The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N

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Summary

A 120 kDa glycoprotein in the larval midgut membrane of the lepidopteran *Manduca sexta*, previously identified as a putative receptor for *Bacillus thuringiensis* CryIA(c) δ-endotoxin, has been purified by a combination of protoxin affinity chromatography and anion exchange chromatography. In immunoblotting experiments, the purified glycoprotein has the characteristics predicted of the receptor: it binds CryIA(c) toxin in the presence of GlcNAc but not GalNAc; it binds the lectin SBA; but it does not bind CryIB toxin. N-terminal and internal amino acid sequences obtained from the protein show a high degree of similarity with the enzyme aminopeptidase N (EC 3.4.11.2). When assayed for aminopeptidase activity, purified receptor preparations were enriched 5.3-fold compared to *M. sexta* brush border membrane vesicles. We propose that the receptor for CryIA(c) toxin in the brush border membrane of the lepidopteran *M. sexta* is the metalloprotease aminopeptidase N.

Introduction

The target of lepidopteran active *Bacillus thuringiensis* (Bt) insecticidal crystal δ-endotoxins is the apical (brush border) membrane of larval midgut cells (Percy and Fast, 1983; Singh *et al.*, 1986; Bauer and Pankratz, 1992). While different Bt toxins have significantly different insecticidal spectra *in vivo*, in most, but not all cases so far studied, the activity spectrum correlates with the presence of specific receptors in the brush border membrane vesicle (BBMV) preparations from susceptible insects (Wolfersberger *et al.*, 1987; Hofmann *et al.*, 1988; Van Rie *et al.*, 1989; 1990; Wolfersberger, 1990). BBMVs from the insect gut thus constitute a powerful system in which receptor

binding and mode of action of Bt toxins can be studied *in vitro*.

Previously we employed ligand blotting to visualize CryIA(c) toxin-binding proteins in BBMVs from the susceptible lepidopteran *Manduca sexta*, and identified a 120 kDa glycoprotein as a putative receptor for this toxin (Knowles *et al.*, 1991). The finding that this glycoprotein bound an N-acetyl galactosamine (GalNAc)-specific lectin was significant, since we also demonstrated that the toxicity of CryIA(c) for a susceptible insect cell line could be reduced by preincubation with GalNAc and that binding of CryIA(c) to *M. sexta* BBMVs was blocked by GalNac but not by N-acetyl glucosamine (GlcNAc) (Knowles *et al.*, 1991). This suggests that the receptor is a GalNac-bearing glycoconjugate, and that GalNAc is directly involved in the binding of CryIA(c) toxin to its receptor in *M. sexta*. In addition, Garczynski *et al.* (1991), using ^{125}I-labelled CryIA(c) toxin, identified the same 120 kDa protein as a putative receptor in *M. sexta* BBMVs.

We now report the purification of this 120 kDa putative receptor by a combination of protoxin affinity chromatography and anion-exchange chromatography. N-terminal and internal amino acid sequencing reveal amino acid sequence similarity with the known sequences of aminopeptidase N (microsomal aminopeptidase; α-amino-acyl-peptide hydrolase (microsomal); EC 3.4.11.2) from a variety of organisms. In order to explore the possibility that the CryIA(c) receptor is in fact aminopeptidase N, purified receptor protein preparations were assayed for aminopeptidase activity.

