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A 106 kDa Form of Aminopeptidase is a Receptor for *Bacillus thuringiensis* CryIC δ-Endotoxin in the Brush Border Membrane of *Manduca sexta*

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A soluble 106 kDa protein with aminopeptidase activity was isolated from *Manduca sexta* using CryIC toxin-affinity and anion-exchange chromatographies. Based on internal amino acid sequence analysis and different mobilities obtained with non-denaturing polyacrylamide gel electrophoresis, the 106 kDa aminopeptidase is distinct from a previously described 115 kDa CryIAc-binding aminopeptidase. The 106 kDa protein was preferentially precipitated by CryIC relative to CryIAc toxin. The 106 kDa form, like the 115 kDa aminopeptidase, has a cross-reacting determinant typical of a cleaved glycosyl-phosphatidylinositol (GPI) anchor. On ligand blots, CryIAc recognized membrane bound 120 and soluble 115 kDa aminopeptidases, but not the soluble 106 kDa form. The results show that CryIC and CryIAc δ-endotoxins recognize functionally related, but structurally distinct 106 kDa and 115 kDa isoforms of aminopeptidase in the *M. sexta* midgut.

A *Bacillus thuringiensis* CryIC δ-endotoxin is a lepidopteran-specific insecticidal protein with a toxic spectrum different from CryIA δ-endotoxins. CryIC has high activity against *Spodoptera* species, which are relatively resistant to CryIA toxins. CryIC remains active against several insect species that have acquired resistance to CryIA toxins (Ferre et al., 1991; Tabashnik et al., 1994). CryIC is released from *B. thuringiensis* crystals as a 135 kDa protoxin and cleaved to an active 62 kDa toxin in the alkaline insect midgut. Insecticidal activity is caused by toxin binding to midgut brush border membranes, then toxin integration into the membrane leading to pore formation, cell lysis and insect mortality (reviewed in Gill et al., 1992; Knowles and Dow, 1993).

CryIC and CryIA toxins recognize different sites in the larval midgut (Van Rie et al., 1990; Escriche et al., 1994). CryIC and CryIAc binding sites are functionally distinct. For example, CryIAc resistant insect species (*Plodia interpunctella* and *Plutella xylostella*) still bind and are killed by CryIC toxin. Several CryIC binding proteins have been described. Oddou et al. (1993) showed that CryIC toxin binds to a 40 kDa protein on ligand blots of brush border membrane vesicles (BBMV) from three *Spodoptera* species. Sanchis and Ellar (1993) observed CryIC binding to 40 and 110 kDa proteins in *S. littoralis* midgut BBMV using a combination of toxin-affinity selection and ligand blotting.

CryIAb and CryIAc binding proteins have been identified in *Manduca sexta* (Vadlamudi et al., 1993; Sangadala et al., 1994; Knight et al., 1994). The CryIAb binding protein is a 210 kDa cadherin-like glycoprotein (Vadlamudi et al., 1995), whereas the CryIAc binding protein is a 120 kDa aminopeptidase N (APN) (Sangadala et al., 1994; Knight et al., 1994). Recently, Knight et al. (1995) cloned a *M. sexta* aminopeptidase that may encode the CryIAc receptor. To date the only functional evidence that aminopeptidase is a toxin receptor comes from Sangadala et al. (1994) who reconstituted a mixture of 120 kDa APN and a 65 kDa protein into membrane vesicles and measured a 30% increase in CryIAc binding and a 1000-fold decrease in the amount...
of CryIAc toxin required to induce \(^{86}\text{Rb}^+\) efflux. The binding characteristics of CryIA toxins to the 115 kDa soluble form of the CryIAc receptor were determined using plasmon resonance spectroscopy (Masson et al., 1995). CryIAc binds at two sites, CryIAb and CryIAc recognize one site and CryIC does not bind to the 115 kDa molecule.

