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Identification of amino acids important for binding of *Clostridium perfringens* epsilon toxin to host cells and to HAVCR1

Susan E. Ivie and **Mark S. McClain***
Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232

Abstract

Clostridium perfringens epsilon toxin belongs to the aerolysin-like family of pore-forming toxins and is one of the most potent bacterial toxins known. The epsilon toxin causes fatal enterotoxemia in sheep, goats, and possibly humans. Evidence indicates that the toxin binds to protein receptors including hepatitis A virus cellular receptor 1 (HAVCR1), but the region of the toxin responsible for cell binding has not been identified. In the present study, we identify amino acids within the epsilon toxin important for this cell interaction. Site-specific mutagenesis was used to investigate the role of a surface-accessible cluster of aromatic amino acids, and purified mutant proteins were tested in a series of cell-culture assays to assess cytotoxic activity and cell binding. When added to cells, four mutant proteins (Etx-Y29E, Etx-Y30E, Etx-Y36E and Etx-Y196E) were severely impaired in their ability to not only kill host cells, but also in their ability to permeabilize the plasma membrane. Circular dichroism spectroscopy and thermal stability studies revealed that the wild-type and mutant proteins were similarly folded. Additional experiments revealed that these mutant proteins were defective in binding to host cells and to HAVCR1. These data indicate that an amino acid motif including Y29, Y30, Y36, and Y196 is important for the ability of epsilon toxin to interact with cells and HAVCR1.

Epsilon toxin, produced by *Clostridium perfringens* types B and D, is one of the most potent bacterial toxins. [(1)] Epsilon toxin can lead to fatal enterotoxemia in a variety of livestock animals. In natural intoxications, the toxin is expressed by *C. perfringens* in the intestine. The toxin disrupts the intestinal epithelium and is believed to enter the bloodstream to disseminate throughout the body. [(2–5)] Once in the bloodstream, the toxin causes widespread vascular permeability. [(6, 7)] Postmortem analysis reveals pathologic changes primarily in the brain and kidneys of intoxicated animals. [(8–10)] Although human exposure is rare, evidence does suggest the toxin may be toxic to humans. [(11–14)] The United States Department of Health and Human Services has categorized epsilon toxin as a select agent.

Epsilon toxin is secreted as an inactive prototoxin. Proteolytic cleavage of small peptides at both the N- and C-termini by proteases such as trypsin or chymotrypsin results in activation of the toxin. [(15)] This proteolytic activation increases the toxicity of epsilon toxin approximately 1000-fold over the minimally toxic prototoxin. [(16)]

Numerous studies suggest that the toxin binds to a specific receptor. Binding of the toxin is saturable and can be inhibited by inactive epsilon prototoxin [(17)] or by excess unlabeled

*Corresponding Author Mark S. McClain, mark.mcclain@vanderbilt.edu, Telephone: 615-343-3033, Fax: 615-343-6160, Division of Infectious Diseases, A2200 Medical Center North, Vanderbilt University School of Medicine, Nashville, TN 37232.

SUPPORTING INFORMATION AVAILABLE

Table S1 and Figure S1 provide additional analyses of the CD spectra and thermal stability. This material is available free of charge via the internet at http://pubs.acs.org.

toxin when labeled and unlabeled toxins are mixed. [8] Though the identity of the receptor has not been definitively determined, evidence from multiple studies suggests that the toxin binds to a glycoprotein. For example, toxin binding to isolated membranes is inhibited by treatment with pronase or neuraminidase. [17] Similarly, analysis of epsilon toxin binding to the canine kidney cell line MDCK and to kidney cryoslices indicates the importance of O-glycoproteins in epsilon toxin binding. [18] Recently, we have shown that hepatitis A virus cellular receptor 1 (HAVCR1) contributes to epsilon-toxin-induced cytotoxicity in MDCK and human kidney ACHN cells, and further that epsilon toxin binds to the extracellular domain of human HAVCR1. [19] These studies suggest that HAVCR1, an extensively O-glycosylated protein, may serve as a receptor or co-receptor for the toxin.

In the present study, we sought to further characterize the interaction between epsilon toxin and HAVCR1 as well as between the toxin and host cells. Using site-specific mutagenesis of the *etxB* gene, mutant proteins were isolated that are defective in their ability to mediate cell death and for the ability to interact with MDCK cells and human HAVCR1.

Experimental Procedures

Expression and purification of recombinant epsilon prototoxin

The gene encoding epsilon prototoxin, *etxB*, from *C. perfringens* type B strain ATCC 3626 was PCR-amplified and cloned into plasmid pET22b (Novagen). This placed the *etxB* gene under the regulation of the bacteriophage T7 RNA polymerase and fused the N-terminal end of the prototoxin to the *pelB* leader peptide and the C-terminal end of the prototoxin to a His_6 affinity tag (to aid in purification of the protein). A derivative plasmid that expressed a GFP-epsilon toxin fusion protein was also constructed. [8, 9, 20] Expression and purification of recombinant epsilon prototoxin were performed as described previously. [21] The concentrations of purified proteins were determined based on absorbance at 280 nm and equal amounts of protein were confirmed by SDS-PAGE and coomassie staining.