Results

Purification of the receptor

M. sexta BBMVs were solubilized in the detergent CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propane-sulphonate) and applied to a CryIA(c) protoxin affinity column. The 120 kDa glycoprotein, which had been previously identified as a possible receptor for CryIA(c) toxin in *M. sexta* BBMVs (Knowles *et al.*, 1991; Garczynski *et al.*, 1991), was specifically eluted with 200 mM GalNAc (Fig. 1A). Immunoblotting experiments demonstrated that this 120 kDa protein bound CryIA(c) toxin (Fig. 1C, lane 6) and also that activated CryIA(c) toxin was being
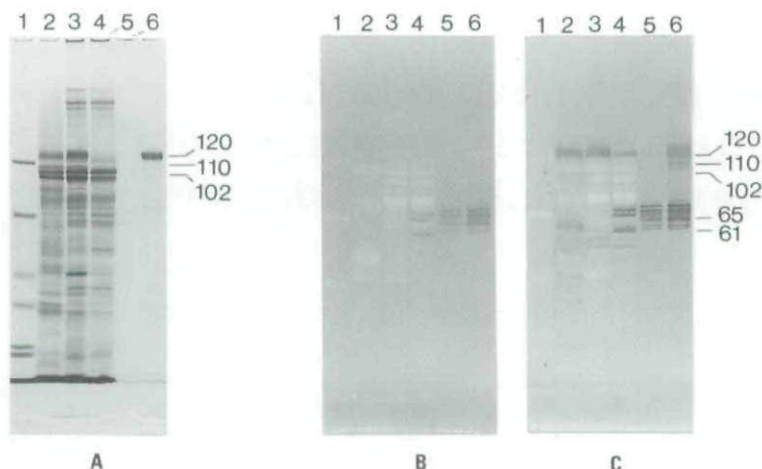
Fig. 1. CrylA(c) protoxin affinity chromatography. Selected fractions from CrylA(c) protoxin affinity chromatography were subjected to SDS (10%)–PAGE and duplicates transferred to nitrocellulose.
A. Coomassie brilliant blue stained gel.
B. Nitrocellulose filter incubated with antibodies alone to detect toxin from the column, as described in the *Experimental procedures*.
C. The same filter re-incubated with CrylA(c) toxin, then developed with antibodies as above to identify toxin-binding proteins. Lane 1, molecular mass markers (116, 66, 45, 36, 29 and 24 kDa); lane 2, CHAPS-solubilized BBMVs, $100\,000 \times g$ pellet; lane 3, CHAPS-solubilized BBMVs, $100\,000 \times g$ supernatant; lane 4, breakthrough (unretarded) fraction from affinity chromatography; lane 5, 0.5 M KCl wash fraction; lane 6, 0.2 M GalNAc-eluted peak.

leached from the column (Fig. 1B, lanes 4–6). One or two minor proteins (110 and 102 kDa) were sometimes co-eluted from the column, depending on the BBMV preparation and the age of the column. Both bind toxin in immunoblots (Fig. 1C, lane 6), and are probably breakdown products of the intact 120 kDa receptor, since neither band can reproducibly be detected in immunoblots of freshly solubilized BBMVs (Fig. 1C, lanes 2 and 3).

Activated toxin affinity columns proved to be inferior to protoxin columns, resulting in very low yields of 120 kDa receptor, probably owing to low concentrations of immobilized toxin (data not shown). The protoxin affinity column suffers the disadvantage of continuously leaching activated CrylA(c) toxin (Fig. 1B; see above), which was subsequently removed from receptor preparations by anion exchange chromatography (Fig. 2). The 120 kDa receptor glycoprotein was recovered in the run-through fractions, whilst toxin was retained on the column, and specifically eluted at 500 mM KCl. As judged by SDS–PAGE and silver staining, the final 120 kDa receptor preparation (post-anion exchange) is greater than 95% pure (Fig. 2, lane 4).

Ligand blotting

BBMVs and purified receptor from *M. sexta* were resolved by SDS–PAGE, transferred to nitrocellulose and characterized according to their ability to bind two toxins under a variety of conditions. CrylA(c) toxin bound to a major band of 120 kDa and, when present (see above), minor bands of 110 and 102 kDa in the purified receptor preparation, and to a major band of 120 kDa and minor bands of 60 and, when present, 110 and 102 kDa in BBMVs (Fig. 3B). Binding to all of these bands in both the purified receptor preparation and the BBMVs was abolished by GalNAc (Fig. 3C), but unaffected by GlcNAc (Fig. 3D). CrylB toxin did not bind any proteins in either BBMVs or purified receptor preparations (Fig. 3E). The GalNAc-specific lectin soybean agglutinin (SBA) bound only to the 120 kDa band in receptor preparations, and to a major band of 120 kDa and minor bands of 62 and 53 kDa in BBMVs (Fig. 3F).

