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Identification, cloning and expression of a *Heliothis virescens* 110 kDa aminopeptidase N that binds *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ-endotoxins

(Under the direction of MICHAEL J. ADANG)

The initial goal of this research was to determine whether Cry1Ac and Cry1Fa δ-endotoxins from the insecticidal bacterium *Bacillus thuringiensis* (Bt) shared binding proteins in the lepidopteran insect pest species, *Heliothis virescens*. The identification of molecules that bind Bt toxins is important, because they have been demonstrated to catalyze pore formation *in vitro*. Further, these binding proteins have been predicted to catalyze toxin mode of action *in vivo*. I demonstrated that Cry1Ac and Cry1Fa toxins shared 110, 120 and 170 kDa binding proteins. This evidence suggests that the cross-resistance between Cry1Ac and Cry1Fa in the Cry1Ac resistant *H. virescens* strain YHD2 is likely related to toxin recognition of common binding proteins.

During the early phase of my research, I discovered that while the 120 and 170 kDa binding proteins had previously been identified as aminopeptidase N (APN) molecules, the 110 kDa binding protein had been described, but remained unidentified. I subsequently identified the 110 kDa binding protein as an APN using N-terminal amino acid sequence analysis, APN antibody, and PCR of an internal region of total *H. virescens* cDNA. With the cooperation of Juan Luis Jurat Fuentes, we established that toxin recognition of the 110 kDa APN was not mediated by N-acetylgalactosamine (GalNAc). This finding was important, because previous research had indicated that the Cry1Ac domain III mutant ^{509}QNR^{511}-AAA that contained an ablated GalNAc binding pocket remained toxic

to *H. virescens*. This suggsted that Cry1Ac toxin mode of action was not entirely GalNAc dependent, as previous research had indicated. Further, these results indicated that Cry1Ac toxicity was likely mediated by a molecule that displayed GalNAc independent toxin recognition. Since the 110 kDa APN fulfilled this criteria, we predicted that it likely mediated toxicity *in vivo*. In order to address this prediction, I then cloned the 110 kDa APN gene, and expressed it in *Drosophila* S2 cells. Microscopy results showed that rhodamine-labeled Cry1Ac toxin bound to some 110 kDa APN transfected cells, but not to cells transcfected with vector-alone. However, microscopy and flow cytometry results both showed that cells expressing the 110 kDa APN remained viable after exposure to toxin, measured as a function of membrane integrity. This indicates that the 110 kDa APN does not mediate cytotoxicity when expressed in S2 cells. Alternatively, it remains possible that 110 kDa APN mediated toxin mode of action may not be dependent on pore formation, but rather on intercellular phenomena that remain unidentified.

INDEX WORDS: *Bacillus thuringiensis*, δ-endotoxin, Cry1Ac, Cry1Fa, *Heliothis virescens*, aminopeptidase N, N-acetylgalactosamine, *Drosophila* S2 cells.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 *History of* Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a Gram positive, spore-forming bacterium that forms parasporal inclusion bodies during the stationary phase of its growth cycle (Schnepf *et al*., 1998). These inclusion bodies consist of proteins that display high specificity and toxicity to an array of insect pest species (Schnepf *et al*., 1998). In particular, Bt toxins are highly active against lepidopteran (moths and butterflies) larvae, which are the main pest species of important crops, such as cotton, corn and soybean. Bt is the most widely used bacterium or bacteria-derived product in the microbial control of insect pest species (Tanada and Kaya, 1993). Due to their crystal-like shape, these inclusion bodies received the colloquial name 'crystal,' which led to the 'Cry' terminology for Bt toxin nomenclature. Different Cry strains were originally categorized by flagellar antigens, but were later classified into four major classes according to both amino acid sequence and insecticidal spectra (Hofte and Whiteley, 1989).

Different Bt strains have been isolated from soil, insects, stored products, and on the surface of leaves (Tanada and Kaya, 1993). In 1901, Ishiwata isolated from diseased silk worm larvae in Japan a bacillus strain that was later named *Bacillus sotto*. Ishiwata further concluded that the disease was due to a toxin that was associated with the *Bacillus* spore (Tanada and Kaya, 1993). In 1911, Berliner isolated a bacillus strain from the Mediterranian flour moth, and he termed this strain *Bacillus thuringiensis* after the Thuringia region in Germany where the strain was isolated (Tanada and Kaya, 1993). In 1931, it was reported

that Bt and the green muscardine fungus, *Metarhizium anisopliae*, were both effective agents for controlling the European corn borer, *Ostrinia nubialis* (Tanada and Kaya, 1993). It was also discovered around the same time period that Bt was pathogenic to the gypsy moth, *Lymantria dispar*, and other pests of cotton and cabbage (Tanada and Kaya, 1993). However, it was not until twenty years later that Bt was widely recognized as a potential biological control agent for insect pest species (Tanada and Kaya, 1993). Angus (1954; 1956) proved that the proteinaceous parasporal inclusion body was the source for toxicity against the silkworm. Subsequently, a Bt crystal-spore mixture became commercially available, and was applied directly to plant surfaces as an effective biological control agent. Bt has become the biological control agent of choice for lepidopteran pests due to its low LC_{50} and high specificity for target organisms. Additionally, since Bt is a proteinaceous toxin, it is not amplified in the food chain like many synthetic, lipophillic pesticides. This characteristic of Bt is particularly appealing to environmentally conscious farmers and consumers that want to support alternative and effective means of pest control.

Unfortunately, the Bt crystal is sensitive to ultraviolet light, and degrades relatively quickly when sprayed directly on the leaf surface. This limitation necessitated constant attention and repeated application. These economic burdens on farmers would not be mitigated until the advent of recombinant DNA technology. Toxin genes were first localized on plasmids when it was discovered that acrystalliferous strains could be transformed into crystal producing strains during transconjugation (Gonzales *et al*., 1982). After further inspection, crystal

genes were shown to occur on large plasmids from 30 to over 225 MDa in size in Bt strains that are toxic to lepidopterans (Tanada and Kaya, 1993). Subsequently, many Bt genes were then cloned (Adang *et al*., 1985; Chambers *et al*., 1991), and it was discovered that codon usage could be optimized with synthetic genes for expression of A + T rich cry genes in plants (Adang *et al*., 1993). Transgenic plants were engineered to produce the Bt toxin, and these recombinant plants in essence protected themselves from insect pest species. This eliminated both the regimented Bt crystal-spore application, and the dependence on synthetic pesticides. Additionally, This step dramatically increased the levels of toxin expression in plants, making them more efficacious for crop protection.

1.2 *Toxin sequence and structure*

Both Cry1Aa (Grochulski *et al*., 1995) and Cry3A (Li and Ellar 1991) toxin crystal structures have been determined. Although Cry1Aa and Cry3A share only 36% amino acid identity, their crystal-derived structures can be virtually superimposed (Schnepf *et al*., 1998). Both toxins contain three separate domains. Domain I consists of seven antiparallel α-helices, in which α-helix 5 is surrounded by the remaining α-helices (Grochulski *et al*., 1995; Li and Ellar 1991). This domain has been postulated to be a pore-forming domain due to its structural similarity with the colicin A pore-forming domain, and the membrane translocating region of diphtheria toxin (Parker and Pattus, 1993). In support of this prediction, Gazit and Shai (1995) have shown that α-helices 5 and 7 assemble into a pore forming domain *in vitro*. Additionally, Schwartz et al (1997) used site-

directed mutagenesis to introduce disulfide bonds within domain I and between domains I and II to restrict intermolecular movement and inhibit toxin activity. The activity of these mutant toxins was rescued under reducing conditions (breaking the disulfide bonds), supporting the predicted role of domain I in toxicity. Domain II consists of three anti-parallel β-sheets joined in a Greek-key topology (Grochulski *et al*., 1995; Li and Ellar 1991). These β-sheets contain three loops that are topologically close to one another and extend outwards from the active toxin. Mutational analysis of these loops indicates that they are directly involved in specific binding of the toxins to putative receptors (Rajamohan *et al*., 1996a; Rajamohan *et al*., 1996b). Domain III contains two twisted, antiparallel β-sheets forming a jelly-roll topology (Grochulski *et al*., 1995; Li and Ellar 1991). Domain III has been predicted to be involved in toxin stability, and has been shown to be involved in toxin binding to putative receptors (Lee *et al*., 1999; de Maagd *et al*., 1999). These observations of toxin structure, domain interactions and domain movement have led to the development of an 'umbrella model' whereupon after toxin domains II and III bind to the receptor, α-helices 4 and 5 of domain I insert into the membrane, and the remaining helices of domain I fold out over the membrane surface like an umbrella (Schwartz *et al*., 1997; Gazit and Shai, 1993). Alternatively, a "penknife" model of Bt toxin pore-formation based on a colicin A toxin mode of action model has been proposed (Hodgman and Ellar 1990).

The modeled toxin structures described above are very informative when considered with the regions of high sequence homology found among different

Cry toxins. In their review of Cry toxin nomenclature, Hofte and Whiteley (1989) aligned most of the Cry toxin amino acid sequences known at the time, and reported that all Cry toxins share five conserved blocks. When compared to the crystal structures of Cry1Aa and Cry3A, these five blocks are found to be located in regions that are either predicted to be involved in toxin pore-formation, lie in regions of contact between domains, or comprise hydrophobic stretches that likely contribute to toxin folding (Schnepf *et al*.,1998). Therefore, it can be concluded that these regions of high homology maintain toxin tertiary structure and ultimately toxin mode of action.

1.3 *Toxin binding*

After the Bt crystal is ingested, it is solubilized in the alkaline insect midgut. The solubilized protein exists in a protoxin form that is typically 130 kDa. The protoxin is then activated by endogenous proteases in the insect midgut that sequentially remove C-terminus portions of the protoxin, yielding a toxin of approximately 65 kDa that is N-terminus in origin (Tanada and Kaya, 1993). Activated Cry toxins bind specifically to putative receptors on the apical brush border surface of insect midgut microvillae of susceptible insects both *in vitro* (Hofmann *et al*., 1988) and *in vivo* (Aranda *et al*., 1996). However, the toxin binding characteristics and the identity of the toxin receptor remained unknown until a toxin binding model system was established with vesicles derived from the apical brush border of midgut epithelial cells. The purification of brush border membrane vesicles (BBMV) from Lepidoptera midguts (Wolfersberger 1987)

provided a system to measure the specific binding of Cry toxins to their putative receptors *in vitro*. Through differential centrifugation and $MgCl_2$ precipitation, BBMV are purified from homogenized midgut tissue (Wolfersberger 1987). BBMV are composed primarily of the brush border of midgut epithelial cells, and are enriched in marker enzyme activity (Wolfersberger 1987). Since Cry toxins were demonstrated to interact directly with the apical surface of these epithelial cells, BBMV provided an excellent model to study the interaction of Cry toxins with their binding proteins. Hofmann et al (1988) demonstrated that radiolabeled toxin bound high affinity sites on BBMV. Further, heterologous and homologous competition experiments indicated that toxin binding was both specific and saturable (Hoffman *et al*., 1988). These lines of evidence indicated that the brush border of midgut epithelial cells likely contained a putative Cry toxin receptor(s). However, some toxins were later found to specifically bind BBMV derived from both susceptible and resistant insects (Wolfersberger 1990; Garczynski *et al*., 1991; Luo *et al*., 1999). Therefore, specific toxin binding to BBMV was not necessarily synonymous with toxicity. Rather, a step downstream of toxin binding was required for toxicity. Indeed, toxin binding was shown to be a two-stage process where initial toxin binding is followed by irreversible binding when toxin inserts into the membrane (Ihara *et al*., 1993; Liang *et al*., 1995). In the same study, irreversible binding of toxin was found to be a good indicator of toxin activity (Ihara *et al*., 1993).

Utilization of BBMV also led to the description of Bt binding proteins with ligand blotting technique, and eventually led to the identification of these

binding proteins. In the ligand blot assay, total BBMV proteins are separated by SDS-PAGE, transferred to a membrane, and then probed with radiolabeled toxin. This procedure led to the description of binding proteins in several different Lepidoptera species (Oddou *et al*., 1991; Garczynski *et al*., 1991).

