ANALYSES OF TOXIN-RECEPTOR INTERACTION IN *MANDUCA SEXTA* USING *BACILLUS THURINGIENSIS* CRY1A MUTANT TOXINS

by

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ABSTRACT

The mechanism of action of *Bacillus thuringiensis* Cry1A toxin in susceptible larvae involves activation of toxin, binding to apical membrane proteins of larval midgut, insertion of toxins into membrane, and pore formation. I studied two aspects of the mechanism of action of Cry1A toxins in this research using Cry1A mutant toxins. The first aspect analyzed pore-forming properties of Cry1A domain I and domain II mutant toxins with *Manduca sexta* brush border membrane vesicles (BBMV) using a light scattering assay. The second aspect focused on identifying alternate *M. sexta* Cry1Ac-binding protein(s) and studying effect of protein denaturation on toxin-receptor binding specificity.

In the light scattering assay, wild-type toxins Cry1Ac and Cry1Ab and mutant toxins A92D and A92E were able to form pores in BBMV in the presence of KCl. However, in the presence of sucrose, mutants A92D and A92E did not form pores, unlike Cry1Ac and Cry1Ab which was probably due to a charge-masking effect by KCl. Our results with the light scattering assay indicated that toxin-structures in membrane resembled pores.

We studied binding of Cry1Ac and Cry1Ac mutant ^{509}QNR^{511}-AAA with *M. sexta* BBMV and MsAPN-1 under denaturing and non-denaturing conditions. Toxin-binding specificity was altered under denaturing conditions indicating that binding epitopes absent on native protein surface were exposed under denaturing conditions.

Finally, the presence of *M. sexta* BBMV protein(s) that could bind Cry1Ac independent of N-acetylgalactosamine (GalNAc) was investigated. Cry1Ac mutant ^{509}QNR^{511}-AAA with a disrupted GalNAc-interaction pocket is toxic to *M. sexta* even though binding to MsAPN-1 is abolished suggesting that protein(s) other than MsAPN-1 mediates GalNAc-independent Cry1Ac toxicity. We isolated a 110 kDa protein (Ms110-APN) by toxin-affinity chromatography and cloned the full-length Ms110-APN cDNA and expressed the protein in *E. coli*. Cry1Ac and ^{509}QNR^{511}-AAA bound *E. coli*-expressed Ms110-APN indicating that Ms110-APN is a GalNAc-independent Cry1Ac-binding protein in *M. sexta*.

The overall conclusions of my studies with Cry1A mutant toxins are that Cry1A toxins utilize different brush border proteins to interact with midgut membrane. Toxins also increase the chance of membrane insertion by utilizing its positively charged residues to interact with negatively charged midgut membrane.

INDEX WORDS: *Bacillus thuringiensis*, δ-endotoxin, Cry1A mutant toxin, Cry1Ac, Cry1Ab, ^{509}QNR^{511}-AAA, *Manduca sexta*, brush border membrane vesicles, light-scattering assay, aminopeptidase N, N-acetylgalactosamine

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DEDICATION

I dedicate this dissertation to my family:

My late father Mr. M. T. Daniel (Papa) and my mother Mrs. Sosamma Daniel (Amma) who believed in me and constantly encouraged me in all my endeavors even during the most difficult of times.

My wife Shobha, for her love, support and constant encouragement.

Our daughter who brings us joys and cheers.

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CHAPTER 1

INTRODUCTION

1.1 *Bacillus thuringiensis:* An introduction

Bacillus thuringiensis is a gram-positive, aerobic, spore-forming bacterium. It is closely related to two other important spore-forming Bacilli, *Bacillus anthracis* and *Bacillus cereus* and is differentiated by the presence of several plasmid-encoded protoxin genes (Aronson and Shai, 2001). *B. thuringiensis* produces parasporal crystalline inclusions during sporulation in addition to other virulence factors such as exotoxins, phospholipases and vegetative insecticidal proteins (Tanada and Kaya, 1993). The crystalline inclusions are composed of one or several pesticidal crystal proteins also called Cry proteins or δ-endotoxins. Cry proteins are toxic to a broad range of insects in the Orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, and Phthiraptera as well as other invertebrates (Nemathelminthes, Platyhelminthes and Sarcomastigophora) (Feitelson, 1993). However, these proteins do not show any activity against vertebrates and are harmless to humans (Adang, 1991). Therefore, *B. thuringiensis*-based biopesticides have become valuable alternatives to synthetic pesticides because of their high toxicity and target insect specificity as well as environmental safety.

By the early 1980s, the HD-1 isolate was commercially produced for the control of lepidopteran insects (Luthy *et al.*, 1982). However, limited field stability caused by the sensitivity of *B. thuringiensis* crystals to ultraviolet light proved to be a major disadvantage of *B. thuringiensis* sprays. This required multiple spray regimens during a crop season. Also, the high cost of *B. thuringiensis* products and narrow spectrum of activity has prevented *B. thuringiensis* sprays from occupying a major share in the

insecticide market (Ferre and Van Rie, 2002). In 1999, the total sales of Bt products constituted <2% of the total value of all insecticides (Shelton *et al.*, 2002).

A number of *cry* genes were cloned into plants (Adang *et al.*, 1993). High levels of toxin expression in plants were achieved by optimization of codon usage (Adang *et al.*, 1993). Since 1996, transgenic plants (e.g. cotton, corn, potato, rice) harboring *cry* genes and expressing high levels of toxin were commercially released in the US and around the world (James, 2000). In 2000, transgenic plants harboring *cry* genes were grown on 11.4 million ha worldwide (Shelton *et al.*, 2002). The percentage of total area of *B. thuringiensis* corn in the United States was approximately 26% (8 million ha) in 2000. The area of *B. thuringiensis* cotton in the United States was 1.78 million ha in 2000 (Shelton *et al.*, 2002).

1.2 *Bacillus thuringiensis* crystal protein genes

The genome size of *B. thuringiensis* strains ranges from 2.4 to 5.7 million bp (Carlson *et al.*, 1994). *B. thuringiensis* isolates typically harbor several large plasmids ranging from 30 to over 200 mDa in size, and the *cry* genes are usually located in these plasmids (Gonzalez *et al.*, 1981). Currently, more than 150 different *cry* genes have been cloned and classified (Crickmore *et al.*, 2002). The classification scheme for Cry proteins is based exclusively on amino acid identity allowing closely related toxins to be ranked together (Crickmore *et al.*, 1998). Cry proteins are assigned Arabic numerals for the primary rank, capital letters for the secondary rank and lowercase letters for the tertiary and quaternary ranks corresponding to the percent amino acid identity (Crickmore *et al.*, 1998).

1.3 The Cry toxin: structure and function

Most Cry toxins possess five conserved blocks of amino acids (Hofte and Whiteley, 1989). The five blocks are located in predicted pore-forming regions and in regions of contact between structural domains. The location of the conserved blocks implied that the overall tertiary structure of Cry toxins is similar (Schnepf *et al.*, 1998). This hypothesis was confirmed when crystal structures of several Cry toxins were solved. Toxins with different insect specificities; for example Cry1Aa (lepidopteran-specific) (Grochulski *et al.*, 1995), Cry2Aa (lepidopteran- and dipteran-specific) (Morse *et al.*, 2001), Cry3Aa (Li *et al.*, 1991), and Cry3Bb (Galitsky *et al.*, 2001) (coleopteran-specific) have similar overall tertiary structures consisting of three distinct domains (Fig. 1-1). The N-terminal domain I consists of a bundle of seven amphipathic α-helices with six of the helices surrounding a central hydrophobic helix α5 (Fig. 1-2). Domain II consists of three antiparallel β-sheets with a 'Greek key' conformation arranged in a β-prism fold. The C-terminal domain III contains two antiparallel β-sheets in a 'jelly-roll' topology (Li *et al.*, 1991).

The amphipathic and hydrophobic helices of domain I suggest that this domain is responsible for membrane insertion and pore formation in insect midgut epithelium. Domain I is structurally similar to the pore-forming domain of other bacterial toxins including diphtheria toxin and *Pseudomonas* exotoxin A (Parker and Pattus, 1993). Based on the mechanism of pore-formation by colicin A and diphtheria toxin, the 'umbrella model' for Cry toxin pore formation was proposed (Li *et al.*, 1991). According to this model helices α4 and α5 inserts into the membrane while the other helices lie flat on the membrane resembling spokes of an umbrella. Using synthetic peptides

corresponding to domain I α-helices Gazit *et al.* (1998) presented evidence in favor of the 'umbrella' model by demonstrating that helices α4 and α5 inserted into the membrane while other helices flattened out on the membrane surface. Schwartz *et al.* (1997a) also presented evidence in favor of the 'umbrella' model by demonstrating that toxins with disulfide bonds that restricted domain I movement failed to insert into the membrane. However, Thompson *et al.* (1995) argued that most domain I α-helices resembled lytic peptides and were long enough to span a membrane. This observation was supported by protease digestion experiments where Aronson *et al.* (1999) demonstrated that upon insertion into the membrane all helices except helix α1 were resistant to protease digestion. This suggests that most of domain I may insert in membrane. Hodgman and Ellar (1990) proposed a second model called the 'penknife model' for membrane insertion based upon the penknife model for colicin A (Lakey *et al.*, 1996). According to this model the strongly hydrophobic helices α5 and α6 of domain I swing out and insert into the membrane.

The surface-exposed loops of domain II are the most variable regions among the Cry toxins (Grochulski *et al.*, 1995). Based on their similarity to immunoglobin antigen-binding sites these loops were proposed to be involved in receptor binding (Li *et al.*, 1991). Site-directed mutagenesis of loop residues supported the hypothesis that the three loops of domain II participate in receptor binding (reviewed in Schnepf *et al.*, 1998). Substitution of loop residues in this domain of Cry1A and Cry1C toxins affect receptor binding and toxicity (Smith and Ellar, 1994; Rajamohan *et al.*, 1996a; Rajamohan *et al.*, 1996b). However, in Cry2A, the receptor binding epitope lies on a core β-sheet rather than the loops when compared with Cry1 toxin (Morse *et al.*, 2001). Domain II is also

described as the specificity-determining domain. Swapping segments of domain II between closely related toxins results in chimeric toxins with altered specificity (Schnepf *et al.*, 1990; Ge *et al.*, 1991). Domain II also shows structural similarity to certain carbohydrate-binding proteins including jacalin (Sankaranarayanan *et al.*, 1996), vitelline membrane outer layer protein I (Shimizu *et al.*, 1994), and *Maclura pomifera* agglutinin (Lee *et al.*, 1996). The carbohydrate-binding properties of domain II have not yet been investigated.

Domain III plays a number of roles in Cry toxin function. This domain protects the toxin from proteolysis and therefore is important for toxin stability (Li *et al.*, 1991). It stabilizes toxin structure by forming salt bridges and hydrogen bonds with adjacent residues (Grochulski *et al.*, 1995). Studies with chimeric toxins indicate that domain III is involved in receptor binding. Domain III exchanges between Cry1Ac and Cry1Aa toxins altered receptor binding in *L. dispar* (Lee *et al.*, 1995). Swapping of domain II and domain III between closely related toxins produced hybrids with altered specificity (de Maagd *et al.*, 1996; Ge *et al.*, 1989). Mutations in domain III of Cry1Ac produced toxins with reduced binding to *H. virescens* and *M. sexta* BBMV and decreased toxicity to both insects (Aronson *et al.*, 1995). Domain III also plays a role in membrane insertion and pore formation. Cry1Aa domain III mutants bound BBMV comparable to wild-type toxin but displayed reduced pore formation (Chen *et al.*, 1993; Wolfersberger *et al.*, 1996). Domain III of Cry1Ac contains a sugar-recognition pocket. The importance of this pocket for the recognition of a Cry1Ac binding protein is discussed later.

1.4 Mode of action of Cry toxins

A number of events are required for Cry toxin to kill susceptible insects. This includes crystal solubilization, proteolytic processing of protoxins by midgut proteases, binding of toxin to proteins present in insect brush border membrane followed by insertion and aggregation in the membrane, and finally formation of lytic pores (reviewed in Schnepf *et al.*, 1998).

The crystals produced by *B. thuringiensis* are made of one or several protoxins subunits. The lepidopteran-specific Cry1 protoxins are 130to 140 kDa in size. Crystals dissolve in the high pH (>10) and reducing environment of the gut lumen (Hofmann *et al.*, 1988b). Solubilized protoxin is activated by trypsin-like midgut proteases to a 55 to 65 kDa toxin core by removal of 28 amino acids from the N-terminus and ~500 amino acids from the C-terminus (Milne and Kaplan, 1993). N-terminal activation is essential for insecticidal activity (Bravo *et al.*, 2002). Activated toxins bind to target proteins present on the apical brush border of midgut columnar cells (Hofmann *et al.*, 1988a). Toxin monomers insert into the membrane and aggregate (Aronson and Shai, 2001) resulting in pore formation in target membranes (reviewed in Schnepf *et al.*, 1998).

The architecture of Cry toxin pores is still not very clear. Masson *et al.* (1999) proposed that 4 toxin monomers make up a pore. This hypothesis was supported by atomic force microscopy experiments in lipid bilayers. The Cry toxin structure in the lipid bilayer resembled an oligomer consisting of four toxin monomers (Vie *et al.*, 2001). The formation of pores disrupts membrane integrity (Carroll and Ellar, 1997). The generally accepted Cry toxin mechanism of action is colloid osmotic lysis (Knowles and Ellar, 1987); however, release of calcium from internal stores is also reported (Potvin *et*

al., 1998). The midgut cells eventually lyse leading to insect mortality. Aronson and Shai (2001) reported that sub-lethal concentrations of toxin cause behavioral changes like avoidance of toxin during feeding. These results suggest that Cry toxin pathology probably involves intra-cellular events as well as gross changes in midgut epithelium ultimately resulting in gut paralysis and death.

1.5 Cry toxin mutants and elucidation of toxin-function

Mutational analyses of key amino acid residues in individual Cry toxin domains have provided insight into their respective roles in Cry toxin action. A comprehensive list of mutants and their associated effects on toxin function are presented in (Schnepf *et al.*, 1998). Based on binding studies, mutants are classified into three groups: 'A' mutants are domain II mutants that have reduced initial binding (see scheme below). Examples include the Cry1Ab^{368}RRP^{370}, R368A/R369A, F440A, and G439A mutants (Rajamohan *et al.*, 1996a; Rajamohan *et al.*, 1996b). 'B' mutants are other domain II mutants that are affected in dissociation but not initial binding. Unlike wild-type toxin, binding of 'B' mutants is reversible. Cry1Ab mutant F371A is a 'B' type mutant (Rajamohan *et al.*, 1995). 'C' mutants are domain I mutants which are defective in membrane insertion and pore formation. Mutants in this class include Cry1Ac mutants A92D and R93G (Wu and Aronson, 1992) and Cry1Ab mutants A92E and Y153D (Chen *et al.*, 1995). This classification does not include domain III mutants. Cry1Ac mutants like Tmut (Burton *et al.*, 1999) or ^{509}QNR^{511}-AAA (Lee *et al.*, 1999) are neither defective in membrane insertion nor pore formation but exhibit altered receptor interaction. The different mutations in each of the three domains are illustrated in Fig. 1-3.

The following diagram of interaction of toxin with receptor was obtained from kinetic analyses of toxin-receptor binding using different Cry1A mutant toxins:

$$T+R \rightleftharpoons T \equiv R \rightarrow {}^{*}T \text{ (or } {}^{*}TR)$$

‘A’ ‘B’ ‘C’ mutants

where, T is a Cry toxin, R is a receptor for this toxin, T ≡ R represents a toxin that is reversibly bound to receptor , *T represents irreversibly bound toxin presumably inserted into the membrane but not associated with the receptor and *TR is an irreversibly bound toxin still associated with the receptor (Liang *et al.*, 1995).

1.6 Toxin binding to brush border membrane vesicles

Both *in vivo* (Bravo *et al.*, 1992) and *in vitro* (Hofmann *et al.*, 1988a) binding experiments revealed that Cry toxins bound to microvilli in the insect midgut epithelium. Brush border membrane vesicles (BBMV) isolated from lepidopteran insect midgut have been a major tool for investigating Cry toxin mode of action and identifying potential Cry toxin receptors (Wolfersberger *et al.*, 1987). Because these vesicles are derived from the apical surface of midgut epithelial cells, they are suitable substrates for binding assays. BBMV are enriched in brush border proteins, some of which function as toxin binding proteins (Wolfersberger *et al.*, 1987).

Cry1 toxins bind saturably and with high affinity to sites in BBMV from susceptible insects (Hofmann *et al.*, 1988a; Van Rie *et al.*, 1989). Generally, the presence of high-affinity binding sites correlates with insecticidal activity (Hofmann *et*

al., 1988a; Hofmann *et al.*, 1988b; Van Rie *et al.*, 1989; Garczynski *et al.*, 1991; Denolf *et al.*, 1993). However, toxin binding does not always correlate with insecticidal activity (Wolfersberger, 1990; Ferre *et al.*, 1991; Garczynski *et al.*, 1991; Sanchis and Ellar, 1993; Luo *et al.*, 1999a). As represented in the kinetic diagram above, binding of Cry toxins to BBMV involves two stages: a reversible binding (Hofmann *et al.*, 1988b; Hofmann *et al.*, 1988a; Sanchis and Ellar, 1993) followed by irreversible binding (Van Rie *et al.*, 1989; Ihara *et al.*, 1993; Chen *et al.*, 1995; Liang *et al.*, 1995). The insecticidal activity of Cry toxins correlates with irreversible binding (Ihara *et al.*, 1993; Liang *et al.*, 1995). For example, toxicity of Cry1Aa and Cry1Ab toxins correlated well with the irreversible binding component in *B. mori* larvae (Ihara *et al.*, 1993). Therefore, post-binding events, including membrane insertion and pore formation are crucial for toxicity.

1.7 Cry toxin binding proteins and potential receptors

Since high-affinity saturable binding sites were present in BBMV, the next step was to identify toxin-binding molecules in BBMV and then establish their function as Cry toxin receptors. In *B. thuringiensis* literature, the term "receptor" has been used incorrectly. A toxin-binding protein is a surface protein that can bind Cry toxin. However, a toxin-binding protein can be considered a true Cry toxin receptor only by demonstrating that it mediates pore formation *in vitro* or toxicity *in vivo*. Initially, toxin-binding proteins in BBMV from different insects were identified using a ligand blot assay (Garczynski *et al.*, 1991; Knowles *et al.*, 1991; Oddou *et al.*, 1991; Martinez-Ramirez *et al.*, 1994; Jurat-Fuentes *et al.*, 2000). In the ligand blot assay, total BBMV proteins are separated by SDS-PAGE, transferred to membrane filters and probed with labeled Cry

toxins. A disadvantage of this technique is that the binding occurs under denaturing conditions and denaturing conditions may expose toxin-binding epitopes not present on the surface of the native protein (Daniel *et al.*, 2002b). Another technique used to isolate binding proteins is affinity chromatography. In affinity chromatography, solubilized non-denatured BBMV proteins are incubated with toxin-affinity columns. Toxin-binding proteins are subsequently eluted from the affinity column (Indrasith and Hori, 1992; Gill *et al.*, 1995; Luo *et al.*, 1996; Denolf *et al.*, 1997; Bagchi, 2000; Banks *et al.*, 2001; Valaitis *et al.*, 2001). A disadvantage of this technique is that some membrane regions can be resistant to detergent and cause non-specific binding. Such detergent resistant microdomains and protein complexes are present in insect midgut membranes (Lu and Adang, 1996; Zhuang *et al.*, 2002).

Numerous Cry1 toxin-binding proteins from different lepidopteran insects have been isolated, identified, cloned, and expressed (Sanchis and Ellar, 1993; Vadlamudi *et al.*, 1993; Sangadala *et al.*, 1994; Knight *et al.*, 1994; Martinez-Ramirez *et al.*, 1994; Cowles *et al.*, 1995; Gill *et al.*, 1995; Valaitis *et al.*, 1995; Luo *et al.*, 1996; Yaoi *et al.*, 1997; Luo *et al.*, 1997a; Nagamatsu *et al.*, 1998a; Banks *et al.*, 2001;). Although Valaitis *et al.* (2001) described a 270 kDa glycoconjugate (BTR-270) in *Lymantria dispar* as a Cry1A toxin-binding protein, most toxin-binding proteins belong to two protein families: cadherin-like proteins and aminopeptidase Ns.

1.7.1 Cadherin-like proteins

Cadherins are calcium-dependent, cell adhesion molecules that play crucial roles in the formation of intercellular junctions, epithelial cell polarization, and maintenance of

tissue architecture (Galan, 2000). Cadherins are composed of extracellular ectodomain repeats, a transmembrane segment, and a cytoplasmic domain (Galan, 2000). Since cadherins are present between cells and not on the cell surface, the process by which cadherin-like proteins function as Cry toxin receptors is still not clearly understood. However, there are reports that cadherins function as receptors or targets for pathogenic bacteria like *Listeria monocytogenes* (Mengaud *et al.*, 1996) and *Bacteriodes fragilis* (Sears, 2001). In the case of *Listeria* pathogenesis, E-cadherin is the receptor for internalin. E-cadherin is expressed on the basolateral side of polarized cells and would not be available for interaction with its bacterial ligand. Lecuit *et al.* (2001) proposed that the transient opening of cell-cell junctions during regeneration of intestinal epithelia allows bacteria to gain access to E-cadherin. Although speculative, *B. thuringiensis* toxin interaction with cadherin-like protein could follow the same mechanism.

Cadherin-like proteins function as Cry toxin-binding proteins (Vadlamudi *et al.*, 1995; Francis and Bulla, 1997; Keeton and Bulla, 1997; Keeton *et al.*, 1998; Nagamatsu *et al.*, 1998a; Nagamatsu *et al.*, 1998b;). In *M. sexta*, a 210 kDa cadherin-like protein binds Cry1Ab (Vadlamudi *et al.*, 1993; Martinez-Ramirez *et al.*, 1994) as well as Cry1Aa and Cry1Ac (Francis and Bulla, 1997; Keeton and Bulla, 1997; Keeton *et al.*, 1998). Cadherin-like proteins expressed in insect cells mediate toxicity to Cry toxins and are potential Cry toxin receptors (Nagamatsu *et al.*, 1998a; Dorsch *et al.*, 2002;).

1.7.2 Aminopeptidase Ns

Aminopeptidase Ns (APN) are ectopeptidases (EC 3.4.11.2) found on the surface of many cell types including the epithelial cell lining of the alimentary canal (Kenny *et*

al., 1987). These enzymes catalyze the cleavage of N-terminal amino acid residues from proteins and peptides and aid in the digestion process. A central Zn^{2+}-coordination site with the sequence $HEXXH_{18}E$ is present in all APNs along with the conserved gluzincin aminopeptidase motif GAMENWG (Taylor, 1996). APNs are involved in protein maturation and turnover, regulation of hormone levels, and degradation and invasion of extracellular matrix and are implicated in tumor progression (Taylor, 1996). They also function as receptors for coronavirus in animals and are important modulators of cell-cell interactions (Taylor, 1996).

APNs are the most studied class of Cry toxin-binding proteins. These proteins have been identified in BBMV from many lepidopteran insects (Knight *et al.*, 1994; Sangadala *et al.*, 1994; Gill *et al.*, 1995; Knight *et al.*, 1995; Masson *et al.*, 1995; Luo *et al.*, 1996; Yaoi *et al.*, 1997; Nagamatsu *et al.*, 1998a; Banks *et al.*, 2001). APNs are attached to the brush border membrane by a glycosylphosphatidylinositol (GPI)-anchor (Tomita *et al.*, 1994; Garczynski and Adang, 1995; Luo *et al.*, 1996; Luo *et al.*, 1997a; Luo *et al.*, 1997b). The GPI-anchor on MsAPN-1, a *M. sexta* APN, is susceptible to phosphatidylinositol phopholipase C (PIPLC) cleavage. A soluble 115-kDa molecule is obtained from the brush border membrane as a result of this cleavage (Lu and Adang, 1996). However, not all GPI-anchored APNs are cleaved by PIPLC (Luo *et al.*, 1997a).

In *M. sexta*, a number of APNs bind Cry toxins. For example, MsAPN-1 binds Cry1Aa (Masson *et al.*, 1995), Cry1Ab (Masson *et al.*, 1995; Denolf *et al.*, 1997), and Cry1Ac (Knight *et al.*, 1994; Sangadala *et al.*, 1994; Masson *et al.*, 1995), while the 106-kDa APN binds Cry1Ca (Luo *et al.*, 1996). A 110 kDa APN from *M. sexta* (Ms110-

APN) binds Cry1Ac (Daniel *et al.*, 2002a), and another *M. sexta* 106-kDa APN (MsAPN-2) binds Cry1Ab (Denolf *et al.*, 1997).

1.7.3 Classification of APNs identified from lepidopteran insects

Knight *et al.* (1994) and Sangadala *et al.* (1994) were first to identify the Cry1Ac-binding protein from *M. sexta* as an APN. Multiple APN isoforms are present in the same insect. Nakanishi *et al.* (2002) reported that eighteen APN isoform cDNAs from eight lepidopteran insects are present in protein databases. These APNs are from *Bombyx mori, Heliothis virescens, Plutella xylostella, Helicoverpa punctigera, Manduca sexta, Lymantria dispar, Plodia interpunctella, and Epiphyas postvittana*. Full-length APN cDNAs from *Helicoverpa armigera* and *Spodoptera litura* are also catalogued in databases. The nomenclature of APNs in literature is ambiguous. The APNs are denoted by number (MsAPN-1, BmAPN-1), letter (PxAPN-A), as well as by molecular mass of the protein (Hv170-APN, Hv110-APN). Phylogenetic analyses based on amino acid homology cluster APNs into four classes I-IV (Oltean *et al.*, 1999; Nakanishi *et al.*, 2002). Consequently, a particular class of APNs clustered by dendrogram analyses contains APNs with different nomenclature. For example, Class I contains MsAPN-1, BmAPN-1, Hv170-APN, PxAPN-A, and LdAPN-4, while class III contains MsAPN-3, BmAPN-3, Hv120-APN, and LdAPN-1. Therefore, for consistency, I have used *M. sexta* APNs with the following numbers throughout my dissertation: 120 kDa APN is MsAPN-1, 110 kDa APN is Ms110-APN, 106-kDa Cry1Ab-binding APN is MsAPN-2, 106-kDa Cry1Ca-binding APN is Ms106-APN, and a fourth APN (Table 1-1; Appendix I) is called

MsAPN-3. The complete list of all cloned and catalogued full-length lepidopteran APN cDNAs along with respective abbreviations is presented in Table 1-1.

1.7.4 Importance of APN glycosylation for Cry toxin function

Glycosylation is an important posttranslational modification of proteins in eukaryotic organisms. Glycosylated proteins play important roles in signal transduction and cell-cell communication (Alberts *et al*., 1994). APNs from different organisms including rat (Takasaki *et al.*, 1991), pig (Danielsen *et al.*, 1983), and human (Look *et al.*, 1989; Noren *et al.*, 1997) are N- and O-glycosylated. N- and O-glycosylation sites are also present in lepidopteran insect APNs (Gill *et al.*, 1995; Knight *et al.*, 1995; Banks *et al.*, 2001; Daniel *et al.*, 2002a).

N-acetylgalactosamine (GalNAc) is an important Cry1Ac toxin-binding determinant since GalNAc specifically inhibits binding of Cry1Ac to MsAPN-1 (Garczynski *et al.*, 1991; Knowles *et al.*, 1991; Masson *et al.*, 1995) and Hv170-APN (Luo *et al.*, 1997a). Lectin blots with soybean agglutinin (SBA) confirmed that GalNAc is present on these APNs (Knowles *et al.*, 1991; Lee *et al.*, 1996). Since GalNAc inhibits binding of Cry1Ac but not Cry1Aa or Cry1Ab toxins to MsAPN-1, a GalNAc-interaction pocket is present in Cry1Ac toxin. Structural analysis indicated that domain III of Cry1Ac shared structural homology with the cellulose-binding domain in β-glucanase of *Cellumonas fimi* (Burton *et al.*, 1999). Cry1Ac mutants ^{509}QNR^{511}-AAA (Lee *et al.*, 1999) and Tmut (Burton *et al.*, 1999) were produced by mutating the GalNAc-binding region in domain III of Cry1Ac. ^{509}QNR^{511}-AAA did not bind purified MsAPN-1 in SPR analyses (Jenkins *et al.*, 1999) confirming that GalNAc mediates binding of Cry1Ac to

MsAPN-1. In another study Cry1Ac did not recognize MsAPN-1 expressed in Sf21 cells (Luo *et al.*, 1999b). The authors reasoned that posttranslational modifications in Sf21 cells are different from *M. sexta* midgut and that the sugar moieties recognized by Cry1Ac are absent on Sf21-expressed APN (Luo *et al.*, 1999b).

