

Mechanisms of v.cholerae cytotoxin (vcc)



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V. cholerae cytolysin (VCC) is a pore-forming toxin secreted by many pathogenic strains of the Gram-negative bacteria *V. cholerae* the causative pathogen of diarrheal disease cholera. VCC display potent cytotoxic activity against the erythrocytes and mammalian cells. It is also reported to possess enterotoxin activity in terms of inducing bloody fluid accumulation in the rabbit ileal loops. Based on these observations, VCC has been believed as a potential virulence factor of *V. cholerae*. VCC, in particular, secreted by the pathogenic strain lacking 'cholera toxin', the primary virulence factor of *V. cholerae* that responsible for inducing the massive dehydrating diarrhea disease during *V. cholerae* infection.

VCC is encoded by the *hlyA* gene present in *V. cholerae* chromosome two. VCC toxin is synthesized as a ~ 81 kDa protein, called Pre-Pro-VCC. During the secretion of toxin, the N-terminal signal peptide composed of 25-residue is removed to generate a nonfunctional precursor form of the VCC molecule, named as Pro-VCC. Following, ~ 15 kDa N-terminal sequences from Pro-VCC is proteolytically deleted that resulting the formation of the functional mature form of the toxin. Proteolytic activation of the toxin is mediated by the HA/protease, which display the major extracellular proteolytic activity of *V. cholerae*. Conversion from Pro-VCC into the mature state of the VCC can also be obtained in vitro by other proteases like trypsin, chymotrypsin, and subtilisin. It has been reported that the activation of the Pro-VCC can be resulted by the proteolytic activity of the proteases present on the surface of the target host cell membrane as well.

Functional mature form of the toxin has been displaying to induce lysis of the erythrocytes and other eukaryotic cells by generating the heptameric

oligomeric pore structure of 1-2 nm diameters. The functional membrane permeabilization ability of the toxin could also be resembled in the membrane lipid bilayer of the synthetic liposomes. Along with its membrane permeabilization activity, VCC toxin also displayed a prominent lectin-like activity by binding to the complex glycoproteins and glycolipids with the terminal β 1-galactosyl component. VCC is characterized as a member of β -PFTs and reported that the toxin follow the overall scheme of the generalized β -PFT mode of action.

1. Structural Features of VCC

VCC is secreted as a water-soluble monomeric form of the toxin, which after the removal of the N-terminal Pro-domain gets converted into the mature functional form of the molecule. VCC induces lysis of its target cells by generating heptameric oligomeric pores on the membrane. The high-resolution structure of the water-soluble, monomeric precursor state Pro-VCC toxin has been determined. Heptameric transmembrane structure of the VCC has also been determine recently. Many previous studies confirmed that the VCC is a β -PFTs family member, and the toxin employed pore-forming activity by generating the transmembrane heptameric β -barrel pores on the target cell membrane. Consistent with the β -PFTs transmembrane pore structure, pore complex of VCC represent a mushroom-shaped organization, which can be divided into two parts: (a) transmembrane b-barrel structural, and (b) membrane interacting rim domain. Structural analysis of the VCC molecule highlights many unique features which are not reported in the archetypical member of β -PFTs family. Consistent with the structural of archetypical β -PFTs, VCC harbors a central cytolysin domain that constitutes

the core structure of the mushroom-shaped oligomeric transmembrane pore structure. The cytolysin domain contains the pore-forming stem-loop of the toxin. Apart from cytolysin domain, VCC structure also contains three additional structural domain which are not commonly documented in any other member of β -PFTs family: an N-terminal Pro-domain in the inactive Pro-VCC precursor state of the toxin, and two lectin-like domain name β -Trefoil domain and β -Prism lectin-like domain at the C-terminal side of the cytolysin domain.

Cytolysin domain

The VCC molecular structure contains 325 amino acid long cytolysin domain that structurally similar with the cytolysin domains present in the member of β -PFTs like *S. aureus* α -hemolysin. Cytolysin domain of the VCC during the membrane pore-formation process inserts its 'pre-stem' region into the lipid bilayer and generates β -barrel structure on the membrane and provides the central scaffold of the pore structure. VCC generate mushroom-shaped oligomeric transmembrane pore structures that can be classify into two major parts: (a) transmembrane region that make the β -barrel pore structure, and, (b) membrane interacting rim-domain that interact with the membrane surface. The membrane inserted β -barrel structure of the VCC pore structure is composed solely of the central cytolysin domain of the toxin. Notably, the majority of the rim-domain is also generated by the cytolysin domain.