Amino acid sequencing

N-terminal amino acid sequence was determined from the partially purified (post-protoxin affinity chromatography)
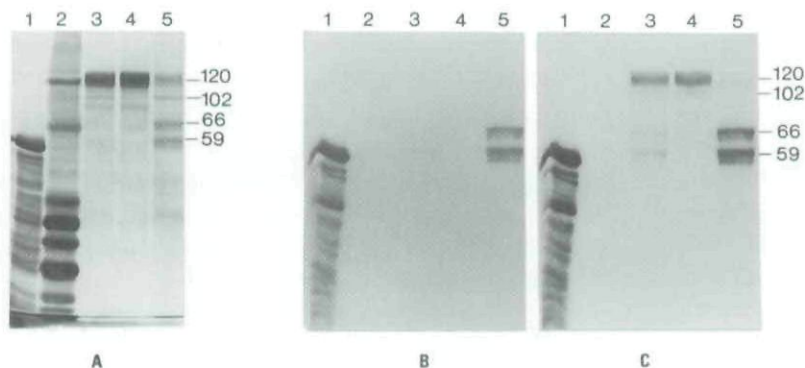
Fig 2. Anion exchange chromatography. Selected fractions from Mono Q anion exchange chromatography were subjected to SDS (10%)–PAGE and duplicates transferred to nitrocellulose.
A. Silver stained gel.
B. Nitrocellulose filter incubated with antibodies alone to detect toxin from the column, as described in the *Experimental procedures*.
C. The same filter re-incubated with CrylA(c) toxin, then developed with antibodies as above to identify toxin-binding proteins. Lane 1, CrylA(c) toxin; lane 2, molecular mass markers (116, 66, 45, 36, 29 and 24 kDa); lane 3, GalNAc-eluted fraction from protoxin affinity column; lane 4, breakthrough (unretarded) fraction from anion exchange chromatography; lane 5, retarded fraction, eluted at 500 mM KCl.
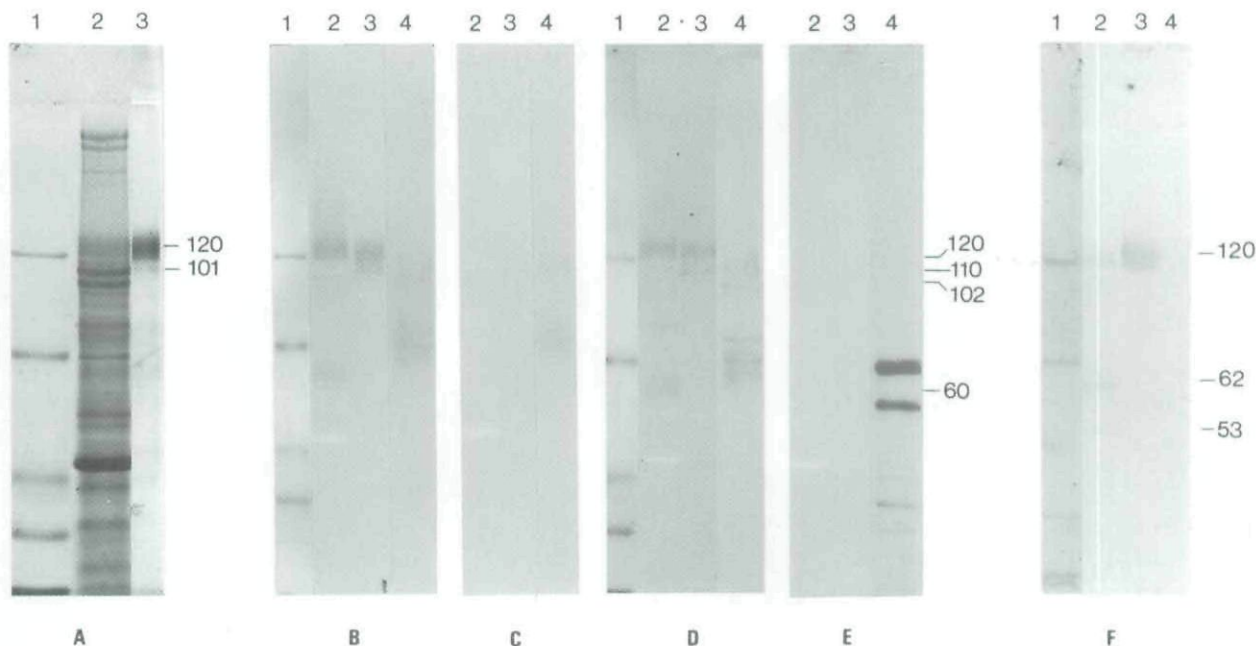
Fig. 3. Ligand blotting. Purified receptor from Mono Q anion exchange chromatography was subjected to SDS (10%)–PAGE and duplicates transferred to nitrocellulose.
A. Coomassie brilliant blue stained (lanes 1 and 2) or silver stained (lane 3) gel.
B–F. Nitrocellulose filters incubated with (B) CryIA(c) toxin, (C) CryIA(c) plus 200 mM GalNAc, (D) CryIA(c) plus 200 mM GlcNAc, (E) CryIB toxin and (F) SBA, peroxidase linked. All filters were developed with antibodies under exactly the same conditions as described in the *Experimental procedures.* Lane 1, molecular mass markers (116, 66, 45, 36 and 29 kDa); lane 2, *M. sexta* BBMVs; lane 3, purified (post-anion exchange) receptor preparation; lane 4, CryIA(c) toxin (B–D and F), or CryIB toxin (E).

