Binding of *Bacillus thuringiensis* δ-endotoxins Cry1Ac and Cry1Ba to a 120-kDa aminopeptidase-N of *Epiphyas postvittana* purified from both brush border membrane vesicles and baculovirus-infected Sf9 cells

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Abstract

A 120-kDa protein was purified from brush border membrane vesicles of the tortricid moth *Epiphyas postvittana* (Walker) based both on its activity as an aminopeptidase and the ability to bind the *Bacillus thuringiensis* δ-endotoxin Cry1Ac. The purified enzyme had a pI of 5.6 and was a leucine aminopeptidase, with some isoleucine, phenylalanine and tryptophan aminopeptidase activity. Further characterisation showed that the protein was also able to bind Cry1Ba. During purification, the molecular weight of the protein decreased from 120 to 115 kDa due to the loss of a glycoprophosphatidyl anchor. The protein was N-terminally sequenced and, using this information and conserved regions within other insect aminopeptidase-N (APN) sequences, redundant primers were designed to amplify the aminopeptidase coding sequence from *E. postvittana* midgut cDNA. The predicted protein sequence from the full-length cDNA was most closely related to the APN protein sequence from *Heliothis virescens* (61% identity) and shared other features of insect APNs including a Zn²⁺ binding site motif and four conserved cysteines. The *E. postvittana* was expressed in Sf9 cells using baculovirus, yielding a protein of molecular weight 130 kDa, but with unchanged N-terminal sequence. Purified recombinant protein bound both Cry1Ac and Cry1Ba by ligand blot assays. However, despite the protein being expressed on the external surface of the Sf9 cells, it bound neither Cry1Ac nor Cry1Ba in vivo. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Aminopeptidase-N; *Bacillus thuringiensis*; Cry1Ac binding protein; Cry1Ba Cry1Ac binding protein; Baculovirus-induced protein expression; *Epiphyas postvittana* (Walker)

1. Introduction

During sporulation, the Gram-positive bacteria, *Bacillus thuringiensis* forms crystalline protein inclusions, which possess insecticidal activity. The mode of action of these inclusion proteins or δ-endotoxins has been partially elucidated (Gill, 1995). The crystals are solubilised within the alkaline midgut of certain insects and cleaved by proteases to release an active toxin. This activated toxin then binds to specific high-affinity receptors on the surface of the midgut epithelia. After binding, the toxin is thought to undergo a conformational change leading to formation of ion pores within the membrane of epithelial cells, eventually causing the death of the insect (Knowles and Dow, 1993).

Proteins which bind to one or more of the δ-endotoxins Cry1Aa, Cry1Ab, Cry1Ac and Cry1C have been purified and characterised from the midguts of *Manduca sexta* (Knight et al., 1994; Valdamudi et al., 1995; Denolf et al., 1997), *Heliothis virescens* (Gill et al., 1995, Luo et al., 1997a), *Lymantria dispar* (Valaitis et al., 1995), *Plutella xylostella* (Denolf et al., 1997; Luo et al., 1997b) and *Bombyx mori* (Yaio et al., 1997). In all but one case, these proteins were found to be members of the aminopeptidase-N (APN) family (EC 3.4.11.2), the exception being a cadherin-like protein which bound Cry1Aa in *M. sexta* (Valdamudi et al., 1995; Francis and Bulla, 1997; Keeton and Bulla, 1997). APN is a 120- or 170-kDa glycoprotein, with a glycosylphosphatidylinositol (GPI) anchor. It has been shown that incorporating

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a mixture of APN and an alkaline phosphatase into phospholipid vesicles enhances Cry1Ac-mediated efflux of $^{86}$Rb+ up to 1000-fold (Sangadala et al., 1994). In Trichoplusia ni brush border membrane vesicles (BBMVs), Cry1Ac pore-forming activity is dependent on the amount of APN activity present on the surface of the BBMV (Lorence et al., 1997). A 170-kDa APN from H. virescens has been shown by surface plasmon resonance to bind Cry1Aa, Cry1Ab and Cry1Ac, and when the protein was reconstituted in phospholipid vesicles, Cry-toxin-mediated $^{86}$Rb+ efflux was related to toxicity of the endotoxin (Luo et al., 1997a).

