Functional Mapping of the Lectin Activity Site on the β-Prism Domain of Vibrio cholerae Cytolysin

IMPLICATIONS FOR THE MEMBRANE PORE-FORMATION MECHANISM OF THE TOXIN

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Background: Vibrio cholerae cytolsin is a pore-forming toxin with lectin-like activity.

Results: β-Prism domain mediates the lectin activity, which determines efficient membrane binding and modulates membrane oligomerization of the toxin.

Conclusion: β-Prism domain-mediated lectin activity acts to play critical regulatory role in the mode of action of the toxin.

Significance: The study elucidates novel implications of the β-Prism domain-mediated lectin activity.

Vibrio cholerae cytolsin (VCC) is a prominent member in the family of β-barrel pore-forming toxins. It induces lysis of target eukaryotic cells by forming transmembrane oligomeric β-barrel channels. VCC also exhibits prominent lectin-like activity in interacting with β1-galactosyl-terminated glycoconjugates. Apart from the cytolsin domain, VCC harbors two lectin-like domains: the β-Trefoil and the β-Prism domains; however, precise contribution of these domains in the lectin property of VCC is not known. Also, role(s) of these lectin-like domains in the mode of action of VCC remain obscure. In the present study, we show that the β-Prism domain of VCC acts as the structural scaffold to determine the lectin activity of the protein toward β1-galactosyl-terminated glycoconjugates. Toward exploring the physiological implication of the β-Prism domain, we demonstrate that the presence of the β-Prism domain-mediated lectin activity is crucial for an efficient interaction of the toxin toward the target cells. Our results also suggest that such lectin activity may act to regulate the oligomerization ability of the membrane-bound VCC toxin. Based on the data presented here, and also consistent with the existing structural information, we propose a novel mechanism of regulation imposed by the β-Prism domain’s lectin activity, implicated in the process of membrane pore formation by VCC.

Vibrio cholerae cytolsin (VCC) is a potent membrane-damaging cytolytic protein toxin produced by many pathogenic strains of V. cholerae (1). It is considered to be a potential virulence factor contributing toward the pathogenesis mechanism of the organism (2–5). In its mode of action, VCC belongs to the family of β-barrel pore-forming toxins (β-PFTs) (6–12). VCC is secreted by the bacteria as water soluble monomeric precursor molecule (Pro-VCC), which after removal of an N-terminal Pro-domain converts into the mature VCC toxin (13). Upon binding to its target cell membrane, mature VCC forms transmembrane oligomeric β-barrel channels thus leading to colloid osmotic lysis of the target cells (14–18).

The mature VCC molecule is composed of a central cytolsin domain (6, 7) that exhibits an overall structural similarity to those found in other archetypal members of the β-PFT family, including Staphylococcus aureus α-hemolysin and staphylococcal LukF toxin (19, 20). Apart from the cytolsin domain, mature VCC molecule possesses two additional C-terminal domains displaying the structural features of the lectin-like folds: a β-Trefoil lectin-like domain, followed by a β-Prism lectin-like domain (6, 7) (Fig. 1A). The β-Trefoil domain is connected to the cytolsin domain through a short linker sequence, whereas the β-Prism domain is connected to the β-trefoil domain via a straight and long linker region (6, 7). Presence of the lectin-like domains is consistent with the previous report showing a lectin-like property of VCC, specifically toward the terminal β1-galactosyl moiety of complex glycoconjugates (21). However, precise contribution of the β-Trefoil and the β-Prism domains toward the lectin-like property of VCC has not been explored yet. Moreover, implication of these lectin-like domains for the overall structure-function mechanism of VCC has not been elucidated.