receptor, resolved by SDS–PAGE and electroblotting. The Coomassie brilliant blue-stained 120 kDa band was excised from the membrane and sequenced (*N*-terminus, Fig. 4). Internal amino acid sequence was obtained from a tryptic digest of the purified (post-anion exchange chromatography) receptor, resolved by reverse-phase chromatography. Two peaks containing single peptides (as judged by capillary electrophoresis) were *N*-terminally sequenced (73 and 77; see Fig. 4). Sixteen heterogeneous peaks were re-chromatographed on a second reverse-phase column under different conditions, and the resulting peaks re-assessed by capillary electrophoresis. Six of these peptides yielded amino acid sequence (45.6, 61.4, 61.11, 61.14, 63.11 and 68.5; see Fig. 4). A suspected overlap between the *N*-terminal and one of the internal sequences (77) was confirmed by 'nested' polymerase chain reactions (PCR) with fully degenerate primers designed from the two stretches of amino acid sequence (Walker *et al.*, 1992; see Fig. 4).
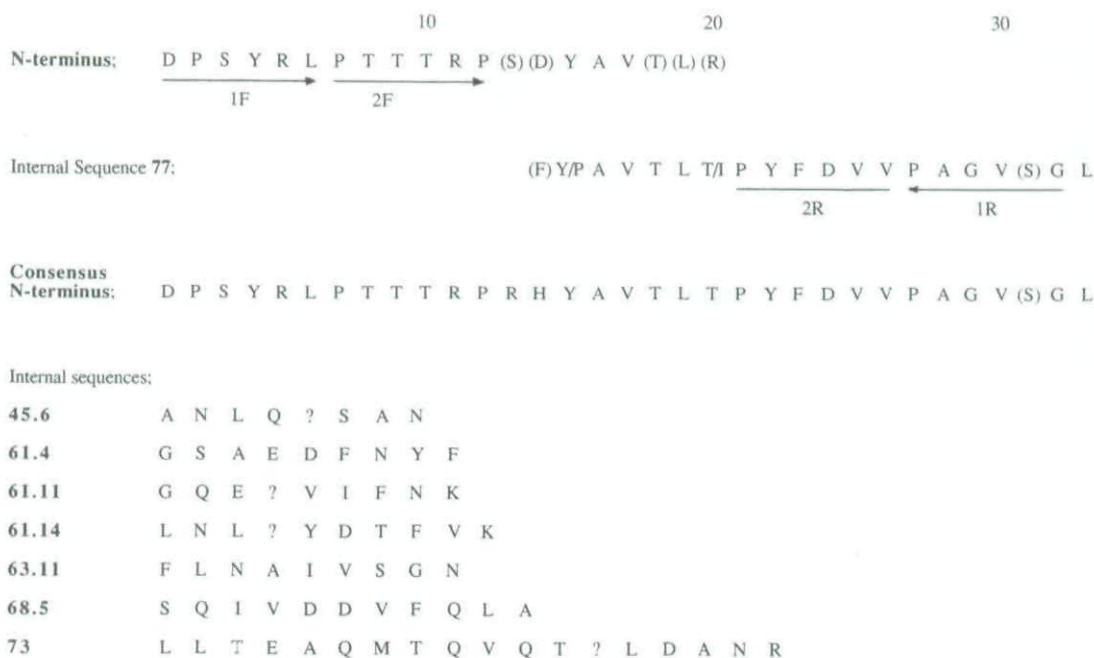
Amino acid sequence comparison

Amino acid sequences from the 120 kDa receptor were used to search the SWISS-PROT protein database. the *N*-terminal and one of the internal amino acid sequences (68.5, see Fig. 4) showed sequence similarity with the aminopeptidase N family (microsomal aminopeptidase; EC 3.4.11.2). This enzyme has been fully or partially cloned from a number of organisms, and a multiple alignment of the protein sequences derived from these clones has been constructed (Tan *et al.*, 1992). The sequence similarities between the receptor-derived peptide fragments and this alignment are shown in Fig. 5.

Assay of aminopeptidase activity

To confirm that the 120 kDa CryIA(c) receptor is aminopeptidase N, we assayed purified (post-anion exchange chromatography) receptor preparations for enzyme activity by the method of Hafkenscheid (1984). Both *M. sexta* BBMVs and the purified *M. sexta* receptor hydrolysed L-leucine-*p*-nitroanilide, a chromogenic substrate specific for aminopeptidase N (Kramer and Robinson, 1979). The specific activity was 7.36 μmol min^{-1} mg protein^{-1} for *M. sexta* BBMVs, and 39.13 μmol min^{-1} mg protein^{-1} for the purified receptor (Table 1). This represents a 5.3-fold enrichment in aminopeptidase activity in the purified receptor preparations compared to BBMVs. The purity of the receptor preparation was indicated by the absence of detectable alkaline phosphatase activity, commonly used as a midgut brush border membrane marker (Wolfersberger, 1984).

