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Identification of tyrosine 71 as a critical residue for the cytotoxic activity of *Clostridium perfringens* epsilon toxin towards MDCK cells

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***Clostridium perfringens* epsilon toxin (Etx) is an extremely potent toxin, causing fatal enterotoxaemia in many animals. Several amino acids in domains I and II have been proposed to be critical for Etx to interact with MDCK cells. However, the critical amino acids in domain III remain undefined. Therefore, we assessed the effects of aromatic amino acids in domain III on Etx activity in this study. All of the results indicated that Y71 was critical for the cytotoxic activity of Etx towards MDCK cells, and this activity was dependent on the existence of an aromatic ring residue in position 71. Additionally, mutations in Y71 did not affect the binding of Etx to MDCK cells, indicating that Y71 is not a receptor binding site for Etx. In summary, we identified an amino acid in domain III that is important for the cytotoxic activity of Etx, thereby providing information on the structure-function relationship of Etx.**

***Keywords*:** *Clostridium perfringens*, epsilon toxin, tyrosine 71, aromatic ring, cytotoxic activity

Introduction

Clostridium perfringens epsilon toxin (Etx), which is produced by type B and type D isolates of *C. perfringens*, is one of the most potent bacterial toxins (Bokori-Brown *et al.*, 2011; Popoff, 2011). Etx causes fatal enterotoxaemia in sheep, goats, calves and other livestock species, resulting in considerable economic losses (Songer, 1996; Uzal and Songer, 2008). Etx is secreted by *C. perfringens* as a prototoxin that is activated when N-terminal amino acids 11–13 and C-terminal amino acids 22–29 are removed by proteases, including trypsin, chymotrypsin, and γ-protease (Worthington and Mulders, 1977; McDonel, 1986; Minami *et al.*, 1997). The toxin is believed to enter the bloodstream via the intestinal mucosal barrier and then disseminate to several organs, preferentially accumulating in the kidneys and the brain, where intoxication causes widespread edema and necrotic lesions (Songer, 1996; Miyamoto *et al.*, 2000; Soler-Jover *et al.*, 2004).

Although Etx is known to be a β-pore-forming toxin, the mechanism underlying Etx-mediated pore formation is poorly understood. The Madin–Darby canine kidney (MDCK) cell line has been extensively used as an *in vitro* model to study the molecular actions of Etx (Lindsay *et al.*, 1995; Petit *et al.*, 1997; Borrmann *et al.*, 2001). Etx binding, oligomerization, and pore formation at the plasma membrane are thought to constitute the basic model of the interaction between Etx and MDCK cells (Lindsay *et al.*, 1995; Petit *et al.*, 1997; Nagahama *et al.*, 1998; Petit *et al.*, 2001; Miyata *et al.*, 2002; Bokori-Brown *et al.*, 2011; Robertson *et al.*, 2011). In two of the three domains of Etx (Cole *et al.*, 2004), certain functional amino acids have already been identified. In domain I of Etx, four aromatic amino acids (i.e., Y29, Y30, Y36, and Y196) have recently been determined to be receptor binding sites for MDCK cells (Fig. 1A), and mutants with substitutions at these residues demonstrate a loss of cytotoxic activity (Ivie and McClain, 2012; Bokori-Brown *et al.*, 2013). Additionally, the amphipathic β-hairpin in domain II (residues 124–146) is considered a membrane insertion structure (Cole *et al.*, 2004; Pelish and McClain, 2009), and H106 in domain II is predicted to play a critical role in the conformational changes that Etx undergoes during cellular interactions, given that the H106P mutant exhibits no detectable cytotoxic activity in MDCK cells (Oyston *et al.*, 1998). However, there have been no reports concerning the amino acids in domain III that are critical for the interaction of Etx with cells, although it is accepted that aromatic amino acids are important for the interactions of β-pore-forming toxins with cells (Tsitrin *et al.*, 2002; Ivie and McClain, 2012).

Thus, in this context, we sought to further identify functional amino acids of Etx involved in the interaction with MDCK cells and focused on a cluster of aromatic amino acids in domain III of Etx. We identified one amino acid residue, Y71, for the first time as a site that is critical for the cytotoxic activity of Etx, and we also found that this cytotoxic activity is dependent on an aromatic ring residue in position 71. Furthermore, by evaluating the ability of Etx mutants to bind to MDCK cells, we determined that the aromatic amino acids in Etx domain III, including Y71, did not serve as receptor binding sites. These results provide additional information regarding the structure and function of Etx.