1.8 Toxin-induced pore versus toxin-induced channel

The membrane permeation activity of Cry toxins has been analyzed by several biophysical techniques. Toxin-induced structures in membranes are categorized as either cation-selective ion channels or non-selective pores. This classification depends on the technique employed to study membrane permeation. In experiments with receptor-free liposomes and planar lipid bilayers (PLB), cation-selective channels formed at high concentrations of toxin (Slatin *et al.*, 1990). The ion channel conductance were 600 pS and exhibited multiple opening and closing states probably due to different conformations in the membrane (Slatin *et al.*, 1990). Channel formation was observed at significantly lower concentrations of toxin when purified receptor complex from *M. sexta* BBMV was incorporated into PLBs (Schwartz *et al.*, 1997b). These channels were also cation-selective and their size was in agreement with the previous report by Slatin *et al.* (1990).

The characteristics of ion-channels were significantly different when total *M. sexta* BBMV were incorporated in PLBs instead of purified receptor complex (Martin and Wolfersberger, 1995). The smallest toxin-dependent increase in conductance representing a single membrane pore was 13 nS. Martin and Wolfersberger (1995) estimated the diameter of the cylindrical pore to be 22 Å using 13 nS as the conductance

of a single membrane pore. This pore size was more than twice the diameter measured in PLBs.

Carroll and Ellar (1993) studied Cry toxin-induced membrane permeation in BBMV with a light scattering technique. In this technique vesicle volume changes are monitored with a spectrofluorometer as a function of change in BBMV size relative to the amount of 90^0 scattered light (Kasai and Nonogaki, 1988). Cry1Ac altered the permeability of *M. sexta* BBMV for cations (K^+, Na^+), anions (Cl^-) and neutral solutes (sucrose, raffinose, polyethylene glycol) (Carroll and Ellar, 1993; 1997). This suggested that the toxin-induced pore was non-selective in nature and allowed both charged and uncharged molecules to pass through the pore. The size of the toxin-induced pore was calculated as ~24 Å at pH 8.7 which was similar to the toxin-induced pore obtained by (Martin and Wolfersberger, 1995).

1.9 Dissertation Objectives

The overall objective of my dissertation research was to study Cry toxin interaction with receptors in *M. sexta* using Cry1A mutant toxins. To achieve this goal I applied three approaches with two sets of Cry1A mutant toxins. The first set of mutant toxins included Cry1Ab and Cry1Ac domain I and II mutants while the second set consisted of a single Cry1Ac domain III mutant ^{509}QNR^{511}-AAA.

In the first approach I studied the relationship between toxicity and pore formation using Cry1Ab and Cry1Ac domain I and II mutants with a light scattering technique. The mutants I studied included Cry1Ab mutants R368A/R369A, F371A, Y153D and A92E, and Cry1Ac mutant A92D. As depicted in the kinetic diagram,

mutant R368A/R369A has reduced reversible binding ('A' mutant); F371A has reduced tight binding ('B' mutant), while Y153D and A92D/A92E have reduced irreversible binding ('C' mutants). The receptor-binding and pore-forming abilities of these mutants were previously studied (Wu and Aronson, 1992; Chen *et al.*, 1995; Rajamohan *et al.*, 1995; Rajamohan *et al.*, 1996b; Rajamohan *et al.*, 1996a; Lee *et al.*, 1999). Using a light scattering technique we investigated the pore forming abilities of these mutants in different osmotic environments. The results of this study are presented in Chapter Two.

In the second approach, we investigated the effect of protein denaturation on Cry toxin binding to BBMV proteins and purified MsAPN-1 because results of a ligand blot assay with Cry1Ac mutant ^{509}QNR^{511}-AAA did not agree with previously reported results (Jenkins *et al.*, 1999). Lack of consistency between ligand blotting and vesicle binding assays was also reported by Lee and Dean (1996). Our hypothesis was that the apparent inconsistency was due to protein denaturation causing altered specificity of interaction. Using a combination of ligand and dot blotting techniques we investigated the role of protein denaturation on binding specificity. The results of this study are presented in Chapter Three.

In the third study, we used Cry1Ac mutant ^{509}QNR^{511}-AAA to identify protein(s) other than MsAPN-1 that could function as a Cry1Ac-binding protein. Disruption of the GalNAc-binding pocket abolished binding of Cry1Ac to MsAPN-1 even though toxicity was comparable to wild-type toxin (Jenkins *et al.*, 1999; Lee *et al.*, 1999). Therefore, our hypothesis was that Cry1Ac bound some *M. sexta* BBMV protein(s) independently of sugar residues. We isolated a previously unidentified *M. sexta* APN (Ms110-APN) that matched this criterion using ^{509}QNR^{511}-AAA and Cry1Ac affinity chromatography. We

cloned the Ms110-APN cDNA from midgut cDNAs, expressed the protein in *E. coli,* and conducted binding analyses under non-denaturing and denaturing conditions. The results of this study are presented in Chapter Four.

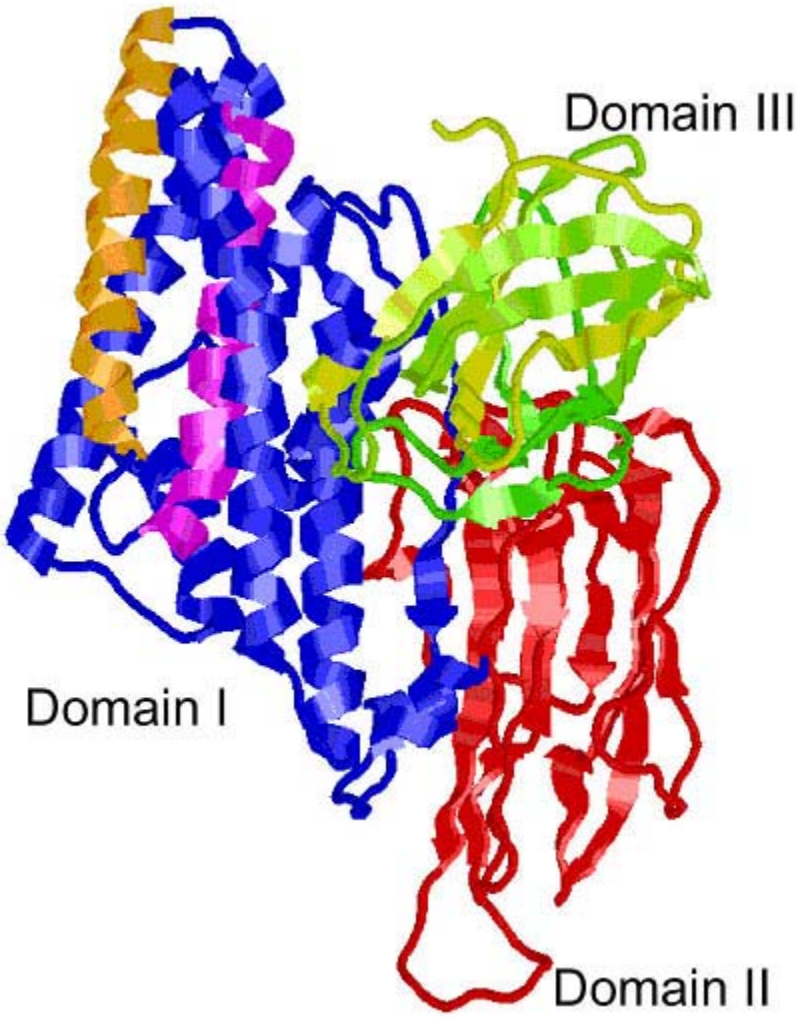

Figure 1-1: Crystal structure of *Bacillus thuringiensis* Cry1Aa toxin showing domains I, II, and III. α-Helices 4 and 5 of domain I are colored orange and magenta, respectively.

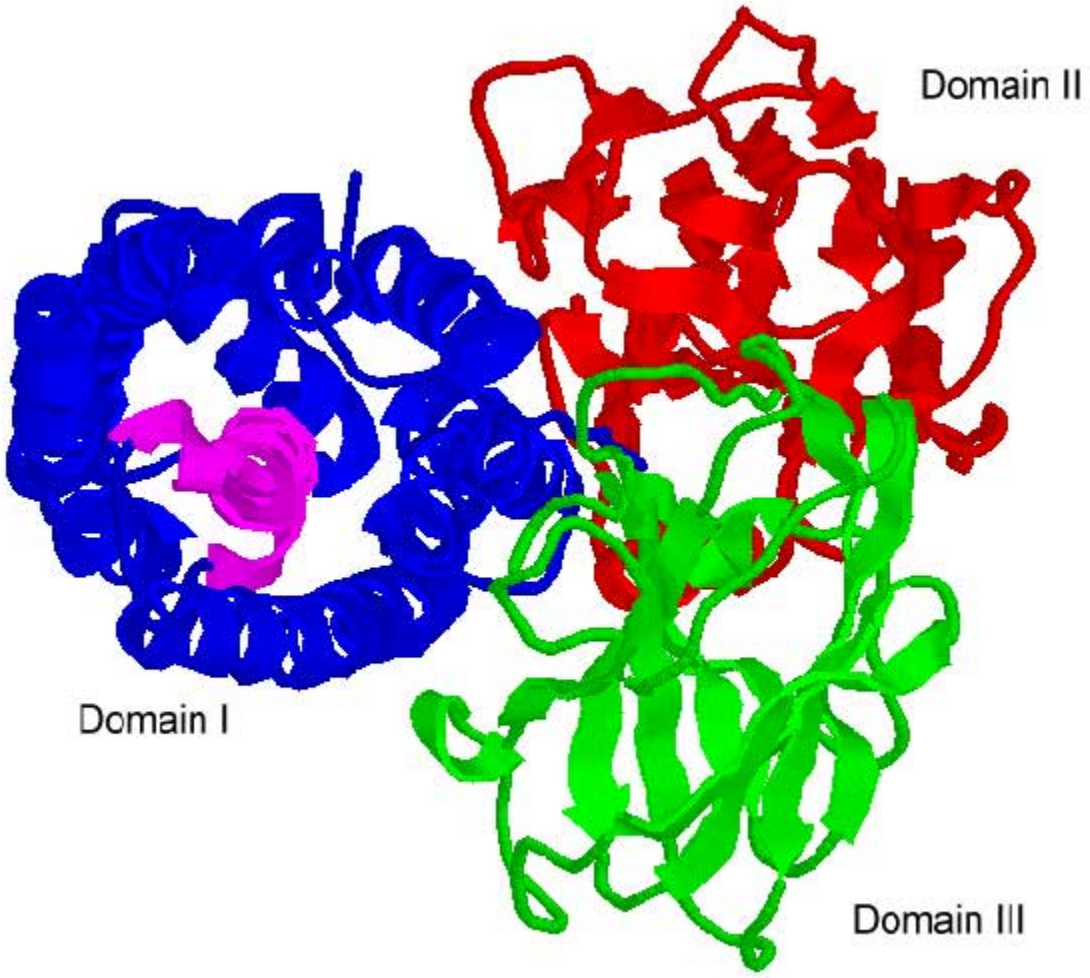

Figure 1-2: Top view of *Bacillus thuringiensis* Cry1Aa toxin showing domains I, II, and III. α-Helix 5 of domain I is colored magenta.

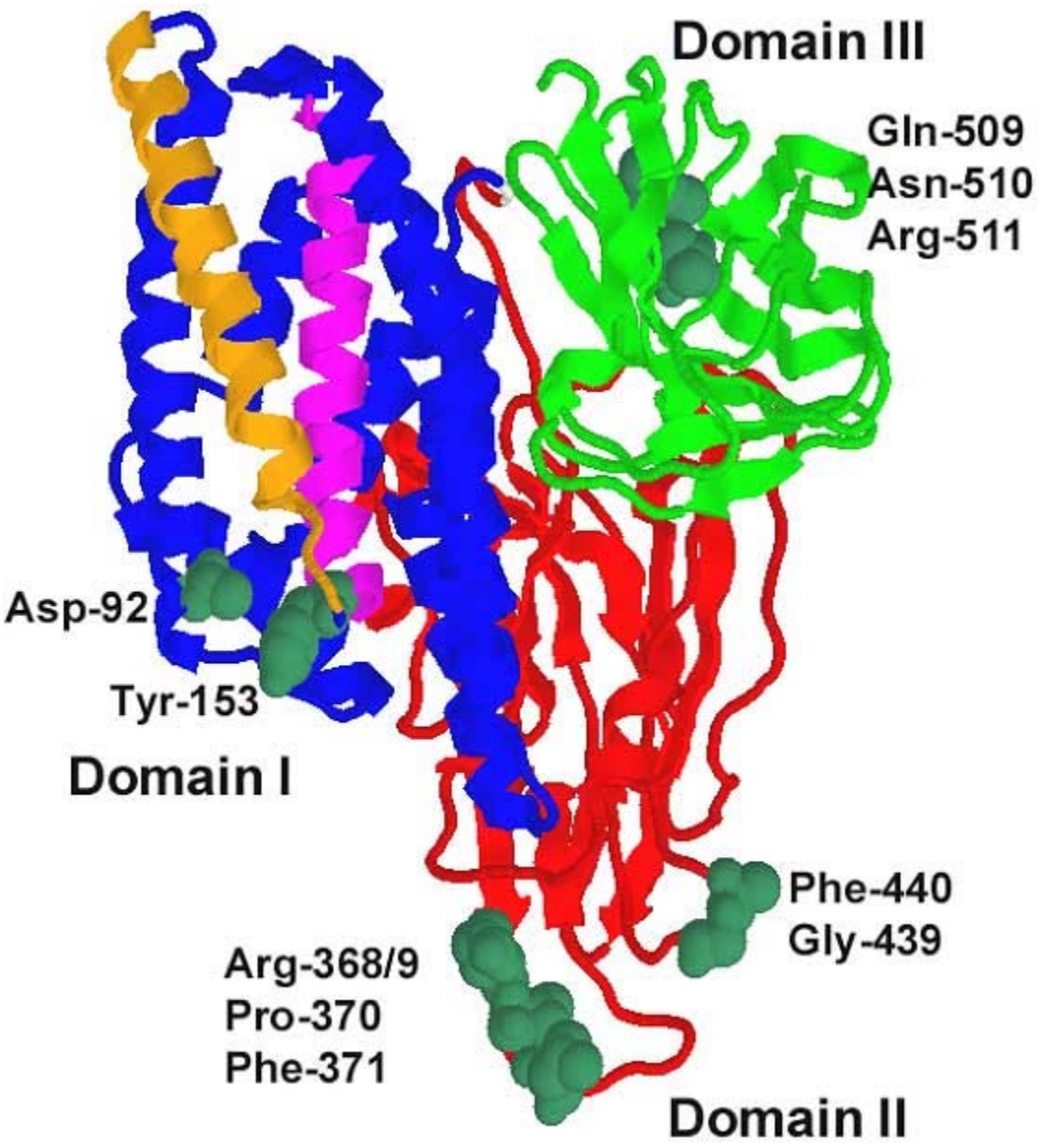

Fig. 1-3. Crystal structure of Cry1Aa toxin with mutations in each domain illustrated by ball and stick models. α-helices 4 and 5 are colored orange and magenta, respectively.

Source of APN	*Previous nomenclature*	*Nomenclature in this dissertation*	*GenBank accession #*	Reference
M. sexta; Ms	120 kDa APN	MsAPN-1	X89081	(Knight et al., 1995)
	120 kDa APN	MsAPN-1A	AF123313	(Luo et al., 1999b)
	110 kDa APN	Ms110-APN	AF498996	This study
	106-kDa APN	MsAPN-2	X97877	(Denolf et al., 1997)
	-	MsAPN-3	AY095259	This study
H. virescens; Hv	Hv-A	Hv170-APN	AF173552	(Oltean et al., 1999)
	120 kDa APN	Hv120-APN	U35096	(Gill et al., 1995)
	110 kDa APN	Hv110-APN	AF378666	(Banks et al., 2001)
H. punctigera; Hp	APN1	HpAPN-1	AF217248	(Emmerling et al., 2001)
	APN2	HpAPN-2	AF217249	(Emmerling et al., 2001)
	APN3	HpAPN-3	AF217250	(Emmerling et al., 2001)
B. mori; Bm	BmAPN1	BmAPN-1	AF084257	(Yaoi et al., 1999)
	BmAPN_	BmAPN-1A	AB007038	(Hua et al., 1998b)
	BmAPN2	BmAPN-2	AB011497	(Hua et al., 1998a)
	BmAPN3	BmAPN-3	AF352574	(Nakanishi et al., 2002)
	BmAPN4	BmAPN-4	AB013400	Unpublished
P. xylostella; Px	PxAPNA	PxAPN-1	AF020389	(Chang et al., 1999)
	PxAPN1	PxAPN-2	X97878	(Denolf et al., 1997)
	PxAPN3	PxAPN-3	AF109692	(Nakanishi et al., 2002)
	PxAPN4	PxAPN-4	AJ222699	Unpublished
L. dispar; Ld	LdAPN1	LdAPN-1	AF126442	(Garner et al., 1999)
	Ld λ APN2	LdAPN-2	AF126443	(Garner et al., 1999)
	APN3	LdAPN-3	AF317619	Unpublished
	APN4	LdAPN-4	AF317620	Unpublished
H. armigera; Ha	APN1	HaAPN-1	AY038607	Unpublished
	APN2	HaAPN-2	AY038608	Unpublished
E. postivttana; Ep	APN	EpAPN-1	AF276241	(Simpson and Newcomb, 2000)
P interpunctella; Pi	APN	PiAPN-1	AF034484	(Zhu et al., 2000)
S. litura; Sl	APN	SlAPN-1	AF320764	Unpublished
D.melanogaster; Dm	dPsa	DmAPN	AF327435	(Schulz et al., 2001)
A. aegypti; Aa	APNRc1	AaAPN-1	AY064078	Unpublished
	APNMc2	AaAPN-2	AY064079	Unpublished

Table 1-1. Nomenclature of the APNs used in this dissertation.

1.10 References

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CHAPTER 2

ANALYSES OF THE PORE FORMING ABILITY OF *BACILLUS THURINGIENSIS* CRY1A MUTANT TOXINS USING A LIGHT SCATTERING TECHNIQUE[1]

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2.1 Abstract

The pore formation properties of *Bacillus thuringiensis* Cry1 wild-type and domain I and II mutant toxins were studied on *Manduca sexta* brush border membrane vesicles (BBMV) using the light scattering technique. Wild-type Cry1Ac, Cry1Ab and Cry1Ba, Cry1Ab mutant toxins A92E, Y153D, R368A/R369A, F371A and Cry1Ac mutant toxin A92D were analyzed. In a direct mixing assay the mutant toxins Y153D, R368A/R369A, F371A and Cry1Ba did not elicit a response in a one-minute signal-monitoring period. Mutant toxins A92D and A92E elicited slight responses. After pre-incubation of toxin with BBMV, the signal recovery response increased for all toxins. The signal recoveries caused by A92D and A92E were greater than Y153D, F371A, and R368A/R369A-induced signal recoveries, which were slightly greater than Cry1Ba. By increasing the monitoring period to 3 minutes in direct mixing experiments, we observed greater pore formation by A92D. A92E had a response similar to, but lower than A92D. The response induced by both wild-type and mutant toxins decreased when the hyperosmotic solution was changed from KCl to sucrose. However, in the presence of sucrose the response induced by A92D and A92E was substantially reduced relative to KCl.

2.2 Introduction

Bacillus thuringiensis produces crystal (Cry) proteins during sporulation and these proteins are toxic to lepidopteran, dipteran, and coleopteran larvae (Schnepf *et al.*, 1998). Based on amino acid sequence homology the Cry proteins are classified into 27 major classes (Crickmore *et al.*, 1998). The site of action of Cry proteins is the larval midgut (Gill *et al.*, 1992; Knowles and Dow, 1993). Upon ingestion, the 130-140 kDa Cry1 protoxins are solubilized in the alkaline midgut lumen of lepidopteran larvae and converted by midgut proteases to active 55 to 65 kDa toxins (Tojo and Aizawa, 1983). Activated toxins diffuse across the peritrophic matrix and bind to specific receptors on the brush-border membrane of midgut columnar cells (Hofmann *et al.*, 1988b; Martin and Wolfersberger, 1995). Binding to the receptor is thought to cause a conformational change in the toxin enabling irreversible association with the membrane (Schwartz *et al.*, 1997). Irreversible binding leads to toxin aggregation (Aronson *et al.*, 1999) and the formation of pores which disrupts the ionic balance causing the loss of membrane potential (Hofmann *et al.*, 1988b; Knowles and Ellar, 1987; Schwartz *et al.*, 1993). The affected larva stops feeding and dies due to starvation.

X-ray crystal structures of Cry3Aa, a coleopteran-active toxin (Li *et al*., 1991) and Cry1Aa toxin (Grochulski *et al*., 1995) a lepidopteran-active toxin, have shown that Cry proteins have similar tertiary structures comprised of three domains. A seven α-helix bundle describes domain I and is involved in membrane insertion and pore formation. Insecticidal specificity of Cry toxins to different insect orders and genera is conferred primarily through domain II consisting of three sets of β-sheets with loops at the apices of the β-hairpin extensions. Domain III, a β-sandwich, whose role in toxin

mode of action is not completely defined, may play a role in pore formation (Chen *et al*., 1993), membrane protein recognition and insecticidal toxicity (de Maagd *et al*., 1996). Domain III of Cry1Ac has lectin-like folds and through mutations in this region was shown to be important for binding of toxin to *M. sexta* aminopeptidase (Burton *et al*., 1999; de Maagd *et al*., 1999; Jenkins *et al*., 1999).

Cry toxin mutants have been used to dissect the mode of action of Cry toxins. Some of the mutants have been used in this study. For example, the domain I mutants A92D, A92E and Y153D were previously tested for toxicity, receptor binding and pore forming ability (Wu and Aronson, 1992). Toxicity of these mutants was abolished to *M. sexta*. Initial receptor binding was unchanged, however irreversible binding and pore formation were significantly reduced. Hussain *et al*. (1996) also showed that substitution of a positively charged residue (R93F) or addition of a negatively charged residue (A92D) in Cry1Ac at the N-terminal of α-helix 3 of Cry1Ac resulted in a substantial reduction of toxicity to *M. sexta*. Cry1Ab domain II loop 2 mutants F371A and R368A/R369A had reduced toxicity to *M. sexta*. While mutant R368A/R369A had reduced reversible binding in binding assays (Rajamohan *et al*., 1995), mutant F371A had reduced irreversible binding and pore formation ability (Rajamohan *et al*., 1995; Rajamohan *et al*., 1996a).

Cry1 toxins inhibited voltage-clamped short circuit current across isolated midguts (Harvey and Wolfersberger, 1979), probably by altering the permeability of midgut apical membranes for monovalent cations (Sacchi *et al*., 1986; Crawford and Harvey, 1988; Hendrickx *et al*., 1990). This assay correlated well with toxicity, but currently can be used only on large larvae. BBMV derived from midgut tissue provided

an alternate approach to measuring toxin effects on membrane permeability. Toxin-induced permeation of BBMV uncoupled K^+-driven amino acid co-transport (Sacchi *et al*., 1986). Membrane potential measurements using a fluorescent dye indicated that Cry toxins active against *Spodoptera frugiperda* caused immediate permeability changes in BBMV (Lorence *et al*., 1995). Both BBMV assays had limitations since the amino acid co-transport assay was indirect and depended on the transport of amino acid across the membrane and the fluorescent dye assay was limited to measuring changes in membrane permeability for specific ions. Carroll and Ellar (1993) developed an assay for measuring Cry1 toxin-induced permeability of BBMV to a variety of solutes using a light scattering assay. The light scattering assay revealed that Cry1Ac toxin significantly increased *M. sexta* permeability of BBMV for cations, anions and neutral solutes. Wolfersberger *et al*. (1996) used the light-scattering assay to differentiate related Cry1A domain III mutant toxins. However, the light scattering assay also has limitations. Relative to physiological conditions high toxin concentrations and high osmotic pressure was necessary to obtain a response. The advantages of the light scattering method were its rapidity and potential for high-throughput screening.

We used the light scattering assay to compare the membrane-permeabilizing ability of Cry1 domain I/II mutant toxins. Cry1Ab mutants Y153D, A92E, and Cry1Ac mutant A92D and Cry1Ab mutant F371A and R368A/R369A were used in this study. These mutated toxins were not lethal to *M. sexta* and the loss of toxicity corresponded to either loss in initial binding (R368A/R369A) or irreversible binding (Y153D, A92D, A92E and F371A) to *M. sexta* BBMV. We found the mutant toxins to be impaired in their capacity to permeabilize membranes as measured by the light scattering assay.

Cry1Ac mutant A92D and Cry1Ab mutant A92E elicited differential responses under different hyperosmotic conditions.

2.3 Materials and Methods

2.3.1 Strains used.

Cry1Ac was purified from *B. thuringiensis* subsp. *kurstaki* HD-73 obtained from the *Bacillus* Genetic Stock Culture Collection (Columbus, Ohio). *E. coli* harboring *cry1Ab* (Masson *et al*., 1990) was provided by Dr. L. Masson (Montreal, Canada). *E. coli* strains producing Cry1Ac mutant A92D, and Cry1Ab mutants A92E, Y153D, F371A and R368A/R369A have been previously described (Wu and Aronson, 1992; Chen *et al*., 1995; Rajamohan *et al*., 1995; Lee *et al*., 2000). A *B. thuringiensis* strain producing Cry1Ba was obtained from Ecogen, Inc. (Langhorne, PA).

2.3.2 Purification of toxins.

Toxins were prepared and purified from *B. thuringiensis* strains as previously described (Luo and Adang, 1994). Cry1A mutant inclusions were prepared from *E. coli* as described by Lee *et al*. (1992). Bio-Rad protein assay reagent was used to determine the toxin concentration. Bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

2.3.3 Insect bioassays.

Insect diet was purchased from Southland Products (Lake Village, AK), *M. sexta* eggs from Carolina Biological Supply and bioassay trays from C-D International

(Pittman, NJ). Toxins were diluted in phosphate-buffered saline (10mM Na_2HPO_4, 1.7 mM KH_2PO_4, 2.7 mM KCl, 136.9 mM NaCl [pH 74.]) containing 0.1% bovine serum albumin (BSA). Diet was surface-coated with 50 µl toxin/cm^2, allowed to dry and hatched larvae applied. Bioassay trays were incubated at 26^0C and 70% relative humidity with a 12:12 photoperiod for 7 days. Mortality was scored and data analyzed with the computer program POLO-PC (Daum, 1970).

2.3.4 Gel electrophoresis

Toxin preparations were analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250.

2.3.5 Preparation of BBMV.

Midguts were dissected from *M. sexta* larvae (fifth instar) and were immediately frozen on dry ice and stored at -70^0C until needed. BBMV were prepared by the $MgCl_2$ precipitation method (Wolfersberger *et al*., 1987) as modified by Ferre *et al*. (1991) except that the final BBMV pellet was suspended in cold 10 mM Tris-HCl (pH 7.5). BBMV were kept on ice and used in light scattering assays the same day as prepared. The amount of protein in BBMV was determined by Bio-Rad protein assay reagent with BSA as the standard.

2.3.6 Permeability assays.

Light scattering assays were performed with a stopped-flow spectrofluorimeter (model RSM 1000; On-line Instrument Systems, Bogart, GA.). BBMV were diluted to a concentration of 0.4 mg protein/ml with iso-osmotic buffer (10 mM Tris-HCl pH 7.5). For all measurements which did not involve pre-incubation of BBMV with toxin, 1.0 ml of BBMV and 1.0 ml of 10 mM Tris-HCl (pH 7.5) were manually loaded into separate reservoirs. Readings were initiated by simultaneously injecting BBMV and 10 mM Tris-HCl (pH 7.5) into the spectrofluorimeter cuvette. Incident 450-nm light scattered at 90^0 from incidence was monitored for 1 or 3 minutes. This reading provided the baseline tracing for iso-osmotic conditions. After the monitoring period, buffer was emptied from the syringe and replaced with hyperosmotic buffer (0.25 M KCl or 0.125 M sucrose in 10 mM Tris-HCl, pH 7.5). Hyperosmotic buffer and BBMV were injected and measurements taken as above. This reading provided a measure of maximal BBMV shrinkage in a hyper-osmotic environment. For direct toxin mixing experiments, an appropriate concentration of toxin was diluted in 0.25 M KCl or 0.125 M sucrose and loaded into the syringe after emptying 0.25 M KCl/0.125 M sucrose. For all pre-incubation studies, aliquots (1.0 ml) of BBMV were incubated with individual toxins at room temperature for different times. Nystatin (400 units final concentration/mg of BBMV) was used as positive control.