There remains some debate over the role of APN as the key binding site for endotoxin activity (Francis and Bulla, 1997). It is a highly abundant protein, yet competitive binding assays suggest that a much lower amount of the binding proteins is present (van Rie et al., 1989; Garczynski et al., 1991). There are also inconsistencies between competitive binding assays and ligand blotting experiments, the latter being used to assay binding protein purification (Lee and Dean, 1996). The cadherin-like receptor from M. sexta has also been expressed in Sf9 cells, and the expressed protein has the same ligand blotting and competitive binding assay characteristics as the native protein, not only for Cry1Ab, but also for Cry1Aa and Cry1Ac (Francis and Bulla, 1997; Keeton and Bulla, 1997). This has not yet been shown for APN.

The tortricid moth Epiphyas postvittana (Walker) is a polyphagous pest mainly affecting horticultural production and export from Australia and New Zealand (Wearing et al., 1991). Cry1Ac is an effective toxin against E. postvittana, while Cry1Ba has moderate insecticidal activity (Gleave et al., 1992; Simpson et al., 1997). Competitive binding studies have shown that each of these toxins interact via two binding sites in the midgut (Simpson et al., 1997). Here we report the characterisation of Cry1Ac-binding proteins of E. postvittana and the purification of one of them, revealing it to be an APN. Furthermore, we describe the isolation of the gene encoding this APN and characterise the protein’s δ-endotoxin binding capacity, comparing endogenous and baculovirus-expressed APN.

2. Materials and methods

2.1. Insects

Epiphyas postvittana were collected from Nelson, New Zealand, in 1975 and maintained in the Insect Rearing Facility, Mt Albert Research Centre, using conditions described previously (Singh, 1974; Clare et al., 1987). The midguts of fifth instar larvae were dissected into liquid nitrogen and stored at −80°C.

2.2. Assays

Cry1Ac and Cry1Ba were prepared as described in Simpson et al. (1997). The ligand blot assay used to detect endotoxin binding was based on a western transfer protocol as described by Sanchis and Ellar (1993), modified to include alkaline phosphatase-linked secondary antibody, and colour development by NBT/BCIP (Gibco BRL). Alkaline phosphatase (AP) and leucine aminopeptidase (LAP) assays were performed as described by Christeller et al. (1989). Aminopeptidase activity using various amino acid amide substrates was determined using the method described by Himmelhoch (1970).

Radiolabelled Cry1Ac and Cry1Ba were prepared as described by Simpson et al. (1997). Competitive binding assays were performed as described by van Rie et al. (1989), except that Sf9 cells were used instead of using BBMVs. The optimal number of Sf9 cells for competitive binding assays was determined by a set of initial experiments varying the number of control Sf9 cells. These were infected with baculovirus-containing pnlb, an external membrane-bound pectin lyase.

2.3. Protein purification

Brush border membrane vesicles (BBMVs) were prepared by the method of Woltersberger et al. (1987). Approximately 1 ml of BBMV suspension was centrifuged in a bench-top centrifuge at 14,000g for 5 min, then resuspended in 200 μl of 1% w/v octylglucoside (OG) in 0.9% sodium chloride in 9.8 mM sodium phosphate buffer, pH 7.4 (PBS) and briefly vortexed. This was incubated at 4°C overnight, then centrifuged again. The supernatant was diluted to 1.2 ml using PBS, left at 4°C for 2 h, then recentrifuged to remove any precipitate, and applied to a gel filtration column (Superose-12 HR10/30; Pharmacia, Uppsala, Sweden; 300 mm long, 10 mm i.d.), using 20 mM Tris–HCl, 100 mM sodium chloride, 0.15% w/v OG, pH 7.4. Fractions (200 μl) containing a single Cry1Ac-binding protein were combined and separated by anion exchange chromatography (Mono Q HR5/5; Pharmacia; 50 mm long, 5 mm i.d.), equilibrated with 20 mM Tris–HCl, 100 mM NaCl, 0.15% OG, pH 7.4, and eluted with a gradient of 0.1 to 1 M NaCl in 20 mM Tris–HCl, 0.15% OG, pH 7.4 in 30 ml.