                                    10   20   30

N-terminus: D P S Y R L P T T T R P (S) (D) Y A V (T) (L) (R)
                 ────────────────→ ─────────────────→
                         1F   2F

Internal Sequence 77:   (F) Y/P A V T L T/I P Y F D V V P A G V (S) G L
                                                                      ──────────────→ ←──────────────
                                                                            2R   1R

Consensus
N-terminus: D P S Y R L P T T T R P R H Y A V T L T P Y F D V V P A G V (S) G L

Internal sequences:

45.6   A N L Q ? S A N

61.4   G S A E D F N Y F

61.11   G Q E ? V I F N K

61.14   L N L ? Y D T F V K

63.11   F L N A I V S G N

68.5   S Q I V D D V F Q L A

73   L L T E A Q M T Q V Q T ? L D A N R

Fig. 4. *N*-terminal and internal amino acid sequences from purified receptor as described in the *Results* section. A suspected overlap between the *N*-terminus and one of the internal sequences (77) was confirmed by PCR (Walker *et al.*, 1992; see the *Experimental procedures*) to give a consensus *N*-terminus. All oligonucleotides were designed from the known amino acid sequence (underlined), and were fully degenerate.
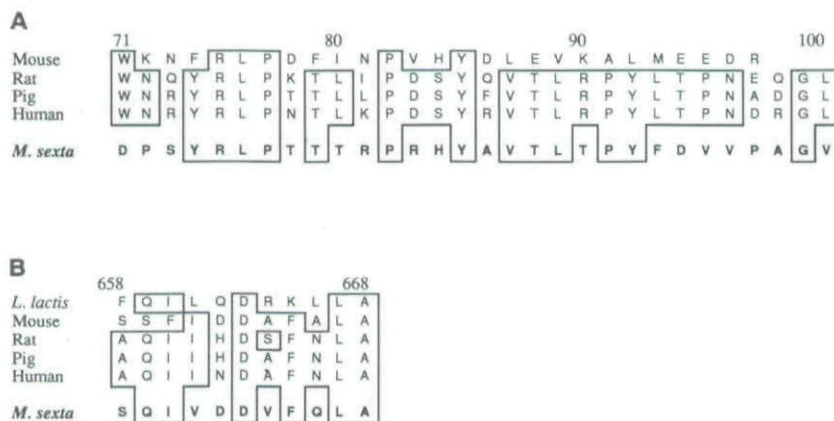
Table 1. Specific activity and enrichment of enzymes in *M. sexta* BBMVs and purified receptor preparations.

	Specific Activity		
	BBMVs	purified receptor	Enrichment
Aminopeptidase N	7.36	39.13	5.3
Alkaline phosphatase	0.56	0.00	–

Specific activity is expressed in μmol substrate hydrolysed min^{-1} mg protein^{-1}. Aminopeptidase N values are the means of four experiments at three (BBMV) or two (purified receptor) protein concentrations. Alkaline phosphatase values are the means of two experiments at one (BBMV) or two (purified receptor) protein concentrations.

Discussion

Radio-labelled toxin-binding assays using insect BBMVs have demonstrated that *M. sexta* has only one CryIA(c) toxin-binding site (Van Rie *et al.*, 1989; Garczyinski *et al.*, 1991). Ligand-blotting studies have shown that CryIA(c) toxin binds principally to a 120 kDa protein in *M. sexta* BBMVs, which was thus a good candidate for the receptor (Garczyinski *et al.*, 1991; Knowles *et al.*, 1991). GalNAc but not GlcNAc abolished binding to this protein in ligand-blotting experiments (Garczyinski *et al.*, 1991), and abolished binding to BBMVs in a direct toxin/BBMV-binding assay (Knowles *et al.*, 1991). The latter authors also

A

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        71              80              90              100
Mouse   W K N F R L P D F I N P V H Y D L E V K A L M E E D R
Rat     W N Q Y R L P K T L I P D S Y Q V T L R P Y L T P N E Q G L
Pig     W N R Y R L P T T L L P D S Y F V T L R P Y L T P N A D G L
Human   W N R Y R L P N T L K P D S Y R V T L R P Y L T P N D R G L

M. sexta D P S Y R L P T T T R P R H Y A V T L T P Y F D V V P A G V
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B