Here, we have studied the involvement of the β-Prism domain in the mode of action of the VCC toxin. In the first part of our study, we have investigated the role of the β-Prism domain in the context of the lectin-like activity of VCC. Using the β-Prism domain-deletion variant of the protein, we have shown that the presence of the β-Prism domain is critically required for the lectin activity of VCC toward β1-galactosyl-terminated complex glycoconjugates. Removal of the β-Prism domain results in complete loss of such lectin activity of the protein, while the isolated β-Prism domain alone exhibits prominent binding toward glycoconjugates possessing terminal β1-galactosyl groups. We have also mapped the critical res-
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idues within the β-Prism domain contributing toward the lectin activity of VCC. Toward exploring further the physiological implication of the lectin activity of the β-Prism domain, we have shown that the β-Prism domain-mediated lectin activity plays a critical role in targeting the VCC toxin toward the erythrocyte membrane. Specific disruption of the β-Prism domain lectin activity is found to have a deleterious effect on the membrane interacting propensity of the VCC protein. Finally, we have examined the role of the β-Prism domain-mediated lectin activity in regulating the membrane oligomerization process of VCC. Based on our result, we hypothesize that the β-Prism domain-mediated lectin activity may act as a crucial triggering mechanism so as to allow formation of the VCC oligomeric assembly in the target cell membrane. Altogether, our study provides novel insights regarding critical implications of the β-Prism domain-mediated lectin activity for the mode of action of VCC.

EXPERIMENTAL PROCEDURES

Protein Reagents—All the recombinant constructs were generated by PCR-based strategy, and were confirmed by DNA sequencing. Purity of the protein samples were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Protein concentrations were measured by monitoring absorbance at 280 nm on the basis of theoretically calculated molar extinction coefficient values obtained from the analysis of the amino acid sequences of the protein constructs.

VCC-Δβ-Prism Variant—Mature form of the recombinant VCC-Δβ-Prism variant was generated following the method as described for the wild type VCC (22).

The β-Prism Variants—Wild type and mutant forms of the isolated β-Prism domain protein were generated using the method described for the wild type VCC protein, with some modification. Briefly, the nucleotide sequences encoding the wild type and mutant β-Prism domain constructs were cloned in the pET14b bacterial expression vector, proteins were overexpressed in Escherichia coli Origami B cells (Novagen), and recombinant His-tagged proteins were purified by Ni-NTA-agarose (Qiagen) affinity chromatography followed by ion exchange chromatography in Q Sepharose (GE Healthcare Life Sciences). Structural integrity of the β-Prism domain variants were assessed by monitoring the secondary structural organization of the proteins using far-UV CD spectroscopy (data not shown).

D617A-VCC Mutant—D617A mutation was introduced in the nucleotide sequence encoding full-length VCC employing the PCR-based strategy. The construct was cloned into the pET14b bacterial expression vector as per the manufacturer’s instructions, and the protein was overexpressed in BL21(DE3)pLysS cells (Novagen). Mature form of the recombinant D617A-VCC protein was generated following the method as described for the wild type VCC (22).

Fluorescence and Far-UV Circular Dichroism (CD)—Intrinsic tryptophan fluorescence spectra of the VCC variants (250–300 nm) were recorded on a Fluoromax-4 (Horiba Scientific, Edison, NJ) spectrofluorimeter, upon excitation at 290 nm using slit widths of 2.5 and 5 nm for excitation and emission, respectively.

Far-UV CD spectra of the VCC variants (0.5–1 μM) were monitored on a Chirascan spectropolarimeter (Applied Photophysics, Leatherhead, Surrey, UK) using 5 mm pathlength quartz cuvette as described before (22).

Assay of Lytic Activity against Human Erythrocytes—The cytolytic activity against human erythrocytes (suspended in PBS (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) corresponding to OD_{650 nm} = 0.8–0.9) was assayed by monitoring the decrease in turbidity of the erythrocyte suspension at 650 nm (21).

Enzyme-linked Immunosorbent Assay (ELISA)—Binding of the VCC variants to the immobilized asialofetuin (Sigma Aldrich) were monitored following an ELISA-based method as described earlier (21). Briefly, 100 μl of asialofetuin (10 μg/ml) in PBS was added into each well of 96-well flat-bottom microtiter plates (Nunc), and incubated for overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween 20 (TPBS), blocked with 200 μl of 3% nonfat dry milk powder in PBS for 1 h and subsequently treated with VCC variants for 2 h at 25 °C. After three washes with TPBS, wells were treated with 100 μl of rabbit anti-VCC antiserum [1:5000 dilution (v/v) for the wild type and β-Prism deleted variants of VCC; 1:50 (v/v) dilution for the wild type and mutant variants of β-Prism domain protein] for 90 min, washed three times with TPBS, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000, v/v) for 1 h at 25 °C. Binding of VCC variants to asialofetuin were detected by color development by adding o-phenylenediamine (10 mg/ml) in 0.1 M sodium citrate buffer (pH 4.5) containing H_2O_2 (2 μl/ml of 30% (v/v) H_2O_2), reactions were stopped with 2 N H_2SO_4, and absorbance values were recorded at 490 nm in a microplate absorbance reader (iMark, Bio-Rad).

