bond potential of the peptide bond is satisfied (100). Therefore, the insertion of the large number of TMHs in a CDC oligomer would be far more energetically favorable if the backbone hydrogen bond potential of the β-strands is fulfilled, as would be the case in a partially or wholly formed preinsertion β-barrel in the prepore complex. Another factor that can be important to stabilizing the interaction of a peptide with the bilayer is the interaction of basic amino acids with the acidic phosphates of the phospholipids headgroups. Both TMHs of PFO contain lysine residues at the beginnings and ends of the hairpins as well as near the hairpin turns on the opposite side of the membrane (87, 88). This characteristic appears to be mostly conserved for the predicted TMHs of other CDCs.

Alignment of the TMHs. The intermolecular alignment of the twin TMHs of each monomer in the membrane oligomer also posed a potential problem in the assembly of the β-barrel. The alignment of β-strands 1 through 4 of the twin TMHs within each of the monomers is probably guided by the core β-sheet of domain 3 since each β-strand of the TMHs is an extension of β-strands 1 through 4 of the core β-sheet (Fig. 1). Therefore, upon their extension, the hydrogen bond pattern of the core β-sheet would essentially act as a template to promote the formation of the hydrogen bonds between these strands in proper register. However, the same scenario does not allow for the proper alignment of the two outer strands, β1 and β4 of the TMHs, with the β4 and β1 strands, respectively, of the adjacent monomers. In PFO and most other CDCs, this alignment is guided by a π-stacking interaction of the aromatic rings of Tyr-181 and Phe-318 (77) that are located in β1 and β4 of the TMHs, respectively.

As described above, Hotze et al. (32) showed that the conversion of Tyr-181 to alanine locked PFO in a prepore complex. Ramachandran et al. (77) subsequently showed that the mechanistic basis of this mutant phenotype resulted from the loss of the π-stacking interaction of Tyr-181 with Phe-318. The mutation of Phe-318 to alanine also exhibits the same prepore-trapped phenotype as the Tyr-181 mutant. The interaction of these two residues is necessary to align the hairpins of adjacent monomers; when either residue is altered to a nonaromatic residue or when the position of either residue is shifted in the peptide chain, the TMHs do not align properly and their membrane insertion does not proceed. However, if both residues are moved together along the β-strands and are kept in register with one another, proper alignment is maintained and pore formation occurs. Interestingly, mixing a sufficient amount of native PFO with either the Tyr-181 or Phe-318 mutant can force either mutant to insert its TMHs (32, 77). This observation suggests that the interaction of just one aromatic pair may be sufficient to properly align the β-hairpins for insertion.

Vertical collapse of the prepore structure. The final stage of the pore assembly is the formation the membrane-spanning β-barrel. Shatursky et al. (87) showed unambiguously that the TMHs of PFO spanned the bilayer, yet the subsequent studies of Ramachandran et al. (75) showed that the monomers were anchored in an upright position on the membrane. If the monomers remained in an upright position in the pore oligomer, then the monomers would stand about 115 Å above the membrane and domain 3 would be about 40 Å above the membrane surface (Fig. 1 and 2). If the TMHs were extended down from domain 3 toward the bilayer, they would be of only sufficient length to reach the membrane surface but could not span the bilayer. This conundrum was resolved by studies that showed that the prepore undergoes a vertical collapse of 35 to 40 Å (12, 76). Czajkowsky et al. (12), using atomic force microscopy (AFM), first showed that the height of the disulfide-trapped PFO prepore complex (31) was approximately 115 Å, indicating that the monomers within the prepore were oriented perpendicular to the membrane in an upright position. The conversion of the prepore structure to the pore resulted in a remarkable decrease in the vertical height of the oligomeric complex by about 40 Å. Ramachandran et al. (76) confirmed these results using fluorescence resonance energy transfer to measure changes in the height of the prepore and pore complexes. The difference in height of the prepore and pore complexes was measured to be 35 Å, in good agreement with the AFM results. Similar results have recently been obtained by three-dimensional reconstructions of cryo-electron microscopy (cryo-EM) data of the pneumolysin prepore and pore complexes (93).