Materials and Methods

Cell culture and bacterial strain

MDCK (Madin-Darby canine kidney cell line) and BHK-21

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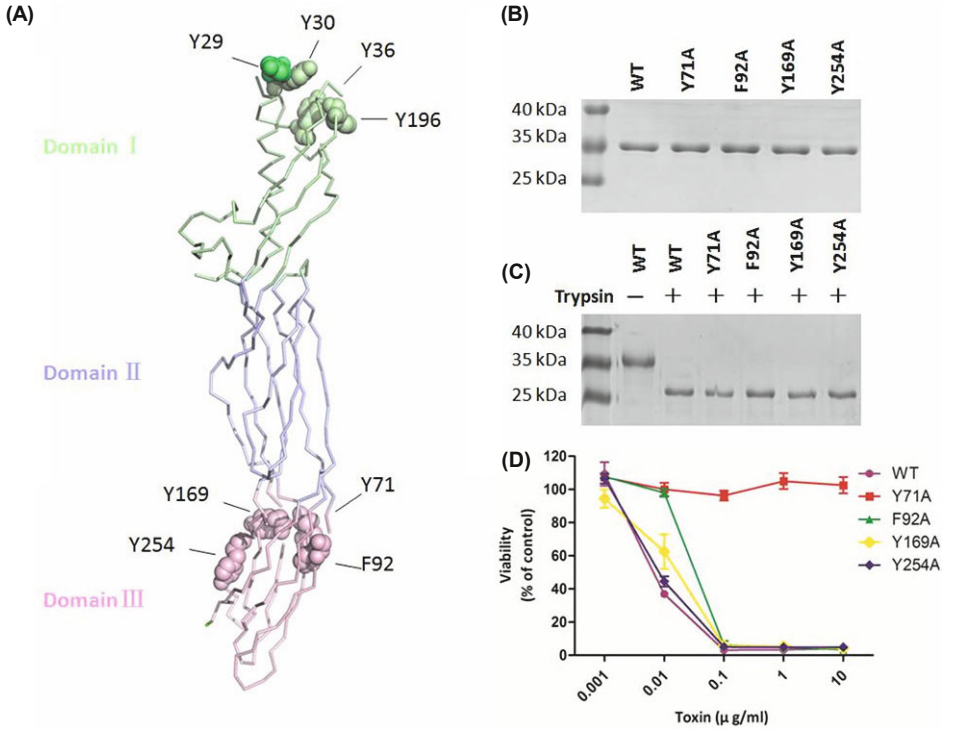

Fig. 1. Expression, activation and cytotoxicity of wild-type Etx and its mutants with single alanine substitutions. (A) Ribbon representation of wild-type *C. perfringens* epsilon prototoxin was colored by domains using Pymol™1.6.x (PDB ID: 1UYJ). Four tyrosines in domain I were considered to be receptor binding sites. Four aromatic amino acids (Y71, F92, Y169, and Y254) in domain III, shown as sphere models, were investigated in this study. (B–D) The aromatic amino acids (Y71, F92, Y169, and Y254) of Etx were mutated to alanines. The prototoxins (B) and their mature forms (C) were analyzed by SDS-PAGE, and the cytotoxicity of these aromatic amino acid mutants was determined in MDCK cells using a CCK-8 kit (D).

(baby hamster kidney cell line) cells were routinely cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 8% fetal bovine serum (Gibco). Cells were maintained at 37°C in 5% atmospheric CO_2 and grown until confluent in 75-cm^2 flasks before seeding for the experiments. *C. perfringens* type D strain CVCC C60-1 was purchased from the China Institute of Veterinary Drug Control (China).