2.4 Results

2.4.1 Toxicity of wild-type and mutant toxins

Toxicity of wild-type Cry1Ac, Cry1Ab, Cry1Ba and Cry1A mutants A92D, Y153D, F371A, and R368A/R369A was determined for *M. sexta* larvae. SDS-PAGE analysis of toxins used in bioassays and subsequent experiments are shown in Fig. 2-1. The protein profile of A92E was similar to Cry1Ac mutant A92D (data not shown). In bioassays, Cry1Ac was the most toxic (LC_{50}, 4.9±3.5 ng/cm^2) consistent with the previously reported value (Garczynski *et al.*, 1991). Cry1Ab was also highly toxic (LC_{50}, 13.9±4.2 ng/cm^2). Mutated toxins were not lethal to *M. sexta* larvae (LC_{50}, >1000ng/cm^2), in agreement with previous studies (Wu and Aronson, 1992; Chen *et al.*, 1995; Rajamohan *et al.*, 1995; Lee *et al.*, 2000). Though not bioassayed in this study, A92E has an LC_{50} value of >100ng/cm^2 (Chen *et al.*, 1995). Cry1Ba, reported to be non-toxic (Hofte and Whiteley, 1989) was slightly toxic (LC_{50}, 656±254 ng/cm^2).

2.4.2 Light scattering assays with M. sexta BBMV and Cry1 toxins

Direct mixing of toxin and BBMV

Light scattering assays were done to measure the pore forming properties of Cry1 domain I and II mutants. Maximal BBMV shrinkage, as measured by the greatest amount of 90^0 scattered light, was induced by co-injecting BBMV and a hyperosmotic solution (0.25 M KCl) (Fig. 2-2A). Due to the rate of vesicle shrinkage and selected parameters for data collection, the initial shrinkage response is not visible in Fig. 2-2A. The amount of light scattered on co-injecting BBMV and iso-osmotic solution (10mM Tris-HCl buffer, pH 7.5) provided a baseline for calculating the shrinkage response

(measured by difference in light scattering with hyperosmotic solution and iso-osmotic solution).

The effect of light scattering by different toxins was analyzed from the point BBMV were mixed with toxin. The increase in membrane permeability of BBMV due to interaction with toxin caused the BBMV to re-swell reducing the amount of light scattered in contrast to when BBMV were exposed to KCl alone. The response was measured as percent signal recovery calculated by the following formula:

$$\text{Percent signal recovery} = \frac{(KCl_0 - T_f)-(KCl_0 - KCl_f)}{(KCl_f - Buf_f)} \times 100 \qquad \text{(A)}$$

where KCl_0 was the maximum response due to vesicle shrinkage when BBMV come in contact with KCl. KCl_f was the value obtained subsequent to reswelling of vesicles after KCl treatment. T_f was the final value obtained due to vesicle reswelling on treatment of BBMV with toxin, and Buf_f was the value obtained on exposing vesicles to 10 mM Tris-Cl buffer, pH 7.5.

The final concentration of toxin used in each case was 5µg toxin/mg BBMV protein. The light scattering signal was monitored for one minute (Fig. 2-2B). Cry1Ac induced the maximum response with ~25% signal recovery followed by Cry1Ab with ~19% signal recovery. Mutants Y153D, F371A and R368A/R369A had no effect on signal recovery, while A92D and A92E showed a signal recovery (~7% and ~5% respectively) slightly above the background signal. Cry1Ba did not induce a response and the signal recovery induced by Cry1Ba corresponded to background signal. One minute measurements distinguished the membrane permeabilizing properties of wild-type Cry1Ac and Cry1Ab toxins from the Cry1A mutants and Cry1Ba. Nystatin was used as

positive control and at a concentration of 400 units/mg of BBMV nystatin elicited a response of ~67% in the one-minute signal monitoring period.

Pre-incubation of BBMV with toxin

Since the highest response by any mutant toxins was only slightly above background in the direct mixing experiments, toxins were preincubated with BBMV for 30 min prior to measuring the response in the light scattering assay. Toxin-treated BBMV were co-injected with iso-osmotic buffer followed by 0.25 M KCl and the signal response was monitored for one minute (Fig. 2-3A). The formula used for calculating the signal recovery for pre-incubation studies was:

$$\text{Percent signal recovery} = \frac{(KCl_0 - KCl_f)}{(KCl_f - Buf_f)} \times 100 \qquad \text{(B)}$$

where KCl_f was the final response due to reswelling of vesicles after KCl treatment, KCl_0 was the initial response due to maximum shrinkage with KCl and Buf_f was the response observed on treatment of vesicles with 10 mM Tris-Cl buffer, pH 7.5. Fig. 3B revealed that pre-incubation of toxin with BBMV at a concentration of 5 μg toxin/mg BBMV protein caused a substantial increase in the signal recovery for all toxins. Cry1Ac showed the highest response with ~90% signal recovery followed by Cry1Ab with ~78% signal recovery. A92D which had a very weak effect in the direct mixing assays, induced a response of ~68% while A92E induced a response of ~56%. Mutants Y153D, F371A and R368A/R369A had effects (28-35%) comparable to Cry1Ba (~27%). Preincubation of BBMV with nystatin induced a response of ~100% indicating that BBMV re-swelled to nearly its original volume due to the pores formed by nystatin.

2.4.3 Effect of toxin concentration on signal recovery

Since preincubating BBMV with toxins induced a large increase in signal recovery, we tested to see if the response was concentration dependent. BBMV were incubated with increasing concentrations of toxin for 30 minutes and then the signal recovery was monitored for one minute. Fig. 2-4 revealed that the signal recovery obtained upon treating BBMV with increasing concentrations of toxins was concentration-dependent. The lowest concentration tested (1.25 μg toxin/mg BBMV protein) induced the smallest response. The response increased until a concentration of 2.5 μg toxin/mg BBMV protein with little additional response at 5μg toxin/mg BBMV protein. Cry1Ac-treated BBMV had the greatest response with a signal recovery of ~80% at 10μg toxin /mg BBMV protein followed by Cry1Ab with a response of ~73% and then A92D which had a response of ~62%. The other mutants Y153D, F371A and R368A/R369A showed very low increase in signal recovery, as was the case with Cry1Ba, which had the lowest response of only ~19%.

2.4.4 Effect of changing the hyperosmotic solution to sucrose

An advantage of light scattering experiments noted by Carroll and Ellar (1993) was that the permeability of different solutes to toxin-treated BBMV could be compared. Preincubation experiments with 0.25 M KCl showed that mutants A92D and A92E were able to induce a response indicating that A92D and A92E were able to permeabilize BBMV. We conducted light scattering assays in the presence of 0.125 M sucrose to see if the change of hyperosmotic environment would alter the pore forming abilities of the toxins. Also, since preincubation of toxin with BBMV caused the background signal to

increase (Cry1Ba, Fig 2-3B) the experiments were performed without preincubating the BBMV with toxins. Instead, direct mixing of BBMV and toxin (5μg toxin/mg BBMV protein) was carried out and signal monitoring time increased to 3 minutes. The percent signal recovery was calculated using formula (A). Since mutants F371A and R368A/R369A had effects comparable to background levels in direct mixing and preincubation experiments, these mutants were not used in this set of experiments.

The response elicited by toxins in the presence of 0.125 M sucrose was lower than in comparison to 0.25 M KCl (Fig. 2-5). This was expected because sucrose is a larger molecule than KCl and sucrose would be less permeable across membranes. Cry1Ac induced a response of ~78% in 0.25 M KCl and ~57% in 0.125 M sucrose. Cry1Ab elicited a response of ~65% and ~40% in KCl and sucrose respectively. However, the response induced by A92D and A92E substantially decreased in the presence of sucrose. A92D caused a signal recovery of ~42% in KCl and only ~12% recovery with sucrose while A92E induced a signal recovery of ~26% in KCl and only ~4% recovery in sucrose. The response induced by Y153D was ~10% in KCl when the signal monitoring was increased to 3 minutes. In the presence of sucrose, Y153D induced a signal recovery of ~4%. Cry1Ba elicited the least amount of signal recovery in both hyperosmotic environments, ~18% in KCl and ~5% in sucrose. Nystatin however was able to induce a response of ~100% in the presence of KCl as seen in the preincubation assays and in the presence of sucrose the BBMV did not reswell after shrinking.

2.5 Discussion

In this study we analyzed the pore forming properties of three types of mutants defined by Schnepf *et al*. (1998). These mutants included a domain II mutant R368A/R369A that affected competition and not dissociation (A mutants), a domain II mutant F371A that affected dissociation but not competition (B mutants), and finally domain I mutants, Y153D, A92D and A92E, which affected insertion of toxin into the membrane (C mutants).

By varying time and medium composition we were able to discriminate between wild-type and some of the mutant toxins. Wild-type Cry1Ab and Cry1Ac toxins were able to rapidly permeabilize BBMV in a high external KCl environment (Fig. 2-2B) in direct mixing assays. Nystatin was the most effective and induced the highest response in the one minute monitoring period. Mutants A92D and A92E were able to induce some response (~5-7%) compared to Y153D (Fig. 2-2B). The domain II mutants R368A/R369A, F371A and wild-type Cry1Ba toxin did not elicit any response during the signal monitoring period. Since nystatin and wild-type Cry1Ac and Cry1Ab were able to induce a response in the one minute signal monitoring period, this period of interaction between nystatin or toxins with BBMV was sufficient for membrane permeation.

The domain II mutants are defective in reversible binding (R368A/R369A) (Lee *et al*., 2000) or irreversible binding F371A (Wu and Aronson, 1992). The absence of pore formation detected in the mixing assay agreed with previous binding and voltage clamp measurements (Wu and Aronson, 1992; Lee *et al*., 2000). The light scattering results are further evidence that decrease of inhibition of short circuit current in midgut tissue caused by Cry toxins is actually a measure of pore formation by toxins. However,

the relatively higher responses induced by A92D and A92E compared to Y153D, F371A and R368A/R369A in the direct mixing experiments indicated that A92D and A92E were able to permeabilize BBMV to some extent.

A preincubation period of toxin with BBMV was carried out to provide a greater opportunity for the toxins to associate with the BBMV. Our experiments revealed that the response induced by both wild-type and mutant toxins was substantially higher in the preincubation assays than in the direct mixing assays. Wild-type Cry1Ac and Cry1Ab and nystatin were able to induce a signal recovery of ~90-100% (Fig. 2-3B) indicating that Cry1Ac, Cry1Ab and nystatin were very potent pore formers and caused almost complete recovery of vesicle volume. Cry1Ba also induced a higher response (~27%, Fig. 2-3B) in the preincubation assays. The limited pore forming property of Cry1Ba agrees with the low toxicity of the toxin. The unexpected observation was that mutants A92D and A92E induced a signal recovery of nearly 56% and 68% respectively, in the presence of high salt concentration (0.25 M KCl) (Fig 2-3B). In contrast, the domain I mutant Y153D pore forming capacity was not enhanced to a comparable extent after pre-incubation. Mutants F371A and R368A/R369A had responses close to Cry1Ba indicating that these mutants were impaired in their ability to permeabilize BBMV. We recognize that insect midguts do not have the high salt conditions used in this study. However, it was necessary to use high salt concentrations to obtain the vesicle shrinkage needed for light scattering. Under the physiological conditions used in voltage clamping, Y153D but not A92E inhibited short circuit current (Chen *et al*., 1995).

Since we observed that A92D and A92E were able to induce signal recovery in the presence of KCl, we carried out the direct mixing experiments in the presence of

sucrose with a signal monitoring period of 3 minutes. This interval was sufficient for maximal association of toxins with BBMV while minimizing BBMV reswelling effects due to endogenous ion channels (Wieczorek *et al*., 1989) and reduced the background noise seen in the preincubation assays. The response induced by the toxins was lower in sucrose relative to KCl under similar signal monitoring periods (Fig. 2-5) presumably because of the larger hydrodynamic radius of sucrose which made sucrose less permeable than KCl. While wild-type toxins caused relatively high signal recovery in sucrose in comparison to Cry1Ba, the responses induced by the mutants A92D and A92E were lower than expected. Though speculative, it is possible that the anomalous behavior of A92D and A92E in KCl could be due to a charge masking effect by KCl. Nystatin did not induce any signal recovery in the presence of sucrose. Nystatin forms cation-channels in membranes and allowed passages of cations like Na^+ and K^+ (Lewis *et al*., 1977). Since sucrose is a larger uncharged molecule, it did not pass through the nystatin-pore. Consequently, BBMV did not reswell after shrinking in the hyperosmotic environment even though nystatin induced pore formation in BBMV.

The experiments with Cry1Ab mutant Y153D in different hyperosmotic solutions revealed that the membrane permeabilizing capacity of Y153D did not increase in KCl when the signal monitoring time was increased to 3 minutes (Fig. 2-5) as seen in direct mixing experiments (Fig. 2-2B) or preincubation experiments (Fig. 2-3B). Even though Y153D and A92E had similar characteristics of reduced irreversible binding, inhibition of short circuit current and reduced toxicity to *M. sexta* larvae (Chen *et al*., 1995), the pore forming ability of Y153D was not altered by KCl unlike A92D or A92E. The absence of an effect in KCl could be explained by the location of the mutation in Y153D. The

position of tyr153 is in the loop region between helix 4 and 5. According to the umbrella model proposed by (Gazit *et al*., 1998) helices 4 and 5 insert into the membrane. Therefore, even though KCl in the hyperosmotic environment may neutralize the negative charge of asp153, it would not compensate for the lack of a hydrophobic side chain and in turn the response of Y153D would not alter in KCl. Sucrose would have no effect on the mutation in Y153D as seen in our experiments.

In this paper we used the light scattering technique to evaluate the pore forming capacities of toxins defective at different stages of membrane interaction and pore formation. We found the light scattering technique was a simple and useful tool for measuring pore-forming abilities of toxins under different solute conditions. A disadvantage of the light scattering technique was the high external solute concentration needed for vesicle shrinkage. Because pore forming properties of Cry toxins were fundamental and central to toxin activity, the light scattering assay was a useful tool for investigating the relationship between Cry toxin structure and function.

Acknowledgements

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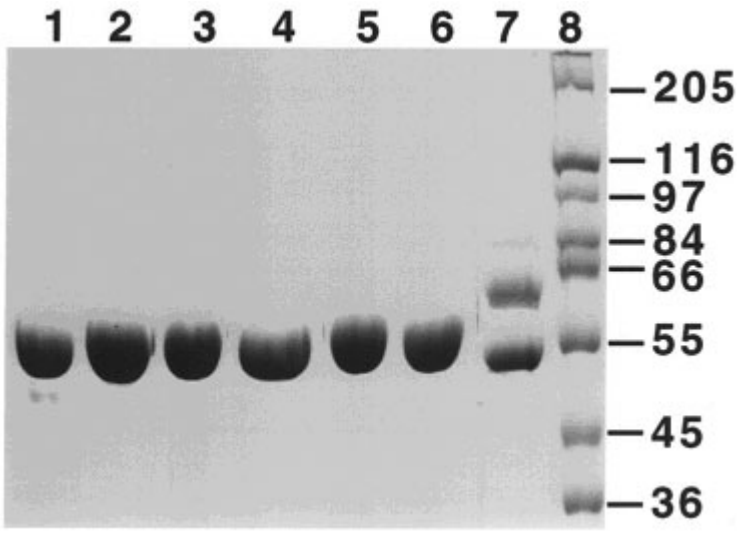

Fig. 2-1. SDS-PAGE analysis of purified Cry1 toxins visualized with Coomassie blue staining. Lane 1, Cry1Ac; lane 2, Cry1Ab; lane 3, Y153D; lane 4, A92D; lane 5, F371A; lane 6, R368A/R369A; lane 7, Cry1B; lane 8, molecular size marker.

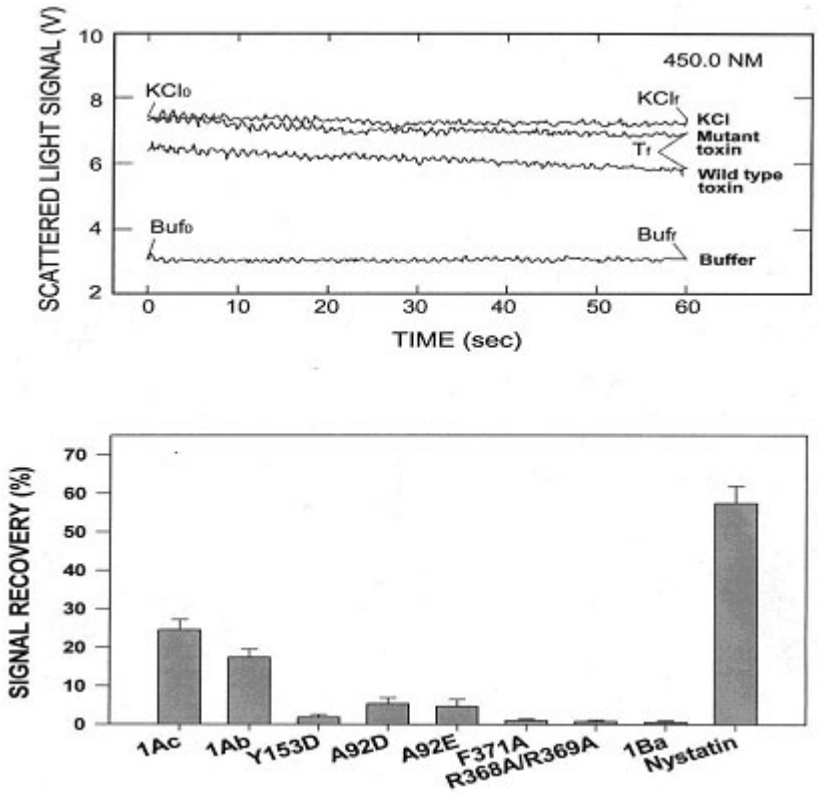

Fig. 2-2. Kinetic trace and percent signal recovery of *M. sexta* BBMV after treatment with toxin. (A) Kinetic trace of scattered light from toxin treated BBMV with no prior incubation of toxin and BBMV. The Buffer sample contained BBMV coinjected with 10 mM Tris-HCl (pH 7.5). In the KCl sample, BBMV were coinjected with 0.25 M KCl. (B) Percent signal recovery for different toxins was calculated from at least three independent assays according to formula (A) given in Results. The standard errors were calculated from the means of the independent assays and are shown as error bars.

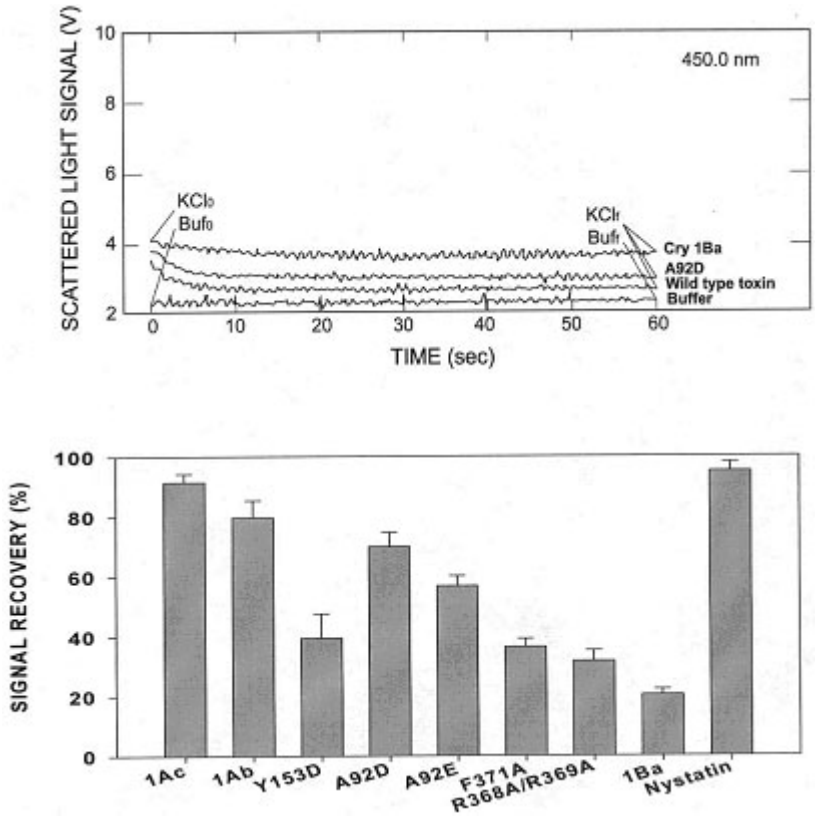

Fig. 2-3. Kinetic trace and percent signal recovery of *M. sexta* BBMV after treatment with toxin. (A) Kinetic trace of scattered light from toxin-treated BBMV. BBMV were resuspended in 10 mM Tris-HCl (pH 7.5) to a concentration of 0.4 mg/ml. BBMV were incubated with 5μg toxin/mg BBMV protein for 30 minutes. Assays were conducted by simultaneously injecting 35 μl of toxin treated BBMV and 35 μl of 0.25 M KCl. (B) Percent signal recovery for the different toxins was calculated according to the formula (B) stated in Results. The standard errors were calculated from the means of at least three independent assays and are shown as error bars.

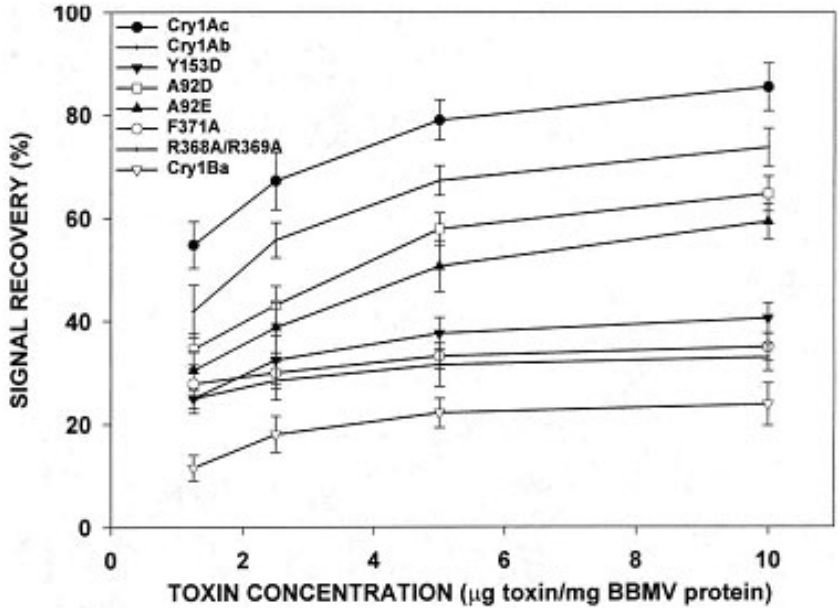

Fig. 2-4. Relationship between toxin concentration and signal recovery. BBMV were preincubated with different concentrations of toxin. Experiments were conducted as described in Fig. 3.

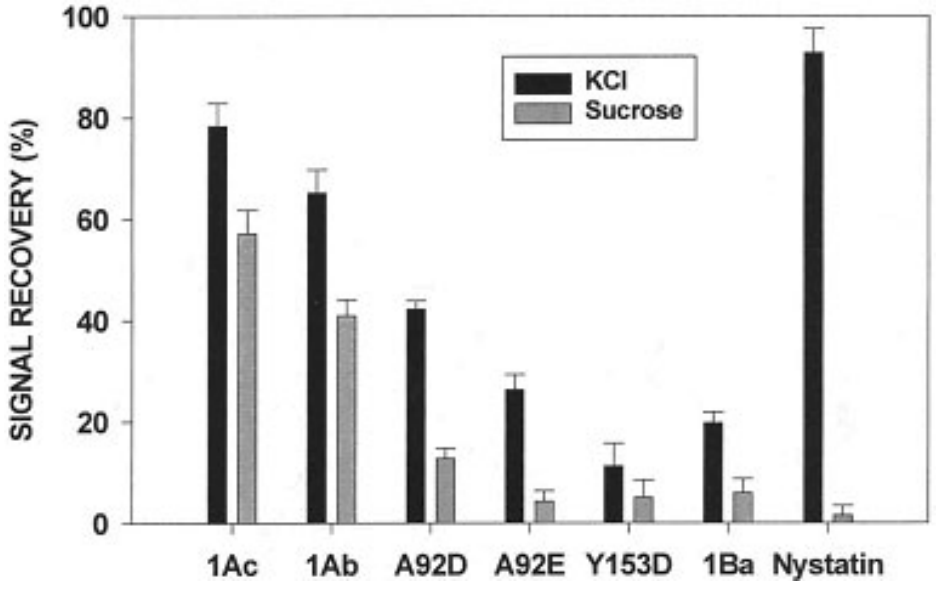

Fig. 2-5. Comparison of signal recovery under different hyperosmotic conditions. Experiments were conducted as described in Fig. 2 with the following modifications. In one set of assays 0.25 M KCl (filled bars) and in the other set 0.125 M sucrose (open bars) was used. The monitoring time was 3 minutes. Signal recovery was calculated according to formula (A). The standard errors were calculated from the means of three independent values.

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CHAPTER 3

DENATURATION OF EITHER *MANDUCA SEXTA* AMINOPEPTIDASE N OR *BACILLUS THURINGIENSIS* CRY1A TOXINS EXPOSES BINDING EPITOPES HIDDEN UNDER NON-DENATURING CONDITIONS[1]

[1] Daniel, A., Sangadala, S., Dean, D. H. and Adang, M. J. 2002. *Applied and Environmental Microbiology* 68: 2106-2112. Reprinted here with permission of publisher.

3.1 Abstract

The effect of polypeptide denaturation of *Bacillus thuringiensis* Cry1A toxins or purified *Manduca sexta* 120 kDa aminopeptidase N (MsAPN-1) on the specificities of their interactions was investigated. Ligand and dot-blotting experiments were conducted with ^{125}I-labeled Cry1Ac, Cry1Ac mutant ^{509}QNR^{511}-AAA or MsAPN-1 as probes. Mutant ^{509}QNR^{511}-AAA does not bind N-acetylgalactosamine moiety on MsAPN-1. Both ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA bound to 210 kDa and 120 kDa proteins from *M. sexta* brush border membrane vesicles and purified MsAPN-1 on ligand blots. However, on dot blots ^{125}I-^{509}QNR^{511}-AAA bound brush border vesicles, but did not bind purified aminopeptidase except when aminopeptidase was denatured. In the reciprocal experiment, ^{125}I-aminopeptidase bound Cry1Ac, but did not bind ^{509}QNR^{511}-AAA. ^{125}I-aminopeptidase bound Cry1Ab to a limited extent, but not the Cry1Ab domain I mutant Y153D, or Cry1Ca. However, denatured ^{125}I-MsAPN-1 detected each Cry1A toxin and mutant, but not Cry1Ca on dot blots. The same pattern of recognition occurred with native (non-denatured) ^{125}I-aminopeptidase probe and denatured toxins as targets. The broader pattern of toxin-binding protein interaction is probably due to peptide sequences being exposed upon denaturation. Putative Cry toxin binding proteins identified by the ligand blot technique need to be investigated under native conditions early in the process of identifying binding proteins that may serve as functional toxin receptors.

3.2 Introduction

Bacillus thuringiensis Cry1 proteins are specifically and highly toxic to lepidopteran insects (Schnepf *et al*., 1998). Cry1 proteins are expressed by the bacterium as 130-kDa protoxins then occluded in large biypyramidal crystals. Feeding larvae ingest crystals that dissolve in the alkaline midgut releasing soluble protoxin. Protoxin is processed by gut proteinases to active toxin that binds to receptors located in the brush border epithelium. Toxin inserts into the membrane, and oligomerizes forming ion channels. These events culminate in midgut cell lysis and insect mortality. Bt toxin mode of action was recently reviewed (Aronson and Shai, 2001).

