AMINOPEPTIDASE N AS A RECEPTOR FOR *BACILLUS THURINGIENSIS* CRY TOXINS

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1. INTRODUCTION

Bacillus thuringiensis, a Gram-positive bacterium, produces various insecticidal proteinaceous crystal inclusions during sporulation (Hofte and Whiteley, 1989). These inclusions consist of one or more protein protoxins that are grouped into 30 classifications (Cry1-32 and Cyt1-2) according to their amino acid sequences (Crickmore et al., 1998, 2001). When susceptible insects ingest this bacterium, the crystal inclusions are solubilized in the alkaline environment of the insect midgut and processed proteolytically to yield smaller active Cry toxins (Gill et al., 1992). The Cry toxins bind specifically to receptor molecules in the midgut epithelial cells of host insects (Hofmann et al., 1988a; Hofmann et al., 1988b; Van Rie et al., 1989, 1990), altering the ion permeability of the midgut cell membranes (Harvey and Wolfersberger, 1979). A net influx of ions and an accompanying influx of water cause the cells to swell and lyse (Luthy and Ebersold, 1981; Knowles and Ellar, 1987). The formation of either cation-selective (Knowles et al., 1989; Lorence et al., 1995; Slatin et al., 1990) or small nonspecific pores in the membrane has been proposed as a possible mechanism for the toxin action (Carroll et al., 1993).

Various APN isoforms or APN-like proteins have been suggested as candidate receptors for *B. thuringiensis* Cry toxins in several insect species (Knight et al., 1994; Valaitis et al., 1995; Gill et al., 1995; Yaoi et al., 1997). For example, 3 such proteins have been reported in *Bombyx mori* (Hua et al., 1998a; Tsukamoto et al., 1998; Yaoi et al., 1999a), 3 in *Manduca sexta* (Knight et al., 1995; Cahng et al., 1999), 3 in *Plutella xylostella* (Denolf et al., 1997; Denolf, 1997; Chang et al., 1999), 2 in *Heliothis virescens* (Gill et al., 1995; Oltean et al., 1999), 2 in *Lymantria dispar* (Garner et al., 1999), and 2 in *Helicoverpa punctigera* (Emmerling et al., 1999a,b,c). A phylogenetic tree of APN isoforms based on sequence-similarity data shows that APN molecules may be divided into at least 4 classes (Oltean et al.,1999). In addition, it has been reported that different classes of Cry toxins bind to APN molecules from different classes. For example, in *M. sexta*, Cry1C binds to a 106-kDa APN but not to a 115-kDa APN, and Cry1Ac binds to a 115-kDa APN but not to a 106-kDa APN (Luo et al., 1996). In *L. dispar*, Cry1Ac binds to the 120-kDa APN1, but Cry1Aa and Cry1Ab bind to a different molecule (Valaitis et al., 1995; Lee et al., 1996). Since APN diversity may affect the susceptibility of an insect to Cry toxins, we have been studying the diversity of APN isoforms of the midgut of *B. mori* and *P. xylostella*.

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Experiments using membranes reconstituted with APN suggest that APN promotes insertion of Cry toxins into membranes and pore formation by these toxins (Sangadala et al., 1994; Luo et al., 1997; Schwartz et al., 1997; Cooper et al., 1998). APN has been shown to function as a Cry toxin receptor *in vitro*, but it is unknown whether APN functions as a receptor on epithelial cell membranes in the environment of the insect midgut. Furthermore, cadherin-like proteins (CLPs) have also been proposed as candidate receptors for Cry1Aa and Cry1Ab toxins (Vadlamudi et al.,1995; Nagamatsu et al., 1998a; Nagamatsu et al., 1998b; Ihara et al., 1998). Our observations have confirmed that Cry1Aa and Cry1Ab bind to both APN and recombinant cadherin-like protein in *B. mori* (data not shown). However, the multifunctional binding mechanism of these toxins is still unknown, and it is uncertain which candidate receptor is important for *B. mori* susceptibility. To elucidate the mechanisms by which Cry toxins show differential toxicities in different insects, we have examined these issues by a variety of approaches.

