BPB Reports

Report

Critical Residues of *Clostridium perfringens* Delta-Toxin for Oligomerization and Receptor Binding

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Received May 16, 2022; Accepted June 21, 2022

Delta-toxin produced by *Clostridium perfringens* types B and C is a β-pore-forming cytotoxin. Here, using site-directed mutagenesis, we identified the amino acid residues that contribute to delta-toxin oligomerization and binding. We replaced Lys-43 and Ser-109 located in the β-sandwich domain and Arg-200 located in the rim domain. Substitution of alanine for Ser-109 caused reductions in both cytotoxicity and oligomerization. However, exchange of Lys-43 for alanine resulted in a reduction in the cytotoxicity. Replacement of Arg-200 with alanine led to drastic reductions cytotoxicity and cell binding. Our results demonstrate that Ser-109 plays a role in oligomerization, and that Arg-200 is critical for binding of the toxin.

Key words *C. perfringens* delta-toxin, pore-forming toxin, key amino acid residue

INTRODUCTION

Delta-toxin is a cytotoxic toxin produced by *C. perfringens* type B and C strains.[1–4] Delta-toxin belongs to the β-pore-forming-toxin (β-PFT) family, which also includes *C. perfringens* beta-toxin, *C. perfringens* NetB toxin, *C. perfringens* NetF toxin, and *Staphylococcus aureus* alpha-toxin.[5–7] Delta-toxin selectively destroys sheep, goat, and pig erythrocytes.[1,8,9] In addition, it is toxic to various cell types including platelets, macrophages, and monocytes.[1,10,11] We previously reported that delta-toxin causes rapid ATP loss and necrosis through pore formation in the lipid raft microdomains of target cells.[12] Furthermore, delta-toxin induces impairments of mitochondrial membrane permeability and cytochrome *c* release.[12]

We previously reported that delta-toxin disrupted the barrier function of human polarized intestinal monolayers of Caco-2 cells.[13] The toxin induced the activation of a disintegrin and metalloprotease (ADAM) 10 [13], similarly to alpha-toxin of *S. aureus*.[14] Activation of ADAM10 causes degradation of E-cadherin, an epithelial adherens junction protein, resulting in impaired epithelial barrier integrity.[13] We subsequently showed that delta-toxin induces fluid accumulation and intestinal damage in mouse-ligated intestinal loops.[15] E-cadherin digestion by delta-toxin-induced ADAM10 upregulation was responsible for intestinal injury in a mouse model.[15]

The crystal structure of delta-toxin is similar to *S. aureus* alpha-toxin and *C. perfringens* NetB toxin,[6,16] comprising three domains: β-sandwich, rim and stem. The heptameric pore of delta-toxin retains a similar structure to those of NetB toxin and *S. aureus* alpha-toxin.[16] It is widely presumed that delta-toxin shares a common cytotoxic mechanism with alpha-toxin and NetB toxin.[5,6,16] In the present study, to clarify the key residues for delta-toxin function, we conducted a site-directed mutagenesis study of delta-toxin on some conserved residues that are important for the biological activities of NetB toxin and alpha-toxin.[5] We sought to determine the critical residues for binding to the cell membrane and oligomer formation by constructing site-directed mutants.

MATERIALS AND METHODS

Materials A delta-toxin gene was provided by Dr. M. Popoff (Institute Pasteur, Paris, France).[5] Efficient expression and purification of recombinant delta-toxin was carried out as described previously.[12] Rabbit polyclonal antibody against anti-delta-toxin was obtained as previously reported.[12] Rabbit anti-β-actin antibody was purchased from Cell Signaling Technology (Tokyo, Japan). Horseradish peroxidase-labeled anti-rabbit IgG and enhanced chemiluminescence (ECL) kits were purchased from Merck (Tokyo, Japan).