Isothermal Titration Calorimetry (ITC)—Interactions of the β-Prism domain variants with asialofetuin were monitored by ITC-based experiments using an iTC200 instrument (MicroCal/GE Healthcare, Piscataway, NJ). All the protein samples were taken in a buffer containing 10 mM Tris-HCl (pH 8.0) and dialyzed extensively against the same buffer. Concentrations of the β-Prism variants were finally measured by monitoring the absorbance at 280 nm as described above. Asialofetuin concentration was also determined by monitoring absorbance at 280 nm (based on the extinction coefficient of 0.45 for 1 mg/ml protein and a molecular mass of 48 kDa). All the buffers and protein reagents were freshly filtered and degassed under vacuum. For each ITC measurement, the sample cell (cell volume of 0.201 ml) was filled with the β-Prism variant protein (47.7 μM), and the reference cell was filled with the buffer containing 10 mM Tris-HCl (pH 8.0). The β-Prism variant protein was titrated with asialofetuin (330 μM) using the following protocol: an initial 0.5 μl injection, followed by 19 injections of 2 μl each were applied with an interval of 3 min. The titration was carried out with a constant stirring at 1000 rpm at 25 °C. The binding isotherm profile was obtained excluding the initial data point. The data were fitted to one-site binding model using Origin 7 software. The values of ΔH (binding enthalpy in kcal/mol), K_a (association constant), and n (number of binding sites per mono-
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β-Prism domain

VCC structural coordinates were obtained from the Protein Data Bank (PDB entry 1XEZ) for the VCC-β-Prism variant to human erythrocytes were quantitatively estimated using a flow cytometry-based assay following the methods as described in (24). Briefly, human erythrocytes were incubated with VCC variants for 30 min at 4 °C in PBS. Cells were then treated with rabbit anti-VCC antiserum, followed by treatment with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody as described previously (24). Cells were analyzed by FACSCalibur (BD Biosciences) flow cytometer. FITC fluorescence was measured at an excitation wavelength of 488 nm, with an emission wavelength of 530 nm. The geometric mean fluorescence (GMF) values were obtained using the FlowJo software (www.flowjo.com). The % binding data were calculated using the expression: [(GMF test - GMF control)/(GMF maximum - GMF control)] × 100. GMF control represents GMF for the cells that were not treated with VCC variants, but stained with anti-VCC and anti-rabbit-FITC; GMF maximum represents GMF for the cells incubated with the highest concentration of full-length VCC (75 nM), followed by treatment with anti-VCC and anti-rabbit-FITC.

Western Blotting—SDS-PAGE and Western blotting was done following standard protocol using rabbit anti-VCC antiserum. The blot was developed using the ECL Western blotting detection kit (GE Healthcare Life Sciences), and images were acquired using ImageQuant LAS 4010 (GE Healthcare Life Sciences).

Liposome Preparation—Cholesterol and Asolectin (from soybean) were obtained from Sigma Aldrich. Asolectin-cholesterol (with weight ratio of 1:1) mixed liposome was prepared following the methods described earlier (25, 26). Briefly, the lipids dissolved in chloroform were evaporated under vacuum, resuspended in PBS. Large unilamellar vesicles were generated by repeatedly passing the liposome suspension through polycarbonate membranes of 0.1 μm pore size using a Mini-Extruder apparatus (Avanti Polar Lipids, Inc.).

RESULTS AND DISCUSSION

Construction of VCC Variant Having Truncation of the β-Prism Domain—Analysis of the VCC molecular structure reveals that the C-terminal region of the toxin encompassing

Merlan were kept as adjustable parameters. The dissociation constant (Kd) was determined from 1/Kd. Experiments were carried out with the c values [= Kc.M(0); M(0) being the initial concentration of the macromolecule in the sample cell] in the range 1<c<500 (23).

