FOR THE RECORD

Vibrio cholerae cytolysin is composed of an α -hemolysin-like core

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Abstract

The enteric pathogen *Vibrio cholerae* secretes a water-soluble 80-kD cytolysin, *Vibrio cholerae* cytolysin (VCC) that assembles into pentameric channels following proteolytic activation by exogenous proteases. Until now, VCC has been placed in a unique class of pore-forming toxins, distinct from paradigms such as Staphylococcal α -hemolysin. However, as reported here, amino acid sequence analysis and three-dimensional structure modeling indicate that the core component of the VCC toxin is related in sequence and structure to a family of hemolysins from *Staphylococcus aureus* that include leukocidin F and α -hemolysin. Furthermore, our analysis has identified the channel-forming region of VCC and a potential lipid head-group binding site, and suggests a conserved mechanism of assembly and lysis. An additional domain in the VCC toxin is related to plant lectins, conferring additional target cell specificity to the toxin.

Keywords: α -hemolysin; cytolysin; lectin; leukocidin; pore-forming bacterial toxins; ricin; *Staphylococcus aureus*; *Vibrio cholerae*

Many pathogenic bacteria secrete pore-forming toxins that bind and assemble on target cell membranes, forming lytic, transmembrane ion channels (Gouaux 1997). A subset of these toxins employ β -barrels to span the bilayer, where the barrel is composed of strands contributed by protomers arranged around a rotational axis of symmetry (Heuck et al. 2001; Menestrina et al. 2001). High-resolution structures of the water-soluble, monomeric leukocidin F (LukF; Olson et al. 1999; Pedelacq et al. 1999) and the detergent-soluble, heptameric channel of α -hemolysin (α HL; Song et al. 1996) from *Staphylococcus aureus* define the initial and final steps on the assembly pathway, illuminating the conformational rearrangements that occur upon pore formation. Although the sequence identity between α HL and LukF is low ($\sim$ 30%), the structural cores of the proteins are superimpos-

able, as previously predicted from sequence analysis (Gouaux et al. 1997).

Vibrio cholerae cytolysin (VCC) is an important pore-forming toxin and, until now, has been classified as functionally and structurally distinct from the *S. aureus* toxins described above. For example, VCC exhibits specificity for membranes containing cholesterol and ceramides (Zitzer et al. 1999) and contains a prodomain that is required for folding and must be cleaved to produce mature toxin (Nagamune et al. 1997). In contrast, α HL and LukF are most active at membranes that contain phosphatidylcholine; they each contain a specific binding site for phosphocholine (Tomita and Kamio 1997), and they do not have a prodomain. Furthermore, the 65-kD mature VCC protein assembles to form pentameric channels (Zitzer et al. 1999), whereas the α HL 33-kD protomer assembles to a homoheptamer, and LukF (34 kD), together with LukS, forms a heteroheptamer (Sugawara-Tomita et al. 2002) or a heterooctamer (Miles et al. 2002). On the basis of functional properties, differences in protomer molecular mass and oligomer subunit stoichiometry, and apparent absence of amino acid sequence homology, the VCC toxin appears to be unrelated in structure and mechanism to LukF and α HL.

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Abbreviations: VCC, *Vibrio cholerae* cytolysin; α HL, α -hemolysin; LukF, leukocidin F component.

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To determine the extent to which VCC may be related in amino acid sequence, three-dimensional structure, and biological function to the α HL family of pore-forming toxins from *Staphylococcus aureus*, we employed ψ -BLAST at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) using the complete VCC protein sequence (NCBI accession number NP_232618) as a search probe. After several rounds of iterative searching, the LukF and α HL sequences were located with E-values of 10^{-63} and 10^{-49} , respectively. This analysis clearly revealed that VCC contains a central region of ~ 250 amino acid residues that is related to the Aerolysin/Hemolysin/Leukocidin family of pore-forming toxins (Bateman et al. 2002), which we hereafter call the cytolytic domain (Fig. 1). A closer examination of the search results indicated that VCC is more closely related to the Staphylococcal α -hemolysin and leukocidin toxins than the aerolysin protein from *Aeromonas hydrophila*, although *Aeromonas* has a separate VCC-like toxin. We also discovered that following the cytolytic domain, VCC has a ricin-like domain (Schultz et al. 1998) spanning residues R484 to T600. After the ricin-like domain there are ~ 140 amino acids of unknown function or structure that can be proteolytically removed without impairing the function of the toxin (Ikigai et al. 1999; Zitzer et al. 1999), and that are missing in related cytolytins from *Vibrio vulnificus* (Yamamoto et al. 1990), *Aeromonas hydrophila* (Hirono and Aoki 1991; Wong et al. 1998), and *Aeromonas salmonicida* (Hirono and Aoki 1993).