Toxin binding to receptors in the insect midgut is an important determinant of insect susceptibility and a mechanism by which insects become resistant to *B. thuringiensis* Cry toxins. For example, the Cry1A resistant YHD2 strain of *Heliothis virescens* has a transposon insertion in a cadherin-like protein, a known class of Cry1 receptors (Gahan *et al*., 2001). The implication is that 'knock-out' of the receptor causes the observed lack of Cry protein binding (Jurat-Fuentes *et al*., 2000) and insect resistance.

In addition to cadherin-like proteins, aminopeptidases, and glycoconjugates are identified as binding molecules that in some cases serve as receptors for *Bacillus thuringiensis* Cry1 proteins in lepidopteran species (reviewed in Garczynski and Adang, 2000). The ligand blotting technique is frequently the first step towards identifying a toxin binding molecule. In this technique brush border proteins are separated by SDS-PAGE and blotted to a membrane filter. Toxin binding molecules are identified on the filter by using radiolabeled toxin or an indirect toxin detection technique. The ligand blotting technique detects Cry toxin binding to molecules under denaturing conditions.

Where investigated, Cry1 proteins often bind the same proteins under denaturing and native conditions. For example, Cry1Ab binds the 210 kDa cadherin-like protein under denaturing and non-denaturing conditions (Vadlamudi *et al*., 1993). Also, Cry1Ac binds to MsAPN-1 under both denaturing and non-denaturing conditions (Sangadala *et al*., 1994). However, Cry1Aa binds to the 175-kDa cadherin-like protein from *Bombyx mori* under native, but not denaturing conditions. As technologies, such as phage display are applied to the investigation of binding epitopes on proteins (Gomez *et al*., 2001) the concept of where the epitope, i.e. peptide, is located on a native protein becomes especially relevant.

Cry1Ac recognizes an N-acetyl galactosamine (GalNAc) on APN via a sugar-binding pocket located in domain III of the toxin (Burton *et al*., 1999; de Maagd *et al*., 1999). Cry1Ac mutant ^{509}QNR^{511}-AAA is a mutant with a disrupted sugar-binding pocket (Jenkins *et al*., 1999). ^{509}QNR^{511}-AAA has reduced binding to BBMV, but is only two-fold less toxic than Cry1Ac to *M. sexta* (Jenkins *et al*., 1999). Surface plasmon resonance (SPR) binding analyses revealed that Cry1Ac mutant ^{509}QNR^{511}-AAA did not bind APN under non-denaturing conditions. Ligand blot analyses with *M. sexta* brush border membrane vesicles (BBMV) and purified APN revealed that ^{509}QNR^{511}-AAA did not bind APN but bound a 210 kDa protein, in agreement with SPR analyses (Jenkins *et al*., 1999). The results that prompted the present study, came from a ligand blot experiment we conducted in which Cry1Ac and ^{509}QNR^{511}-AAA were probed against *M. sexta* BBMV and purified APN. Unexpectedly, both Cry1Ac and ^{509}QNR^{511}-AAA bound 210 kDa protein and MsAPN-1.

Since our ligand blotting results disagreed with results reported elsewhere (Jenkins *et al*., 1999), we designed experiments to investigate this apparent inconsistency. We investigated the possibility that denaturation of target binding molecule was altering binding specificity. The hypothesis that denatured (unfolded) proteins have binding epitopes not exposed on native proteins was tested. Native and denatured target proteins (BBMV and purified MsAPN-1) were probed with Cry1Ac and ^{509}QNR^{511}-AAA. For comparison, we included Cry1Ab, which also binds MsAPN-1 (Masson *et al*., 1995), and Cry1Ab mutant Y153D. Mutant Y153D is impaired in irreversible binding and membrane insertion and is not toxic to *M. sexta* (Hussain *et al*., 1996; Daniel *et al*., 2001). When denaturation of target molecules caused altered binding specificity, we reversed the approach by denaturing toxin probe (Cry1Ac or ^{509}QNR^{511}-AAA). Denaturation of either toxin or target proteins caused altered, but not a complete loss, of specificity.

3.3 Materials and methods

3.3.1 Bacterial strains and toxin purification.

B. thuringiensis strain HD-73 producing Cry1Ac was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH). *B. thuringiensis* strain producing Cry1Ca was obtained from Ecogen Inc. (Langhorne, PA). *Escherichia coli* strain NRD-12 expressing a *cry1Ab* gene was kindly provided by Dr. Luke Masson (Biotechnology Research Institute, Montreal, Canada). *E. coli* strains producing Cry1Ac mutant toxin ^{509}QNR^{511}-AAA and Cry1Ab mutant toxin Y153D were obtained from Dr. Donald Dean (Ohio State University, Columbus, OH).

Toxins from *B. thuringiensis* were isolated and purified as described previously (Daniel *et al*., 2001). Cry1Ab, ^{509}QNR^{511}-AAA and Y153D toxins were purified from *E. coli* according to Lee *et al*. (1992). Protein concentration was determined by the Bradford protein assay using bovine serum albumin as standard Bradford (1976).

3.3.2 Iodination of toxins and APN.

Cry1Ac, Cry1Ac mutant ^{509}QNR^{511}-AAA and MsAPN-1 were iodinated with Na^{125}I using IODO-Beads (Pierce Co.) according to manufacturer's instructions. Either 1 μg toxin or 5 μg MsAPN-1 were labeled with 0.5 mCi of Na^{125}I (Amersham Pharmacia). Based on input protein the specific activities of labeled Cry1Ac and ^{509}QNR^{511}-AAA were 27.3 μCi/μg and 25.6 μCi/μg respectively, while the specific activity of MsAPN-1 was 10.3 μCi/μg.

3.3.3 Gel electrophoresis.

Purity of toxin and MsAPN-1 preparations was analyzed by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE). 5 μg of purified protein or 10^5 cpm of radiolabeled toxin or APN per lane were electrophoresed. Gels were stained with Coomassie brilliant blue R-250 or exposed to Kodak XAR-5 film with intensifying screen at –80^{0}C for autoradiography.

3.3.4 Preparation of BBMV.

Midguts were dissected from fifth instar *M. sexta* larvae, washed with ice-cold MET buffer (250 mM mannitol, 17 mM Tris-HCl (pH 7.5), 5 mM EGTA) and

immediately frozen on dry ice and stored at –80^{0}C until needed. BBMV were prepared by the $MgCl_2$ precipitation method (Wolfersberger *et al*., 1987). The final BBMV pellet was suspended in ice-cold 10 mM Tris-HCl (pH 7.5). Rat intestinal BBMV were prepared according to Steiger and Murer (1983).

BBMV proteins were solubilized by mixing BBMV with 1% CHAPS in TBS (pH 7.5) for 30 min at 4^{0}C. The mixture was centrifuged at 15,000 x g for 10 min and the supernatant collected. Solubilized BBMV for dot blotting were prepared immediately before use.

3.3.5 Purification of MsAPN-1 from M. sexta BBMV.

MsAPN-1 was purified by anion exchange chromatography followed by hydrophobic interaction chromatography to remove associated lipids as reported previously (Sangadala *et al*., 2001).

3.3.6 Ligand and reverse- ligand blotting.

BBMV (5 μg) or toxins (5 μg) were separated by SDS-PAGE then electro-blotted onto polyvinylidene difluoride Q (PVDF) membrane filters (Millipore). Filters were blocked with 3% BSA in TBST (25 mM Tris-HCl (pH 7.4), 3 mM KCl, 135 mM NaCl, 0.1% Tween 20) for one hour. After incubation with 10^6 cpm of ^{125}I-labeled toxin or MsAPN-1 in 10 ml of 0.1% BSA-TBST for 3 hrs (1 hr for ^{125}I-APN) filters were washed in 0.1% BSA-TBST three times for 15 min and exposed to film overnight at –80^{0}C.

3.3.7 Dot and Reverse-dot blotting.

Varying concentrations of protein diluted in 100 µl TBS, were dot blotted on PVDF filters under vacuum for 10 minutes using a BioRad apparatus. Filters were incubated with 10^6 cpm of ^{125}I-labeled probe (toxin or MsAPN-1) in the same manner as for ligand blotting. Proteins were denatured by adding SDS to 1% and boiling for 5 min. Samples were clarified by centrifugation at 10,000 x g for 10 min prior to dot blotting.

3.4 Results

3.4.1 Autoradiography of ^{125}I-labeled proteins and ligand blotting with ^{125}I-labeled Cry1Ac and ^{509}QNR^{511}-AAA toxins.

^{125}I-labeled MsAPN-1, Cry1Ac and ^{509}QNR^{511}-AAA appeared as single bands when separated by SDS-PAGE and visualized on autoradiograms (Fig. 3-1A).

The differences between ligand blot results shown in Figure 1 and previously published results prompted us to undertake this investigation. In previous studies, Cry1Ac recognized MsAPN-1 and 210 kDa cadherin-like protein on ligand blots (Garczynski and Adang, 2000). Additionally, Cry1Ac mutant ^{509}QNR^{511}-AAA (which is impaired in GalNAc recognition) bound the 210 kDa protein, but not the MsAPN-1 (Jenkins *et al*., 1999). In this study, ^{125}I-Cry1Ac bound proteins as expected including the absence of Cry1Ac binding to rat BBMV and competition of Cry1Ac binding by GalNAc (Fig. 3-1B). However, ^{509}QNR^{511}-AAA binding results shown in Fig. 1C were not expected. ^{125}I-^{509}QNR^{511}-AAA bound to 210 kDa protein, MsAPN-1, a 110 kDa protein and a 45-kDa protein. GalNAc did not compete ^{509}QNR^{511}-AAA binding and apparently increased binding to the 110-and 45-kDa proteins. Apparently, our ligand

blotting conditions contributed to an apparent change in ^{509}QNR^{511}-AAA binding specificity. Since surface plasmon resonance analyses showed ^{509}QNR^{511}-AAA did not bind *M. sexta* MsAPN-1 under non-denaturing conditions (Jenkins *et al*., 1999), we considered the possibility that denatured proteins on ligand blots have binding sites not exposed on the native protein.

3.4.2 Dot blotting with ^{125}I-labeled toxins.

The effect of denaturation (SDS-heat treatment) on Cry1Ac and ^{509}QNR^{511}-AAA binding specificity was tested by dot blot experiments. The results of Cry1Ac and ^{509}QNR^{511}-AAA binding to native proteins are shown in Fig. 3-2A. *M. sexta* BBMV, CHAPS-solubilized BBMV and purified MsAPN-1 were spotted onto PVDF membrane then probed with ^{125}I-Cry1Ac or ^{125}I-^{509}QNR^{511}-AAA. ^{125}I-Cry1Ac bound BBMV, CHAPS-solubilized BBMV and purified MsAPN-1. ^{125}I-^{509}QNR^{511}-AAA bound strongly to BBMV, and less strongly to CHAPS-solubilized BBMV, but did not bind purified MsAPN-1.

The effect of denaturation on ^{509}QNR^{511}-AAA binding is visible in Fig. 3-2B. Denaturation did not alter the binding pattern of Cry1Ac, although the amount of Cry1Ac binding to denatured BBMV and MsAPN-1 was reduced. However, ^{125}I-^{509}QNR^{511}-AAA bound denatured MsAPN-1 in addition to BBMV and CHAPS-solubilized BBMV. ^{125}I-labeled Cry1Ac or ^{509}QNR^{511}-AAA did not bind denatured rat BBMV. Denaturation, in this case by SDS and heat treatment, altered ^{509}QNR^{511}-AAA-binding specificity.

3.4.3 Reverse-dot blotting with native and denatured ^{125}I-MsAPN-1.

Next, we probed a series of toxins on dot blots with either native or denatured MsAPN-1 to determine the extent to which specificity for toxins was changed. The results of native MsAPN-1 binding are shown Fig 3-3A and B. ^{125}I- MsAPN-1 bound strongly to Cry1Ac, weakly to Cry1Ab (note, the faint signal), but not to ^{509}QNR^{511}-AAA, the Cry1Ab domain I mutant Y153D or Cry1Ca. As expected, GalNAc decreased ^{125}I- MsAPN-1 binding to Cry1Ac. Both dot blotting and reverse-dot blotting results were in agreement: MsAPN-1 has a specific pattern of binding specificity for wild-type Cry1 toxins and native MsAPN-1 does not bind mutant ^{509}QNR^{511}-AAA.

Since BBMV proteins on ligand blots are denatured, we probed toxins on dot blots with denatured MsAPN-1. ^{125}I-MsAPN-1 was boiled in 1% SDS for 5 min before probing the dot blot. Denatured ^{125}I-MsAPN-1 bound strongly to all the toxins except Cry1Ca (Fig 3-3C). While denaturation exposed binding sites on MsAPN-1 recognized by Cry1Ac and Cry1Ab toxins, binding sites were apparently not present on Cry1Ca. Since ^{509}QNR^{511}-AAA bound denatured MsAPN-1 in reverse-dot blot experiments, it agreed with the observation that denaturation altered ^{509}QNR^{511}-AAA binding specificity (ligand and dot blot experiments).

Since denatured MsAPN-1 had an increased range of binding, we reversed the procedure by denaturing toxins before dot blotting then tested for altered binding of MsAPN-1. ^{125}I-MsAPN-1 was probed against toxins that were denatured by boiling in 1% SDS before dot blotting. ^{125}I-MsAPN-1 bound denatured Cry1Ac, Cry1Ab and the Cry1A mutant toxins. ^{125}I-MsAPN-1 did not bind denatured Cry1Ca (Fig. 3-3D). Denaturation of either MsAPN-1 or toxin decreased binding specificity. However,

absence of binding to denatured Cry1Ca is evidence that binding has some level or degree of specificity.

3.4.4 Reverse-ligand blotting with ^{125}I-labeled APN.

Cry1 proteins on ligand blots were probed with ^{125}I-MsAPN-1. This experiment allowed us to test binding specificity under actual ligand blot conditions. ^{125}I-MsAPN-1 bound Cry1Ac, Cry1Ac mutant ^{509}QNR^{511}-AAA, Cry1Ab and Cry1Ab mutant Y153D, but not Cry1C (Fig 3-4). Labeled MsAPN-1 binding to these toxins was not competed by GalNAc (data not shown). The reverse-ligand blot results are further evidence that denaturation of either toxin or brush border proteins induces protein interactions that do not occur under native conditions.

3.5 Discussion

We report that denaturation of either Cry1 toxin or brush border proteins alters toxin binding specificity. Previous reports have suggested that alteration of toxin binding specificity occurs under denaturing conditions. Nagamatsu *et al*. (1998) observed that Cry1Aa bound the 175-kDa protocadherin of *Bombyx mori* under native, but not denaturing conditions. Vadlamudi *et al*. (1993) demonstrated that Cry1Ab toxin could interact with both native and denatured 210 kDa cadherin-like protein. Lee and Dean (1996) reported inconsistencies in results between BBMV binding studies and ligand blot results.

For identification of putative toxin receptors, native toxins and denatured BBMV proteins on ligand blots have been used (Vadlamudi *et al*., 1993; Luo *et al*., 1996). In this report we attempted to determine if the state of polypeptide (both toxin and receptor)

affects their interaction. To address this issue, we performed combinations of binding experiments involving labeled receptor and toxins in their native and denatured forms. Table 3-1 summarizes the results from these experiments; the state of polypeptide in experiments we conducted and resulting alteration in binding specificity. Table 3-1 shows whether toxin bound to MsAPN-1 and vice versa under different experimental conditions.

In the present study, Cry1Ac bound 210- and120 kDa proteins in BBMV and purified MsAPN-1 on ligand blots consistent with published results (Fig 3-2) (Garczynski and Adang, 2000). Jenkins *et al*. (1999) reported ^{509}QNR^{511}-AAA binding only to 210 kDa protein on ligand blots, whereas, we detected ^{509}QNR^{511}-AAA binding to the 210-, 120- and 110 kDa proteins using the same technique. We also observed that ^{509}QNR^{511}-AAA, relative to Cry1Ac, gave a stronger signal on ligand blots.

The Cry1Ac mutant ^{509}QNR^{511}-AAA bound BBMV, but not MsAPN-1 under native conditions (Jenkins *et al*., 1999). The lack of QNR-AAA binding to native MsAPN-1 on dot blots agreed with these reports. Since ^{509}QNR^{511}-AAA is toxic to *M. sexta* (Jenkins *et al*., 1999), another protein must be a functional receptor *in vivo*. Evidence supports the 210 kDa Bt-R1 as being the remaining functional receptor (Vadlamudi *et al*., 1995). The possibility of other protein (s) (e.g. 110 kDa protein) being ^{509}QNR^{511}-AAA-binding protein (s) is currently being investigated in our lab. The reason for ^{509}QNR^{511}-AAA binding to the 45-kDa protein and increased binding to the 110 kDa protein in the presence of GalNAc are unknown.

Since ^{509}QNR^{511}-AAA bound to purified MsAPN-1 only under denaturing conditions (Table 3-1), it appears that ^{509}QNR^{511}-AAA recognizes an epitope on MsAPN-

1 that is exposed upon denaturation. From our ligand blot results conducted in the presence of GalNAc, it also appears that this second toxin-recognition site on MsAPN-1 is GalNAc-independent. Jenkins *et al*. (1999) suggested that toxin interacts with the MsAPN-1 initially via the GalNAc-dependent site on domain III followed by domain II interactions. Though speculative, it appears that toxin can interact with MsAPN-1 under denaturing conditions directly via the GalNAc-independent domain II site.

Using labeled MsAPN-1, we designed reverse dot blot and reverse ligand blot experiments to further test the hypothesis that denaturation affected binding specificity. Labeled-MsAPN-1 bound strongly to Cry1Ac (Fig. 3-3A) and this binding was reduced in the presence of GalNAc (Fig. 3-3B). This result confirmed the ligand blotting results with Cry1Ac and GalNAc and was evidence that radiolabeling of native MsAPN-1 did not alter the major binding properties of MsAPN-1.

Labeled-MsAPN-1 bound weakly to Cry1Ab but did not bind to ^{509}QNR^{511}-AAA, Cry1Ab mutant Y153D or Cry1Ca on dot blots (Fig. 3-3A). Absence of binding of labeled- MsAPN-1 to ^{509}QNR^{511}-AAA agreed with dot blot results in which labeled ^{509}QNR^{511}-AAA did not bind purified MsAPN-1. The limited MsAPN-1binding to Cry1Ab was surprising as Cry1Ab bound to MsAPN-1 in SPR analysis (Masson *et al*., 1995). However, Keeton *et al*. (1998) did not detect Cry 1Ab binding to MsAPN-1 on ligand blots. Y153D, the Cry1Ab domain I mutant, did not bind MsAPN-1. As expected from Luo *et al*. (1996) Cry1Ca did not bind MsAPN-1.

When denatured toxins were probed with labeled MsAPN-1 on reverse dot blots, MsAPN-1 bound to Cry1Ac, Cry1Ab and the tested Cry1A mutants (Fig. 3-3D). These results were confirmed in reverse ligand blots in which SDS-PAGE separated Cry1A

toxins were probed with labeled-MsAPN-1 (Fig 3-4). In both cases, MsAPN-1 did not bind to denatured Cry1Ca, indicating that the binding of MsAPN-1 to toxin was altered, yet retained specificity to Cry1A toxins. This is evidence that Cry1A toxins, but not Cry1Ca toxin, possess MsAPN-1-binding epitope (s) that becomes accessible upon denaturation. Denaturation apparently causes toxin to bind BBMV proteins that would not bind in the native toxin confirmation. Whether this phenomenon occurs naturally as toxin unfolds *in vivo* is unknown.

The summary conclusion is that while ligand blotting technique is a useful technique to identify putative toxin-binding proteins in brush border membranes, the technique exposes unique binding epitope (s) not present on the surface of the native BBMV proteins due to denaturation. Therefore, interpretation of ligand blot results towards identification of putative binding proteins should be carried out with caution and warrants confirmation with other techniques.

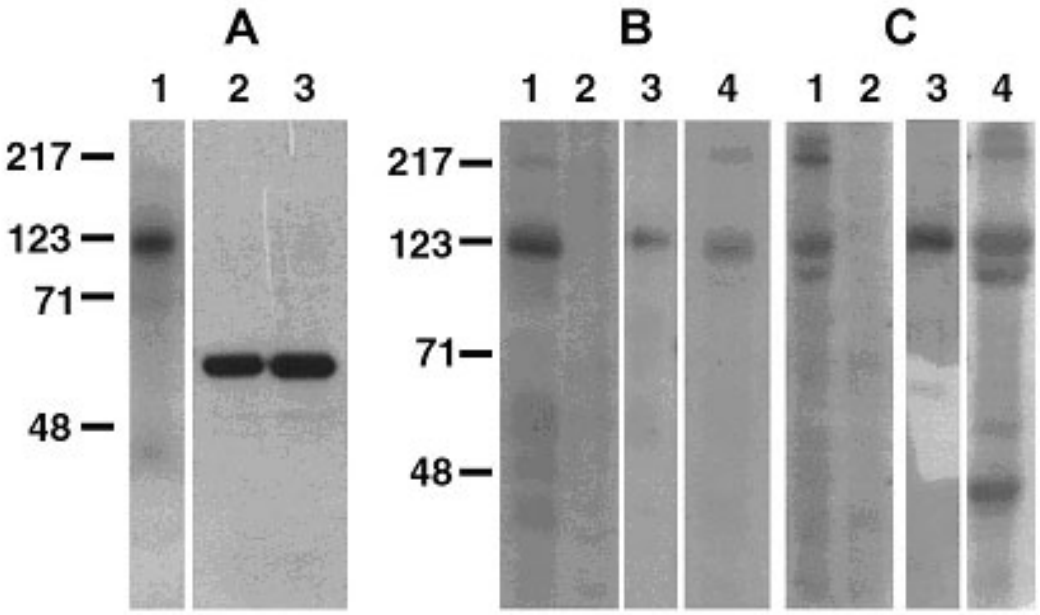

Fig 3-1. Autoradiography of ^{125}I-labeled proteins (A) and ligand blot analyses of SDS-PAGE separated BBMV proteins and purified MsAPN-1 with ^{125}I-Cry1Ac (B) or ^{125}I-^{509}QNR^{511}-AAA (C). Panel A. Lanes 1-3 contained 100,000 cpm radiolabeled MsAPN-1, Cry1Ac and QNR-AAA respectively. Panels B and C. 5 μg each *M. sexta* (lane 1) and rat (lane 2) BBMV proteins and 1 μg of purified MsAPN-1 (lane 3) were transferred to PVDF membrane and incubated with ^{125}I-toxin (0.5 nM) and detected with autoradiography. *M. sexta* BBMV proteins were incubated with 0.5 nM ^{125}I-toxin in the presence of 100 mM GalNAc and autoradiographed (lane 4).

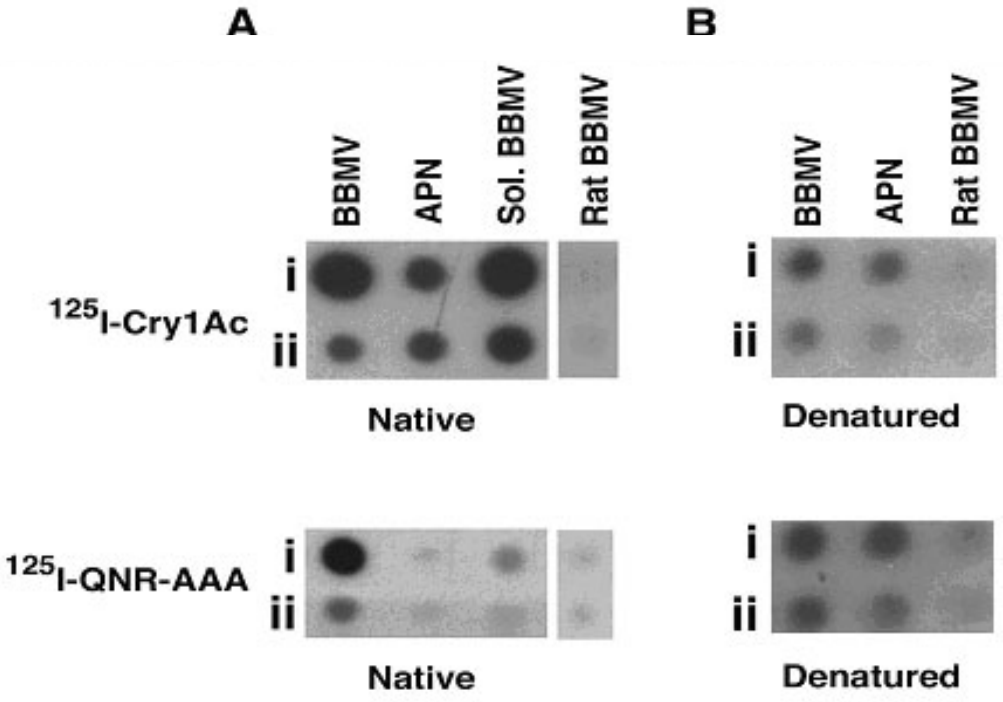

Fig. 3-2. Dot blot analyses of *M. sexta* BBMV, CHAPS-solubilized BBMV and purified APN with ^{125}I-Cry1Ac or ^{125}I-^{509}QNR^{511}-AAA. The amount of proteins in row i) is 5 μg and in row ii) is 1 μg except purified MsAPN-1 with proteins amounts of 2 μg and 1 μg in rows i and ii, respectively. Native (Panel A) or denatured (Panel B) proteins were spotted on PVDF membrane and damp-dried under vacuum. The membranes were incubated with ^{125}I-Cry1Ac (0.5 nM) or ^{125}I-^{509}QNR^{511}-AAA (0.5 nM) for 1 hr and autoradiographed as described in methods.

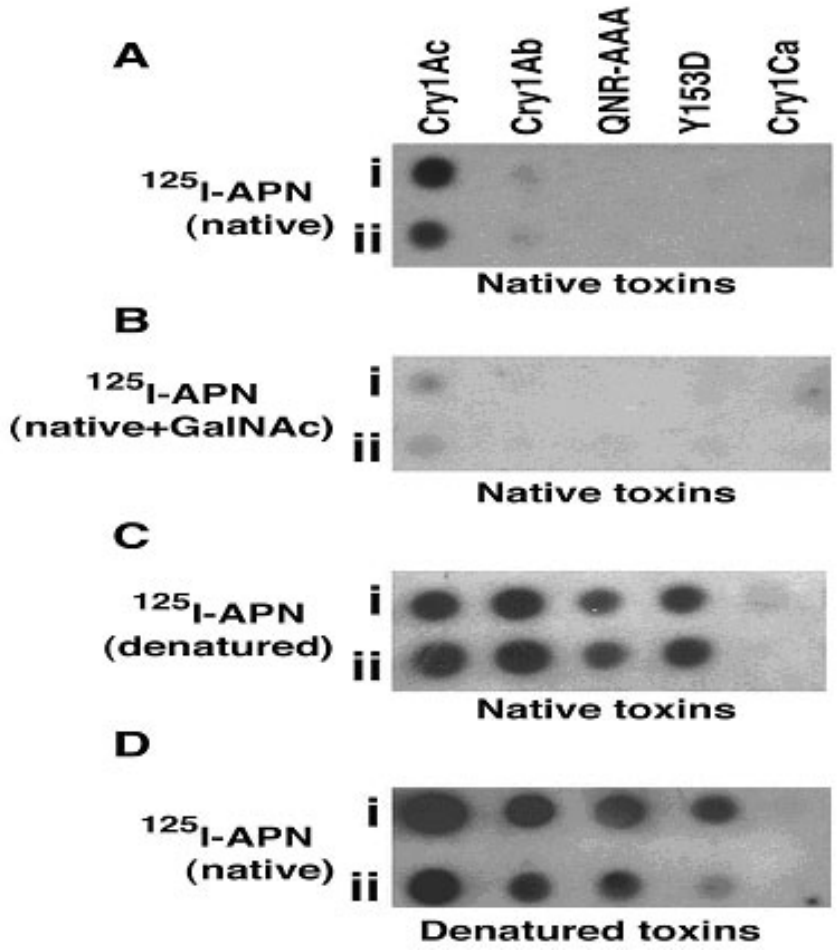

Fig. 3-3. Reverse-dot blot analyses of purified toxins. 5 μg (row i) and 1 μg (row ii) of purified toxins were dot blotted as described above. (A) Toxin-membrane exposed to ^{125}I-MsAPN-1. (B) Toxin-membrane exposed to ^{125}I-MsAPN-1 + 100 mM GalNAc (B). (C) Toxin-membrane exposed to denatured ^{125}I-MsAPN-1. ^{125}I-MsAPN-1 was denatured as described in methods. (D) Purified toxins were denatured as described in methods before dot-blotting onto membrane and exposed to ^{125}I-MsAPN-1. All incubations were done for 1 hr, washed and autoradiographed.

