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Isolated CyaA-RTX subdomain from *Bordetella pertussis*: Structural and functional implications for its interaction with target erythrocyte membranes

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ABSTRACT

The 126-kDa *Bordetella pertussis* CyaA-hemolysin (CyaA-Hly) was previously expressed in *Escherichia coli* as a soluble precursor that can be acylated to retain hemolytic activity. Here, we investigated structural and functional characteristics of a ~100-kDa isolated RTX (Repeat-in-ToXin) subdomain (CyaA-RTX) of CyaA-Hly. Initially, we succeeded in producing a large amount with high purity of the His-tagged CyaA-RTX fragment and in establishing the interaction of acylated CyaA-Hly with sheep red blood cell (sRBC) membranes by immuno-localization. Following pre-incubation of sRBCs with non-acylated CyaA-Hly or with the CyaA-RTX fragment that itself produces no hemolytic activity, there was a dramatic decrease in CyaA-Hly-induced hemolysis. When CyaA-RTX was pre-incubated with anti-CyaA-RTX antisera, the capability of CyaA-RTX to neutralize the hemolytic activity of CyaA-Hly was greatly decreased. A homology-based model of the 100-kDa CyaA-RTX subdomain revealed a loop structure in Linker II sharing sequence similarity to human WW domains. Sequence alignment of Linker II with the human WW-domain family revealed highly conserved aromatic residues important for protein–protein interactions. Altogether, our present study demonstrates that the recombinant CyaA-RTX subdomain retains its functionality with respect to binding to target erythrocyte membranes and the WW-homologous region in Linker II conceivably serves as a functional segment required for receptor-binding activity.

1. Introduction

Adenylate cyclase toxin (CyaA) is one of the major virulence factors of *Bordetella pertussis*, a Gram-negative pathogen that causes whooping cough in humans [1]. The toxin is synthesized as a single polypeptide of 1706 residues and consists of an N-terminal adenylate cyclase (AC) domain of 400 residues and a pore-forming/hemolysin (Hly) domain of 1306 residues [2]. The CyaA-Hly domain contains of a hydrophobic pore-forming segment (residues 500–800) [3,4] and an acylation region (residues 800–1000) [5]. There is also an RTX (Repeat-in-ToXin) segment, harboring ~40 repeats of Gly-Asp-rich nonapeptides that serve as Ca^{2+}-binding sites (residues 1006–1600) [6], along with an unprocessed C-terminal secretion signal (residue 1600–1706) [7]. The CyaA toxin is stabilized by extracellular Ca^{2+} ions which act as a structural stabilizing bridge in a β-roll motif within the RTX region [8,9]. For biological activity, this toxin requires a palmitoyl group be added at Lys^{983} by CyaC acyltransferase [10].

The RTX subdomain of CyaA is reported to harbor a target receptor-binding region that is required for cell targeting and for AC-domain translocation across the host cell membrane [11]. The AC-catalytic domain would generate cAMP in an uncontrolled manner, which in turn would disrupt transcription of many inflammatory-associated genes in apoptotic pathways leading to the target cell death [12]. CyaA preferentially binds to target cells

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through the $\alpha_M\beta_2$-integrin (CD11b/CD18) receptor expressed on the surface of cells in the myeloid lineage, *e.g.* macrophages [13]. It is noteworthy that the CyaA toxin also exerts its hemolytic activity against sheep erythrocytes, even though they lack the $\alpha_M\beta_2$-intergrin receptor, suggesting an alternative mechanism of target cell binding [14]. Moreover, the 126-kDa truncated CyaA-Hly fragment retains hemolytic activity independent of the N-terminal AC domain [15].

The requirement for Ca^{2+} binding to the RTX subdomain for its structural stabilization has been clearly established [8,9]. Furthermore, other studies have provided some insights into a plausible receptor-binding segment in the C-terminal domain of CyaA [16]. However, the identity of CyaA-receptor binding residues crucial for target cell recognition remains ambiguous. In the present study, structural and functional characteristics of a ~100-kDa RTX subdomain (CyaA-RTX) were investigated and the results suggested that the CyaA-RTX subdomain can be highly produced as an isolated soluble form in *Escherichia coli* and that the purified product retains its intrinsic capability to functionally interact with erythrocyte membranes. Moreover, a 3D-modeled structure of the CyaA-RTX subdomain revealed conserved aromatic residues in the Linker II loop homologous to the human WW domain family that is important for protein–protein interactions, suggestive of its significance for CyaA-RTX functional activity.