Based on ligand blotting results and N-terminal sequencing, Bt toxin binding proteins were identified as aminopeptidase N (APN) (Sangadala *et al*., 1994; Knight *et al*., 1994) and cadherin-like molecules (Vadlamudi et al., 1993; Nagamatsu *et al*., 1997). In addition to the denaturing conditions utilized during ligand blotting, the non-denaturing conditions of affinity chromatography have also been utilized to purify APN molecules that bind Bt toxins (Luo *et al*., 1996; Banks *et al*., 2001).

1.4 *Aminopeptidase N and cadherin*

APN molecules are metal-binding exopeptidases that sequentially cleave N-terminal amino acid residues from proteins and peptides. All APNs contain a central Zn^{2+} ion, which is bound at a conserved HEXXH site, and is required for enzyme activity (Taylor, 1996). APNs are involved in various functions in prokaryotes and eukaryotes, including both plants and animals (Taylor, 1996). APNs are found in subcellular organelles, in the cytoplasm, and as membrane components (Taylor, 1996). Although APN molecules display a highly conserved enzymatic function, they are versatile in the physiological roles they serve. APNs are involved in a variety of physiological processes such as protein processing and turnover, regulation of peptide hormone action, viral infection, tissue invasion and

cell cycle control (Taylor, 1996). Recently, APN has been demonstrated to have a pivotal role in tumor growth (Pasqualini *et al*., 2000). APN transcription and protein levels are up-regulated in cell lines that are exposed to conditions characteristic of a growing tumor, such as elevated angiogenic growth factor concentration and hypoxia (Bhagwat *et al*., 2001). Furthermore, drugs coupled to a peptide that targets APN in tumor vasulature has been shown to posess greater antitumor activity than free drug alone (Pasqualini *et al*., 2000). These studies highlight the versatile physiological roles in which APNs participate. Currently, crystal structures exist for several different aminopeptidases (Lowther and Matthews, 2000; Burley *et al*., 1992; Olsen et al., 1997). Crystal structure determination of an APN that binds Bt toxin would contribute greatly to research in many different areas including the Bt field.

Cadherins mediate Ca^{2+} dependent cell-cell adhesion, and occur on the plasma membrane of cells. Cadherin is usually concentrated in adhesion belts in mature epithelial cells, where it connects the cortical actin cytoskeletons of neighboring cells (Alberts *et al*., 1994). Cadherin-like molecules have not been localized in insect midgut epithelial cells, but presumably they occur between cells, like other cadherins, and not on the apical surface. Investigators in the Bt field unfortunately have not addressed the paradox of how a Bt toxin can access cadherin if it is located between cells, rather than on the apical surface, as is the case for APN. However, research in a distantly related field of bacterial pathogenesis may provide some insight into solving this paradox. *Listeria monocytogenes* is responsible for the food-borne infection listerosis, and

expresses the surface protein internalin. The internalin molecule in turn interacts with the host receptor, E-cadherin, to promote cell entry into human epithelial cells (Lecuit *et al*., 2001). Lecuit et al (2001) query how internalin could reach E-cadherin since it is not exposed on the apical surface. The authors speculate that since the intestinal mucosa is a fast-renewing tissue, enterocytes at the tip of the villi are replaced by ascending enterocytes produced in intestinal crypts, and that the transient opening of cell-cell junctions may allow E-cadherin to interact with bacteria (Lecuit *et al*., 2001). Whether or not a similar phenomenon occurs in insect midgut epithelial cells remains a matter of speculation. Further work on cadherin-like molecule localization in insect midgut epithelial cells is needed to elucidate its physical accessibility to Bt toxins.

1.5 *Toxin mode of action*

Bt toxin mode of action *in vitro* has been demonstrated to be both cytolytic and cytotonic in nature, depending on the method of investigation. Bt cytotoxicity has been shown to be mediated by disruption of the plasma membrane by formation of large aqueous pores (Carroll and Ellar 1997). Alternatively, Bt toxins have also been demonstrated to open cation-selective, rectifying channels (Schwartz *et al*., 1993; Schwartz *et al*., 1997) and to release internal calcium stores (Potvin et al., 1998). When Bt mode of action is measured as a function of the change in BBMV size relative to the amount of side-scattered light with a spectrofluorometer, evidence indicates that Bt toxin creates large aqueous pores (Carroll and Ellar 1997). For example, light scattering assays

indicate that sugars up to 2nm in diameter can enter BBMV that have been exposed to toxin. However, when purified APN molecules are integrated into planar lipid bilayers, small rectifying and cation-selective channels approximately 0.2 nm in diameter are opened by the toxin (Schwartz *et al*., 1993; Schwartz *et al*., 1997). Therefore, evidence of Bt toxin mode of action are equivocal in terms of the size of the toxin-induced pore, whether or not a pore is formed *de novo* or a pre-existing channel is opened, and whether toxins are cytotonic or cytolytic. Despite these results, there is strong evidence that APNs mediate Bt pore formation *in vitro*. For example, when incorporated into phospholipid vesicles loaded with Rb^+, the *Heliothis virescens* 170 kDa APN mediated toxin-induced Rb^+ efflux (Luo *et al*., 1997). However, no published evidence has implicated an APN molecule in mediating *in vivo* cytotoxicity.

1.6 *APN glycosylation*

Eukaryotic cell plasma membranes contain proteins that are often highly glycosylated, and these glycoproteins are typically involved in cell-cell communication and adhesion. The plasma membrane-bound proteins of insect midgut epithelial cells are no exception. APN molecules line the brush border of the insect midgut epithelial cells, and are involved primarily in digestion and amino acid uptake (Terra and Ferreira 1994; Wolfersberger 2000). Therefore, if APN molecules that bind Bt toxins are indeed Bt receptors, Bt toxins have subverted a membrane-bound digestive enzyme to mediate their pathology. Since Bt toxins recognize glycosylated APN molecules, researchers examined whether

toxins behave like lectins and bind sugar moieties on APNs. Indeed, GalNAc was demonstrated to specifically inhibit Cry1Ac toxin binding to the *M. sexta* 120 kDa APN (Garczynski *et al*., 1991) and to the *H. virescens* 170 kDa APN (Luo *et al*., 1997), and lectin blotting further indicated that these APNs contained GalNAc (Lee *et al*., 1996; Knowles *et al*., 1991). In order to determine the role of the lectin-like epitope of Cry1Ac in toxin binding and activity, its putative GalNAc binding pocket was altered with site directed mutagenesis, yielding the Cry1Ac domain III mutant ^{509}QNR^{511}-AAA (Lee *et al*., 1999; see also Burton et al., 1999). While this mutant toxin no longer bound the *M. sexta* 120 kDa APN recognized by wild toxin, it unexpectedly remained toxic to insects (Lee *et al*., 1999; Jenkins *et al*., 1999). Therefore, toxicity should be mediated by a receptor where toxin recognition of a GalNAc moiety is not essential. This receptor may or may not be an APN. Jenkins et al (1999) showed that a previously identified cadherin-like molecule in *M. sexta* fulfilled these criteria. Additionally, native Cry1Ac and the ^{509}QNR^{511}-AAA mutant both bound a novel 110 kDa APN, as well as the 170 kDa APN in *H. virescens* (Banks *et al*., 2001). Moreover, the *H. virescens* 110 kDa APN was not recognized by the GalNAc-binding lectin, soybean agglutinin (SBA) (Banks *et al*., 2001). This evidence supports the prediction that Bt toxin binding and activity may be mediated by both carbohydrate-dependent and carbohydrate-independent recognition of the putative receptor (Carroll *et al*., 1997).

1.7 *Bt Resistance*

Since insects have acquired resistance to numerous synthetic pesticides, a legitimate concern for the use of Bt in transgenic crops is the prevention of resistance. Currently, the incidence of resistance to Bt toxins in the field by insect pests is extremely low. Only *Plutella xylostella*, the diamondback moth (DBM), has developed resistance in the field (Tabashnik *et al*., 1994). Although the biochemistry and genetics of resistance in the DBM remains unknown, laboratory strains resistant to Bt toxins have been intensively studied. Results suggest that Bt resistance in insects may be mediated by different mechanisms, including a decrease in overall toxin binding affinity to BBMV (Van Rie *et al*., 1990), a change in amino acid sequence of toxin binding APNs (Zhu *et al*, 2000), reduced midgut protease activities that activate toxins (Oppert *et al*., 1997), disruption of a cadherin gene by transposon-mediated insertion (Gahan *et al*., 2001). Additionally, the absence of a galactosyltransferase has also been recently implicated as a source of Bt resistance in nematodes (Griffitts *et al*., 2001).

1.8 *Objectives*

The initial objective of the research described herein was to determine whether Cry1Ac and Cry1Fa shared binding proteins in *H. virescens*. Based on two lines of evidence, I predicted that Cry1Ac and Cry1Fa would share binding proteins in *H. virescens*. First, the Cry1A and Cry1Fa toxins share high sequence homology in domain II loops (Tabashnik *et al*., 1996), which are involved in toxin binding and activity (Rajamohan *et al*., 1996a, 1996b). Second, cross-resistance

between Cry1Ac and Cry1Fa has been described in the *H. virescens* Cry1Ac resistant YHD2 strain (Gould *et al*., 1995). This means that the insect line YHD2 was selected for Cry1Ac resistance, but was not exposed to Cry1Fa during the selection process. After Cry1Ac resistance was acquired, it was subsequently discovered that YHD2 was also resistant to Cry1Fa. A mechanistic explanation for Cry1Fa cross-resistance in YHD2 is that Cry1Ac and Cry1Fa toxins recognize the same binding proteins. That is, if resistance in *H virescens* to Cry1Ac toxin is due receptor alteration and subsequent loss of toxin binding, then cross-resistance to Cry1Fa may also be related to loss of binding at the same altered binding site. Indeed, Lee et al (1995) show that Cry1A resistance in *H. virescens* is related to the loss of binding. Further, heterologous competition binding experiments have shown that Cry1Ab and Cry1Fa toxins share a high affinity binding site in the DBM (Granero *et al*., 1996; Ballester *et al*., 1999). Additionally, binding assays have shown that Cry1Fa competes with low affinity for Cry1Ac binding sites in *H. virescens* brush border membrane vesicles (BBMV) (Jurat-Fuentes and Adang, 2001). These lines of evidence suggested that Cry1Ac and Cry1Fa should share binding proteins in H. *virescens*.

This first objective was fulfilled when it was discovered that Cry1Ac and Cry1Fa both bind to 110, 120, 170 and 210 kDa proteins in BBMV. The 120 and 170 kDa molecules were previously identified as APNs (Gill *et al*., 1995; Luo *et al*., 1997). The *H. virescens* 210 kDa binding protein has not been identified, but is similar in size to the Cry1Ab binding cadherin-like molecule in *M. sexta* (Vadlamudi *et al*., 1993). The 110 kDa molecule was described as a Cry1Ac

binding protein (Gill *et al*., 1995), but had not been identified. I identified the 110 kDa binding protein as a novel APN based on N-terminal amino acid sequence, and PCR-based amplification using this N-terminal amino acid sequence and a highly conserved downstream APN motif.

The project then shifted to a second objective, which was to determine whether the 110 kDa APN toxin binding protein was a true Bt receptor. In order to receive 'receptor' status, a Bt toxin binding protein must be demonstrated to mediate Bt pore formation *in vitro* or cytotoxicity *in vivo*. Therefore, I set out to clone the 110 kDa APN and express it in the *Drosophila* S2 cell line. The intention of this phase of research was to determine whether expression of the 110 kDa APN could transform an insect cell line having innate Bt toxin resistance into a Bt susceptible cell line. Positive *in vivo* data could change the status of the 110 kDa APN from a molecule that binds toxin to a receptor that mediates Bt toxicity.