2. CLONING AND CLASSIFICATION OF APN ISOFORMS FROM *B. MORI* AND *P. XYLOSTELLA*

A phylogenetic tree for available APN isoforms was constructed with ClustalX (Thompson et al., 1997) by the neighbor-joining method based on sequence similarity data. APN isoforms from insects were shown to separate into at least 4 classes (Figure 1). The group including *B. mori* APN1 (110 kDa) (Yaoi et al., 1999), *M. sexta* APN1 (120 kDa) (Knight et al., 1995; Luo et al., 1999), and *H. virescens* APN (170 kDa) (Oltean et al., 1999) was tentatively named class 1. The isoform group including *P. xylostella* APN1 and *M. sexta* APN2 (Denolf et al., 1997) was named class 2, the group including *H. virescens* BTBP1 (Gill et al., 1995) and *L. dispar* APN1 (120 kDa) (Garner et al., 1999) was named class 3, and the group including APN-like protein from *P. xylostella* (GenBank accession no. AJ222699) (Denolf, 1997) was named class 4. As shown in Figure 1, in all insect species examined, 1, 2, or 3 classes of APN isoforms or APN-like proteins are present, and no insect has been reported to have all 4 isoforms. Accordingly, we addressed the question of whether every insect species has APNs from all 4 classes, and if not, how many APN isoforms an insect species typically possesses.

When amino acid sequences of APNs from insects and mammals were compared, several conserved regions were observed. Cloning of an APN isoform cDNA from the *B. mori* midgut was conducted by PCR with degenerate primers based on the conserved amino acid sequences. The 5' and 3' ends of the resulting cDNA fragment were amplified by RACE, resulting in acquisition of the complete cDNA. Alignment of the deduced amino acid sequence with those of other APNs followed by construction of a phylogenetic tree showed that the cloned cDNA encodes a novel class 3 APN (BmAPN3) (Figure 1). Thus, a novel cDNA encoding a class 3 APN from *B. mori* was cloned, adding to the complement of APN class 1, 2, and 4 cDNA fragments already reported from *B. mor I* (Nakanishi et al., unpublished).

We next cloned a complete APN isoform cDNA from *P. xylostella* midgut in the same way. Alignment of the deduced amino acid sequence with those of other APNs followed by construction of a phylogenetic tree showed that the cDNA encodes a novel class 3 APN (PxAPN3) (Figure 1). Thus, an APN class 3 cDNA fragment was cloned, adding to the class 1, 2, and 4 APN cDNA fragments already reported. These results show that *B. mori* and *P. xylostella* express in the midgut all 4 APN classes, suggesting that closely-related insect species, and possibly even *Manduca*, *Lymantria*, *Heliothis*, and *Helicoverpa*, might express all 4 classes as well.

Although BmAPN4, HpAPN2, and PxAJ222699 were all grouped into the same class, the similarity between PxAJ222699 and the BmAPN4 and HpAPN2 subclass is not high (Figure 1). As more APNs are discovered, a reclassification of some of these APNs may become necessary, and class 3 may be split into 2 or more classes. In fact, we have cloned a partial cDNA fragment encoding a new APN-like protein that appears to be a fifth APN isoform in *P. xylostella*. Further,

as described later in this report, we have found in *B. mori* a 120-kDa protein that binds to Cry1Aa toxin but does not react with any class-specific APN antisera. Therefore, *B. mori* and *P. xylostella* may have 5 or more APN isoforms in their midgut epithelial cells.