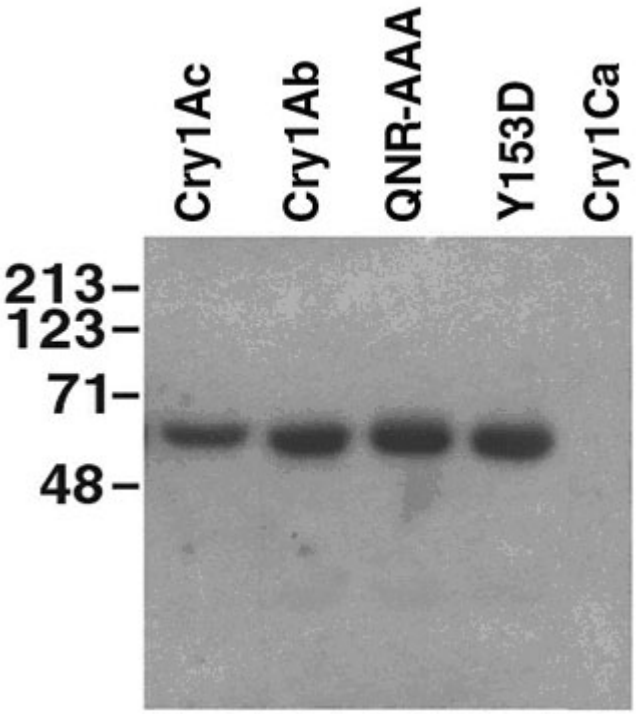

Fig. 3-4. Reverse-ligand blot analyses of SDS-8%PAGE separated purified toxins with ^{125}I-MsAPN-1 (1 nM). 5 μg of each of purified toxin were transferred to PVDF membrane filters and incubated with ^{125}I-MsAPN-1, washed and autoradiographed.

TABLE 3-1. Summary of experiments conducted to investigate the interaction of *Bacillus thuringiensis* Cry1A toxins with purified *M. sexta* MsAPN-1

Experimental Method (*radiolabeled protein[s])	State of MsAPN-1	State of toxin	Binding of toxin to MsAPN-1	Binding of MsAPN-1 to Cry1Ac /^{509}QNR^{511}-AAA
Ligand blot (*Cry1Ac/*^{509}QNR^{511}-AAA)	Denatured	Native	Yes / Yes [a]	—
Dot blot (*Cry1Ac/*^{509}QNR^{511}-AAA)	a) Native b) Denatured	Native	a) Yes / No [a] b) Yes / Yes [a]	—
Reverse-dot blot (*MsAPN-1)	Native	a) Native b) Denatured	—	a) Yes / No b) Yes / Yes
Reverse-dot blot (Denatured *MsAPN-1)	Denatured	Native	—	Yes / Yes
Reverse-ligand blot (*MsAPN-1)	Native	Denatured	—	Yes / Yes

[a] Results for Cry1Ac/results for ^{509}QNR^{511}-AAA

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CHAPTER 4

A NOVEL *MANDUCA SEXTA* AMINOPEPTIDASE N FUNCTIONS AS A N-ACETYLGALACTOSAMINE-INDEPENDENT BINDING PROTEIN FOR *BACILLUS THURINGIENSIS* CRY1Ac TOXIN [1]

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4.1 Abstract

A novel 110 kDa protein was isolated from *Manduca sexta* brush border membrane vesicles (BBMV) by toxin-affinity chromatography using Cry1Ac-domain III mutant toxin ^{509}QNR^{511}-AAA. ^{509}QNR^{511}-AAA has nearly wild-type toxicity to *M. sexta* larvae but unlike Cry1Ac, does not bind MsAPN-1. The full-length cDNA encoding 110 kDa protein was cloned from *M. sexta* midgut cDNAs using rapid amplification of cDNA ends (RACE) cloning. The 110 kDa protein, a previously unidentified *M. sexta* APN (Ms110-APN) (GenBank accession #: AF498996) has a deduced molecular mass of 108.3 kDa and contains putative N- and O-glycosylation sites, an N-terminal signal sequence, and a potential C-terminal glycosylphosphatidylinositiol-anchor sequence. Ms110-APN shared ~65% identity and ~80% similarity with *Helicoverpa punctigera* APN2, *Heliothis virescens* 110-APN and *Bombyx mori* APN4 and ~30% identity with previously described MsAPN-1, which binds Cry1Ac, and MsAPN-2, which binds Cry1Ab. Anti-110-APN antibody recognized the Ms110-APN in BBMV and did not cross-react with MsAPN-1 or MsAPN-2. Cry1Ac and ^{509}QNR^{511}-AAA recognized the *E. coli*-expressed Ms110-APN in affinity chromatography and ligand blot experiments. Since *E. coli* proteins are not glycosylated recognition of *E. coli*-expressed Ms110-APN by Cry1Ac and ^{509}QNR^{511}-AAA was independent of N-acetylgalactosamine. These results indicate that the Ms110-APN is a sugar-independent Cry1Ac-binding protein and is a potential receptor for Cry1Ac toxin in *M. sexta*.

4.2 Introduction

The 120 kDa aminopeptidase N (APN; E.C. 3.4.11.2) of *M. sexta* (MsAPN-1) was the first molecule identified as a *Bacillus thuringiensis* (Bt) Cry1 toxin-binding protein (Knight *et al*., 1994; Sangadala *et al*., 1994). Since Bt Cry1 proteins require receptors located in the insect brush border membrane for toxicity, these reports focused attention on APNs as possible receptors. Typically, lepidopteran larvae have 4 isoforms of APN and one or more of these APNs bind Cry1 toxins. Each APN is an ectopeptidase that is tethered to the luminal surface of the brush border membrane by a glycosylphosphatidyl inositol (GPI) anchor. MsAPN-1 is released from brush border membrane as a soluble 115 kDa molecule by phosphatidylinositol phopholipase C cleavage. The 115 kDa APN is 5% carbohydrate and has an N-acetylgalactosamine (GalNAc) moiety (Sangadala *et al*., 2001). Cry1Ac binds MsAPN-1 via the GalNAc moiety, whereas Cry1Aa and Cry1Ab toxins bind the peptide backbones of APNs.

Garczynski and Adang (2000) and Nakanishi *et al*. (2002) summarized the status of APN isoforms and their relationships to Cry1 toxin binding in lepidopteran insects. Briefly, in *H. virescens* Cry1Ac binds distinct 170, 120 and 110 kDa APNs. Cry1Aa and Cry1Ab bind 170 and 110 kDa APNs (Gill *et al*., 1995; Luo *et al*., 1997; Banks *et al*., 2001). In *M. sexta* Cry1Aa, Cry1Ab, and Cry1Ac bind MsAPN-1 (Knight *et al*., 1994; Sangadala *et al*., 1994), Cry1Ab also binds 106 kDa APN (MsAPN-2) (Denolf *et al*., 1997), and Cry1Ca binds a different 106 kDa APN (Luo *et al*., 1996). In *B. mori* Cry1Aa, Cry1Ab, and possibly Cry1Ac bind 120 kDa APN (Yaoi *et al*., 1997). In ligand blot experiments, Cry1Aa binds *E. coli*-expressed peptide fragments of the following APNs: 120 kDa (BmAPN-1), 90 kDa (BmAPN-2), 110 kDa (BmAPN-3), and 100 kDa

(BmAPN-4) (Nakanishi *et al*., 2002). A cautionary note is that Cry1 binding detected by the ligand blot technique can involve epitopes not present on the surface of native protein (Daniel *et al*., 2002).

The soluble 115 kDa form of MsAPN-1 from *M. sexta* has an exclusive Cry1Ac binding site and another Cry1Ac site shared by Cry1Ab and Cry1Aa (Masson *et al*., 1995). GalNAc inhibits binding of Cry1Ac but not Cry1Ab to MsAPN-1 (Masson *et al*., 1995). Cooper *et al*. (1998) reported that GalNAc inhibited only one Cry1Ac-binding site on MsAPN-1.

Cry1Ac and Cry1Ab toxins share significant amino acid identity in domains I and II but differ in domain III (Van Rie *et al*., 1989). Domain III of Cry1Ac has a region of structural similarity with the N-terminal cellulose-binding domain of 1,4-β-glucanase from *Cellulomonas fimi* (Burton *et al*., 1999). GalNAc is associated with domain III in this region of Cry1Ac (Derbyshire *et al*., 2001). This structural analysis explains the GalNAc-inhibition effect detected for Cry1Ac but not for Cry1Ab.

The GalNAc interaction motif in Cry1Ac was also described by mutations of amino acid residues in domain III (Burton *et al*., 1999; Jenkins *et al*., 1999). Two such mutants ^{509}QNR^{511}-AAA (Jenkins *et al*., 1999) and Tmut (Burton *et al*., 1999) with a disrupted GalNAc-binding motif do not bind MsAPN-1. In toxicity assays however ^{509}QNR^{511}-AAA exhibited only 2- to 3-fold decrease in toxicity to *M. sexta* when compared to Cry1Ac (Lee *et al*., 1999). This observation and the fact that ^{509}QNR^{511}-AAA binds a 210 kDa protein in *M. sexta* BBMV (Jenkins *et al*., 1999), suggests that the 210 kDa protein is an important Cry1Ac receptor in *M. sexta*. The 210 kDa protein is probably the cadherin-like Cry1Ab receptor called BtR1 (Vadlamudi *et al*., 1995).

Recently, Banks *et al*. (2001) identified a 110 kDa APN in *H. virescens* (Hv110-APN) that binds Cry1Ac and mutant ^{509}QNR^{511}-AAA independent of GalNAc. We reasoned that similar Cry1Ac binding molecules might exist in *M. sexta* brush border membranes.

The major goal of this study was to isolate BBMV protein(s) from *M. sexta* that bind Cry1Ac in a GalNAc-independent manner using Cry1Ac mutant ^{509}QNR^{511}-AAA. A novel 110 kDa APN was isolated using ^{509}QNR^{511}-AAA and Cry1Ac affinity chromatography. The cDNA encoding Ms110-APN has high sequence homology to specific *H. punctigera*, *H. virescens,* and *B. mori* APNs. Cry1Ac and ^{509}QNR^{511}-AAA and anti-110 kDa APN antibody recognized the Ms110-APN in BBMV and in extracts of recombinant *E. coli*. Cry1Ac and ^{509}QNR^{511}-AAA bind native Ms110-APN in a GalNAc-independent manner via a peptide motif. This interaction is distinct from Cry1Ac recognition of MsAPN-1.

4.3 Materials and methods

4.3.1 Bacterial strains and toxin purification

B. thuringiensis strain HD-73 producing Cry1Ac was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH). *E. coli* producing Cry1Ac mutant ^{509}QNR^{511}-AAA was kindly provided by Dr. Donald Dean (Ohio State University, Columbus, OH).

B. thuringiensis was cultured and Cry1Ac toxin prepared as described previously (Daniel *et al*., 2000). ^{509}QNR^{511}-AAA was purified from *E. coli* inclusions according to (Lee *et al*., 1992). Protein concentration was determined by the Bradford protein assay using bovine serum albumin as standard (Bradford, 1976).

4.3.2 ^{125}I-labeling of toxins

Purified Cry1Ac and ^{509}QNR^{511}-AAA (1 μg) were radiolabeled with 0.5 mCi of Na^{125}I (Amersham Pharmacia) using the Chloramine-T method (Garczynski *et al*., 1991). Based on input toxin, the specific activities of Cry1Ac and ^{509}QNR^{511}-AAA were 25.7 μCi/μg and 20.2 μCi/μg respectively.

4.3.3 BBMV preparation

M. sexta eggs were obtained from Carolina Biological Supply Co. (Burlington, NC) and larvae reared on artificial diet (Southland Products, Inc., Lake Village, AK). Midguts were dissected from early fifth instar *M. sexta* larvae, washed with ice-cold MET buffer (250 mM mannitol, 17 mM Tris-HCl pH 7.5, 5 mM EGTA) and immediately frozen on dry ice, and stored at –80^{0}C until needed. BBMV were prepared by $MgCl_2$ precipitation method (Wolfersberger *et al*., 1987). The final BBMV pellet was suspended in ice-cold 10 mM Tris-HCl pH 7.5. BBMV for affinity chromatography were prepared the same day as needed.

4.3.4 Affinity purification of toxin-binding protein(s)

BBMV proteins were solubilized by incubating BBMV with 1% CHAPS in phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 2.7 mM KCl, 136.9 mM NaCl, pH 7.4) in the presence of protease inhibitors (Roche) (1 tablet/10ml) on ice for 30 min. Unsolubilized materials were removed by centrifugation at 16,000 x g for 10 min.

Affinity purification of toxin-binding protein(s) was performed according to Luo *et al*. (1996) with slight modifications. Five to eight mg of toxin were coupled to 1 g

activated cyanogen bromide (CNBr) sepharose beads. Fifteen mg of CHAPS-solubilized BBMV were added to the toxin-bead slurry and the mixture rotated overnight at 4^0C. The mixture was washed three times with 20 mM Na_2CO_3 pH 9.6 and bound proteins eluted with 2 M NaSCN in 20 mM Na_2CO_3 pH 9.6. The eluate volume was reduced to 200 µl using a Centricon concentrator (MWCO: 30,000) (Millipore).

4.3.5 Ligand blotting and Western blot analyses

Proteins were separated by SDS-8%PAGE and electro-blotted onto polyvinylidene difluoride Q (PVDF) membrane filters (Millipore). Filters were blocked with 3% BSA in TBST (25 mM Tris-HCl pH 7.4, 3 mM KCl, 135 mM NaCl, 0.1% Tween 20) for 1 hr. For ligand blot analyses, filters were incubated with 10^6 cpm of ^{125}I-labeled toxin in 0.1% BSA-TBST for 1 hr. Filters were washed three times in 0.1% BSA-TBST then exposed to film overnight at -80^0C.

Filters for Western blot analyses were blocked as above, then incubated with anti-APN antibody (1:5,000 in 0.1%BSA TBST) for 1 hr. Anti-MsAPN-1 serum is described in Luo *et al*. (1999). A second anti-Ms110-APN serum is described below. After three washes in 0.1% BSA-TBST, filters were incubated with goat-anti rabbit-HRP conjugate (1:30,000 in 0.1%BSA TBST) for 1 hr. Filters were washed in 0.1% BSA-TBST three times and APN detected by enhanced chemiluminescence (ECL kit, Amersham Pharmacia). Western blot analyses with anti-His-HRP antibody (Invitrogen) were detected directly without secondary antibody conjugate.

4.3.6 N-terminal amino acid sequencing

Proteins eluted from the ^{509}QNR^{511}-AAA affinity column were separated on 8% SDS-PAGE and transferred to PVDF membrane filter. The filter was stained with Coomassie Blue and then destained with 40% (v/v) methanol, 10% (v/v) acetic acid for 1 hr. The band corresponding to the 110 kDa protein was excised and submitted for N-terminal amino acid sequencing (Dr. Michael Berne, Tufts University, Boston, MA).

4.3.7 Cloning of internal fragment of 110 kDa protein from M. sexta midgut cDNAs

Midguts freshly dissected from *M. sexta* larvae (early 5th instar) were incubated in RNAlater (Ambion) on ice for 1 hr. Total RNA was isolated from 100 mg midgut tissue with an RNeasy kit (Qiagen). First-strand cDNA was synthesized from total RNA using *Not*I-dT_{18} primer, 0.25 mM each dNTPs, and Superscript reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

A PCR strategy was designed to generate a cDNA fragment that includes the determined N-terminal amino acid sequence TNLDEP and the APN consensus residues GAMENWG. The degenerate forward primer was 5'AC(A/C/T/G)AA(C/T)CT-(A/C/T/G)GA(C/T)GA(A/G)CC3' and the reverse primer was 5'CCCCA(A/G)TT(C/T)-TCCAT(A/T/G/C)GC(A/T)CC3'). Codon usage was optimized according to Frohlich and Wells (1994). PCR assay mixture contained 0.25 mM of each dNTP and 1U Taq polymerase (Eppendorf) in 50 mM KCl, 10 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$. PCR consisted of 35 cycles (94^0C, 1 min; 56^0C, 1 min; 72^0C, 1 min) and a 7 min 72^0C extension period. The 0.9 kb and 0.45 kb products unique to the combination of forward and reverse primer amplifications were purified from an agarose gel with a Qiaex II gel

extraction kit (Qiagen) and cloned into pCR 2.1 TA vector (Invitrogen). The plasmid carrying the 0.9 kb insert was called pTA-0.9 and the plasmid with the 0.45kb insert was called pTA-0.45. Inserts for all clones were sequenced completely from both strands at the Molecular Genetics Instrumentation Facility (University of Georgia).

4.3.8 3' and 5' RACE cloning to obtain full length cDNA encoding Ms110-APN

To amplify the 3' and 5' regions of cDNA encoding Ms110-APN, non-degenerate primers were designed from pTA-0.9. Single-primer controls were included in all amplification reactions. cDNA for 3' RACE was synthesized from *M. sexta* total RNA using *Not*I-dT_{18} adapter primer (Amersham Pharmacia) with Superscript reverse transcriptase (Invitrogen). The 3' RACE primers were Gene Specific Primer 1 (GSP1) (5'CCAGCACCAATACGCTGCTGAAATCG3') and *Not*I-dT_{18} primer (Amersham Pharmacia). Amplification was conducted in 50 mM KCl, 10 mM Tris-HCl, 15 mM $MgCl_2$, 1U Taq Polymerase (Eppendorf), 0.25 mM each dNTP, pH 8.3 and consisted of 30 cycles of 94^0C for 1 min, 55^0C for 1 min, 72^0C for 2 min, and a 7 min 72^0C extension period. A ~2.0 kb 3' RACE fragment was gel purified with a Qiaex II gel extraction kit (Qiagen) and cloned into pCR 2.1 TA vector (Invitrogen) yielding plasmid pTA-110-3'.

cDNA for 5' RACE was synthesized from *M. sexta* total RNA using GSP2 primer (GSP2: 5'CCTCCGACCAGTTCATGGGCGTGGCGCC3') with Superscript reverse transcriptase (Invitrogen). 5' RACE was conducted with abridged anchor primer (AAP) (Invitrogen): 5'GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG3' and GSP3: 5'GCTGTGTACGTAGCTAGTGACAAACTGG3' designed from pTA-110-3' sequence. Amplification was conducted in 50 mM KCl, 10 mM Tris-HCl, 15 mM

$MgCl_2$, 1U Taq Polymerase (Eppendorf), 0.25 mM each dNTP pH 8.3 with 30 cycles of 94^0C for 1 minute, 55^0C for 1 min, 72^oC for 1 min, and a 7 min 72^0C extension period. The 1.4 kb 5' RACE fragment was gel purified with a Qiaex II gel extraction kit (Qiagen) and cloned into pCR 2.1 TA to yield pTA-110-5'.

The full-length Ms110-APN cDNA (pTA-110) was constructed using inserts from plasmids pTA-110-5' and pTA-110-3' by utilizing a unique *BstB*I restriction site in the overlapping region of the clones plus unique 5' *Sal*I and 3' *Xho*I sites.

4.3.9 Production of polyclonal antibody against Ms110-APN

Anti-Ms110-APN antibody was prepared using a ~27 kDa truncated APN as immunogen. A 756 nt *Sac*I-*Kpn*I fragment encoding amino acids 58 through 309 was generated by PCR with primers (*Sac*I-pTA-110) 5'CCTGGAGCTC-TACTTGGATGACGCCAGG3' and (*Kpn*I-pTA-110) 5'CAGACGGTACCTCTCA-CAAGGCAATATG3' and pTA-110 as template. The resulting 0.75 kb DNA was digested with *Sac*I and *Kpn*I and cloned into pET30A (Novagen) in-frame with the His_6 tag. The plasmid was designated pET110-Ab.

E. coli strain BL21(DE3) harboring pET110-Ab was cultured and expression induced by addition of isopropyl-1-β-D-galactopyranoside (IPTG) according to the manufacturer's instructions (Novagen). Recombinant His_6-tagged 27-kDa APN fragment was purified using a Histrap affinity column (Amersham Pharmacia). Eluted fractions were successively dialyzed against 4, 2, 1, and 0 M urea in 50 mM Na_2CO_3 pH 9.6. The dialyzed sample was centrifuged at 16000 x g for 10 min. Protein concentration of the supernatant was 1.2 mg/ml. Anti-Ms110-APN sera were prepared in rabbits at the

Polyclonal Antibody Production Service, University of Georgia using the regime of Luo *et al*. (1999).

4.3.10 Construction of pTrcHis-110

Mature Ms110-APN was expressed in *E. coli* using plasmid pTrcHisA (Invitrogen). Mature APN extends from amino acid 33 to 947 and does not include the signal peptide or C-terminus past the predicted GPI-anchor processing site. The cDNA encoding mature APN was cloned in-frame with the N-terminal His_6 tag in pTrcHisA in 3 steps. First, a 0.2 kb *Xho*I-*Pst*I fragment was PCR amplified from pTA-110 using forward primer (*Xho*I-pTA-110) 5'GGAACTCGAGACTAACTTGGATGAGCCG3' and reverse primer (*Pst*I-pTA-110) 5'CAGGGTTGCCTGCAGAGTTCAGTACG3'. The PCR product was cloned into pTrcHisA (pTrcHis-1). Next, a 0.6 kb *Kpn*I-*Hind*III fragment was PCR amplified from pTA-110 using forward primer (*Kpn*I-2-pTA110) 5'CGTTGGTACCGGTCAACGCTCGCCGC3' and reverse primer (*Hind*III-pTA-110) 5'TAATAAGCTTTTAACGCAGCATTTGCGC3'. The PCR product was cloned into pTrcHis-1 (pTrcHis-2). Finally, plasmid pTrcHis-110 was constructed by inserting a 2.0 kb *Pst*I-*Kpn*I fragment from pTA-110 into pTrcHis-2.

4.3.11 Expression of Ms110-APN in E. coli and toxin-affinity chromatography with E. coli-expressed APN

Recombinant His_6-tagged Ms110-APN was expressed in *E. coli* DH5α cells from pTrcHis-110 according to the manufacturer's instructions (Invitrogen). Bacterial cells from the 800 ml culture were harvested 4 hours after induction with IPTG and washed

once with distilled water, and then resuspended in 50 ml PBS pH 7.4. An aliquot of induced cells (1 ml) was centrifuged, and the pellet resuspended in 200 μl SDS-PAGE sample buffer. The sample was boiled for 5 min and centrifuged at 16000 x g for 10 min. Protein extracts of *E. coli* DH5α with plasmid pTrcHisA were also prepared. Protein samples (10 μl) were separated by SDS-8%PAGE.

E. coli extract for affinity chromatography was prepared by incubating IPTG-induced cells with 0.25 mg/ml lysozyme for 30 min at 27^0C for 1 hr. The cells were sonicated and the supernatant obtained by centrifugation at 15,000 x g for 20 min. Affinity chromatography was performed as mentioned above except that 1 mg toxin was coupled to 100 mg Sepharose beads. The toxin-coupled beads were incubated with 5 mg *E. coli* extract and the selected proteins eluted with 2 M NaSCN.

4.4 Results

4.4.1 Purification of toxin-binding proteins by affinity chromatography

Cry1Ac and ^{509}QNR^{511}-AAA binding proteins were purified from solubilized *M. sexta* BBMV using affinity chromatography. Toxin-bead mixtures were incubated with CHAPS-solubilized BBMV. Bound proteins were eluted with 2 M NaSCN, separated by SDS-PAGE and silver-stained or blotted to PVDF membrane filter. Cry1Ac and ^{509}QNR^{511}-AAA affinity eluates contained 120- and 110 kDa proteins (Fig.4-1, lanes 2 and 3 respectively). A ~90 kDa protein was visible in the ^{509}QNR^{511}-AAA eluate (Fig.4-1, lane 3). The 60-kDa protein was probably non-covalently bound toxin that leached from the column.

Blots of eluted binding proteins were probed with anti-MsAPN-1 antibody (Fig 4-1, lanes 5 and 6). MsAPN-1 (120 kDa) was detected in the eluates from both toxin columns. Anti-MsAPN-1 antibody did not cross-react with the 110 kDa protein or with the 90 kDa protein from the ^{509}QNR^{511}-AAA column.

4.4.2 N-terminal sequencing of the 110 kDa protein

The N-terminal amino acid sequence of the 110 kDa protein from the ^{509}QNR^{511}-AAA column was TNLDEPK(Y)RL(R). A search of GenBank using the BLAST program and the determined N-terminal sequence as probe extracted *H. punctigera* APN2 (GenBank accession #: AF217249) and Hv110-APN (GenBank accession #: AF378666). Both proteins shared ~90% identity with the N-terminal sequence of 110 kDa protein. These data suggested that the 110 kDa protein was a previously unidentified Ms110-APN from *M. sexta*.

4.4.3 PCR amplification of cDNA encoding Ms110-APN

A cDNA encoding Ms110-APN was cloned by PCR and RACE. We amplified an internal region of Ms110-APN cDNA from *M. sexta* midgut cDNAs using a degenerate forward primer designed from the N-terminal amino acids TNLDEP. The degenerate reverse primer was designed from the conserved lepidopteran APN motif GAMENWG located ~0.9 kb downstream from the start methionine (Gill *et al*., 1995; Oltean *et al*., 1999; Chang *et al*., 1999). This conserved sequence is also present in eucaryotic alanine/arginine APNs (Taylor, 1996). PCR products of 0.9 kb and 0.45 kb were generated (Fig 4-2A, lane 3), cloned (pTA-0.9 and pTA-0.45), and sequenced. The 0.45

kb sequence shared ~90% identity with MsAPN-1 (GenBank accesson #: X89081, Knight *et al*., 1994), while the 0.9 kb fragment shared ~80% identity with *H. punctigera* APN2 and Hv110-APN. The open reading frame of pTA-0.9 encodes TNLDEP and GAMENWG sequences. This was further evidence that the 0.9 kb insert corresponded to an internal region of Ms110-APN cDNA. The 3' and 5' ends of cDNA for Ms110-APN were obtained by RACE cloning. Primers, using pTA-0.9 sequence, generated ~2 kb and ~1.4 kb PCR products from 3' and 5' RACE reactions (Fig. 4-2B and C). A full-length ~3.0 kb cDNA clone (pTA-110) was assembled using pTA-110-3' and pTA-110-5' inserts.

The full-length 110-cDNA is 2959 nt with a 2938 nt open reading frame. The predicted 947 amino acid APN has an estimated molecular mass of 108.3-kDa and a pI of 6.31. The cDNA has a 20 nt 5' untranslated region and a 60 nt 3' untranslated region with a polyadenylation consensus signal (AAUAAA) (Fig. 4-3). The predicted APN has 4 potential O-glycosylation sites (predicted by NetOGlyc 2.0 program; Hansen *et al*., 1998), 7 potential N-glycosylation sites, and a GPI-anchor attachment site at the C-terminus (predicted by big-PI predictor program; Eisenhaber *et al*., 1999). Amino acids S_{926}, S_{927}, and S_{928} precede the 20 amino acid hydrophobic region with a cleavage site between S_{925} and S_{926} and attachment of GPI-anchor to S_{925}. The zinc-binding motif $HEXXHX_{18}E$ and gluzincin APN motif, GAMEN, are located at positions typical for lepidopteran APNs (Fig. 4-3).

4.4.4 Expression of Ms110-APN cDNA in E. coli

Heterologous expression of Ms110-APN cDNA in *E. coli* was accomplished using the plasmid pTrcHisA. The expressed protein begins with a His_6 tag proximal to the mature protein (beginning with residues TNLDEP) and terminates at the predicted GPI anchor cleavage site (Fig. 4-3). *E. coli* DH5α pTrcHis-110 was induced with IPTG and cells harvested 4 h post-induction. *E. coli* samples were separated by SDS-PAGE and proteins visualized by Coomassie blue staining or blot analyses. *E. coli* pTrcHis-110 cells had a 100 kDa protein visible in the stained gel (Fig. 4-4A, lane 2) and on the blot probed with anti-His-HRP antibody (Fig. 4-4A, lane 4).