Flow Cytometry—Binding of the full-length VCC and the VCC-Δβ-Prism variant to human erythrocytes were quantitatively estimated using a flow cytometry-based assay following the methods as described in (24). Briefly, human erythrocytes were incubated with VCC variants for 30 min at 4 °C in PBS. Cells were then treated with rabbit anti-VCC antiserum, followed by treatment with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody as described previously (24). Cells were analyzed by FACSCalibur (BD Biosciences) flow cytometer. FITC fluorescence was measured at an excitation wavelength of 488 nm, with an emission wavelength of 530 nm. The geometric mean fluorescence (GMF) values were obtained using the FlowJo software (www.flowjo.com). The % binding data were calculated using the expression: [(GMF test - GMF control)/(GMF maximum - GMF control)] × 100. GMF control represents GMF for the cells that were not treated with VCC variants, but stained with anti-VCC and anti-rabbit-FITC; GMF maximum represents GMF for the cells incubated with the highest concentration of full-length VCC (75 nM), followed by treatment with anti-VCC and anti-rabbit-FITC.

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Structural Models—VCC structural coordinates were obtained from the Protein Data Bank (PDB) (PDB entry 1XEZ for water-soluble monomeric form of VCC; PDB entry 3O44 for the VCC oligomer). Superimposition of structural coordinates were performed using the program COOT (27). The structural models were visualized using PyMOL [DeLano, W. L. (2002) The PyMOL Molecular Graphics System, found online (www. pymol.org)].

RESULTS AND DISCUSSION

Construction of VCC Variant Having Truncation of the β-Prism Domain—Analysis of the VCC molecular structure reveals that the C-terminal region of the toxin encompassing
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![Graph showing binding to immobilized asialofetuin](image)

**FIGURE 2. Binding to immobilized asialofetuin.** A, binding of VCC-Δβ-Prism protein to immobilized asialofetuin was compared with that of the wild type VCC protein using an ELISA-based assay. Each data point represents the average of three independent readings. Error bars indicate the standard deviations. Data were compared using non-linear curve fitting (one site binding model) of the data using GraphPad Prism 4 (GraphPad Software, Inc.). For the VCC-Δβ-Prism protein no detectable interaction was observed, and therefore $K_d$ value was not determined (denoted as ND). B, binding of wild type and mutant variants of β-Prism domain protein to immobilized asialofetuin was estimated as described above. C, structural model of the β-Prism domain highlights the three residues (Asp-617, Tyr-654, and Tyr-679) critically associated with the lectin activity of the protein toward asialofetuin. The βOG molecule, bound to the β-Prism domain (as observed in the crystal structure of VCC (7)), is also shown in the model.

the residues Gln-584 to Asn-716 constitutes a β-Prism lectin-like domain (6, 7). In our study, we generated the truncation variant of VCC lacking the β-Prism domain (VCC-Δβ-Prism; having deletion in the region Gln-584 to Asn-716 of wild type VCC molecule) (Fig. 1, B and C). Consistent with the earlier reports (7, 28), the VCC-Δβ-Prism variant was found to have severely compromised cytolytic activity. The VCC-Δβ-Prism protein, at the concentration range of 100 nM, displayed <10% of the wild type hemolytic activity against human erythrocytes (Fig. 1D). Previous studies have reported noticeable hemolytic activity, although significantly reduced as compared with the full-length VCC, for the β-Prism domain-deleted variant of VCC (7, 28). Such studies have used rabbit erythrocytes for monitoring hemolytic activity, and rabbit erythrocytes are known to display significantly enhanced susceptibility toward VCC-induced hemolysis (29). Therefore, the observed differences in the hemolytic activity of the VCC-Δβ-Prism proteins, as reported in the present study and in the earlier reports, could be attributed to the differential susceptibility of the human and rabbit erythrocytes toward VCC-mediated cytotoxicity. When compared with those of the full-length VCC protein, the VCC-Δβ-Prism variant displayed overall similar intrinsic tryptophan fluorescence emission spectra (full-length VCC contains 11 tryptophan residues distributed throughout the protein structure; two of these are located within the β-Prism domain) and far-UV circular dichroism (CD) profile (Fig. 1E). Moreover, when subjected to thermal denaturation, VCC-Δβ-Prism displayed unfolding propensity similar to that of the full-length VCC protein, as monitored by the intrinsic tryptophan fluorescence measurements (data not shown). Altogether, these data suggested that the removal of the β-Prism domain did not affect the folding and the structural integrity of the truncated VCC variant. It is also important to note here that the VCC-Δβ-Prism protein, despite having critically compromised hemolytic activity, could form SDS-stable oligomeric assembly in the membrane lipid bilayer of human erythrocytes, a characteristic feature of the archetypical β-PFTs including VCC (Fig. 1D, inset) (24).

