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Structural requirement of the hydrophobic region of the *Bordetella pertussis* CyaA-hemolysin for functional association with CyaC-acyltransferase in toxin acylation

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ABSTRACT

Previously, we demonstrated that the ~130-kDa CyaA-hemolysin (CyaA-Hly, Met^{482}-Arg^{1706}) from *Bordetella pertussis* was palmitoylated at Lys^{983} when co-expressed with CyaC-acyltransferase in *Escherichia coli*, and thus activated its hemolytic activity. Here, further investigation on a possible requirement of the N-terminal hydrophobic region (HP, Met^{482}-Leu^{750}) for toxin acylation was performed. The ~100-kDa RTX (Repeat-in-ToXin) fragment (CyaA-RTX, Ala^{751}-Arg^{1706}) containing the Lys^{983}-acylation region (AR, Ala^{751}-Gln^{1000}), but lacking HP, was co-produced with CyaC in *E. coli*. Hemolysis assay indicated that CyaA-RTX showed no hemolytic activity. Additionally, MALDI-TOF/MS and LC-MS/MS analyses confirmed that CyaA-RTX was non-acylated, although the co-expressed CyaC-acyltransferase was able to hydrolyze its chromogenic substrate–*p*-nitrophenyl palmitate and acylate CyaA-Hly to become hemolytically active. Unlike CyaA-RTX, the ~70-kDa His-tagged CyaA-HP/BI fragment which is hemolytically inactive and contains both HP and AR was constantly co-eluted with CyaC during IMAC-purification as the presence of CyaC was verified by Western blotting. Such potential interactions between the two proteins were also revealed by semi-native PAGE. Moreover, structural analysis *via* electrostatic potential calculations and molecular docking suggested that CyaA-HP comprising α1-α5 (Leu^{500}-Val^{698}) can interact with CyaC through several hydrogen and ionic bonds formed between their opposite electrostatic surfaces. Overall, our results demonstrated that the HP region of CyaA-Hly is conceivably required for not only membrane-pore formation but also functional association with CyaC-acyltransferase, and hence effective palmitoylation at Lys^{983}.

1. Introduction

Adenylate cyclase-hemolysin toxin (CyaA) is a major virulence factor secreted from *Bordetella pertussis*, a Gram-negative pathogen that causes whooping cough (also known as 'pertussis') in humans, a serious respiratory infectious disease [1]. CyaA, which belongs to the class of RTX (Repeat-in-ToXin) cytolysins, is able to facilitate respiratory tract colonization of *B. pertussis* by impairing defense function of host macrophages [2]. Whooping cough has now re-

Abbreviations: AR, acylation region; CyaA, adenylate cyclase-hemolysin toxin; IMAC, immobilized-metal ion affinity chromatography; HP, hydrophobic region; pNPP, *p*-nitrophenyl palmitate; RTX, Repeat-in-ToXin.

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emerged worldwide as a consequence of declining immunity, following vaccination and imperfect vaccination of populations [3], hence requiring novel approaches for treatment. Recently, we have successfully generated CyaA-specific humanized VH/V_HH nanobodies that would have potential applications in developing a novel anti-pertussis agent [4].

Unlike other RTX cytolysins, the 1706-residue CyaA toxin (~180-kDa) consists of two functionally different domains: the ~40-kDa N-terminal adenylate cyclase (AC) and the ~130-kDa C-terminal pore-forming or hemolysin (Hly) domains [5] (*see* Supplementary Fig. 1). While translocation of the AC domain into the target cell interior is known to induce an increase in pathological levels of cAMP, resulting in apoptosis [6], multiple sources of evidence revealed that the Hly domain is responsible for hemolysis against sheep erythrocytes and ion-channel formation in planar lipid bilayers [7,8]. Of particular interest in the Hly domain (residues ~400–1700), there are four important regions, including (*i*) a pore-forming hydrophobic region (HP, residues ~400–750) containing transmembrane helices of which α2 and α3 are likely to participate in membrane-pore formation [9–12], (*ii*) an acylation region (AR, residues ~750–1000) with the Lys^{983}-acylation site [13,14], (*iii*) a receptor-binding RTX region (residues ~1000–1700) containing Gly-Asp nonapeptide repeats that serve as Ca^{2+}-binding sites [15,16] and (*iv*) a secretion signal [17]. The binding of Ca^{2+} ions has been independently demonstrated to play a key role in providing either structural stability against proteolytic degradation [18] or proper folding into a β-roll structure of the CyaA-RTX region [19].

CyaA is initially produced as an inactive pre-protoxin of ~180 kDa and after removal of the signal sequence, it becomes activated by a post-translational palmitoylation of Lys^{983} mediated by the endogenous CyaC-acyltransferase [13]. According to functional requirements of lipid acylation at Lys^{983} for both cytotoxic and hemolytic activities [7,20], the Lys^{983}-linked palmitoyl moiety was proposed to enhance membrane association of the full-length CyaA toxin *via* receptor binding [21]. However, our recent studies showed that such toxin modification *via* Lys^{983}-palmitoylation was not required for binding of the 130-kDa CyaA-Hly domain to target erythrocyte membranes, but it appeared to be involved in stabilizing toxin-induced ion-leakage pores [22].