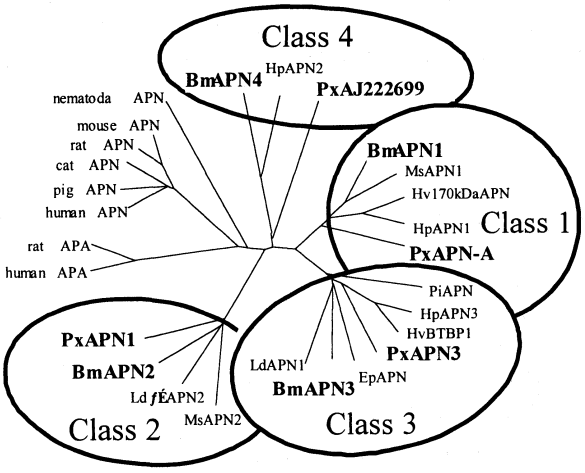

Figure 1. Protein sequence similarity among insect and mammalian aminopeptidase N isoforms. Sequences were aligned using ClustalX (Thompson et al., 1997), and a phylogenetic tree was drawn using PHYLIP (Joe Felsenstein). GenBank accession numbers for the amino acid sequences shown are: *B. mori* APN1 (BmAPN1), AF084257 (Yaoi, 1999); *B. mori* APN2 (BmAPN2), AB011497 (Hua, 1998 CBPBB); *B. mori* APN3 (BmAPN3), AF352574; *B. mori* APN AB013400 (BmAPN4), AB013400; *M. sexta* APN1a (MsAPN1a), AF123313 (Lou, 1999); *M. sexta* APN2 (MsAPN2), X97877 (Denolf et al., 1997); *H. virescens* 170-kDa APN (Hv170kDaAPN), AF173552 (Oltean, 1999); *H. virescens* BTBP1 (HvBTBP1), U35096 (Gill, 1995); *Helicoverpa punctigera* APN1 (HpAPN1), AF217248 (Emmerling et al., 1999a); *H. punctigera* APN2 (HpAPN2), AF217249 (Emmerling et al., 1999b); *H. punctigera* APN3 (HpAPN3), AF2172450 (Emmerling et al., 1999c); *P. xylostella* APN-A (PxAPN-A), AF020389 (Chang, 1999); *P. xylostella* APN1 (PxAPN1), X97878 (Denolf et al., 1997); *P. xylostella* APN (PxAJ222699), AJ222699 (Denolf, 1997); *L. dispar* APN1 (LdAPN1), AF126442 (Garner, 1999); *L. dispar* APN2 (LdλAPN2), AF126443 (Garner, 1999); *P. interpunctella* APN (PiAPN), AF034483 (Zhu, 2000); *Epiphyas postvittana* APN (EpAPN), AF276241 (Simpson, 2000); *H. contortus* APN (nematoda APN), X94187 (Smith, 1997); *M. musculus* APN (mouse APN), BC005431; *R. norvegicus* APN (rat APN), M26710 (Malfroy, 1989); *F. catus* APN (cat APN), U58920 (Tresnan, 1996); *S. scrofa* APN (pig APN), Z29522 (Delmas, 1994); *H. sapiens* APN (human APN), X13276 (Olsen, 1988); *R. norvegicus* aminopeptidase A (rat APA), AF146044 (Lee, 2000); *H. sapiens* aminopeptidase A (human APA), L14721 (Nanus, 1990).

3. STRUCTURE OF *B. MORI* APN ISOFORMS

The structural characteristics of 4 APN isoforms from *B. mori* are shown schematically in Figure 2. In each of the mature peptides, Zn^{2+}-binding motifs of the gluzincin metalloprotease type are found at amino acid positions 310 - 320 (Hooper et al., 1994). This motif consists of His-Glu-X-X-His, followed 19 residues downstream by a Glu residue. A Gly-Ala-Met-Glu-Gln motif at position –36 upstream of the His-Glu-X-X-His sequence distinguishes gluzincin aminopeptidases from other gluzincins (Laustsen et al., 1997). APN activities have been reported for BmAPN1, BmAPN2, and BmAPN4 (Yaoi et al., 1997; Hua et al., 1998a,b,c).

Glycosylphosphatidylinositol (GPI) anchor signal sequences (Englund et al., 1993), consisting of 3 small amino acids and a stretch of 19 - 21 hydrophobic residues, are found at the C termini of all 4 *B. mori* APNs. Since *B. mori* BmAPN1 can be cleaved from the midgut membrane by phosphatidylinositol-specific phospholipase C (PI-PLC) (Yaoi et al., 1997; Hua et al., 1999b), BmAPN1 is probably linked to the membrane by a GPI anchor.

Potential *n*-glycosylation sites of differing types are present in all 4 APN isoforms of *B. mori*,

and NetOglyc 2.0 (Hansen, 1995), a program for *o*-glycosylation site prediction, predicts potential *o*-glycosylation sites in all four isoforms. For the class I APN of *H. virescens*, Oltean et al. (1999) reported a discrepancy between the molecular mass estimated by SDS-PAGE (170,000) and that deduced from the cDNA sequence (113,000). This discrepancy may be due to extensive *o*-glycosylation. For BmAPN3, 11 such sites are predicted. The molecular weight of BmAPN3, as deduced from the cDNA sequence, is 107,500. Its molecular weight has not yet been examined by SDS-PAGE, but it is likely that it will appear much larger by this method due to glycosylation at its many predicted sites.

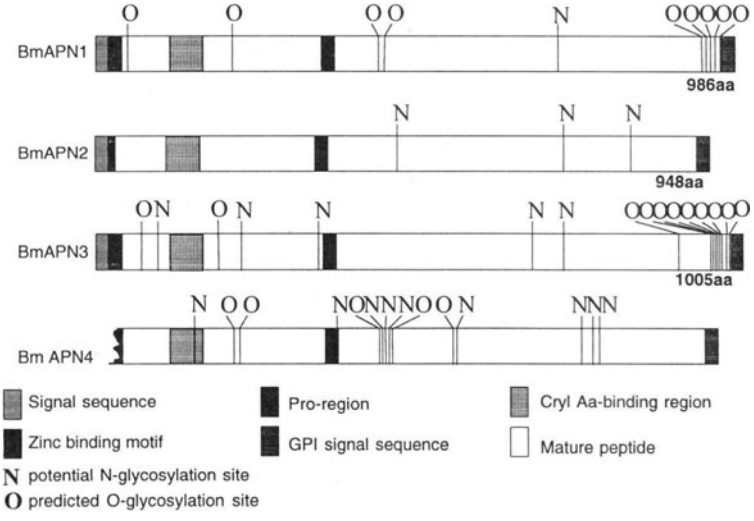

Figure 2. Structural comparison of 4 APN isoforms from *Bombyx mori*.