The cloning strategy was to PCR amplify an internal region of the 110 kDa APN gene using first strand cDNA template, and then design gene specific primers from this internal region to clone the entire 110 kDa APN gene using the 5' and 3' rapid amplification of cDNA ends (RACE) technique (Frohman *et al*., 1988). RACE yielded two amplimers from which a single clone containing the entire 3.0 kb 110 kDa APN gene was constructed. This gene contained a 3.0 kb open reading frame having a N-terminal signal sequence, start methionine, Zn^{2+} HEXXH binding site, putative GPI anchor signal sequence, stop codon, poly-adenylation signal, and a poly-A tail (GENBANK accession # AF378666). BLAST analysis with the 110 kDa nucleotide sequence indicated 89% similarity

to the *Helicoverpa punctigera* APN 2 (GENBANK accession # AF217249) (Emmerling *et al*., 2001). These two APNs are the only members of a branch within a lepidopteran APN dendrogram (Emmerling *et al*., 2001). The most closely related lineage of lepidopteran APNs share 26% nucleotide sequence similarity with *H. virescens* 110 kDa APN and *H. punctigera* APN2.

The next step was to express the 110 kDa APN in insect cells and test transfected cells for Bt susceptibility. Although investigations have previously attempted to demonstrate that APN expression in cell lines confers Bt susceptibility, none has conclusively shown an APN to mediate Bt toxicity (Denolf *et al*., 1997; Simpson *et al*., 2000). However, Denolf et al (1997) and Simpson et al (2000) both used a baculovirus to express APN in insect cells, and evidence suggested that glycosylation patterns of baculovirus expressed APN may be different from BBMV-derived APN (Denolf *et al*., 1997). Denolf et al (1997) also observed only non-specific binding of Cry toxins to *Plutella xylostella* APN expressed in baculovirus-transformed Sf9 cells. Second, it was recently discovered that baculovirus-mediated expression of GPI anchored proteins in insect cells results in a deficiency of the de-acylation step of GalNAc during synthesis of the GPI anchor in the endoplasmic reticulum (Azzous *et al*., 2000). This could prevent proper downstream assembly of the GPI glycan core. When considered together, these results suggested that baculovirus-mediated expression of recombinant APN could cause improper glycosylation and/or glycan core assembly. This evidence indicated that an alternative expression method could mitigate problems due to baculovirus-mediated expression. Therefore, the 110

kDa APN gene was expressed using the pIZT vector (Novagen) that utilizes the constitutive baculovirus IE2 promoter to drive expression of gene of interest, but does not require baculovirus infection for gene expression. Additionally, this vector also contains a separate cassette for green fluorescent protein (GFP) expression as a marker protein for transfection. S2 cells transfected with the 110 kDa APN gene expressed membrane-bound APN that had four-fold increased enzymatic activity, relative to vector alone transfected cells. This indicated that biologically active APN was expressed in 110 kDa transfected S2 cells. Additionally, 110 kDa APN transfected S2 cells bound rhodamine-labeled Cry1Ac. However, 110 kDa APN transfected S2 remained resistant to Cry1Ac and Cry1Fa toxins, measured as a function of membrane integrity. This indicates the *H. virescens* 110 kDa APN does not mediate cytotoxicity in S2 transfected cells, yet functions as a binding protein.

It was incidentally discovered that the frequency of an elongated cell phenotype, which is indicative of increased cell motility and migration, was enhanced when S2 cells were transfected with the 110 kDa APN. These results are suggestive in light of human CD13/APN function. The activity of this molecule has been shown to be correlated with angiogenesis (Pasqualini *et al*., 2000), which involves the migration and motility of endothelial cells involved in the formation of new blood vessels that sustain tumor growth (Rubanyi 2000). Therefore, the *H. virescens* 110 kDa APN may be pleiotropic in that it may function as both a Bt binding protein, as well as a protease involved in cellular phenotype determination.

2.0 REFERENCES

Adang, M.J., Staver, M.J., Rochleau, T.A., Leighton, J., Barker, R.F., Thompson, D.V. 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene 36, 289-300.

Adang, M.J., Brody, M.S., Cardineau, G., Eagan, N., Roush, R.T., Shewmaker, C.K., Jones, A., Oakes, J.V., McBride, K.E. 1993. The reconstruction and expression of a *Bacillus thuringiensis cry*IIIA gene in protoplasts and potato plants. Plant Molec. Biol. 21, 1131-1145.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D. 1994. Molecular Biology of the Cell. Garland Publishing Inc.

Angus T.A. 1954. A bacterial toxin paralyzing silkworm larvae. Nature 73, 5454-546.

Angus, T.A. 1956. Association of toxicity with protein-crystalline inclusions of *Bacillus sotto Ishiwata*. Can. J. Microbiol. 2, 122-131.

Aranda, E., Sanchez, J., Peferoen, M., Guereca, L., Bravo, A. 1996. Interactions of *Bacillus thuringiensis* crystal proteins with the midgut epithelial cells of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 68, 203-212.

Azzouz, N., Kedees, M.H., Gerold, P., Becker, S., Dubremetz, J. Klenk, H., Eckert, V., Schwarz R.T. 2000. Glycobiology. An early step of glycosylphosphatidyl-inositol anchor biosynthesis is abolished in lepidopteran insect cells following baculovirus infection. 10(2) 177-183.

Ballester, V., Granero, F., Tabashnik, B. E., Malvar, T., Ferre, J. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamond back moth (*Plutella xylostella*). Appl. Environ. Microbiol. 65, 1413-1419.

Banks, D.J., Jurat-Fuentes, J. L., Dean, D.H., Adang, M.J. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ–endotoxin binding to a novel 110 kDa

aminopeptidase in *Heliothis virescens* in not N-acetylgalactosamine mediated. Insect Biochem. Molec. Biol. 31, 909-918.

Bhagwat, S.V., Lahdenranta, J., Giordano, R., Arap, W., Pasqualini, R., Shapiro, L.H. 2001.CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. Blood, 97 No. 3, 652-659.

Burley, S.K., David, P.R., Sweet, R.M., Taylor, A., Lipscomb, W.N. 1992. Structure determination and refinement of bovine lens leucine aminopaptidase and its complex with bestatin. J. Mol. Biol. 224, 113-140.

Burton, S. L., Ellar, D. J., Li, J., Derbyshire, D. J., 1999. N- acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Molec.. Biol. 287, 1011-1022.

Carroll, J., Wolfersberger, M. G., Ellar, D. J., 1997. The *Bacillus thuringiensis* Cry1Ac toxin-induced permeability change in *Manduca sexta* midgut

brush border membrane vesicles proceeds by more than one mechanism. J. Cell Sci. 110, 3099-3104.

Chambers, J. A., Jelen, A., Gilbert, M. P., Jany, C. S., Johnson, T. B., Gawron-Burke, C., 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. aizawai. J. Bacteriol. 173, 3966-3976.

Chang, W. X. Z., Gahan, L. J., Tabashnik, B. E., Heckel, D. G., 1999. A new aminopeptidase from diamondback moth provides evidence for a gene duplication event in Lepidoptera. Insect Molec. Biol. 8, 171-177.

De Maagd, R.A., Bakker, P.L., Masson, L., Adang, M.J., Sangadala, S., Stiekema, W., Bosch, D. 1999. Domain III of the *Bacillus thuringiensis* δ–endotoxin Cry1Ac in involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Molec. Microbiol. 31(2), 463-471.

Denolf, P., Jansens, S., Van Houdt, S., Peferoen, M., Degheele, D., Van Rie, J., 1993. Biotinylation of *Bacillus thuringiensis* insecticidal crystal proteins. Appl. Environ. Microbiol. 59,1821-1827.

Emmerling, M., Chandler, D., Sandeman, M. 2001. Molecular cloning of three cDNAs encoding aminopeptidases from the midgut of *Helicoverpa punctigera*, the Australian native budworm. Insect Biochem. Molec. Biol. 31, 899-907.

Frohman, M.A., Dush, M.K., Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. 85, 8998-9002.

Gahan, L.J., Gould, F., Heckel, D G. 2001. Identification of a gene associated with Bt resistence in *Heliothis virescens*. Science. 293, 857-860.

Garczynski, S. F., Crim, J.W., Adang, M. J., 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus*

thuringiensis δ–endotoxin by protein blot analysis. Appl. Environ. Microbiol. 57, 2816-2820.

Gazit, E., Shai, Y. 1995. The assembly and organization of the α5 and α7 helices from the pore-forming domain of *Bacillus thuringiensis* δ–endotoxin. J. Biol. Chem. 270, 2571-2578.

Gill, S. S., Cowles, E. A, Francis, V., 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J. Biol. Chem. 270, 27277-27282.

Gonzalez, J.M., Brown, B.J., Carlton, B.C. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ–endotoxin among strains of. *B. uringiensis* and *B. cereus*. Natl. Acad. Sci. 79, 6951-6955.

Gould, F., Anderson, A., Reynolds, A., Bumgarner, L., Moar, W., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae)

strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545-1559.

Granero, F., Ballester, V., Ferre, J. 1996. *Bacillus thuringiensis* crystal protein Cry1Ab and Cry1Fa share a high affinity binding site in *Plutella xylostella* (L.). Biochem. Biophys. Res. Commun. 224, 779-783.

Griffiths, J.S., Whitacre, J.L., Stevens, D.E., Aroian, R.V. 2001. Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science 293, 860-864.

Grochulski, P.L., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J-L., Brousseau, R., Cygler, M. 1995. *Bacillus thuringiensis* Cry1Aa insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. 254, 447-464.

Hodgman, T.C., Ellar, D.J. 1990. Models for the structure and function of *Bacillus thuringiensis* δ–endotoxins determined by compilational analysis. DNA Seq. 1, 97-106.

Hofte, H., Whitely, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53, 242-255.

Hofmann, C., Vanderbruggen, H., Hofte, H., Van Rie, J., Jasens, S., Van Mellaert, H., 1988. Specificity of *Bacillus thuringiensis* δ–endotoxin is correlated with the presence of high affinity binding sites in the brush border membrane of target insect midgets. Proc. Natl. Acad. Sci. 85, 7844-7848.

Ihara, H. E., Kuroda, A., Wadano, A., Himeno, M. 1993. Specific toxicity of δ–endotoxins from *Bacillus thuringiensis* to *Bombyx mori*. Biosci. Biotechnol. Biochem. 57, 200-204.

Jenkins, J. L., Lee, M. K., Sangadala, S., Adang, M. J., Dean, D. H., 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Letters 462, 373-376.

Jurat-Fuentes, J.L., Adang, M.J. 2001. Importance of Cry1 δ–endotoxin domain II loops for binding specificity in *Heliothis virescens.* Appl. Environ. Microbiol. 67, 323-329.

Knight, P. J. K., Crickmore, N., Ellar, D. J., 1994. The receptor for *Bacillus thuringiensis* Cry1Ac delta-endotoxin in the brush border of the lepidopteran *Manduca sexta* is aminopeptidase J. Molec. Microbiol. 11, 429-436.

Knowles, B. H., Knight, P. J. K., Ellar, D. J., 1991. N-acetylgalactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. Proc. R. Soc. Lond. B 245, 31-35.

Knowles, B. H., Dow, J. A. T., 1993. The crystal δ–endotoxin of *Bacillus thuringiensis*: models for their mechanisms of action on the insect gut. Bioessays 15, 469-476.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., Cossart, P. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science. 292, 1722-1275.

Lee, M. K., You, T. H., Young, B. A., Cotril, J. A., Valaitis, A. P., Dean, D. H., 1996. Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* Cry1Ac toxin. Appl. Environ. Microbiol. 62, 2845-2849.

Lee, M. K., You, T. H., Gould, F. L., Dean, D. H., 1999. Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. Appl. Environ. Microbiol. 65, 4513-4520.

Li, J., Carroll, J., Ellar, D.J. 1991. Crystal structure of insecticidal δ–endotoxin from *Bacillus thuringiensis* at 2.5 angstroms resolution. Nature. 353, 815-821.

Liang, Y., Patel, S.S., Dean, D.H. 1995. Irreversible binding kinetics of *Bacillus thuringiensis* Cry1A δ–endotoxins to gypsy moth brush border membrane

vesicles is directly correlated to toxicity. J. Biol. Chem. 270(42), 24719-24724.

Lowther, T.W., Matthews, B.W. 2000. Structure and function of methionine aminopeptidases. Biochimica et Biophysica Acta. 1477, 157-167.

Luo, K., Yang-Jiang, L., Adang, M. J., 1996. A 106 kDa form of aminopepidase is a receptor for *Bacillus thuringiensis* Cry1C δ–endotoxin in the brush border membrane of *Manduca sexta*. Insect Biochem. Molec. Biol. 26, 783-791.

Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R., Adang, M. J., 1997. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* δ–endotoxin binding and pore formation. Insect Biochem. Molec. Biol. 27, 735-743.

Luo, K., Banks, D. J, Adang, M. J., 1999a. Toxicity, binding and permeability analyses of four *Bacillus thuringiensis* Cry1 δ–endotoxins using brush

border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. Appl. Environ. Microbiol. 65, 457-464.

Luo, K., McLachlin, J. R., Brown, M. R., Adang, M. J., 1999b. Expression of a glycosylphosphatidylinositol-linked *Manduca sexta* Aminopeptidase N in insect cells. Protein Expression and Purification 17, 113-122.

Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S., Kogure, M., 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal Cry1Aa toxin. Biosci. Biotechnol. Biochem. 62, 727-734.

Oddou, P., Hartmann, H., Geiser, M. 1991. Identification and charachterization of *Heliothis virescens* midgut membrane proteins binding *Bacillus thuringiensis* delta-endotoxins. Eur. J. Biochem. 188, 209-217.

Olsen, J., Kokholm, K., Noren, O., Sjostrom, H. 1997. Structure and expression of aminopeptidase N. Adv. Exp. Med. Biol. 421, 47-57.

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H. 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J. Biol. Chem. 272, 23473-23476.

Parker, M.W., Pattus, F. 1993. Rendering a membrane protein soluble in water: a common packing motif in bacterial protein toxins. Trends Biochem. Sci. 18, 391-395.

Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Shapiro, L.H., Arap, W. Ruoslahti, E. Aminopeptidase N is a receptor for tumor-homing peptides and a target inhibiting angiogenesis. Cancer Res. 60(3), 722-727.

Peferoen, M., 1997. Progress and prospects for field use of Bt genes in crops. Trends in Biotechnology 15, 173-177.

Rajamohan, F., Cotrill, J. A., Gould, F., Dean, D. H., 1996a. Role of domain II, loop 2 residues of *Bacillus thuringiensis* Cry1Ab δ–endotoxin in

reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J. Biol. Chem. 271, 2390-2396.

Rajamohan, F., Alzate, O., Cotrill, J. A., Curtiss, A., Dean, D. H., 1996b. Protein engineering of *Bacillus thuringiensis* δ–endotoxin: mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. Proc. Natl. Acad. Sci. USA 93, 14338-14343.

Sangadala, S., Walters, F. S., English, L. H., Adang, M. J., 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal Cry1Ac toxin binding and 86Rb+-K+ efflux in vitro. J. Biol. Chem. 269, 10088-10092.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. And Molec. Biol. Rev. 62(3), 775-806.

Schwartz, J.L., Garneau, L., Savaria, D., Masson, L., Brousseau, R., Rousseau, E. 1993. Lepidopteran specific crystal toxins from *Bacillus thuringiensis*

form cation and anion selective channels in planar lipid bilayers. J. Membr. Biol. 132, 53-62.

Schwartz, J.L., Yiang-Jiang, L., Sohnlein, P., Brousseau, R., Laprade, R., Masson, L., Adang, M.J. 1997. Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. FEBS Letters 412, 270-276.

Schwartz, J.L., Juteau, M., Grochulski, P., Cygler, M., Prefontaine, G., Brousseau, R., Masson, L. 1997. Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. FEBS Lett. 410, 397-402.

Simpson, R.M., Newcomb, R.D. 2000. Binding of *Bacillus thuringiensis* δ–endotoxins Cry1Ac and Cry1Ba to a 120 kDa aminopeptidase N of Epiphyas postvittana purified from both brush border membrane vesicles and baculovirus infected Sf9 cells. Insect Biochem. And Molec. Biol. 30, 1069-1078.

Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol.39, 47-79.

Tabashnik, B. E., Malvar, T., Liu, Y. B., Finson, N., Borthakur, D., Shin, B. S., Park, S. H., Masson, L., De Maagd, R. A., Bosch, D., 1996. Cross-resistance of the diamondback moth indicates altered interaction with domain II of *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 62, 2839-2844.

Tanada, Y., Kaya, H.K. 1993. Insect Pathology. Academic Press. Inc.

Taylor, A. 1996. Aminopeptidases. R.G. Landes Company and Chapman & Hall.

Terra, W.R., Ferreira, C. 1994. Insect digestive enzymes: properties compartmentalization and function. Comp. Biochem. Physiol. 109B, 1-62.

Vadlamudi, R. K., Weber, E., Inhae, J., Tae, H. J., Bulla, L. A ., 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270, 5490-5494.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D., Van Mellaert, H., 1989. Specificity of *Bacillus thuringiensis* δ–endotoxins: importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. 186, 239-247.

Wolfersberger, M. G., Luthy, P., Maurer, A., Parenti, P., Sacchi, V. F., Giordana, B., Hanozet, G. M., 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol.86, 301-308.

Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., Iwahana, H., Sato R., 1997.Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. Eur. J. Biochem. 246, 652-657.

Zhu, Y-C., Kramer, K.J., Oppert, B., Dowdy, A.K. cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins. Insect Biochem. Molec. Biol. 30, 215-224.

CHAPTER 2

BACILLUS THURINGIENSIS CRY1AC AND CRY1FA δ-ENDOTOXIN BINDING TO A NOVEL 110 KDA AMINOPEPTIDASE IN *HELIOTHIS VIRESCENS* IS NOT N-ACETYLGALACTOSAMINE MEDIATED [1]

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[1] Banks, D. J., Jurat-Fuentes, J. L., Dean, D. H., Adang M. J. 2001. Insect Biochemistry and Molecular Biology 31, 909-918.

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ABSTRACT

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

Key words: *Bacillus thuringiensis*, *Heliothis virescens*, Cry1, δ-endotoxin, aminopeptidase, binding protein, N-acetylgalactosamine, GalNAc

1.0 INTRODUCTION

Bacillus thuringiensis Cry1Fa and Cry1Ac δ-endotoxins have overlapping yet distinct ranges of insecticidal activities. Cry1Fa δ-endotoxin is highly toxic to *Heliothis virescens*, *Spodoptera exigua*, and *Ostrinia nubilalis*, but not *Helicoverpa zea* (Chambers *et al.,* 1991). Cry1Ac δ-endotoxin is also highly toxic to *H. virescens, O. nubilalis* and *H. zea* (Van Rie *et al*., 1989, Garczynski *et al.,* 1991), but not *S. exigua* (Luo *et al.,* 1999a). Surface contamination bioassays indicate that the LC50 values for Cry1Ac and Cry1Fa in *H. virescens* are 0.040 and 0.031 ng of toxin per square millimeter, respectively (Chambers *et al*., 1991).

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

A Cry1Ac binding protein was first identified as a 120 kDa aminopeptidase N (APN) (EC. No. 3.4.11.2) in *Manduca sexta* (Sangadala *et al.,* 1994; Knight *et al.,* 1994). APN is an exopeptidase that hydrolyzes neutral amino acids from the amino terminus of proteins. Cry1Ac binds to a N-acetylgalactosamine (GalNAc) residue on 120 kDa APN. This conclusion is based on two lines of evidence. First, Cry1Ac binding to the 120 kDa APN is specifically inhibited by N-acetylgalactosamine, but not other sugars (Garczynski *et. al*., 1991; Sangadala *et al.* 1994; Knight *et al.,* 1994). Second, Cry1Ac domain III mutants such as ^{509}QNR^{511}-AAA (Jenkins *et al*., 1999) and Tmut (Burton *et*

al., 1999) that are defective in GalNAc binding do not recognize the 120 kDa APN.

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

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

approach to delay the onset of field resistance is utilization of multiple toxins to target different binding proteins (Peferoen, 1997).

In this study we determined that Cry1Ac and Cry1Fa recognize common 110, 120, and 170 kDa binding proteins in *H. virescens*. We identify the 110 kDa binding protein as a novel APN with unusual characteristics. The mature 110 kDa APN has a variant APN consensus sequence unlike other described lepidopteran APNs that bind Cry toxins. Perhaps most interesting is that the 110 kDa APN appears to lack GalNAc, yet is recognized by Cry1Ac and Cry1Fa. This finding adds credence to the proposal that Cry1Ac-induced membrane permeation proceeds by both GalNAc dependent and GalNAc independent mechanisms (Carroll *et al.*,1997). We further show that a Cry1Ac mutant which was designed with an altered GalNAc binding pocket, yet maintains toxicity (Lee *et al* 1999), still binds to the 110 and 170 kDa APNs. These two APNs are therefore implicated in Cry1Ac toxicity to *H. virescens*. Additionally, a *H. virescens* >210 kDa molecule shown to be recognized by Cry1Ac and Cry1Fa is similar in size to a previously described cadherin-like binding protein in *M. sexta* (Vadlamudi *et al*., 1995).

2.0 MATERIALS AND METHODS

2.1 Toxin purification

A *B. thuringiensis* strain harboring the *cry1Fa* gene (GenBank accession number M63897) was provided by Ecogen, Inc. (Chambers *et al.,* 1991). *B. thuringiensis* strain HD-73 carrying the *cry1Ac* gene was obtained from the *Bacillus* Genetic Stock Culture Collection (Columbus, Ohio). *B. thuringiensis* strain MR522 producing Cry1Ea was obtained from Dow Agrosciences (San Diego, CA). *B. thuringiensis* strains were grown at 28°C in ½-strength tryptic soy broth (Difco) until cell sporulation and lysis. The crystal-spore mixture was washed two times in 1M NaCl, 0.1% Triton X-100 and two times in de-ionized water. Toxins were purified according to Luo *et al.* (1999). The Cry1Ac domain III mutant ^{509}QNR^{511}-AAA was purified according to Lee *et. al*. (1999). Protein concentrations were determined with a Bio-Rad protein assay kit: bovine serum albumin (BSA) was used as the standard, as described by Bradford (1976).

2.2 Toxin biotinylation

Cry1Ac and Cry1Fa toxins were biotinylated according to Denolf *et al.* (1993) with the following modifications. Sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce) was dissolved in 20 mM Na_2CO_3, pH 9.7, mixed with toxin at a molar ratio of 1:30 for 30 minutes at room temperature. Free biotin was removed with overnight dialysis in 20mM Na_2CO_3, 150 mM NaCl, pH 9.7 at 4ºC. Biotinylation was confirmed by western blot analysis. Briefly, 2µg of biotinylated toxins were separated on sodium dodecyl sulfate (SDS) 10%

polyacrylamide gel electrophoresis (PAGE) and electroblotted to polyvinylidene difluoride (PVDF) (Bio-Rad) membrane in 50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol, pH 8.3. The membrane was blocked with phosphate buffered saline (PBS; 140 mM NaCl, 2.5 mM KCl, 5.5 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) containing 0.1% Tween 20 (PBST) and 3% BSA, then incubated with anti-biotin conjugated alkaline phosphatase antibody for one hour at room temperature. The membrane was then washed three times with PBST plus 0.1% BSA and color developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate.

2.3 Toxin iodination

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

2.4 Preparation of brush border membrane vesicles

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

2.5 Affinity chromatography

The affinity purification method of Luo *et al.* (1996) was followed with minor modifications. Between 8 and 10 mg of toxin was bound to 1g of activated CNBr sepharose beads. The identity of toxins used for affinity chromatography was confirmed by Western blot using anti-Cry1Fa antiserum. Fifteen mg of BBMV proteins prepared within 1 day of binding protein purification were solubilized in CHAPS-containing buffer (Luo *et al*., 1996). This buffer also contained 100 mM NaCl and the following proteinase inhibitor cocktail: 4 mM Pefabloc SC AEBSF (Boehringer Mannheim), 5 μM leupeptin (Pierce), 5 μM pepstatin (Pierce), and 5 μM antipain (Pierce). Bound proteins were eluted with 10 ml of 2 M NaSCN in 20 mM Na_2 CO_3, pH 9.6. The eluate was diluted to 20 ml in 20 mM Na_2CO_3 pH 9.6, then concentrated using a Centriprep-30 ultra-filtration device (Amicon) at 4°C and finally stored at -80°C until needed for experimentation.

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

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

for one hour. Cry1Ac binding proteins that were recognized by biotinylated Cry1Fa were visualized with NBT/BCIP substrate.