4.4.5 Immunoreactivity of antibody raised against Ms110-APN

Anti-MsAPN-1 antibodies did not recognize Ms110-APN (Fig 4-1). Therefore, we expressed a 27-kDa fragment of Ms110-APN in *E. coli* and raised anti-Ms110-APN rabbit antibodies. Anti-Ms110-APN antibody recognized a 110 kDa protein in *M. sexta* BBMV but did not cross-react with MsAPN-1 (Fig. 4-4B, lane 1). The anti-Ms110-APN antibody recognized the 100 kDa protein expressed in *E. coli* pTrcHis-110 and two proteins of less than 25-kDa (Fig. 4-4B, lane 2). The two small proteins were cross-reactive proteins detected in *E. coli* pTrcHis control (Fig. 4-4B, lane 3).

4.4.6 Affinity chromatography with toxin and E. coli-expressed Ms110-APN

We investigated if Cry1Ac and ^{509}QNR^{511}-AAA-coupled to beads could bind *E. coli*-expressed Ms110-APN. Toxin-coupled beads were incubated with *E. coli* extracts, washed, and bound proteins eluted with 2 M NaSCN. Eluted proteins were separated by

SDS-PAGE, blotted, and probed with anti-Ms110-APN antibody. Both Cry1Ac and ^{509}QNR^{511}-AAA selected the 100 kDa APN expressed in *E. coli* (Fig. 4-4C, lanes 1 and 2). The 100-kDa band was not detected in the no-toxin control sample (Fig. 4-4C, lane 3). Cry1Ac and ^{509}QNR^{511}-AAA bound *E. coli*-expressed 100 kDa APN under non-denaturing conditions.

4.4.7 Ligand blot analyses of E. coli-expressed Ms110-APN

Protein blots with *E. coli* pTrcHis-110 and *E. coli* pTrcHis control were probed with ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA. BBMV from *M. sexta* larvae were included for comparison. ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA recognized proteins of >210 kDa, 120, and 110 kDa proteins in BBMV (Fig. 4-4D, lanes 1 and 4). Based on previous studies, the 210 kDa protein is cadherin-like protein and the other proteins APNs. ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA detected the 100 kDa APN in *E. coli* pTrcHis-110 extract (Fig. 4-4D, lanes 2 and 5). Additionally, both Cry1Ac and ^{509}QNR^{511}-AAA also recognized smaller-sized proteins in *E. coli* pTrcHis-110. These proteins were similar in size to truncated APN detected with anti-His antibody (Fig. 4-4A, lane 4). Cry1Ac and ^{509}QNR^{511}-AAA did not recognize proteins of corresponding sizes in vector alone control (Fig. 4-4D, lanes 3 and 6).

4.5 Discussion

In this study a 110 kDa APN was identified in *M. sexta* BBMV that bound Cry1Ac and mutant ^{509}QNR^{511}-AAA. Cry1Ac recognizes GalNAc on MsAPN-1 (Masson *et al*., 1995). Yet, when the GalNAc binding site on Cry1Ac was eliminated, as in the case of mutant ^{509}QNR^{511}-AAA, Cry1Ac does not bind MsAPN-1 (Jenkins *et al*., 1999; Daniel *et al*., 2002) but ^{509}QNR^{511}-AAA toxicity to *M. sexta* was only reduced 2- to 3-fold relative to wild-type Cry1Ac (Lee *et al*., 1999). ^{509}QNR^{511}-AAA bound 210 kDa protein on a ligand blot of *M. sexta* BBMV (Jenkins *et al*., 1999). Therefore, the 210 kDa cadherin-like protein became the most likely candidate as the dominant receptor for Cry1Ac in *M. sexta*. We reasoned that undiscovered Cry1Ac binding molecules may exist in *M. sexta* and used ^{509}QNR^{511}-AAA to identify the Ms110-APN that matches these criteria.

Our approach used affinity chromatography with Cry1Ac and ^{509}QNR^{511}-AAA to isolate Cry1Ac binding protein(s) from solubilized BBMV. This approach has the advantage in that binding occurs under non-denaturing conditions. However, the GPI anchorage of lepidopteran APNs complicates the affinity selection approach because APN co-purifies with other GPI-linked proteins (Lu and Adang, 1996; Schwartz *et al*., 1997). Recently, the GPI-protein complex in *M. sexta* was identified as a lipid raft (Zhuang *et al*., 2002). It is likely that the MsAPN-1 was extracted by ^{509}QNR^{511}-AAA because the protein was associated with Ms110-APN in a lipid raft complex. The absence of 210 kDa cadherin-like protein in the column eluates is probably due to the small amount of 210 kDa protein in BBMV preparations.

Cry1Ac recognition of 110 kDa APN in *M. sexta* BBMV does not involve GalNAc. This statement is supported by data from published studies, ^{509}QNR^{511}-AAA binding of Ms110-APN, and analyses of *E. coli*-expressed Ms110-APN. Soybean agglutinin, a lectin specific for GalNAc, recognizes only MsAPN-1 in BBMV (Knowles *et al*., 1991; Lee *et al*., 1996). The GalNAc on MsAPN-1 predicted by lectin blot analyses was recently confirmed by quantitative mass spectroscopy (Sangadala *et al*., 2001).

We have shown here that Cry1Ac and ^{509}QNR^{511}-AAA bound *E. coli*-expressed Ms110-APN using affinity selection and ligand blot approaches. Since *E. coli* proteins are not glycosylated, toxin binding is a result of direct protein-to-protein interaction. In Fig. 4-4C, toxin-bead mixtures selected mature Ms110-APN from an *E. coli* extract under non-denaturing conditions. Then, using ligand blots probed with ^{125}I-toxins, we showed that Cry1Ac and ^{509}QNR^{511}-AAA also bound mature 100 kDa protein (Fig 4-4D). There is precedent for Cry1A toxin binding to an APN expressed in *E. coli*. Yaoi *et al*. (1999) demonstrated that Cry1Aa bound an *E. coli*-expressed BmAPN-1. In their experiments, Cry1Ac bound expressed *B. mor*i BmAPN-1, but to a much lesser extent than Cry1Aa. We noted that Cry1Ac and ^{509}QNR^{511}-AAA bound fragments of expressed Ms110-APN (Fig. 4-4D). In a similar fashion, Cry1Aa bound a peptide fragment of *E. coli*-expressed BmAPN-1, and the Cry1Aa binding region is between Ile135 and Pro198 (Yaoi *et al*., 1999). The location of the Cry1Ac-binding region in Ms110-APN was not determined in this study.

The Ms110-APN has characteristics similar to other lepidopteran APNs. An N-terminal signal sequence, putative GPI-anchor signal sequence, and potential N- and O-

glycosylation sites in addition to conserved gluzincin aminopeptidase motif and metalloprotease zinc-binding motif are present (Fig. 4-3). The 4 conserved Cys residues, first noted by Denolf *et al*. (1997), are present in Ms110-APN. Among the 4 conserved peptides discussed by Denolf *et al*. (1997) only one peptide Y^{594}RVNYD (594 refers to corresponding amino acid residue in Ms110-APN) was conserved in Ms110-APN while the other three: AFPCYDEP, GAMENWGL and WLNEFGFA were present as A^{180}FPCFDEP, G^{114}AMENWGM and W^{370}LNESFA respectively (Fig. 4-3).

The Ms110-APN has greatest similarity to *H. punctigera* APN2 and Hv110-APN and *B. mori* APN4 (GenBank accession #: AB012400). Multiple sequence alignment of the 4 highly similar APNs was performed by the Clustalx (version1.8) method reported by Higgins and Sharp (1988) (Fig. 4-5). The percentage of full-length Ms110-APN sequence identity was ~65% and similarity ~80% for *H. punctigera* APN2, Hv110-APN and *B. mori* APN4. Blocks of highly conserved amino acid regions were present in the gluzincin and zinc-binding motifs (Fig. 4-5). Multiple sequence alignments of all known *M. sexta* APNs revealed that Ms110-APN shared only ~30% identity and ~50% similarity with other known *M. sexta* APNs (data not shown).

Phylogenetic analyses of all known APNs from *M. sexta, H. virescens, B. mori* and *H. punctigera* support the suggestion by Chang *et al*. (1999) that gene duplication events occurred in aminopeptidases of Lepidoptera (Fig. 4-6). Oltean *et al*. (1999) suggested the presence of 4 APN homology groups in insects. The Ms110-APN falls into the class shared by the Hv110-APN, *H. punctigera* APN2, and *B. mori* APN4. Banks *et al*. (2001) suggested that a gene duplication event and subsequent divergence in ancestral Noctuidae lineage led to occurrence of similar APN genes in 2 geographically isolated

species. However, the presence of genes homologous to *H. punctigera* and *H. virescens* in *M. sexta* and *B. mori* suggest that a gene duplication event occurred even before the Sphingoidea, Noctuoidea, and Bombycoidea families diverged. The isolation of homologous genes in the 3 families prompts us to believe that homologs are yet to be identified in other lepidopteran families.

Recently, Banks *et al*. (2001) demonstrated that Cry1Ac bound the Hv110-APN in a GalNAc-independent manner. The Hv110-APN was purified as a Cry1Fa-binding protein that was also recognized by ^{509}QNR^{511}-AAA. Cry1Ac and Cry1Fa share high sequence homology in loops of domain II (Rajamohan *et al*., 1996a; Rajamohan *et al*., 1996b; Tabashnik *et al*., 1996). It is possible that Cry1Ac may bind a conserved structure on APN shared by the *H. virescens* and *M. sexta* 110 kDa APNs, and the interaction may be mediated by domain II rather than domain III. This is the first report of Cry1Ac binding to a recombinant *M. sexta* APN independent of GalNAc.

In this paper we report the isolation of a novel *M. sexta* APN with unique toxin-binding properties. At the outset, we had expected to isolate the 210 kDa cadherin-like protein as the GalNAc-independent Cry1Ac binding protein. Even though we did not detect the 210 kDa protein in our affinity purifications, we isolated a protein with a high degree of homology to APNs from phylogenetically distant insects but with similar binding characteristics. The interaction of Cry1Ac with proteins present in the *M. sexta* brush border membrane is more complex than anticipated and possibly involves the participation of several different proteins. Further work is necessary to completely understand the role of different BBMV proteins in the toxin intoxication process.

Acknowledgements

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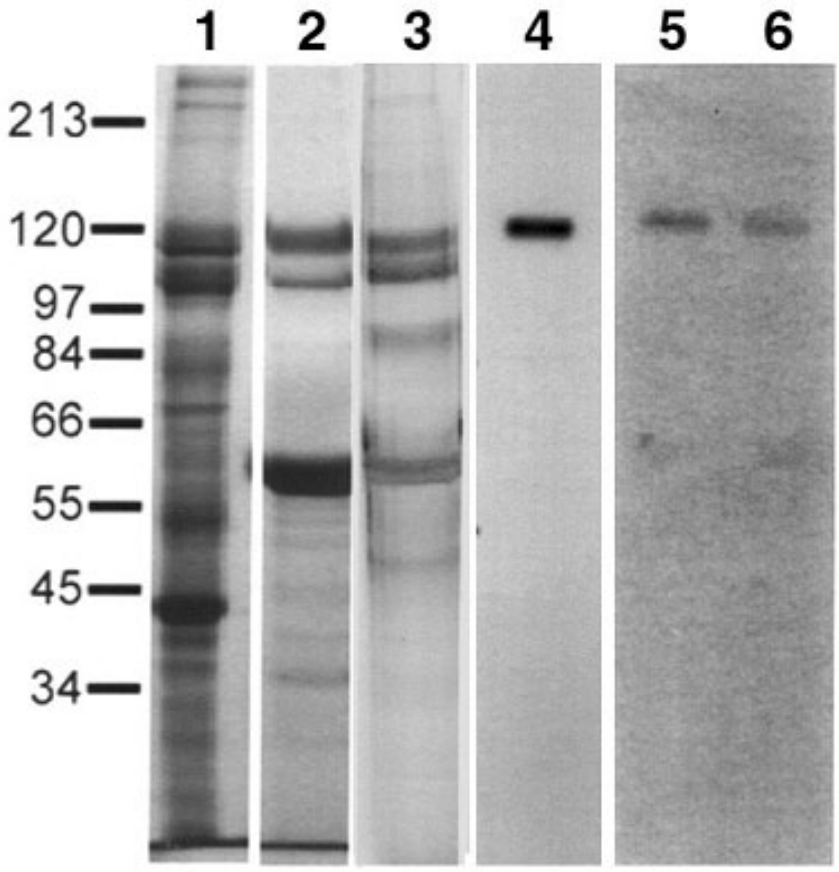


Fig. 4-1. SDS-PAGE analyses of BBMV proteins from *M. sexta* selected by Cry1Ac and ^{509}QNR^{511}-AAA affinity chromatography. *M. sexta* BBMV proteins (lane 1), Cry1Ac-affinity eluate (lane 2), and ^{509}QNR^{511}-AAA-affinity eluate (lane 3) separated by SDS-8%PAGE and silver-stained. *M. sexta* BBMV proteins (lane 4), Cry1Ac-affinity eluate (lane 5), and ^{509}QNR^{511}-AAA-affinity eluate (lane 6) probed with anti-MsAPN-1 antibody, anti-rabbit-HRP secondary antibody, and visualized with enhanced chemiluminescence (ECL Plus, Amersham Pharmacia).

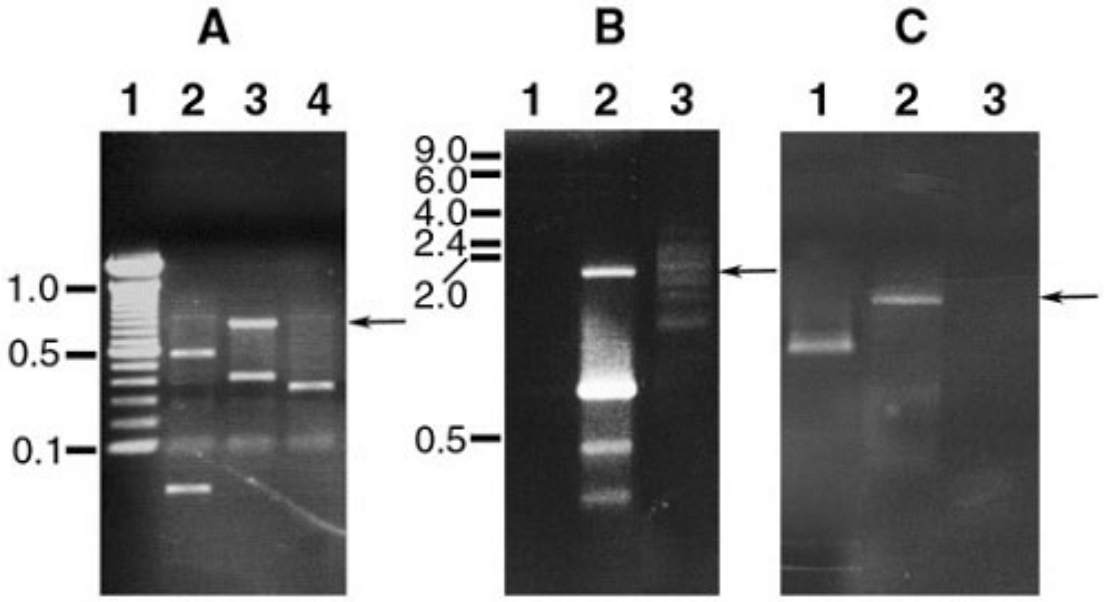
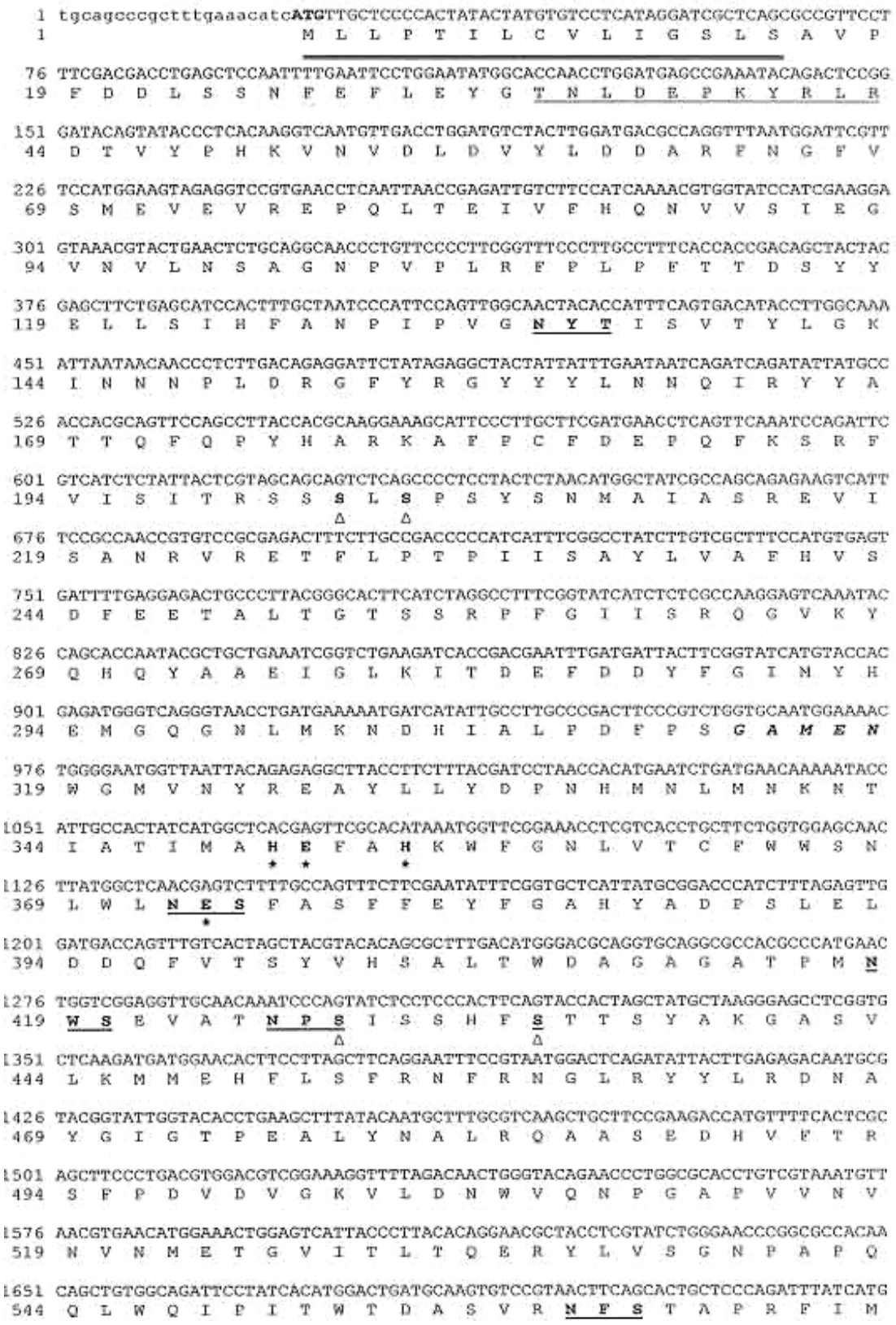


Fig. 4-2. PCR amplification of 0.9 kb fragment and cloning of 3' and 5' cDNA ends by RACE-PCR. (A) Amplification of 0.9 kb fragment of 110 kDa protein using degenerate forward and reverse primers. Lane 1: 100-bp ladder; lane 2: forward primer alone; lane 3: both forward and reverse primers; lane 4: reverse primer alone. (B) 3' RACE PCR to obtain 3' end of cDNA encoding Ms110-APN using GSP1 and *Not*I-dT_{18} adapter primer. Lane 1: GSP1 primer alone; lane 2: both GSP1 and *Not*I-dT_{18} adapter primers; lane 3: *Not*I-dT_{18} adapter primer alone. (C) 5' RACE PCR to obtain 5' end of Ms110-APN cDNA using AAP and GSP3 primers. Lane 1: AAP alone; lane 2: both AAP and GSP3 primers; lane 3: GSP3 primer alone. Arrows designate expected fragment following PCR amplification.



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1 M L L P T I L C V L I G S L S A V P

76 TTCGACGACCTGAGCTCCAATTTTGAATTCCTGGAATATGGCACCAACCTGGATGAGCCGAAATACAGACTCCGG
19 F D D L S S N F E F L E Y G T N L D E P K Y R L R

151 GATACAGTATACCCTCACAAGGTCAATGTTGACCTGGATGTCTACTTGGATGACGCCAGGTTTAATGGATTCGTT
44 D T V Y P H K V N V D L D V Y L D D A R F N G F V

226 TCCATGGAAGTAGAGGTCCGTGAACCTCAATTAACCGAGATTGTCTTCCATCAAAACGTGGTATCCATCGAAGGA
69 S M E V E V R E P Q L T E I V F H Q N V V S I E G

301 GTAAACGTACTGAACTCTGCAGGCAACCCTGTTCCCCTTCGGTTTCCCTTGCCTTTCACCACCGACAGCTACTAC
94 V N V L N S A G N P V P L R F P L P F T T D S Y Y

376 GAGCTTCTGAGCATCCACTTTGCTAATCCCATTCCAGTTGGCAACTACACCATTTCAGTGACATACCTTGGCAAA
119 E L L S I H F A N P I P V G N Y T I S V T Y L G K

451 ATTAATAACAACCCTCTTGACAGAGGATTCTATAGAGGCTACTATTATTTGAATAATCAGATCAGATATTATGCC
144 I N N N P L D R G F Y R G Y Y Y L N N Q I R Y Y A

526 ACCACGCAGTTCCAGCCTTACCACGCAAGGAAAGCATTCCCTTGCTTCGATGAACCTCAGTTCAAATCCAGATTC
169 T T Q F Q P Y H A R K A F P C F D E P Q F K S R F

601 GTCATCTCTATTACTCGTAGCAGCAGTCTCAGCCCCTCCTACTCTAACATGGCTATCGCCAGCAGAGAAGTCATT
194 V I S I T R S S S L S P S Y S N M A I A S R E V I
Δ Δ

676 TCCGCCAACCGTGTCCGCGAGACTTTCTTGCCGACCCCCATCATTTCGGCCTATCTTGTCGCTTTCCATGTGAGT
219 S A N R V R E T F L P T P I I S A Y L V A F H V S

751 GATTTTGAGGAGACTGCCCTTACGGGCACTTCATCTAGGCCTTTCGGTATCATCTCTCGCCAAGGAGTCAAATAC
244 D F E E T A L T G T S S R P F G I I S R Q G V K Y

826 CAGCACCAATACGCTGCTGAAATCGGTCTGAAGATCACCGACGAATTTGATGATTACTTCGGTATCATGTACCAC
269 Q H Q Y A A E I G L K I T D E F D D Y F G I M Y H

901 GAGATGGGTCAGGGTAACCTGATGAAAAATGATCATATTGCCTTGCCCGACTTCCCGTCTGGTGCAATGGAAAAC
294 E M G Q G N L M K N D H I A L P D F P S G A M E N

976 TGGGGAATGGTTAATTACAGAGAGGCTTACCTTCTTTACGATCCTAACCACATGAATCTGATGAACAAAAATACC
319 W G M V N Y R E A Y L L Y D P N H M N L M N K N T

1051 ATTGCCACTATCATGGCTCACGAGTTCGCACATAAATGGTTCGGAAACCTCGTCACCTGCTTCTGGTGGAGCAAC
344 I A T I M A H E F A H K W F G N L V T C F W W S N
* * *

1126 TTATGGCTCAACGAGTCTTTGCCAGTTTCTTCGAATATTTCGGTGCTCATTATGCGGACCCATCTTTAGAGTTG
369 L W L N E S F A S F F E Y F G A H Y A D P S L E L
*

1201 GATGACCAGTTTGTCACTAGCTACGTACACAGCGCTTTGACATGGGACGCAGGTGCAGGCGCCACGCCCATGAAC
394 D D Q F V T S Y V H S A L T W D A G A G A T P M N

1276 TGGTCGGAGGTTGCAACAAATCCCAGTATCTCCTCCCACTTCAGTACCACTAGCTATGCTAAGGGAGCCTCGGTG
419 W S E V A T N P S I S S H F S T T S Y A K G A S V
Δ Δ

1351 CTCAAGATGATGGAACACTTCCTTAGCTTCAGGAATTTCCGTAATGGACTCAGATATTACTTGAGAGACAATGCG
444 L K M M E H F L S F R N F R N G L R Y Y L R D N A

1426 TACGGTATTGGTACACCTGAAGCTTTATACAATGCTTTGCGTCAAGCTGCTTCCGAAGACCATGTTTTCACTCGC
469 Y G I G T P E A L Y N A L R Q A A S E D H V F T R

1501 AGCTTCCCTGACGTGGACGTCGGAAAGGTTTTAGACAACTGGGTACAGAACCCTGGCGCACCTGTCGTAAATGTT
494 S F P D V D V G K V L D N W V Q N P G A P V V N V

1576 AACGTGAACATGGAAACTGGAGTCATTACCCTTACACAGGAACGCTACCTCGTATCTGGGAACCCGGCGCCACAA
519 N V N M E T G V I T L T Q E R Y L V S G N P A P Q

1651 CAGCTGTGGCAGATTCCTATCACATGGACTGATGCAAGTGTCCGTAACTTCAGCACTGCTCCCAGATTTATCATG
544 Q L W Q I P I T W T D A S V R N F S T A P R F I M

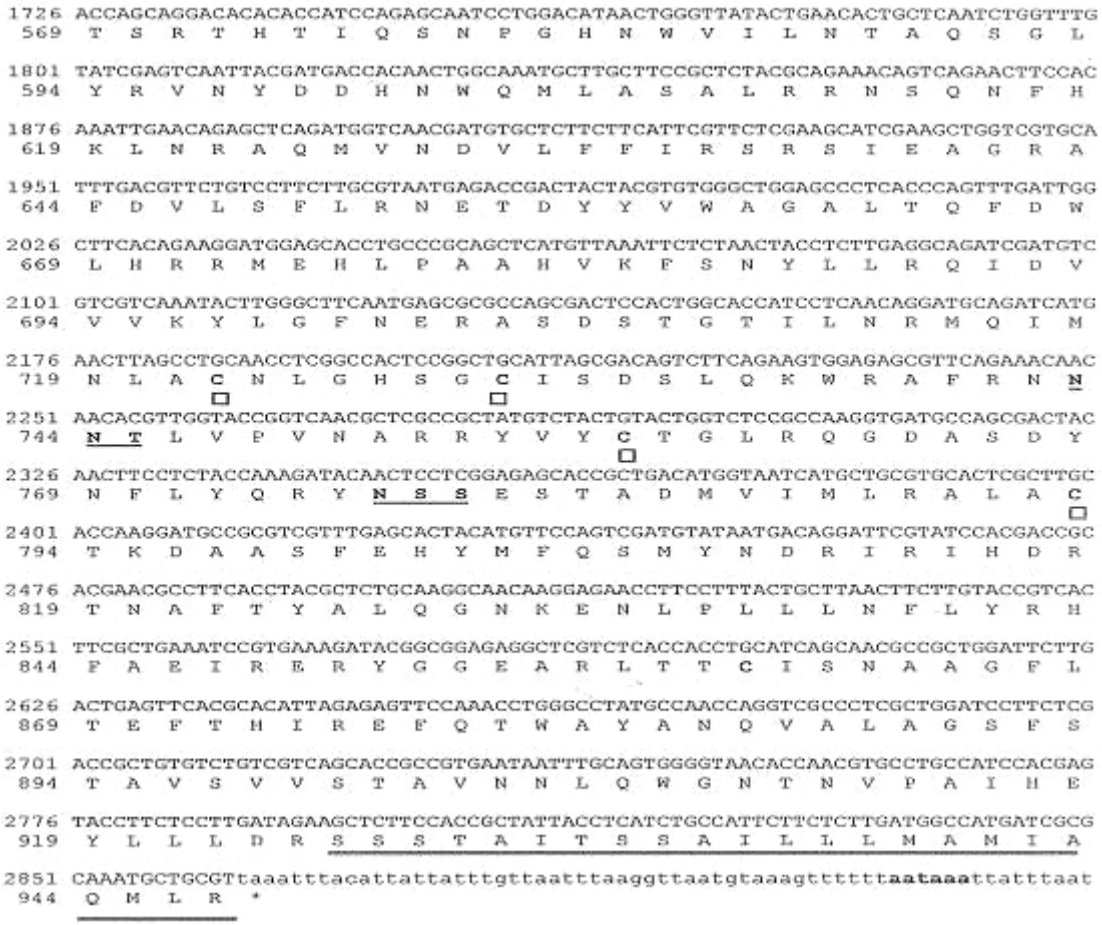

Fig. 4-3. Nucleotide and predicted amino acids of Ms110-APN cDNA. The nucleotide sequence of the ORF is uppercase while the 5' and 3' untranslated regions are lowercase. The putative translation start-site and polyadenylation consensus sequence are shown in bold, the putative signal peptide and potential GPI-anchor signal peptide are double underlined and the N-terminal sequence is underlined. Potential N-glycosylation sites are bold underlined, while potential O-glycosylation sites are marked by triangles. The zinc-binding site $HEXXHX_{18}E$ is indicated by asterisks and the GAMEN motif is in bold and italics. The four conserved Cys residues conserved between eukaryotic APN genes are in bold and marked by squares.