Previously, we demonstrated that a ~100-kDa truncated fragment of CyaA-Hly (*i.e.*, CyaA-RTX, Ala^{751}-Arg^{1706}), recombinantly produced without an acylation-mediating CyaC, could still bind to sheep erythrocyte membranes, although it showed no hemolytic activity [23]. However, there was no direct evidence to support whether the lack of HP region (Met^{482}-Leu^{750}) at the N-terminus of the CyaA-RTX truncate would indeed lead to the disappearance of toxin acylation, hence limiting its hemolytic activity. In this study, this truncated CyaA-RTX fragment with the Lys^{983}-acylation region (Ala^{751}-Gln^{1000}) was therefore co-produced with CyaC-acyltransferase in order to determine if the HP region would contribute to toxin acylation. Our results suggested a potential requirement of such HP region for specific binding of CyaC prior to its effective acylation. Additionally, an apparent association during protein purification was observed between CyaC and a smaller CyaA-Hly truncate, *i.e.*, 70-kDa CyaA-HP/BI which is hemolytically inactive and consists of HP, AR and the first block of the RTX region (*see* Supplementary Fig. 1). Moreover, structural analysis *via* molecular docking revealed several hydrogen bonding and ionic interactions formed between CyaC-acyltransferase and the ~20-kDa CyaA-HP segment comprising α1-α5 (Leu^{500}-Val^{698}), as supporting evidence of their potential association for toxin acylation.

2. Materials & methods

2.1. Construction of recombinant plasmid

pCyaAC-PF plasmid encoding both ~130-kDa CyaA-Hly and ~22-kDa CyaC-acyltransferase under control of the T_7 promoter [7] was used as a template for gene manipulation. Construction of pCyaAC-RTX plasmid co-expressing CyaA-RTX (Ala^{751}-Arg^{1706}) and CyaC was accomplished by deletion of the gene segment encoding the ~30-kDa N-terminal HP region (*i.e.*, Met^{482}-Leu^{750}) from the original pCyaAC-PF to which an additional *Nde*I site was introduced by site-directed mutagenesis. The 6747-bp *Nde*I-digested DNA fragment corresponding to pCyaAC-RTX was gel-purified and re-ligated. The resulting plasmid was transformed into *E. coli* strain BL21(DE3)pLysS and verified by DNA sequencing.

2.2. Expression and preparation of CyaA-RTX

E. coli cells strain BL21(DE3)pLysS harboring the pCyaAC-RTX plasmid were grown at 30 °C in Luria-Bertani medium containing 100 μg/mL ampicillin until OD_{600} of the culture reached 0.5–0.6. After addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, incubation was continued under the same condition for another 6 h. The IPTG-induced *E. coli* samples were analyzed for protein expression level by sodium dodecyl sulfate-(12% w/v) polyacrylamide gel electrophoresis (SDS-PAGE).

After centrifugation, *E. coli* cells expressing CyaA-RTX together with CyaC were washed twice with hemolysis buffer (5 mM $CaCl_2$, 125 mM NaCl, 20 mM HEPES, pH 7.4). The washed cell pellets were re-suspended in the buffer containing 1 mM phenylmethylsulfonyl fluoride and subsequently disrupted using an ultrasonic processor. The resulting cell lysate was centrifuged (8000×*g*, 4 °C, 15 min) and the supernatant, referred as crude lysate, was then carefully transferred to a new tube. Concentrations of total proteins (including CyaA-RTX) in the crude lysate were determined by Bradford-based microassay.

2.3. Expression and purification of CyaA-HP/BI

The ~70-kDa His-tagged CyaA-HP/BI protein was expressed and subsequently purified as described previously [24]. Briefly, after 6-h IPTG-induction, cell pellets were harvested and incubated with lysozyme at 4 °C overnight followed by ultrasonication. Inclusion bodies were separated by centrifugation (8000×*g*, 4 °C, 15 min) and subsequently denatured with 4 M urea. His-tagged soluble protein samples were purified by immobilized-metal ion affinity chromatography (IMAC) using a Ni^{2+}-NTA (nickel-nitrilotriacetic acid) column and subsequently desalted through a PD10 column.

2.4. Hemolytic activity assay

In vitro hemolytic activity of the tested samples against sheep red blood cells (sRBC) was assessed as described previously [7], with some modifications. Soluble *E. coli* lysate expressing CyaA proteins (CyaA-RTX or CyaA-Hly) was diluted to desired concentrations and the volume was adjusted to 980 μL with hemolysis buffer before addition of 20-μL sRBC (~10^8 cells). The sample-sRBC mixtures were incubated at 37 °C for 5 h. Unlysed erythrocytes were removed by centrifugation (12,000 × *g*, 2 min) and released hemoglobin in the supernatant was measured at OD_{540}. Crude lysate containing pET17b was used as a negative control. OD_{540} value corresponding to complete hemolysis was obtained by lysing

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