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

Eluted Cry1 binding proteins were separated on SDS-8 % PAGE and transferred to PVDF membrane as above. The membrane was probed with anti-APN antiserum for one hour. Anti-APN serum was raised in rabbits against a 31 kDa truncated form of the *M. sexta* 120 kDa APN expressed in *E.coli* (Luo *et al.,* 1999b). The membrane was then washed and probed with goat-anti-rabbit-conjugated alkaline phosphatase. Molecules recognized by anti-APN antibody were visualized with NBT/BCIP substrate.

2.8 Lectin blot of H. virescens BBMV proteins

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

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

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

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

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

2.11 Amino acid sequence analyses

H. virescens BBMV proteins (15 μg) were separated on SDS-8% PAGE and electroblotted to PVDF membrane. The membrane was rinsed in water, briefly stained in amido black, destained in 5% acetic acid for 20 minutes, and washed three times in water. The 110 kDa band was excised and submitted to Dr.

Michael Berne (Department of Physiology and Medicine, Tufts University, Boston, MA) for N- terminal sequencing.

H. virescens BBMV proteins (320 μg) were separated by SDS-8% PAGE. The 110 kDa band was excised and digested with *Staphylococcus aureus* V8 protease according to the method of Cleveland *et al*. (1977). The digested protein was then separated by SDS-12% PAGE and blotted to PVDF membrane as above. Bands of 19 kDa, 28 kDa and 32 kDa were excised and submitted for N- terminal sequencing.

2.12 PCR cloning of a 110 kDa binding protein fragment

Midguts from early fifth instar *H. virescens* larvae were dissected and incubated in RNAlater (Ambion) on ice for one hour. Total RNA was prepared from 100 mg midguts with a RNeasy kit (Qiagen), and cDNA was made with a $Nd(T)_{18}$ primer, 0.25 mM each dNTP, and Superscript reverse transcriptase (Life Technologies) according to the conditions suggested by the manufacturer. Degenerate primers (synthesized by IDT, Inc.) for amplification of internal sequence of the 110 kDa binding protein were based on the peptide sequence TNLDEPA (5' AC(A/C/G/T) AA(C/T) CT(A/C/G/T) GA(C/T) GA(A/G) CC(A/G/C/T) GC3'; forward primer) and the APN consensus sequence GAMENWG (5' CCC CA(A/G) TT(C/T) TCC AT(A/T/G/C) GC(A/T) CC 3'; reverse primer). Codon usage was based on the 120 kDa APN sequence (Gill *et al*., 1995). PCR was conducted in 50 mM KCl, 10 mM Tris-HCl, 15mM $MgCl_2$, 0.25 mM each dNTP (pH 8.3), 1U Taq polymerase (Eppendorf) with 40 cycles of

94°C for 1 minute, 57°C for 1 minute, and 72°C for one minute. PCR products that were unique to forward plus reverse primer amplifications were gel purified with a Qiaex II gel extraction kit (Qiagen), and cloned into the pCR 2.1 TA vector (Invitrogen). Plasmid inserts were sequenced at the Molecular Genetics Instrumentation Facility (University of Georgia).

3.0 RESULTS

3.1 Affinity chromatography purification of H. virescens binding proteins

Cry1Ac and Cry1Fa toxin affinity chromatographies were used to purify binding proteins from *H. virescens* midgut brush border membranes. CHAPS-solubilized BBMV were applied to toxin affinity columns. Bound proteins were eluted with 2M NaSCN, separated by SDS-8 % PAGE and coomassie stained. Proteins of 110, 120 and 170 kDa were eluted from Cry1Ac and Cry1Fa columns (Fig. 1.1A, lanes 3 and 5 respectively). Additionally, Cry1Ac and Cry1Fa selected small amounts of two proteins of about 100 kDa. The 60 kDa protein present in both the Cry1Ac and Cry1Fa eluates corresponds to toxin leached from the beads during elution with high salt. Cry1Ea was used as a negative control toxin because Cry1Ea does not to bind to *H. virescens* BBMV (Van Rie *et al.,* 1990). No *H. virescens* BBMV proteins were purified by Cry1Ea affinity chromatography (data not shown).

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

We confirmed that Cry1Fa recognized proteins eluted from the Cry1Ac column by ligand blotting. The 110, 120 and 170 kDa proteins present in the Cry1Ac eluate were recognized by biotinylated Cry1Fa. (Fig. 1.1B, lane 2). As a control, Cry1Ac toxin affinity chromatography was conducted in the absence of solubilized BBMV. The affinity eluate contained only 60 kDa Cry1Ac. Ligand blot analysis showed that the Cry1Ac was recognized by biotinylated Cry1Fa (data not shown). This result indicates that the signal at 60 kDa resulted from biotinylated Cry1Fa interacting with Cry1Ac that eluted from the column. Additionally, minor proteins below 60 kDa are visible in this ligand blot. These smaller proteins are likely degradation products of purified binding proteins that are still recognized by toxin. Alternatively, these bands could be binding proteins that were not visible after coomassie staining, but were visualized with more sensitive ligand blotting. Unexpectedly, biotinylated Cry1Fa recognized a molecule of >210 kDa, although a molecule of that molecular size was not visible on coomassie stained gels of either total BBMV or Cry1-selected molecules (Fig. 1.1A). A >210 kDa molecule was previously detected when ligand blots of *H. virescens* BBMV were probed with Cry1Ac and Cry1Fa (Jurat Fuentes and Adang, 2001).

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

Since the 170 and 120 kDa molecules are forms of APN (Gill *et al.,* 1995; Luo *et al.,* 1997), proteins eluted from the Cry1Fa column were probed with anti-APN antibody. In addition to the expected recognition of 170 and 120 kDa proteins, the 110 kDa protein was also recognized by anti-APN antiserum (Fig. 1.1B, lane 1). The two minor binding proteins near 100 kDa that were identified in the Cry1Ac and Cry1Fa eluates with coomassie stained SDS PAGE (Fig. 1A, lanes 3 and 5) were also identified by anti-APN antibody (Fig. 1.1B, lane 1). These two proteins are also visible in total BBMV (Fig. 1.1A, lane 1), and are apparently additional binding proteins that are also APN's.

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

Lee et al. (1996) previously showed that of all *H. virescens* BBMV proteins, only the 170 and 120 kDa proteins strongly cross-reacted with the lectin, soybean agglutinin. When we probed *H. virescens* BBMV proteins on blots with soybean agglutinin, we also observed strong cross-reactivity to 170 and 120 kDa proteins (Fig. 1.2, lane 2). Since soybean agglutinin specifically recognizes vicinal GalNAc moieties, the 170 and 120 kDa proteins are probably glycoproteins containing terminal GalNAc residues. Although a faint signal is visible in the 110 kDa region, the relative degree of recognition compared to the 170 and 120 kDa APNs was nominal.

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

To examine the relationship between GalNAc on APN molecules and binding of Cry1Ac toxin to these molecules, we performed ligand blot experiments using ^{125}I-labeled Cry1Ac and the Cry1Ac mutant ^{509}QNR^{511}-AAA. Our reasoning was based on evidence that Cry1Ac binding to 170 kDa APN is mediated by GalNAc (Luo et al. 1997) and that the Cry1Ac mutant ^{509}QNR^{511}-AAA is impaired in GalNAc recognition (Jenkins et al. 1999). Figure 1.2 shows ^{125}I-Cry1Ac (lane 3) and ^{125}I- ^{509}QNR^{511}-AAA ligand blots (lane 4) of *H. virescens* BBMV. Relative to Cry1Ac, ^{509}QNR^{511}-AAA recognition of the 120 kDa APN is nearly abolished, recognition of the 170 kDa APN is reduced, and recognition of the 110 kDa APN is enhanced. Additionally, a >210 kDa molecule was also recognized by ^{509}QNR^{511}-AAA, and to a lesser extent by Cry1Ac. These results support a model whereby Cry1Ac binding to the 120 and 170 kDa APNs, but not the 110 kDa APN, is primarily mediated by GalNAc.

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

Periodate treatment was used to determine if Cry1Ac binds to carbohydrates on proteins in *H. virescens* BBMV. Periodate treatment, which oxidizes and disrupts sugar structures on glycoproteins, has been used to map carbohydrate epitopes of antibodies by disrupting the sugar ring structure without destroying the peptide chain (Woodward *et al*., 1985). Garczynski (1999) discovered that periodate treatment eliminated Cry1Ac binding to 120 kDa APN

from *M. sexta*, indicating Cry1Ac dependence on carbohydrate recognition. Since GalNAc was not detected on the 110 kDa APN (Fig 1.2., lane 2) and ^{509}QNR^{511}-AAA bound to 110 kDa APN (Fig 1.2., lane 4), we predicted that ^{125}I-Cry1Ac and ^{125}I-^{509}QNR^{511}-AAA should bind periodate-treated 110 kDa APN independent of carbohydrate recognition. Figure 1.2 shows that ^{125}I-Cry1Ac bound to the 110 kDa APN and the >210 kDa molecule, but not to 120 APN (Fig 1.2., lane 5). Periodate treatment reduced ^{125}I-Cry1Ac binding to the 170-kDa APN. In contrast, periodate treatment had little, if any effect, on ^{125}I-^{509}QNR^{511}-AAA binding to BBMV molecules (Fig. 1.2, lane 6). To confirm that periodate treatment modified GalNAc moieties, periodate-treated BBMV proteins were probed with soybean agglutinin. No BBMV molecules were detected by this lectin after periodate treatment (data not shown). These results obtained using periodate treated blots provide further support for the conclusion that Cry1Ac binding to the 120 and 170 kDa APNs is primarily GalNAc mediated. However, this is not the case for 110 kDa APN and >210 kDa molecule recognition.

3.7 110 kDa APN N-terminal sequencing

N-terminal sequencing revealed that the 110 kDa protein from total BBMV contains the residues VIQTGQ(C)NENIQVVTGFNLD. No homologous sequences were identified with database searches of this sequence. Gill et al. (1995) describe the N-terminal sequence of a 105 kDa Cry1Ac-binding protein from *H. virescens* as being different from other APN sequences. It is likely that the 105 kDa and 110 kDa proteins are synonymous. To obtain additional amino

acid sequence, the 110 kDa molecule from total BBMV was subjected to V8 protease digestion. This procedure yielded three major fragments of 19, 28 and 32 kDa (data not shown). The N-terminal sequence of the 19 kDa fragment contained the same sequence as the intact 110 kDa. The 28 kDa fragment contained the N-terminal residues ETATVAGIPNFNI. No sequence homologies were identified with database searches of either of these sequences. The 32 kDa fragment contained the N-terminal residues TNLDEPAYRLRDVVY. This fragment sequence shares 100% homology with amino acids 37-51 of *H. punctigera* APN 2 (Emmerling *et al*., GenBank accession # AAF37559). The presence of APN sequence in the 32 kDa fragment released from the 110 kDa protein accounts for detection by anti-APN antiserum.

3.8 SDS-6% PAGE and silver staining of sub-microgram quantities of H. virescens BBMV proteins and Cry1Ac affinity chromatography eluate

The amino acid sequences derived from proteinase digestion of the 110 kDa protein led us to suspect that two BBMV proteins were co-migrating on SDS-8% PAGE. To resolve the two proteins, sub-microgram concentrations of BBMV were separated with SDS-6% PAGE and silver stained. Fig. 1.2C shows 0.25 μg of BBMV separated with SDS-6% PAGE (lane 1). The 110 kDa band that appears as one protein when separated by SDS-8% PAGE (see Fig. 1.2A, lane 1) is resolved as two distinct bands under these conditions. For comparison, sub-microgram concentrations of Cry1Ac eluate were also separated by SDS-6% PAGE and silver stained (Fig. 1.2C, lane 2). The Cry1Ac eluate contains the 170

and 120 kDa proteins, but only the lower co-migrating 110 kDa protein. The 110 kDa binding protein in the Cry1Ac eluate was also recognized by anti-APN antibody (see Fig. 1.2B, lane 1), indicating that toxin binds to the 110 kDa APN, and not the other unidentified protein.