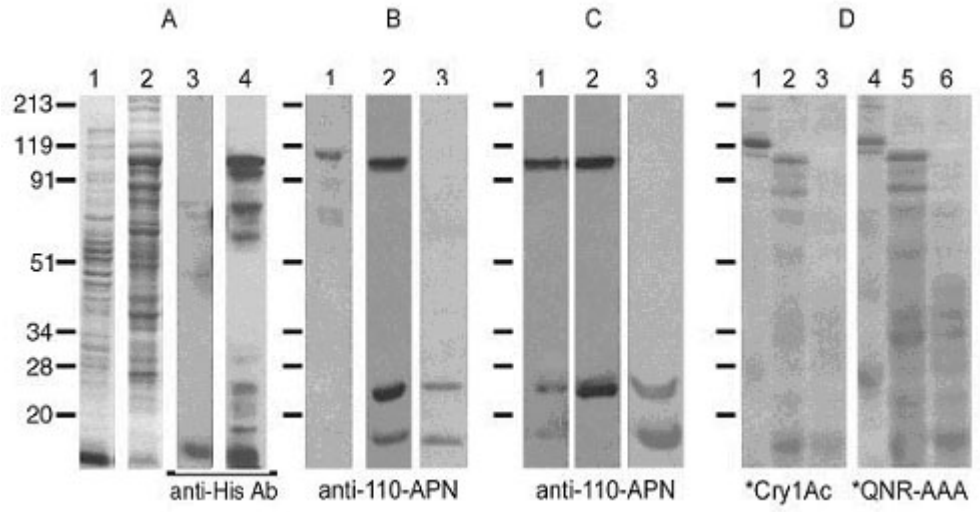

Fig. 4-4. Ligand and western blot analyses of SDS-PAGE separated proteins. (A) *E. coli* DH5α-pTrcHisA (lanes 1, 3) and DH5α-pTrcHis-110 (lanes 2, 4) separated by SDS-PAGE and Coomassie blue stained (lanes 1, 2) or probed with anti-His-HRP antibody (lanes 3, 4). (B) Protein blots incubated with anti-Ms110-APN antibody followed by anti-rabbit HRP secondary antibody. Lane 1: *M. sexta* BBMV, lane 2: DH5α -pTrcHis-110, lane 3: DH5α-pTrcHisA. (C) Protein blots of affinity chromatography-selected *E. coli* proteins probed with anti-Ms110-APN antibody and anti-rabbit HRP antibody. Lane 1: Cry1Ac-selected proteins, lane 2: ^{509}QNR^{511}-AAA selected proteins, lane 3: no-toxin control. (D) Ligand blot of *M. sexta* BBMV (lanes 1, 4), DH5α-pTrcHis-110 (lanes 2, 5) and DH5α-pTrcHisA (lanes 3, 6) probed with ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA.

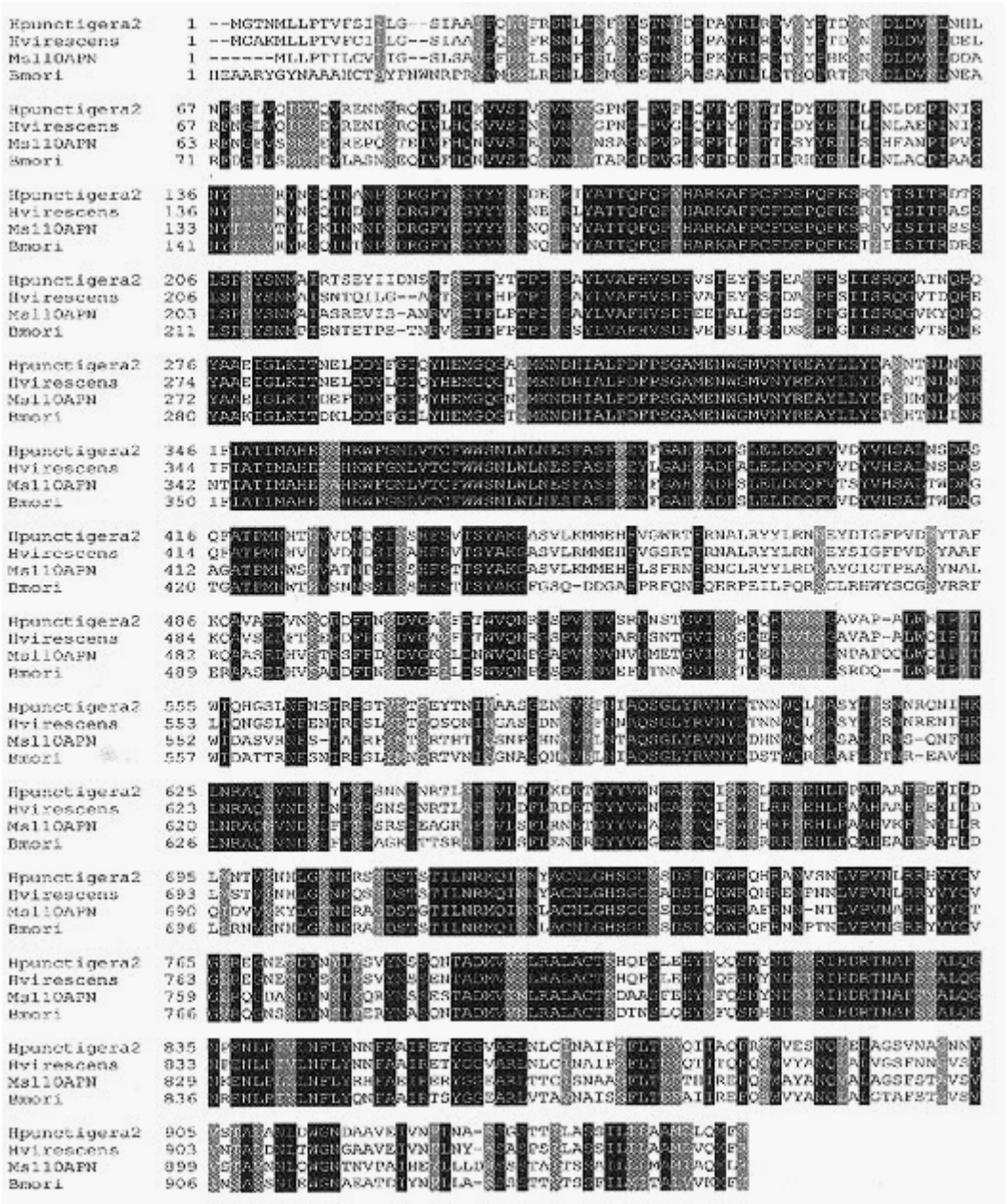

Fig. 4-5. Multiple sequence alignment of Ms110-APN, *H. punctigera* APN2, Hv110-APN and *B. mori* APN4. Alignment was performed using Clustwalx program (version 1.8) and results presented with BOXSHADE (version 2.7). Amino acids boxed in black are identical in all 4 sequences; those in grey boxes are identical in 3 sequences and gaps are indicated by dashes.

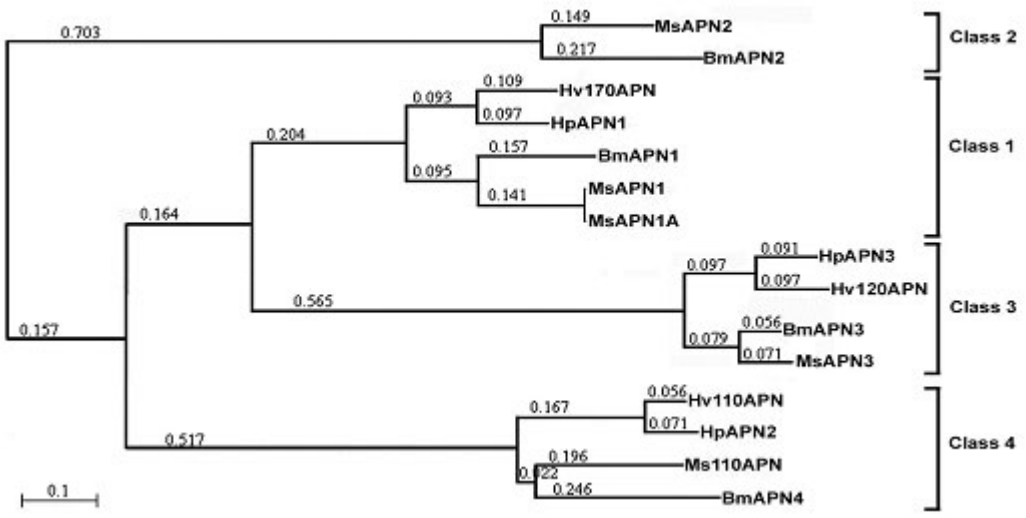

Fig. 4-6. Phylogenetic analyses of all known APN isoforms of *M. sexta*, *H. punctigera*, *H. virescens,* and *B. mori*. Using multiple aligned sequence the genetic distances were calculated with the program PROTDIST of J. Felsenstein's PHYLIP 3.5 phylogeny inference package (Felsenstein, 1993). Subsequently, phylogenetic relationship was determined by the method of Fitch and Margoliash (Fitch and Margoliash, 1967) with the least squares criterion and the FITCH program (Felsenstein, 1993). The analyses were bootstrapped 100 times using the SEQBOOT program (Felsenstein, 1993). The GenBank accession numbers of APN sequences are: MsAPN-1: X89081 (Knight *et al*., 1994); MsAPN-1A: AF123313 (Luo *et al*., 1999); BmAPN-1: AF084257 (Yaoi *et al*., 1999); Hv170APN: AF173552 (Oltean *et al*., 1999); HpAPN-1: AF217248 (Emmerling *et al*., 2001); MsAPN-2: X97877 (Denolf *et al*., 1997); BmAPN-2: AB011497 (Hua *et al*., 1998); MsAPN-3: AY095259 (unpublished data); BmAPN-3: AF352574 (Nakanishi *et al*., 2002); HvAPN-1: U35096 (Gill *et al*., 1995); HpAPN-3: AF217250 (Emmerling *et al*., 2001); Ms110-APN: AF498996 (this study); BmAPN-4: AB012400; Hv110-APN: AF378666 (Banks *et al*., 2001); HpAPN-2: AF217249 (Emmerling *et al*., 2001). The different classes of APNs are indicated in brackets.

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CHAPTER 5

CONCLUSIONS AND DISCUSSION

The insecticidal activity of Cry toxins has been commercially exploited for more than four decades. The extremely high toxicity and target insect specificity makes these toxins the most attractive alternative to synthetic pesticides. The biochemistry of toxin action can be summarized in a series of steps that includes toxin activation, toxin binding to membrane proteins, and insertion into membranes leading to pore formation (reviewed in Garczynski and Adang, 2000).

The three-domain architecture is evident from X-ray crystallography of Cry toxins (Grochulski *et al.*, 1995; Li *et al.*, 1991; Morse *et al.*, 2001). Mutations of specific amino acid residues in the three domains confer unique characteristics to these mutants. For example, Cry1Ab domain II mutant R368A/R369A displays reduced toxicity and reversible binding (Lee *et al.*, 1999) while Cry1Ab domain I mutants A92D and Y153D have significantly reduced toxicity and irreversible binding (Chen *et al.*, 1995; Rajamohan *et al.*, 1995; Wu and Aronson, 1992). Cry1Ac domain III mutant (^{509}QNR^{511}-AAA) with near wild-type toxicity against *M. sexta* has altered receptor specificity (Jenkins *et al.*, 1999; Lee *et al.*, 1999). Therefore, using different available Cry1A mutants I analyzed toxin-receptor and toxin-membrane interactions. In my research I also explored the hypothesis of alternate receptors for Cry toxins.

In the first study described in Chapter Two, my research focused on Cry1Ac domain I mutant A92D (Wu and Aronson, 1992), Cry1Ab domain I mutants A92E and Y153D (Chen *et al.*, 1995), Cry1Ab domain II mutants F371A (Rajamohan *et al.*, 1995), and R368A/A369A (Lee *et al.*, 1999). I chose these mutants because each of them has different membrane binding properties. I used a light scattering assay to analyze pore-forming properties of these mutants under different osmotic conditions in *M. sexta*

BBMV. The light scattering assay was used because changes in vesicle size in the presence of mutant toxins could be studied with cations, anions, and neutral solutes (Carroll and Ellar, 1993). In direct mixing and preincubation assays, A92D/E induced solute permeation. Mutants Y153D, F371A, and R368A/R369A did not induce solute permeation and therefore could not be differentiated (Fig. 2-2). A92D/E induced solute permeation in the presence of KCl but not sucrose (Fig. 2-5). The anomalous behavior of A92D/E under different osmotic conditions was probably due to charge masking by KCl. KCl, a charged molecule, could compensate the negative charge on Asp-92 and allow A92D/E to interact with the membrane. The integrity of the brush border membrane was maintained because nystatin, a cation channel-forming antibiotic, did not induce permeability to sucrose (Fig. 2-5). However, Y153D did not induce signal recovery in either osmotic condition (Figs. 2-2, 2-3, 2-5). In the case of Y153D, the presence of a hydrophobic residue (Tyr-153) was probably crucial for pore formation. Also, my results support the observation of Carroll and Ellar (1997) and Martin and Wolfersberger (1995) that toxin-induced structures resemble pores that allow passage of both charged and uncharged molecules through brush border membranes.

In Chapter Three, I describe the effect of protein denaturation on toxin binding to *M. sexta* BBMV proteins and purified MsAPN-1. Ligand blotting is a commonly used technique in Cry toxin research and has often been the first step in identifying toxin-binding molecules (Garczynski *et al.*, 1991; Oddou *et al.*, 1991; Vadlamudi *et al.*, 1993). In these assays, binding of Cry toxin to molecules from BBMV occurs under denaturing conditions. The results from a ligand blot assay in which *M. sexta* BBMV were probed with ^{125}I-^{509}QNR^{511}-AAA did not agree with previously reported results (Jenkins *et al.*,

1999). I observed that ^{509}QNR^{511}-AAA bound MsAPN-1 and a 210 kDa protein (Fig. 3-1). Jenkins *et al.* (1999) reported that ^{509}QNR^{511}-AAA bound the 210 kDa protein, but not MsAPN-1. Therefore I designed experiments to explain the inconsistency and investigate the possibility that denaturation of binding proteins altered toxin-binding specificity. Previous reports have suggested that alteration of toxin-binding specificity occurs under denaturing conditions (Lee and Dean, 1996; Nagamatsu *et al.*, 1998; Vadlamudi *et al.*, 1993).

Under non-denaturing dot blotting conditions, I observed that, unlike Cry1Ac ^{509}QNR^{511}-AAA did not bind MsAPN-1 (Fig. 3-2A), similar to results obtained in SPR assays (Jenkins *et al.*, 1999). However, when denatured proteins were dot-blotted, MsAPN-1 was recognized by ^{509}QNR^{511}-AAA (Fig. 3-2B) in agreement with my ligand blot results. Similarly, in reciprocal dot and ligand blots with ^{125}I-MsAPN-1, alteration of binding specificity was observed with denatured toxins while binding specificity was maintained with non-denatured (native) toxins (Figs. 3-3, 3-4). In a dot blot experiment where denatured ^{125}I-MsAPN-1 was probed against non-denatured toxins, MsAPN-1 recognized all Cry1A toxins, similar to ligand blot results (Fig. 3-3C). Interestingly, binding specificity of MsAPN-1 was altered for Cry1A toxins but not for Cry1Ca toxin (Figs. 3-3D, 3-4) indicating that only Cry1A toxins possessed APN-binding epitope(s) that were exposed upon denaturation. From these results I conclude that denaturation exposes binding epitopes that are not present on the surface of native proteins. This suggests that results from ligand blotting when used for identifying putative toxin-binding proteins in BBMV should be confirmed by other techniques.

In ligand blots ^{509}QNR^{511}-AAA recognized MsAPN-1, 210 kDa protein, and 110 kDa protein (Fig. 3-1C). ^{509}QNR^{511}-AAA is a Cry1Ac domain III mutant with a disrupted GalNAc-interaction domain (Burton *et al.*, 1999; Jenkins *et al.*, 1999). In surface plasmon resonance assays, ^{509}QNR^{511}-AAA does not bind MsAPN-1 (Jenkins *et al.*, 1999) although toxicity was only 2- to 3-fold lower than for Cry1Ac (Lee *et al.*, 1999). These observations and the results from my ligand blots suggested that a protein(s) other than MsAPN-1 was the dominant Cry1Ac receptor(s) in *M. sexta*. The 210 kDa protein identified as a cadherin-like Cry1Ab receptor BT-R1 (Vadlamudi *et al.*, 1993) seemed to be a suitable candidate receptor for Cry1Ac. In order to identify ^{509}QNR^{511}-AAA binding protein(s) I conducted affinity chromatography experiments with toxin-coupled columns and solubilized *M. sexta* BBMV.

Affinity chromatography with Cry1Ac and ^{509}QNR^{511}-AAA selected a 110 kDa protein along with MsAPN-1 (Fig. 4-1). The 210 kDa cadherin-like protein was not detected in the affinity eluates probably because of low concentrations in BBMV. Banks *et al.* (2001) recently identified a Cry1Ac- and Cry1Fa-binding 110 kDa APN (Hv110-APN) in *H. virescens* by affinity selection. ^{509}QNR^{511}-AAA recognizes Hv110-APN, but the GalNAc-specific lectin, soybean agglutinin, does not recognize Hv110-APN. Thus, the potential GalNAc-independent Cry1Ac-binding protein in *H. virescens* most likely is the Hv110-APN. Since this type of Cry1Ac-binding protein has not been identified in *M. sexta*, I cloned the ^{509}QNR^{511}-AAA affinity-selected 110 kDa protein cDNA by RACE PCR (Fig. 4-2). The 110 kDa protein is a previously unidentified APN (Ms110-APN). It has all the characteristic features of a typical lepidopteran APN (Fig. 4-3). This protein shares ~60% identity and ~80% similarity with Hv110-APN, *H. punctigera* APN2

(HpAPN-2), and *B. mori* APN4 (Bm APN-4). In phylogenetic analyses, Ms110-APN clustered with Class IV APNs that included BmAPN-4, HpAPN-2, and Hv110-APN as well as PxAPN-4, HaAPN-1, LdAPN-3, and SlAPN-1 (Appendix 1, Fig. A-4). Antibodies against Ms110-APN were raised in rabbits using an internal 27 kDa fragment of this protein as antigen. Anti-Ms-110 sera recognized the 110 kDa protein in *M. sexta* BBMV and *E. coli*-expressed Ms110-APN, but did not cross-react with other *M. sexta* APNs (Fig. 4-4B). Both Cyr1Ac and ^{509}QNR^{511}-AAA bound *E. coli*-expressed Ms110-APN in affinity chromatography and ligand blot assays (Fig. 4-4). Since *E. coli*-expressed proteins lack glycosylation, toxin binding was due to direct protein-to-protein interaction. These results suggest that Ms110-APN is the GalNAc-independent toxin-binding protein in *M. sexta*.

The two major approaches I used to study Cry toxin mechanism of action focused on two distinct steps of this process. Using BBMV and a light scattering assay I studied interaction of wild-type and mutant toxins with membranes under different osmotic conditions. The anomalous behavior of mutants A92D and A92E in KCl and sucrose emphasizes the importance of charge on residues involved in interaction with brush border membranes. My results also support the observation that Cry toxins form pores in membranes. The second aspect of my research focused on interaction of Cry toxins with proteins in brush border membrane. The presence of proteins that bound Cry1Ac in a sugar-independent manner came from studies with the Cry1Ac mutant ^{509}QNR^{511}-AAA. I identified, cloned, and expressed such a toxin-binding protein (Ms110-APN). The role of Ms110-APN *in vivo* as a Cry 1Ac receptor still needs to be established. However, the presence of proteins displaying sugar-independent toxin-binding indicates that Cry1Ac

interacts with proteins in insect midgut via domain II as well as domain III. A binding model for interaction of Cry1A toxins with *M. sexta* APNs is shown in Fig. 5-1. It is likely that MsAPN-1 along with the 210 kDa cadherin-like protein functions as the receptor for these toxins. Two lines of evidence support this statement. MsAPN-1 reconstituted into membrane vesicles promotes Cry1Ac induced $^{86}Rb^{2+}$ release (Sangadala *et al.*, 1994). The 210 kDa cadherin-like protein promotes Cry1Ab-mediated cytotoxicity in cells (Dorsch *et al.*, 2002). Though speculative, the other toxin-binding APNs could function as 'null' receptors and promote initial toxin binding but not participate in post-binding events like irreversible binding and membrane insertion. A model for interaction of Cry1A toxins with the lepidopteran insect midgut membrane is illustrated in Fig. 5-2. Cry toxins bind to proteins present in lipid rafts on the brush border membrane (Zhuang *et al*., 2002). The lipid rafts contain GPI-anchored proteins and in the case of *M. sexta* probably MsAPN-1 and Ms110-APN which allows the toxin to insert into the membrane. Also, though speculative, the toxin could gain access to cadherin-like proteins between cells and subsequently insert into the membrane.

In summary, my studies with different Cry1A mutant toxins and *M. sexta* BBMV indicate that Cry toxins interact with a number of proteins in *M. sexta*. Even though toxin-receptor interaction is studied at the protein level, it would be interesting to characterize toxin-binding epitopes in the proteins. Application of new techniques such as phage display and receptor display will facilitate characterization of critical toxin-binding epitopes.

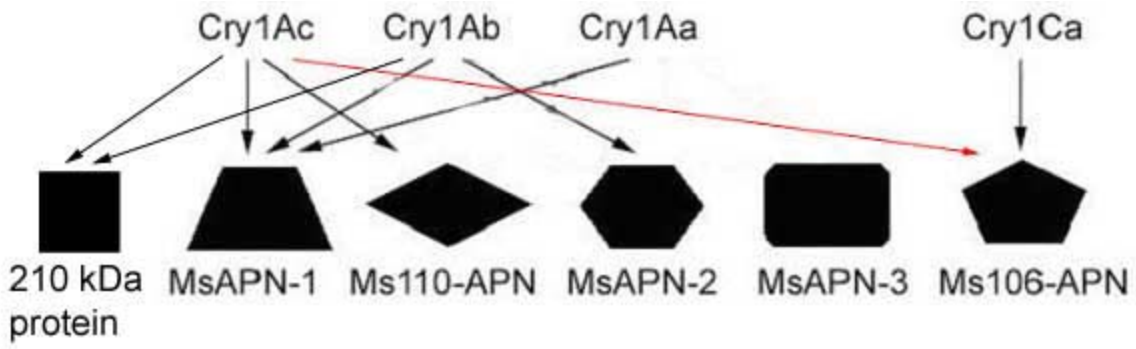

Fig. 5-1. A toxin-binding model for Cry1 toxins with *M. sexta* APNs. Known interactions are shown in black arrows. The red arrow indicates that Cry1Ac does not bind to Ms106-APN.

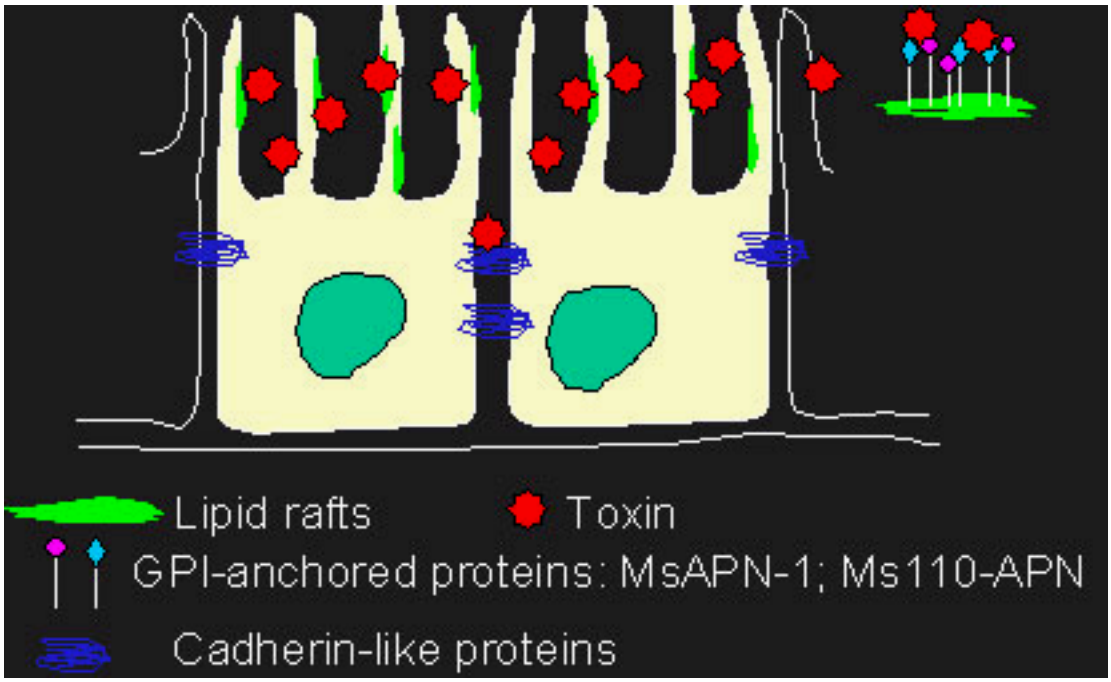

Fig. 5-2. A model for the interaction of Cry1A toxins with lepidopteran insect midgut membrane.

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APPENDIX I

CLONING OF A *MANDUCA SEXTA* AMINOPEPTIDASE N BELONGING TO CLASS III OF LEPIDOPTERAN AMINOEPEPITDASES

A1.1 Introduction

Aminopeptidase N (APN) (EC 3.4.11.2) is an ectopeptidase located in the midgut of insect larvae (Kenny *et al.*, 1987). Lepidopteran larvae have multiple APNs (Emmerling *et al.*, 2001; Gill *et al.*, 1995; Luo *et al.*, 1996; Oltean *et al.*, 1999; Tabashnik *et al.*, 1996; Valaitis *et al.*, 1997). They cleave the N-terminal amino acids from peptides allowing co-transport into epithelial cells.

APNs in Lepidoptera received attention because they bind *Bacillus thuringiensis* Cry1 toxins (Banks *et al.*, 2001; Chang *et al.*, 1999; Denolf *et al.*, 1997; Gill *et al.*, 1995; Hua *et al.*, 1998; Knight *et al.*, 1994; Luo *et al.*, 1996; Luo *et al.*, 1997a; Nagamatsu *et al.*, 1998; Oltean *et al.*, 1999; Sangadala *et al.*, 1994; Yaoi *et al.*, 1997; Zhu *et al.*, 2000). Typically, a Cry1 toxin was used as an affinity reagent to select binding proteins from solubilized brush border membrane vesicles. In each case the selected protein was an APN (Bagchi, 2000; Banks *et al.*, 2001; Chang *et al.*, 1999; Denolf *et al.*, 1997; Gill *et al.*, 1995; Indrasith and Hori, 1992; Luo *et al.*, 1996; Valaitis *et al.*, 2001). Cry1 toxin-binding APNs are reported in nine genera of Lepidoptera (Table 1-1).