Site-specific mutagenesis

Mutations were introduced into the cloned *etxB* gene using the QuickChange Lightning Multi Site Directed Mutagenesis Kit (Stratagene). DNA sequence analysis was performed to confirm that the desired mutation had been introduced successfully. Two different amino acid numbering systems have historically been used to designate specific amino acids within the primary sequence of the epsilon toxin. [22, 23] We are following the precedent established in the earliest of these studies in which the first amino acid of the mature epsilon toxin is defined as amino acid 1 [21, 23]; adding 13 (the number of amino acids removed from the N-terminus of the secreted prototoxin upon trypsin activation) to a given amino acid position within this numbering system will yield the position described in Cole *et al.* [22]

Activation of epsilon toxin by trypsin

Purified epsilon prototoxin was activated with trypsin to form the activated epsilon toxin. [23] Activation was performed as described previously [21] with minor modifications. Briefly, trypsin-coated agarose beads (Pierce) were washed and resuspended in 5 mM Tris-HCl, pH 7.5. Preparations containing the epsilon prototoxin were incubated with the trypsin beads for approximately 90 minutes and the beads were removed by centrifugation. Conversion to epsilon toxin was assessed based on SDS-PAGE and coomassie stain.

Circular dichroism

Circular dichroism (CD) spectroscopy was performed in the Vanderbilt University Center for Structural Biology Biophysical Instrumentation Core facility. CD spectra in the far UV region (190–260 nm) were obtained with a Jasco J-810 spectropolarimeter using a 0.1 cm

path length cuvette. Protein was buffer exchanged into 5 mM Tris-HCl (pH 7.5). Spectra were obtained using the average of 6 scans with a data pitch of 1 nm, scan speed of 50 nm/min, and bandwith of 1 nm. The temperature was controlled at 37°C throughout data acquisition using an integrated Peltier cooling device.

Thermal stability

Thermal stability of wild-type and mutant epsilon toxin proteins was assessed using the Protein Thermal Shift Dye Kit (Applied Biosystems) according to the manufacturer's instructions. Preliminary experiments were used to titrate the dye and protein concentrations to identify the dye:protein ratio that minimized the initial fluorescent signal and that resulted in an estimated melting temperature (T_m) that was independent of the protein concentration. Following optimization, purified proteins (3 μg per well) were mixed with 1x Thermal Shift dye (Applied Biosystems) in quadruplicate reactions, and fluorescence was monitored using a StepOnePlus rt-PCR instrument (Applied Biosystems) with a 1% thermal gradient from 37°C to 95°C. Fluorescence data were fitted to the Boltzmann equation (1) using Protein Thermal Shift software (Applied Biosystems) to determine the T_m.

$$I=(A+\frac{(B-A)}{1+e^{(Tm-T)/C}}) \quad (1)$$

where I is the fluorescence intensity at temperature T, A and B are pre-transitional and post-transitional fluorescence intensities, respectively, and C is a slope factor.

Cytotoxicity

For cytotoxicity assays, MDCK cells were plated in Leibovitz L-15 medium supplemented with 10% fetal bovine serum at 5×10^3 cells per well in 384-well dishes. [24] Epsilon toxin was added and the cells were incubated at 37°C overnight. Cytotoxicity was determined by treating cells with resazurin (CellTiter Blue, Promega) at 37°C for 4 hours. Fluorescence was measured at 590 nm, following excitation at 560 nm using a BioTek FLx800 plate reader. Results were normalized to the fluorescent signal from cells incubated in the absence of toxin (100%) and in 0.1% Triton X-100 (0%).

Calcium flux assay

MDCK cells were plated at 2×10^4 cells per well in black, clear-bottomed 96 well plates. Cells were incubated for 10 minutes at 37°C in HBSS (25 mM HEPES, pH 7.5; 5 mM KCl; 125 mM NaCl; 6 mM glucose; 12 mM $MgCl_2$) containing 1 mM Probenecid. Cells then were incubated for 45 minutes at room temperature in the dark with 5 μm Asante Calcium Red-AM dye (TEFLabs) in HBSS containing 1 mM Probenecid. Cells were washed three times with HBSS containing 1 mM Probenecid and then were incubated for 20 minutes at 37°C with HBSS containing 1 mM Probenecid. Wild-type and mutant epsilon toxin was added to cells in HBSS containing 2 mM $CaCl_2$ and 1 mM Probenecid. Fluorescence was measured at 5 minute intervals at 645 nm following excitation at 528 nm using a BioTek FLx800 plate reader at 37°C.

Binding and oligomerization

MDCK cells were plated at 2.5×10^5 cells per well in a 24 well plate. GFP-tagged epsilon toxin was diluted in L-15 medium pre-warmed to 37°C and added to cells for 30 minutes at 37°C. Toxin was removed and the cell monolayer was washed twice with PBS. Cells were lysed in a mixture of 2% SDS lysis buffer (containing 1.5% Tris-HCl, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.002% bromophenol blue) and 5 mM Tris-HCl (pH 7.5) containing a protease inhibitor cocktail (Roche Complete-mini, EDTA free). The samples

were heated at 100°C for 5 minutes, electrophoresed on a 10% SDS-polyacrylamide gel, and analyzed by immunoblotting using an anti-GFP (sc-9996, Santa Cruz Biotechnology) monoclonal antibody followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. SuperSignal West Femto (Thermo Scientific) was used for enhanced chemiluminescent detection. Anti-β-actin (ab8227, Abcam) was used as a loading control.

