Trapping of *Vibrio cholerae* Cytolysin in the Membrane-bound Monomeric State Blocks Membrane Insertion and Functional Pore Formation by the Toxin*

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*Running Title: Oligomerization of *Vibrio cholerae* cytolsyn

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**Background:** *Vibrio cholerae* cytolsyn (VCC) kills target eukaryotic cells by forming transmembrane oligomeric β-barrel pores.

**Results:** Alteration of key residue(s) in VCC arrests oligomerization and membrane insertion, and compromises membrane pore formation.

**Conclusion:** Trapping of VCC in its membrane-bound monomeric state blocks membrane insertion step.

**Significance:** The study provides novel insights regarding the membrane pore formation mechanism of VCC.

**ABSTRACT**

*Vibrio cholerae* cytolsyn (VCC) is a potent membrane-damaging cytolytic protein toxin that belongs to the family of β-barrel pore-forming protein toxins (β-PFTs). VCC induces lysis of its target eukaryotic cells by forming transmembrane oligomeric β-barrel pores. Mechanism of membrane pore formation by VCC follows overall scheme of the archetypical β-PFT mode of action, in which water-soluble monomeric form of the toxin first binds to the target cell membrane, then assembles into a pre-pore oligomeric intermediate, and finally converts into the functional transmembrane oligomeric β-barrel pore. However, there exists a vast lacuna in our understanding regarding the intricate details of the membrane pore-formation process employed by VCC. In particular, membrane oligomerization and membrane insertion steps of the process have been described only to a limited extent. In the present study, we have determined the key residue(s) in VCC that are critical to trigger membrane oligomerization of the toxin. Alteration of such key residue(s) traps the toxin in its membrane-bound monomeric state, and abrogates subsequent oligomerization, membrane insertion, and functional transmembrane pore-formation events. Results obtained from our study also suggest that the membrane insertion of VCC depends critically on the oligomerization process, and it cannot be initiated in the membrane-bound monomeric form of the toxin. In sum, our study for the first time dissects membrane binding from the subsequent oligomerization and membrane insertion steps, and thus defines the exact sequence of events in the course of membrane pore formation process by VCC.

**INTRODUCTION**

*Vibrio cholerae* cytolsyn (VCC) is a potent membrane-damaging cytolytic protein toxin produced by many pathogenic strains of the Gram
negative bacteria *V. cholerae* (1-5). In its mode of action, VCC belongs to the family of β-barrel pore-forming protein toxins (β-PFTs)(1,6-8). VCC is secreted by the bacteria in the form of a water-soluble, monomeric, inactive precursor molecule, termed as Pro-VCC. Proteolytic removal of the N-terminal Pro-domain from this precursor generates the mature active form of the VCC toxin (9-12). VCC causes colloid-osmotic lysis of the target eukaryotic cells by forming transmembrane heptameric β-barrel pores/channels (6,13). High-resolution structural informations are available for the water-soluble monomeric state (7), as well as for the transmembrane oligomeric pore form of VCC (6). Analysis of the structural models suggests that VCC follows the overall scheme of the archetypical β-PFT mode of action. However, the discrete intermediate events leading toward membrane pore formation by VCC have been described only to a limited extent.

Consistent with the generalized β-PFT mode of action, mechanism of membrane pore formation by VCC is proposed to follow three distinct steps: (a) binding of the toxin monomers onto the target cell membrane, (b) formation of transient meta-stable pre-pore oligomeric intermediates on the membrane, and (c) conversion of the pre-pore oligomers into the transmembrane oligomeric β-barrel pores (6,8,14-17). Studies on several β-PFTs including VCC also suggest that the formation of the functional transmembrane oligomeric pore structure involves membrane insertion of the pore-forming stem loop from each of the toxin protomers toward generation of the transmembrane β-barrel segments (18,19). However, it has not been tested experimentally, at least in case of VCC, whether the membrane insertion of the stem loop could occur in the membrane-bound monomeric state before the pre-pore oligomer formation, or whether the pre-pore oligomer formation precedes membrane insertion. Even in case of generalized β-PFT mechanisms, such sequence of events has not been established unambiguously. Previous studies have employed engineered β-PFT variants (for example, staphylococcal LukF, and VCC) incapable of inserting their pore-forming stem loop into the membrane lipid bilayer (18,20). Such toxin variants, having their stem loop in a locked configuration via engineered disulphide linkage, are found to remain trapped in their pre-pore oligomeric state (18,20). These observations, however, do not address the issue whether oligomerization is absolutely essential to initiate membrane insertion, or whether membrane insertion could be initiated before pre-pore formation. Such notions can only be examined by trapping the β-PFT molecules in their membrane-bound monomeric state, without allowing formation of the oligomeric structures. In this direction, direct correlation between oligomerization and membrane insertion has been shown in case of staphylococcal α-toxin, an archetypical member in the β-PFT family (21). Staphylococcal α-toxin harbouring a point mutation has been shown to display defective oligomerization of the membrane-bound toxins, with abortive membrane insertion of the pore-forming stem loop (21). Such observation suggests that, in case of staphylococcal α-toxin, membrane insertion event critically depends on the prior oligomerization step. Similar mode of action has been documented in case of perfringolysin O, a prominent member in the subclass of cholesterol-dependent cytolsins (CDCs) in the β-PFT family (22). Notably, streptolysin O, another important member in the CDC type β-PFT category, highlights a distinct mechanism of membrane pore formation that may involve different sequence of events (23). In case of streptolysin O, it has been suggested that progressive assembly of the membrane-inserted monomeric units may act toward generation of the oligomeric pore structures of varying sizes. Similar assembly mechanism has also been observed in a very recent study done on another CDC class of β-PFT, pneumolysin (24). Thus, it appears that the members of the β-PFT family may not necessarily follow a common generalized scheme toward exerting their membrane pore-forming activity. It is, therefore, critical to explore the sequence of the membrane insertion and oligomerization events for each individual member in the β-PFT family, so as to elucidate the mechanistic detail of their membrane pore-formation process.

