Biochemical and Biophysical Research Communications 461 (2015) 300–306

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Two specific membrane-bound aminopeptidase N isoforms from *Aedes aegypti* larvae serve as functional receptors for the *Bacillus thuringiensis* Cry4Ba toxin implicating counterpart specificity

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ARTICLE INFO

Article history:
Received 25 March 2015
Available online 12 April 2015

Keywords:
Baculovirus expression system
Cry δ-endotoxin
Cytotoxicity assay
Membrane-bound APN
Double immunolocalization
APN structure

ABSTRACT

The interaction between *Bacillus thuringiensis* Cry toxins and their receptors on midgut cells of susceptible insect larvae is the critical determinant in toxin specificity. Besides GPI-linked alkaline phosphatase in *Aedes aegypti* mosquito-larval midguts, membrane-bound aminopeptidase N (AaeAPN) is widely thought to serve as a Cry4Ba receptor. Here, two full-length AaeAPN isoforms, AaeAPN2778 and AaeAPN2783, predicted to be GPI-linked were cloned and successfully expressed in *Spodoptera frugiperda* (*Sf*9) cells as 112- and 107-kDa membrane-bound proteins, respectively. In the cytotoxicity assay, *Sf*9 cells expressing each of the two AaeAPN isoforms showed increased sensitivity to the Cry4Ba mosquito-active toxin. Double immunolocalization revealed specific binding of Cry4Ba to each individual AaeAPN expressed on the cell membrane surface. Sequence analysis and homology-based modeling placed these two AaeAPNs to the M1 aminopeptidase family as they showed similar four-domain structures, with the most conserved domain II being the catalytic component. Additionally, the most variable domain IV containing negatively charged surface patches observed only in dipteran APNs could be involved in insect specificity. Overall results demonstrated that these two membrane-bound APN isoforms were responsible for mediating Cry4Ba toxicity against AaeAPN-expressed *Sf*9 cells, suggesting their important role as functional receptors for the toxin counterpart in *A. aegypti* mosquito larvae.

1. Introduction

Cry δ-endotoxins produced by Gram-positive spore-forming *Bacillus thuringiensis* (*Bt*) are highly toxic toward a wide range of insect larvae [1]. Of particular interest, the Cry4Ba toxin from *Bt* subsp. *israelensis* is specifically active against mosquito larvae of the genus *Aedes* and *Anopheles,* vectors of various human diseases including dengue fever, chikungunya, yellow fevers and malaria [2,3]. After ingestion by susceptible insect larvae, these Cry toxins which are produced as insoluble protoxins are solubilized in the larval midgut lumen (generally alkaline pH for dipteran and lepidopteran larvae) prior to their activation by gut proteases to yield ~65-kDa active toxins [2,4]. According to all the known Cry structures, the activated toxins consist of three distinct functional domains: an α-helical bundle (DI), a β-sheet prism (DII) and a β-sheet sandwich (DIII). DI and DII have been revealed as membrane-pore formation and receptor recognition, respectively [5]. Recently, we have demonstrated that charge-reversal mutations at Asp^{454} (D454R and D454K) located within a receptor-binding loop of Cry4Ba-DII markedly increases the toxin activity against less-susceptible *Culex* larvae, suggesting a role of the charged side-chain in determining target specificity [6].

A number of activated Cry toxins are shown to bind to a variety of specific receptors located on the midgut epithelium cells [7]. This toxin-receptor interaction would promote toxin oligomerization and membrane-pore formation, resulting in midgut cell lysis and the eventual death of the target larvae [7]. Specific binding to their individual receptors is believed to be the critical determinant in Cry

Abbreviations: AaeAPN, *Aedes aegypti* membrane-bound aminopeptidase N; AeaALP, *Aedes aegypti* membrane-bound alkaline phosphatase; CAT, chloramphenicol acetyltransferase; GPI, glycosylphosphatidylinositol.

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http://dx.doi.org/10.1016/j.bbrc.2015.04.026
0006-291X/

toxin specificity. In mosquito larvae, various membrane-bound proteins were identified as functional receptors for several Cry toxins, including *Aedes* and *Anopheles* cadherin-like proteins for Cry11Aa and Cry4Ba, respectively [8,9], *Aedes* alkaline phosphatases (ALP) for Cry11Aa and Cry4Ba [10–12], *Anopheles* ALP for Cry11Ba [13], *Aedes* aminopeptidases N (APN) for Cry4Ba and Cry11Aa [14,15] and *Anopheles* APN for Cry11Ba [16,17]. Recently, the Cyt2Aa toxin from *Bt* subsp. *darmstadiensis* has also been reported as another binding protein for Cry4Ba to exert synergistic toxicity against *Aedes aegypti* larvae [18]. However, the structural basis for Cry4Ba toxin-receptor interaction is still unclear and need to be explored.

The APN family is a class of M1 zinc-metalloprotease/peptidase superfamily that cleaves neutral amino acids from the N-terminus of polypeptides [19]. Members of this family are classified as gluzincins that contain a consensus Zn^{2+}-binding sequence [HEXXH-(X18)-E, where X stands for any amino acid] and a GAMEN sequence in the active site [19]. To date, crystal structures of the M1 superfamily are available for various species, *e.g. Escherichia coli* (PDB ID: 2HPT), *Plasmodium falciparum* (PDB ID: 3EBG), *Thermoplasma acidophilum* (PDB ID: 1Z5H) and human (PDB ID: 2YD0) [20]. They all show similar overall structures, with domain II being the most conserved and domain IV the most variable [21]. In addition to being studied for their typical role in digestion, APNs have also been extensively studied as tumor-related targets, receptors for coronavirus and bacterial toxins [21,7].