HAVCR1 binding assay

The extracellular domain of recombinant human HAVCR1 (R & D Systems) was conjugated to tosyl-activated Dynabeads according to the manufacturer's instructions (Invitrogen). For capturing epsilon toxin from solution, coated beads in Tris-buffered saline supplemented with 0.1% tween-20 and 0.1% BSA were mixed with purified epsilon toxin. Beads were incubated with the toxin at 37°C for 1 hour with agitation. The beads then were captured, and washed three times in Tris-buffered saline containing 0.1% tween-20. The washed beads were heated at 100°C in SDS-sample buffer to elute bound toxin, and samples were analyzed by SDS-PAGE followed by immunoblotting with anti-epsilon-toxin 4D7 [25] and HRP-conjugated rabbit anti-mouse secondary antibody. SuperSignal West Femto was used for enhanced chemiluminescent detection.

Select agent

Plasmid DNA capable of expressing the epsilon prototoxin (or epsilon toxin) is considered a select agent by the U.S. Department of Health and Human Services.

Results

Introduction of substitution mutations in epsilon toxin

Though the identity of the epsilon toxin receptor has not been definitively determined, we previously demonstrated epsilon toxin binds to recombinant HAVCR1 [19], an extensively O-linked glycoprotein. Earlier studies demonstrated that the binding site of epsilon toxin may be a sialoglycoprotein. [17] Furthermore, recent work has implicated an O-glycoprotein in the binding of epsilon toxin. [18] The binding of a protein ligand to a glycoprotein receptor often involves stacking interactions between sugar rings on the glycoprotein and aromatic amino acids in the protein ligand. [26, 27] Two or three coplanar, closely-spaced, surface-accessible aromatic amino acids are frequently involved in glycan binding. [28] In the present study, we investigate the role of clustered, surface-accessible aromatic amino acids within epsilon toxin in mediating binding of the toxin to cells and to its putative receptor, HAVCR1.

To identify surface-exposed clusters of aromatic amino acids, we first determined the relative accessible surface area of each amino acid within the epsilon toxin (Fig. 1). We utilized a conservative threshold such that amino acids with greater than 20% relative accessible surface area are considered to be surface (and solvent) accessible. [28, 29] Based on this threshold, 14 aromatic amino acids were considered to be surface accessible. We next determined the distances between beta-carbons for each pairwise combination of these 14 amino acids, selecting 7 of the surface-accessible aromatic amino acids (Y16, Y20, Y29, Y30, Y36, F37, and Y196) that are within 10.5 A of at least one other surface-accessible aromatic. [28] This set of amino acids is similar to a set of amino acids (Y29, Y30, Y36, Y196 and F199) previously suggested as a possible cell-binding motif. [22]

We used site-specific mutagenesis to investigate the role of these 7 amino acids, as well as F199 [as suggested by Cole, *et al.* [22]], in cytotoxicity, cell binding, and binding to HAVCR1. Mutations were designed to replace the aromatic amino acids with glutamate - an amino acid of similar size and accessible surface area compared with phenylalanine and

tyrosine (and similar surface probability compared with tyrosine) [30], but which eliminates the aromatic ring. Plasmids designed to express the mutant proteins were transformed into *E. coli*, and the recombinant proteins were purified as described in Experimental Procedures. As a preliminary indication of whether the purified mutant proteins were folded, we treated the purified prototoxins with trypsin to yield the mature forms of the proteins. Compared to wild-type epsilon-prototoxin as a control, a 30-kDa protein similar in size to the mature wild-type epsilon toxin was detected for each of the trypsin-treated mutant proteins (Fig. 2A), suggesting that these proteins may not be grossly misfolded.

We next investigated whether the mutant epsilon toxin proteins retained cytotoxic activity. Equal amounts of wild-type epsilon toxin and the mutant epsilon toxin proteins were added to MDCK cells. As expected, MDCK cells were effectively killed by wild-type epsilon toxin (Fig. 2B). Mutant proteins containing substitutions at Y16, Y20, or F37 exhibited cytotoxicity similar to wild-type toxin. In contrast, a mutant protein containing a substitution at Y196 exhibited reduced but detectable activity, and mutant proteins containing substitutions at Y29, Y30, Y36, or F199 lacked detectable cytotoxic activity. These results suggest that several positions within the identified cluster of surface-accessible aromatic amino acids are important for toxin activity.