Cytolysin domain of the toxin harbors the 42-residue long 'pore-forming loop' loop that involve in the formation of the transmembrane β -barrel pore

structure. In the water soluble monomeric form of the toxin, this region remains completely folded against the cytolysin domain, in the form of a so named 'pre-stem' motif. During the process of the functional pore-formation, the 'pre-stem' loop from each of the participating protomers undergoes enormous structure recognition to obtain a so-called 'stem' configuration, and inserted into the lipid bilayer of the membrane. Stem region from each of the protomers contributes two β -strands towards the formation of the stem region of the heptameric β -barrel pore structure. Heptameric oligomer highlights that the stem regions make the extensive interaction between the neighboring protomers and hence contribute towards the robust stability of the transmembrane oligomeric assembly. Apart from the pore-forming stem-loop segment, other part of the cytolysin domain contains the membrane-proximal rim-domain of the transmembrane pore structure. Structural analysis of the β -PFTs pore, suggests that the membrane-proximal rim-domain work as the structure motif for transmembrane pores. Rim-domain acts as structural scaffolds that mediate interaction of the protein with the lipid head-group of the target membrane lipid bilayer. Cytolysin domain of the VCC contributes towards the interaction of the toxin with the lipid head-group of the membrane.

Pro-domain

As mentioned previously, VCC toxin is secreted by yet bacteria as the water-soluble inactive precursor state called Pro-VCC. The high resolution three-dimensional structure of Pro-VCC molecule shown the presence of ~ 15 kDa Pro-domain, which make contact to the N-terminal of the core cytolysin domain through a 29-residue long flexible linker. The linker region harbors ~

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amino acid long structural motif that act as the cleavage site(s) for a group of proteases. Proteolytic removal of the Pro-domain at this linker sequence resulted in the generation of a mature form of the toxin. The presence of the Pro-domain in the precursor form of the toxin has been reported to be critical for the efficient secretion and the appropriate folding of the VCC molecule. One earlier study has been reported that the recombinant *V. cholerae* cells, containing the deleted variant of *hlyA* gene lacking the sequence for the Pro-domain, unable to secrete the protein outside the bacterial cells. In vitro denaturation/renaturation, assay have demonstrated that without the Pro-domain VCC fails to refold back to its active conformation, whereas Pro-VCC can obtain proper refolding. Recent study on Pro-domain, suggested that the presence of Pro-domain increase the unfolding property of the Pro-VCC molecule in response to many denaturing conditions, whereas mature active form of the toxin display considerable resistant towards the unfolding of the toxin. Overall, these studies suggested, the Pro-domain show an intramolecular chaperone-like activity in term of providing significant level of structural plasticity in the VCC structure, which probably essential for the efficient secretion of the toxin in its precursor from across the bacterial membrane. However, it's not clear so far how the presence of the Pro-domain keep the protein in its precursor form.

β -Trefoil lectin-like domain:

VCC harbors a β -Trefoil lectin-like domain (~ 15 kDa) at the C-terminal edge of the center cytolysin domain. This β -Trefoil lectin-like domain is also present in related cytolysin from *Vibrionaceae* bacteria, but not present in the archetypical β -PFTs protein for example *S. aureus* α -hemolysin. The β -

Trefoil lectin-like domain is associated with the cytolysin domain through a short linker sequence constitute of Gly-Gly-Arg-Pro. The β -Trefoil lectin-like domain of VCC display structural similar to the carbohydrate-interacting domain of the plant toxin ricin, and featured the presence of the QXW conserved carbohydrate-interacting motif (s) observed in the archetypical β -Trefoil lectin domains of carbohydrate binding lectins. However, the carbohydrate binding propensity of the β -Trefoil domain of VCC has not been elucidated. Also, the implications of the β -Trefoil domain in the structure-function mechanism of the VCC need to be explored in future.