Previously, we have demonstrated in *A. aegypti* mosquito larvae that knockdown of three different GPI-anchored APN isoforms (AaeAPN2778, AaeAPN2783 and AaeAPN5808) *via* RNA interference resulted in the decrease of Cry4B toxicity [14]. Here, we provide further insights into the molecular structure and specific binding to the Cry4Ba toxin of two functionally expressed AaeAPN isoforms, AaeAPN2778, AaeAPN2783, on the *Sf*9 insect cell membrane.

2. Materials and methods

2.1. Amplification of GPI-anchored APN-coding regions from A. aegypti larval midgut cDNA

Three pairs of AaeAPN-specific primers were designed (*see* Supplementary Table 1) based on three putative GPI-anchored APN sequences from *A. aegypti* genome database (www.vectorbase.org): AaeAPN2778, AaeAPN2783 and AaeAPN5808, where the numbering is referred to the last four digits (underlined) of transcript identification from VectorBase (AAEL012778-RA, AAEL012783-RA and AAEL005808-RA). Then, potential GPI-anchoring sites were predicted by four different GPI-prediction programs: PredGPI (gpcr2.biocomp.unibo.it/gpipe/pred.htm), big-PI Predictor (mendel.imp.univie.ac.at/sat/gpi/gpi_server.html), FragAnchor (http://navet.ics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html) and GPI-SOM (http://gpi.unibe.ch/). Signal peptide and N-glycosylation sites were further predicted by SignalP and NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

First-strand cDNA was synthesized from RNA transcripts of the 5th instar *A. aegypti* larval midgut using oligo-dT primer. Coding sequences of these three AaeAPNs were individually amplified *via* the cDNA product with Phusion DNA polymerase (Finnzymes). The expected size-amplicons were purified, ligated to the pENTR/SD-TOPO vector and transformed into One-Shot TOP10 competent cells, following the manufacturer's protocol (Invitrogen). Recombinant pENTR/AaeAPN plasmids were randomly selected and their sequences verified by DNA sequencing.

2.2. Transient expression of recombinant AaeAPN isoforms in insect cells

Each of recombinant pENTR/AaeAPN plasmids was selected for an *in vitro* Lamda Recombination (LR). Individual AaeAPN fragments were transferred from pENTR/AaeAPN plasmids to Baculodirect Linear DNA (Invitrogen) by site-specific recombination. Briefly, ~200 ng of pENTR/AeAPN plasmids were mixed with Baculodirect C-term Linear DNA (~200 ng) and LR Clonase II for 18 h at 26 °C. The recombinant AaeAPN expression vectors were subsequently transfected into the insect *Sf*9 (*Spodoptera frugiperda* 9) cells using Cellfectin, following manufacturer's protocol (Invitrogen).

2.3. Mass spectrometry (MS) analysis of AaeAPNs proteins

Cell lysates collected from *Sf*9 cells expressing each recombinant AaeAPN were separated by SDS-PAGE. The protein bands corresponding to AaeAPN proteins were excised from the gel and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis (MaXis, Bruker).

2.4. Expression and preparation of the Cry4Ba active toxin

Cry4Ba-R203Q mutant toxin (one tryptic cleavage site was eliminated by substitution at Arg^{203} with Gln) which still retains high larvicidal activity [22] was used in this study. *E. coli* strain JM109 expressing the R203Q mutant was grown in LB medium containing 100 μg/mL ampicillin at 37 °C until OD_{600} reached 0.3–0.6. Toxin expression was induced with 0.1 mM isopropyl-β-D thiogalactopyranoside for 4 h and harvested by centrifugation at 6000× *g*. The cell pellet was re-suspended in 100 mM KH_2PO_4 (pH 5.0) containing 0.1% Triton X-100 and 0.5% NaCl, and further disrupted by sonication (VCX750-Sonics Vibra Cell™) with the following parameters: 5 cycles of amplitude 60%, 10-s ON, 30-s OFF with a total time ON = 1 min/cycle. Protein inclusions were collected by centrifugation at 6000× *g*, washed and subsequently re-suspended in distilled water. The concentration of the partially purified inclusion was determined using the Bradford-based protein microassay. Cry4Ba toxin inclusions were solubilized in carbonate buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.0) at a final concentration of 1 mg/mL for 1 h at 37 °C. The solubilized toxin was activated by trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin; Sigma) at a ratio of 1:10 enzyme/toxin (w/w). The 65-kDa activated toxin was further purified by size-exclusion chromatography (Superose 12 10/300, GE Healthcare) and eluted with the carbonate buffer (pH 9.0) at a flow rate of 0.4 mL/min. The purified Cry4Ba protein fraction was then concentrated with a 10-kDa-MWCO Millipore membrane (Merck KGaA) and analyzed by SDS-PAGE.

2.5. Cytotoxicity assays of the Cry4Ba toxin on Sf9 cells-expressing AaeAPNs

100 μg/mL of Cry4Ba toxin (mixed with 1 mL Sf-900 medium (Invitrogen) was applied to the 5-day post-infected *Sf*9 cells expressing recombinant AaeAPNs, AaeALP (*Aedes* membrane-bound alkaline phosphatase) and CAT control (chloramphenicol acetyltransferase) at ~8×10^5 cells/mL in a 6-well culture plate and further incubated at 26 °C for 2 h. The cell morphology was observed under light microscope. To determine viability of cells, PrestoBlue™ cell viability assay (Life Technologies, USA) was performed. Briefly, 100 μL PrestoBlue™ reagent was added directly to cells in 900 μL culture medium, incubated for 10 min at 37 °C and then spectrofluorometrically quantitated (excitation at 530 nm;