4. BINDING OF CRY1 TOXINS TO APN ISOFORMS OF *B. MORI*

To determine whether each of the 4 APN isoforms of *B. mori* can act as a receptor for Cry1A, we studied the binding of each of these molecules to Cry1Aa, Cry1Ab, and Cry1Ac. Since the 4 APN isoforms are similar in molecular weight to each other and to other unidentified toxin-binding proteins, it is difficult to identify each APN isoform by SDS-PAGE. Hence, protein fragments corresponding to non-conserved regions of the APN isoforms (amino acids 57 - 173 of BmAPN1) were expressed as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* cells. The fusion proteins were purified and used to raise APN class-specific antisera in mice. Proteins from midgut brush border membrane vesicles (BBMVs) of *B. mori* were separated by SDS-PAGE and immunoblotted with the APN class-specific antisera to identify the APN isoforms. The anti-BmAPN1, -BmAPN2, and -BmAPN4 antisera bound to 110-kDa, 90-kDa, and 100-kDa proteins, respectively (Figure 3) (Nakanishi et al., unpublished). These observations are consistent with previous reports (Yaoi et al., 1997; Hua et al., 1999a,b,c).

We used ligand blotting to identify the proteins binding to Cry1Aa, Cry1Ab, and Cry1Ac. Cry1Aa bound mainly to 230-, 120-, and 110-kDa proteins at 10 nM, suggesting that Cry1Aa binds to BmAPN1 and other unidentified proteins (Figure 3). Cry1Ab was not observed to bind to any protein at 10 nM, but at 50 nM, it bound to 230- and 110-kDa proteins, suggesting that Cry1Ab binds to BmAPN1 and one unidentified protein. Cry1Ac was not observed to bind to any protein at 10 nM, but at 50 nM, it bound to a 230-kDa protein, suggesting that Cry1Ac does not bind to any of the identified APN isoforms at low concentration. These observations indicate that the 3 Cry1A

toxins bind APN isoforms in different manners with different affinities. On the other hand, CLP, which yields a band of approximately 180 kDa by SDS-PAGE (Nagamatsu et al., 1998b; Ihara et al., 1998), did not give a signal when ligand blotted with Cry1Aa, b, or c, although the band stained in Western blots with anti-cadherin antiserum. The IC_{50} values for Cry1Aa and Cry1Ac protoxins against *B. mori* 3rd instar larvae were reported to be 1.9 and 174.5 μg/g diet respectively (Shinkawa et al., 1999), and the IC_{50} value for Cry1Ab protoxin against *B. mori* 4th instar larvae was reported to be 175.8 μg/g (Ihara et al., 1993). It seems likely that this variation in susceptibility is at least partially due to differences in the Cry-binding properties of the various APN isoforms

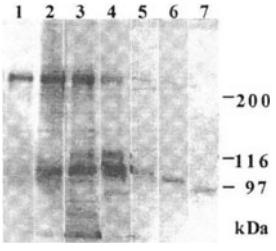

Figure 3. Identification of Cry1A toxin-binding proteins. BBMV proteins were separated on a 6 % polyacrylamide SDS-PAGE gel and ligand or Western blotting was conducted. Lanes 1-3, ligand blotting with 50 nM Cry1Ac, 50 nM Cry1Ab, and 10 nM Cry1Aa respectively; lane 4, Western blotting with anti-full-length BmAPN1 antiserum that cross-reacts with all APN isoforms; lanes 5-7, Western blotting with BmAPN1-specific antiserum, BmAPN4-specific antiserum, and BmAPN2-specific antiserum respectively.