β -Prism lectin-like domain:

The VCC harbors an additional ~ 15 kDa domain that is linked to the C-terminal of the β -Trefoil domain through the long linker sequence. This domain is not present in any other member of the β -PFTs family, including the cytolysin secreted by *V. vulnificus* and *Aeromonas hydrophilia*. The C-terminal domain of the VCC display structural similarity to several β -Prism lectins including jacalin and *Maclura pomifera* agglutinin (MAP). VCC β -Prism lectin-like domain possess a binding pocket similar to the carbohydrate-binding site of the jacalin and MPA lectins. Recently, we have conclusively established the role of β -Prism domain in the lectin activity of the toxin. In the absence of the β -Prism domain, VCC toxin did not show lectin activity towards β -1 galactosyls terminated glycoconjugates. We have identified the critical site within the β -Prism domain which responsible for the lectin activity of the toxin. We reported that the amino acid triad (composed of Asp617, Tyr654, and Tyr679) located within the putative carbohydrate-interacting pocket generate the crucial element for the VCC lectin activity. Overall, it

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has been established that the β -Prism domain of the VCC act as structural scaffold playing a critical role in the lectin-like activity of the toxin. During the process of functional pore-formation in the lipid bilayer of the target host membrane, VCC molecule undergoes enormous structural reorganization. The β -Prism domain of the VCC obtained two different positions with respect to the core cytotoxin domain, in the monomeric precursor form (Pro-VCC) and the transmembrane pore structure. In monomeric water-soluble inactive precursor Pro-VCC, the β -Prism domain positioned on the opposite side of the Pro-domain on top of the pre-stem region, whereas in the transmembrane pore structure it is relocated in the place of the Pro-domain. This structural rearrangement of the β -Prism domain is mandatory for the membrane insertion, and the functional oligomeric pore-formation procedure. In the absence of such structural reorganization of the β -Prism domain, it would be located in such a way that would generate steric hindrance between the contributing protomers and subsequently block the oligomerization of the toxin. Also, without such reorganization of the β -Prism domain, the pre-stem loop would not be able to unfold for the membrane insertion of the toxin. Overall it appears that the β -Prism domain-mediated lectin activity of the toxin might act as a triggering mechanism to allow such structural reorganization of the β -Prism domain with respect to core cytotoxin domain. Our study suggested that the presence of the β -Prism domain in VCC molecule is critical for the efficient membrane pore-formation of the toxin. The β -Prism domain truncated variant of the toxin display aborted membrane pore-formation. However, in the absence of β -Prims domain, VCC molecule could generate membrane-associate oligomers but does not show any functional membrane pore-forming activity.

2. Structural reorganizations during oligomeric pore-formation:

Structural analysis of the water-soluble monomeric form and the transmembrane oligomeric structure of VCC reveal that the VCC molecule undergoes structural reorganization within the toxin monomer during the process of the oligomeric transmembrane pore-forming procedure. The most critical structural change is the unfolding the 'pre-stem' region from the cytolysin domain, and its insertion into the lipid bilayer to generate 'stem' configuration. In the water soluble monomeric structure of Pro-VCC, the 'pre-stem' region remains packed between the β -Prism domain and the cytolysin domain of the toxin. Hence, the movement of β -Prism domain is essential for the conversion of 'pre-stem' to the 'stem' region of the toxin. During the formation of the functional pore-formation of the toxin on the membrane, the β -Prism domain of the toxin reorients with respect to the central cytolysin domain by almost 180° angle, and attends the location where the Pro-domain was located in the Pro-VCC molecule structure. This reorganization of the β -Prism domain of the VCC represents the second most critical structural change involved in the membrane pore-formation of the VCC toxin. The structural change in the position of the β -Prism allows the 'pre-stem' to undergo the reorganization for the following membrane insertion and the functional heptameric pore-formation process.

