Bacillus thuringiensis Cry1Ac and Cry1Fa δ-endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* is not *N*-acetylgalactosamine mediated

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Abstract

We determined that *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ-endotoxins recognize the same 110, 120 and 170 kDa aminopeptidase N (APN) molecules in brush border membrane vesicles (BBMV) from *Heliothis virescens*. The 110 kDa protein, not previously identified as an APN, contained a variant APN consensus sequence identical to that found in *Helicoverpa punctigera* APN 2. PCR amplification of *H. virescens* cDNA based on this sequence and a conserved APN motif yielded a 0.9 kb product that has 89% sequence homology with *H. punctigera* APN 2. Western blots revealed that the 110 kDa molecule was not recognized by soybean agglutinin, indicating the absence of GalNAc. A ^{125}I labeled-Cry1Ac domain III mutant (^{509}QNR^{511}-AAA) that has an altered GalNAc binding pocket (Lee et al., Appl. Environ. Microbiol. 65 (1999) 4513) showed abolished binding to the 120 APN, reduced binding to the 170 kDa APN, and enhanced binding to the 110 kDa APN. Periodate treated *H. virescens* BBMV blots were also probed with ^{125}I labeled-Cry1Ac and ^{509}QNR^{511}-AAA toxins. Both toxins still recognized the 110 kDa APN and a >210 kDa molecule which may be a cadherin-like protein. Additionally, ^{125}I-^{509}QNR^{511}-AAA recognized periodate treated 170 kDa APN. Results indicate that the 110 kDa APN is distinct from other Cry1 toxin binding APNs and may be the first described Cry1Ac-binding APN that does not contain GalNAc. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Bacillus thuringiensis Cry1Fa and Cry1Ac δ-endotoxins have overlapping yet distinct ranges of insecticidal activities. Cry1Fa δ-endotoxin is highly toxic to *Heliothis virescens*, *Spodoptera exigua*, and *Ostrinia nubilalis*, but not *Helicoverpa zea* (Chambers et al., 1991). Cry1Ac δ-endotoxin is also highly toxic to *H. virescens*, *O. nubilalis* and *H. zea* (Van Rie et al., 1989; Garczynski et al., 1991), but not *S. exigua* (Luo et al., 1999a). Surface contamination bioassays indicate that the LC50 values for Cry1Ac and Cry1Fa in *H. virescens*

are 0.040 and 0.031 ng of toxin per square millimeter, respectively (Chambers et al., 1991).

Cry1 protoxins are solubilized from crystals in the alkaline midgut and processed by proteinases to an active toxin of about 60 kDa. *B. thuringiensis* Cry1 toxins bind to the brush border of midgut epithelial cells and insert into the membrane. This event leads to permeation of the membrane and insect death (Knowles and Dow, 1993).

A Cry1Ac binding protein was first identified as a 120 kDa aminopeptidase N (APN) (EC. No. 3.4.11.2) in *Manduca sexta* (Sangadala et al., 1994; Knight et al., 1994). APN is an exopeptidase that hydrolyzes neutral amino acids from the amino terminus of proteins. Cry1Ac binds to a *N*-acetylgalactosamine (GalNAc) residue on 120 kDa APN. This conclusion is based on two lines of evidence. First, Cry1Ac binding to the 120 kDa APN is specifically inhibited by *N*-acetylgalactosa-

mine, but not other sugars (Garczynski et al., 1991; San-gadala et al., 1994; Knight et al., 1994). Second, Cry1Ac domain III mutants such as [509]QNR[511]-AAA (Jenkins et al., 1999) and Tmut (Burton et al., 1999) that are defective in GalNAc binding do not recognize the 120 kDa APN.

Cry1Ac binds to multiple brush border membrane vesicle (BBMV) proteins in *H. virescens* (Luo et al., 1997; Gill et al., 1995). The 120 and 170 kDa Cry1Ac binding proteins have been cloned and identified as APNs (Gill et al., 1995; Oltean et al., 1999). The 130 and 140 kDa proteins are also APNs, and may be processed products of 170 kDa APN (Luo et al., 1997; Oltean et al., 1999). Finally, BBMV from *H. virescens* contains 105 (Gill et al., 1995) and 110 kDa (Luo et al., 1997) Cry1Ac binding proteins that remain unidentified. Relative to the role of APN as a receptor, the 170 kDa APN mediated Cry1Ac binding and pore formation as measured with both surface plasmon resonance and toxin-induced $^{86}Rb^+$ efflux from phospholipid vesicles (Luo et al., 1997).

Cry1A and Cry1Fa toxins share high sequence homology in the domain II loops involved in toxin binding and activity (Tabashnik et al., 1996; Rajamohan et al., 1996a,b). Furthermore, binding assays have shown that Cry1Fa competes with low affinity for Cry1Ac sites in BBMV from *H. virescens* (Jurat-Fuentes and Adang, 2001). Additionally, the Cry1Ac-resistant *H. virescens* strain YHD2 is cross-resistant to Cry1Fa (Gould et al., 1995). In the diamond back moth *Plutella xylostella*, heterologous competition binding experiments have also shown that Cry1Ab and Cry1Fa toxins share a high affinity binding site (Granero et al., 1996; Ballester et al., 1999). These observations led to our hypothesis that Cry1Ac and Cry1Fa would share binding proteins in *H. virescens*. Identification of these binding proteins is important for resistance management, since an approach to delay the onset of field resistance is utilization of multiple toxins to target different binding proteins (Peferoen, 1997).