5. IDENTIFICATION OF THE CRY TOXIN-BINDING DOMAIN OF *B. MORI* APN ISOFORMS

Cry1Aa toxin can bind to full-length recombinant BmAPN1 produced in *E. coli* (Yaoi et al., 1997), indicating that its native glycosylation state is not required for formation of the binding epitope. Thus, the recombinant protein has been used as a model for studying the binding epitope. Protein fragments from various parts of BmAPN1 were produced as GST fusion proteins in *E. coli*, and Cry1Aa-binding ability was assessed by ligand blotting. As a result, the Cry1Aa binding region of BmAPN1 was narrowed to the region Ile135 - Pro198 (Yaoi et al., 1999b) (Figure 4). The same region of the *P. xylostella* class 3 APN, PxAPN3, was also shown to bind to Cry1Aa (Nakanishi et al., 1999) (Figure 4). Thus, the conserved residues in this region may be important for Cry1Aa binding. A comparison of this region of BmAPN1 and PxAPN3 with sequences from other APNs from several insects showed that this region includes both conserved and variable subdomains; about 81% of the residues common to BmAPN1 and PxAPN3 are highly conserved in the other insect APNs (Nakanishi et al., 1999). Since Cry1Aa toxin does not bind to the 120-kDa APN, LdAPN1, from *L. dispar* BBMV (Lee et al., 1996), small sequence differences in this region are likely to have a crucial effect on Cry1Aa-binding affinity and insect susceptibility.

In the present study, toxin-binding regions from each APN class of *B. mori* and *P. xylostella* were produced as GST fusion proteins, and ligand blotting was conducted to compare their binding affinities for Cry1Aa. At 10 nM, Cry1Aa bound to the regions derived from class 1, 2, and 4 molecules of both *B. mori* (BmAPN1, BmAPN2, and BmAPN4, respectively) and *P. xylostella* (PxAPN-A, PxAPN1, and PxAJ222699, respectively). At a higher concentration (100 nM), Cry1Aa also bound to the class 3-derived binding regions from both *B. mori* (BmAPN3) and *P. xylostella* (PxAPN3) (Nakanishi et al., unpublished). Therefore, the conserved region in each of the 8 APN isoforms appears capable of recognizing Cry1Aa. No significant differences in amino acid sequence of the conserved subdomain are present in the toxin-binding regions of the eight isoforms, and

therefore the reason for the low affinity of the class 3 molecules (BmAPN3 and PxAPN3) for Cry1Aa is unclear (Figure 4). Most of the conserved amino acids within the *B. mori* and *P. xylostella* APNs are also conserved in human APN, suggesting that Cry1Aa may bind not only to APN from insects other than Bombycidae and Plutellidae, but also to mammalian (human) APN, although this supposition has not yet been tested.

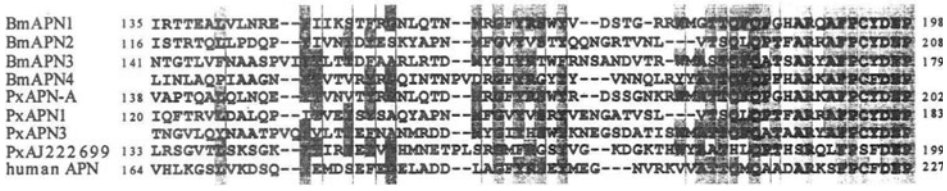

Figure 4. Comparison of the amino acid sequences of the Cry1Aa-binding regions of APN isoforms from *B. mori*, *P. xylostella*, and *H. sapiens*. Residues conserved in more than half of the 9 APN isoforms are shaded.

Cry1Aa bound with high affinity to the toxin-binding regions from BmAPN2 and BmAPN4 when they were produced as GST-fusion proteins, but it did not bind to full-length BmAPN2 and BmAPN4 separated from BBMV by SDS-PAGE. Midgut BBMV may contain only a small amount of BmAPN2 and BmAPN4, or some feature of the full-length APNs may disturb the binding of Cry1Aa to their toxin-binding regions. In any case, this result demonstrates that APN isoforms do not always function as receptors even in the presence of the potential binding epitopes.

Molecules of the integrin family bind to specific amino acid motifs. For example, integrin $\alpha V\beta 3$ binds to the Arg-Gly-Asp motif of many proteins (Cheresh et al., 1989; Gehlsen et al., 1988). Similarly, Cry1Aa and Cry1Ab can bind not only to APN but also to CLP (Vadlamudi et al., 1995; Nagamatsu et al., 1998a, 1999; Ihara et al., 1998), suggesting that CLP and APN might have consensus motifs responsible for Cry1A binding. However, we did not find any consensus motifs for the 2 molecules. Hence, we conclude that Cry1Aa recognition does not require a specific sequence motif, or, alternatively, that Cry1Aa toxin has two distinct binding sites, one for APN and the other for CLP.