This decrease in vertical height would bring the bottom of domain 3 close to the membrane surface, and therefore, upon their extension, the TMHs would easily cross the bilayer. The collapse of the domain 2 structure likely leads to the decrease in the height of the membrane oligomer (Fig. 1 and 2). The extrication of the α-helical bundle, which ultimately forms TMH1, from the domain 2 and 3 interface would likely result in the destabilization of the extended β-sheet structure of

domain 2, resulting in its collapse and leading to the movement of domains 1 and 3 downward to the membrane surface (12).

The dramatic decrease in the complex height is thus far unique to the CDCs. We suggest that the initial position of domain 3 40 Å above the membrane surface allows the prepore the vertical space to organize a preinsertion β-barrel (Fig. 2). As indicated earlier in this review, the formation of a preinsertion β-barrel would satisfy the hydrogen bond potential of the TMHs, which would decrease the energetic cost of inserting the TMHs into the bilayer.

Conformational coupling of domains 3 and 4. It is clear that domain 4 is the first to interact with the cell surface, and so it is not entirely unexpected that this initial interaction directs subsequent events that lead to oligomerization of the monomers and pore formation. In support of this scenario, Heuck et al. (27) have shown that domains 3 and 4 are conformationally coupled, even though they are not in direct contact with one another. Mutations within the domain 3 TMHs of PFO that altered their rate of insertion into the membrane correspondingly altered the rate of insertion of the domain 4 undecapeptide tryptophans into the bilayer surface. Since membrane insertion of the domain 4 undecapeptide precedes the insertion of the domain 3 TMHs (27), the conformational changes that occur in domain 3 must be conformationally coupled to the insertion of the domain 4 undecapeptide. The purpose of this coupling seems obvious at first, in that this coupling may trigger changes in the structure of the membrane-bound monomers so that they can oligomerize into the prepore complex. It would appear that the interaction of domain 4 (75) with the membrane triggers the movement of the domain 3 β5-α1 loop (Fig. 1) that is required to initiate oligomerization of the monomers via the edge-on interactions of β-strands 1 and 4 of the domain 3 core β-sheet (77).

The conformational coupling, however, may be more complex than is immediately obvious. As described above in the section on the undecapeptide, the studies by Polekhina et al. (72) and Nagamune et al. (53) have shown that, unlike PFO and other CDCs, ILY membrane binding has been uncoupled from the undecapeptide. Yet, the ILY undecapeptide still controls prepore-to-pore conversion; mutation of Trp-491 of the ILY undecapeptide to alanine blocks prepore-to-pore conversion. Therefore, ILY binding to CD59 appears to trigger conformational changes that lead to oligomerization (presumably the rotation of the β5α1 loop away from β4), but membrane insertion is still controlled by its undecapeptide. In CDCs like PFO, both binding to cholesterol-rich membranes and prepore-to-pore conversion appear to depend on the undecapeptide. Therefore, the study of the conformational coupling between domains 3 and 4 may be best studied in the ILY system since its undecapeptide appears to exhibit only a single function, that of controlling prepore-to-pore conversion.