APNs have been classified based on sequence identity into four classes (Nakanishi *et al.*, 2002; Oltean *et al.*, 1999). APN cDNAs belonging to three classes (I, II, and IV) have been isolated from *M. sexta* midguts (Denolf *et al.*, 1997; Knight *et al.*, 1994); Chapter 3). The presence of APN belonging to the Class III in *M. sexta* was investigated. Since Cry1A toxins recognize APNs grouped in Class III (Gill *et al.*, 1995; Nakanishi *et al.*, 2002; Simpson and Newcomb, 2000; Valaitis *et al.*, 1995) identification and cloning of a Class III *M. sexta* APN will allow characterization of its toxin-binding properties and its role as a Cry1A toxin-receptor.

A1.2 Methods

A1.2.1 Preparation of M. sexta midgut cDNAs

M. sexta midgut cDNAs were prepared as described in the methods section of Chapter 4.

A1.2.2 Multiple sequence alignment of known lepidopteran APNs

Multiple sequence alignment of 32 APNs from different lepidopteran species was performed by the Clustalx (version 1.8) method reported by (Higgins and Sharp, 1988). The complete list of APNs used in sequence alignment is presented in Table 1-1.

A1.2.3 PCR amplification

A PCR strategy was designed to generate a small cDNA fragment from each APN in *M. sexta* midgut. Based on multiple sequence alignment of APNs, degenerate forward and reverse primers representing conserved APN amino acid sequences FPCYDEP and WLNEGFA were designed. The forward primer for the sequence FPCYDEP was 5'TT(C/T)CC(A/T/C/G)TG(C/T)TA(C/T)GA(C/T)GA(A/G)CC3' and the reverse primer for the sequence WLNEGFA was 5'(A/G)C(G/A)AA(A/T/C/G)CC(C/T)TC(A/G)-TT(A/T/C/G)AGCC3'. PCR was conducted using midgut cDNA as template as described in Chapter 4 except the primer extension period was 30 sec. The PCR fragment was cloned into pCR2.1 vector as described in Chapter 4. Ten colonies were randomly selected and inserts from clones were sequenced completely from one strand at the Molecular Genetics Instrumentation Facility (University of Georgia).

A1.2.4 BLAST search using 0.6 kb sequences

The determined nucleotide sequences were used in NCBI's BLAST program for nucleotide sequences (BLASTn) (http://www.ncbi.nlm.nih.gov/BLAST) for identification of cDNA clones that displayed high sequence identity to APNs belonging to Class III. Sequence of one clone out of ten displayed ~80% identity with LdAPN-1 and EpAPN-1. Since these are Class III APNs, the clone was designated pTA-MsAPN-3.

A1.2.5 3'-RACE and 5'-RACE cloning to obtain a full length cDNA coding for 110 kDa binding protein

To amplify the 3' region of cDNA encoding MsAPN-3, non-degenerate primers were designed from pTA-MsAPN-3. RACE PCR was conducted according to methods in Chapter Four with primers MsAPN-3 Primer-1 (P-1): (5'CAATGGACCC-CAACCTTAAG3') and Not I-dT_{18} primer (Amersham Pharmacia). A ~2.3 kb 3'RACE fragment was cloned into pCR 2.1 TA vector (Invitrogen) to yield plasmid pMsAPN-3-3'. cDNA for 5' RACE was synthesized from *M. sexta* total RNA using MsAPN-3 Primer-2 (P-2): 5'CCACCATTCGCAAGTGACGAGGTTTCC3') with Superscript reverse transcriptase (Invitrogen). 5' RACE was conducted with abridged anchor primer (Invitrogen) (AAP) (5'GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG3' and MsAPN-3 Primer-3 (P-3): 5'GTTTGTAATAACTGCTTGTGTGATTCGC3' designed from pMsAPN-3-3' sequence. 5' RACE amplification conditions are described in Chapter 4. The ~1.4 kb 5' RACE fragment was cloned into pCR 2.1 TA to yield plasmid pMsAPN-3-5'.

A1.3 Results

A1.3.1 PCR amplification of MsAPN-3 cDNA

A cDNA encoding MsAPN-3 was cloned from *M. sexta* midgut cDNAs by PCR and RACE. A 0.6 kb internal region of MsAPN-3 cDNA was amplified using a degenerate forward and reverse primer designed from conserved sequences FPCYDEP and WLNEGFA, respectively (Fig. A-1). Ten random colonies were picked and the inserts sequenced. Nine out of ten sequences shared ~90% identity with MsAPN-1 (belonging to Class I) (GenBank accesson #: X89081; Knight *et al.* (1994) while one sequence shared ~80% identity with EpAPN-1 and LdAPN-1 and was named pTA-MsAPN-3. The 3' and 5' ends of cDNA for MsAPN-3 were obtained by 5' and 3' RACE cloning.

A1.3.2 Sequence analyses of MsAPN-3

The full-length MsAPN-3 cDNA is 3057 nt with a 3030 nt open reading frame. The predicted 1010 amino acid APN has an estimated molecular mass of 113.8-kDa and a pI of 5.89. A signal sequence cleavage site after Ser20 was predicted (Nielsen *et al.*, 1998). The predicted APN has 2 potential N-glycosylation sites and a GPI-anchor attachment site at the C-terminus (predicted by big-PI predictor program; (Eisenhaber *et al.*, 1999). Amino acids S989, A990 and A991 precede the 19 amino acid hydrophobic region with a cleavage site between S989 and S990 and attachment of GPI-anchor to S989. A stretch of threonine residues is present at the C-terminus and contains predicted sites for O-glycosylation from Thr957 to Thr979. Several other O-glycosylation sites are predicted in MsAPN-3 (predicted by NetOGlyc 2.0 program; Hansen *et al.* 1998). The

zinc-binding motif HEXXHX_{18}E and gluzincin APN motif GAMENWG are also present in MsAPN-3 (Fig. A-2).

A1.4 Discussion

Phylogenetic analyses of lepidopteran APNs indicated the presence of at least four classes of APNs (Nakanishi *et al.*, 2002; Oltean *et al.*, 1999). To date three APNs from *M. sexta* midgut have been identified and cloned; MsAPN-1, MsAPN-2 and Ms110-APN (Denolf *et al.*, 1997; Knight *et al.*, 1994; Chapter 4). These three APNs belong to Class I (MsAPN-1), II (MsAPN-2), and IV (Ms110-APN). Four APN isoforms have been isolated from other lepidopteran insects such as *B. mori* and *L. dispar*. I cloned a cDNA for MsAPN-3 from *M. sexta*.

The strategy I employed to amplify the cDNA encoding MsAPN-3 was based on the presence of highly conserved regions in all APNs. PCR amplification with degenerate primers designed from these conserved sequences should potentially amplify all APNs from *M. sexta* midgut cDNAs. Several highly conserved sequences are present in lepidopteran APNs (Denolf *et al.*, 1997). I designed degenerate primers from FPCYDEP and WLNEGFA sequences and conducted PCR amplification with midgut cDNAs as template (Fig. A-1). FPCYDEP and WLNEGFA sequences are nearly 600 nt apart in lepidopteran APNs and a 0.6 kb fragment was obtained following PCR amplification. Nine out of ten randomly selected clones matched MsAPN-1. However, one clone (pTA-MsAPN-3) had ~80% identity with LdAPN-1 and EpAPN-1. APN phylogeny clusters LdAPN-1 and EpAPN-1 in Class III (Nakanishi *et al.*, 2002; Oltean *et al.*, 1999). Therefore, the insert in pTA-MsAPN-3 most likely represents an *M. sexta*

APN that belongs to Class III. RACE-PCR was conducted with non-degenerate primers designed from pTA-MsAPN-3 sequence.

A ~3 kb open reading frame was predicted for MsAPN-3. Sequence analysis revealed that MsAPN-3 has features similar to lepidopteran APNs. Like other cloned APNs, MsAPN-3 has an N-terminal signal sequence, putative GPI-anchor signal sequence and potential N- and O-glycosylation sites in addition to conserved gluzincin aminopeptidase motif GAMENWG and zinc-binding motif HEXXH (Fig. A-2). Interestingly, the C-terminus of MsAPN-3 contains a stretch of threonine residues with extensive predicted O-glycosylation (Fig. A-2). A similar polythreonine stretch with extensive predicted O-glycosylation at the C-terminus is present in Hv170-APN (Oltean *et al.*, 1999). The cDNA encoding Hv170-APN predicts a protein of 113 kDa and the higher molecular mass of the mature protein is attributed to the extensive glycosylation (Oltean *et al.*, 1999). Therefore, it is possible that even though the cDNA encoding MsAPN-3 predicts an APN of 113-kDa, the actual molecular mass of the mature protein in midguts could be much higher and possibly similar to that of Hv170-APN.

Thirty two lepidopteran APN sequences are deposited in GenBank (Table 1-1). Phylogenetic analyses of these APNs were performed using the PHYLIP inference package 3.5 (Felsenstein, 1993) (Fig. A-1). The APNs clustered in four groups (Classes I-IV) as reported by Nakanishi *et al.* (2002) and Oltean *et al.* (1999). MsAPN-3 belonged to Class III along with BmAPN-3, EpAPN-1, LdAPN-1, PiAPN-1, Hv120-APN, PxAPN-3, and Hp-APN-3 (Fig. A-1). Identification and cloning of Ms APN-3 confirms that *M. sexta* contains at least four APNs in the midgut. Three other lepidopteran insects have APNs belonging to all four classes identified and cloned. These are *B. mori, L. dispar,*

and *P. xylostella* (Fig. A-1). Hence, it is likely that other species also contain APNs that remain to be identified.

APNs belonging to Class III display different toxin-binding properties. Cry1Aa and Cry1Ab bind PxAPN-3 while Cry1Aa, Cry1Ab, or Cry1Ac do not recognize BmAPN-3 (Nakanishi *et al.*, 2002). Cry1Ac binds Hv120-APN (Gill *et al.*, 1995) while EpAPN-1 is recognized by Cry1Ac as well as Cry1Ba (Simpson and Newcomb, 2000). Nakanishi *et al.* (2002) demonstrated that Cry1Aa recognized a fragment of BmAPN-3 but not intact BmAPN-3 and suggested that APNs may contain a common structure that is recognized by Cry1 toxins. Even though toxin-binding studies have not been conducted with MsAPN-3, it is possible that MsAPN-3 can function as another Cry1A toxin-binding protein.

1/1 31/11
ATG GCA GGC CTA AGA CGA CAA ATC TTC GCT TTG GCA TGC GTT CTA TCC AAC GTC GCG TCC
M A G L R R Q I F A L A C V L S N V A S
61/21 91/31
TTC GAC CCG CCG GTG ATG CGG ACA GCG TCC ACC ATC TTC GGT GAT GAA AAA CTC AAA GGC
F D P P V M R T A S T I F G D E K L K G
121/41 151/51
GAG ATT TTC GAA GAC ATC GAC GAA CAA GAA GTC GCG ATG TCA TCA GCT GTG ACG CGT AAT
E I F E D I D E Q E V A M S S A V T R N
181/61 211/71
TCT GCC TAT CGT CTA CCT ACC ACC ACA AGA CCT TCG CGG TAC AAC GTT CAT TGG ACC ATA
S A Y R L P T T T R P S R Y N V H W T I
•
241/81 271/91
GAT ATG TCC AGA AGG ACA TAC ACC GGT AAT GTG GCA ATC CAG CTC TTC GCT ACA CAG TCT
D M S R R T Y T G N V A I Q L F A T Q S
301/101 331/111
GGC GTC AAC GAA ATT GTC ATC CAT TCT GAC CAC GTG ACA ATC CAA TCC GTG GTC CTG CAG
G V N E I V I H S D H V T I Q S V V L Q
361/121 391/131
CAA GGA TCC GCC ATC ATT CCT CAA ACC TAC AGA CTG GAT CAG CAG TAC CAG TTC TTG AGA
Q G S A I I P Q T Y R L D Q Q Y Q F L R
421/141 451/151
GTT CGC TTG ACC AGC GGC ACG TTG AAT TAC AAT CCT TCG ACC CCT GTT ATC TAC ACT CTG
V R L T S G T L N Y N P S T P V I Y T L
∇ •
481/161 511/171
ACC ATC AAC TTT GGT GCC GCT ATG CGC ACT GAC ATG TAT GGT ATT TAC GAG AGT TGG TTC
T I N F G A A M R T D M Y G I Y E S W F
541/181 571/191
AGG AAC AAC CCT AAC AGC GAA ACA GTC AGT TGG ATG GCT ACC ACC CAG TTT CAA GCG ACG
R N N P N S E T V S W M A T T Q F Q A T
601/201 631/211
TCT GCG CGC TAC GCC TTC CCT TGC TAC GAC GAA CCC AGT TTC AAG GCC AAT TTC GAC ATA
S A R Y A **F P C Y D E P** S F K A N F D I
•
661/221 691/231
ACC ATC ACT CGA CCT AAT AAT TTC AGA AGC TGG TCC TGT ACC AGG ATT AAG GAG ACC AGA
T I T R P N N F R S W S C T R I K E T R
721/241 751/251
GCC TCT AGT GTC TTG AAC TTC CAG GAT GAC ATC TAC CAC ACG ACG CCC TGC ATG TCA ACT
A S S V L N F Q D D I Y H T T P C M S T
781/261 811/271
TAC CTC ATC GCT CTG ATC GTT GCG GAG TAC GAT TCG CTA GAA TTA AGA CAA AAC AAT GTC
Y L I A L I V A E Y D S L E L R Q N N V
841/281 871/291
GTC ATG TAT GAG GTT ATT GCC CGA CCT GGC GCA CTT TCT GCT GGT CAG GGT CAA TAC GCT
V M Y E V I A R P G A L S A G Q G Q Y A
901/301 931/311
TTC GAC GTC GGT CAA GAG CTT CTC GCT GAG ATG AGT AAG CAC ACA GCC ATG GAC TTC TAT
F D V G Q E L L A E M S K H T A M D F Y
961/321 991/331
ACC ATG GAT CCC AAC CTT AAG ATG ACT CAA GCC TCC ATC CCT GAC TTC TCA GCT GGT GCT
T M D P N L K M T Q A S I P D F S A **G A**
1021/341 1051/351
ATG GAA AAT TGG GGT CTT CTT ACG TAC AGA GAA GCG TAC CTG ATG TAC GAT GCG AAT CAC
M E N W G L L T Y R E A Y L M Y D A N H
1081/361 1111/371
ACA AGC AGT TAT TAC AAA CAG TTG ATT GCT TAC AGT CTA TCT CaC GAG ATC GCC CAC ATG
T S S Y Y K Q L I A Y S L S ***H E I A H*** M

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1141/381                                                1171/391
TGG TTC GGA AAC CTC GTC ACT TGC GAA TGG TGG GAC GTA GTT TGG CTG AAC GAA GGC TTT
 W   F   G   N   L   V   T   C   E   W   W   D   V   V   W   L   N   E   G   F
1201/401                                                1231/411
GCT AGA TAC TAC CAG TAC TTC CTC ACT GAT TGG GTG GAA ACT GAC ATG GGC TTA GGA GTA
 A   R   Y   Y   Q   Y   F   L   T   D   W   V   E   T   D   M   G   L   G   V
1261/421                                                1291/431
CGT TTC ATC ACC GAG CAA GTC CAC GCC TCA TTA CTT AGT GAT TCC GCC AAC AAC CCT CAC
 R   F   I   T   E   Q   V   H   A   S   L   L   S   D   S   A   N   N   P   H
1321/441                                                1351/451
GCC CTC TCG ACC TCT GGT ATC AAC ACT CCA GCG CAA GTC AGC GGG ATG TTT TCT ACC ATC
 A   L   S   T   S   G   I   N   T   P   A   Q   V   S   G   M   F   S   T   I
             •
1381/461                                                1411/471
TCG TAC AAC AAA GGC GCT GCT GTC ATT AGA ATG ACT GAG CAC CTT CTC GGC TTT AAT GTT
 S   Y   N   K   G   A   A   V   I   R   M   T   E   H   L   L   G   F   N   V
1441/481                                                1471/491
CAC AGA CAA GGA CTT AGG AAC TAT TTG GTC GAA AGG GCC TTT AAT ATG GCT TCC CCG ATC
 H   R   Q   G   L   R   N   Y   L   V   E   R   A   F   N   M   A   S   P   I
1501/501                                                1531/511
GAC CTC TTC CAA TCT CTG GAG AGA GCT GCC AAC GCA ACC GGA GCC ATT TCT GAG TAC GGA
 D   L   F   Q   S   L   E   R   A   A   N   A   T   G   A   I   S   E   Y   G
1561/521                                                1591/531
AGA GAC TTC GAT TTT ATT GAA TAC TAC AGA AGT TGG ACA GAG CAA AGT GGT CAT CCA GTG
 R   D   F   D   F   I   E   Y   Y   R   S   W   T   E   Q   S   G   H   P   V
1621/541                                                1651/551
CTT AAC GTC GAT GTT AAT CAT CGA ACC GGT CAA ATG ACT GTC TAT CAG CGC CGC TTC AAC
 L   N   V   D   V   N   H   R   T   G   Q   M   T   V   Y   Q   R   R   F   N
1681/561                                                1711/571
ATT AAC ACC GGG TAC TCT AAT GTT AAC ACT AAC TAC ATC GTG CCG ATA TCT TTC GCG ACC
 I   N   T   G   Y   S   N   V   N   T   N   Y   I   V   P   I   S   F   A   T
1741/581                                                1771/591
GCC AGC AAC CCC GAC TTC GCT AAT ACC AAG CCC ACG CAC ATC TTG TCA AAG GCT GTG CAG
 A   S   N   P   D   F   A   N   T   K   P   T   H   I   L   S   K   A   V   Q
1801/601                                                1831/611
ATT ATC AAC CGC GGT TCC GTC GGT GAT GAA CGG GTT ATT TTC AAC AAA CAA CAG ACA GGT
 I   I   N   R   G   S   V   G   D   E   R   V   I   F   N   K   Q   Q   T   G
1861/621                                                1891/631
TTC TAC CGC GTG AAC TAT GAT GAT TAT ACT TGG GAT CTC AAT ATT ATG GCT CTT CGA GGC
 F   Y   R   V   N   Y   D   D   Y   T   W   D   L   N   I   M   A   L   R   G
1921/641                                                1951/651
GCA CAG AGG ACT CAA ATC CAC GAG TAC AAC AGA GCC CAG ATT GTG AAC GAT GTC TTC CAA
 A   Q   R   T   Q   I   H   E   Y   N   R   A   Q   I   V   N   D   V   F   Q
1981/661                                                2011/671
TTC GCT CGC TCC GGG CTC ATG ACC TAC AAC AGA GCG TTC AAC ATC CTC TCG TTC TTG GAG
 F   A   R   S   G   L   M   T   Y   N   R   A   F   N   I   L   S   F   L   E
2041/681                                                2071/691
AAC GAG ACT GCC TAC ACC CCA TGG GTT GCC GCG GTA ACT GGT TTT AAC TGG ATC AGA AAT
 N   E   T   A   Y   T   P   W   V   A   A   V   T   G   F   N   W   I   R   N
2101/701                                                2131/711
CGT CTC GCC GGA ACC CCT GAA TTG GCT CGT CTT CAT ACA ACG ATC GCC CAA TGG GCA TCA
 R   L   A   G   T   P   E   L   A   R   L   H   T   T   I   A   Q   W   A   S
2161/721                                                2191/731
AGG GTC ATG TCG GAG CTG ACC TAC TAC CCG GTC GCG AAT GAG AGC TTC ATG AGG TCG TAC
 R   V   M   S   E   L   T   Y   Y   P   V   A   N   E   S   F   M   R   S   Y
                                                         ∇
2221/741                                                2251/751
CTC AGG TAC CAG CTG GCT CCA CTG ATG TGT AAT GTC AAC GTC GCC GCG TGT CGT ACA GCT
 L   R   Y   Q   L   A   P   L   M   C   N   V   N   V   A   A   C   R   T   A
2281/761                                                2311/771
GCC ACC GCG CAG TTC CAA GCT CTA CGC AAC AAC GCA GTC GAA GTA CCA GTA GAC AGC CGC
 A   T   A   Q   F   Q   A   L   R   N   N   A   V   E   V   P   V   D   S   R
2341/781                                                2371/791
```

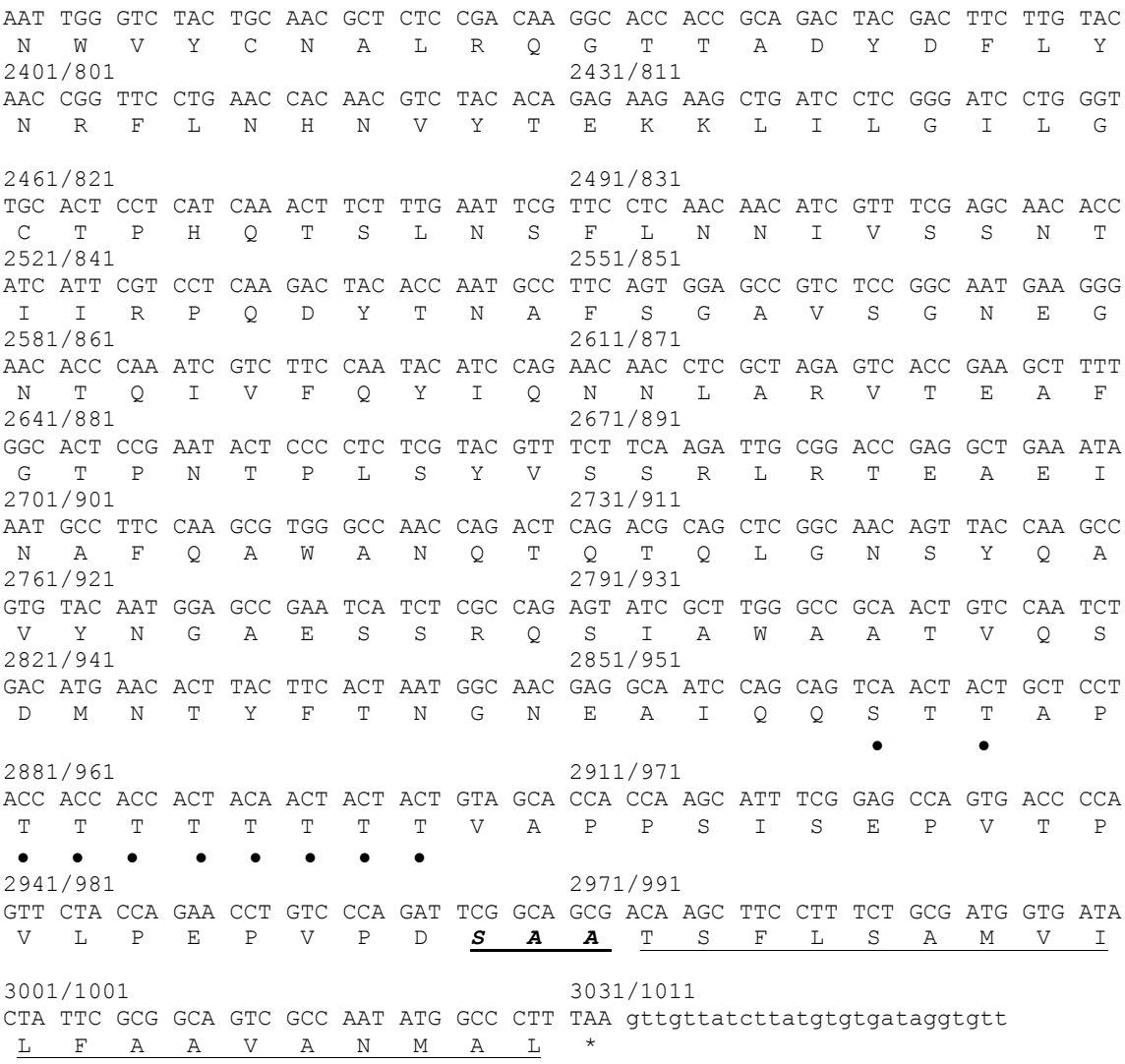

Fig. A-1. Nucleotide and predicted amino acid sequence of MsAPN-3 cDNA. The putative signal peptide and potential GPI-anchor signal peptide is underlined. Potential N-glycosylation sites are marked by inverted triangles (∇) while potential O-glycosylation sites are marked by filled circles (•). The zinc-binding site HEXXH is indicated in bold and italics. The GAMENWG motif is in bold. The primers used to amplify the internal 0.6 kb fragment are in bold and underlined. The GenBank accession number for this sequence is AY095259.

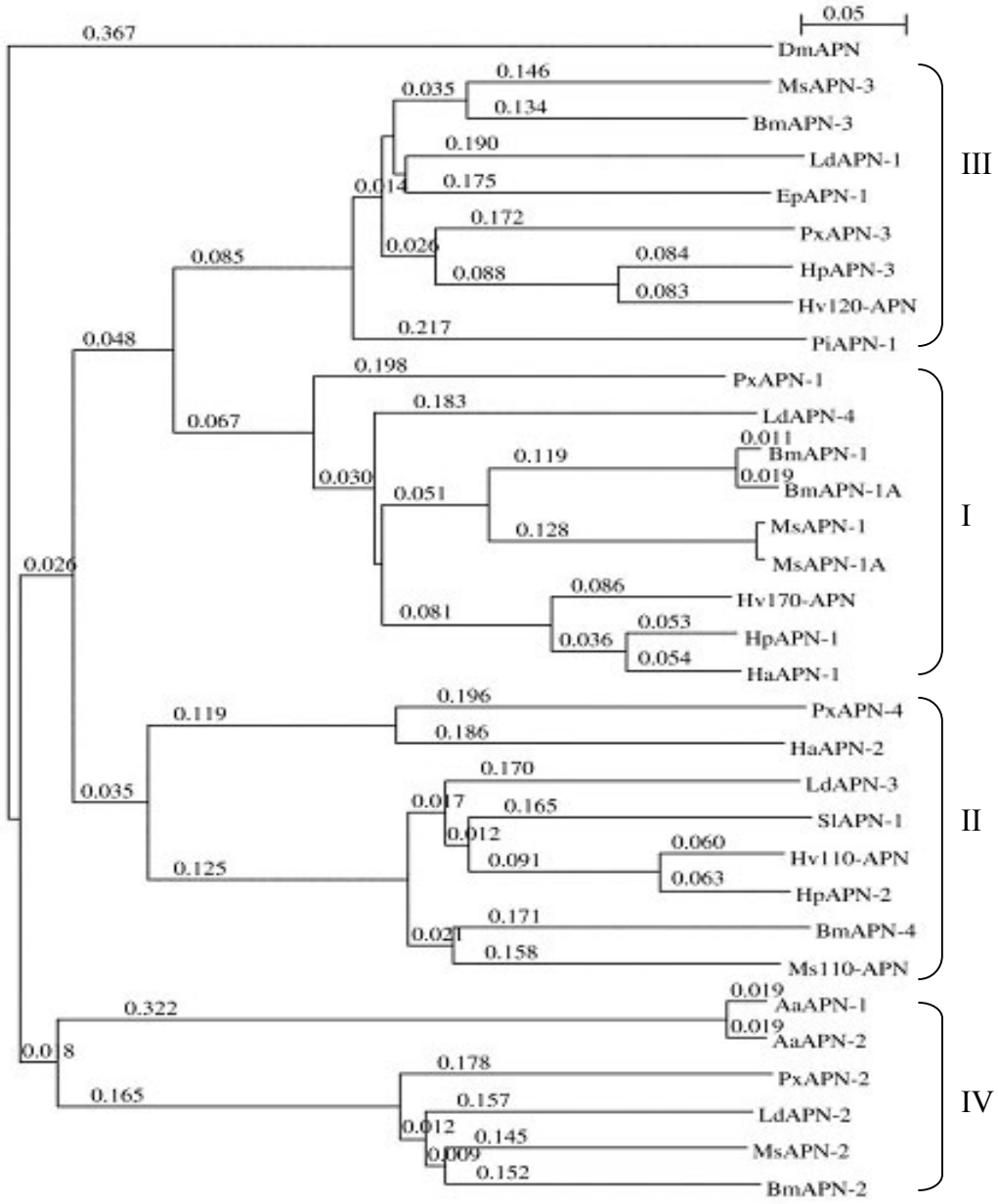

Fig. A-2. Phylogenetic analyses of APNs using the PHYLIP phylogeny inference package 3.5. The numbers on the branches indicate the branch length corresponding to the genetic distance (arbitrary units). The abbreviations for the different species are presented in Table 1-1. The APN classes (I-IV) are shown in brackets.

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