In this study we determined that Cry1Ac and Cry1Fa recognize common 110, 120, and 170 kDa binding proteins in *H. virescens*. We identify the 110 kDa binding protein as a novel APN with unusual characteristics. The mature 110 kDa APN has a variant APN consensus sequence unlike other described lepidopteran APNs that bind Cry toxins. Perhaps most interesting is that the 110 kDa APN appears to lack GalNAc, yet is recognized by Cry1Ac and Cry1Fa. This finding adds credence to the proposal that Cry1Ac-induced membrane permeation proceeds by both GalNAc dependent and GalNAc independent mechanisms (Carroll et al., 1997). We further show that a Cry1Ac mutant which was designed with an altered GalNAc binding pocket, yet maintains toxicity (Lee et al., 1999), still binds to the 110 and 170 kDa APNs. These two APNs are therefore implicated in

Cry1Ac toxicity to *H. virescens*. Additionally, a *H. virescens* >210 kDa molecule shown to be recognized by Cry1Ac and Cry1Fa is similar in size to a previously described cadherin-like binding protein in *M. sexta* (Vadlamudi et al., 1995).

2. Materials and methods

2.1. Toxin purification

A *B. thuringiensis* strain harboring the *cry1Fa* gene (GenBank accession number M63897) was provided by Ecogen, Inc. (Chambers et al., 1991). *B. thuringiensis* strain HD-73 carrying the *cry1Ac* gene was obtained from the *Bacillus* Genetic Stock Culture Collection (Columbus, OH). *B. thuringiensis* strain MR522 producing Cry1Ea was obtained from Dow Agrosciences (San Diego, CA). *B. thuringiensis* strains were grown at 28°C in half-strength tryptic soy broth (Difco) until cell sporulation and lysis. The crystal–spore mixture was washed two times in 1 M NaCl, 0.1% Triton X-100 and two times in de-ionized water. Toxins were purified according to Luo et al. (1999a). The Cry1Ac domain III mutant [509]QNR[511]-AAA was purified according to Lee et al. (1999). Protein concentrations were determined with a Bio-Rad protein assay kit: bovine serum albumin (BSA) was used as the standard, as described by Bradford (1976).

2.2. Toxin biotinylation

Cry1Ac and Cry1Fa toxins were biotinylated according to Denolf et al. (1993) with the following modifications. Sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce) was dissolved in 20 mM Na_2CO_3, pH 9.7, mixed with toxin at a molar ratio of 1:30 for 30 min at room temperature. Free biotin was removed with overnight dialysis in 20 mM Na_2CO_3, 150 mM NaCl, pH 9.7 at 4°C. Biotinylation was confirmed by Western blot analysis. Briefly, 2 μg of biotinylated toxins was separated on sodium dodecyl sulfate (SDS) 10% polyacrylamide gel electrophoresis (PAGE) and electroblotted to polyvinylidene difluoride (PVDF) (Bio-Rad) membrane in 50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol, pH 8.3. The membrane was blocked with phosphate buffered saline (PBS; 140 mM NaCl, 2.5 mM KCl, 5.5 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) containing 0.1% Tween 20 (PBST) and 3% BSA, then incubated with anti-biotin conjugated alkaline phosphatase antibody for 1 h at room temperature. The membrane was then washed three times with PBST plus 0.1% BSA and color developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate.

2.3. Toxin iodination

Labeling of purified Cry1Ac and [509]QNR[511]-AAA toxin with Na[125]I was done using the Chloramine-T method (Garczynski et al., 1991). Toxin (1 μg) was labeled with 0.5 mCi of Na[125]I. The specific activities of Cry1Ac and [509]QNR[511]-AAA were 23.8 and 18 μCi/μg, respectively (based on input toxin).

2.4. Preparation of brush border membrane vesicles

Midguts were dissected from early fifth-instar *H. virescens* larvae, rinsed in 0.3 M mannitol, 5 mM EDTA, 17 mM Tris–Cl (pH 7.5) (MET) and frozen on dry ice. BBMV were prepared according to the $MgCl_2$ precipitation method (Wolfersberger et al., 1987). The final BBMV pellets were suspended in PBS and frozen at −80°C until used.

2.5. Affinity chromatography

The affinity purification method of Luo et al. (1996) was followed with minor modifications. Between 8 and 10 mg of toxin was bound to 1 g of activated CNBr sepharose beads. The identity of toxins used for affinity chromatography was confirmed by Western blot using anti-Cry1Fa antiserum. Fifteen milligrams of BBMV proteins prepared within 1 day of binding protein purification were solubilized in CHAPS-containing buffer (Luo et al., 1996). This buffer also contained 100 mM NaCl and the following proteinase inhibitor cocktail: 4 mM Pefabloc SC AEBSF (Boehringer, Mannheim), 5 μM leupeptin (Pierce), 5 μM pepstatin (Pierce), and 5 μM antipain (Pierce). Bound proteins were eluted with 10 ml of 2 M NaSCN in 20 mM Na_2CO_3, pH 9.6. The eluate was diluted to 20 ml in 20 mM Na_2CO_3 pH 9.6, then concentrated using a Centriprep-30 ultra-filtration device (Amicon) at 4°C and finally stored at −80°C until needed for experimentation.