Folding and thermal stability of mutant proteins

To further investigate the folding of the mutant proteins, circular dichroism (CD) spectroscopy was performed. The CD spectra of all the mutants exhibited a minimum at 216–217 nm and a maximum at 199 nm, similar to wild-type epsilon toxin (Fig. 3) and as expected for proteins rich in β-sheet. [22, 31] A small deviation (between 195 and 210 nm) was noted in the spectrum of some mutant proteins compared to the wild-type toxin, particularly for the Etx-F199E mutant protein (Fig. 3). The deviation observed in the spectrum for the Etx-F199E mutant protein may be the result of introducing the charged glutamic acid side chain in a buried amino acid position. To further evaluate the CD spectra, the spectra were analyzed using the CDSSTR algorithm on the DICHROWEB website (http://dichroweb.cryst.bbk.ac.uk). [32–35] The wild-type and mutant proteins were predicted to have similar compositions of α-helix, β-strand, turns, and unordered structure, in close agreement to the composition of the epsilon-prototoxin (Table S1 of the Supporting Information).

To further evaluate the mutant proteins, a thermal stability assay was performed as described in Experimental Procedures. In this assay, a fluorescent dye is used that exhibits minimal fluorescence in aqueous solution, but is highly fluorescent in non-polar environments. When a protein is unfolded, the dye binds to exposed hydrophobic regions of the protein resulting in a significant increase in the fluorescent signal. Thus, the melting temperature of a protein (a characteristic of protein stability) can be determined by monitoring the change in fluorescence versus changes in temperature. This approach has been shown to yield T_m estimates that closely approximate T_m estimates obtained from thermal denaturing during CD spectroscopy, but is more easily adapted to analysis of larger sample numbers, including experimental replicates. [36] Using this approach, the melting temperature of Etx-F199E was determined to be markedly different than that of wild-type epsilon toxin (Table 1, and Figure S1 of the Supporting Information). Based on this and the deviation observed in the CD spectrum, we conclude that the Etx-F199E mutant protein may be misfolded and this mutant protein was excluded from further analyses. However, the melting temperature of wild-type epsilon toxin and the Etx-Y16E, Etx-Y20E, Etx-Y29E, Etx-Y30E, Etx-Y36E, Etx-F37E, and Etx-Y196E mutant proteins were similar (Table 1, and Figure S1 of the Supporting Information). Based on these results, the similarities in the CD spectra, and the stability of the mature proteins to trypsin activation, we conclude that the wild-type toxin and these mutant proteins are structurally similar.

Mutant proteins do not mediate pore formation

It has been previously reported that cell death caused by epsilon toxin is correlated with the formation by the toxin of large pores in the target cell plasma membrane, leading to dysregulated ion homeostasis including a rapid decrease of intracellular K^+ and an increase of intracellular Ca^{2+}. [37–39] To further understand the behavior of the mutant toxins, we next monitored changes in intracellular Ca^{2+} in response to the mutant proteins.

MDCK cells were treated with wild-type epsilon toxin, Etx-Y29E, Etx-Y30E, Etx-Y36E, and Etx-Y196E as described in Experimental Procedures. The Etx-Y20E and Etx-F37E mutants were excluded from this and subsequent studies because they exhibit cytotoxicity similar to wild-type toxin; the Etx-Y16E mutant was used as a representative mutant that retained cytotoxic activity. Results indicated an increase in intracellular Ca^{2+} in cells treated with wild-type epsilon toxin or Etx-Y16E (Fig. 4). However, no increase in intracellular Ca^{2+} was observed in cells treated with Etx-Y29E, Etx-Y30E, Etx-Y36E, or Etx-Y196E (Fig. 4). As expected based on the cytotoxicity assay (Fig. 2), a 10-fold higher concentration of Etx-Y196E resulted in an increase in intracellular Ca^{2+}, whereas a 10-fold higher concentration of Etx-Y29E, Etx-Y30E, or Etx-Y36E did not lead to an increase in intracellular Ca^{2+} (Fig. 4 and data not shown). These results suggest that the Etx-Y29E, Etx-Y30E, Etx-Y36E, and Etx-Y196E mutant proteins are defective in the ability to form membrane pores.

Binding and oligomerization on MDCK cells

Results from the cytotoxicity and cell permeabilization assays suggest that the Etx-Y29E, Etx-Y30E, Etx-Y36E, and Etx-Y196E mutant proteins may be impaired in the ability to interact with host cells. To investigate the toxin-cell interaction further, a GFP-tagged form of the wild-type and mutant epsilon toxin proteins was used (we do not have an epsilon toxin antibody that detects the toxin in association with host cells). To investigate the interaction of the mutant proteins with MDCK cells, equal amounts of wild-type or mutant proteins were added to cells for 30 minutes at 37°C. As has been demonstrated previously, [21, 37] wild-type epsilon toxin bound to cells and formed a heat-and SDS-resistant oligomeric complex (Fig. 5A–D). Similarly, the active mutant Etx-Y16E also bound to cells and formed oligomeric complexes (Fig. 5A–D). In contrast, the Etx-Y29E, Etx-Y30E, Etx-Y36E, and Etx-Y196E mutant proteins were defective in the ability to interact with cells (Fig. 5A–D). Binding and oligomerization of Etx-Y196E was detected when a 10-fold excess of this mutant protein was added to cells (Fig. 5E–G); binding and oligomerization were not detected when a 10-fold excess of Etx-Y29E, Etx-Y30E, or Etx-Y36E was added to cells (data not shown).