Cryo-EM structure of the pneumolysin pore. It is unlikely that a high-resolution atomic structure of a CDC prepore or pore will be solved in the near future due to the heterogeneity in the number of monomers that can be incorporated into the CDC pore complex. Cryo-EM, however, offers an approach to obtain a low-resolution structure for the CDC pore. Tilley et al. (93) have recently presented a three-dimensional reconstruction of the PLY pore by fitting the crystal structure of the PFO monomer (80) to the electron density map of the PLY pore complex. Their cryo-EM structure of the PLY pore complex correlated well with previous models of CDC pore structure derived from the monomer crystal structure of PFO and the previous biophysical and AFM studies described above (12, 27, 28, 32, 33, 75–77, 80, 87–89). Gilbert et al. (21) had previously used cryo-EM to determine the pore structure PLY, but unlike the current PLY pore structure by Tilley et al. (93), they did not see membrane penetration. It is now well known that twin transmembrane TMHs span the bilayer (87, 88), and based on studies by Shepard et al. (89) and Hotze et al. (33), who demonstrated the CDCs first oligomerize into a prepore structure, and the atomic force microscopy data of the PFO prepore and pore oligomers (12), it is now accepted that Gilbert et al. (21) had actually identified the prepore structure of PLY. Based on these cryo-EM studies, PLY exhibits about a 40 Å collapse of the oligomer height upon prepore-to-pore conversion, similar to that shown previously for PFO by AFM (12) and fluorescence resonance energy transfer (76). The collapse of the prepore structure was predicted to occur as a result of structural changes in the domain 2 structure (12), since withdrawal of the domain 3 α-helical bundle, which ultimately forms TMH1, from its interface with domain 2 would remove a major stabilizing influence on the extended two-stranded β-sheet structure of domain 2. The cryo-EM results are consistent with this model of prepore collapse, since it appears that domain 2 bulges out slightly on the outside of the PLY monomer structure upon the prepore-to-pore transition.

CDC STRUCTURAL DIVERSITY AND PATHOGENESIS

The CDCs are beta-hemolytic proteins and were originally termed hemolysins, although to date, it does not appear that they are used as hemolysins by the bacterial pathogens during an infection. The CDCs are also capable of the lysis of a wide variety of nucleated cell types in vitro, and this capacity has in turn has been used by many investigators to permeabilize various eukaryotic cell types with CDCs (7). Despite the ability of these toxins to perform as general cell-lytic agents in vitro, it has not yet been demonstrated that cell lysis is a primary function of the CDCs during an infection. The contribution of CDCs to infection has been studied to various extents in *Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Arcanobacterium pyogenes*, and *Clostridium perfringens*. The results of some of these studies suggest that the bacteria use the CDCs in more sophisticated ways than as general cytolytic agents. It also appears that the CDC structure has undergone some unique evolutionary transformations that facilitate the pathogenic mechanism of these bacterial species. These changes to the basic CDC structure alter the mechanistic behavior of the CDC without affecting the ability of the protein to assemble into a pore-forming complex. These evolutionary changes in the CDC structure are likely beneficial to the bacterial pathogen during disease establishment and/or progression. The structural changes and their possible impact on pathogenesis are discussed below.

Listeriolysin O. The contribution of listeriolysin O (LLO) to the pathogenic mechanism of *Listeria monocytogenes* is probably one of the best understood of the CDCs, but many fundamental aspects of its contribution to listerial disease remain unexplained. *L. monocytogenes* is a facultative intracellular

pathogen in humans, and it was established nearly two decades ago that the expression of LLO was required for the vacuolar escape of *L. monocytogenes* (15) and pathogenesis (10, 73). *L. monocytogenes* mutants that do not express LLO are avirulent in animal models of listerosis. Purified LLO exhibits a pH-dependent cytolytic activity (18), wherein it exhibits little or no activity near neutral pH and maximal activity at about pH 5.5. This characteristic is not observed in any other nonlisterial CDC. It appears that the pH-dependent nature of LLO is important in the intracellular survival of the bacterial cell. Beauregard et al. (5) showed that neutralization of the acidic environment of the phagosome prevents the LLO-dependent perforation of the phagosome and the escape of the bacterium from the phagosome. Also, if *L. monocytogenes* expresses a mutant of LLO that remains highly active at neutral pH (22), or if LLO is replaced by the non-pH-dependent PFO (39), the eukaryotic cell membrane is lysed. The lysis of the cell membrane often correlates with a decreased in vivo virulence in the mouse model, presumably because the bacterial cell can no longer replicate within the protected environment of the eukaryotic cell cytosol. These observations suggest that the LLO cytolytic activity must be turned off after the escape of the bacterial cell from the phagosome in order to prevent the lysis of the eukaryotic cell plasma membrane, thereby allowing the bacterial cells to replicate within the protected environment of the eukaryotic cytosol. Therefore, it is likely that the activity of LLO would remain high in the acidic environment of the endosome, but it would be largely inactive in the neutral pH of the host cell cytosol.