Expression and purification of recombinant epsilon prototoxin

The gene encoding epsilon prototoxin, *etxD*, from *C. perfringens* type D strain CVCC C60-1 was PCR-amplified using the following primers with *Nco*I/*Xho*I restriction sites (underlined): Etx-F (GAGCCATGGAAAAGGAAATATCTAATAC) and Etx-R (AGGCTCGAGTTTTATTCCTGGTGCCTT). The *etx*D gene was cloned into plasmid pET-22b(+) (Novagen), which 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. The correct recombinant plasmids were transformed into Rosetta(DE3)pLysS *E. coli* competent cells, and transformants were grown to an OD_{600} of 0.6 in broth supplemented with antibiotics. Expression of recombinant prototoxin was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) at 30°C for 6 h. A Ni^{2+} NTA affinity column (Qiagen) was used to purify the expressed prototoxin according to the manufacturer's instructions. The concentrations of purified proteins were determined using a BCA kit (Tiangen), and the purity of the proteins was assessed by SDS-PAGE.

Site-directed mutagenesis

Mutations were introduced into the cloned *etxD* gene using the Quick Change Lightning Multi Site-Directed Mutagenesis Kit (Stratagene) with synthetic oligonucleotide primer pairs. DNA sequence analysis was performed to determine whether the desired mutation was correctly introduced. According to the literature (Ivie and McClain, 2012), the N-terminal amino acid of the mature toxin (following trypsin treatment) was numbered as 1.

Activation of Etx by trypsin

Aliquots of the purified recombinant epsilon prototoxin (mutated or not) were activated by incubation with 12.5 μg of trypsin (Sigma) per μg of toxin for 1 h at 37°C. Following this activation, Protease Inhibitor Cocktail (Promega) was added to inhibit trypsin activity. The conversion to mature Etx was assessed by SDS-PAGE.

Cytotoxicity assay

The cytotoxic activity of Etx towards MDCK cells was determined using a Cell Counting Kit-8 (CCK-8) (Beyobime) according to the manufacturer's protocol. In brief, a ten-fold dilution series of each activated toxin (ranging from 10 μg/ml to 0.001 μg/ml) was prepared in DMEM and added to cells seeded into 96-well plates (3×10^4 cells/well). Following incubation at 37°C for 16 h, a CCK-8 solution was added to each well at a 1:10 (v/v) ratio and incubated at 37°C for 1 h. The amount of the reagent reduced to formazan by cellular dehydrogenase, which is indicative of cell viability, was assayed by reading the absorbance at 450 nm using an EnVision Multilabel Plate Reader.

Calcium flux assay

A calcium flux assay was performed using a Fluo-4 Direct™

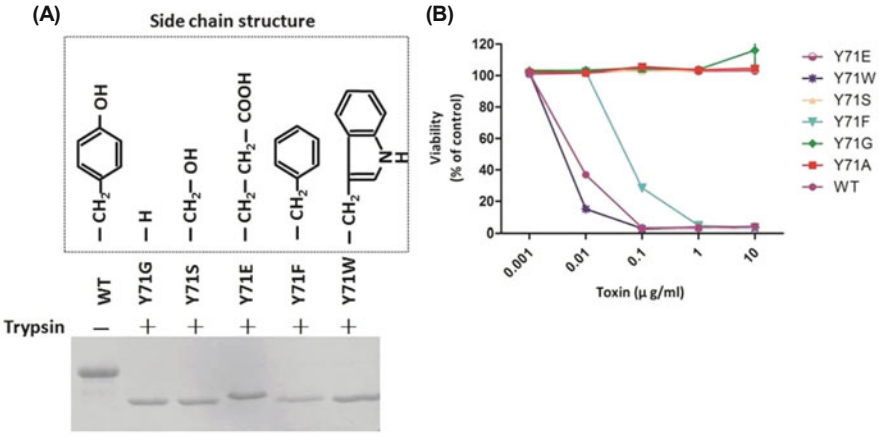

Fig. 2. Y71 of Etx was replaced with amino acids containing different side chain structures. (A) The mature forms of Y71 mutants (Y71E, Y71G, Y71S, Y71F, and Y71W) were analyzed by SDS-PAGE. (B) The cytotoxicity of the Y71 mutants was determined in MDCK cells using a CCK-8 kit.