A large number of studies have explored the mechanism(s) of membrane oligomerization process associated with the mode of actions of the
β-PFT family members, including VCC. It is commonly proposed that the interactions of the β-PFT toxin monomers with the membrane components of the target cells act as the triggering mechanism so as to initiate the subsequent events leading toward membrane oligomerization, membrane insertion and functional transmembrane β-barrel pore generation (1). In particular, membrane lipid components like cholesterol have been widely implicated for regulating the membrane pore formation process (25,26). In case of VCC, presence of cholesterol in the membrane lipid bilayer has been shown to be an obligatory requirement for efficient membrane oligomerization and functional membrane pore formation by the toxin (27-33). Cholesterol appears to regulate the mode of action of VCC by physically interacting with the toxin molecule, and not by simply altering the physicochemical environment of the target membrane (32). It is important to note here that the VCC toxin molecule itself has been examined only to a limited extent, in the context of exploring its oligomerization mechanism. In particular, interaction(s) between the toxin monomers, that might be instrumental to mediate the oligomerization process of VCC, has not been elucidated so far. Analysis of the VCC oligomer structure (6) highlights extensive inter-protomer interactions between the neighbouring subunits. The most prominent interactions are observed between the residues within the pore-forming stem region. Interestingly, trapping of the stem loop in its pre-stem configuration has been shown to block functional transmembrane oligomeric pore generation (SDS-stable oligomeric structures), without affecting pre-pore oligomer formation (SDS-labile oligomers) (18). It has also been shown that, even in the absence of the stem region, a truncated variant of VCC can form the pre-pore oligomer structure on the membrane (34). These observations clearly suggest that the inter-protomer interactions involving the stem region of VCC are critically involved in generating the functional transmembrane oligomeric pore state, without playing any significant role in initiating the oligomerization step of the membrane-bound toxin molecules. Therefore, it appears that the additional interactions, that are not part of the pore-forming stem region, might be playing key role(s) in triggering membrane oligomerization of VCC.

Toward elucidating the detail molecular mechanism of oligomeric membrane pore-formation process of VCC, in the present study we have identified the key residue(s) in the VCC structure that are critical to trigger oligomization of the membrane-bound toxin molecules. Alteration of such key residue(s) blocks the membrane oligomerization step, arrests the protein in its membrane-bound monomeric state, and does not even allow membrane insertion of the pore-forming stem region from the toxin monomers. Our study, for the first time, separates the membrane binding step from the subsequent oligomerization and membrane insertion event for VCC as a prototype in the β-PFT family. The study also establishes that the membrane insertion indeed requires oligomerization of the membrane-bound VCC toxin protomers on the target membrane.

**EXPERIMENTAL PROCEDURES**

*Purification of recombinant VCC variants*- Recombinant form of wild type VCC (WT-VCC) was generated as described previously (34,35).

Recombinant VCC variants harbouring single point mutation of Asp214Ala, Trp318Phe, Arg330Ala, or Phe581Ala were generated by polymerase chain reaction (PCR)-based method. Recombinant nucleotide constructs were verified by DNA sequencing. The VCC variants (Asp214Ala-VCC, Trp318Phe-VCC, Arg330Ala-VCC, and Phe581Ala-VCC) were purified following the method as described for WT-VCC. Homogeneity of the purified proteins were analysed by SDS-PAGE and Coomassie staining. Protein concentrations were estimated by measuring absorbance at 280 nm using the extinction coefficients of the proteins calculated from the corresponding amino acid compositions.

*Intrinsic tryptophan fluorescence and far-UV circular dichroism (CD)*- Intrinsic tryptophan fluorescence spectra were recorded using Fluoromax-4 (Horiba Scientific, Edison, NJ, USA) spectrofluorimeter upon excitation at 290 nm. The excitation and emission slits were set at 2.5 and 5 nm, respectively. All the experiments were
performed at 25 °C using protein concentration of 500 nM in 10 mM Tris-HCl buffer (pH 8.0).

Chirascan spectropolarimeter (Applied Photophysics, Leatherhead, Surrey, UK) was used to monitor the far-UV CD spectra of the VCC variants (~400 nM).

**Assay of hemolytic activity**- Work with human blood has been approved by the Institutional Bioethics Committee of IISER Mohali.

Hemolytic activity of the VCC variants against human erythrocytes was determined as described previously (35). Kinetics of hemolysis was monitored by recording the decrease in turbidity of the human erythrocyte suspension in PBS [20 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.4)] at 650 nm. Human erythrocyte concentration was adjusted in the reaction mixture corresponding to A_{650} = 0.9. Protein concentrations used were 100 nM.

**Flow cytometry**- Binding of the VCC variants with human erythrocytes were monitored using a flow cytometry-based assay as described previously (33). Briefly, human erythrocytes (10^6 cells) were incubated with 75 nM protein for 30 min at 4 °C in PBS. The cells were washed and treated with rabbit anti-VCC antiserum, followed by treatment with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody. The cells were analysed for FITC fluorescence using FACSCalibur (BD Biosciences) flow cytometer. Cells that were not incubated with VCC variants, but stained with anti-VCC and anti-rabbit-FITC were taken as control.

**Preparation of liposomes**- Asolectin-cholesterol (1:1 weight ratio) liposomes with or without trapped calcein dye was prepared as described previously (33). Asolectin-cholesterol liposomes labelled with DPH (1,6-Diphenyl-1,3,5-Hexatriene) was prepared as described in (34).

**Calcein-release assay**- Membrane permeabilization activity of the VCC variants (1 μM) against the membrane lipid bilayer of Asolectin-cholesterol liposomes (25 μg/ml) was probed by monitoring the release of calcein trapped within the liposome vesicles, as described previously (33). Calcein fluorescence was recorded on a Perkin-Elmer LS 55 spectrofluorimeter at 520 nm upon excitation at 488 nm, using excitation and emission slit widths of 2.5 nm and 5 nm, respectively.

**Pull down assay to monitor association of VCC variants with liposomes**- Association of the VCC variants with Asolectin-cholesterol liposomes was monitored using a pull down-based method. Liposomes (6.5 μg) was incubated with 1 μM protein in 100 μl reaction volume at 25 °C for 30 min, subjected to ultracentrifugation at 1,05,000 x g for 30 min at 4 °C. After collecting the supernatant, liposome pellets were washed with PBS and then resuspended in 100 μl PBS. Equal volume of samples from the supernatant fraction and the resuspended lipidosome fractions were analysed by SDS-PAGE/Coomassie staining to probe for the free and the liposome-bound VCC variants, respectively.

**Detection of SDS-stable oligomer formation by membrane-bound VCC variants**- For detection of SDS-stable oligomer formation in human erythrocyte cell membrane, cells (in PBS; corresponding to A_{550}=0.9) were treated with the VCC variants (100 nM) in a reaction volume of 100 μl at 25 °C, subjected to ultracentrifugation at 1,05,000 x g, pellet fractions were washed with PBS, and were resuspended in 50 μl SDS-PAGE sample buffer. Dissolved pellets were divided into two equal parts; one half was incubated at room temperature while the other half was boiled for 10 min, and subsequently analysed by immunoblotting. Sample without boiling would allow detection of SDS-stable oligomers formed by the membrane-bound fractions of the VCC variants.