Binding to HAVCR1

We have previously shown that HAVCR1 contributes to epsilon-toxin-induced cytotoxicity in both MDCK and in ACHN cells and that the toxin is able to bind to the extracellular domain of human HAVCR1. [19] We therefore investigated the ability of the mutant proteins to bind to HAVCR1. The extracellular domain of human HAVCR1 was conjugated to magnetic beads. The protein conjugated beads then were mixed with wild-type epsilon toxin or mutant proteins Etx-Y16E, Etx-29E, Etx-30E, Etx-Y36E, and Etx-Y196E. As expected, wild-type epsilon toxin bound to HAVCR1-coated beads (Fig. 6). Similarly, the active mutant Etx-Y16E also bound to HAVCR1-coated beads (Fig. 6). In contrast, the Etx-Y29E, Etx-Y30E, Etx-Y36E, and Etx-Y196E mutant proteins were defective in the ability to interact with HAVCR1-coated beads (Fig. 6).

Discussion

Several studies using chemical modification of epsilon toxin have revealed amino acids that are essential for toxin activity, including tryptophan, tyrosine, and histidine. [40–42] However, whereas unlabeled epsilon toxin competes for binding of ^{125}I-labeled toxin, an epsilon toxin preparation in which tryptophan, tyrosine, and histidine have been chemically modified with *N*-bromosuccinimide did not inhibit binding of ^{125}I-labeled epsilon toxin. These results suggest that the oxidation of tryptophan, tyrosine, or histidine prevents the toxin from binding. [17, 40, 43] A mutant epsilon toxin in which the sole tryptophan was replaced by leucine retained cytotoxic activity, indicating that the aromatic side chain of the tryptophan was not essential for toxin activity. [23] Thus far, specific amino acids that may be important for binding of epsilon toxin to host cells have not been identified. In the present study, we used site-specific mutagenesis to determine the role of a surface-accessible cluster of aromatic amino acids in mediating binding of the toxin to MDCK cells.

Amino acids Y29, Y30, Y36, and Y196 are closely spaced and surface accessible in domain I of epsilon toxin. [22] Domain I of epsilon toxin exhibits structural similarity with domain II of aerolysin, which also contains surface-accessible aromatic amino acids. This motif in aerolysin is important for the interaction of the toxin with its receptor. [44] The receptor-interacting domains of *Clostridium septicum* alpha toxin and *C. perfringens* enterotoxin, additional members of the aerolysin-like family of pore-forming toxins, also include surface-exposed aromatic amino acids [45–47]. For each of these pore-forming toxins, mutagenesis of the aromatic amino acids to non-aromatic amino acids greatly reduces binding. [44–47] However, although aerolysin and *C. septicum* alpha toxin have been proposed to interact with the sugar component of glycoproteins, [45, 48] the aromatic amino acids that contribute to binding of *C. perfringens* enterotoxin appear to interact with the amino acid side chains of claudin 4. [49, 50] Our analysis indicates that Y29, Y30, Y36, and Y196 of epsilon toxin contribute to cytotoxic activity. Mutation of these amino acids also resulted in proteins that were severely impaired in binding to host cells.

Although a receptor for epsilon toxin has not been definitively identified, it has been suggested that the receptor may be a sialoglycoprotein. [17] Recent work has shown that epsilon toxin binding to MDCK cells or mouse kidney sections is greatly diminished following removal of O-linked oligosaccharides by β-elimination. [18] We have previously shown that HAVCR1 contributes to epsilon-toxin-induced cytotoxicity, and that the toxin can bind to the extracellular domain of human HAVCR1 but not to the extracellular domains of structurally-related proteins. [19] It remains to be determined whether HAVCR1 is a receptor for the toxin on cells. Although we have demonstrated that disrupting expression of HAVCR1 in MDCK and in ACHN cells results in decreased cytotoxicity and that the toxin binds to the recombinant extracellular domain, we have not yet demonstrated an association between the toxin and HAVCR1 on cells. [19] There are alternative explanations, in addition to HAVCR1 acting as a toxin receptor, that could explain previous results. [19] Despite the uncertain role of HAVCR1, the identification of mutant proteins defective in cytotoxicity, cell interaction, and binding to HAVCR1 provides additional circumstantial evidence implicating HAVCR1 as a receptor or co-receptor for the toxin. In addition to an immunoglobulin-like domain, the extracellular domain of HAVCR1 has a mucin-like domain with approximately 57 O-linked glycosylation sites. [51] Previous studies have demonstrated that surface-accessible clusters of aromatic amino acids (like Y29, Y30, Y36, and Y196 identified in the present study) are important for the binding of proteins to carbohydrates. [26, 27] However, soluble sialic acid does not inhibit epsilon toxin binding or cytotoxicity. [52] Furthermore, another recent study indicated that sialidase treatment of MDCK cells enhanced epsilon toxin binding and cytotoxicity. [53] Thus, the role protein glycosylation plays in epsilon toxin binding also remains to be determined. It will be

important in future studies to explore whether the binding of epsilon toxin to HAVCR1 is a result of binding to the protein or to the sugars and what additional amino acid positions within both the toxin (aromatic or non-aromatic amino acids) and HAVCR1 contribute to binding.