Cell Culture and Cytotoxicity Assay MDCK, A431 and Caco-2 cells were purchased from RIKEN (Tsukuba, Japan) and cultured in DMEM supplemented with 10% fetal calf serum (FCS). The media included streptomycin (100 μg/mL), penicillin (100 U/mL) and glutamine (2 mM) (FCS-DMEM). Cells were cultured at 37°C in a 5% CO_2 atmosphere. To investigate the cytotoxic activity of delta-toxin, cells were inoculated into 48-well tissue culture plates and treated with varying amounts of wild-type or variant toxins for 8 h. Cytotoxicity was evaluated with lactate dehydrogenase (LDH) release assay. LDH activity in cell culture supernatants was determined using LDH-Cytotoxic Test (Wako Chem., Osaka, Japan) according to the protocol of the manufacture. A positive control for cell lysate was prepared using 0.2% Triton X-100 (= 100% cell death).

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Table 1. Primer Sequences Used for the Generation of Delta-Toxin Mutants

Primer	Primer sequence (5'-3')
K43A-For	CTCATAACAGTAGATGCTAG
K43A-Rev	CGAATTACTTTTTGTAGTAGCTG
S109A-For	GCAGTTCCTAAGAATACTATTTCAA
S109A-Rev	ATCAACTATTTTTACAGAACTATCC
R200A-For	GCCTATTCAGGTACAAGTACTAC
R200A-Rev	ACTTCTCATAAACATTTGATTGCC

Construction and Purification of Delta-Toxin Variants The following delta-toxin variants were created by site-directed mutagenesis: K43A, S109A, and R200A. Variants were obtained using QuickChange II with a site-directed mutagenesis kit (Agilent Technologies, Tokyo, Japan). Primers utilized for generation of site-directed mutagenesis were listed in Table 1. The mutations were confirmed by sequence analysis. Delta-toxin variants were expressed and purified in a similar manner to wild-type delta-toxin.[12] The purified proteins were observed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting Analysis Immunoblotting was carried out as described previously.[12] Protein concentrations in cell lysate samples were determined using Takara BCA protein assay kit (Tokyo, Japan). Cell lysate samples containing 20 μg of total protein were incubated at 37°C for 10 min, subjected to SDS-PAGE, and transferred to PVDF membrane. Immunoblotting was performed with antibodies against delta-toxin and β-actin, as described previously.[12]

RESULT

Mutations of Delta-Toxin To elucidate the mechanism for the binding and oligomer formation of delta-toxin, three amino acid residues of delta-toxin were selected for alanine substitution: Lys-43 and Ser-109 which lie within the β-sandwich domain, and Arg-200 located at the rim domain. The results of other mutagenic studies of three structurally-conserved residues (Lys-41, Ser-106, and Arg-200) of NetB toxin provided insights into their individual functions.[13,16,17] Mutants of delta-toxin were created as described in Materials and Methods by site-directed mutagenesis and confirmed by DNA sequencing. As shown in supplementary Fig. S1, the three purified variant toxins replaced with alanine each appeared as a single band on SDS-PAGE, and the molecular masses of the variant toxins were identical to that of the wild-type toxin (approximately 32 kDa).

Cytotoxic Activities of Wild-Type and Variant Toxins To evaluate the cytotoxic activities of wild-type and variant toxins, the LDH release assay was performed with MDCK, Caco-2 and A431 cells (Fig. 1). We previously reported that wild-type delta-toxin caused cytotoxicity toward these cells.[12] These cells exhibit different susceptibilities to the actions of delta-toxin. By examining the cytotoxic activity of variant toxin using various cell lines, the role of amino acid residues in the toxin can be elucidated more accurately. In the present study, we evaluated the action of variant toxins in three cell lines. The mutant K43A showed approximately 5-20 fold less than the activities of wild-type toxin against MDCK, Caco-2 and A431 cells, respectively. The cytotoxic activities of S109A against MDCK, Caco-2 and A431 cells were 150-300-fold lower than that of wild-type toxin. R200A showed a loss of cytotoxic activity. These observations indicate that Lys-43, Ser-109 and Arg-200 in delta-toxin are important for the cytotoxic activity of the toxin, to varying degrees.