For detection of SDS-stable oligomers in Asolectin-cholesterol liposomes, liposome suspension (6.5 μg) were treated with the VCC variants (1 μM) in 100 μl reaction volume as described above. Liposome-bound proteins were pelleted by ultracentrifugation at 1,05,000 x g, pellets were washed with PBS, and total pellet fractions were dissolved in 50 μl SDS-PAGE sample buffer. Dissolved pellets were divided into two equal parts; one half was incubated at room temperature while the other half was boiled for 10 min, and subsequently analysed either by SDS-PAGE/Coomassie staining, or immunoblotting. Sample without boiling would allow detection of...
SDS-stable oligomers formed by the liposome-bound fraction of the VCC variants.

Immunoblots were probed using rabbit anti-VCC antiserum, followed by reacting with horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoblots were developed using the ECL western blotting detection kit (GE Healthcare Life Sciences), and images were acquired on ImageQuant LAS 4010 (GE Healthcare Life Sciences).

**Fluorescence resonance energy transfer (FRET)** - FRET from the tryptophan residue in the VCC variants to DPH embedded in the Asolectin-cholesterol liposome membranes was monitored on a Perkin-Elmer LS 55 spectrofluorimeter following the method described previously (34). Briefly, DPH-labelled Asolectin-cholesterol liposomes (50 μg/ml) were treated with the VCC variants (1 μM) at 25 °C, and the FRET signal at 470 nm was recorded upon excitation at 280 nm, with excitation and emission slit widths of 2.5 nm and 5 nm, respectively. Tryptophan-to-DPH FRET signal was represented in terms of relative change in DPH fluorescence at 470 nm using the following expression: [Fluorescence intensity at any time point – Fluorescence intensity of the control]/ Fluorescence intensity of the control]. The DPH-labelled Asolectin-cholesterol liposomes without protein treatment were taken as control.

**BS³ cross-linking of liposome-associated VCC variants** - Covalent cross-linking of SDS-labile pre-pore oligomers of the VCC variants in Asolectin-cholesterol liposomes was carried out using BS³ (bis[sulfo succinimidyl] suberate; Thermo Pierce) following the methods as described previously (34). Briefly, Asolectin-cholesterol liposomes (6.5 μg) incubated with the VCC variants (1 μM) at 0.1 μl reaction volume were subjected to ultracentrifugation at 1,05,000 x g, pellets were washed and resuspended in 50 μl PBS with or without 5 mM BS³, and incubated for 30 min at 25 °C. The reactions were quenched by 50 mM Tris-HCl (pH 8.0) at room temperature for 15 min. After ultracentrifugation at 1,05,000 x g, the pellet fractions were analysed by SDS-PAGE and Coomassie staining.

**Surface plasmon resonance measurements** - Surface plasmon resonance (SPR) measurements were performed on a Biacore 3000 platform (GE Healthcare Life Sciences) at 25 °C using a L1 sensor chip. The surface of the chip was conditioned with HBS (20 mM HEPES, 150 mM NaCl, pH 7.5). The liposome-coated chip surface was prepared by injecting Asolectin-cholesterol liposome suspension (0.5 mM lipid concentration) at the flow rate of 1 μl/min for 15 min, and was washed with one injection of 20 mM NaOH for 12 seconds at a flow rate of 100 μl/min. Nonspecific binding were blocked by one injection of 0.1 mg/ml BSA for 5 min at flow rate of 10 μl/min.

For the binding experiments, proteins were injected for 10 min at 5 μl/min flow rate. Ten concentrations were used for each VCC variant. After each binding experiment, L1 chip was regenerated by stripping of the liposomes and bound proteins using one injection of 40 mM Octyl β-D-glucopyranoside for 5 min at flow rate of 10 μl/min. No loss of liposome capturing efficiency was observed upon regeneration. The corrected sensogram plots were generated using BIACore 4.1.1. software (GE Healthcare Life Sciences).

**Amino acid sequence alignment** - Amino acid sequences were acquired from the NCBI server (found online at http://www.ncbi.nlm.nih.gov/protein). Sequence alignment was generated using ClustalW (36) within the Biology Workbench server available online at http://workbench.sdsc.edu (37). The sequence alignment was rendered with the ESPript server (http://espript.ibcp.fr/ESPript/ESPript/) (38).

**Visualization of structural models** - VCC protein structure coordinates (PDB codes 1XEZ and 3O44) were obtained from the Protein Data Bank (www.pdb.org). Structural model of the transmembrane oligomeric form of VCC was generated using the OPM server found online (http://opm.phar.umich.edu/server.php). Protein structural models were visualized using PyMOL [DeLano WL, The PyMOL Molecular Graphics System (2002) found online (http://pymol.org)].

**RESULTS AND DISCUSSION**

Characterization of the VCC variants harbouring single point mutations of Asp214Ala, Arg330Ala and Phe581Ala - Analysis of the inter-
protomer interfaces of the VCC oligomer (6) shows presence of an aspartate residue at position 214 (Asp214), an arginine residue at position 330 (Arg330) and a phenylalanine residue at position 581 (Phe581)(Fig. 1A-B). These residues, Asp214, Arg330, and Phe581, are found to be highly conserved in the related cytolyzin/hemolysin proteins of Vibrionaceae bacteria (Fig. 1A). Asp214 is positioned within a unique loop structure in the cytolyzin domain of VCC. It appears to participate in salt-bridge interaction with a conserved Lys269 residue located in the neighbouring protomer within the VCC oligomer structure (Fig. 1B). The Arg330 residue is located within the first β-strand next to the membrane spanning stem region. In the VCC oligomer structure, Arg330 is involved in the hydrogen bond interactions with the side chain of a conserved residue Ser380, and the main chain carbonyl group of another conserved residue Ala218 from the neighbouring protomer (Fig. 1B). The Phe581 residue is located at the C-terminal boundary of the β-Trefoil domain of VCC, and it appears to participate in the van der Waals interaction with a conserved residue Val197 within the so called ‘cradle loop’ of the adjacent VCC protomer (Fig. 1B). In order to examine the role of these three conserved residues, Asp214, Arg330 and Phe581, in regulating the oligomerization mechanism of VCC, we generated three recombinant variants of the toxin harbouring the single point mutation of Asp214Ala (Asp214Ala-VCC), Arg330Ala (Arg330Ala-VCC) and Phe581Ala (Phe581Ala-VCC) (Fig. 1C). All the three mutants displayed intrinsic tryptophan fluorescence emission and far-UV CD spectra that overlapped with those of the wild type VCC protein (WT-VCC) (Fig. 1D-E). Overlapping intrinsic tryptophan fluorescence emission spectra of the wild type toxin and its mutants (Fig. 1D) indicated similar environment for the 11 tryptophan residues within the molecular structures of the VCC variants. This, in turn, suggested overall similar global tertiary structures of Asp214Ala-VCC, Arg330Ala-VCC and Phe581Ala-VCC as compared to that of WT-VCC. Likewise, nearly similar far-UV CD profile of the wild type and the three VCC mutants (Fig. 1E) also indicated their similar secondary structural organization. Altogether, these data suggested that the mutations of Asp214Ala, Arg330Ala and Phe581Ala in VCC did not noticeably alter the overall secondary and tertiary structural arrangements of the proteins.