Calcium Assay Kit (Invitrogen) according to the manufacturer's protocol. In brief, MDCK cells were plated at 3×10^4 cells/well in black, clear-bottomed 96-well plates. Then, 2× Fluo-4 Direct™ calcium reagent loading solution mixed with DMEM at a 1:1 (v/v) ratio was added to the plates (100 µl/well) for incubation at 37°C for 30 min and then at room temperature for 30 min. The cells were washed three times with HBSS (25 mM HEPES; pH 7.5, 125 mM NaCl, 5 mM KCl, 6 mM glucose, 12 mM $MgCl_2$), and then 100 µl of wild-type Etx and its mutants were added to the cells (10 µg/ml) in HBSS containing 2 mM $CaCl_2$ (Ivie and McClain, 2012). Fluorescence was measured at 5-min intervals at an excitation of 485 nm and emission of 516 nm using an EnVision Multilabel Plate Reader at 37°C. The data were analyzed using GraphPad Prism Version 5.01 software (GraphPad Software). Two-way ANOVA was used to compare variables between different groups.

Confocal microscopy

Wild-type Etx and its mutants (10 µg/ml) were added to MDCK cells grown on glass coverslips, respectively. Following incubation at 37°C for 5 min, the toxins were removed, and the cells were washed three times with PBS. The cells were then fixed with ice-cold anhydrous ethanol for 20 min at room temperature and air-dried. The cells were incubated with anti-Etx hyper-immune mouse serum followed by staining with goat anti-mouse IgG conjugated with FITC (Sigma). After mounting on slides using Antifade-4,6-diamidino-2-phenylindole (DAPI) mounting solution (Sigma), the cells were visualized using a Leica DM-IRE2 confocal microscope. The Y30E mutant, previously reported to lose its ability to bind MDCK cells, were constructed and expressed according to the method described by Ivie and McClain (2012) to serve as a negative control in this assay. In addition, wild-type Etx and the Y71A mutant were added to BHK-21 cells, which served as control cells, to determine the specificity of the interaction between the toxins and MDCK cells.

Results

Y71 is critical for the cytotoxic activity of Etx

According to the structure of Etx (Cole *et al.*, 2004), the upper boundary of domain III is delineated by a ring of four aromatic residues, Y71, F92, Y169, and Y254, which are reminiscent of the 'aromatic belts' found in other membrane proteins that are thought to anchor and stabilize those proteins in the bilayer (Fig. 1A) (Killian and von Heijne, 2000; Tsitrin *et al.*, 2002). Thus, to investigate the role of these four residues in MDCK cell interactions, mutations were designed to replace them with alanine residues.

Recombinant wild-type Etx and its mutants were expressed in *E. coli* and then were purified using a Ni^{2+} NTA affinity column. These purified prototoxins (Fig. 1B) were treated with trypsin to yield the mature ~30 kDa form of the Etx protein (Fig. 1C). To investigate whether the trypsin-treated

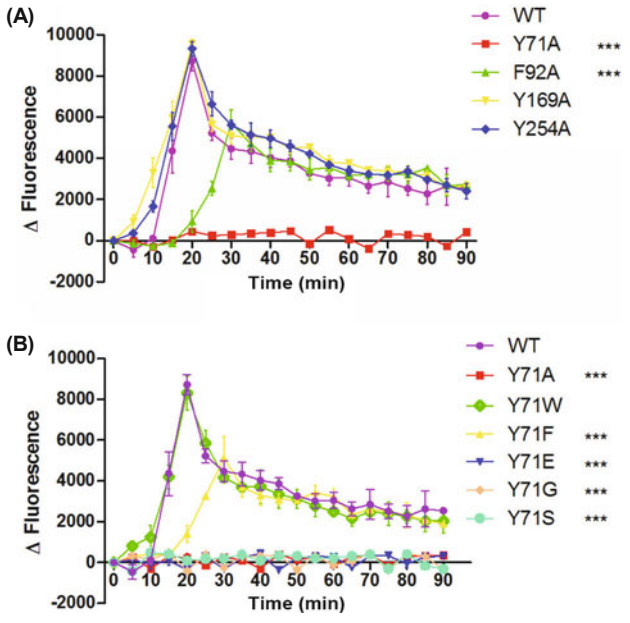

Fig. 3. Pore formation by Etx and its mutants in MDCK cells. MDCK cells were inoculated with wild-type Etx or one of four aromatic amino acid mutants (Y71A, F92A, Y169A, and Y254A) (A) or five Y71 mutants (Y71E, Y71G, Y71S, Y71F, and Y71W) (B). Changes in intracellular calcium were monitored using the Fluo-4 calcium indicator, and the results represent the mean change in fluorescence (± SEM) based on triplicate samples. ***, Highly significant difference from wild-type Etx group (two-way ANOVA; $P < 0.001$).