Sf9 cells expressing recombinant E. postvittana APN, prepared as described below, were briefly centrifuged, and an equal volume of PBS was added. The cells were resuspended and 10% w/v OG added to give a final detergent concentration of 0.8%. This was left at 4°C overnight, and then recentrifuged. The supernatant was diluted to give a final OG concentration of 0.15% using PBS, left at 4°C for 2 h, then centrifuged to remove any precipitate. The remainder of the purification scheme for recombinant APN was as that used for the APN from BBMVs.
2.4. PCR template preparation

Total RNA was prepared from midguts using a modified protocol of Chirgwin et al. (1979). Approximately 200 *E. postvittana* midguts were thoroughly homogenised in liquid nitrogen using a mortar and pestle. The resulting powder was resuspended in 15 ml of 4 M guanidine thiocyanate, 5 mM sodium citrate pH 7.0, 0.5% sarkosyl and 0.1 M β-mercaptoethanol (solution D) and 6 ml layered over 5 ml of 4.8 M CsCl, 10 mM EDTA, pH 8. The RNA was separated by centrifugation at 150,000g for 16 h at 15°C. The RNA pellet was resuspended in 400 µl of diethylpyrocarbonate (DEPC)-treated dH₂O. It was then precipitated by the addition of 800 µl of ethanol and 10 µl of 4 M sodium chloride and stored under ethanol at −20°C. The RNA pellet was washed in 75% ethanol and air dried before resuspension in DEPC-treated dH₂O. PolyA⁺ RNA was prepared by affinity chromatography on oligo-dT cellulose (Sambrook et al., 1989) using a QuickPrep Micro mRNA purification kit (Pharmacia). cDNA was made from 10 of the 400 µl of polyA⁺ mRNA using reverse transcriptase (Superscript II, BRL) as per the manufacturer’s instructions in a 20 µl reaction volume using Rₜ Rₗ dT₁₆ (Ma et al., 1994) as a primer.

2.5. PCR and sequencing

cDNA encoding the 120-kDa Cry1Ac-binding protein was amplified from midgut cDNA using Boehringer Mannheim Taq DNA polymerase following the manufacturer’s instructions. Fifty-µl amplification reactions included 2.5 U Taq DNA polymerase, 10 mM Tris–HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTPs and 100 pmol of each primer. All amplification reactions were conducted in a Progene Thermal Cycler and included single primer and negative controls. Redundant PCR primers were designed from the N-terminal amino acid sequence (APNf1) and conserved regions of other insect aminopeptidases (APNrl, APNnr2) to amplify a central region of the Cry1Ac-binding protein gene. The sequences of all primers used in this study are given in Table 1. Amplification using 2/20 µl of *E. postvittana* midgut cDNA included a “hot start” of 94°C 3 min after which the Taq DNA polymerase was added followed by 30 cycles of 94°C 1 min, 50°C 1 min, 72°C 2 min. The 3’ end of the gene was amplified using a nested approach with primers designed from sequence of the APNf1–APNr2 fragment (APNf2, APNf3) and primers (Rₜ Rₗ; Ma et al., 1994) that bound within the cDNA synthesis primer region (Rₜ Rₗ dT₁₆). Conditions for the PCR from midgut cDNA (2/20 µl) using the Rₜ and APNf2 primers included a hot start of 94°C 3 min followed by 35 cycles of 94°C 1 min, 58°C 1 min, 72°C 3 min. Two µl of this reaction was used in the second round of PCR which included a hot start of 94°C 3 min followed by 35 cycles of 94°C 1 min, 65°C 30 s, 72°C 1 min. The major product (1.4 kb) was gel purified using Qiagen’s QIAquick Gel Extraction Kit and cloned into a t-tailed pBluescript SK⁻ plasmid vector (Stratagene, Marchuk et al., 1991) using standard techniques (Sambrook et al., 1989). A Rapid Amplification of cDNA Ends (RACE) procedure was employed to amplify the 5’ end of the coding region using a modified version of Clonetech’s CapFinder™ PCR cDNA Library Construction Kit. cDNA was synthesised as above using the primer APNr3 in the presence of 10 pmol of a primer that binds to the cap region of mRNA (CAP). PCR using primers CAPCR and APNr4 (Table 1) was then used to amplify the 5’ region of the APN gene. Conditions included a hot start of 94°C 2 min followed by 35 cycles of 94°C 10 s, 60°C 30 s, 72°C 1 min. The resulting PCR products were isolated and cloned as above.

Primers designed to the extreme 5’ and 3’ ends of the APN coding region (EpAPN5’ and EpAPN3’) were used to amplify the entire coding region of the *E. postvittana* APN gene from 2 µl of the midgut cDNA preparation using High Fidelity Taq DNA polymerase (Boehringer Mannheim) according to the manufacturer’s instructions. This was cloned into pBSK⁻ as described above. Four clones (pEpAPN1–4) were characterised on the basis of restriction enzyme cleavage sites. EpAPN1–2 contains a single internal BamHI site while pEpAPN3–4 contains two BamHI sites.