2.6. Ligand blot of eluted Cry1Ac H. virescens binding proteins with biotinylated Cry1Fa

Eluted Cry1Ac binding proteins were separated on SDS–8% PAGE and transferred to PVDF membrane as described above. The membrane was probed with 0.75 μg/ml biotinylated Cry1Fa for 1 h in PBST plus 0.1% BSA. The membrane was incubated with goat-anti-biotin-conjugated alkaline phosphatase for 1 h. Cry1Ac binding proteins that were recognized by biotinylated Cry1Fa were visualized with NBT/BCIP substrate.

2.7. Western blot of eluted Cry1Fa H. virescens binding proteins with anti-APN antibodies

Eluted Cry1 binding proteins were separated on SDS–8% PAGE and transferred to PVDF membrane as above.

The membrane was probed with anti-APN antiserum for 1 h. Anti-APN serum was raised in rabbits against a 31 kDa truncated form of the *M. sexta* 120 kDa APN expressed in *E. coli* (Luo et al., 1999b). The membrane was then washed and probed with goat-anti-rabbit-conjugated alkaline phosphatase. Molecules recognized by anti-APN antibody were visualized with NBT/BCIP substrate.

2.8. Lectin blot of H. virescens BBMV proteins

H. virescens BBMV proteins (15 μg) were separated on SDS–8% PAGE and transferred to PVDF membrane as described above. The membrane was then incubated with soybean agglutinin-conjugated peroxidase (0.1 μg/ml) in PBST plus 0.1% BSA for 1 h followed by thorough washing and detection with ECL reagents (Amersham Pharmacia Biotech) according to the manufacturer's directions.

2.9. ^{125}I-Cry1Ac and [125]I-[509]QNR [511]-AAA ligand blots

H. virescens BBMV proteins (15 μg) were separated by SDS–8% PAGE and transferred to PVDF membrane as described above. For periodate treatment, the membrane was incubated in 0.2 M NaOAc, pH 4.5 containing 50 mM sodium-m-periodate overnight at 4°C with shaking before incubation with toxins. For both periodate and non-periodate treatments, membranes were blocked and washed as described above, then incubated with 0.1 nM ^{125}I-Cry1Ac or [125]I-[509]QNR[511]-AAA for 1.5 h at room temperature. Membranes were then washed three times, air dried and exposed to XAR-5 photographic film (Kodak) at −80°C for 48 h.

2.10. Sub-microgram quantities of BBMV proteins separated with SDS–6% PAGE

Sub-microgram quantities of protein from Cry1Ac affinity eluate and *H. virescens* BBMV were separated by SDS–6% PAGE. The gel was fixed overnight and stained according to the Amersham-Pharmacia Biotech silver staining kit instructions.

2.11. Amino acid sequence analyses

H. virescens BBMV proteins (15 μg) were separated on SDS–8% PAGE and electroblotted to PVDF membrane. The membrane was rinsed in water, briefly stained in amido black, destained in 5% acetic acid for 20 min, and washed three times in water. The 110 kDa band was excised and submitted to Dr Michael Berne (Department of Physiology and Medicine, Tufts University, Boston, MA) for N-terminal sequencing.

H. virescens BBMV proteins (320 μg) were separated by SDS–8% PAGE. The 110 kDa band was excised and

digested with *Staphylococcus aureus* V8 protease according to the method of Cleveland et al. (1977). The digested protein was then separated by SDS–12% PAGE and blotted to PVDF membrane as above. Bands of 19, 28 and 32 kDa were excised and submitted for N-terminal sequencing.

2.12. PCR cloning of a 110 kDa binding protein fragment

Midguts from early fifth instar *H. virescens* larvae were dissected and incubated in RNAlater (Ambion) on ice for 1 h. Total RNA was prepared from 100 mg midguts with a RNeasy kit (Qiagen), and cDNA was made with a Nd(T)$_{18}$ primer, 0.25 mM each dNTP, and Superscript reverse transcriptase (Life Technologies) according to the conditions suggested by the manufacturer. Degenerate primers (synthesized by IDT, Inc.) for amplification of internal sequence of the 110 kDa binding protein were based on the peptide sequence TNLDEPA (5′ AC(A/C/G/T) AA(C/T) CT(A/C/G/T) GA(C/T) GA(A/G) CC(A/G/C/T) GC3′; forward primer) and the APN consensus sequence GAMENWG (5′ CCC CA(A/G) TT(C/T) TCC AT(A/T/G/C) GC(A/T) CC 3′; reverse primer). Codon usage was based on the 120 kDa APN sequence (Gill et al., 1995). PCR was conducted in 50 mM KCl, 10 mM Tris–HCl, 15 mM MgCl$_2$, 0.25 mM each dNTP (pH 8.3), 1U Taq polymerase (Eppendorf) with 40 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. PCR products that were unique to forward plus reverse primer amplifications were gel purified with a Qiaex II gel extraction kit (Qiagen), and cloned into the pCR 2.1 TA vector (Invitrogen). Plasmid inserts were sequenced at the Molecular Genetics Instrumentation Facility (University of Georgia).