APN molecules themselves are of interest due to their important role in digestion. APN cleaves an amino acid from the N-terminus of proteins (Kenny et al., 1987). In bovine renal BBMV, APN appears to be associated with Na\(^+\)-dependent amino acid co-transport (Plakidou-Dymock et al., 1993). The enzyme is found in both soluble and membrane-bound ectoenzyme forms. The ectoenzyme form is attached to the intestinal brush border cells of many animals by a hydrophobic N-terminal stalk (Kenny et al., 1987; Takasaki et al., 1991). In insects, however, attachment is via a glycosyl-phosphatidylinositol (GPI) anchor (Tomita et al., 1994; Garcia and Adang, 1995). This observation agrees with the predicted amino acid sequence of the cloned aminopeptidase which has a typical carboxy terminal signal for attachment of the GPI anchor (Knight et al., 1995). M. sexta brush border cells have a phosphatidylinositol-specific phospholipase C (PIPLC) which can cleave the lipid anchor from the 120 kDa CryIAc-binding APN, releasing a 115 kDa form (Lu and Adang, 1996).

In this study we report that CryIC toxin binds to a 106 kDa form of APN in M. sexta midgut that is distinct from the CryIAc-binding APN previously identified in that species. The identification and purification of a CryIC-binding APN enables the comparison of the two APNs and elucidation of the determinants of toxin binding.

**MATERIAL AND METHODS**

**Materials**

All chemicals, except where noted, were purchased from Sigma. \(N,N'\)-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS), glycine, Triton X-100, SDS molecular weight protein marker, polyvinylidene difluoride (PVDF) membrane, EconoPac Q column and Bradford protein assay reagents were from Bio-Rad; BCA protein assay reagents were from Pierce.

**Toxin purification**

*B. thuringiensis* strains were grown at 28°C in 1/2-strength L-broth until sporulation and cell lysis. Toxin was purified by a modified procedure of Luo and Adang (1994). Prototoxin was released from the crystal–spore mixture with 50 mM Na\(_2\)CO\(_3\), pH 9.6, 0.1% 2-mercapto-ethanol for 2 h at room temperature, then activated to toxin by treating with trypsin (0.5%, w/w) at room temperature for 15 min. After passing through a 0.2 \(\mu\)m filter (Nalge), the trypsin-treated material was loaded on an EconoPac Q column pre-equilibrated with 20 mM Na\(_2\)CO\(_3\), pH 9.6. Toxin was eluted with a 0-0.6 M NaCl gradient in the equilibration buffer using fast-performance liquid chromatography (FPLC, Pharmacia). Fractions containing toxin were pooled, dialysed against 20 mM Na\(_2\)CO\(_3\), pH 9.6, overnight, and re-fractonated using the same condition as above on a Mono Q HR 5/5 column (Pharmacia). Purified toxin was stored in 20 mM Na\(_2\)CO\(_3\), pH 9.6, containing 120 mM NaCl at \(-20°C\).

**Preparation of BBMV**

*M. sexta* larvae were reared on artificial diet (Southland Products, Lake Village, AR) at 26°C, 70% r.h. with a photoperiod of 12:12 (L:D) h. Midguts were dissected from second day fifth instar larvae, and either immediately used to prepare BBMVs or stored at \(-80°C\). BBMVs were prepared according to Ferre et al. (1991) and stored in 0.3 M mannitol, 5 mM EGTA, 17 mM Tris–Cl, pH 7.5, at \(-80°C\) until needed.