Characterizing the interactions between toxins and target cells is critical to understanding events leading to cytotoxicity and may lead to the development of a variety of different therapeutic or diagnostic tools. For example, antibodies, aptamers or other molecules designed to inhibit interaction of the identified motif with the toxin receptor could be used to treat individuals exposed to epsilon toxin. [54, 55] Similarly, inactive mutant toxins such as those described in the present study might also be an effective vaccine. [23] More broadly applicable might be an engineered toxin with altered binding specificity developed for therapeutic use to kill specific cellular targets. [56–59] Conversely, the receptor binding domain of an inactivated epsilon toxin may be used to target therapeutic or diagnostic compounds to tissues such as the kidney or brain where epsilon toxin naturally accumulates. [10, 60, 61]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

HAVCR1	Hepatitis A Virus Cellular Receptor 1
CD	circular dichroism spectroscopy
T_m	melting temperature

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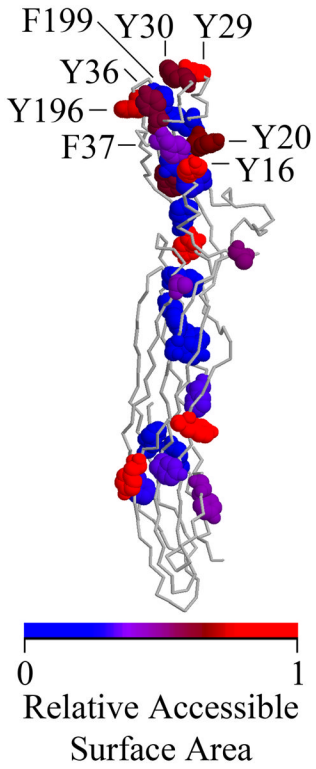

FIGURE 1. Aromatic amino acids within epsilon-toxin

A ribbon diagram of epsilon-toxin is shown, with each aromatic amino acid (F, H, W, and Y) shown in space-filling representation. The aromatic amino acids are shaded based on a heat map generated from the relative accessible surface area for each amino acid within epsilon-toxin. Specific amino acids mutated in the present study are labeled.

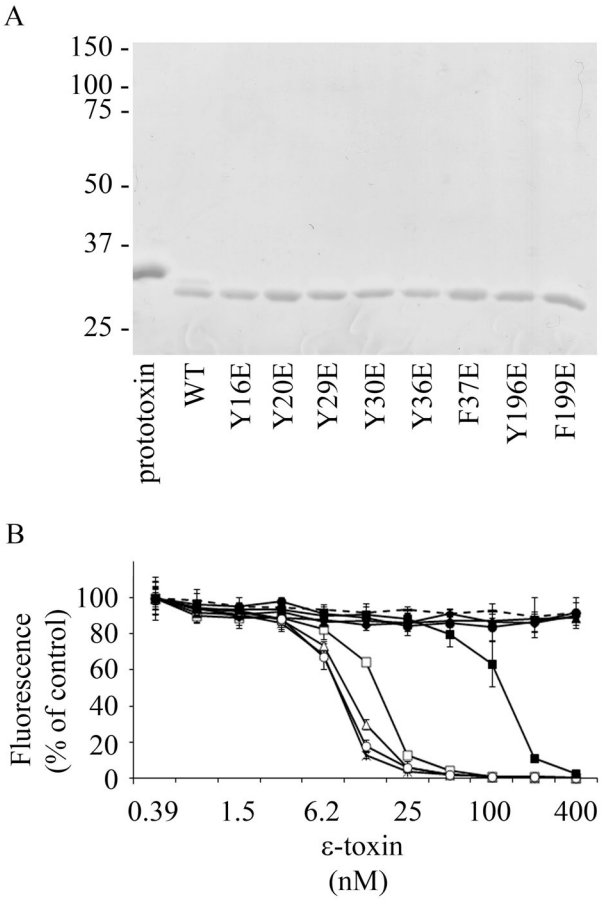

FIGURE 2. Purification and cytotoxicity of wild-type and mutant epsilon-toxins

(A) Wild-type (WT) epsilon toxin and mutant proteins were expressed, purified, and treated with trypsin to convert proteins from the immature prototoxin to the active toxin form as described in Experimental Procedures. Trypsin-treated proteins were analyzed by coomassie stain. (B) Wild-type epsilon-toxin (*), Etx-Y16E (△) Etx-Y20E (□), Etx-Y29E (▲), Etx-Y30E (◆), Etx-Y36E (●), Etx-F37E (○), Etx-Y196E (■), and Etx-F199E (- - -) were purified and activated. Equal amounts of protein were added to MDCK cells in serial two-fold dilutions. Cytotoxicity was measured by resazurin. Results were normalized to the fluorescent signal from untreated MDCK cells (100%) and cells treated with 1% Triton (0%). Results represent the mean ± SD from quadruplicate samples.