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        658             668
L. lactis  F Q I L Q D R K L L A
Mouse      S S F I D D A F A L A
Rat        A Q I I H D S F N L A
Pig        A Q I I H D A F N L A
Human      A Q I I N D A F N L A

M. sexta   S Q I V D D V F Q L A
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Fig. 5. Similarities between (A) *N*-terminal and (B) internal partial amino acid sequences from the 120 kDa CryIA(c) toxin receptor and a multiple aminopeptidase N protein sequence alignment (Tan *et al.*, 1992). The boxes indicate positions where three or more amino acid residues are conserved between species. The numbering shown corresponds to that of rat aminopeptidase N (Watt and Yip, 1989).

found that GalNAc, but not GlcNAc, specifically decreased the activity of CryIA(c) toxin against a *Choristoneura fumiferana* (CF-1) cell line. In contrast, CryIB toxin does not exhibit *in vivo* activity against *M. sexta* (Hofte and Whiteley, 1989), and has no effect on the permeability of *M. sexta* BBMVs in a sensitive light-scattering assay (Carroll and Ellar, 1993).

From these results it was predicted that the CryIA(c) receptor from *M. sexta* would be a 120 kDa GalNAc-bearing glycoconjugate, able to bind both CryIA(c) toxin and the lectin SBA. Binding of toxin to the receptor would be inhibited by GalNAc, but not by GlcNAc. The receptor would not bind the CryIB toxin. The glycoprotein we have purified from *M. sexta* BBMVs satisfies all these criteria.

Two of the 120 kDa glycoprotein sequences show a high degree of similarity with the aminopeptidase N family of enzymes. Aminopeptidase N is a highly glycosylated zinc metalloenzyme that catalyses the removal of *N*-terminal, neutral amino acids (reviewed in Semenza, 1986). It is a common component of lepidopteran larval midguts, and is used as a marker enzyme to assess the purity of BBMVs from insect larval guts (Wolfersberger, 1984).

A multiple aminopeptidase N protein sequence alignment shows that the first 40 residues of the human, rat, pig and mouse enzymes have the characteristics of a signal sequence, including a stretch of 20 or more hydrophobic residues, which may function as a membrane anchor (Semenza, 1986; 1989; Geirasch, 1989). The receptor *N*-terminal protein sequence similarity with the aminopeptidase N multiple alignment lies to the *C*-terminal side of the mammalian hydrophobic signal/membrane anchor sequence, suggesting either that it has undergone processing or that it may be anchored in an alternative way. Recently, Takesue *et al.* (1992) found partial release of aminopeptidase N activity from larval BBMVs of the lepidopteran *Bombyx mori* by treatment with phosphatidylinositol-specific phospholipase C, and concluded that at least some of the membrane-bound aminopeptidase N in *B. mori* is linked via a glycosyl-phosphatidylinositol (GPI) anchor. If aminopeptidase N in the closely related lepidopteran *M. sexta* is also linked to the membrane by a GPI anchor, then this would explain the apparent absence of an *N*-terminal hydrophobic signal sequence.

On the basis of these results we propose that the receptor for the Bt CryIA(c) toxin in the brush border membrane of the lepidopteran *M. sexta* is the metalloprotease aminopeptidase N (EC 3.4.11.2). Recent reports (Delmas *et al.*, 1992; Yeager *et al.*, 1992) have shown that human and pig coronaviruses also use aminopeptidase N as a receptor in their target tissues. Aminopeptidase N and related peptidases are commonly found in the brush border membranes of the alimentary tract, and may constitute a family of receptors for various viruses, fungi and bacteria.

Experimental procedures

Materials

M. sexta eggs were obtained from Dr S. Reynolds, University of Bath, School of Biological Sciences, UK. All reagents were of analytical grade. Unless otherwise stated, the sources of materials and the experimental procedures were as previously described (Knowles *et al.*, 1991; Carroll and Ellar, 1993).

Protein estimation

Protein concentrations of BBMV preparations were determined by the method of Smith *et al.* (1985) using BCA protein assay reagent (Pierce) and bovine serum albumin (Sigma) as a standard. Protein concentrations of purified receptor preparations were determined by amino acid analysis, assuming a molecular mass of 120 kDa for the intact receptor protein.

SDS–PAGE and immunoblotting

SDS–PAGE and immunoblotting was performed as described by Knowles *et al.* (1991). CryIA(c) toxin bound to the nitrocellulose filter was first detected by sequential incubation with primary (rabbit anti-toxin) antibody, followed by secondary (peroxidase-conjugated goat anti-rabbit) antibody. The peroxidase colour reaction was developed as described by Hawkes *et al.* (1982). In order to identify CryIA(c) toxin binding to *M. sexta* proteins, the same filter was then incubated with activated CryIA(c) toxin at 1 μg ml⁻¹ for 60 min, then bound toxin was detected as described above. All filters were pre-blocked with 3% (w/v) bovine haemoglobin as described by Knowles *et al.* (1991).