3. Structural Features of the VCC β -Barrel Pore

Earlier study based on the Transmission electron microscopy (TEM) characterized the transmembrane oligomer of VCC as typical ring-like structures with the inner diameter of almost 1-2 nm. Inhibitions in the cell cytotoxic ability by the osmoprotectants of defined molecular sizes have also

advised similar pre diameter for VCC oligomer pore. Single channel conductance measurement by using the VCC oligomeric pore generated in the synthetic lipid bilayer suggested that VCC produce anion-selective diffusion channels. This analysis also indicated that the VCC pore is having asymmetric pore geometry: larger opening in the '*cis-side*' than in the '*trans-side*' with a narrow region at the central part of the human. The high-resolution structure of the VCC oligomer suggests 'cup-shaped' lumen geometry of the pore. Analysis of the oligomeric pore structure also suggests that the narrow constriction near the central of the pore lumen is generated by the aromatic ring of a tryptophan residue contributed by each of the participating protomers during heptameric pore-formation.

4. Mechanism of Membrane Pore-formation

The functional pore-formation of the β -PFTs involve on the membrane lipid bilayer of the target cells involves three distinct steps: (i) interaction of the water-soluble monomeric form of the toxin towards the target cell membrane; (ii) self-assembly of the membrane-associated monomeric toxin to generate the intermediate 'pre-pore' oligomeric assembly on the membrane surface; (iii) conversion from the transient 'pre-pore' oligomeric assembly to the functional transmembrane pore structure. During the process of the pore-formation, the pore-forming 'stem-loop' of the toxin inserted into the membrane lipid bilayer and generates the transmembrane β -barrel structure. Many structural studies reported that the member of β -PFTs follow the similar way of pore-formation on the membrane. However, each member of the β -PFTs family differs from each other in the definite step towards the pore-formation process. Membrane interaction step displays

enormous range of variation in term of receptor specificity, the role of different lipid component and presence of specific carbohydrate receptor on the membrane. Notably, the molecular mechanism that involve the discrete steps for the functional pore-formation are not properly elucidate for most of the β -PFTs members. The pore-formation on the membrane by VCC can resemble in the synthetic lipid bilayer liposomes indicating that the membrane association step does not critically required any particular non-lipid components. However, the membrane pore-formation is reported more efficient in the biomembrane as compared to that in the synthetic lipid bilayer of the liposomes, indicating the role of extra molecule present on the cell membrane plays significant role in the pore-formation process. For example, erythrocytes are more susceptible compared to the liposome. Notably, VCC toxin displays a different level of hemolytic activity towards the erythrocytes of the different species. Rabbit erythrocytes are found to exhibit more sensitive as compared to the human erythrocytes. Earlier studies have suggested the role of many cell surface receptor proteins (e. g., glycophorin B on the human erythrocytes) as a potential receptor molecule for the VCC toxin. VCC displayed potent lectin-like activity towards the interacting with the cell membrane. However, the specific receptor for the VCC has not been identified. The formation of the transmembrane oligomeric pore structure can be induced in the presence of the synthetic lipid bilayer liposomes. A previous study suggested that the binding of the VCC molecule with the liposomes driven mostly by global amphiphilicity of the monomeric state of the toxin. However, the self-assembly of the toxin and membrane pore-formation has been observed more specific events required the specific components of the membrane. More importantly, the presence of the

cholesterol in the lipid bilayer of the membrane has been reported to play critical role in the membrane pore-formation of the toxin. In our recent study, we identified the specific lipid-binding structure motif present within the cytolysin domain of the toxin. However, our study suggested that the specific motif is responsible for the lipid association in general not specific towards the cholesterol presence in the lipid bilayer of the membrane. In the pore-formation process of the VCC, the ' pore-forming loop' of the toxin unfolds and insert into the membrane toward the generation of the functional pore-formation. It is reported that the trapping of the pore-forming stem-loop in its pre-stem configuration through engineered disulfide linkage could arrest the toxin in its pre-pore oligomeric assembly. Also, a VCC variant without the pre-stem loop is found to remain arrest in the pre-pore oligomer on the membrane surface. Overall these studies suggested that the VCC follows the archetypical β -PFTs mechanism of pore-formation. A previous study indicated that the membrane interaction of the VCC precedes membrane oligomerization. Many environmental factors also affect the binding and oligomerization events of the toxin. For example, membrane association can occur even at a low temperature of 4 °C while the membrane oligomerization and functional pore-formation blocked under the similar condition. This observation clearly indicated that the association of the toxin with the target cell membrane is distinct step from its subsequent oligomerization and pore-formation steps.