**CryIC toxin affinity chromatography**

The affinity purification method of Knight et al. (1994) was adapted to CryIC toxin with modifications. CryIC toxin (10 mg) was coupled to 3 ml of cyanogen bromide-activated Sepharose 4B in 20 mM Na\(_2\)CO\(_3\), pH 9.6, according to the manufacturer’s instructions (Sigma). The gel was re-suspended in 20 mM Na\(_2\)CO\(_3\), pH 9.6, 200 mM NaCl, 5 mM EGTA, 0.1% 3-[(cholamidopropyl) dimethylammonio]-1-propane-sulphonate (CHAPS) (buffer A) and stored at 4°C. BBMV (15 mg) was solubilized in 10 ml of 1% CHAPS in buffer A containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 4 \(\mu\)M leupeptin, 10 \(\mu\)M aprotinin, and 100 \(\mu\)M pepstatin A, at 4°C for 30 min. The sample was centrifuged at 100 000 \(\times\) g at 4°C for 1 h and the supernatant applied to 3 ml of CryIC toxin affinity column. The gel–BBMV mixture was incubated overnight at 4°C with gentle rotation. After washing with 200 ml of buffer A, bound proteins eluted with 5 ml of 1 M NaSCN in buffer A. The eluate was immediately diluted to 10 ml with buffer A and then concentrated to 0.2 ml using a Centriprep-30 ultra-filtration device (Amicon) at 4°C. The sample was either used for analysis by SDS-PAGE or stored at \(-20°C\). Protein amounts were measured by Bio-Rad or BCA protein assay (for samples containing detergents) using bovine serum albumin as a standard.

**Purification of 106 kDa protein by anion-exchange chromatography**

BBMVs (15 mg) were solubilized in 20 mM MES buffer, pH 5.5, containing 2% Polyoxyethylene 23 lauryl ether (Brij 35) at 4°C for 30 min and centrifuged at
Purification of the 120 kDa and 115 kDa APNs

BBMVs were solubilized with 1% CHAPS in 20 mM Tris–HCl, pH 7.4, and centrifuged at 27,000 xg for 60 min. The supernatant was loaded onto an Econo Pac Q column pre-equilibrated with 20 mM Tris–HCl, pH 7.4, containing 0.1% Brij 35, and the sample loaded onto a DE 52 column pre-equilibrated with 20 mM Tris–HCl, pH 8.0, containing 0.1% Brij 35 (buffer B). The column flow-through containing 0.1% Brij 35. Column flow-through containing 106 kDa protein was dialysed against 20 mM Tris-HCl, pH 7.4, containing 0.1% Brij 35 (buffer C), and bound proteins eluted with 0–600 mM NaCl gradient in buffer C, analysed by SDS–PAGE and fractions containing 106 kDa protein stored at −80°C.

Indirect immunoprecipitation of toxin binding proteins

Indirect immunoprecipitation was done according to Oddou et al. (1991) with modifications. Anti-CryIC antisera (10 μl) was incubated with 200 μl of protein A-Sepharose 4B equilibrated at room temperature for 1 h. After three washes with buffer A, CryIC toxin (50 μg) was added, and then the mixture was incubated at room temperature for 2 h. The toxin–anti-toxin–bead complex was washed three times with buffer A and then mixed with CHAPS-solubilized BBMV (100 μg) or 125I–106 kDa protein (10^5 cpm). The mixture was incubated at 4°C for 2 h with rotation. After three washes with buffer A, radioactivity in the binding protein–toxin–antisera complex was measured on a Beckman Gamma 4000 counter, and then the binding protein–toxin–antisera complex was dissociated from the beads by heating in SDS–PAGE sample buffer at 100°C for 5 min and analysed by 10% SDS–PAGE. In control experiments, precipitation was performed as described above except that CryIC toxin or anti-CryIC antisera was omitted. Precipitation was also carried out in the presence of various concentrations of GalNAc or GlcNAc and APN inhibitors (see legend to Fig. 6).

RESULTS

Electrophoresis and ligand blot analysis

SDS–PAGE was performed according to Laemmli (1970). Non-denaturing PAGE was performed according to Souther and Wade (1989) using the gel electrophoresis conditions described in Lu and Adang (1996). Ligand blotting was performed as described in Garczynski et al. (1991).

N-Terminal amino acid sequence analysis

Purified 106 kDa protein was resolved by SDS–PAGE and electrophorobotted onto PVDF membrane. The 106 kDa protein was partially digested with Staphylococcus aureus V8 protease according to Cleveland et al. (1977). The digested peptides were separated by SDS–PAGE, and then blotted to PVDF membrane. The blotted filter was stained with amido black and stained bands were excised and submitted for amino acid sequencing, as described by Sangadala et al. (1994).