Etx mutants retained their cytotoxic activity towards MDCK cells, equal amounts of wild-type Etx and its mutants were added to MDCK cells. As shown in Fig. 1D, the mutants Y169A and Y254A exhibited cytotoxic activity similar to that of wild-type Etx, which effectively killed MDCK cells, and the mutant F92A exhibited slightly reduced but detectable cytotoxic activity. Only the mutant Y71A lacked detectable cytotoxic activity, indicating that Y71 in domain III of Etx is critical for cytotoxic activity.

The aromatic ring of residue 71 is essential for the cytotoxic activity of Etx

To further evaluate the effects of amino acids with different side chain structures on the cytotoxic activity of Etx, Y71 of Etx was mutated to glutamate, glycine, serine, phenylalanine, and tryptophan to generate the mutants Y71E, Y71G, Y71S, Y71F and Y71W (Fig. 2A). Cytotoxicity assays (Fig. 2B) revealed that the mutants Y71E, Y71G, and Y71S lacked detectable cytotoxic activity, similar to the mutant Y71A, whereas the Etx mutants that contained only aromatic amino acid (W or F) substitutions at Y71 exhibited cytotoxic activity. In contrast to the mutant Y71A, the mutant Y71W demonstrated complete restoration of Etx cytotoxicity, whereas the mutant Y71F demonstrated partial restoration of cytotoxicity. These results indicate that the aromatic ring of residue 71 in domain III is essential for the cytotoxic activity of Etx.

Pore formation by Etx in MDCK cells depends on an aromatic ring residue at position 71

Previous studies reported that cell death caused by Etx results from pore formation in the target cell plasma membrane, leading to a rapid decrease in intracellular K^+ and an increase in intracellular Ca^{2+} (Petit *et al.*, 2001; Ivie and McClain, 2012). To determine the ability of the Etx mutants to form pores in MDCK cells, changes in intracellular Ca^{2+} were monitored in a calcium flux assay. First, MDCK cells were treated with four Etx mutants containing alanine substitutions at aromatic amino acids in domain III (Y71A, F92A, Y169A, and Y254A, Fig. 3A). Increased intracellular Ca^{2+} was observed in cells treated with wild-type Etx or with