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

PCR was conducted with *H. virescens* cDNA as template, TNLDEPA forward degenerate primer, and GAMENWG reverse degenerate primer. The forward primer was designed from internal 110 kDa APN sequence that shared 100% homology with *H. punctigera* APN 2. The reverse primer was designed from the lepidopteran internal APN consensus sequence GAMENWG. We were confident that the 110 kDa molecule would contain this sequence once we determined that this molecule was an APN. All lepidopteran APN sequences share this motif, which is always located between 960 and 985 nucleotides from the start methionine (Gill *et al.,* 1995; Oltean *et al.,* 1999; Chang *et al.,* 1999; Yaoi *et al.,* 1999). Additionally, all eucaryotic alanine/arginine aminopeptidases share this motif (Taylor, 1996). The PCR should thus yield an approximately 0.9 kb product. Amplification with forward primer alone yielded 0.65 kb product (Fig. 1.3, lane 1) and PCR with reverse primer alone yielded 0.35 kb and 1.0 kb products (Fig. 1.3, lane 2). PCR with both forward and reverse primers yielded unique 0.45 kb and 0.9 kb products (Fig. 1.3, lane 3). Lane 4 shows a 2.0 kb PCR product generated with non-degenerate internal primers designed from the 120

kDa APN sequence (Gill et al., 1995), which served as a positive control. The 0.45 kb and 0.9 kb products were cloned into the pCR2.1 TA vector yielding pCR0.45 and pCR0.9 plasmids. Sequence derived from pCR0.45 insert shares 98% homology with *H. virescens* 170 kDa APN. Sequence derived from pCR0.9 insert shares 89% homology with *H. punctigera* APN 2. Sequence derived from both the pCR0.45 and pCR0.9 inserts contained the nucleotides corresponding to the GAMENWG sequence. However, only the pCR0.9 insert contained the nucleotides corresponding to the TNLDEPA sequence found in the V8 digested 32 kDa fragment N-terminus. These results indicate that the 0.9 kb PCR product represents an internal sequence from the 110 kDa APN.

4.0 DISCUSSION

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

Our results support the multiple receptor model proposed by Van Rie *et al*. (1989) which characterizes Cry1Ac as binding to at least three populations of receptors in the brush border membrane of *H. virescens*. Subsequent studies by Luo *et al*. (1997) and Oltean *et al*. (1999) identify Receptor A as being composed of the 170 kDa APN and/or the 130 kDa APN. The 120 kDa APN characterized in detail by Gill et al. (1995), and the 110 kDa APN described here are candidates for the other two populations of receptors, as well as the >210 kDa molecule visualized on our ligand blots. Detailed binding experiments performed with various Cry1 toxins are required before assignments of receptor type can be made for the 110, 120 and >210 kDa molecules.

The abundance of the 110, 120 and 170 kDa APNs were relatively equivalent in *H. virescens* BBMV (see Fig. 1.1). However, the 110 and 120 kDa binding proteins constituted the main binding proteins purified with Cry1Ac and Cry1Fa affinity chromatography, while the 170 kDa APN was purified at a relatively much lower level. The low abundance of the 170 kDa APN could be related to elution conditions. For example, Luo *et al*. (1997) eluted *H. virescens* BBMV proteins from a Cry1Ac affinity column with GalNAc instead of NaSCN, yielding a larger yield of the 170 kDa protein relative to the 110 and 120 kDa proteins. Therefore, the relatively low abundance of the 170 kDa APN in the eluate could be related to elution with high salt, rather than GalNAc. A second possibility is that Cry1Ac and Cry1Fa have greater affinity for the 110 and 120 kDa proteins relative to the 170 kDa protein. This explanation is supported by

Oltean et al (1999), who found that Cry1Ac has low affinity binding to the 170 kDa APN.

Where examined previously, all Cry1Ac-binding APNs contain GalNAc moieties (Garczynski *et al*., 1991; Knight *et al*., 1994; Lee *et al*., 1996). However, in this study we present evidence that GalNAc is not required for Cry1Ac binding to the *H. virescens* 110 kDa APN. While Cry1Ac and Cry1Fa bound the 110 kDa APN, this molecule was not recognized by the GalNAc-binding lectin soybean agglutinin. These results were further supported by our experiments with the Cry1Ac mutant ^{509}QNR^{511}-AAA. This mutant was designed to remove the functional groups lining the lectin-like pocket predicted to be involved in GalNAc binding (Lee *et al*., 1999). While ^{509}QNR^{511}-AAA no longer binds the GalNAc-containing *M. sexta* 120 kDa APN (Jenkins *et al*, 1999), it does bind *M. sexta* BBMV and remains toxic to *M. sexta*, *H. virescens* and *Lymantria dispar* (Lee *et al.,* 1999). Therefore, ^{509}QNR^{511}-AAA binding and toxicity are not dependent on GalNAc recognition. A logical corollary follows. Namely, GalNAc-independent Cry1Ac recognition of a binding protein must exist. This binding protein may or may not be an APN. Here we show that GalNAc independent Cry1Ac binding to the *H. virescens* 110 kDa APN, 170 kDa APN, and an unidentified > 210 kDa molecule fulfil this requirement. Further work is required to determine the possible roles of the 110-kDa APN and >210 kDa molecule relative to the 170 kDa APN in pathogenicity. It should also be noted that both radiolabeled Cry1Ac and ^{509}QNR^{511}-AAA weakly recognized several BBMV proteins, as yet unidentified, smaller than 100 kDa.

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

The >210 kDa molecule bound by Cry1Fa and Cry1Ac did not cross react with anti-APN antibody, suggesting that this molecule is not an APN. Since ^{509}QNR^{511}-AAA bound to the >210-kDa molecule and periodate treatment did not reduce binding, it is likely that carbohydrates are not binding determinants on this molecule. Though the identity of the >210 kDa molecule in *H. virescens* BBMV is unknown, it is similar in molecular size to the Cry1A-binding cadherin-like molecule of *M. sexta* (Vadlamudi *et al*., 1997).

Our results may also explain the phenomenon described by Lee et al. (1999) where ^{509}QNR^{511}-AAA binding affinity to *H. virescens* BBMV was

reduced 15-22 fold, while toxicity was only reduced 3-4 fold. The reduction in ^{509}QNR^{511}-AAA binding to BBMV appears to be correlated with loss of binding to the 120 kDa APN and reduced binding to the 170 kDa APN, while the maintenance of toxicity may be related to continued binding to 110 and 170 kDa APNs.

Typically, the residues between the APN start methionine and the mature protein N-terminus consensus sequence are cleaved during post-translational modification, and are revealed only through cDNA analysis (Gill *et al*., 1995; Oltean *et al*., 1999). This modification yields the YRLPTTT APN consensus sequence at the N-terminus of the mature protein. Therefore, we were surprised when the 110 kDa molecule from total BBMV lacked the APN consensus sequence, yet this protein as well as the 110 kDa binding protein purified with toxin affinity chromatography were both recognized by APN antibody. This led us to suspect that two BBMV proteins co-migrated to 110 kDa on SDS-PAGE, one of which is an APN. Amino acid sequencing of peptides released by V8 protease digestion of the 110 kDa band yielded a 32 kDa peptide containing a variant APN N-terminal consensus sequence, as well as two other peptides that contained sequence with no known amino acid sequence homologies. The co-migrating 110 kDa proteins were resolved by separating a sub-microgram quantity of BBMV protein on SDS-6% PAGE followed by detection with silver staining. The smaller sized protein had the same electrophoretic mobility as the Cry1Ac-binding protein on SDS-6% PAGE. Since the 110 kDa binding protein purified from affinity chromatography was recognized by anti-APN antibody, this

indicates that Cry1Ac and Cry1Fa recognize the 110 kDa APN, and not the unidentified 110 kDa protein.

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

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

It is interesting to note that *H. punctigera* is confined to Australia, while *H. virescens* is restricted to North America. The most parsimonious explanation

for the APN sequence similarities between these two geographically isolated species is that a single APN gene duplication event occurred in an ancestral Noctuidae lineage before *Helicoverpa* and *Heliothis* diverged (*Heliothis* and *Helicoverpa* are both in Noctuidae). Chang *et al*. (1999) describe an APN gene duplication event in the ancestral Plutellidae and Sphingidae lineage that resulted in APN genes that are more similar between two species in these two families than within a single species. Although they compared APN genes in separate families, a similar phenomenon may explain the high sequence homology between the *H. virescens* 110 kDa APN and *H. punctigera* APN 2. That is, a gene duplication event in the ancestral Noctuidae lineage may have resulted in an APN gene that is more similar between *Heliothis* and *Helicoverpa* than it is with other APNs within either of these taxa alone.

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

5.0 ACKNOWLEDGEMENTS

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6.0 FIGURES

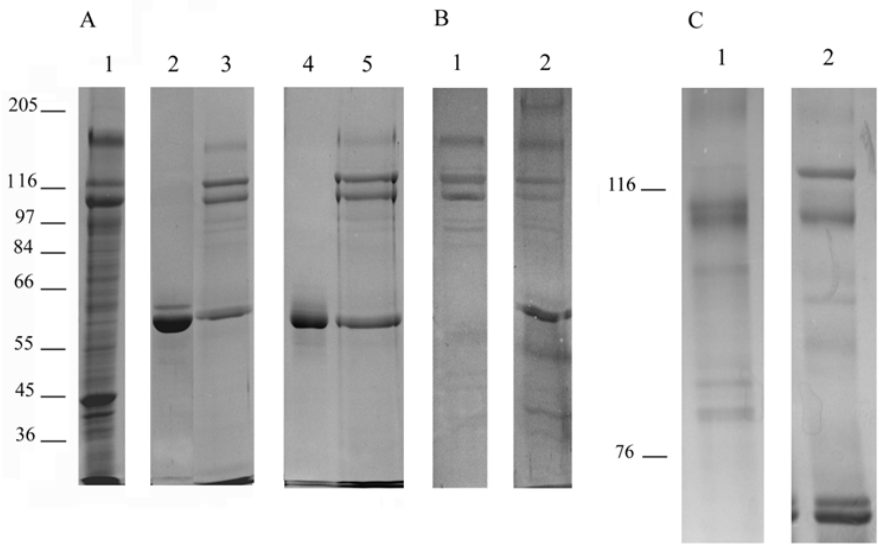

Fig. 1.1. SDS-8% PAGE of Cry1Ac and Cry1Fa toxin affinity chromatography purified *H. virescens* binding proteins, western blot analysis of Cry1Fa toxin affinity chromatography purified *H. virescens* BBMV binding proteins with anti-APN antibody, and ligand blot analysis of Cry1Ac toxin affinity chromatography purified *H. virescens* binding proteins with biotinylated Cry1Fa. SDS-6% PAGE silver staining of sub-microgram quantities of *H. virescens* BBMV and Cry1Ac toxin affinity chromatography purified *H. virescens* binding proteins. (A) *H. virescens* BBMV proteins (lane 1), Cry1Ac toxin (lane 2), eluted Cry1Ac *H. virescens* binding proteins (lane 3), Cry1Fa toxin (lane 4), eluted Cry1Fa *H. virescens* binding proteins (lane 5). (B) Eluted Cry1Fa *H. virescens* binding proteins were probed with anti-APN antibody (1°), goat-anti-rabbit conjugated alkaline phosphatase antibody (2°), and visualized with NBT/BCIP substrate (lane 1). Eluted Cry1Ac *H. virescens* binding proteins were probed with biotinylated Cry1Fa toxin (0.75 µg/mL), rabbit-anti-biotin conjugated alkaline phosphatase antibody, and visualized with NBT/BCIP substrate (lane 2). (C) *H. virescens* BBMV proteins (0.25 µg) (lane 1) and Cry1Ac affinity chromatography eluate (0.5µg) (lane 2) were separated by SDS-6% PAGE, fixed overnight and silver stained the following day.