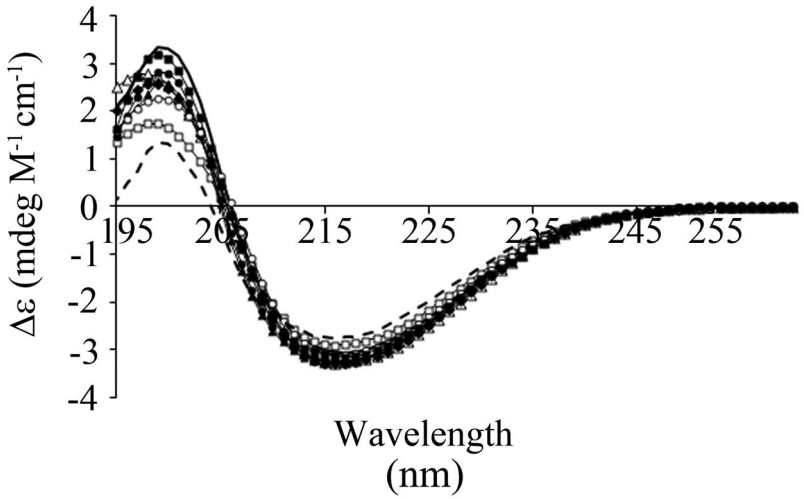

FIGURE 3. CD spectroscopy of wild-type and mutant epsilon-toxins

Circular dichroism spectra of wild-type epsilon toxin (—), Etx-Y16E (△), Etx-Y20E (□), Etx-Y29E (▲), and Etx-Y30E (◆), Etx-Y36E (●), Etx-F37E (○), Etx-Y196E (■), Etx-F199E (- - -) are shown. Results represent the mean of six scans; the background spectrum from buffer alone was subtracted.

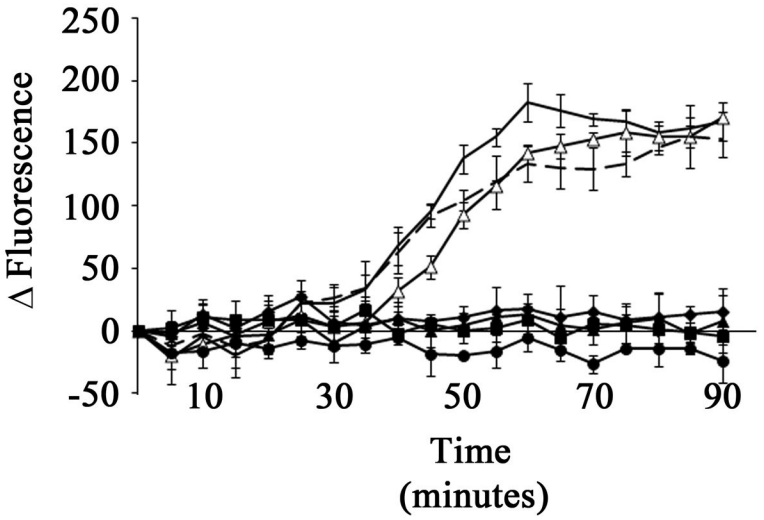

FIGURE 4. Functional activity of epsilon-toxin mutant proteins

Changes in intracellular calcium were monitored using Asante Calcium Red as described in Experimental Procedures. At time zero, triplicate wells received 25 nM toxin [wild-type epsilon toxin (—), Etx-Y16E (△), Etx-Y29E (▲), Etx-Y30E (◆), Etx-Y36E (●), Etx-Y196E (■)] or 250 nM Etx-Y196E (---) and fluorescence was measured at 5 minute intervals at 37°C. Results represent the mean change in fluorescence (± standard deviation) based on triplicate samples.

