Insect Biochemistry and Molecular Biology 43 (2013) 1201–1208

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

A 104 kDa *Aedes aegypti* aminopeptidase N is a putative receptor for the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*

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ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form
20 September 2013
Accepted 27 September 2013

Keywords:
Bacillus thuringiensis
Cry11Aa toxin
Aminopeptidase
Receptor
Binding affinity
Midgut
Aedes aegypti

ABSTRACT

The Cry11Aa protein produced in *Bacillus thuringiensis* subsp. *israelensis*, a bacterial strain used worldwide for the control of *Aedes aegypti* larvae, binds midgut brush border membrane vesicles (BBMV) with an apparent K_d of 29.8 nM. Previously an aminopeptidase N (APN), named AaeAPN2, was identified as a putative Cry11Aa toxin binding protein by pull-down assays using biotinylated Cry11Aa toxin (Chen et al., 2009. Insect Biochem. Mol. Biol. 39, 688–696). Here we show this protein localizes to the apical membrane of epithelial cells in proximal and distal regions of larval caeca. The AaeAPN2 protein binds Cry11Aa with high affinity, 8.6 nM. The full-length and fragments of AaeAPN2 were cloned and expressed in *Escherichia coli*. The toxin-binding region was identified and further competitive assays demonstrated that Cry11Aa binding to BBMV was efficiently competed by the full-length AaeAPN2 and the fragments of AaeAPN2b and AaeAPN2e. In bioassays against *Ae. aegypti* larvae, the presence of full-length and a partial fragment (AaeAPN2b) of AaeAPN2 enhanced Cry11Aa larval mortality. Taken together, we conclude that AaeAPN2 is a binding protein and plays a role in Cry11Aa toxicity.

1. Introduction

Aedes aegypti is the principle vector for dengue and yellow fever diseases, both of which have seen recent reemergence. Control of *Aedes* mosquitoes during their larval stages has increasingly used *Bacillus thuringiensis* subsp. *israelensis* formulations. This bacterium produces inclusions that contain crystalline (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and cytolytic (Cyt1Aa, Cyt2Ba and Cyt1Ca) proteins, which are produced during the sporulation phase (Berry et al., 2002). Among them, Cry11Aa is one of the most active toxins against *Ae. aegypti* larvae (Chilcott and Ellar, 1988).

The mechanism of action of Cry toxins has been best studied in lepidopteran insects, where presently four major protein receptors have been identified for Cry1A toxins – cadherins, ABCC transporters, aminopeptidases (APNs) and alkaline phosphatases (ALPs) (for reviews see Bravo et al., 2005; Pardo-Lopez et al., 2013; Pigott and Ellar, 2007; Soberon et al., 2009). The activated Cry toxins bind first to the cadherin receptor or ABCC transporter in the microvilli of midgut epithelial cells. Binding to the former is known to trigger toxin oligomerization, and then the toxin oligomers bind to GPI-anchored receptors, APN and/or ALP, leading to membrane insertion and pore formation (Bravo et al., 2005, 2007; Soberon et al., 2009). It is possible a similar process is involved after Cry toxin binding to the ABCC transporter. Membrane insertion and pore formation are thought to lyse the midgut cells ultimately killing larval insect (Soberon et al., 2009). Alternately, in another model, the cadherin alone initiates an intracellular cascade that leads to cell toxicity (Zhang et al., 2006).

Since APNs were identified as Cry1 toxin-binding proteins (Gill et al., 1995; Knight et al., 1995), numerous lepidopteran APNs have been reported to bind Cry1 toxins (Pigott and Ellar, 2007). Cry1Ac toxin interaction with APNs is generally thought to involve glycosylated moieties. For example, Cry1Ac interacts with APNs from *Manduca sexta* (Burton et al., 1999; Masson et al., 1995), *Heliothis virescens* (Gill et al., 1995) and *Lymantria dispar* (Jenkins et al., 2000) through *N*-acetyl galactosamine residues (GalNAc). But *Bombyx mori* APNs are believed to bind toxins in a glycan-independent manner (Atsumi et al., 2005; Yaoi et al., 1997, 1999). However, only a few APNs apparently mediate *in vivo* toxin activity, for example, the silencing of midgut APNs results in reducing *Spodoptera litura* and *Helicoverpa armigera* sensitivity to Cry1C and Cry1Ac, respectively (Rajagopal et al., 2002; Sivakumar et al., 2007). More recent evidence shows that a mutation in APN is associated with Cry1Ac resistance in *H. armigera* (Zhang et al., 2009) and down-regulation of APN is correlated with cabbage looper resistance to Cry1Ac toxins (Tiewsiri and Wang, 2011). These evidences support a functional role for APN in mediating Cry1 toxicity. In the