PCR products and cloned fragments (e.g. EpAPN1) were sequenced using dye-terminator chemistry and reactions run on an ABI 377 automated sequencer according to the manufacturer’s instructions (Applied BioSystems). Sequencing primers included those mentioned above as well as APNf2, APNf4, APNf5, APNf6, APNr5, APNr6, APNr7 and APNr8, see Table 1. Sequence data were analysed using the GCG package, version 3.0, 1995 (Gene codes Corporation, Inc.).

2.6. Baculovirus expression

The entire APN coding region of PEpAPN1 was subcloned into the *SalI/XbaI* sites of pFastbac of the Bacto-Bac system (Life Technologies). A region containing the APN coding region downstream of the baculovirus polyhedrin promoter within pFastbac was transposed into the baculovirus genome and used to transfect SF9 cells maintained in SF900 II media (Life Technologies) according to the manufacturer’s instructions. Protein was expressed in 75-cm² static culture vessels containing 15 ml of SF900 II media seeded with 2 million cells and infected with 2 million pfu of virus. Cultures containing baculovirus expressing pnlb (a fungal pectin lyase) and no virus (cells only) were also raised as controls. Cells were harvested 3–4 days after infection and collected by low-speed centrifugation at 500g for 5 min. Recombi-
nant *E. postvittana* APN protein purification is described in the protein purification section.

**3. Results**

**3.1. Binding protein purification**

The δ-endotoxin ligand blot assay revealed three major Cry1Ac binding proteins in *E. postvittana* BBMVs of molecular weights 120, 180 and 200 kDa (Fig. 1b, Lane 1); binding of Cry1Ac was not altered in the presence of 200 mM N-acetylgalactosamine (data not shown). All three major Cry1Ac-binding proteins were also able to bind Cry1Ba. The binding proteins were solubilised using 1% OG (Fig. 1a, Lane 2), although on solubilisation the molecular weight of the 120-kDa binding protein decreased to 115 kDa. Purification of the 115-kDa protein by gel filtration followed by ion exchange chromatography yielded a single peak with both Cry1Ac-binding and leucine aminopeptidase activity. IEF and SDS–PAGE showed that the protein was purified to homogeneity (Fig. 1a, Lane 4 and Fig. 1c, Lane 1). The pI of the protein was 5.6 as determined.

![Fig. 1. Silver stained 7.5% SDS–polyacrylamide gel (a), ligand blot (b) and IEF (c) analysis of purification of APN. In both gels Lane 1 is BBMV proteins, Lane 2 solubilised BBMV proteins, Lane 3 after gel filtration and Lane 4 after anion exchange chromatography. The ligand blot was probed with Cry1Ac. For the IEF gel, a pH 3–10 Bio-Rad ReadyGel was used, and only the fully purified protein was focused.](image-url)
from the IEF gel. The N-terminal amino acid sequence was determined by gas-phase sequencing as XNVDPALYRLPT (single letter amino acid code, X as any amino acid). Ligand blot assays demonstrated that this protein was able to bind Cry1Ba toxin in addition to Cry1Ac (Fig. 4). This contains the YRLPT sequence, which is conserved in all Cry1Ac-binding APNs (Medof et al., 1996).

The relative rates of hydrolysis of amino acid amides by the enzyme are given in Table 2. The enzyme was most active with leucinamide, but also was able to hydrolyse isoleucinamide, tryptophanamide, phenylalaninamide, valinamide and histidinamide to a lesser extent. There was no significant hydrolysis of serinamide and argininamide.

### 3.2. cDNA sequence analysis

Sequencing of pEposAPN and the 5′ and 3′ RACE products were aligned to form a contig of 3450 bp in length (Fig. 2); GenBank accession number AF276241). This sequence contained an open reading frame of 1007 amino acids (EposAPN) and includes the N-terminal amino acid sequence attained from the purified 115-kDa Cry1Ac-binding protein described above. The predicted start codon is embedded in a consensus Kozac translation initiation sequence (UCAACUCAGAGGC, nts -10-5, Fig. 2) Cavener and Ray, 1991). There is a putative polyadenylation signal (AAUAAA) 14 bp before the polyA tail. Analysis of the predicted protein EposAPN for protein localisation by PSORT predicts with the possibility that the protein is located in the plasma membrane (Nakai and Kanehisa, 1992). The inferred protein contains a putative signal peptide (MAALKLLVFALACYCASS, residues 1–18) and a GPI anchor including a GPI signal sequence (GSA, residues 750, 757, 785, 821). The inferred EposAPN protein contains no predicted N-linked glycosylation sites, but has many possible O-linked glycosylation sites. It has a predicted molecular weight of 108 kDa once the predicted signal and other leader peptides are removed (amino acids 51–990) and assuming no glycosylation. Blast searches using protein sequence against the NCBI database reveal that EposAPN is most similar to other insect glutamyl aminopeptidases from H. virescens (61% identical; Gill et al., 1995) and M. sexta APN1 (42% identical; Knight et al., 1995). EposAPN is less related to M. sexta APN2 and P. xylostella APN (Denolf et al., 1997). EposAPN shares conserved Zn\(^{2+}\) binding and catalytic residues with other metallopeptidases (amino acid residues 374, 375, 378, 397; Rawlings and Barrett, 1995).