Cry1Aa, Cry1Ac, and Cry8Ca can bind to at least 9 non-APN-like proteins, including bovine pancreatic RNase A, bovine carbonic anhydrase, and *E. coli* ß-galactosidase. Bovine carbonic anhydrase inhibits binding of Cry1Aa to BmAPN1, and phosphatidyl inositol-specific phospholipase c digests of BBMV, which should contain solubilized APN, inhibit the binding between Cry1Aa and bovine pancreatic RNase A or *E. coli* β-galactosidase. Furthermore, carbonic anhydrase reduces Cry1Aa cytotoxic activity *in vitro* when preincubated with Cry1Aa (Kadotani et al., unpublished). From these results, we conclude that all these proteins bind to the same or neighboring sites on Cry1Aa.

In order to determine the Cry1Aa toxin-binding regions of bovine carbonic anhydrase and bovine pancreatic RNase A, these proteins were hydrolyzed with acid or protease to yield small fragments. Testing of these fragments showed that Leu189 - Lys261 of bovine carbonic anhydrase and Arg10 - Thr70 of bovine pancreatic RNase A contain Cry1Aa-binding epitopes (data not shown). No consensus sequence was found for these Cry1Aa-binding epitopes. As shown in Figure 5, Arg10 - Thr70 of bovine pancreatic RNase A contains helices, β-strands, a random coil, and loops (Birdsall and Mcpherson, 1992). No contribution of carbohydrate to the binding of Cry1Aa has been reported (Masson et al., 1995; Luo et al., 1997), and *E. coli* β-galactosidase does not contain mammalian-type glycosylation. These observations suggest that the Cry1A-binding epitope of bovine pancreatic RNase A must consist of a simple structure made of non-glycosylated amino acids.

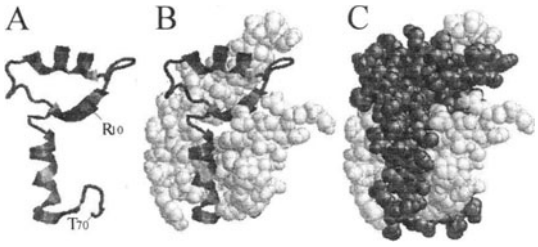

Figure 5. Molecular model of the Cry toxin-binding structure of bovine RNase A. The model was generated from PDB file 1RTB (Birdsall and McPherson, 1992) with RasMol v. 2.6 (Sayle and Milner-White, 1995). A, secondary structure of the Cry1Aa-binding fragment Arg10 - Thr70. B and C, full-length RNase A molecule with the Cry1Aa binding surface highlighted.

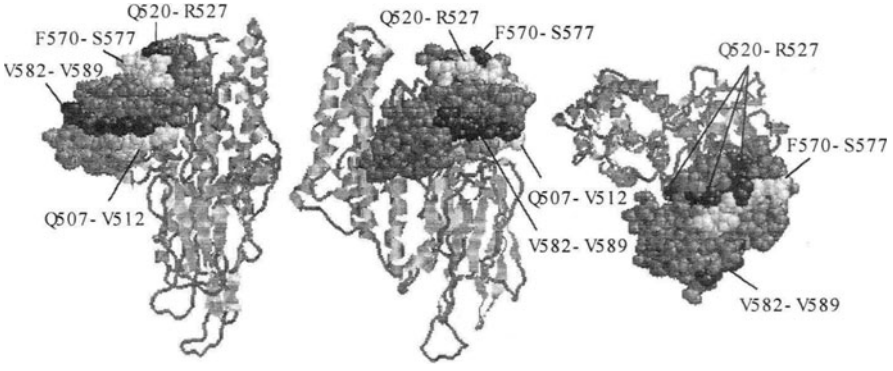

Figure 6. Candidate epitope for binding of monoclonal antibodies 1B10 and 2C2 to Cry1Aa toxin. The putative binding domain at Ile514 - Asp615 is shown in gray in a space-filling representation. Other amino acids that may contribute to formation of the epitope are shown in black or white.

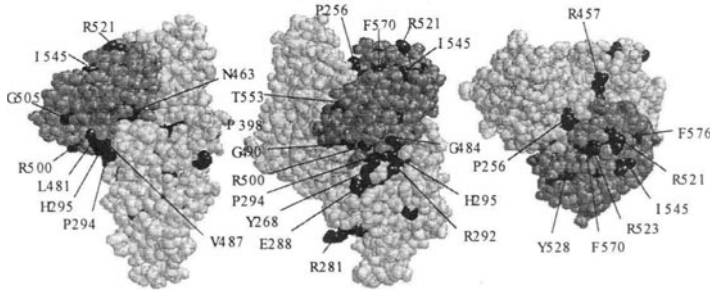

Figure 7. Amino acid residues conserved for *B. mori* Cry1Aa, Cry1Ac, and Cry9Da toxins. The 3-dimensional structure of Cry1Aa is shown (PDB accession no. 1CBY) (Grochulski et al., 1995), with the consensus amino acids superimposed in black. Domain III is shown in gray.