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http://dx.doi.org/10.1016/j.ibmb.2013.09.007

sequential toxin binding model, cadherin-induced toxin oligomers can bind APN (Bravo et al., 2004).

Recent evidence suggests mosquitocidal toxins also bind a similar set of proteins in the mosquito midgut, and toxin binding APNs have been identified. For example, APNs from *Anopheles quadrimaculatus* and *Anopheles gambiae* bind Cry11Ba (Abdullah et al., 2006; Zhang et al., 2008) and APNs from *Ae. aegypti* bind Cry11Aa (Chen et al., 2009b). Furthermore, all these Cry11A-bound APNs from *Ae. aegypti*, along with a number of other GPI-anchored proteins are localized in lipid rafts (Bayyareddy et al., 2012). In the case of the 110 kDa APN (AaeAPN1) from *Ae. aegypti* and the 106 kDa APN (AgAPN2) from *An gambiae*, partial protein fragments expressed in *Escherichia coli* bind Cry11Aa and Cry11Ba toxins, respectively (Chen et al., 2009b; Zhang et al., 2008). Moreover, two partial APN fragments show synergistic and inhibitory effects on the Cry11B toxicity in the *An. gambiae* (Zhang et al., 2010). In addition, recently three predicted glycosylphosphatidylinositol (GPI)-anchored APNs transcript knockdown by RNAi cause Cry4B toxicity decrease in *Ae. aegypti* (Saengwiman et al., 2011).

Previously we reported another *Ae. aegypti* APN, named AaeAPN2, also binds Cry11Aa (Chen et al., 2009b). Here we report further characterization of this protein, including its expression and characterization in *E. coli*, and its immunolocalization in the larval midgut. Cry11Aa toxin binds AaeAPN2 with high affinity, and we also identified a toxin binding region in this APN. Here this cloned APN is referred to as AaeAPN2, which has five amino acids that differ from that of AAEL008155.

2. Materials and methods

2.1. Purification and activation of Cry11Aa toxin

Cry11Aa inclusions were isolated from a recombinant *B. thuringiensis* strain (Chang et al., 1993), grown in nutrient broth sporulation medium containing 12.5 μg/ml erythromycin at 30 °C (Lereclus et al., 1995). Following cell autolysis spores and inclusions were harvested, washed 3× with 1 M NaCl, 10 mM EDTA, pH 8.0 and centrifuged. The resulting pellet was resuspended in 30 ml of the same buffer and purified by NaBr gradients as previously described (Cowles et al., 1995). The purified inclusions were solubilized in 50 mM Na_2CO_3, pH 10.5 and activated by trypsin (1:20, w/w), and the activated Cry11Aa toxin was purified by ion exchange chromatography (Mono Q, FPLC).

2.2. Preparation of membranes from Aedes midguts

Brush border membrane vesicles (BBMV) were prepared from early fourth instar *Ae. aegypti* larvae midguts as described (Nielsen-Leroux and Charles, 1992). The cell suspension was sonicated, centrifuged at 700 *g* for 10 min, and the supernatant was then centrifuged at 16,000 *g* for 1 h at 4 °C. The membrane pellet obtained was used fresh.