### 3.3. Baculovirus expressed EposAPN

Sf9 cells infected by baculovirus expressing EposAPN from the clone pEposAPN1 had significantly increased APN activity compared to uninfected and pnlb-infected Sf9 cells (Table 3). When the cells were lysed, there was no significant increase in APN activity, suggesting that nearly all the enzyme was expressed on the surface of the cells. EposAPN was purified from Sf9 cells using a method that was essentially the same as the purification of APN from midgut material. The purified recombinant EposAPN protein had a molecular weight of 130 kDa, and was able to bind both Cry1Ac and Cry1Ba in ligand blot assays (Fig. 4). The N-terminal amino acid sequence was determined by gas-phase sequencing as XDNVDPALYRLPTT.

The results of competitive binding assays of Sf9 cells are shown in Fig. 5. Cells expressing the pectin lyase, pnlb, were able to saturably bind both Cry1Ac and Cry1Ba (filled symbols). Using Sf9 cells expressing recombinant EposAPN showed no change in the binding of either Cry1Ac or Cry1Ba as compared to cells expressing the pectin lyase pnlb (the hollow symbols).

### 4. Discussion

Competitive binding assays using \(^{125}\text{I}\)-labelled \(\delta\)-endotoxins of \(B.\ thuringiensis\) show that \(E.\ postvittana\) BBMVs possess at least two binding sites for Cry1Ac and Cry1B (Simpson et al., 1997). One binding site had a high affinity with both Cry1Ac and Cry1Ba (dissociation constant in the high nM range) while there was a distinct low-affinity site (dissociation constants in the high nM range) for each toxin. Ligand blot binding assays revealed three binding proteins from \(E.\ postvittana\) BBMVs with molecular weights 120, 180, and 200 kDa which all bound both Cry1Ac and Cry1Ba. The 120-kDa Cry1Ac/Cry1Ba-binding protein was purified, using the
ligand blot assay to follow purification. During purification the molecular weight, by SDS-PAGE, dropped to 115 kDa. This decrease in size is probably due to the loss of a glycosylphosphatidylinositol (GPI) anchor. Further evidence for the presence of a GPI anchor was our observation of ethanolamine in low molecular weight gel filtration fractions (data not shown, Ferguson and Williams, 1988). The purified binding protein has aminopeptidase activity and the gene encoding the binding protein is most similar to other lepidopteran APN genes isolated to date.

The inferred protein contains conserved features of aminopeptidases such as the conserved Zn$^{2+}$-binding cysteines and catalytic residues. Similar to the lepidopteran APNs from H. virescens and M. sexta (Lu and Adang, 1996; Luo et al., 1997b), Epos APN contains a GPI anchor; during membrane solubilisation this is cleaved resulting in the 115-kDa protein. In contrast, it has no sites for N-linked glycosylation, although there are many potential sites for O-linked glycosylation, including a large number of serine and threonine residues near the C-terminus. If these C-terminal sites are glycosylated, the carbohydrate chains are probably involved in holding the enzyme proud from the membrane.

After purification, baculovirus-expressed recombinant EposAPN retained its aminopeptidase activity. The recombinant EposAPN had a higher molecular weight than BBMV-purified protein (130 kDa compared with 120 kDa); a result similar to the findings of Denolf et al. (1997), who found evidence for altered glycosylation on the expressed protein. N-terminal sequencing confirmed the identity of the recombinant protein as Epos-APN, thus the higher molecular weight could be due to either the lack of cleavage of the GPI anchor, extra glycosylation of the protein or a combination of both in Sf9 cells as compared with BBMVs.
Fig. 3. Alignment of the amino acid sequence of aminopeptidase-N from *E. postvittana* (this study), *H. virescens* (Gill et al., 1995), *M. sexta* APN1 (Knight et al., 1995), *M. sexta* APN2 (Denolf et al., 1997) and *P. xylostella* (Denolf et al., 1997). Amino acids with dark backgrounds are conserved residues of the Zn$^{2+}$ binding motif, potential signal peptide sequences are underlined, sequenced N-terminus of *Epos* APN is boldly underlined, highly conserved residues have a grey background, and residues potentially part of the GPI anchor signal sequence and hydrophobic core sequence are boxed.

