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Acylation of the *Bordetella pertussis* CyaA-hemolysin: Functional implications for efficient membrane insertion and pore formation

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ABSTRACT

Previously, the ~130-kDa CyaA-hemolysin domain (CyaA-Hly) from *Bordetella pertussis* co-expressed with CyaC-acyltransferase in *Escherichia coli* was demonstrated to be palmitoylated at Lys^{983} and thus activated its hemolytic activity against target erythrocytes. Here, we report the functional importance of Lys^{983}-palmitoylation for membrane insertion and pore formation of CyaA-Hly. Intrinsic fluorescence emissions of both non-acylated CyaA-Hly (NA/CyaA-Hly) and CyaA-Hly were indistinguishable, suggesting no severe conformational change upon acylation at Lys^{983}. Following pre-incubation of sheep erythrocytes with NA/CyaA-Hly, there was a drastic decrease in CyaA-Hly-induced hemolysis. Direct interactions between NA/CyaA-Hly and target erythrocyte membranes were validated *via* membrane-binding assays along with Western blotting, suggestive of acylation-independent capability of NA/CyaA-Hly to interact with erythrocyte membranes. As compared with CyaA-Hly, NA/CyaA-Hly displayed a slower rate of incorporation into DOPC:DOPE:Ch or DiPhyPC bilayers under symmetrical conditions (1 M KCl, 10 mM HEPES, pH 7.4) and formed channels exhibiting different conductance. Further analysis revealed that channel-open lifetime in DOPC:DOPE:Ch bilayers of NA/CyaA-Hly was much shorter than that of the acylated form, albeit slightly shorter lifetime found in DiPhyPC bilayers. Sequence alignments of the Lys^{983}-containing CyaA-segment with those of related RTX-cytolysins revealed a number of highly conserved hydrophobic residues and a Lys/Arg cluster that is predicted be important for toxin-membrane interactions. Altogether, our data disclosed that the Lys^{983}-linked palmitoyl group is not directly involved in either binding to target erythrocyte membranes or toxin-induced channel conductivity, but rather required for efficient membrane insertion and pore formation of the acylated CyaA-Hly domain.

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1. Introduction

Adenylate cyclase-hemolysin toxin (CyaA) is a key virulence factor of *Bordetella pertussis* which is a causative agent of human whooping cough (pertussis)-an acute respiratory infection that is more serious among infants [1]. CyaA is an RTX (Repeat-in-ToXin) cytolysin that facilitates respiratory tract colonization of this human pathogen by impairing host defense function of alveolar macrophages [2]. Unlike other RTX cytolysins, CyaA is typically produced as a secreted bi-functional protein (1706 residues, ~180 kDa) comprising adenylate cyclase (AC, residues 1–400) and pore-forming/hemolysin (Hly, residues 401–1706) domains. The Hly domain consists of four fundamental regions (*see* Fig. 1A), including a hydrophobic pore-forming segment (HP, residues 500–700) [3,4], an acylation region (residues 800–1000) [3], an RTX segment (residues 1000–1600) which harbors ~40 repeats of Gly-Asp-rich nonapeptides (Gly-Gly-X-Gly-X-Asp-X-U-X, X for any amino acids and U for hydrophobic residues) that serve as Ca^{2+}-binding sites [4–6] and an unprocessed secretion signal (residues 1600–1706) [6,7]. In addition, the RTX subdomain segment is organized into five structurally similar blocks (Blocks I-V) joined by linker sequences (Linkers 1–4) of variable lengths (20–50 residues) [6,8]. The requirement of Ca^{2+} binding to individual blocks for their structural stabilization [9] and proper folding into β-rolls for accelerating toxin secretion [10] has been clearly demonstrated. Very recently, we have shown for the first time that CyaA-RTX/Linker 1 could serve as a potential neutralizing epitope of CyaA-protective antigen that possibly will be useful for development of peptide-based pertussis vaccines [11].

Human immune cells expressing the $\alpha_M\beta_2$-integrin receptor (known as CD11b/CD18), *e.g.* macrophages and neutrophils, are primary target cells of the CyaA toxin [12]. Upon binding to the receptor, CyaA

Abbreviations: CyaA-Hly, CyaA-hemolysin; Ch, cholesterol; DiPhyPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine; NA/CyaA-Hly, non-acylated CyaA-Hly; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; PLBs, planar lipid bilayers; RTX, Repeat-in-ToXin.