APN assays

APN activity was assayed using L-leucine-β-nitroanilide (LpNA) as substrate as described by Garczynski and Adang (1995). APN activity staining of non-denaturing gels was according to Ward (1975). L-leucine-β-naphthylamide was freshly prepared as a 0.5 M stock solution in 95% ethanol then diluted to 5 mM in 20 mM sodium phosphate buffer, pH 8.0. The non-denaturing gel was immersed in 5 mM L-leucine-β-naphthylamide for 5 min and bands were visualized with 0.15% fast garnet GBC in 20 mM sodium phosphate buffer, pH 8.0.

PIPLC digestion and GPI anchor detection

Purified 106 kDa protein (1 μg) was digested with 1 unit of PIPLC at 30°C for 1 h and then separated by SDS–PAGE. After transfer onto nitrocellulose membrane, the presence of a cleaved GPI group was detected using a polyclonal antibody against a cross-reacting determinant (CRD) of GPI anchored proteins as described in Garczynski and Adang (1995) (anti-CRD sera was kindly provided by K. Mensa-Wilmot, University of Georgia).

Purification of CryIC binding protein

CryIC toxin affinity chromatography was used to purify a toxin binding protein from M. sexta midgut brush border membranes. CHAPS-solubilized BBMV were applied to the CryIC affinity column, and bound proteins were eluted with 1 M NaSCN. Fig. 1 (Lane 5) shows a 106 kDa protein that was present in the eluate. A considerable amount of 106 kDa protein was also visible in Lane 4 (not bound). The purification of a 106 kDa protein was not expected, based on previous studies identifying a 40 kDa CryIC binding protein in several insect species. CryIC toxin from the column and a slight amount of a 115 kDa protein were also present in the eluate.
formed the same experiment using ~251-106 kDa protein and less than 5% of the labeled protein was precipitated, and CryIAc toxin-anti-CryIAc Sepharose beads. In this experiment, approximately 16% of the input ~251-106 kDa protein was precipitated. To test the specificity of toxin binding we performed autoradiography showing that the labeled 106 kDa protein bound to CryIC [Fig. 2(B), Lane 2]. Approximately 16% of the input 125I-106 kDa protein was precipitated. To test the specificity of toxin binding we performed the same experiment using 125I-106 kDa protein and CryIAc toxin-anti-CryIAc Sepharose beads. In this case, less than 5% of the labeled protein was precipitated, and a faint band was visible on the autoradiograph [Fig. 2(B), Lane 3]. When anti-CryIC antiserum was omitted from the precipitation step, only a trace of 125I-106 kDa protein was detected [Fig. 2(B), Lane 4]. The 106 kDa protein purified by conventional chromatography was used in subsequent experiments.

Since CryIAc toxin binds to the PIPLC cleaved soluble 115 kDa form of APN (Garzynski and Adang, 1995; Lu and Adang, 1996), we were concerned that the 106 and 115 kDa proteins were isoforms of the same molecules. We compared the migration of purified 115 kDa APN, 106 kDa protein and the membrane bound 120 kDa APN by SDS–PAGE [Fig. 3(A)] and 125I-CryIAc ligand blotting [Fig. 3(B)]. Each protein had a unique migration pattern and 125I-CryIAc toxin recognized only the 120 and 115 kDa proteins, but not the 106 kDa protein [Fig. 3(B), Lane 3].

**Identification of the 106 kDa protein as an APN**

The 106 kDa protein had an N-terminus of DPAYRLPTTTT. This amino acid sequence is nearly identical to the N-terminus of DPSYRLPTTTT of the 120 kDa (Sangadala et al., 1994; Knight et al., 1994) and processed 115 kDa APN (Lu and Adang, 1996). The sequencing result was confirmed by testing for aminopeptidase activity. The 106 kDa protein hydrolysed LpNA with a specific activity of 48.61 µmol/min/mg, representing a 19.3-fold enrichment compared to M. sexta crude BBMV (Table 1). The APN activity of the 106 kDa protein was inhibited by amastatin and EDTA.