Careful analysis of the sequence alignments among VCC, α HL, and LukF indicates that these toxins have a common cytolytic core with a similar overall fold. We have outlined the putative membrane-crossing region and suggest that the VCC pore is an antiparallel β -barrel. We also hypothesize that the ricin-like domain confers the carbohydrate-binding activity upon VCC. Our new observations define the architecture of the VCC protein, identify the critical pore-forming residues, and suggest that VCC and the *Staphylococcus aureus* toxins share a common mechanism of pore formation.

Cytolytic domain

The cytolytic cores of VCC, α HL, and LukF were aligned using the ClustalW program (Thompson et al. 1994), as

illustrated in Figure 2. The first 47 residues of α HL and 46 residues of LukF did not show significant similarity to VCC and were excluded from the alignment. The sequence identity values between VCC and α HL and VCC and LukF were 16.5% and 12.9%, respectively, with 19 out of 236 residues conserved in all three proteins. Sequence similarity was $\sim 25\%$ between VCC and each of the *S. aureus* toxins, and $\sim 45\%$ between α HL and LukF.

Although the level of sequence identity between VCC and α HL/LukF is low, a close examination of the alignment, in light of the α HL and LukF structures, provides substantiating evidence for a similar fold, and suggests a common architecture for the transmembrane pore. Indeed, we see that hydrophobic and aromatic residues occupy key positions in the protein core (Chen and Stites 2001). There are five strictly conserved aromatics (VCC numbering: Y338, W367, Y438, Y445, W475) with six additional aromatics conservatively substituted (VCC numbering: Y260, Y279, F313, W343, Y454, F471). When mapped onto the LukF structure, most of these residues are located in the core of the β -sandwich and rim domains (Fig. 3). An exception is W367 (LukF W177), which is solvent-exposed and forms a lipid head-group binding pocket in both α HL and LukF, and is found in all three sequences, thus identifying a likely lipid-head group binding site in VCC. A number of non-aromatic hydrophobic residues scattered throughout the VCC sequence are either identical or conservatively replaced in the three proteins: L251, I292, I353, I398, V407, I434, V447, and V473. On the basis of these sequence and structure relationships, we propose that the main core (β -sandwich and rim) of the VCC cytolytic domain shares structural homology with the β -sandwich and rim domains of the α HL and LukF toxins from *S. aureus*.

Stem region

Using the alignment shown in Figure 2, we can pinpoint the stem or pore-forming region of VCC. The stem is flanked by highly conserved residues in the triangle region, is glycine-rich, and contains alternating polar and nonpolar residues. Residues in the triangle and stem of α HL make important contacts between protomers in the oligomeric state,

Figure 1. Domain outline of the *Vibrio cholerae* cytotoxin gene, showing the location of the α -hemolysin-like cytolytic domain in green.

Figure 2. Sequence alignment of the related domains of LukF, α HL, and VCC. Hydrophobic residues (F, I, L, V, W, Y) identical or similar in all three sequences are marked by magenta and green circles, respectively. The secondary structure of LukF from the crystal structure is illustrated above the sequences using the same coloring scheme as in Figure 3. The alignment was generated with ClustalW version 1.74 and displayed using Boxshade. Residues identical in two or three sequences are boxed in black, and similar residues are in gray. Green arrows designate alternating hydrophobic residues that could potentially contact the membrane.

and specific mutations in this region arrest α HL in various stages of heptamer assembly (Walker and Bayley 1995). A key proline residue (P102 in LukF) adopts a *cis*-peptide bond in the α HL and LukF structures, is located near a hinge point in the triangle, and is present in VCC (Olson et al. 1999).

Although the triangle region is extremely sensitive to mutations, the stem has been removed (Cheley et al. 1997), replaced (Gu et al. 2000), and even reversed (Cheley et al. 1999) without substantially affecting assembly of the α HL heptamer. In the LukF structure, the stem is folded into a three-strand β -sheet that packs against a hydrophobic patch on the β -sandwich domain. In the α HL heptamer, the stem is unfurled and each protomer contributes two strands to a 14-strand β -barrel. The stem must be flexible so that it can undergo these dramatic conformational changes, and the stem has an abundance of glycine residues: eight in α HL, seven in LukF, and six in VCC.

Like other β -barrel membrane proteins, a ring of aromatic residues is found near the apparent membrane-aqueous interface in α HL and LukF at positions Y117 and F119 (LukF). Although these residues are not identical in VCC, the phenylalanine and leucine (F313 and L315 in VCC) residues are conservative substitutions. LukF residues Y117 and F119, along with F139 (isoleucine in α HL and leucine in VCC), contact hydrophobic residues in the amino-latch in the water-soluble state (Olson et al. 1999), and may help coordinate stem unfolding with latch movement during assembly of the channel.