**Removal of the β-Prism Domain Abolishes the Lectin-like Activity of VCC toward β1-Galactosyl-terminated Complex Glycoconjugate—**VCC has been reported to possess potent lectin-like property with specificity toward β1-galactosyl-terminated complex glycoconjugates (21). However, it is still not known which of the two lectin-like domains, namely the β-Trefoil and the β-Prism domains, of VCC contribute toward this lectin-like property. To explore the potential contribution of the β-Prism domain in the lectin-like activity of VCC, we employed an ELISA-based assay to test and compare the ability of the VCC-Δβ-Prism variant to bind to the immobilized β1-galactosyl-terminated glycoprotein asialofetuin. It has been shown previously that VCC binds specifically to the terminal β1-galactosyl moiety of asialofetuin (21). Consistent with the earlier report (21), wild type VCC molecule showed prominent interaction with immobilized asialofetuin with a qualitative dissociation constant ($K_d$) in the range of ~16 nM (Fig. 2A). In contrast, the VCC-Δβ-Prism variant displayed no detectable binding to the immobilized asialofetuin (Fig. 2A). The result, therefore, suggested that the β-Prism domain of VCC acted as the structural scaffold supporting the lectin activity of the VCC toxin. This also ruled out the contribution of the β-Trefoil domain for the lectin activity of VCC in terms of its specific interaction with β1-galactosyl-terminated complex glycoconjugates. However, it needs to be explored in future whether VCC possesses additional lectin activity with distinct specificity that might be contributed by the β-Trefoil domain.

**β-Prism Domain of VCC in Isolation Could Display Prominent Lectin Activity toward β1-Galactosyl-terminated Glycoconjugate—**We wanted to test whether the β-Prism domain in isolation could display any lectin-like activity in terms of specific binding to immobilized asialofetuin. For this, we generated a His$_6$-tagged β-Prism domain protein corresponding to the region Phe-581 to Asn-716 of the wild type VCC molecule. This β-Prism domain protein displayed detectable binding activity toward immobilized asialofetuin (with a qualitative $K_d$ value in the range of 2 μM) as monitored by the ELISA-based assay (Fig. 2B). This result supported the notion that the residues Gln-584 to Asn-716 constitutes a β-Prism lectin-like domain (6, 7).
that the β-Prism domain alone was capable of displaying the lectin-like activity toward β1-galactosyl-terminated complex glycoconjugates. It must be noted here that the qualitative assessment of the data obtained from our ELISA-based assay indicated that the β-Prism domain protein bound to immobilized asialofetuin with an affinity, at least 100-fold weaker as compared with that of the full-length VCC protein. It, therefore, appeared that the presence of the complete VCC structure was required to strengthen the lectin activity imposed by the β-Prism domain. Future studies would be required to investigate the mechanistic basis of such regulation of the lectin activity of the β-Prism domain.

Mapping of the Lectin Activity Site onto the β-Prism Domain of VCC—Next, we explored the presence of specific site(s) onto the β-Prism domain responsible for its lectin activity. Crystal structure of the VCC molecule has shown that the β-Prism domain harbors, in its solvent-exposed surface, a potential pocket that binds to a detergent molecule β-octyl glucoside (βOG)(Fig. 2C) (7). Similar binding pocket is found to be present in other members of the β-Prism lectin family, including Maclura pomifera agglutinin (MPA) (30) and jacalin (31). More interestingly, the amino acid residues within this pocket in the VCC β-Prism domain making side chain interactions with the βOG molecule has been found to be remarkably identical with that of the MPA and jacalin. These residues are Asp-617, Tyr-654, and Tyr-679 within the VCC β-Prism domain (Fig. 2C). Based on the structural similarity with the ligand-binding pocket of MPA and jacalin, we explored the possibility whether the amino acid triad composed of Asp-617, Tyr-654, and Tyr-679 residues in the β-Prism domain would contribute toward the lectin-like activity of VCC. We generated alanine substitution mutations of Asp-617, Tyr-654, and Tyr-679 in the β-Prism domain protein, and compared their ability to bind to the immobilized asialofetuin using the ELISA-based assay.