Production and purification of crystal δ-endotoxin

Bt crystal δ-endotoxins were purified and solubilized as previously described (Thomas and Ellar, 1983; Knowles *et al.*, 1991; Carroll and Ellar, 1993). Solubilized protoxins were activated by incubation with trypsin (50 : 1) for 60 min at 37°C (CryIA(c)), or with either 5 or 10% (v/v) *Pieris brassicae* gut extract for 3–20 h at 37°C (CryIB). Activated toxins were subjected to further centrifugation at 11 600 × *g* for 5 min at room temperature prior to use.

Brush border membrane vesicle (BBMV) preparation

M. sexta BBMVs were prepared as described by Wolfersberger *et al.* (1987). The final BBMV preparation was resuspended at 5 mg ml⁻¹ in 10 mM Tris-HCl, pH 8, 150 mM KCl, 5 mM EGTA, 1 mM PMSF (buffer I), and stored at −80°C.

Solubilization of BBMVs

Five milligram aliquots of BBMVs (1 ml) were mixed with 1 ml buffer I containing 48 mM CHAPS (Sigma), to give final concentrations of 24 mM CHAPS and 2.5 mg ml⁻¹ BBMVs.

Detergent suspensions were briefly sonicated, incubated on ice for 30 min, then centrifuged at $110\,000 \times g$ for 60 min at 4°C to pellet detergent-insoluble material.

Immobilization of CryIA(c) protoxin

One-hundred milligrams of purified CryIA(c) crystal inclusions were solubilized at $5\,\text{mg ml}^{-1}$ as described above, then dialysed overnight against $2 \times 5\,\text{l}$ of 50 mM Na_2CO_3/HCl, pH 10.5 to remove DTT. The solubilized protoxin (70 mg) was coupled to CNBr-activated Sepharose 4B (Sigma) according to the manufacturer's instructions. The gel was resuspended in 10 mM Tris-HCl, pH 8, 150 mM KCl, 5 mM EGTA, 1 mM PMSF, 8 mM CHAPS (buffer II), and stored at 4°C. The amount of immobilized protoxin was found to be $5.6\,\text{mg ml}^{-1}$ gel.

Protoxin affinity chromatography of solubilized BBMVs

Affinity chromatography was performed at 4°C in a cold room. In a typical run, CHAPS-solubilized BBMVss (5 mg protein; 2 ml) were loaded onto the protoxin column (13.5 ml) at a linear flow rate of $10\,\text{cm h}^{-1}$. The column effluent was monitored at 278 nm, and fractions (2 ml) were collected. The column was washed with two column volumes of buffer II, two column volumes of buffer II made 0.5 M in KCl, and another two column volumes of buffer II. Protoxin-binding proteins were eluted with 20 ml of buffer II made 0.2 M in GalNAc. The column was regenerated by washing three times with 40 ml 0.1 M Tris-HCl, pH 8, 0.5 M NaCl, 8 mM CHAPS followed by 40 ml 0.1 M CH_3COONa/NaOH, pH 4, 0.5 M NaCl, 8 mM CHAPS. Selected fractions were analysed by SDS–PAGE and immunoblotting, or were concentrated 10-fold to a final volume of 1 ml using Centriprep-30 ultra-filtration devices (Amicon) prior to ion exchange chromatography.

Anion exchange chromatrography

Concentrated, partially purified receptor preparations were adjusted by dilution to be 10 mM Tris/HCl, pH 8, 75 mM KCl, 5 mM EGTA, 1 mM PMSF, 8 mM CHAPS (buffer III). Samples (2 ml) were loaded at a flow rate of $0.25\,\text{ml min}^{-1}$ onto an HR5/5 Mono Q column (Pharmacia) pre-equilibrated in buffer III. The column effluent was monitored at 280 nm, and fractions (1 ml) were collected. After 40 min (10 ml) the flow rate was increased to $1\,\text{ml min}^{-1}$, and the column was developed with a linear 20 min gradient of 75 mM–1 M KCl in buffer III. The receptor was recovered in the run-through fractions, and was concentrated using Centriprep-30 ultra-filtration devices (Amicon).