6. BINDING SITE FOR BMAPN1 ON CRY1AA

BALB/c mouse was immunized with Cry1Aa toxin and monoclonal antibodies were screened. The binding of Cry1Aa to BmAPN1 is inhibited by 2 different monoclonal antibodies, 1B10 and

2C2, suggesting that their binding sites and the BmAPN1 binding site are located very close together on Cry1Aa. To localize these epitopes on Cry1Aa, Cry1Aa deletion mutant proteins were produced in *E. coli* and used in Western blotting with 1B10 and 2C2. The 2C2 antibody bound to the fragment Ile514 - Asp615 in domain III of Cry1Aa and to fragment Met1 - Val512, but not to Met1 - Gln506 (Atsumi et al., unpublished). A reasonable interpretation of these results is that the 2C2 epitope is composed of amino acids from both Ile514 - Asp615 and Ile507-Val512. In fact, the Ile507 - Val512 segment is adjacent to Ile514 - Asp615 in the structure. A similar epitope is probable for 1B10, since 1B10 bound to Met1-Val512 and Ile514 - Asp615, but not to Met1-Gln506 or Met1-Pro256.

To further map the 1B10 and 2C2 epitopes, a series of 8-amino acid polypeptides (overlapping by 6 residues) from domain III of Cry1Aa were synthesized using a MULTIPIN™ peptide synthesis kit (CHIRON). The ability of these peptides to bind to 1B10 and 2C2 was assessed by an enzyme-linked immunosorbent assay. Antibody 1B10 bound to the Val582-Val589 peptide (Atsumi et al., unpublished). Since this peptide abuts a segment (Ile507 - Val512) of the candidate epitope, the amino acids contained in these 2 strands may constitute the 1B10 epitope (Figure 6). On the other hand, the 2C2 epitope is clearly different from that of 1B10, since 2C2 bound to synthesized peptides Gln520-Arg527 and Phe570-Ser577 in addition to Val582-Val589. Thus, amino acids from both Val582-Val589 and Ile507-Val512 may form the 2C2 epitope, since these 2 segments are close together. Since 1B10 and 2C2 inhibit each other's binding to Cry1Aa, and each antibody almost completely inhibits binding of Cry1Aa toxin to BmAPN1, these antibodies appear to bind to single sites within close proximity on Cry1Aa. Hence, it is most likely that Val582-Val589 contains the epitope-forming amino acids. On the other hand, Gln520-Arg527 and Phe570-Ser577 are contained in Ile514-Asp615 but far from Ile507-Val512. However, even if Gln520-Arg527 or Phe570-Ser577 is part of the 2C2 epitope, 2C2 might be able to inhibit the binding of both APN and 1B10 to the toxin. Consequently, we cannot rule out the involvement of amino acids from Gln520-Arg527 or Phe570-Ser577 in forming the 2C2 epitope.

These experiments suggest that the binding sites for 1B10 and 2C2 are on domain III of Cry1Aa (Figure 6). On the other hand, several other monoclonal antibodies that bound to domain III but did not inhibit the binding of the toxin to BmAPN1 were found in the same experiment. Consequently, our results with antibodies 1B10 and 2C2 suggest that the BmAPN1 binding site is on the specific segment of domain III, on the domain adjacent to domain III, or at the inter-domain region consisting of domain III and another domain. Domain III is reported to be important for binding specificity or host specificity (Lee et all., 1995; de Maagd et al., 1996a,b; 1999). Jenkins et al. (1999) reported that mutagenesis of Gln509, Arg511, or Tyr513 of Cry1Ac toxin resulted in elimination of binding to the *M. sexta* 120-kDa APN. In addition, Burton et al.(1999) reported that mutagenesis of Asn506, Gln509, or Tyr513 of the toxin reduced binding to APN. These reports are not in conflict with our data discussed above.

In our ligand blotting experiments, in which BBMV proteins were separated by SDS-PAGE, Cry1Aa, Cry1Ac, and Cry9Da bound to BmAPN1, and binding of each was inhibited reciprocally by the other 2 toxins (Shinkawa et al., 1999). These results suggest that these distantly related Cry toxins bind to the same site on BmAPN1. If this is true, then the structures conserved in these three toxins may be important for APN binding. The sequences of Cry1Aa, Cry1Ac, and Cry9Da were compared, and the consensus amino acid residues were plotted onto the 3-dimensional structure of Cry1Aa (Figure 7). Five main islands of consensus were observed : (1) an interdomain region (domains I, II, and III) consisting of Leu236, Arg265, Glu266, Tyr268, Glu288, Ile291, Gly490 and Pro491; (2) an adjacent interdomain region (domains II and III) consisting of Arg292, Pro294, His295, Leu296, His433, Leu481, Gly484, Val487, and Leu481; (3) an interdomain region on the opposite face (domains I, II, and III) consisting of Gln262, Arg430, His456, Arg457, Gln472, and Lys477; (4) a region in domain III containing Arg521, Tyr522, Arg523, Arg525, and Phe570; and (5) a region in domain II containing Asp409, Leu411, Pro416, Arg424, and Thr269. Conserved region 3, which lies between domains II and III, forms a concave site consisting of Arg292, Pro294,