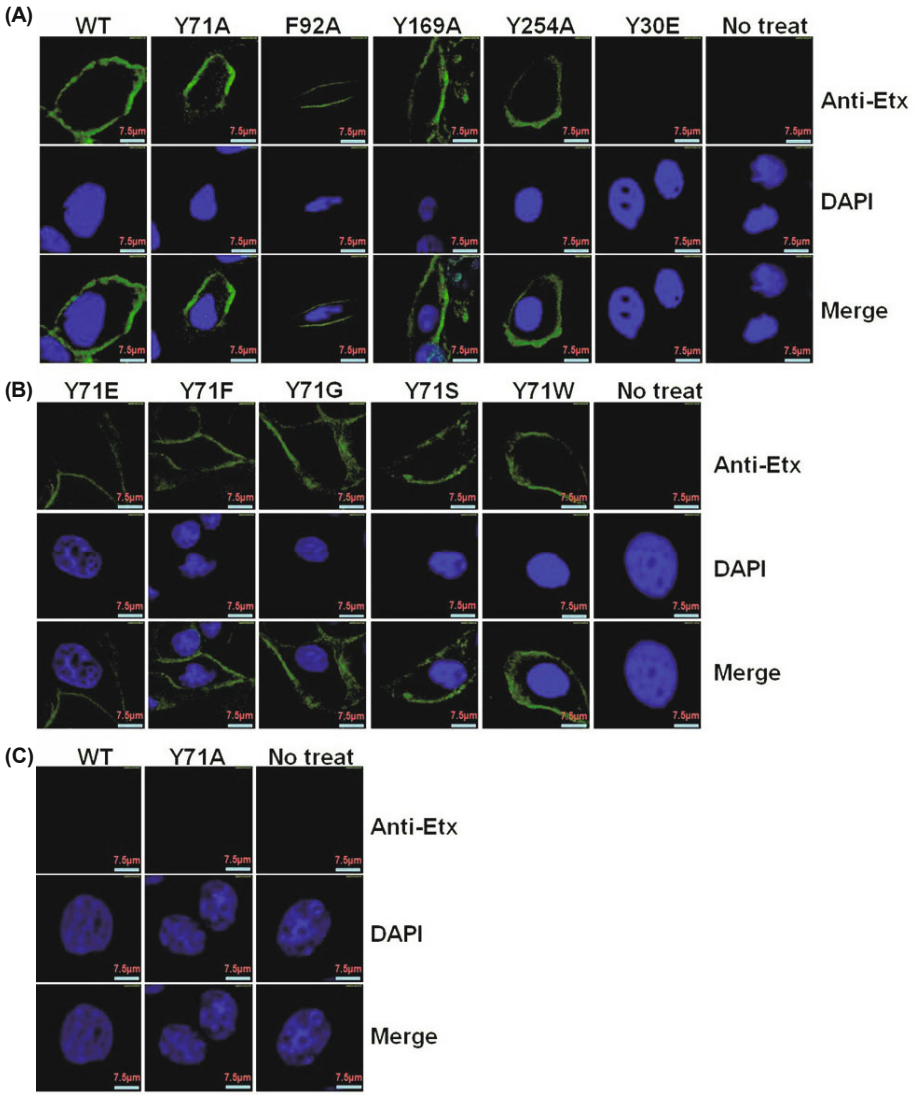

Fig. 4. Analysis of the binding of Etx and its mutants to MDCK cells. MDCK cells were inoculated with wild-type Etx or one of four aromatic amino acid mutants (Y71A, F92A, Y169A, and Y254A) (A) or five Y71 mutants (Y71E, Y71G, Y71S, Y71F, and Y71W) (B) at 37°C for 30 min. The cells were fixed and incubated with anti-Etx hyper-immune serum, followed by staining using secondary antibodies conjugated with FITC (green) for the detection of Etx and its mutants; nuclei were stained with DAPI (blue). Subsequently, cells were analyzed by confocal microscopy. (C) BHK-21 cells were inoculated with wild-type Etx or the Y71A mutant, which served as a control to determine the specificity of the interaction between the toxins and MDCK cells.

the mutants F92A, Y169A, and Y254A. Compared with the increase in intracellular Ca^{2+} mediated by wild-type Etx, there is no significant difference in that mediated by the mutant Y169A or Y254A (two-way ANOVA, $P > 0.05$), but the mutant F92A mediated a lower level of the increase (two-way ANOVA, $P < 0.001$). However, no increase in intracellular Ca^{2+} was observed in cells treated with the Y71A mutant. Furthermore, changes in intracellular Ca^{2+} were also monitored in MDCK cells treated with five Etx Y71 mutants (Y71E, Y71G, Y71S, Y71W, and Y71F, Fig. 3B). Increased intracellular Ca^{2+} was observed only in cells treated with mutants with an aromatic amino acid (W or F) substitution at Y71, and the increase mediated by the Y71W mutant was higher than that mediated by the Y71F mutant (two-way ANOVA, $P < 0.001$). However, when Y71 was replaced with E, G or S, which do not contain an aromatic ring in the side chain, no increase in intracellular Ca^{2+} was observed. These data agree with that obtained in the cytotoxicity assay (Figs. 2B and 1D), indicating that Y71 in domain III is critical for pore formation by Etx and that the aromatic ring of residue 71 is essential for Etx activity.