3. Results

3.1. Affinity chromatography purification of H. virescens binding proteins

Cry1Ac and Cry1Fa toxin affinity chromatographies were used to purify binding proteins from *H. virescens* midgut brush border membranes. CHAPS-solubilized BBMV were applied to toxin affinity columns. Bound proteins were eluted with 2 M NaSCN, separated by SDS–8% PAGE and coomassie stained. Proteins of 110, 120 and 170 kDa were eluted from Cry1Ac and Cry1Fa columns (Fig. 1A, lanes 3 and 5, respectively). Additionally, Cry1Ac and Cry1Fa selected small amounts of two proteins of about 100 kDa. The 60 kDa protein present in both the Cry1Ac and Cry1Fa eluates corresponds to toxin leached from the beads during elution with high salt. Cry1Ea was used as a negative control toxin

because Cry1Ea does not bind to *H. virescens* BBMV (Van Rie et al., 1989). No *H. virescens* BBMV proteins were purified by Cry1Ea affinity chromatography (data not shown).

3.2. Ligand blot of Cry1Ac eluted binding proteins probed with biotinylated Cry1Fa

We confirmed that Cry1Fa recognized proteins eluted from the Cry1Ac column by ligand blotting. The 110, 120 and 170 kDa proteins present in the Cry1Ac eluate were recognized by biotinylated Cry1Fa (Fig. 1B, lane 2). As a control, Cry1Ac toxin affinity chromatography was conducted in the absence of solubilized BBMV. The affinity eluate contained only 60 kDa Cry1Ac. Ligand blot analysis showed that the Cry1Ac was recognized by biotinylated Cry1Fa (data not shown). This result indicates that the signal at 60 kDa resulted from biotinylated Cry1Fa interacting with Cry1Ac that eluted from the column. Additionally, minor proteins below 60 kDa are visible in this ligand blot. These smaller proteins are likely degradation products of purified binding proteins that are still recognized by toxin. Alternatively, these bands could be binding proteins that were not visible after coomassie staining, but were visualized with more sensitive ligand blotting. Unexpectedly, biotinylated Cry1Fa recognized a molecule of >210 kDa, although a molecule of that molecular size was not visible on coomassie stained gels of either total BBMV or Cry1-selected molecules (Fig. 1A). A >210 kDa molecule was previously detected when ligand blots of *H. virescens* BBMV were probed with Cry1Ac and Cry1Fa (Jurat-Fuentes and Adang, 2001).

3.3. Western blot of Cry1Fa eluted binding proteins probed with anti-APN antiserum

Since the 170 and 120 kDa molecules are forms of APN (Gill et al., 1995; Luo et al., 1997), proteins eluted from the Cry1Fa column were probed with anti-APN antibody. In addition to the expected recognition of 170 and 120 kDa proteins, the 110 kDa protein was also recognized by anti-APN antiserum (Fig. 1B, lane 1). The two minor binding proteins near 100 kDa that were identified in the Cry1Ac and Cry1Fa eluates with coomassie stained SDS PAGE (Fig. 1A, lanes 3 and 5) were also identified by anti-APN antibody (Fig. 1B, lane 1). These two proteins are also visible in total BBMV (Fig. 1A, lane 1), and are apparently additional binding proteins that are also APNs.

3.4. Lectin blot of H. virescens BBMV proteins probed with soybean agglutinin

Lee et al. (1996) previously showed that of all *H. virescens* BBMV proteins, only the 170 and 120 kDa pro-
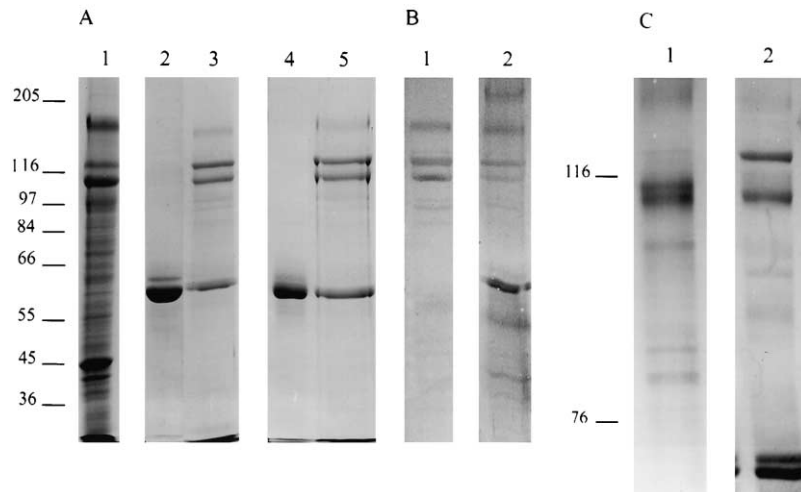
Fig. 1. SDS–8% PAGE of Cry1Ac and Cry1Fa toxin affinity chromatography purified *H. virescens* binding proteins, Western blot analysis of Cry1Fa toxin affinity chromatography purified *H. virescens* BBMV binding proteins with anti-APN antibody, and ligand blot analysis of Cry1Ac toxin affinity chromatography purified *H. virescens* binding proteins with biotinylated Cry1Fa. SDS–6% PAGE silver staining of sub-microgram quantities of *H. virescens* BBMV and Cry1Ac toxin affinity chromatography purified *H. virescens* binding proteins. (A) *H. virescens* BBMV proteins (lane 1), Cry1Ac toxin (lane 2), eluted Cry1Ac *H. virescens* binding proteins (lane 3), Cry1Fa toxin (lane 4), eluted Cry1Fa *H. virescens* binding proteins (lane 5). (B) Eluted Cry1Fa *H. virescens* binding proteins were probed with anti-APN antibody (1°), goat-anti-rabbit-conjugated alkaline phosphatase antibody (2°), and visualized with NBT/BCIP substrate (lane 1). Eluted Cry1Ac *H. virescens* binding proteins were probed with biotinylated Cry1Fa toxin (0.75 µg/ml), rabbit-anti-biotin-conjugated alkaline phosphatase antibody, and visualized with NBT/BCIP substrate (lane 2). (C) *H. virescens* BBMV proteins (0.25 µg) (lane 1) and Cry1Ac affinity chromatography eluate (0.5 µg) (lane 2) were separated by SDS–6% PAGE, fixed overnight and silver stained the following day.