N-*terminal amino acid sequence analysis*

GalNAc-eluted fractions from the CryIA(c) protoxin affinity column were trichloroacetic acid (TCA) precipitated, resolved by 10% SDS–PAGE and electroblotted to ProBlot membrane (Applied Biosystems). The blotted membrane was extensively washed with water, then stained with Coomassie brilliant blue R (Sigma) according to the manufacturers' instructions. Stained bands were excised from the membrane and placed

in the cartridge of an Applied Biosystems 477A automated pulse-liquid phase sequencer. Amino acid phenylthiohydantoin derivatives were analysed on line with a 120A amino acid analyser. Fractions from reverse-phase high-performance liquid chromatography (HPLC) runs were thawed and washed three times with water by repeated concentration under vacuum, resuspended in trifluoroacetic acid (TFA) and spotted onto pre-cycled, Polybrene-impregnated glass fibre discs prior to automated sequencing as described above.

Internal amino acid sequence analysis

Internal amino acid sequence was determined according to Stone *et al.* (1989). Briefly, concentrated receptor samples obtained following anion exchange chromatography were precipitated with TCA (final concentration 10%, w/v), washed twice with acetone and dried under vacuum. The dried protein was carboxamidomethylated and digested with trypsin exactly as described by Stone *et al.* (1989).

Tryptic peptides were resolved on a Varian MicroPak-SP C18 analytical reverse-phase column ($4.6\,\text{mm} \times 15\,\text{cm}$), using a Hewlett Packard HP1090 HPLC equipped with an HP3396A Integrator. Tryptic peptide solutions were made 0.1% in TFA, injected ($400\,\mu\text{l}$), and the column was developed at a flow rate of $0.5\,\text{ml min}^{-1}$, as follows: 0% B (0–6 min); 0–30% B (6–66 min); 30–60% B (66–96 min); 60–80% B (96–111 min). Buffer A was 0.1% (v/v) TFA in water, and buffer B was 0.1% (v/v) TFA in acetonitrile. Fractions (1 ml) were collected and analysed by capillary electrophoresis using a Beckman P/ACE System 2100 and a 50 cm by $75\,\mu\text{m}$ (internal diameter) capillary. Fractions judged to consist of a single peptide were frozen at $-80°\text{C}$ prior to N-terminal sequence analysis as described above.

Selected heterogeneous fractions were re-chromatographed on a Hypersil C18 narrow-bore reverse-phase column ($2.1\,\text{mm} \times 15\,\text{cm}$). Fractions (1 ml) from the MicroPak C18 column were concentrated under vacuum, diluted into 20 mM NH_4HCO_3, pH 6.5 (buffer C) and injected ($500\,\mu\text{l}$) at a flow rate of $0.2\,\text{ml min}^{-1}$. The column was developed with a non-linear gradient of 20 mM NH_4HCO_3, pH 6.5, 80% (v/v) acetonitrile (buffer D) as follows; 0% D (0–20 min), 0–18.7% D (20–25 min), 18.7–37.5% D (22–55 min), 37.5–100% D (55–80 min). Fractions were collected manually, assessed by capillary electrophoresis and sequenced as described above.

Polymerase chain reaction

'Booster' PCR were carried out exactly as described by Walker *et al.* (1992), using a Techne PHC-3 programmable DriBlock. A nested pair of forward primers (1F and 2F; see Fig. 4) were used with a reverse primer (1R; see Fig. 4), whilst a fourth oligonucleotide (2R) was used as a hybridization probe to identify the correct PCR product. All oligonucleotides were fully degenerate, containing all possibilities predicted by the genetic code from the known protein sequence. The target DNA was a single-stranded cDNA (ss-cDNA) preparation from *M. sexta* midgut membranes, prepared using a 'Quickprep' mRNA purification kit and a 'Timesaver' cDNA synthesis kit (both from Pharmacia) according to the manufacturer's instructions.

Enzyme assays

Aminopeptidase activity was assayed at 25°C (Hafkenscheid, 1984) in 250 mM Tris-HCl, pH 7.8, 250 mM NaCl, using 1 mM L-leucine-*p*-nitroanilide (Sigma) as the substrate. The initial rate of increase in absorbance at 405 nm was used to calculate specific enzymatic activity. The absorption coefficient of *p*-nitroanilide was taken to be 9.9×10^{-3} mol l^{-1}. Alkaline phosphatase activity was assayed at 25°C (Lowry *et al.*, 1954) in 10 mM diethanolamine, pH 9.8, using 1.25 mM 4-nitrophenyl phosphate (Sigma) as the substrate. The initial rate of increase in absorbance at 407 nm was used to calculate specific enzymatic activity. The absorption coefficient of *p*-nitrophenol was taken to be 18.33×10^{-3} mol l^{-1}.

Acknowledgements

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