2. Material and methods

2.1. Construction of recombinant plasmid with His-tagged fusion

Incorporation of a 6 × His tag in the previously constructed pCyaA-RTX plasmid [9] was performed by using the pCyaAC-PF/H_6 plasmid [17] as a template. pCyaA-RTX were digested with *Nde*I and *Sac*I, yielding the CyaA-RTX-coding fragment (1211 bp) which was gel purified and ligated with pCyaAC-PF/H_6 (6358 bp) pre-cut with *Nde*I and *Sac*I. CyaC-coding gene from pCyaAC-PF/H_6 was deactivated by using *Not*1 which deleted a 103-bp 3′-end segment (encoding 34 amino acids) of the *cyaC* gene. The resulting pCyaA-RTX/H_6 plasmid with a 6 × His-tag was transformed into *E. coli* strain JM109 and verified by restriction digestion. The plasmid was re-transformed into the expression host, a protease-deficient *E. coli* strain BL21(DE3)pLysS and the target gene segment was verified by DNA sequencing.

2.2. Expression of His-tagged proteins

All His-tagged recombinant toxins were expressed in *E. coli* strain BL21(DE3)pLysS cells at 30 °C in Luria-Bertani medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at final concentration of 0.1 mM, and *E. coli* cells were harvested by centrifugation, re-suspended in 20 mM HEPES buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mM protease inhibitors (phenylmethylsulfonylfluoride, PMSF, and 1,10-phenanthroline, PNT). After addition of lysozyme (0.1 mg/mL final concentration), cell suspension was incubated on ice overnight and subsequently disrupted by sonication. The cell lysate was centrifuged at 10,000 × *g*, 4 °C for 45 min. Concentrations of soluble proteins in the supernatant were determined by Bradford-based protein microassay.

2.3. Purification of His-tagged proteins

6 × His-tagged toxins were purified *via* immobilized metal affinity chromatography (IMAC). 15-mL lysate supernatant (5 mg/mL) was loaded onto an affinity-based Ni^{2+}-NTA column (5 mL-HisTrap HP, GE Healthcare Bio-sciences) and run at a flow rate of 1 mL/min. The washing step was performed *via* elution with 75 mM imidazole (IMZ) in 50 mM HEPES buffer (pH 7.4) containing 2 mM $CaCl_2$. The target His-tagged protein was then eluted with 250 mM IMZ. Elution fractions containing the His-tagged toxin were pooled, analyzed for homogeneity by SDS–PAGE and desalted through a desalting column prior to additional analysis. Concentrations of the purified His-tagged protein were determined using the Bradford-based microassay.

2.4. Western blot analysis

Toxin samples separated on SDS-PAGE were transferred to a nitrocellulose membrane blocked with 5% skim milk-PBS (120 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4) and probed with rabbit anti-RTX polyclonal antisera (1:40,000 dilution) which was raised against the 100-kDa purified CyaA-RTX fragment as described previously [10]. Toxin-antibody complexes were detected with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG antibodies (Pierce; 1:7000 dilution) and visualized by incubation with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium). The presence of 6 × His tag was verified by probing with AP-conjugated anti-His C-term antibodies (Invitrogen; 1:2000 dilution) and BCIP/NBT detection.

2.5. Immuno-localization assay

Immuno-localization was performed to determine the binding of CyaA-Hly to erythrocyte membranes. A 30-μl suspension of sRBCs (5 × 10^8 cells/mL) were diluted in 80 μL of PBS buffer (pH 7.4) containing 50 mM glycine, 5 mM glucose and 75 mM sucrose, followed by addition of CyaA-Hly toxin (~10 μg). The treated cells were pelleted, washed (3 times), re-suspended in 200 μL of PBS-Gly/sugar buffer (pH 7.4) and incubated with anti-RTX polyclonal antisera (1:200 dilution) at 37 °C for 30 min. Cell complexes were collected by centrifugation, washed 3 times and followed by another 30-min incubation with FITC-conjugated anti-rabbit IgG antibodies (Sigma–Aldrich; 1:50 dilution). After washing, the stained cells were collected by centrifugation, deposited on the glass slide, mounted with a cover slip, and then examined on a confocal laser scanning microscope (Olympus FV1000) under an oil-immersion lens (60×).

2.6. Hemolytic activity assay

In vitro hemolytic activity of the protein toxin against sRBCs was assayed in 1.5-mL microcentrifuge tube as previously described [15], with some modifications. 200 μL of purified toxins (~10 μg) were gently mixed with sRBCs (5 × 10^8 cells) in 800 μL of assay buffer (120 mM Tris–HCl, pH 7.4, 50 mM $NaCl_2$ and 2 mM $CaCl_2$) and incubated at 37 °C for 6 h. At the end of the incubation, the mixture was centrifuged at 12,000 × *g* for 2 min to remove non-lysed sRBCs and cell debris, and the supernatant was transferred to a flat-bottom 96-well microtiter plate for measuring the released hemoglobin by spectrophotometer at OD_{540}. The same amount of total proteins in soluble cell lysate containing pET-17b was used as a negative control while an equal amount of erythrocytes lysed with 0.1% Triton-X 100 was defined as 100% hemolysis. Percent hemolysis for each toxin sample was calculated by {[OD_{540} sample − OD_{540} negative control]/[OD_{540} of 100% hemolysis − OD_{540} negative control]} × 100. All samples were tested in triplicate for three independent experiments.