LLO also exhibits another unique structural feature: an additional amino-terminal sequence deemed the proline-glutamate-serine-threonine (PEST)-like sequence (13, 43). PEST sequences typically promote the degradation of proteins (78). This sequence is comprised of about 16 amino-terminal residues and appears to regulate LLO activity. *L. monocytogenes* that expresses a PEST-deficient version of LLO is significantly less efficient at intracellular replication and is less virulent. Also, when an LLO-deficient *L. monocytogenes* expresses a PFO chimera, in which the LLO PEST-like sequence is fused to the amino terminus of PFO, the bacterium appears to partially regain the ability to replicate in macrophage (13). Presumably the PEST sequence of LLO would promote degradation of LLO in the cytoplasm and therefore add another layer of control to LLO cytolytic activity to ensure that it does not lyse the plasma membrane. It is, however, not entirely clear that this region of LLO functions as a PEST sequence, as there is evidence that it does not function as a PEST sequence (42). Although the mechanism by which the PEST sequence functions to regulate LLO activity is ambiguous, its presence is clearly important to listerial pathogenesis.

It is interesting to note that the mechanism by which LLO actually facilitates the escape of the bacterial cell from the phagosome remains an enigma. The LLO pore is large, but it is not large enough to allow passage of the bacterial cell out of the phagosome. Furthermore, phagosomes cannot be lysed by a colloid-osmotic mechanism in the intracellular environment of the eukaryotic cell. It is likely that the phagosomal contents are quickly equilibrated with the cytosolic constituents as the large LLO pores are formed, but how pore formation results in the escape of the bacterial cell from the phagosome is likely more complex than previously imagined. Many possibilities can be envisioned, but as of yet, there is no compelling evidence that provides any specific insight into how bacterial escape is facilitated by the LLO pore.

Streptolysin O. *Streptococcus pyogenes* is a pathogen frequently associated with dermatological infections, and SLO has been shown to be important to the pathogenesis of *S. pyogenes* in a mouse model of invasive disease (44). Secreted SLO contains about 60 extra amino acids at its amino terminus that are not found on any other sequenced CDC. It has been shown in a set of elegant experiments by Madden et al. (45) that SLO mediates the translocation of the extracellular NAD+ glycohydrolase (SPN), an enzyme that is also produced by *S. pyogenes*, into keratinocytes. It has been further shown that these additional amino-terminal residues contribute to the SLO-mediated translocation of the SPN into human keratinocytes (46). Hence, it was proposed that SLO functioned as an analog to the gram-negative bacterial type III secretion systems since it appears to direct the transfer of SPN directly into the eukaryotic cell cytosol. Removal of the extra amino-terminal residues from SLO abolished SPN translocation (without affecting its pore-forming activity), but the addition of these residues to PFO does not convert PFO into a functional substitute for SLO in this translocation process (46). Thus, there appear to be additional differences in the structure of SLO that are important in the translocation of SPN (46).

It has been shown that the SLO-linked translocation of SPN inhibits bacterial internalization, induces keratinocyte apoptosis, and rushes the cell to death (9, 45). Surprisingly, cells expressing SLO alone are not nearly as cytotoxic as when its expression is coupled with SPN, showing that SLO does not appear to be an effective agent for general cell killing by SLO-dependent lysis. Although little is known about the mechanistic details of how SPN translocation is coupled to the SLO pore, many intriguing questions remain about this system, such as how SPN is directed to the SLO pore and how its translocation is biased to the inside of the keratinocyte without the lysis of the eukaryotic cell.