teins strongly cross-reacted with the lectin, soybean agglutinin. When we probed *H. virescens* BBMV proteins on blots with soybean agglutinin, we also observed strong cross-reactivity to 170 and 120 kDa proteins (Fig. 2, lane 2). Since soybean agglutinin specifically recognizes vicinal GalNAc moieties, the 170 and 120 kDa proteins are probably glycoproteins containing terminal GalNAc residues. Although a faint signal is visible in

Fig. 2. Ligand blot analysis of *H. virescens* BBMV proteins. SDS–8% PAGE separated BBMV proteins alone are shown in lane 1. Ligand blots were conducted with soybean agglutinin (lane 2), ^{125}I-Cry1Ac (lane 3), and [125]I-[509]QNR[511]-AAA (lane 4). Ligand blots of periodate treated *H. virescens* total BBMV were also conducted with ^{125}I-Cry1Ac (lane 5), and [125]I-[509]QNR[511]-AAA (lane 6).

the 110 kDa region, the relative degree of recognition compared to the 170 and 120 kDa APNs was nominal.

3.5. ^{125}I-Cry1Ac and [125]I-[509]QNR[511]-AAA ligand blots

To examine the relationship between GalNAc on APN molecules and binding of Cry1Ac toxin to these molecules, we performed ligand blot experiments using ^{125}I-labeled Cry1Ac and the Cry1Ac mutant [509]QNR[511]-AAA. Our reasoning was based on evidence that Cry1Ac binding to 170 kDa APN is mediated by GalNAc (Luo et al., 1997) and that the Cry1Ac mutant [509]QNR[511]-AAA is impaired in GalNAc recognition (Jenkins et al., 1999). Fig. 2 shows ^{125}I-Cry1Ac (lane 3) and [125]I-[509]QNR[511]-AAA ligand blots (lane 4) of *H. virescens* BBMV. Relative to Cry1Ac, [509]QNR[511]-AAA recognition of the 120 kDa APN is nearly abolished, recognition of the 170 kDa APN is reduced, and recognition of the 110 kDa APN is enhanced. Additionally, a >210 kDa molecule was also recognized by [509]QNR[511]-AAA, and to a lesser extent by Cry1Ac. These results support a model whereby Cry1Ac binding to the 120 and 170 kDa APNs, but not the 110 kDa APN, is primarily mediated by GalNAc.

3.6. ^{125}I-Cry1Ac and [125]I-[509]QNR[511]-AAA ligand blots of periodate treated BBMV proteins

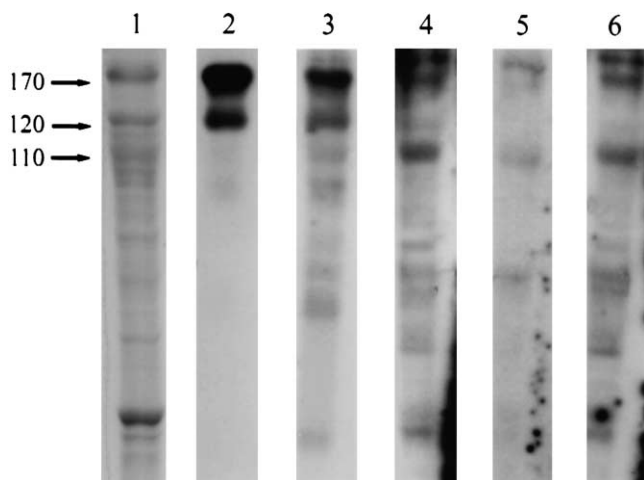Periodate treatment was used to determine if Cry1Ac binds to carbohydrates on proteins in *H. virescens*

BBMV. Periodate treatment, which oxidizes and disrupts sugar structures on glycoproteins, has been used to map carbohydrate epitopes of antibodies by disrupting the sugar ring structure without destroying the peptide chain (Woodward et al., 1985). Garczynski (1999) discovered that periodate treatment eliminated Cry1Ac binding to 120 kDa APN from *M. sexta*, indicating the Cry1Ac dependence on carbohydrate recognition. Since GalNAc was not detected on the 110 kDa APN (Fig. 2, lane 2) and ^{509}QNR^{511}-AAA bound to 110 kDa APN (Fig. 2, lane 4), we predicted that ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA should bind periodate-treated 110 kDa APN independent of carbohydrate recognition. Fig. 2 shows that ^{125}I-Cry1Ac bound to the 110 kDa APN and the >210 kDa molecule, but not to 120 APN (Fig. 2, lane 5). Periodate treatment reduced ^{125}I-Cry1Ac binding to the 170 kDa APN. In contrast, periodate treatment had little, if any, effect, on ^{125}I-^{509}QNR^{511}-AAA binding to BBMV molecules (Fig. 2, lane 6). To confirm that periodate treatment modified GalNAc moieties, periodate-treated BBMV proteins were probed with soybean agglutinin. No BBMV molecules were detected by this lectin after periodate treatment (data not shown). These results obtained using periodate treated blots provide further support for the conclusion that Cry1Ac binding to the 120 and 170 kDa APNs is primarily GalNAc mediated. However, this is not the case for 110 kDa APN and >210 kDa molecule recognition.