Single point mutations of Asp214Ala, Arg330Ala and Phe581Ala in VCC abrogate functional pore formation in the membrane lipid bilayer of human erythrocytes and synthetic lipid vesicles- VCC forms transmembrane oligomeric β-barrel pores in the membrane lipid bilayer of erythrocytes, thereby leading to colloid osmotic lysis of the cells. VCC-induced lysis of erythrocytes is considered as the quantitative measure of the membrane pore-forming efficacy of the VCC toxin. Therefore, functional membrane pore-forming property of Asp214Ala-VCC, Arg330Ala-VCC, and Phe581Ala-VCC was tested by assaying their ability to trigger membrane-damaging cytolytic activity against human erythrocytes. We monitored lysis of human erythrocytes upon treatment with the VCC variants, over a period of 1 hour at 25 °C. We observed that at a concentration of 100 nM, Phe581Ala-VCC showed only about 30% of the wild type lytic activity, while Arg330Ala-VCC could not display any noticeable lytic activity (<5% of the wild type activity) (Fig. 2A). Asp214Ala-VCC displayed nearly 55% lytic activity under such condition (Fig. 2A). When tested over a prolonged duration up to 6 hours, Phe581Ala-VCC showed ~60% of the wild type activity, whereas the Arg330Ala mutant was still devoid of any lytic activity against human erythrocytes (Fig. 2A). Notably, Asp214Ala-VCC started showing wild type-like activity at time points of 3 to 4 hours (Fig. 2A).

We tested whether the reduced activities were due to compromised ability of the VCC variants to associate with the erythrocyte cells. For this, we monitored binding of the VCC variants with human erythrocytes by using a flow cytometry-based assay. Our data showed that all the three mutants, Asp214Ala-VCC, Arg330Ala-VCC and Phe581Ala-VCC, bound to the human erythrocytes with equal efficiency as compared to that of the wild type VCC toxin (Fig. 2B). These results, therefore, suggested that the single point mutations of Asp214Ala, Arg330Ala and Phe581Ala in VCC could abrogate the membrane-
damaging pore-forming efficacy of the protein in human erythrocytes, without affecting their ability to associate with the cells to any noticeable extent. The effect of the Arg330Ala mutation on membrane pore-forming activity appeared to be more severe as compared to that of the Phe581Ala mutation. Asp214Ala mutation appeared to have a marginal effect on the pore-forming activity of VCC.

We also tested the functional pore-forming ability of Asp214Ala-VCC, Arg330Ala-VCC and Phe581Ala-VCC in the membrane lipid bilayer of the Asolectin-cholesterol liposomes, in terms of triggering the release of trapped calcein from within the liposome vesicles. As reported earlier, WT-VCC displayed ~90% calcein-release activity within 30 min of incubation with the Asolectin-cholesterol liposomes (Fig. 3A). In contrast, the VCC mutants displayed severely compromised calcein release from the Asolectin-cholesterol liposome vesicles, when tested over a period of 30 min (Fig. 3A). Prolonged exposure of liposomes with Asp214Ala-VCC and Phe581Ala-VCC, however, resulted in significant extent of calcein release. When tested at 6 hour time-point, Asp214Ala-VCC and Phe581Ala-VCC could induce ~80% and ~50% calcein release from the Asolectin-cholesterol liposomes, respectively (Fig. 3A). Notably, the Arg330Ala mutant could not trigger any prominent calcein release even after 6 hours of treatment (Fig. 3A).

Consistent with our data with human erythrocytes, all the three mutated variants of VCC displayed wild type-like binding with the membrane lipid bilayer of the Asolectin-cholesterol liposome vesicles. A pull down-based assay showed that the Asp214Ala, Arg330Ala, and Phe581Ala variants of VCC could efficiently associate with the Asolectin-cholesterol liposomes (Fig. 3B), as observed with the WT-VCC protein. We also employed a quantitative SPR-based assay to monitor interactions of the VCC variants with the Asolectin-cholesterol membrane lipid bilayer. Steady state binding sensograms showed that the Asp214Ala-VCC, Arg330Ala-VCC and Phe581Ala-VCC proteins possessed wild type-like interaction efficacies with the Asolectin-cholesterol membrane lipid bilayer (Fig. 3C). Analysis of the end point response units (as obtained from the stable phase of the respective sensograms after completion of the protein injections) also revealed nearly similar irreversible membrane association for the wild type and mutant VCC variants (Fig. 3D).

These data, altogether, suggested that the single point mutation of the Asp214Ala, Arg330Ala and Phe581Ala in VCC abrogated the membrane pore formation ability of the toxin, without significantly affecting the membrane interaction ability of the protein. Our results also suggested that while the Asp214Ala and Phe581Ala mutations affected membrane pore-forming activity to a moderate extent, the mutation of Arg330Ala was having a drastic deleterious effect on the process of membrane pore formation by VCC.