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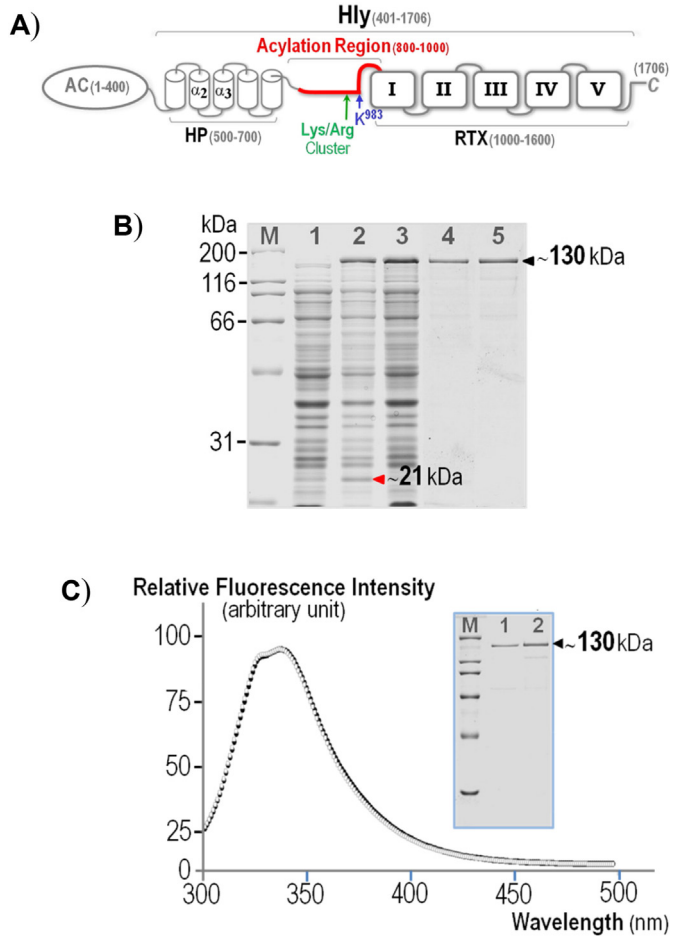

Fig. 1. (**A**) Schematic diagram of the CyaA toxin, illustrating two functional domains of which the Hly domain contains a hydrophobic region (HP) with five predicted α-helices (cylinders) including transmembrane helices 2 and 3, an acylation region with Lys^{983}-acylation site as well as a Lys/Arg cluster, and an RTX region with five numerical boxes representing individual Ca^{2+}-binding β-roll blocks. (**B**) SDS-PAGE (Coomassie brilliant blue-stained, 10% gel) analysis of lysates extracted from *E. coli* (~10^7 cells) expressing the ~130-kDa CyaA-Hly domain together with the 21-kDa CyaC-acyltransferase (lane 2) or the non-acylated protein (NA/CyaA-Hly, lane 3). Lanes 4 and 5 are IMAC-purified CyaA-Hly and NA/CyaA-Hly, respectively. Lane 1, cell lysates containing the pET17b vector. M, molecular mass standards. (**C**) Fluorescence emission spectra of CyaA-Hly and NA/CyaA-Hly on excitation of 280 nm. The spectra are representative of two independent measurements. *Inset*, SDS-PAGE (Coomassie brilliant blue-stained, 10% gel) analysis of the purified proteins used in the measurement: lane 1, CyaA-Hly; lane 2, NA/CyaA-Hly.

would translocate its AC domain into the target's cytosol where this catalytic domain massively produces cAMP, a key signaling molecule that triggers cell apoptosis [2]. The full-length CyaA toxin, as well as its ~130-kDa isolated Hly domain, was also found to be hemolytically active against sheep erythrocytes which lack the CD11b/CD18 receptor [4,13], thus suggesting the possibility of an alternative mechanism for target cell recognition. Although earlier studies have provided some insight into the pore-forming process of the CyaA toxin [14], the actual mechanism underlying its lytic activity remains to be investigated in details. In our recent studies, we have shown that the Gly^{530}_Gly^{533}_Gly^{537} cluster in α_2 within the HP region is important for toxin-induced hemolysis, conceivably involved in helix-helix association of the lytic pore-forming helices [15]. We have also shown that two key amino acid side-chains in α_3, *i.e.* Glu^{570} and Glu^{581}, could play an important role in hemolytic activity of the CyaA-Hly domain, plausibly lining the pore lumen to regulate the toxin-induced pore functions [16]. We have recently demonstrated that CyaA-Hly could cooperatively form a functional trimeric pore in the target cell membrane [17]. However, the precise structural details and determinants of membrane-pore formation by CyaA-Hly still need further investigation.