2.3. Assembly, cloning and analysis of AeaAPN2 gene

Five pairs of primers listed in Table 1 were designed based on AAEL008155 transcript, and used for PCR for isolation of full-length or partial AaeAPN2 cDNA. Total RNA was extracted from early 4th instar larvae midguts using Trizol reagent (Invitrogen). For 5′-rapid amplification of cDNA ends (RACE), a poly(A) tail was added to the 5′ termini of cDNAs transcribed from mRNA by using the gene-specific primer, APN2-C. A 0.2 kb PCR product was amplified from the tailed cDNA by the primers, APN2-5R and adapter primer. For 3′ RACE, first strand cDNA was synthesized from total RNA with an $(dT)_{17}$-adapter primer. Using an adapter primer and a gene-specific primer, APN2-3F, a 0.7 kb PCR product was obtained by nested PCR. Based on sequences of 5′ RACE and 3′ RACE amplified products, two partial overlapping fragments of ORF were amplified by gene-specific primers of AaeAPN2, APN2-1F/1R and APN2-2F/2R, respectively and then were assembled as full-length AaeAPN2 ORF using a unique restriction site, *Bsi*WI, present in both products. In addition, a 815 bp product from the 3′ end of the AaeAPN2 gene was obtained using primers APN1-eF/eR and the protein obtained from this product was used for antibody preparation. All PCR products were cloned into TA cloning vector, pCR2.1 TOPO (Invitrogen) and fully sequenced (Institute of Integrative Genome Biology (IIGB), University of California Riverside).

Table 1
Primers used in this study.

Primer	Primer sequence (5′–3′)
$(dT)_{17}$-Adapter	5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3′
Adapter	5′-GACTCGAGTCGACATCGA-3′
APN2-C	5′-GGCATATTAGCAACCGCATT-3′
APN2-5R	5′-CGTTCGTTTTCATGGATCG-3′
APN2-3F	5′-AAAGTGTCGGCCAATACCG-3′
APN2-1F	5′-GGATCCATGTTGTTGAAGGAAGTGTTTTTGTGCGC-3′
APN2-1R	5′-CGTACGCAACTGTGTCAAACAGGCCAG-3′
APN2-2F	5′-CGTACGATAAGTCGGGAAGTGTTCT-3′
APN2-2R	5′-TCTAGATCACTGTTTGTACGCCGCGAAGAACTC-3′
APN2-eF	5′-GGATCCAAGTTGACAACAACTATCCCTG-3′
APN2-eR	5′-AAGCTTACCGAGAAACTCGATAACG-3′

Sequence alignments and other sequence analysis were performed using NCBI blast programs and Lasergene (DNAstar). The signal peptide and GPI anchor were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and big PI predictor (http://mendel.imp.ac.at/gpi/gpi_server.html), respectively. The OGPET v1.0 (http://ogpet.utep.edu/OGPET/) and NetNGlyc v1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) programs were used to determine potential O- and N-glycosylation sites, respectively.

2.4. Expression of AaeAPN2 in E. coli

The 815 bp PCR product and full-length AaeAPN2 orf, together with four partial overlapping gene fragments cleaved from this full-length AaeAPN2 orf by using appropriate restriction enzymes or obtained by PCR, were cloned into pQE series expression vector (Qiagen). The constructs were transformed into *E. coli* M15(pREP4) strain, and protein expression induced by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). Expressed proteins were purified by Ni-NTA resin (Qiagen) under denaturing conditions and resolved in SDS-PAGE.

Alternatively, inclusion bodies were purified from bacterial cultures expressing AaeAPN2 fragments using a B-PER Bacterial Protein extraction reagent following the manufacturer's instructions (Pierce). The inclusion bodies were dissolved in 0.1 M NaOH buffer for 1 h and then dialyzed against 50 mM Na_2CO_3 (pH 10.5), and protein concentration was measured using the BCA assay (Pierce). Total proteins extracted were analyzed by SDS-PAGE gels and the percentage of protein consisting of AaeAPN2 fragments measured by NIH ImageJ software.

2.5. Antibody production and immunolocalization of AaeAPN2 in larval midgut of Ae. aegypti

For antibody production a variable region 29 kDa fragment, AaeAPN2e, was expressed and purified by Ni-NTA resin (Qiagen) under denaturing conditions and resolved in SDS-PAGE. Gels were stained and destained, and the purified protein bands were excised, washed three times and used for antibody development in rabbits.