Binding of Variant Toxins to Cells Wild-type delta-toxin forms an oligomer in the membrane of sensitive cells.[1–3] We examined whether the changes of amino acid residues

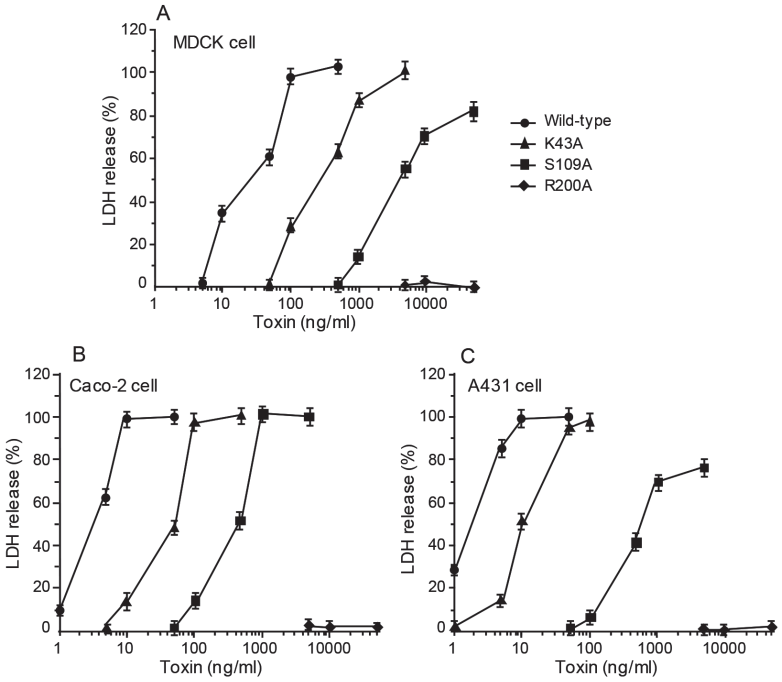

Fig. 1. Cytotoxic Activities of Wild-Type and Variants

Cells were incubated with wild-type delta-toxin and variant toxins at 37°C for 8 h, Cytotoxic activity was determined via LDH release assay. The mean ± standard deviation (SD) (n = 4) were indicated.