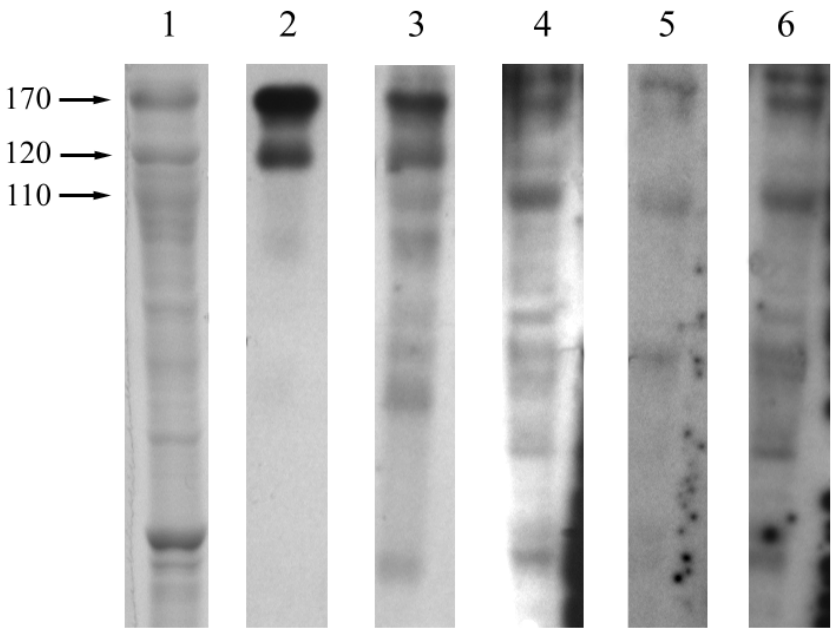

Fig. 1.2. Ligand blot analysis of *H. virescens* BBMV proteins. SDS-8% PAGE separated BBMV proteins alone are shown in lane 1. Ligand blots were conducted with soybean agglutinin (lane 2), ^{125}I- Cry1Ac (lane 3), and ^{125}I-^{509}QNR^{511}-AAA (lane 4). Ligand blots of periodate treated *H. virescens* total BBMV were also conducted with ^{125}I- Cry1Ac (lane 5), and ^{125}I- ^{509}QNR^{511}-AAA (lane 6).

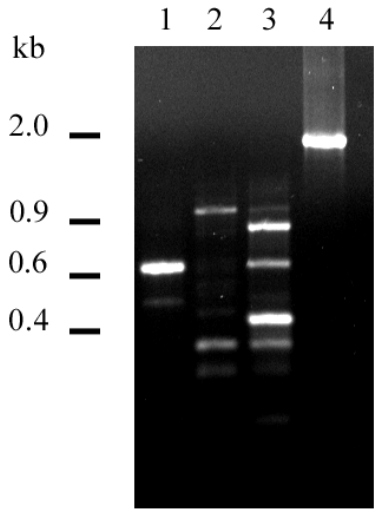

Fig. 1.3. 1.0 % agarose gel separated PCR products. PCR products of forward primer alone (lane 1), reverse primer alone (lane 2), forward plus reverse primers (lane 3), and non-degenerate primers for amplification of an internal sequence of *H. virescens* 120 kDa APN (lane 4) were agarose gel separated and stained with ethidium bromide.

7.0 REFERENCES

Bradford, M.M., 1976. A rapid and sensitive method for the quantitaation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Ballester, V., Granero, F., Tabashnik, B. E., Malvar, T., Ferre, J. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamond back moth (*Plutella xylostella*). Appl. Environ. Microbiol. 65, 1413-1419.

Burton, S. L., Ellar, D. J., Li, J., Derbyshire, D. J., 1999. N- acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Molec.. Biol. 287, 1011-1022.

Carroll, J., Wolfersberger, M. G., Ellar, D. J., 1997. The *Bacillus thuringiensis* Cry1Ac toxin-induced permeability change in *Manduca sexta* midgut brush border membrane vesicles proceeds by more than one mechanism. J. Cell Sci. 110, 3099-3104.

Chambers, J. A., Jelen, A., Gilbert, M. P., Jany, C. S., Johnson, T. B., Gawron-Burke, C., 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J. Bacteriol. 173, 3966-3976.

Chang, W. X. Z., Gahan, L. J., Tabashnik, B. E., Heckel, D. G., 1999. A new aminopeptidase from diamondback moth provides evidence for a gene duplication event in Lepidoptera. Insect Molec. Biol. 8, 171-177.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., Laemmli, U. K., 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102-1106.

Denolf, P., Jansens, S., Van Houdt, S., Peferoen, M., Degheele, D., Van Rie, J., 1993. Biotinylation of *Bacillus thuringiensis* insecticidal crystal proteins. Appl. Environ. Microbiol. 59,1821-1827.

Garczynski, S.F., 1999. Detection and characterization of Cry1Ac binding molecules in brush border membrane vesicles isolated from the midguts of *Manduca sexta* larvae. PhD dissertation, University of Georgia.

Garczynski, S. F., Crim, J.W., Adang, M. J., 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ–endotoxin by protein blot analysis. Appl. Environ. Microbiol. 57, 2816-2820.

Gill, S. S., Cowles, E. A, Francis, V., 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J. Biol. Chem. 270, 27277-27282.

Gould, F., Anderson, A., Reynolds, A., Bumgarner, L., Moar, W., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545-1559.

Granero, F., Ballester, V., Ferre, J. 1996. *Bacillus thuringiensis* crystal protein Cry1Ab and Cry1Fa share a high affinity binding site in *Plutella xylostella* (L.). Biochem. Biophys. Res. Commun. 224, 779-783.

Jenkins, J. L., Lee, M. K., Sangadala, S., Adang, M. J., Dean, D. H., 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Letters 462, 373-376.

Knight, P. J. K., Crickmore, N., Ellar, D. J., 1994. The receptor for *Bacillus thuringiensis* Cry1Ac delta-endotoxin in the brush border of the lepidopteran *Manduca sexta* is aminopeptidase J. Molec. Microbiol. 11, 429-436.

Knowles, B. H., Knight, P. J. K., Ellar, D. J., 1991. N-acetylgalactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. Proc. R. Soc. Lond. B 245, 31-35.

Knowles, B. H., Dow, J. A. T., 1993. The crystal δ–endotoxin of *Bacillus thuringiensis*: models for their mechanisms of action on the insect gut. Bioessays 15, 469-476.

Lee, M. K., You, T. H., Young, B. A., Cotril, J. A., Valaitis, A. P., Dean, D. H., 1996. Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* Cry1Ac toxin. Appl. Environ. Microbiol. 62, 2845-2849.

Lee, M. K., You, T. H., Gould, F. L., Dean, D. H., 1999. Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. Appl. Environ. Microbiol. 65, 4513-4520.

Luo, K., Yang-Jiang, L., Adang, M. J., 1996. A 106 kDa form of aminopepidase is a receptor for *Bacillus thuringiensis* Cry1C δ–endotoxin in the brush border membrane of *Manduca sexta*. Insect Biochem. Molec. Biol. 26, 783-791.

Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R., Adang, M. J., 1997. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* δ–endotoxin binding and pore formation. Insect Biochem. Molec. Biol. 27, 735-743.

Luo, K., Banks, D. J, Adang, M. J., 1999a. Toxicity, binding and permeability analyses of four *Bacillus thuringiensis* Cry1 δ–endotoxins using brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. Appl. Environ. Microbiol. 65, 457-464.

Luo, K., McLachlin, J. R., Brown, M. R., Adang, M. J., 1999b. Expression of a glycosylphosphatidylinositol-linked *Manduca sexta* Aminopeptidase N in insect cells. Protein Expression and Purification 17, 113-122.

Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S., Kogure, M., 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal Cry1Aa toxin. Biosci. Biotechnol. Biochem. 62, 727-734.

Oltean, D. I., Pullikuth, A.K., Lee, H. K., Gill, S. S., 1999. Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. Appl. Environ. Microbiol. 65, 4760-4766.

Peferoen, M., 1997. Progress and prospects for field use of Bt genes in crops. Trends in Biotechnology 15, 173-177.

Rajamohan, F., Cotrill, J. A., Gould, F., Dean, D. H., 1996a. Role of domain II, loop 2 residues of *Bacillus thuringiensis* Cry1Ab δ–endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J. Biol. Chem. 271, 2390-2396.

Rajamohan, F., Alzate, O., Cotrill, J. A., Curtiss, A., Dean, D. H., 1996b. Protein engineering of *Bacillus thuringiensis* δ–endotoxin: mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. Proc. Natl. Acad. Sci. USA 93, 14338-14343.

Sangadala, S., Walters, F. S., English, L. H., Adang, M. J., 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal Cry1Ac toxin binding and 86Rb+-K+ efflux in vitro. J. Biol. Chem. 269, 10088-10092.

Tabashnik, B. E., Malvar, T., Liu, Y. B., Finson, N., Borthakur, D., Shin, B. S., Park, S. H., Masson, L., De Maagd, R. A., Bosch, D., 1996. Cross-resistance of the diamondback moth indicates altered interaction with domain II of *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 62, 2839-2844.

Taylor A. (1996) Aminopeptidases. R.G Landes Company and Chapman & Hall.

Vadlamudi, R. K., Weber, E., Inhae, J., Tae, H. J., Bulla, L. A ., 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270, 5490-5494.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D., Van Mellaert, H., 1989. Specificity of *Bacillus thuringiensis* δ–endotoxins: importance of specific

receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. 186, 239-247.

Wolfersberger, M. G., Luthy, P., Maurer, A., Parenti, P., Sacchi, V. F., Giordana, B., Hanozet, G. M., 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol.86, 301-308.

Woodward, M.R., Young, W.W., Bloodgood R.A., 1985. Detection of monoclonol antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78, 143-153.

Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., Iwahana, H., Sato R., 1997.Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. Eur. J. Biochem. 246, 652-657.

CHAPTER 3

CLONING OF A *HELIOTHIS VIRESCENS* 110 KDA AMINOPEPTIDASE N AND EXPRESSION IN *DROSOPHILA* S2 CELLS [1]

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ABSTRACT

We previously identified a novel *Heliothis virescens* 110 kDa aminopeptidase N (APN) that binds *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ-endotoxins, and cloned an internal region of the 110 kDa APN gene (Banks *et al*., 2001). Here we describe the RACE-PCR cloning and sequence of a cDNA encoding 110 kDa APN. The 110 kDa APN gene was transiently co-expressed with green fluorescent protein (GFP) in *Drosophila* S2 cells using the pIZT expression vector. Enrichment of total membranes purified from S2 cells transfected with the 110 kDa APN gene had four-fold increased APN enzymatic activity relative to enriched total membranes purified from S2 cells transfected with vector-alone. Some S2 cells transfected with the 110 kDa APN gene bound rhodamine-labeled Cry1Ac toxin, whereas no S2 cells transfected with vector-alone bound rhodamine-labeled Cry1Ac toxin. This indicates that toxin binding to whole cells is APN mediated. However, flow cytometry and microscopy indicated that 110 kDa APN transfected S2 cells exposed to Cry1Ac or Cry1Fa toxin did not experience an increase in membrane permeability, indicating APN transfected cells were resistant to toxin. This suggests while the *H. virescens* 110 kDa APN functions as a Bt toxin binding protein, it does not mediate cytotoxicity when expressed in S2 cells.

Key words: *Bacillus thuringiensis*, toxin, Cry1Ac, Cry1Fa, *Heliothis virescens*, binding protein, aminopeptidase, APN, *Drosophila* S2 cells

1.0 INTRODUCTION

Bacillus thuringiensis (Bt) δ-endotoxins, the only recombinant proteins used in commercially available transgenic plants for insect control, are highly effective against target insect pest species (Schnepf, *et al*., 1998). To maintain this efficacy and to prevent the resistance of target species to transgenic Bt crops, the identity of putative Bt receptors and the interaction of Bt toxins with these receptors must be elucidated. This is based on the strategy of targeting different Bt receptors with different toxins to maintain susceptibility in the field (Peferoen, 1997).