**GPI anchor property of the 106 kDa APN**

Garzynski and Adang (1995) demonstrated that the 120 kDa CryIAc-binding APN has an intact C-terminal GPI anchor. In the 115 kDa APN, this GPI anchor was cleaved. The 106 kDa APN was tested for a GPI anchor by probing a filter blot of PIPLC-treated and untreated 106 kDa APN with anti-CRD antibody. Anti-CRD antibody detect the modified inositol product generated by PIPLC cleavage. As shown in Fig. 4, both PIPLC-treated and untreated 106 kDa APN reacted with the anti-CRD antibody [Fig. 4(B), Lane 2], indicating that 106 kDa APN has a GPI anchor and the anchor has been pre-cleaved.

**Comparison of 106 and 115 kDa forms of APN**

The 106 and 115 kDa APN molecules both have cleaved GPI anchors, yet bind CryIC and CryIAc, respectively. We used non-denaturating PAGE to reveal physical differences between purified APNs. The 120 kDa APN with an intact anchor was included for comparison. The gel stained for APN activity [Fig. 5(B)] shows the position of the proteins more clearly than the Coomassie blue stained gel [Fig. 5(A)]. The 120 and 115 kDa APNs had the slowest and fastest mobilities with the 106 kDa protein migrating to an intermediate position. The 106 kDa protein Lane also shows two faint bands on the stained gel (panel A) that had high APN activity.

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**FIGURE 1. SDS-PAGE (10%) of CryIC toxin-affinity and anion-exchange purified 106 kDa protein.** (A) Lane 1, molecular size marker (kDa); Lane 2, M. sexta BBMV protein; Lane 3, CHAPS-solubilized BBMV protein; Lane 4, non-bound fraction from the CryIC affinity column; and Lane 5, proteins eluted from toxin column with 1 M NaSCN. (B) Lane 1, molecular size markers. Lane 2, the 106 kDa protein yield from DE 52 flow-through/60°C treatment/anion exchange column (Mono Q) procedure. Lane 3, the 106 kDa protein enriched fraction after DE 52 flow-through and 60°C treatment.

Our previous experience in purifying the 115 kDa CryIAc binding protein suggested that conventional chromatography can be more efficient than CryIC affinity columns (Lu and Adang, 1996). We developed an alternative method combining heat treatment and conventional chromatographies to isolate the 106 kDa protein. Solubilized BBMV were passed through a DE 52 column at pH 5.5, a condition where most proteins, but not the 106 kDa protein, bound to the column [Fig. 1(B), Lane 3]. Since Hajjou and Le Gal (1994) and Klinkowstrom et al. (1994) reported aminopeptidase was heat stable at 50–60°C, we included a 60°C treatment of the DE 52 flow-through and 60°C treatment. After a second ion-exchange chromatography (Mono Q) procedure, Lane 3, the 106 kDa protein enriched fraction after DE 52 flow-through and 60°C treatment.

Indirect immunoprecipitation was used to verify that the 106 kDa protein isolated by conventional chromatography was a CryIC binding protein. CryIC bound to anti-CryIC Sepharose precipitates a 106 kDa protein from CHAPS-solubilized BBMV [Fig. 2(A), Lane 1]. The same CryIC precipitation was performed with 125I-labeled 106 kDa protein. Precipitates were counted in a gamma counter then analysed by SDS-PAGE followed by autoradiography showing that the labeled 106 kDa protein bound to CryIC [Fig. 2(B), Lane 2]. Approximately 16% of the input 125I-106 kDa protein was precipitated. To test the specificity of toxin binding we performed the same experiment using 125I-106 kDa protein and CryIAc toxin-anti-CryIAc Sepharose beads. In this case, less than 5% of the labeled protein was precipitated.
FIGURE 2. Indirect immunoprecipitation of a 106 kDa protein with CryIC toxin. CHAPS-solubilized BBMV (A) or \(^{125}\)I-106 kDa protein (B) was added to a complex of toxin–antibody–protein A–Sepharose. Precipitate was analysed by SDS–PAGE and autoradiography. (A) Coomassie blue stained gel. Lane 1, anti-CryIC toxin precipitate; Lane 2, CryIC toxin. (B) Autoradiograph. Lane 1, \(^{125}\)I-106 kDa protein; Lane 2, anti-CryIC toxin precipitate; Lane 3, anti-CryIAc toxin precipitate. Lane 4, anti-CryIC antibody was omitted from the precipitation step. The protein markers are the same as in Fig. 1.