3.7. 110 kDa APN N-terminal sequencing

N-terminal sequencing revealed that the 110 kDa protein from the total BBMV contains the residues VIQTGQ(C)NENIQVVTGFNLD. No homologous sequences were identified with database searches of this sequence. Gill et al. (1995) describe the N-terminal sequence of a 105 kDa Cry1Ac-binding protein from *H. virescens* as being different from other APN sequences. It is likely that the 105 and 110 kDa proteins are synonymous. To obtain additional amino acid sequence, the 110 kDa molecule from total BBMV was subjected to V8 protease digestion. This procedure yielded three major fragments of 19, 28 and 32 kDa (data not shown). The N-terminal sequence of the 19 kDa fragment contained the same sequence as the intact 110 kDa. The 28 kDa fragment contained the N-terminal residues ETATVAG-IPNFNI. No sequence homologies were identified with database searches of either of these sequences. The 32 kDa fragment contained the N-terminal residues TNLDEPAYRLRDVVY. This fragment sequence shares 100% homology with amino acids 37–51 of *H. punctigera* APN 2 (GenBank accession no. AAF37559). The presence of an APN sequence in the 32 kDa fragment released from the 110 kDa protein accounts for detection by the anti-APN antiserum.

3.8. SDS–6% PAGE and silver staining of sub-microgram quantities of H. virescens BBMV proteins and cry1ac affinity chromatography eluate

The amino acid sequences derived from proteinase digestion of the 110 kDa protein led us to suspect that two BBMV proteins were co-migrating on SDS–8% PAGE. To resolve the two proteins, sub-microgram concentrations of BBMV were separated with SDS–6% PAGE and silver stained. Fig. 1C shows 0.25 µg of BBMV separated with SDS–6% PAGE (lane 1). The 110 kDa band that appears as one protein when separated by SDS–8% PAGE (see Fig. 1A, lane 1) is resolved as two distinct bands under these conditions. For comparison, sub-microgram concentrations of Cry1Ac eluate were also separated by SDS–6% PAGE and silver stained (Fig. 1C, lane 2). The Cry1Ac eluate contains the 170 and 120 kDa proteins, but only the lower co-migrating 110 kDa protein. The 110 kDa binding protein in the Cry1Ac eluate was also recognized by the anti-APN antibody (see Fig. 1B, lane 1), indicating that toxin binds to the 110 kDa APN, and not the other unidentified protein.

3.9. PCR amplification and sequence of an internal region from the 110 kDa aminopeptidase

PCR was conducted with *H. virescens* cDNA as template, TNLDEPA forward degenerate primer, and GAMENWG reverse degenerate primer. The forward primer was designed from an internal 110 kDa APN sequence that shared 100% homology with *H. punctigera* APN 2. The reverse primer was designed from the lepidopteran internal APN consensus sequence GAMENWG. We were confident that the 110 kDa molecule would contain this sequence once we determined that this molecule was an APN. All lepidopteran APN sequences share this motif, which is always located between 960 and 985 nucleotides from the start methionine (Gill et al., 1995; Oltean et al., 1999; Chang et al., 1999; Yaoi et al., 1997). Additionally, all eucaryotic alanine/arginine aminopeptidases share this motif (Taylor, 1996). The PCR should thus yield an approximately 0.9 kb product. Amplification with forward primer alone yielded 0.65 kb product (Fig. 3, lane 1) and PCR with reverse primer alone yielded 0.35 and 1.0 kb products (Fig. 3, lane 2). PCR with both forward and reverse primers yielded unique 0.45 and 0.9 kb products (Fig. 3, lane 3). Lane 4 shows a 2.0 kb PCR product generated with non-degenerate internal primers designed from the 120 kDa APN sequence (Gill et al., 1995), which served as a positive control. The 0.45 and 0.9 kb products were cloned into the pCR2.1 TA vector yielding pCR0.45 and pCR0.9 plasmids. The sequence derived from the pCR0.45 insert shares 98% homology with *H. virescens* 170 kDa APN. The sequence derived
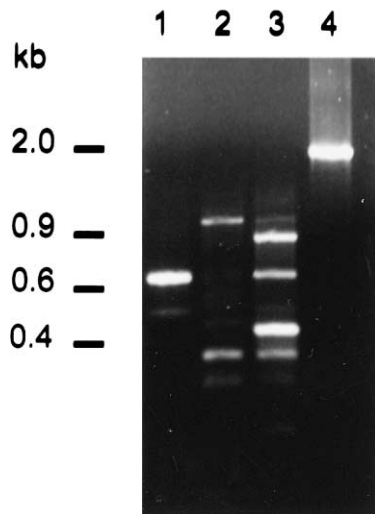
Fig. 3. 1.0% agarose gel separated PCR products. PCR products of forward primer alone (lane 1), reverse primer alone (lane 2), forward plus reverse primers (lane 3), and non-degenerate primers for amplification of an internal sequence of *H. virescens* 120 kDa APN (lane 4), were agarose gel separated and stained with ethidium bromide.

from the pCR0.9 insert shares 89% homology with *H. punctigera* APN 2. The sequence derived from both the pCR0.45 and pCR0.9 inserts contained the nucleotides corresponding to the GAMENWG sequence. However, only the pCR0.9 insert contained the nucleotides corresponding to the TNLDEPA sequence found in the V8 digested 32 kDa fragment N-terminus. These results indicate that the 0.9 kb PCR product represents an internal sequence from the 110 kDa APN.