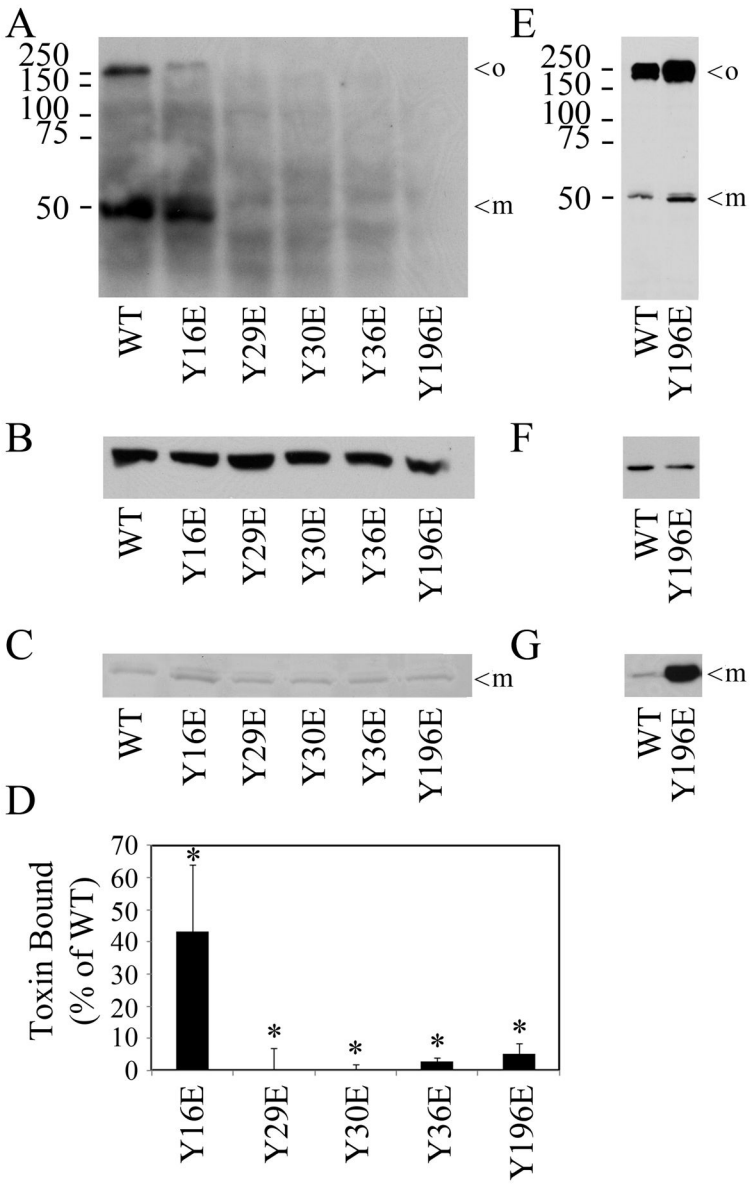

FIGURE 5. Analysis of oligomer formation by epsilon-toxin mutant proteins

(A–C) MDCK cells were treated with 125 nM GFP-tagged epsilon toxin for 30 minutes at 37°C. Samples were solubilized, analyzed by SDS-PAGE, and immunoblotted with an anti-GFP antibody (A) or β-actin antibody as a loading control (B). A coomassie-stained gel showing the toxins before addition to cells is also shown (C). (D) Quantitative analysis was performed using Quantity One software (Bio-Rad). Signals of bound toxin were normalized to the signals of proteins added to cells, and the relative amount of epsilon-toxin bound to MDCK cells was determined. Results represent the means and standard deviations from triplicate gels and are expressed relative to the amount of wild-type epsilon-toxin bound. Data were analyzed by analysis of variance followed by Dunnett's post hoc test. Asterisks denote results significantly different from the control ($P < 0.05$). (E–G) MDCK cells were treated with 125 nM GFP-tagged epsilon toxin or 1250 nM GFP-tagged Etx-Y196E for 30 minutes at 37°C. Samples were solubilized, analyzed by SDS-PAGE, and immunoblotted with an anti-GFP antibody (E) or β-actin antibody as a loading control (F). An immunoblot

of the toxins before addition to cells is also shown (G). Molecular weight markers (in kDa), the GFP-tagged monomer (m), and the GFP-tagged oligomer (o) are indicated.

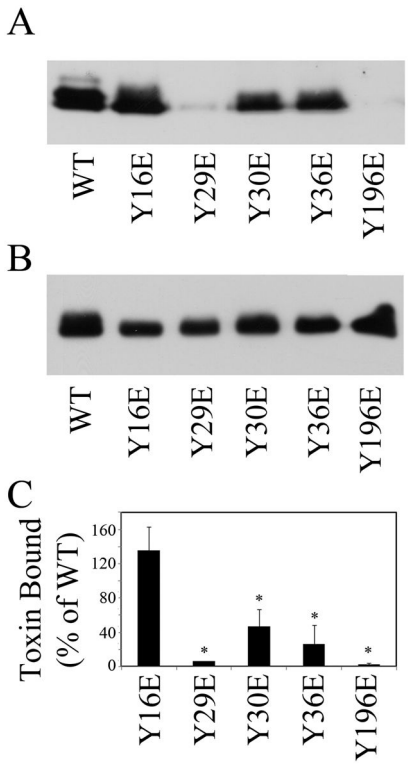

FIGURE 6. Analysis of epsilon-toxin binding to HAVCR1

The extracellular domain of human HAVCR1 was conjugated to paramagnetic beads as described in Experimental Procedures. (A) The bound proteins were eluted by boiling in SDS and analyzed by immunoblotting. (B) An immunoblot showing the toxins before addition to beads is shown. (C) Quantitative analysis was performed using Quantity One software (Bio-Rad). Signals of bound toxin were normalized to the signals of proteins added to beads, and the relative amount of epsilon-toxin bound to beads was determined. Results represent the means and standard deviations from triplicate gels and are expressed relative to the amount of wild-type epsilon-toxin bound. Data were analyzed by analysis of variance followed by Dunnett's post hoc test. Asterisks denote results significantly different from the control ($P < 0.05$).

Table 1

Thermal stability analysis

Protein	T_m [a] (±SD)
Etx	58.24 (0.04)
Etx-Y16E	57.32 (0.10)
Etx-Y20E	55.55 (0.14)
Etx-Y29E	58.15 (0.06)
Etx-Y30E	57.58 (0.03)
Etx-Y36E	56.71 (0.10)
Etx-F37E	57.00 (0.05)
Etx-Y196E	52.05 (0.03)
Etx-F199E	47.33 (0.08)

[a]Fluorescence data were fitted to the Boltzmann equation (1) using Protein Thermal Shift software (Applied Biosystems) to determine the T_m. Results represent the mean and standard deviation of quadruplicate samples.