Single point mutation of Arg330Ala in VCC drastically abrogates membrane oligomerization of the toxin, while oligomer formation is only moderately affected by the Asp214Ala and Phe581Ala mutations. Oligomerization of VCC in the membrane lipid bilayer is considered to be a key step toward generating the transmembrane pore structure. Consistent with the generalized β-PFT mode of action, interaction of VCC with the target membrane lipid bilayer leads to the formation of transient, SDS-labile, ‘pre-pore’ oligomeric intermediates, followed by their conversions into robust, SDS-stable transmembrane oligomeric pore structures. Therefore, toward exploring the mechanistic basis of the abortive membrane pore-formation process caused by the Asp214Ala, Arg330Ala, and Phe581Ala mutations in VCC, we analysed the oligomerization efficacy of Asp214Ala-VCC, Arg330Ala-VCC, and Phe581Ala-VCC in the membrane lipid bilayer of human erythrocytes and Asolectin-cholesterol liposome system. For this, we monitored the ability of the membrane-bound VCC variants to generate SDS-stable oligomeric assembly, a property commonly recognized as the signature of the transmembrane oligomeric pore structures of the archetypical β-PFT family members including wild type VCC. As reported earlier, WT-VCC could form SDS-stable oligomeric assembly in the membrane lipid bilayer of human erythrocytes (Fig. 4A). In comparison, Phe581Ala-VCC was found to display reduced ability to generate SDS-
stable oligomers in the human erythrocyte membrane (Fig. 4A). Phe581Ala-VCC also displayed markedly reduced oligomer formation when incubated in presence of the Asolectin-cholesterol liposomes, as compared to that of WT-VCC (Fig. 4B). Whatsoever, Phe581Ala-VCC showed noticeable extent of oligomerization in the membrane lipid bilayer of human erythrocytes and Asolectin-cholesterol liposomes (Fig. 4A-C), that corresponded with its moderate extent of membrane-damaging pore-forming activity. As reflected in its membrane pore-forming activity, oligomerization efficacy of Asp214Ala-VCC was found to be only nominally reduced as compared to that of the wild type toxin (Fig. 4A-C).

Notably, Arg330Ala-VCC did not form any detectable amount of SDS-stable oligomeric assembly in the membrane lipid bilayer of human erythrocytes and Asolectin-cholesterol liposomes (Fig. 4A-B). Even after 6 hours of interaction with Asolectin-cholesterol liposomes, membrane-bound fraction of Arg330Ala-VCC failed to generate SDS-stable oligomeric species (Fig. 4C). We also explored whether the Arg330Ala mutant of VCC could form any SDS-labile ‘pre-pore’ oligomeric assembly in the membrane lipid bilayer of Asolectin-cholesterol liposome vesicles. In order to trap any SDS-labile oligomer of the proteins generated in the membrane lipid bilayer of liposomes, we used the cross-linking agent BS³. BS³-mediated covalent cross-linking could efficiently trap the oligomers of WT-VCC generated in the liposome within 30 min of interaction (Fig. 4D). In contrast, BS³ cross-linking could not arrest any such oligomeric species for Arg330Ala-VCC in presence of the Asolectin-cholesterol liposomes within the said time frame (Fig. 4D). These data, therefore, suggested that the mutation of Arg330Ala in VCC not only abrogated the SDS-stable oligomeric assembly generation, but it also critically affected the SDS-labile pre-pore oligomer formation.

Taken together, our results established that the mutation of Arg330Ala and Phe581Ala in VCC critically affected the oligomerization step of the membrane pore-formation process. Asp214Ala mutation affected oligomerization of VCC only to a nominal extent. While Phe581Ala mutation imposed only a modest extent of oligomerization defect, Arg330Ala mutation caused severe blockade on the oligomerization step, thereby arresting the membrane-bound form of the protein in an abortive monomeric state.

The mutation of Asp214Ala, Arg330Ala, and Phe581Ala in VCC affects membrane insertion step of the toxin. We examined whether the Asp214Ala-VCC, Arg330Ala-VCC and Phe581Ala-VCC variants could insert their pore-forming stem-region into the membrane lipid bilayer, in absence of efficient membrane oligomerization. For this, we monitored FRET from the tryptophan residue (Trp318) located within the stem region of VCC to the DPH fluorophore embedded within the hydrophobic core of the membrane lipid bilayer, as described previously in (34). An increased tryptophan-to-DPH FRET signal, upon incubation of the VCC variants in presence of the DPH-labelled Asolectin-cholesterol liposomes, would indicate membrane insertion of the pore-forming stem region. It has been shown previously that a truncated variant of VCC lacking the stem region could not display any noticeable tryptophan-to-DPH FRET in presence of the DPH-labelled Asolectin-cholesterol liposomes, as compared to that observed with the wild type VCC toxin (34). To further confirm the specific role of the Trp318 in mediating such FRET process, we generated a Trp318Phe mutant of VCC (Trp318Phe-VCC). The Trp318Phe-VCC showed nearly overlapping intrinsic tryptophan fluorescence emission (Fig. 5A) and far-UV CD spectra (Fig. 5B) as compared to that of the wild type VCC, suggesting no major structural defect in the mutant. More importantly, Trp318Phe-VCC displayed wild type-like membrane pore-forming activity against human erythrocytes (Fig. 5C) and Asolectin-cholesterol liposomes (Fig. 5C, Inset), suggesting that the mutation of Trp318Phe did not induce any defect in the toxin in terms of oligomeric membrane pore-formation efficacy. Based on our proposition, however, alteration of Trp-to-Phe at position 318 within the pore-forming stem region would be expected to abrogate the tryptophan-to-DPH FRET signal. Indeed, in our assay we found that Trp318Phe-VCC did not show any time-dependent increase in the tryptophan-to-DPH FRET, when incubated in presence of the DPH-labelled Asolectin-cholesterol liposomes (Fig. 5D). As reported earlier, WT-VCC showed prominent
increase in the tryptophan-to-DPH FRET signal under that identical experimental condition (Fig. 5D). These data, thus, validated that the above-mentioned tryptophan-to-DPH FRET-based assay could be considered as a robust method to monitor membrane insertion of the VCC stem loop. Consistent with such notion, Asp214Ala-VCC and Phe581Ala-VCC showed considerably reduced tryptophan-to-DPH FRET signal as compared to that of WT-VCC (Fig. 5D), suggesting that the mutations of Asp214Ala and Phe581Ala not only reduced the efficacy of membrane oligomerization, but it also affected membrane insertion step to a moderate extent. Interestingly, no significant time-dependent increase in the tryptophan-to-DPH FRET signal was documented for the Arg330Ala-VCC variant (Fig. 5D). This data, therefore, suggested that the mutation of Arg330Ala could not only block membrane oligomerization of VCC, but it also arrested membrane insertion of the pore-forming stem region from the membrane-bound monomeric toxin molecules.