4. Discussion

The original purpose of this study was to examine the prediction that Cry1Ac and Cry1Fa share binding proteins in *H. virescens*. This hypothesis was based on cross-resistance between Cry1Ac and Cry1Fa in resistant *H. virescens* (Gould et al., 1995) and shared sequence homologies in domain II loops (Tabashnik et al., 1996) critical to toxin binding and toxicity (Rajamohan et al., 1996a,b). Our affinity chromatography experiments revealed that both Cry1Ac and Cry1Fa recognize 110, 120 and 170 kDa binding proteins. Ligand blot analysis showed that biotinylated Cry1Fa cross-reacted with eluted Cry1Ac binding proteins, and also revealed a >210 kDa binding molecule. These results support our hypothesis that shared binding proteins are likely the source of cross-resistance between Cry1A toxins and Cry1Fa.

Our results support the multiple receptor model proposed by Van Rie et al. (1989) which characterizes Cry1Ac as binding to at least three populations of receptors in the brush border membrane of *H. virescens*. Sub-

sequent studies by Luo et al. (1997) and Oltean et al. (1999) identify Receptor A as being composed of the 170 kDa APN and/or the 130 kDa APN. The 120 kDa APN characterized in detail by Gill et al. (1995), and the 110 kDa APN described here are candidates for the other two populations of receptors, as well as the >210 kDa molecule visualized on our ligand blots. Detailed binding experiments performed with various Cry1 toxins are required before assignments of receptor type can be made for the 110, 120 and >210 kDa molecules.

The abundances of the 110, 120 and 170 kDa APNs were relatively equivalent in *H. virescens* BBMV (see Fig. 1). However, the 110 and 120 kDa binding proteins constituted the main binding proteins purified with Cry1Ac and Cry1Fa affinity chromatography, while the 170 kDa APN was purified at a relatively much lower level. The low abundance of the 170 kDa APN could be related to elution conditions. For example, Luo et al. (1997) eluted *H. virescens* BBMV proteins from a Cry1Ac affinity column with GalNAc instead of NaSCN, yielding a larger yield of the 170 kDa protein relative to the 110 and 120 kDa proteins. Therefore, the relatively low abundance of the 170 kDa APN in the eluate could be related to elution with high salt, rather than GalNAc. A second possibility is that Cry1Ac and Cry1Fa have greater affinity for the 110 and 120 kDa proteins relative to the 170 kDa protein. This explanation is supported by Oltean et al. (1999), who found that Cry1Ac has low affinity binding to the 170 kDa APN.

Where examined previously, all Cry1Ac-binding APNs contain GalNAc moieties (Garczynski et al., 1991; Knight et al., 1994; Lee et al., 1996). However, in this study we present evidence that GalNAc is not required for Cry1Ac binding to the *H. virescens* 110 kDa APN. While Cry1Ac and Cry1Fa bound the 110 kDa APN, this molecule was not recognized by the GalNAc-binding lectin soybean agglutinin. These results were further supported by our experiments with the Cry1Ac mutant [509]QNR[511]-AAA. This mutant was designed to remove the functional groups lining the lectin-like pocket predicted to be involved in GalNAc binding (Lee et al., 1999). While [509]QNR[511]-AAA no longer binds the Gal-NAc-containing *M. sexta* 120 kDa APN (Jenkins et al., 1999), it does bind *M. sexta* BBMV and remains toxic to *M. sexta*, *H. virescens* and *Lymantria dispar* (Lee et al., 1999). Therefore, [509]QNR[511]-AAA binding and toxicity are not dependent on GalNAc recognition. A logical corollary follows. Namely, GalNAc-independent Cry1Ac recognition of a binding protein must exist. This binding protein may or may not be an APN. Here we show that GalNAc independent Cry1Ac binding to the *H. virescens* 110 kDa APN, 170 kDa APN, and an unidentified >210 kDa molecule fulfil this requirement. Further work is required to determine the possible roles of the 110 kDa APN and >210 kDa molecule relative to the 170 kDa APN in pathogenicity. It should also be

noted that both radiolabeled Cry1Ac and [509]QNR[511]-AAA weakly recognized several BBMV proteins, as yet unidentified, smaller than 100 kDa.

Ligand blots with [509]QNR[511]-AAA and periodate treated BBMV revealed that the 170 kDa APN displays GalNAc independent binding properties, even though it also has been shown that GalNAc inhibits Cry1Ac binding in surface plasmon resonance experiments (Luo et al., 1997). These results are actually in agreement, since maximal GalNAc inhibition of Cry1Ac binding to 170 kDa APN was less than 70% (Luo et al., 1997). The present results support the proposal of Luo et al. (1997) that one of the two Cry1Ac binding sites on 170 kDa APN involves a GalNAc sugar moiety. This further supports our hypothesis that the 170 kDa APN displays both GalNAc dependent and independent binding properties. Periodate treatment had relatively little effect on [509]QNR[511]-AAA recognition of the 170 kDa protein, while Cry1Ac recognition of periodate treated 170 kDa APN and other proteins was greatly reduced. Since [509]QNR[511]-AAA recognition of GalNAc has been altered, periodate treatment should not effect GalNAc recognition by this mutant.