FIGURE 3. SDS–PAGE and ligand blot analyses of purified 120, 115 and 106 kDa proteins. (A) Coomassie blue stained SDS–PAGE. Lane 1, M. sexta BBMV protein; Lane 2, 120 kDa protein; Lane 3, 115 kDa protein; Lane 4, 106 kDa protein and Lane 5, molecular size markers. (B) \(^{125}\)I CryIAc ligand blot. Lane 1, BBMV protein; Lane 2, 120 kDa protein; Lane 3, 106 kDa protein; Lane 4, 115 kDa protein.

activity (panel B). These fast-migrating species may be degradation products of the 106 kDa protein.

The differences between 106 and 115 kDa APNs were further analysed by peptide mapping and internal amino acid sequence analysis. Both proteins were digested by V8 protease and resolved by SDS–PAGE. The peptide mapping results showed different band patterns for the two APNs (data not shown). The N-terminal amino acid sequence was determined for the three largest peptides from the digested 106 kDa APN sample. The N-termini of the 70, 60 and 45 kDa peptides were QNMIALE. This sequence is not present in the published M. sexta APN sequence (Knight et al., 1995). Based on these data, we conclude that the 106 and 115 kDa are isoforms of APN that differ in physical, structural and toxin-binding characteristics.

Molecules that do not block CryIC binding

Since an amino sugar side chain (GalNAc) is involved in CryIAc binding to 120/115 kDa APNs (Masson et al., 1995; Garczynski and Adang, 1995; Knowles et al., 1991), we tested for amino sugar involvement in CryIC binding. Unlike CryIAc affinity chromatography of the 115 kDa APN, the 106 kDa APN could not be eluted from the CryIC column by GalNAc (data not shown). Neither GalNAc or GlcNAc significantly reduced CryIC binding to \(^{125}\)I-106 kDa protein in the indirect immunoprecipitation assay [Fig. 6(A), Lanes 3 and 4].

We also investigated whether the binding of CryIC toxin was to the active site of the 106 kDa APN. Toxin precipitation of \(^{125}\)I-106 kDa APN was performed in the presence or absence of amastatin and EDTA. While both inhibitors greatly reduced APN activity (Table 1), neither inhibitor significantly decreased CryIC binding to the 106 kDa APN [Figure 6(B), Lane 3 and 4]. Figure 6(B) (Lane 5) is a negative control showing when CryIC toxin was omitted. \(^{125}\)I-106 kDa protein was not precipitated by anti-CryIC antiserum Sepharose beads.
TABLE 1. Effects of inhibitors on aminopeptidase activity in the BBMV and purified protein preparations

| Treatment                  | M. sexta BBMV Specific activity(μmol/min/mg) | CryIC column eluate | Purified 106 kDa protein | Porcine microsomal APN |
|----------------------------|---------------------------------------------|--------------------|--------------------------|-------------------------|
| No inhibitor               | 2.52±0.02                                   | 15.05±1.10         | 48.61±0.01               | 21.23±1.78              |
| 1 mM amastatin             | 0.01±0.01                                   | 0.10±0.01          | 0.37±0.01                | 0.05±0.00               |
| 5 mM EDTA                  | 0.24±0.06                                   | 2.85±0.54          | 7.88±0.21                | 5.29±0.31               |

CryIC binding enhances aminopeptidase activity

CryIC was added in the concentration range 0–500 nM to LpNA assay mixture containing either 106 kDa APN, 115 kDa APN or porcine microsomal APN (Fig. 7). The relative 106 kDa APN activity increased with increasing concentration of CryIC toxin up to 20 nM. A further increase in toxin above 80 nM lowered the enzyme activity. We tested the same amounts of CryIAc toxin with the three APNs and detected enhancement of 115 kDa APN activity as reported previously (Sangadala et al., 1994). There was some enhancement of 106 kDa APN activity by CryIAc. The effect of BSA was negligible relative to the stimulatory effects of CryIC on 106 kDa APN and CryIAc on 115 kDa APN activity.