Remarkably, mutations of Y654A and Y679A resulted in ~30–40% reduction in its binding to asialofetuin, whereas D617A mutation caused ~100% inhibition of binding activity as compared with that of the wild type β-Prism domain protein (Fig. 2B). These data clearly confirmed the role of the three amino acid residues, Asp-617, Tyr-654, and Tyr-679, toward the lectin activity of the VCC toxin, with the Asp-617 residue having the most critical contribution in the process.

We also confirmed the binding activity of the wild type and the D617A mutant of β-Prism domain protein toward β1-galactosyl-terminated glycoprotein asialofetuin using ITC-based experiments. Consistent with the results described above, the β-Prism domain protein showed profound interaction with asialofetuin as revealed by the binding isotherm profile (Fig. 3). Analysis of the ITC data revealed a $K_d$ value of ~4.717 μM for the asialofetuin:β-Prism interaction, that was consistent with the qualitatively estimated $K_d$ value obtained from the ELISA-based assay. The D617A mutation in the β-Prism domain protein resulted in complete disruption of asialofetuin binding, as the mutant protein did not display any heat release while being titrated with asialofetuin (Fig. 3). Alto-
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FIGURE 5. Effect of the D617A mutation in the full-length VCC protein. A, far-UV CD spectrum and intrinsic tryptophan fluorescence emission profile (inset) of the D617A mutant of VCC. B, lytic activity of the D617A-VCC mutant (○) against human erythrocytes. Lytic activity of wild type full-length VCC (●) is shown as control. C, binding of D617A-VCC mutant protein (●) to immobilized asialofetuin was compared with that of the wild type VCC protein (○) using an ELISA-based assay. Each data point represents the average of three independent readings. Error bars indicate the standard deviations. D, binding of D617A-VCC (dashed line) and the full-length wild type VCC (solid line) to human erythrocytes were determined by the flow cytometry-based assay using the protein concentration of 75 nM. Shaded peak represents the control sample. E, oligomerization propensity of the D617A-VCC mutant in the human erythrocyte membrane. Human erythrocytes in PBS (corresponding to OD650 nm = 0.8) were incubated with the VCC variant (100 nM) for 1 h at 25 °C in a final reaction volume of 0.1 ml, reaction mixture was subjected to ultracentrifugation at 105,000 × g, pellet was washed with PBS, dissolved in SDS-PAGE sample buffer with or without boiling, subjected to SDS-PAGE/Western blotting using anti-VCC. Full-length wild type VCC-treated sample was used as a control. Unboiled samples allowed detection of any SDS-stable oligomeric assembly. SDS-stable oligomer of wild type VCC was indicated with an arrow. Data presented here are the representatives of at least three independent experiments.

Together, the results of our ITC-based experiments confirmed the mapping of the β1-galactosyl-terminated glycoconjugate-specific lectin activity onto the β-Prism domain of VCC.

Implication of the β-Prism Domain-mediated Lectin Activity for the Binding of VCC toward Erythrocytes—In the next part of our study we explored the physiological implication of the lectin activity displayed by the β-Prism domain of VCC. In this direction, we examined the possibility whether such lectin activity played any role in the membrane-binding process of the toxin, presumably via recognition of, as yet unknown, cell surface glycan receptor(s).

(i) Removal of the β-Prism Domain Critically Compromised Interaction of VCC with Erythrocytes—We tested whether removal of the β-Prism domain had any effect on the binding efficacy of VCC with human erythrocytes. As noted above, pull-down assays with human erythrocytes qualitatively indicated that the VCC-Δβ-Prism variant could associate with the target cell membrane, when treated at 25 °C. However, such association may not necessarily represent specific mode of interaction of VCC with the human erythrocytes, as VCC is known to display nonspecific hydrophobicity-driven interactions with the lipid components of the target cell membrane (26). Therefore, toward obtaining a more quantitative estimation of specific mode of interaction of the VCC variants with erythrocytes, binding of the VCC-Δβ-Prism variant to human erythrocytes was monitored and compared with that of the full-length VCC protein using a quantitative flow cytometry-based assay (24, 32). In this assay, we monitored the binding ability of the VCC variant with intact human erythrocytes at low temperature of 4 °C. It has been shown previously that at such low temperature efficacy of the membrane oligomerization and cytotoxicity is drastically suppressed, without having any effect on the membrane binding propensity of the VCC toxin (29). Also, incubation at low temperature of 4 °C would minimize the hydrophobicity-driven nonspecific, and presumably abortive association of the toxin with the membrane lipid bilayer (26). Therefore, under such condition, binding of the VCC variants with erythrocytes would be expected to be the result of the specific interaction(s) of the toxin molecule with the erythrocyte cell surface component(s). Remarkably, in this experiment VCC-Δβ-Prism variant (in the protein concentration range of 25–75 nM) displayed significantly reduced binding ability to human erythrocytes, as compared with that of the wild type VCC (Fig. 4A). At a protein concentration of 75 nM, the VCC-Δβ-Prism variant showed only about 15% of the wild type binding activity. This data, therefore, suggested that the absence of the β-Prism domain critically compromised the efficient binding of the VCC toxin toward erythrocyte membrane.