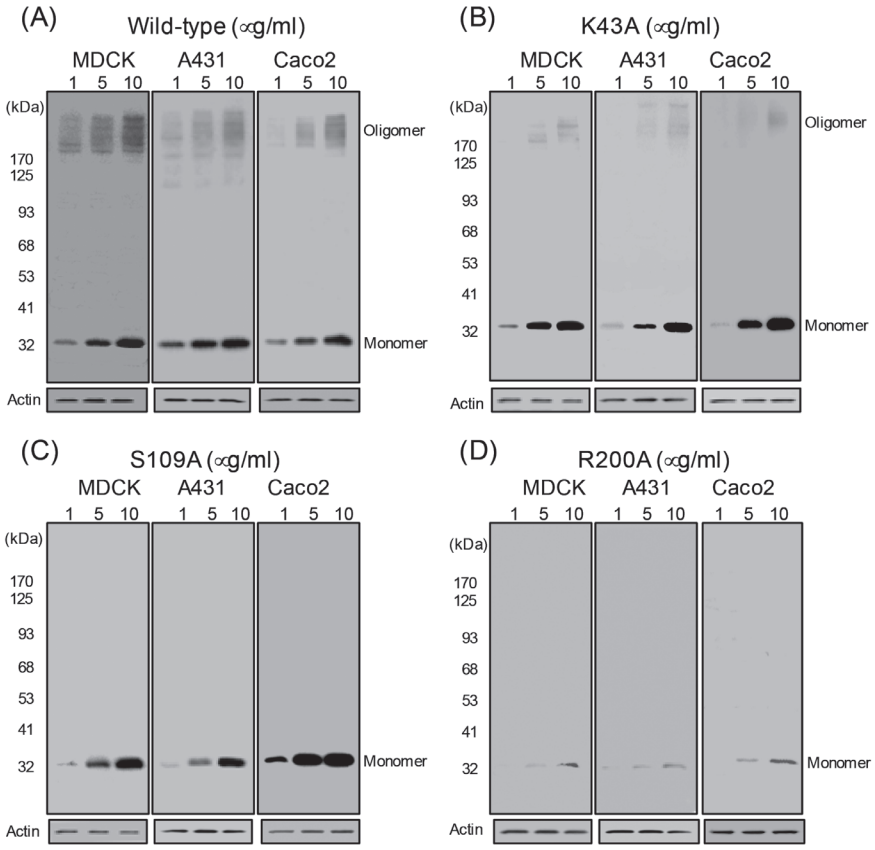

Fig. 2. Binding of Wild-Type Delta-Toxin and Variant Toxins to MDCK, A431 and Caco-2 Cells

Cells were incubated with wild-type delta-toxin and variant toxins at 37°C for 15 min, washed, and assessed by western blotting analysis of delta-toxin and β-actin (control). A typical example from three experiments is shown.

had any effect on the binding and oligomerization of the toxin to cells. Cells were treated with wild-type or variant toxins for 30 min at 37°C. Next, cells were dissolved and analyzed by immunoblotting utilizing antibody to delta-toxin. As shown in Fig. 2, the analysis results for wild-type delta-toxin revealed the presence of monomer and oligomers of the toxin in all three cell lines. Similar profiles were observed with K43A, but oligomerization was reduced compared with wild-type toxin (Fig. S2). S109A was able to bind to all cell lines, but failed to form oligomers. R200A did not bind well to any of the three cell lines.

DISCUSSION

C. perfringens delta-toxin is a pore-forming cytolytic toxin that injures the host cell membrane.[1,6,12,16] Delta-toxin is a member of the β-pore forming toxin family that also includes *S. aureus* alpha-toxin, *C. perfringens* NetB and beta-toxin.[1,5,6] Many amino acid residues have been identified as being functionally critical for this family. In this study, we defined key amino acid residues for binding and oligomer formation within the β-sandwich and rim domains of delta-toxin.

We replaced Lys-43 and Ser-109 in delta-toxin; these are present within the β-sandwich domain involved in oligomer formation of NetB toxin[17,18] and alpha-toxin.[19,20] Replacement of Lys-43 with alanine caused a reduction in cytotoxic activity, although the activity was still detectable. Binding of K43A monomer to the three cell lines did not change, but formation of K43A oligomer was reduced compared with wild-type delta-toxin. Thus, Lys-43 plays a partial role in the oligomerization of delta-toxin. The cytotoxic activity of S109A variant was lower than that of K43A. Binding of S109A monomer to cells did not change, but oligomer of S109A was not detected. On the basis of these findings, the β-sandwich domain of delta-toxin is important for its oligomerization.

The rim domain of β-pore forming toxins is predominantly composed of aromatic and hydrophobic residues and is necessary for toxin binding to the cell membrane.[6,16,17] The rim domain contains an arginine residue that is highly conserved among β-pore forming toxins,[7,19] corresponding to Arg-200 in delta-toxin. Replacement of the arginine residue in β-pore forming toxins impairs the binding of the toxin to the cell surface.[17,19,20] In the present study, substitution of Arg-200 with alanine in delta-toxin led to a complete loss of cytotoxic activity and toxin binding. Arg-200 within the rim domain in the toxin is important for the recognition of cell surface receptors. From these results of the amino acid substitution of the toxin, it was found for the first time that the three domains of delta-toxin have the similar function as other PFTs.

Conflict of interest The authors declare no conflict of interest.

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