Pneumolysin. The *Streptococcal pneumoniae* PLY structure exhibits two unique traits; it has lost its signal peptide for secretion by the type II pathway, and it contains a region that is capable of activating complement via the classical pathway (71). All known CDCs, except for PLY, are secreted via a type II secretion pathway and therefore carry a typical signal peptide at their amino terminus. Why PLY has lost its signal peptide is not known, although it is clear that PLY must be released from the cell since it exerts specific effects outside of the bacterial cytoplasm during an infection. It has been shown that PLY contributes to the pathogenesis of various pneumococcal disease syndromes in a number of ways (reviewed in reference 30). PLY accumulates in the cytoplasm of the bacterial cell and is released during log growth, and its release increases significantly when stationary phase is reached (41). It was hypothesized (70) that PLY is released by cell lysis and in some strains, the release of PLY appears to coincide with cell lysis (6). In another strain of *S. pneumoniae*, however, the release of PLY does not appear to coincide with cell lysis (4), suggesting that another mode of lysis-independent release of PLY may be operative.

PLY-dependent complement activation involves residues

Tyr-384 and Asp-385 of domain 4, and PLY appears to bind to the F_c region of immunoglobulin G (IgG) (49). Mutation of either of these residues abolishes complement activation; however, it does not completely eliminate IgG binding by PLY (49). Activation of complement by IgG requires the clustering of the IgG molecules on the cell surface or in vitro-aggregated IgG. As noted earlier in this review, PLY has a tendency to oligomerize to some extent in solution. It has been hypothesized that aggregated PLY may be more effective in activating complement than monomeric toxin (49), presumably since the bound IgG would be clustered on the toxin oligomer. Therefore, as opposed to most other CDCs, PLY may have evolved in such a way as to be more easily triggered to oligomerize in solution so that a fraction of the PLY can activate complement but that some of the toxin would still be capable of binding to membranes and forming pores. This scenario is also consistent with the observations that the pore-forming and complement activation activities of PLY contribute in different ways to the pathogenesis of *S. pneumoniae* (40, 82).

Intermedilysin. ILY is expressed by *Streptococcus intermedius*, and as described above, it exhibits the unique characteristic of being specific for human cells (54, 55) based on the ability to specifically bind to human CD59 (20). No other CDC appears to bind to a nonsterol receptor, although it is probably too early to dismiss the possibility that other CDCs might use specific receptors other than cholesterol. Does the ability to specifically bind human cells via CD59 contribute in some way to the establishment of *S. intermedius* infections? No studies have been performed that directly address the contribution of ILY to the establishment and progression of *S. intermedius* disease, which is typified by abscess formation. It is unlikely that an animal model will be established in the near future, since ILY is known to bind only to human cells. There are, however, some aspects of abscess formation that allude to a role for ILY in the establishment of the abscess by *S. intermedius*. Abscesses are defined by an abundance of bacteria and neutrophils. It has been shown that *S. intermedius* isolates from abscesses express sixfold- to tenfold-higher levels of ILY than isolates from the oropharyx (56), the normal habitat for the organism. Also, activated neutrophils upregulate CD59 expression (23). Hence, the neutrophils would provide target rich environment for ILY. Whether ILY directly kills the neutrophils and/or modifies their behavior likely depends on the concentration of ILY that binds to the cell surface of the neutrophil.

FUTURE PERSPECTIVES

Many aspects of the pore-forming mechanism of the CDCs have been revealed over the last decade, resulting in the establishment of new paradigms for pore-forming toxins. Despite the advances made in the last decade on the CDC pore-forming mechanism, there are many fundamental questions that still remain. For instance, we do not yet have a clear understanding of the structure that is assumed by the TMHs during their initial penetration of the membrane, nor do we know the fate of the comparatively large amount of lipid that disappears from the central pore of the CDC oligomer or whether incomplete rings can form transmembrane pores. Another area of CDC research that promises to be of interest is that of cellular recognition: like ILY, can other CDCs use cellular receptors other than cholesterol? Equally interesting are questions that concern the actual contribution of CDCs to disease establishment by the various bacterial pathogens and how the changes in the basic structure of the CDC might benefit the pathogen. The study by Madden et al. (45), who showed that SLO can be used as a protein translocation channel, is an excellent example of a paradigm shift that challenges us to look more closely at how CDCs are used during an infection. How CDCs form a hole in the membrane is only the beginning of the story; the ways in which the bacterial pathogens use these extraordinary pore-forming proteins promise to be fascinating.

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