Mutations of aromatic amino acids in domain III do not affect the binding of Etx to MDCK cells

To determine whether the aromatic amino acids in domain III are possible receptor binding sites on Etx for MDCK cells, the binding of Etx to MDCK cells with these sites substituted was characterized through confocal microscopy. As expected, wild-type Etx bound to the plasma membrane of MDCK cells, whereas the mutant Y30E did not bind to MDCK cells, as demonstrated previously (Ivie and McClain, 2012) (Fig. 4A). All four aromatic amino acid mutants (Y71A, F92A, Y169A, and Y254A) and all five Y71 mutants (Y71E, Y71G, Y71S, Y71W, and Y71F) of Etx also bound to the plasma membrane of MDCK cells (Fig. 4A and B). These results indicate that the aromatic amino acids in domain III do not play a role in the binding of Etx to MDCK cells and that the loss of cytotoxic activity of the Y71 mutants (Y71A, Y71E, Y71G, and Y71S) does not result from the loss of their ability to bind to MDCK cells. Additionally, both wild-type Etx and the Y71A mutant did not bind to BHK-21 cells, indicating that the interaction between the toxins and MDCK cells is specific (Fig. 4C).

Discussion

Etx is the third-most potent clostridial toxin in existence after the botulinum and tetanus neurotoxins (Payne and Oyston, 1997). Identification of the amino acids required for Etx activity sheds light on the structure and function of Etx and provides additional information regarding the mechanism by which the toxin interacts with cells. The present study is the first report to identify Y71 in domain III as a critical amino acid residue for Etx activity and to determine the importance of the aromatic side chain. Previous studies indicated that certain mutants with the H149 substituted in domain III exhibited reduced but detectable cytotoxic activity, suggesting that H149 in domain III may participate in mediating the interaction between Etx and MDCK cells (Oyston *et al.*, 1998; Bokori-Brown *et al.*, 2013). However, H149 does not play as a decisive role in the cytotoxicity of Etx compared with Y71, as identified in this study.

Using site-directed mutagenesis, we adopted a two-step strategy to mutate the cluster of aromatic amino acids in domain III of Etx. First, we replaced four aromatic amino acids with alanine to determine whether their side chains contributed to the cytotoxic activity that Etx exerted towards MDCK cells. Among the constructed Etx mutants, only the Y71A mutant demonstrated undetectable cytotoxic activity. Although the other three mutants retained cytotoxic activities similar to that of the wild-type toxin, the F92A mutant exhibited weaker cytotoxic activity than the Y169A and Y254A mutants at a concentration of 0.01 μg/ml (Fig. 1D), suggesting that F92 participates in the interaction of Etx with MDCK cells to some extent but is not essential for cytotoxic activity. The second step of the site-directed mutagenesis focused on Y71. Amino acids with different properties were selected to replace Y71 on the basis of their side chain structures. Only the Etx mutants containing Y71 replaced with an aromatic amino acid (W or F) retained cytotoxic activity (Fig. 2), indicating that the aromatic ring of Y71 was essential for the cytotoxic activity of Etx. The Y71W mutant clearly exhibited greater cytotoxic activity than Y71F (Fig. 2B), and the polarity of the amino acid may have affected the activity of the toxin to a certain extent, as both tyrosine and tryptophan are polar, whereas phenylalanine is non-polar.

Although some Y71 mutants lost all detectable cytotoxic activity towards MDCK cells, these mutations did not affect the binding of Etx to MDCK cells (Fig. 4). The results suggest that the loss of activity occurs during the steps after receptor binding, for instance oligomerization or pore formation; this represents a different mechanism compared to the effect caused by the mutation of four tyrosines in domain I of Etx, as described in previous studies (Ivie and McClain, 2012; Bokori-Brown *et al.*, 2013). Additionally, a previous study showed that the Y221G mutation within a cluster of aromatic amino acids in domain IV of *Aeromonas hydrophila* aerolysin blocked the hemolytic activity of aerolysin by converting the hydrophobic heptamer formed by wild-type aerolysin into a fully hydrophilic heptamer, leading to loss of pore formation (Tsitrin *et al.*, 2002). Domain IV of *A. hydrophila* aerolysin shares structural similarities with domain III of Etx (Cole *et al.*, 2004); however, whether Y71 in domain III of Etx in this study shares the same mechanism with Y221 of aerolysin remains to be confirmed.

In conclusion, in the present study we determined for the first time that Y71 in domain III and the aromatic ring residue at position 71 are critical for the cytotoxicity of Etx. Furthermore, we determined that Y71 does not serve as the receptor binding site for MDCK cells. These findings have important implications for our further understanding of the structure and function of Etx.

Acknowledgements

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