CONCLUSION

Generalized mechanism of membrane pore formation by the β-PFTs proposes three distinct steps: (a) membrane binding, (b) oligomerization, (c) membrane insertion of the pore-forming stem region from each of the toxin protomers to generate the transmembrane β-barrel pore. Despite such overall general scheme, individual members of the β-PFT family differ from each other in the intricate mechanistic detail of the process. Although oligomerization processes of the β-PFTs have been studied in some detail, structural mechanism(s) that drives oligomerization remains poorly described in most of the cases. Also, the sequence of events in the process of oligomeric membrane pore formation by the β-PFTs remains to be validated in every case, as generalization of such issue has not been established yet. In case of VCC, the high-resolution structures have been determined for the water-soluble monomeric state, as well as for the oligomeric pore form. However, the snapshots of the discrete intermediate events leading toward membrane pore formation by VCC have not been depicted earlier in mechanistic detail. Membrane interaction mechanisms of VCC have been studied previously in some detail. It has also been shown that VCC follows the archetypical β-barrel membrane pore-formation mechanism via formation of the pre-pore oligomeric intermediates. Albeit, the critical interactions, that governs the formation of the oligomeric structure on the membrane surface, has not been elucidated earlier in mechanistic detail. Also, it has not been established conclusively before whether membrane insertion step of VCC precedes pre-pore formation, or it occurs only upon formation of the pre-pore intermediate. It has also not been tested earlier for VCC whether membrane binding, oligomerization and membrane insertion steps are strictly discrete events, or they proceed concomitantly in a concerted manner.

In the present study, we examined the role of the conserved Asp214, Arg330 and Phe581 residues in regulating the oligomerization mechanism of VCC. Our study showed that the single point mutation of Asp214Ala, Arg330Ala and Phe581Ala compromised membrane oligomerization efficacy of VCC, without significantly affecting the membrane-binding property of the toxin. While mutations of Asp214Ala and Phe581Ala appeared to have modest effects, Arg330Ala mutation exerted more drastic blockade on the process. Our data demonstrated that the mutation of Arg330Ala in VCC not only blocked oligomerization of the membrane-bound toxins, but it also abrogated the membrane insertion of the pore-forming stem region from the membrane-associated toxin monomers. This is the first study that highlights the key residue(s) in VCC, alteration of which blocks the oligomerization of the membrane-bound toxin monomers, without even allowing formation of the pre-pore oligomeric structures. Our study also enriches our insights regarding the membrane oligomerization mechanism of β-PFT family of proteins, in general. Oligomerization processes of large number of β-PFTs have been explored previously. However, exact structural mechanism that drives such processes has been elucidated only to a limited extent. One classic example is available in case of staphylococcal α-toxin. It has been shown previously that alteration of a His35-residue in the oligomerization surface
of \( \alpha \)-toxin abrogates functional oligomeric pore-formation, and membrane insertion steps of the toxin (21,39). Interestingly, His35 is located in a position within the \( \alpha \)-toxin structure that matches with that of Asp214 in the VCC structure (6,15). In case of VCC, however, Asp214 appeared to play a nominal role in the oligomerization process. It is also important to note that the mutation of His35 in \( \alpha \)-toxin does not block the pre-pore oligomer formation (39). Altogether, such observations clearly entail diverse variation in the structural mechanisms associated with the oligomeric membrane pore formation by the \( \beta \)-PFT family members.

In sum, our study for the first time reveals the interactions, and some of the associated key residues, implicated for the membrane oligomerization process of VCC. Blockade of such interactions traps the protein in its membrane-bound monomeric state, and arrests the critical membrane insertion step toward generation of the functional transmembrane oligomeric \( \beta \)-barrel pore structures by the toxin. The study provides novel insights regarding the membrane pore-formation mechanism of VCC, and also enriches our insights in the broader context of the generalized \( \beta \)-PFT mode of actions.
REFERENCES

1. Alouf, J. E., and Popoff, M. R. (2006) *The Comprehensive Sourcebook of Bacterial Protein Toxins*, Third Edition Ed., Academic Press.
2. Honda, T., and Finkelstein, R. A. (1979) Purification and characterization of a hemolysin produced by *Vibrio cholerae* biotype El Tor: another toxic substance produced by cholera vibrios. *Infection and immunity*, 26, 1020-1027
3. Ichinose, Y., Yamamoto, K., Nakasone, N., Tanabe, M. J., Takeda, T., Miwatani, T., and Iwanaga, M. (1987) Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. *Infection and immunity*, 55, 1090-1093
4. Kaper, J., Fasano, A., and Trucksis, M. (1994) Toxins of *Vibrio cholerae*. *Vibrio cholerae*, 145-176
5. Kaper, J. B., Morris, J. G., Jr., and Levine, M. M. (1995) Cholera. *Clin Microbiol Rev*, 8, 48-86
6. De, S., and Olson, R. (2011) Crystal structure of the *Vibrio cholerae* cytolysin heptamer reveals common features among disparate pore-forming toxins. *Proc Natl Acad Sci U S A*, 108, 7385-7390
7. Olson, R., and Gouaux, E. (2005) Crystal structure of the *Vibrio cholerae* cytolysin (VCC) pro-toxin and its assembly into a heptameric transmembrane pore. *J Mol Biol*, 350, 997-1016
8. Iacovache, I., Bischofberger, M., and van der Goot, F. G. (2010) Structure and assembly of pore-forming proteins. *Current Opinion in Structural Biology*, 20, 241-246
9. Paul, K., and Chattopadhyay, K. (2011) Unfolding distinguishes the *Vibrio cholerae* cytolysin precursor from the mature form of the toxin. *Biochemistry*, 50, 3936-3945
10. Nagamune, K., Yamamoto, K., and Honda, T. (1997) Intramolecular chaperone activity of the pro-region of *Vibrio cholerae* El Tor cytolysin. *J Biol Chem*, 272, 1338-1343
11. Nagamune, K., Yamamoto, K., Naka, A., Matsuyama, J., Miwatani, T., and Honda, T. (1996) In vitro proteolytic processing and activation of the recombinant precursor of El Tor cytolysin/hemolysin (pro-HlyA) of *Vibrio cholerae* by soluble hemagglutinin/protease of *V. cholerae*, trypsin, and other proteases. *Infection and immunity*, 64, 4655-4658
12. Yamamoto, K., Ichinose, Y., Shinagawa, H., Makino, K., Nakata, A., Iwanaga, M., Honda, T., and Miwatani, T. (1990) Two-step processing for activation of the cytolysin/hemolysin of *Vibrio cholerae* O1 biotype El Tor cytolysin. *Infection and immunity*, 58, 4106-4116
13. Zitzer, A., Walev, I., Palmer, M., and Bhakdi, S. (1995) Characterization of *Vibrio cholerae* El Tor cytolysin as an oligomerizing pore-forming toxin. *Med Microbiol Immunol*, 184, 37-44
14. Olson, R., Nariya, H., Yokota, K., Kamio, Y., and Gouaux, E. (1999) Crystal structure of staphylococcal LukF delineates conformational changes accompanying formation of a transmembrane channel. *Nat Struct Biol*, 6, 134-140
15. Song, L., Hobaux, M. R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J. E. (1996) Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science*, 274, 1859-1866
16. Bayley, H., Jayasinghe, L., and Wallace, M. (2005) Prepore for a breakthrough. *Nat Struct Mol Biol*, 12, 385-386
17. Walker, B., Braha, O., Cheley, S., and Bayley, H. (1995) An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch. *Chem Biol*, 2, 99-105
18. Lohner, S., Walev, I., Boukhallouk, F., Palmer, M., Bhakdi, S., and Valeva, A. (2009) Pore formation by *Vibrio cholerae* cytolysin follows the same archetypical mode as {beta}-barrel toxins from gram-positive organisms. *Faseb J*, 23, 2521-2528
19. Valeva, A., Walev, I., Boukhallouk, F., Wassenaar, T. M., Heinz, N., Hedderich, J., Lautwein, S., Mocking, M., Weis, S., Zitzer, A., and Bhakdi, S. (2005) Identification of the membrane
penetrating domain of Vibrio cholerae cytolysin as a beta-barrel structure. Molecular microbiology, 57, 124-131