For both cytotoxic and hemolytic activities, the ~180-kDa full-length CyaA inactive precursor requires post-translational modification *via* lipid acylation at Lys^{983} by the CyaC acyltransferase [3,18]. An acyl group predominantly found to be attached to CyaA is a $C_{16:0}$-hydrocarbon chain (*i.e.* palmitoyl) [18,19]. The added palmitoyl group was suggested to enhance membrane affinity of the CyaA toxin required for efficient attachment to target cell membranes by acting either as a mediator of membrane association or a determinant of specific receptor-toxin interactions [20]. However, the exact role in toxin function of such conjugated palmitoyl at Lys^{983} is yet unclear. In our previous studies, the ~130-kDa CyaA-Hly domain co-expressed with CyaC-acyltransferase in *E. coli* was found to be palmitoylated at Lys^{983} and thus retained its high hemolytic activity [3]. In the present report, we provide pivotal evidence of functional importance of such post-translational modification with respect to membrane-inserting and pore/channel-forming activities of CyaA-Hly.

2. Materials and methods

2.1. Construction of the recombinant plasmid with His-tagged fusion

The pCyaAC-PF/H_6 plasmid encoding both ~130-kDa 6 × His-tagged CyaA-Hly and 21-kDa CyaC-acyltransferase under control of the *T7* promoter [17] was used for production of an acylated form of CyaA-Hly which is linked with a 6 × His tag at its C-terminal end. Construction of pCyaA-PF/H_6 plasmid that encodes only 6 × His-tagged non-acylated CyaA-Hly (NA/CyaA-Hly) was accomplished by deletion of the *cyaC* gene encoding acylation-mediating enzyme (CyaC-acyltransferase) from the original pCyaAC-PF/H_6 plasmid *via* double digestion at *Hin*dIII and *Bam*HI cloning sites. The 6947-bp digested DNA fragment without the *cyaC* gene was gel-purified and both cohesive ends were filled-in by Klenow DNA polymerase prior to blunt-end ligation. The resulting plasmid was initially transformed into *E. coli* strain JM109 to verify the absence of the *cyaC* gene *via* restriction endonuclease digestion and subsequently re-transformed into an expression host, *E. coli* strain BL21 (DE3) pLysS.

2.2. Protein expression and purification

Both 6 × His tagged recombinant proteins (CyaA-Hly and NA/CyaA-Hly) were expressed in *E. coli* strain BL21 (DE3) pLysS as described previously [3]. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (0.1 mM final concentration). *E. coli* cells were harvested by centrifugation, re-suspended in ice-cold 20 mM HEPES buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mM phenylmethyl-sulfonylfluoride, and subsequently disrupted by sonication. After centrifugation, the lysate supernatant was analyzed by SDS-PAGE and concentrations of soluble proteins in the supernatant were determined by Bradford-based microassay.

6 × His-tagged proteins were purified *via* immobilized metal affinity chromatography (IMAC). 10-mL lysate supernatant (~5 mg/mL total proteins) added with 200 mM NaCl and 7.5 mM imidazole (IMZ) was loaded onto an affinity-based Ni^{2+}-NTA column (5-mL HisTrap™ HP) pre-equilibrated with 20 mM HEPES (pH 7.4) containing 2 mM $CaCl_2$, 200 mM NaCl and 7.5 mM IMZ. Target His-tagged proteins were stepwise-eluted at a flow rate of 1 mL/min with 100 mM and 250 mM IMZ, respectively. All eluted fractions were analyzed by SDS-PAGE and fractions containing the His-tagged toxins were pooled and desalted through a PD-10 desalting column, and their concentrations were determined as described above.

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