A hallmark of β -barrel membrane proteins is the alternating pattern of hydrophilic and hydrophobic residues that contact the lumen of the channel and lipid moiety, respectively (Schulz 2000). This pattern is clear in the stem domains of LukF and α HL, even though the sequence identity

Figure 3. Ribbon representation of the LukF crystal structure showing conserved aromatic residues. Magenta residues are identical in all three proteins, and green residues are aromatic (F, W, Y). The amino latch (pink) and first two β -strands (gray) do not appear to be present in VCC and were not included in the alignment in Figure 2.

is low, and is also evident in the stem of VCC, reinforcing our hypothesis that this region forms a transmembrane β -barrel.

Interestingly, the putative membrane-spanning region of VCC is five residues shorter than the corresponding region of α HL, and three residues shorter than the equivalent segment of LukF. The shorter transmembrane β -strands in VCC may be explained by VCC having a shorter assembled stem. However, the shorter length of the VCC β -strands may also be due to the fact the VCC barrel is probably pentameric (Zitzer et al. 1999), and thus has a smaller diameter (Chothia and Murzin 1993), requiring shorter β -strands. A smaller β -barrel for the assembled VCC toxin is also consistent with its lower single-channel conductance (Menzl et al. 1996; Miles et al. 2001), relative to the larger barrels of α HL and leukocidin.

Ricin-like domain

Ricin-like lectin domains are found in many A-B type plant toxins and participate in binding β -D-galactopyranoside molecules on the surface of cells (Olsnes and Kozlov 2001). Structures have been determined for several plant lectins with a portion of the B-subunit forming a β -trefoil structure with three binding sites thought to have arisen from ancestral gene duplication (Rutenber et al. 1987). Most members of this family have either two or three conserved disulfide bonds, as well as three QxW motifs (α , β , and γ) around a pseudo-threefold axis that marks the carbohydrate binding sites.

The presence of a QxW motif in VCC was suggested earlier (Hazes 1996) although the carbohydrate binding capability was questioned due to low sequence homology

within these domains. The carbohydrate binding ability has since been demonstrated (Saha and Banerjee 1997; Zhang et al. 1999), and the ricin-like domain is the likely site of binding. An alignment using ClustalW was performed with VCC and the lectin domains from ricin and abrin-a (Fig. 4A). Although the sequence identity is low (17.5%) between VCC and abrin-a, and 19.8% between VCC and ricin, there is conservation of four cysteines that form disulfide bonds in the ricin family.

As was the case with the cytolytic domains, several hydrophobic residues located in the hydrophobic core are conserved in all three proteins (Fig. 4B). These include F520 (in VCC), W557, W559, Y570, and Y587. Also conserved in VCC were two of the three QxW repeats, suggesting that VCC might possess up to two carbohydrate binding sites. Although most β -trefoil lectins have three QxW repeats, many have lost activity in one or more of these sites (Notenboom et al. 2002), and it is not clear whether both repeats are able to bind sugars in VCC.

Neither α -HL nor LukF contain a lectin domain, reflecting their difference in cellular targets. The C-terminus of LukF is on the side opposite the stem domain, and attaching the lectin domain to the C-terminus of the cytotoxin would not interfere with binding of the rim to the membrane (see Fig. 3), or with assembly of the channel. The last 15-kD domain in VCC may make additional membrane contacts, augmenting toxin specificity.

Conclusions

We have demonstrated that *Vibrio cholerae* cytotoxin is related to α HL and LukF in amino acid sequence and almost certainly in three-dimensional structure, and thus may uti-

Figure 4. Sequence alignment of the lectin domains of abrin-a, ricin (b-domain), and VCC. (A) Coloring of conserved residues follows the same scheme as in Figure 2. Note the four cysteines, which are predicted to form two disulfide bonds. The QxW domains are boxed in cyan. The secondary structure shown in red denotes the two conserved domains, and that in blue outlines the less well-conserved domain. (B) Crystal structure of the lectin domain of abrin-a (b-chain). Identical and similar hydrophobic residues are shown in magenta and in green, respectively. Conserved disulfide bonds are colored yellow. The coloring of the secondary structure is the same as that in (A).

lize a common mechanism for cell lysis. The lectin domain provides a precise mechanism by which the toxin may target specific cells. Sequence homology with conserved families account for the majority of the VCC polypeptide, leaving only the 15-kD C-terminal tail with unknown function and structure.

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Note added in proof

While this manuscript was in press, Harris et al. (*J. Struct. Biol.* **139**: 122–135) reported that the assembled VCC oligomer is a heptamer, like the α -HL heptamer, thus suggesting that the transmembrane domain of the VCC oligomer is a 14-strand β -barrel.

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