20. Nguyen, V. T., Higuchi, H., and Kamio, Y. (2002) Controlling pore assembly of staphylococcal gamma-haemolysin by low temperature and by disulphide bond formation in double-cysteine LukF mutants. Molecular microbiology, 45, 1485-1498

21. Valeva, A., Palmer, M., Hilgert, K., Kehoe, M., and Bhakdi, S. (1995) Correct oligomerization is a prerequisite for insertion of the central molecular domain of staphylococcal alpha-toxin into the lipid bilayer. Biochim Biophys Acta, 1236, 213-218

22. Shepard, L. A., Shatzisky, O., Johnson, A. E., and Tweten, R. K. (2000) The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane beta-hairpins. Biochemistry, 39, 10284-10293

23. Palmer, M., Harris, R., Freytag, C., Kehoe, M., Tranum-Jensen, J., and Bhakdi, S. (1998) Assembly mechanism of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization. The EMBO journal, 17, 1598-1605

24. Sonnen, A. F.-P., Plitzko, J. M., and Gilbert, R. J. C. (2014) Incomplete pneumolysin oligomers form membrane pores. Open Biology, 4,

25. Harris, J. R., and Palmer, M. (2010) Cholesterol specificity of some heptameric beta-barrel pore-forming bacterial toxins: structural and functional aspects. Subcell Biochem, 51, 579-596

26. Soltani, C. E., Hotze, E. M., Johnson, A. E., and Tweten, R. K. (2007) Structural elements of the cholesterol-dependent cytolysins that are responsible for their cholesterol-sensitive membrane interactions. Proc Natl Acad Sci U S A, 104, 20226-20231

27. Zitzer, A., Bittman, R., Verbicky, C. A., Erkulla, R. K., Bhakdi, S., Weis, S., Valeva, A., and Palmer, M. (2001) Coupling of cholesterol and cone-shaped lipids in bilayers augments membrane permeabilization by the cholesterol-specific toxins streptolysin O and Vibrio cholerae cytolysin. J Biol Chem, 276, 14628-14633

28. Zitzer, A., Harris, J. R., Kemminer, S. E., Zitzer, O., Bhakdi, S., Muething, J., and Palmer, M. (2000) Vibrio cholerae cytolysin: assembly and membrane insertion of the oligomeric pore are tightly linked and are not detectably restricted by membrane fluidity. Biochim Biophys Acta, 1509, 264-274

29. Harris, J. R., Bhakdi, S., Meissner, U., Scheffler, D., Bittman, R., Li, G., Zitzer, A., and Palmer, M. (2002) Interaction of the Vibrio cholerae cytolysin (VCC) with cholesterol, some cholesterol esters, and cholesterol derivatives: a TEM study. J Struct Biol, 139, 122-135

30. Chattopadhyay, K., Bhattacharyya, D., and Banerjee, K. K. (2002) Vibrio cholerae hemolysin. Implication of amphiphilicity and lipid-induced conformational change for its pore-forming activity. Eur J Biochem, 269, 4351-4358

31. Zitzer, A., Westover, E. J., Covey, D. F., and Palmer, M. (2003) Differential interaction of the two cholesterol-dependent, membrane-damaging toxins, streptolysin O and Vibrio cholerae cytolysin, with enantiomeric cholesterol. FEBS Lett, 553, 229-231

32. Ikigai, H., Otsuru, H., Yamamoto, K., and Shimamura, T. (2006) Structural requirements of cholesterol for binding to Vibrio cholerae hemolysin. Microbiol Immunol, 50, 751-757

33. Paul, K., and Chattopadhyay, K. (2012) Single point mutation in Vibrio cholerae cytolysin compromises membrane pore-formation mechanism of the toxin. Fems J, 279, 4039-4051

34. Paul, K., and Chattopadhyay, K. (2014) Pre-pore oligomer formation by Vibrio cholerae cytolysin: insights from a truncated variant lacking the pore-forming pre-stem loop. Biochem Biophys Res Commun, 443, 189-193

35. Rai, A. K., Paul, K., and Chattopadhyay, K. (2013) Functional mapping of the lectin activity site on the beta-prism domain of vibrio cholerae cytolysin: implications for the membrane pore-formation mechanism of the toxin. J Biol Chem, 288, 1665-1673
36. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673-4680

37. Subramaniam, S. (1998) The Biology Workbench—a seamless database and analysis environment for the biologist. *Proteins*, **32**, 1-2

38. Gouet, P., Courcelle, E., Stuart, D. I., and Metoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics*, **15**, 305-308

39. Jursch, R., Hildebrand, A., Hobom, G., Tranum-Jensen, J., Ward, R., Kehoe, M., and Bhakdi, S. (1994) Histidine residues near the N terminus of staphylococcal alpha-toxin as reporters of regions that are critical for oligomerization and pore formation. *Infection and immunity*, **62**, 2249-2256
FOOTNOTES

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The abbreviations used are: VCC, Vibrio cholerae cytolsin; β-PFT, β-barrel pore-forming protein toxin; FRET, fluorescence resonance energy transfer; CD, circular dichroism; DPH, 1,6-Diphenyl-1,3,5-Hexatriene; BS3, bis[sulfosuccinimidyl] suberate; SPR, surface plasmon resonance.