(ii) Pre-treatment of VCC with β1-Galactosyl-terminated Glycoprotein Asialofetuin Abolished Binding of VCC with Erythrocytes—Pre-incubation of the full-length VCC molecule in presence of asialofetuin inhibited the binding activity of the
toxin to the erythrocytes to a significant extent (Fig. 4B). As VCC could interact only with the glycan part of asialofetuin (21), presumably through the β-Prism domain-mediated lectin activity of the toxin, it appeared that the inhibition of the β-Prism domain-mediated lectin activity was deleterious for an efficient interaction of VCC with the erythrocyte membrane. (iii) Pre-incubation of Erythrocytes with β-Prism Domain Protein Inhibited VCC-induced Hemolytic Activity—The implication of the β-Prism domain-mediated lectin activity for the interaction process of VCC with erythrocytes was further evidenced from the fact that the pre-treatment of human erythrocytes with wild type β-Prism domain protein resulted in significant inhibition of VCC-induced hemolytic activity. In contrast, the D617A mutation in the β-Prism domain protein completely reversed such inhibitory effect. As described above, the D617A mutation in the β-Prism domain protein resulted in complete loss of its lectin activity (Fig. 4C). Therefore, these data altogether confirmed that the β-Prism domain-mediated lectin activity of VCC played a critical role for an efficient targeting of the toxin toward the erythrocyte membrane.

D617A Mutation in VCC Critically Affected the Lectin Activity of the Protein, and Drastically Abrogated Its Erythrocyte Binding, Membrane Oligomerization as Well as Hemolytic Activity—As described above, mutation of D617A critically affected the lectin activity of the isolated β-Prism domain protein. Therefore, to conclusively establish the physiological implication of the lectin property contributed by the β-Prism domain, we examined the effect of the D617A mutation in the full-length VCC protein. Incorporation of D617A mutation did not alter the structural integrity of the protein as revealed by its intrinsic tryptophan fluorescence emission as well as far-UV CD profiles (Fig. 5A). Interestingly, VCC protein harboring the D617A mutation showed severely compromised hemolytic activity against human erythrocytes. At a protein concentration of 100 nM, the mutant protein displayed <5% of the wild type hemolytic activity, under the experimental conditions described in the present study (Fig. 5B). Even at a protein concentration of 1000 nM, the D617A mutant of VCC did not display any detectable hemolytic activity against human erythrocytes (data not shown). Consistent with our observation with the isolated β-Prism domain variant, D617A mutation resulted in critical abrogation of the lectin activity of the full-length VCC protein toward asialofetuin (Fig. 5C). When tested for its ability to bind to erythrocytes using the flow cytometry-based assay carried out at low temperature of 4 °C, D617A mutant of VCC (at a protein concentration of 75 nM) showed severely diminished interaction with the human erythrocytes; the binding was found to be in the range of <5% of the wild type interaction propensity (Fig. 5D). These data, once again, established...
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a strong correlation between the β-Prism domain-mediated lectin activity of VCC and its functional interaction with the erythrocyte membrane.