His295, Leu481, Gly484, and Val487 that is in close proximity to the segments comprising the candidate monoclonal antibody epitope, Gln506-Val512 and Val582-Val589 (Figures 6 and 7). In addition, the conserved region in domain III contains 2 conserved amino acid residues, Arg521 and Arg523, which are contained in the other candidate epitope for antibody 2C2. Hence, these conserved amino acid residues may form at least part of the APN recognition site on Cry1A.

7. DISTRIBUTION OF APN IN THE MIDGUT OF *B. MORI*

Functional receptors for the Cry toxin must exist on the surface of the brush border membrane of midgut epithelial cells and in the epithelial cell region that undergoes disruption upon ingestion of Cry toxin. These Cry toxin receptors must be capable of assembling toxin molecules into a multimeric ion channel. Hence, we examined the distribution of APN in the midgut of *B. mori* and the affinity of this APN for Cry toxins.

Deparaffinated and hydrated midgut tissue sections from 2nd instar *B. mori* larvae were treated with anti-BmAPN1 antiserum and immunocytochemically stained using the chromogenic reagent VECTOR^{R} VIP Substrate (Vector Laboratories). Only the brush border of the midgut columnar cells was stained (Hara et al., unpublished). Furthermore, when an entire midgut tissue was excised, fixed, and immunocytochemically stained as above, all areas of the inner surface of the midgut were stained. Thus, APN seems to be distributed in the brush border of all areas of the midgut. When the 2nd instar larvae ingested Cry1Aa, columnar cells from every part of their midguts were damaged. The distribution of susceptible midgut cells appeared to correlate with the distribution of APN. Since the antiserum against BmAPN1 cross-reacts with at least 3 APN classes (classes 1, 2, and 4) in Western blots, the staining observed in the above experiments could be due the presence of APN molecules of any of these classes or a combination thereof.

When an entire midgut was immunocytochemically stained using anti-CLP antiserum, all areas of the inner surface of the midgut were stained. In addition, staining of a midgut section showed that only the brush border from midgut columnar cells was stained (Hara et al., unpublished). Cadherins are believed to mediate calcium-dependent cell-cell adhesion (Takeichi, 1991), but the CLP of *B. mori* appears to be present only at the midgut brush border. Transfected Sf9 cells expressing CLP are reported to acquire Cry1Aa susceptibility (Nagamatsu et al., 1999). Hence, CLP may function as a receptor for Cry1Aa toxin in the midgut of *B. mori*.

Dissociation constants for Cry1Aa binding to BmAPN1 and CLP from *B. mori* were reported to be approximately 7 (Yaoi et al., 1997; Shinkawa et al., 1999) and 2.6 nM (Ihara et al., 1998), respectively. Although these values cannot be directly compared, since the methods used in the 2 studies were different; they do demonstrate that the affinity of the receptors for the toxin may be high enough to allow receptor-mediated assembly of toxin molecules into functional ion channels at the cell surface.

8. CONCLUSIONS

Cry toxins can bind to many kinds of proteins, including APN, CLP, pancreatic RNase A, carbonic anhydrase, and others. Indications are that the binding sites of APN and other proteins on Cry toxins overlap or exist in close proximity. Localization of the binding sites on the toxin and elucidation of the mechanism for binding of multiple proteins to the toxin are important in understanding the origin of specificity of Cry toxin binding.

APN isoforms are abundant in the brush border membrane distributed throughout the insect midgut, where they correlate with sites damaged by the toxin. Furthermore, APN isoforms can promote membrane insertion or pore formation by Cry toxins. Consequently, it is possible that APN isoforms function as Cry receptors in the midgut of some or all Cry-susceptible insects. At least 4

kinds of APN isoforms are known, and each isoform binds with different affinity to each toxin. Further complicating matters, CLP may also contribute to insect Cry toxin susceptibility. This complexity makes the determinants of Cry toxin susceptibility difficult to explicate. As further steps in understanding Cry toxin specificity, the identities of the functional Cry toxin receptors in the midgut of susceptible insects must be determined and their roles must be studied.

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