Unlike the APN expressed by Denolf et al. (1997), ligand blot assays with recombinant *Epos*APN showed that it was able to bind both Cry1Ac and Cry1Ba. However, in competitive binding assays using radiolabelled toxins Sf9 cells expressing *Epos*APN showed no difference in the ability to bind either Cry1Ac or Cry1Ba as compared to control Sf9 cells. Inconsistencies between ligand blot and competitive binding assays have been reported previously (Lee and Dean, 1996), but this result is more distinct: a protein which binds endotoxins by the ligand blot assay does not bind at all in competitive binding assay. This suggests that binding to aminopeptidase that is not bound to a lipid bilayer is artifactual. There are a number of possible explanations for the difference.
Table 3
Leucine aminopeptidase activities in Sf9 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>APN activity (nmol min$^{-1}$ mg cell protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected cells</td>
<td>9</td>
</tr>
<tr>
<td>pnlb-infected cells</td>
<td>11</td>
</tr>
<tr>
<td>EposAPN-infected cells</td>
<td>1720</td>
</tr>
<tr>
<td>Lysed EposAPN-infected cells</td>
<td>1744</td>
</tr>
</tbody>
</table>

Fig. 4. Ligand blot analysis of BBMV-purified EposAPN and recombinant EposAPN. The BBMV-purified protein is in Lanes 1 and 3, the recombinant protein from Sf9 cells in Lanes 2 and 4. Lanes 1 and 2 were probed with Cry1Ac; Lanes 3 and 4 with Cry1Ba.

APN and alkaline phosphatase increased Cry1Ac-induced efflux of Rb$^{2+}$ from phospholipid vesicles. Mason et al. (1995) showed, using surface plasmon resonance, that M. sexta APN was able to specifically bind Cry1Ac with a $K_D$ approximately the same as that determined by competitive binding assay. However, the study showed the existence of two binding sites on APN, whereas competitive binding studies showed only one (van Rie et al., 1989). These results have been criticised as being inconsistent and unqualitative (Schuck and Minton, 1996). Lee et al. (1996) showed that adding purified APN inhibited binding of Cry1Ac to L. dispar BBMV, but again free APN was used. Other studies have tried to show the role of APN by indirect methods, such as showing that pore-forming activity is related to the amount of APN activity, which was varied by cleavage of the GPI anchor (Lorence et al., 1997), or that binding of radiolabelled toxin decreases with incubation with enzymes that cleave GPI anchors or N-linked carbohydrate side chains (Denolf et al., 1997). These studies only show that the binding protein probably has a GPI anchor, and that binding involves N-linked carbohydrates, not that APN is the binding protein.

The competitive binding assay results suggest that the E. postvittana 120-kDa APN is not the specific receptor for δ-endotoxins. Other evidence which suggests that the 120-kDa APN does not act as a specific endotoxin receptor is given by Luo et al. (1997b), who found that the ligand blot binding of Cry1Ac to APN from P. xylostella was essentially unchanged between susceptible larvae and ones with over one thousand-fold resistance to Cry1Ac. In contrast, the cadherin-like receptor from M. sexta has been expressed in Sf9 cells, and both ligand blot and competitive binding assay results of the recombinant protein gave the same results as native protein (Francis and Bulla, 1997). This shows that expression of putative receptors to δ-endotoxins in Sf9 cells is able to
yield proteins able to bind the toxins. Thus the lack of binding seen by Denolf et al. (1997) and in this study is likely to be caused by an inherent lack of specific binding by APN rather than problems caused by expression within the SF9 cells.

The role APN plays in the mode of action of endotoxin toxicity remains questionable. Although it is clear that \(\delta\)-endotoxins do bind to at least some APNs in the gut of Lepidoptera it is not obvious whether this binding results in pore formation. APN might be part of a more complex array of proteins that together allow binding and pore formation, or perhaps the endotoxins are transferred to and bind other less-abundant proteins after first being associated with the carbohydrate side chains of APN. Or possibly APN has no role at all in the mode of action of \textit{B. thuringiensis} \(\delta\)-endotoxins.

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