When tested for its ability to associate with the human erythrocytes using the pull down-based assay performed at 25°C, the D617A mutant of full-length VCC displayed considerable propensity to associate with the erythrocyte membrane (Fig. 5E), presumably via the hydrophobicity-driven interaction with the erythrocyte membrane. Remarkably, the mutant protein was found to display severely compromised ability to form any SDS-stable oligomeric assembly in the erythrocyte membrane, under the experimental condition tested. Unlike wild type VCC toxin, the D617A mutant did not form any detectable extent of SDS-stable oligomer in the human erythrocyte membrane, while tested at protein concentration of 100 nM (Fig. 5E). Even at a higher concentration of 1000 nM, the mutant protein could not form any SDS-stable oligomeric assembly in the human erythrocyte membrane (data not shown). These observations appeared to be rather surprising. Since the D617A mutation was located within the β-Prism domain, any direct involvement of the Asp-617 residue in the membrane oligomerization process of VCC would be unexpected as the removal of β-Prism domain did not affect the ability of the truncated protein to form the SDS-stable oligomeric assembly in the erythrocyte membrane (Fig. 1D). Therefore, the observed effect of the D617A mutation on the membrane oligomerization of VCC would possibly be the reflection of a far more complicated regulatory mechanism(s).

Comparison of the monomeric and the oligomeric structures of VCC reveal that the β-Prism domain undergoes prominent structural reorganization with respect to the core cytolsin domain in the process of membrane oligomerization of the toxin (6, 7). It appears that such rearrangement is prerequisite for the subsequent conformational rearrangement of the pre-stem loop toward forming the stem region of the transmembrane β-barrel. More importantly, repositioning of the β-Prism domain is also appearing to be a necessary condition for the oligomerization of the membrane-associated VCC monomers. Unless the β-Prism domain undergoes such spatial rearrangement, it would create steric clashes between the β-Prism domains of the neighboring subunits during the formation of the oligomeric assembly (supplemental Fig. S1). Therefore, to accommodate the participating protomers in the heptameric assembly, the β-Prism domain of each subunit must reorient itself with respect to the core VCC structure. Now, based on the available structural data as well as the data presented in our study, it is possible to hypothesize that the interaction of the β-Prism domain with the cell surface glycan receptor(s) is not only allowing the efficient functional targeting of VCC toward cell membrane, but it is also acting as a triggering mechanism for the structural repositioning of the β-Prism domain so as to allow formation of the transmembrane oligomeric assembly (Fig. 6). From our present study, it appears that the incorporation of the D617A mutation is affecting the interaction of VCC with the cell surface glycan receptors presumably via abrogating its lectin activity. This effect, in turn, would block the spatial rearrangement of the β-Prism domain, thus ultimately abrogating the membrane oligomerization process of the VCC toxin. In case of the Δβ-Prism variant, absence of the β-Prism domain would remove the above-mentioned steric constraint(s) toward membrane oligomerization of the truncated protein. As a result, the membrane oligomerization of the protein would not be regulated anymore via the lectin activity of the β-Prism domain. Consistent with such notion, VCC-Δβ-Prism was found to form oligomeric assembly in the erythrocyte membrane.

Overall, as discussed above, such regulatory role(s) of the β-Prism domain-mediated lectin activity on the membrane binding and oligomerization process of VCC appear to suggest a novel mode of regulatory mechanism modulating the pore-forming activity of the VCC toxin. However, more detail structural studies would be required in future to gain deeper insights regarding the exact structural and molecular description of this proposed regulatory mechanism.

CONCLUSION

The present study provides novel molecular insights regarding the role of the β-Prism lectin-like domain of VCC in the mode of action of the toxin. From our study, we have established the β-Prism domain as the structural module determining the lectin activity of VCC toward β1-galactosyl terminated complex glycoconjugate. We have also mapped the critical amino acid residues within the β-Prism domain defining the lectin activity of the protein. Our data suggest that the β-Prism domain of VCC employs a canonical structural mechanism, similar to those involved in defining the lectin activity of the other β-Prism family proteins. Toward exploring the physiological significance of the β-Prism domain-mediated lectin activity of VCC, we have established a critical involvement of the β-Prism domain in regulating the interaction efficacy of VCC with the target cells, presumably through binding to the glycan moiety of the, as yet unidentified, glycoprotein/glycolipid receptor(s) present on the cell surface. Also, our results indicate that the β-Prism domain-mediated lectin activity may act to regulate the oligomerization process of the membrane-associated VCC molecules. It appears from our study that the presence of the β-Prism domain serves critical regulatory role(s) in terms of modulating the multiple steps of membrane channel formation mechanism of the VCC toxin. Since the β-Prism domain is not commonly present in other members of the β-PFT family, it would be worth exploring in future how the VCC structure-function relationship has evolved to accommodate such a regulatory module within the β-PFT family of proteins.

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