The >210 kDa molecule bound by Cry1Fa and Cry1Ac did not cross react with anti-APN antibody, suggesting that this molecule is not an APN. Since [509]QNR[511]-AAA bound to the >210 kDa molecule and periodate treatment did not reduce binding, it is likely that carbohydrates are not binding determinants on this molecule. Though the identity of the >210 kDa molecule in *H. virescens* BBMV is unknown, it is similar in molecular size to the Cry1A-binding cadherin-like molecule of *M. sexta* (Vadlamudi et al., 1995).

Our results may also explain the phenomenon described by Lee et al. (1999) where [509]QNR[511]-AAA binding affinity to *H. virescens* BBMV was reduced 15–22 fold, while toxicity was only reduced 3–4 fold. The reduction in [509]QNR[511]-AAA binding to BBMV appears to be correlated with loss of binding to the 120 kDa APN and reduced binding to the 170 kDa APN, while the maintenance of toxicity may be related to continued binding to 110 and 170 kDa APNs.

Typically, the residues between the APN start methionine and the mature protein N-terminus consensus sequence are cleaved during post-translational modification, and are revealed only through cDNA analysis (Gill et al., 1995; Oltean et al., 1999). This modification yields the YRLPTTT APN consensus sequence at the N-terminus of the mature protein. Therefore, we were surprised when the 110 kDa molecule from total BBMV lacked the APN consensus sequence, yet this protein as well as the 110 kDa binding protein purified with toxin affinity chromatography were both recognized by APN antibody. This led us to suspect that two BBMV proteins co-migrated to 110 kDa on SDS-PAGE, one of which is an APN. Amino acid sequencing of peptides released

by V8 protease digestion of the 110 kDa band yielded a 32 kDa peptide containing a variant APN N-terminal consensus sequence, as well as two other peptides that contained a sequence with no known amino acid sequence homologies. The co-migrating 110 kDa proteins were resolved by separating a sub-microgram quantity of BBMV protein on SDS–6% PAGE followed by detection with silver staining. The smaller sized protein had the same electrophoretic mobility as the Cry1Ac-binding protein on SDS–6% PAGE. Since the 110 kDa binding protein purified from affinity chromatography was recognized by anti-APN antibody, this indicates that Cry1Ac and Cry1Fa recognize the 110 kDa APN, and not the unidentified 110 kDa protein.

Further identification of the 110 kDa binding protein as an APN was based on amino acid sequence homology with *H. punctigera* APN 2, and high sequence homology of the 0.9 kb PCR product to *H. punctigera* APN 2. We conclude that the *H. virescens* 110 kDa APN is a homologue of *H. punctigera* APN 2 based on two lines of evidence. First, both of these molecules have lost the PTTT residues found in the lepidopteran APN consensus sequence YRLPTTT. Instead, both contain the sequence YRLRDVVY. Second, BLAST database searches with the 0.9 kb PCR product sequence yielded alignment with only *H. punctigera* APN 2, and no other lepidopteran APNs.

PCR of *H. virescens* cDNA template also yielded an unexpected 0.45 kb product with 98% sequence homology to the *H. virescens* 170 kDa APN. Amplification of the 170 kDa cDNA would not be unusual, since the conserved APN GAMENWG sequence was utilized as the reverse primer, and all lepidopteran APNs including the 170 kDa APN share this sequence. The 0.45 kb PCR product is likely an artifact due to primer degeneracy and forward primer annealing at a non-target sequence. This hypothesis is supported by the absence of nucleotide sequence corresponding to the TNLDEPA amino acids utilized for forward primer design in the 0.45 kb PCR product and the presence of this sequence in the 0.9 kb PCR product.

It is interesting to note that *H. punctigera* is confined to Australia, while *H. virescens* is restricted to North America. The most parsimonious explanation for the APN sequence similarities between these two geographically isolated species is that a single APN gene duplication event occurred in an ancestral Noctuidae lineage before *Helicoverpa* and *Heliothis* diverged (*Heliothis* and *Helicoverpa* are both in Noctuidae). Chang et al. (1999) describe an APN gene duplication event in the ancestral Plutellidae and Sphingidae lineage that resulted in APN genes that are more similar between two species in these two families than within a single species. Although they compared APN genes in separate families, a similar phenomenon may explain the high sequence homology between the *H. virescens* 110 kDa

APN and *H. punctigera* APN 2. That is, a gene duplication event in the ancestral Noctuidae lineage may have resulted in an APN gene that is more similar between *Heliothis* and *Helicoverpa* than it is with other APNs within either of these taxa alone.

The results presented in this paper suggest a mechanistic explanation for the cross-resistance observed between Cry1A toxins and Cry1Fa in the *H. virescens* YHD2 strain (Gould et al., 1995). The data further indicate that the 110 kDa APN is novel, particularly with respect to GalNAc-independent recognition by Cry1Ac. Additional research is needed to examine the role of the 110 kDa APN in Cry1Ac and Cry1Fa toxicity.

Acknowledgements

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