DISCUSSION

A 106 kDa form of APN in M. sexta brush border membranes was purified and shown to function as a CryIC binding protein. This protein is an isoform of M. sexta 120/115 kDa APN identified as a CryIAc toxin binding protein. CryIC binding to the 106 kDa APN was not blocked by GalNAc or GlcNAC, indicating that these carbohydrates are not involved in binding, as reported for the CryIAc binding APN. APN inhibitors greatly reduced the activity of the 106 kDa APN but had no
FIGURE 6. Effects of amino sugars and APN inhibitors on CryIC toxin binding to the 106 kDa APN. 125I-106 kDa protein was precipitated using anti-CryIC-toxin–protein A-Sepharose in the presence or absence of amino sugars and APN inhibitors. The precipitate was analysed by SDS-PAGE followed by autoradiography. (A) Lane 1, 125I-106 kDa APN; Lane 2, precipitation without amino sugar; Lane 3, with 200 mM GalNAc; Lane 4, with 200 mM GlcNAc. (B) Lane 1, 125I-106 kDa APN; Lane 2, without APN inhibitor; Lane 3, with 1 mM amastatin; Lane 4, with 5 mM EDTA; Lane 5, CryIC toxin was omitted from the precipitation step.

Effect on CryIC toxin binding. CryIC toxin enhances enzyme activity in the nanomolar toxin range. The 106 kDa APN was also shown to have a cleaved GPI anchor.

Previous studies showed that CryIC recognized a 40 kDa protein in three Spodoptera species and Heliothis virescens (Oddou et al., 1993; Sanchis and Ellar, 1993). The difference between our results and prior studies may be due to several factors. (A) CryIC binding proteins may differ between insect species. (B) Previous investigations of CryIC binding studies were based on ligand blotting. We were unsuccessful using this technique either with 125I-labeled CryIC toxin or anti-CryIC antibody detection. (C) It is possible a 40 kDa binding protein was not solubilized. We believe that was not the case, since previous investigators also used CHAPS for BBMV solubilization (Sanchis and Ellar, 1993). (D) The CryIC binding APN in the previous studies may have been partially degraded. Additional studies are needed to resolve apparent differences in CryIC binding proteins across insect taxa.

APN is an endopeptidase which preferentially cleaves peptides with N-terminal neutral amino acids (Kenny et al., 1987). The N-termini of most activated CryI toxins are neutral amino acids such as leucine and isoleucine (Hofte and Whiteley, 1989; Luo and Adang, unpublished data). One possibility is that APN binding to CryI toxins may be a consequence of the enzyme's N-terminal trimming reaction following endoprotease cleavage. Sangadala et al. (1994) and Knight et al. (1994) argued against this model and proposed that CryIAc toxin binds elsewhere on the APN. In this study, we showed that amastatin, a competitive active-site inhibitor, did not block CryIC binding to 106 kDa APN (Fig. 6), suggesting that the toxin does not bind to the active site. A more complicated explanation of the amastatin experiment involves a consideration of how toxin binding activates APN. CryIC and CryIAc binding to 106 kDa and 115 kDa APN molecules enhanced enzymatic activity against LpNA (Fig. 7). This agrees with our previous study of CryIAc and 120 kDa APN (Sangadala et al., 1994). Toxin-induced enzyme enhancement may be analogous to the effect cer-
tain peptide inhibitors have on microsomal aminopeptidase. Certain analogs of APN inhibitors stimulate rather than decrease enzymatic activity (Taylor, 1993). DiGregorio et al. (1988) explains this unexpected activation as due to the existence of two substrate binding sites in close proximity on APN. CryI toxin binding to APN may alter the fit of LpNA into the active site. This could be due to binding of the toxin’s N-terminus at an active site or near the active site. Similarly, a swine-specific coronavirus uses intestinal APN as an adventitious receptor without involving the enzyme’s active site (Delmas et al., 1994).