FIGURE LEGENDS

Figure 1. A. Amino acid sequence alignment of VCC and its closely related cytolysins/hemolysins from three other Vibrio species. Locations of the conserved residues, Asp214, Arg330 and Phe581, in VCC are indicated. Other conserved residues, Lys269 (making interaction with Asp214), Ser380 and Ala218 (making interactions with Arg330), and Val197 (making interaction with Phe581) at the inter-protomer interface of VCC oligomer, are also marked. B. Structural model of the transmembrane oligomeric form of VCC showing the locations of the conserved residues (as mentioned in Fig. 1A) at the interface of the two neighbouring protomers. C. SDS-PAGE/Coomassie staining of the purified form of wild type and the mutant VCC variants. Proteins were treated in presence of SDS-PAGE sample buffer with (marked as B) or without boiling (marked as UB). Protein standards are shown in lane M. D. Intrinsic tryptophan fluorescence emission spectra of the VCC variants. E. Far-UV CD spectra of the VCC variants.

Figure 2. A. Pore-forming activity of the VCC variants monitored by estimating the hemolytic activity of the proteins (100 nM) against human erythrocytes. B. Binding of the VCC variants (75 nM) with human erythrocytes determined by the flow cytometry-based assay. Solid line, WT-VCC; dashed line, mutant VCC variants as indicated on top of the panel; shaded curve, control.

Figure 3. A. Pore-forming activity of the VCC variants (1 μM), as determined by estimating the calcein release from the Asolectin-cholesterol liposomes. B. Binding of the VCC variants (1 μM) to the Asolectin-cholesterol liposome vesicles determined by pull down-based assay. Pellet fractions containing liposome-bound proteins and the sup fraction containing unbound proteins were analyzed by SDS-PAGE/Coomassie staining. Protein standards are shown in lane M. C-D. Binding of the VCC variants to the membrane lipid bilayer of Asolectin-cholesterol determined by SPR-based assay. Various concentrations (ten different concentrations starting from 100 nM to 1 μM) of wild type and mutant proteins were injected over the membrane lipid bilayer of Asolectin-cholesterol liposomes generated on the SPR sensor chips. Subsequently, buffer was injected for definite time period, at the end of which residual sensogram response units were monitored to estimate the extent of irreversible binding of the VCC variants to the liposome membrane. Overlay of the binding sensograms shows steady state binding of the VCC variants (C). Analysis of the end point response units shows concentration-dependent increase in irreversible binding the VCC variants toward the Asolectin-cholesterol membrane lipid bilayer (D).

Figure 4. A. SDS-stable oligomer formation by the VCC variants in the human erythrocytes membranes. Erythrocyte membrane-bound proteins were pelleted by ultracentrifugation, and were probed by immunoblotting. Samples treated with SDS-PAGE sample buffer without boiling (UB) allowed detection of the SDS-stable oligomers. Phe581Ala-VCC showed ~0.25-fold reduced oligomer formation, as compared to WT-VCC. No SDS-stable oligomer formation was noticed for Arg330Ala-VCC.
Asp214Ala-VCC showed marginally reduced oligomer formation as compared to that of WT-VCC. WT-VCC was included in each of the immunoblots as control. Oligomer band intensities were compared using the Gel Analysis tool within the program ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). B. SDS-stable oligomer formation by the VCC variants in presence of Asolectin-cholesterol liposomes. Proteins were treated in presence of liposomes, and were probed by immunoblotting. Samples treated with SDS-PAGE sample buffer without boiling (UB) allowed detection of the SDS-stable oligomers. Phe581Ala-VCC showed ~0.4-fold reduced oligomer formation, as compared to WT-VCC. No significant extent of SDS-stable oligomer formation was observed for Arg330Ala-VCC. Asp214Ala-VCC showed marginally reduced oligomer formation as compared to WT-VCC. WT-VCC was included as control in each of the immunoblots. Oligomer band intensities were compared as described in A. C. Arg330Ala-VCC failed to generate SDS-stable oligomers in Asolectin-cholesterol liposomes even after prolonged incubation for 6 hours, whereas WT-VCC, Asp214Ala-VCC, and Phe581Ala-VCC formed considerable amount of oligomers in the liposome membranes. VCC variants were incubated with the liposomes, liposome-bound proteins were pelleted by ultracentrifugation, and were analyzed by SDS-PAGE/Coomassie staining. Samples treated with SDS-PAGE sample buffer without boiling (UB) allowed detection of the SDS-stable oligomers. D. BS3 cross-linking of SDS-labile pre-pore oligomers formed in the Asolectin-cholesterol liposome membrane. Liposome-bound proteins were subjected to BS3 cross-linking, and were analyzed by SDS-PAGE/Coomassie staining. For Arg330Ala-VCC cross-linked oligomer could not be detected, whereas for WT-VCC SDS-labile pre-pore oligomers could be trapped by BS3 cross-linking. Bands of oligomeric and monomeric species are marked with double and single arrow marks, respectively.

Figure 5. Membrane insertion of the pore-forming stem loop of the VCC variants probed by the FRET-based assay. A-C, Trp318Phe-VCC was taken as control for the FRET-based assay. Trp318Phe-VCC showed wild type-like intrinsic tryptophan fluorescence emission (A) and far-UV CD spectra (B). Also, Trp318Phe-VCC displayed similar extent of hemolytic activity (C), and liposome permeabilization (inset; C), as compared to that of WT-VCC. D. Incubation of WT-VCC in presence of DPH-labelled Asolectin-cholesterol liposomes triggered time-dependent increase in the tryptophan-to-DPH FRET signal, presumably due to an efficient FRET from the Trp318 located within the stem region of the protein to the membrane embedded DPH. Such notion was confirmed by the observation that mutation of Trp318Phe in VCC completely blocked time-dependent increase in the tryptophan-to-DPH FRET signal. For Asp214Ala-VCC and Phe581Ala-VCC, efficiency of the process was modestly compromised. For Arg330Ala-VCC, no significant time-dependent increase in the FRET signal was observed, suggesting severe blockade on the membrane insertion step for the mutant.
Figure 3
Figure 4

A

Pull down and Western Blot:
Human erythrocytes
Protein concentration: 100 nM
Time: 1 hour

B

Western Blot:
Asolectin-cholesterol liposomes
Protein concentration: 1 μM
Time: 30 min

C

Pull down and SDS-PAGE/Coomassie staining:
Asolectin-cholesterol liposomes
Protein concentration: 1 μM
Time: 6 hours

D

Pull down and SDS-PAGE/Coomassie staining:
Asolectin-cholesterol liposomes
Protein concentration: 1 μM
Time: 30 min
Figure 5
Trapping of Vibrio cholerae Cytolysin in the Membrane-bound Monomeric State Blocks Membrane Insertion and Functional Pore Formation by the Toxin
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