GalNAc is part of the structure on the 120/115 kDa APN recognized by CryIAc toxin (Garczynski et al., 1991; Knowles et al., 1991; Lu and Adang, 1996), but this amino sugar is not involved in CryIC binding to the 106 kDa protein [Fig. 6(A)]. This result is similar to those obtained by Masson et al. (1995) showing that GalNAc blocks CryIAc, but not CryIAc and CryIAb binding to 115 kDa APN. CryIAc may interact with an unidentified sugar residue on 106 kDa APN, based on a recent paper by Shimizu et al. (1994). The authors proposed that the unusual ‘Greek key’ structure of CryIIIA domain II (Li et al., 1991) is folded like vitelline membrane protein I from hens’ eggs. This unusual folding pattern is also present in CryIAb toxin and probably a conserved topology of CryI toxins (Grocshulski et al., 1995). Vitelline membrane protein I reportedly can bind epithelial cells and has oligosaccharide transferase activity (Shimizu et al., 1994).

Three forms of APN have been identified in M. sexta. Garczynski and Adang (1995) showed that the 120 kDa membrane-bound form is released as a soluble 115 kDa form by PIPLC treatment. Lu and Adang (1996) showed an endogenous PIPLC makes the same conversion in solubilized BBMV. Since the 106 kDa form has a cleaved GPI anchor, M. sexta PIPLC is also acting on the membrane-bound form of the 106 kDa APN. We have not yet determined the molecular size of the 106 kDa APN with the intact GPI anchor.

The 106 and 115 kDa soluble aminopeptidases share common features yet have fundamental differences. Common features included: the similarity of their N-termi- nini; both APN forms are attached to the membrane via a GPI anchor, and both forms have high LpNA activity (48.6 μmol/min/mg for the 106 kDa APN compared to 70.4 μmol/min/mg for 115 kDa APN). Toxin binding is the obvious distinguishing feature: 106 kDa APN binds CryIC and not CryIAc, while the 115 kDa APN binds CryIAc and not CryIC. Also, their migrations are slightly distinguishable by SDS–PAGE. However, non-denatur- ing PAGE revealed distinct mobilities for the 115 and 106 kDa APNs (Fig. 5). The internal sequence for the 106 kDa protein was not present in a published APN sequence (Knight et al., 1995). Based on these data, we conclude that the 106 and 115 kDa proteins are isoforms of APN. Multiple aminopeptidase isoforms in insects have been described (Klinkowstrom et al., 1994; Ferreira et al., 1994), but these proteins have not been isolated or characterized in detail.

The molecular basis of isoforms is well studied for a few proteins (Fishman, 1990). The diversity of alkaline phosphatases in rats arises from two genes for individual isozymes being differentially regulated in intestinal tissue (Engle and Alpers, 1992) and expressed as membrane-bound forms attached via a GPI linkage (Engle et al., 1995). Another factor in isozyme diversity is that the native translation products of two genes can be similar in molecular size, but due to slight differences in amino acid sequence, the proteins are glycosylated in dramatically different ways (Engle et al., 1995). In the case of M. sexta, we favor a hypothesis that two mRNA encode the related APN molecules, based on the peptide sequence from the 106 kDa protein not being present in the published sequence. This conclusion is also supported by the physical properties (including different heat stability and mobility on non-denaturing PAGE) of the 106 and 115 kDa proteins.

The successful purification of the CryIC-binding APN, and its identification as an isoform unique from the CryIA-binding APN, will facilitate future comparative studies of these CryIA and CryIC receptors. An understanding of the mechanisms in insects which produce multiple APNs may clarify the complicated relationship between δ-endotoxins and susceptible lepidopteran larvae.

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