The identity of the functional Bt receptor(s) remains unsettled. Both aminopeptidase N (APN) molecules (Sangadala *et al*., 1994; Knight *et al*., 1994; Gill *et al*., 1995; Luo *et al*., 1997) and cadherin-like molecules (Vadlamudi *et al*., 1993; Nagamatsu *et al*., 1999) derived from target insect brush border membrane vesicles (BBMV) have been shown to specifically bind Bt toxins. The evidence in support of cadherin-like molecules as Bt receptors that mediate toxicity is compelling. For example, recombinant *Manduca sexta* BT-R_1 cadherin-like molecule has been shown to specifically bind Cry1Aa toxin (Keeton *et al*., 1997; Meng *et al*., 2001). Additionally, Cry1Aa increased cell membrane currents as measured by whole-cell patch-clamp in Sf9 cells expressing the *Bombyx mori* cadherin-like molecule, BtR175, on the cell membrane (Nagamatsu *et al*., 1999). Evidence that APNs function as Cry1 receptors is based on *in vitro* binding and membrane reconstitution experiments. The *H. virescens* 170 kDa APN mediates pore formation in membrane vesicles (Luo *et al*., 1997), and the *M. sexta* 120 kDa

APN complex mediates channel formation in planar lipid bilayers (Schwarz *et al*., 1997). Since both of these phenomena are indicative of toxicity, it is logical to predict that APNs also mediate cytotoxicity *in vivo*. However, previous research addressing this hypothesis has not yielded results that implicate APNs as mediating cytotoxicity *in vivo* (Denolf *et al*., 1997; Simpson *et al*., 2000).

Three APNs that bind Cry1 toxins have been identified in *H. virescens*: 120 kDa APN (Gill *et al*., 1995), 170 kDa APN (Luo *et al*., 1997; Oltean *et al*., 1999) and 110 kDa APN (Banks *et al*., 2001). The *H. virescens* 110 kDa APN shares 89% sequence identity with *Helicoverpa punctigera* APN 2 (Emmerling *et al*., 2001), yet these two APNs share only 27% sequence identity with the most closely linked group of APNs (Emmerling *et al*., 2001). We predicted that the *H. virescens* 110 kDa APN mediates Cry1Ac and Cry1Fa toxicity *in vivo*. This prediction was based on the results of our previous research that demonstrated Cry1Ac and Cry1Fa toxin binding to the 110 kDa APN was not carbohydrate-dependent (Banks *et al*., 2001). This is relevant because Cry1Ac toxin binding to the *Manduca sexta* 120 kDa APN can be inhibited by free N-acetylglucosamine (GalNAc) in ligand blots (Garczynski *et al*., 1991), and in surface plasmon resonance experiments (Masson *et al*., 1995). Additionally, GalNAc inhibited 70% of Cry1Ac binding to the *H. virescens* 170 kDa APN in surface plasmon resonance (Luo *et al*., 1997). These results indicated that Cry1Ac toxin contains a lectin-like epitope that recognizes a sugar moiety on APN, and that toxin binding is dependent on carbohydrate recognition. However, data also indicated that toxin recognition of GalNAc on putative receptors is not essential to mediate toxicity.

For example, Lee et al (1999) constructed a Cry1Ac domain III ^{509}QNR-AAA^{511} mutant with an altered GalNAc binding pocket. Unexpectedly, this mutant retained toxicity to both *M. sexta* and *H. virescens*, suggesting that Cry1Ac toxicity is mediated by a receptor independent of GalNAc recognition. We then demonstrated that Cry1Ac, Cry1Fa, and the Cry1Ac domain III mutant^{509}QNR-AAA^{511} recognize a *H. virescens* 110 kDa APN, and that this APN does not cross-react with the GalNAc-recognizing lectin, soybean agglutinin (SBA) (Banks *et al*., 2001). This indicated that the 110 kDa APN was a Cry1Ac toxin binding protein that displayed GalNAc-independent toxin binding. Based on this evidence, we hypothesized that the 110 kDa APN binding protein was possibly a functional Cry1Ac toxin receptor.

To address this hypothesis, we cloned the 110 kDa APN gene, and expressed it in a *Drosophila* S2 insect cell line that displays innate Cry1Ac and Cry1Fa toxin resistance. The objective was to transform toxin resistant cells into toxin susceptible cells via APN expression. This approach allowed us to determine whether or not the 110 kDa APN binding protein mediates Bt toxicity *in vivo*, thereby demonstrating its role as a true Bt receptor. While rhodamine-labeled Cry1Ac bound APN transfected cells, the cells remained resistant to toxin. These results indicated that the *H. virescens* 110 kDa APN does not mediate cytotoxicity when expressed in S2 cells.

2.0 METHODS

2.1 Cloning the 110 kDa APN cDNA

As previously described (Banks *et al*., 2001), an internal region of the 110 kDa APN cDNA was amplified using degenerate primers and a *H. virescens* cDNA template to yield a 0.9 kb PCR product. Based on the sequence of the 0.9 kb PCR product, non-degenerate gene specific primers (GSPs) were designed to amplify the remaining 5' and 3' regions according to the rapid amplification of cDNA ends (RACE) method (Frohmann *et al*., 1989). For 3' RACE, RNA was prepared from *H. virescens* total midgut with an RNeasy kit (Qiagen). cDNA was then synthesized with the Not I-d(T)18 adapter primer (Amersham Pharmacia) and with Superscript reverse transcriptase (Gibco-BRL) according to the manufacturer. The 3' region of the 110 kDa APN gene was amplified with GSP1 5' GTT CCA GCC TTA CCA CGC CAG GAA GGC 3' and the bifunctional Not I-d(T)18 adapter primer (Amersham Pharmacia). All primers were manufactured by Integrated DNA Technologies, Inc (IDT). Amplification was conducted in 50 mM KCl, 10 mM Tris-HCl, 15 mM Mg Cl_2, 0.25 mM each dNTP (pH 8.3), 1U Taq Polymerase (Eppendorf) with 30 cycles of 94 °C for 1 minute, 52°C for 1 minute and 72°C for 1 minute. 3' RACE produced a 2.5 kb fragment that was gel purified with a Qiaex II gel extraction kit (Qiagen) and cloned into pCR 2.1 TA vector (Invitrogen), yielding the 3' construct pCR2.5. The 5' region of the 110 kDa APN gene was amplified with the Gibco BRL-5' RACE kit. Superscript reverse transcriptase was used to make first strand cDNA with the GSP2 5' GGA GGG GAA GTC AGG AAG AGC 3'. The cDNA was then tailed with dCTP by

terminal deoxynucleotidyl transferase. This cDNA was then used as template for amplification with GSP3 5' C CAT GGG ACT ACT TCT TAC TGG 3' and the oligo-dG abridged anchor primer (Gibco-BRL). 5' RACE produced a 0.9 kb fragment that was gel purified with a Qiaex II gel extraction kit (Qiagen) and cloned into the pCR 2.1 TA vector (Invitrogen), yielding the 5' construct pCR0.9. The entire gene was assembled from the constructs pCR2.5 and pCR0.9, yielding the construct pCR3.0.

2.2 DNA sequencing

DNA sequencing of pCR3.0 was conducted at the Molecular Genetics Instrumentation Facility (MGIF) (University of Georgia) in both forward and reverse directions to obtain the complete sequence of the 110 kDa APN gene. Primers were designed such that contigs overlapped at least 50 nucleotides. Contigs with overlapping sequences were aligned with the Sequencher program (Gene Code Corporation). Alignment with the 110 kDa APN sequence with the *H. punctigera* APN 2 sequence (Emmerling *et al*., 2001) revealed a missing nucleotide at position 1655, which introduced a stop codon. Site directed mutagenesis was used to insert the missing nucleotide, restoring the predicted 3.0 kb ORF. Codon choice at the mutation site was based on the homologous codon found in *H. punctigera* APN 2 (Emerling *et al*., 2001), since this gene shares 89% sequence similarity with the *H. virescens* 110 kDa APN. The homologous primer method utilized by the Quick Change (Stratagene) site directed mutagenesis method was used to introduce a cytosine at position 1655. The forward

homologous primer containing the introduced cytosine (underlined) 5' CAG ATT CCT CTC ACC TTG ACT CAA AAT GG 3'and the reverse homologous primer containing the introduced guanine (underlined) 5' CC ATT TTG AGT CAA GGT GAG AGG AAT CTG 3' were both were 5' phosphorylated. Screening was conducted by sequencing across the mutagenized region. Once a clone was discovered that contained the introduced nucleotide, the insert was completely sequenced in both directions to confirm that no unintentional mutations affected codon usage. The new construct containing the introduced cytosine was denoted pCR110.

2.3 in vitro *expression* of the 110 kDa APN

The 110 APN gene was cloned into both pTrcHis and pET30 for expression in *E. coli*. However, neither of these constructs produced recombinant protein. Therefore, the pCITE4a vector (Novagen) was used for *in vitro* transcription and translation of the 110 kDa APN gene. PCR was conducted with pCR110 as template to introduce 5' Nde I site and a 3' Xho I site flanking the 110 kDa APN gene. The 5' primer used to introduce the Nde I site was 5' GTA CTG CAG GGG GCC CAT ATG GGT GCC 3'. The 3' primer used to introduce the Xho I site was 5' CAC AAC CTC GAG CAA AAC GAC GGA ACG 3'. Amplification was conducted with pCR110 template in 50 mM KCl, 10 mM Tris-HCl, 15 mM Mg Cl_2, 0.25 mM each dNTP (pH 8.3), 1U Taq Polymerase (Eppendorf) with 30 cycles of 94 °C for 1 minute, 52 °C for 1 minute and 72 °C for 1 minute. PCR produced the predicted 3.0 kb fragment that was subsequently

gel purified (data not shown), and cloned into the pCR 2.1 TA vector (Invitrogen), yielding the construct pCR110-NX. This 110 kDa APN gene with introduced flanking restriction sites was cloned into pCITE4a, and the resulting *E. coli* clone was called pCITE4a-110.

The Single Tube Protein system 3 (STP3) (Novagen) was used for *in vitro* protein expression according to specifications suggested by the manufacturer. The construct pCITE4a-110 and the control pCITE4 vector containing the β-galactosidase gene were used as template during *in vitro* transcription. According to the directions suggested by Novagen, ^{35}[S] methionine (40 μCi; Amersham-Pharmacia) was added to the translation reaction for incorporation into expressed protein. Total protein from translation reactions was separated by SDS-8% PAGE. The gel was then briefly rinsed in water, and exposed to XAR-5 photographic film (Kodak) for 12 hours at room temperature. Molecular sizes were estimated from molecular size standards using AlphaImager 2000 (Alpha Innotech) software.

2.4 Expression vector construction

The 110 kDa APN gene was excised from pCR110 with EcoRI and gel purified with a Qiaex II gel extraction kit (Qiagen). The pIZT vector (Invitrogen) was cut with EcoRI, dephosphorylated with calf intestine alkaline phosphatase (Promega), and gel purified as above. Insert and vector were ligated overnight with T4 DNA ligase (Promega) at 14 °C. Electrocompetent DH5α cells were transformed with 1 μl of the ligation reaction in a Gene Pulser (BioRad) at 25 μF,

2.0 V and 200 Ω in a 0.2 cm Gene Pulser Cuvette (BioRad). Plasmid was purified from cultured colonies according to Sambrook et al (1989), and screened with endonuclease digestion for proper insert orientation, yielding the construct pIZT-110.

2.5 Transient expression in Drosophila *S2 cells*

Drosophila S2 cells were grown to confluency in 25 cm^2 polystyrene flasks (Corning) in serum-free insect cell media (Hyclone). Approximately 1.5 x 10^6 cells were resuspended in 5 ml fresh media warmed to room temperature, and allowed to adhere to 60 mm^2 polystyrene petri dishes (Falcon) overnight. A transfection mixture was prepared by mixing plasmid DNA (2 μg) with 1 ml of serum-free insect cell medium (Hyclone) and 20 μl Cellfectin reagent (Invitrogen). The transfection mixture was incubated for 30 minutes at room temperature before use. Medium was removed from the seeded cells, and gently replaced with the transfection mixture. Cells were gently rocked twice per minute for 4 hours at room temperature. Four ml of room temperature medium containing 50 units of a penicillin-streptomycin cocktail/ml media (Invitrogen) was then added to the cells, which were incubated at 27°C for 48 hours.

2.6 Aminopeptidase N activity assay

The aminopeptidase N activity of enriched total membranes purified from *Drosophila* S2 cells transfected with pIZT vector alone or with the pIZT-110 construct was assayed according to Luo et al (1999). Briefly, cells were

homogenized on ice with a Polytron homogenizer (Brinkman Instruments) using a 0.7 cm diameter probe. Cells were then centrifuged at 27,000 g for 15 minutes at 4°C. The pellet was resuspended in 1 ml 10 mM Tris-HCl, 150 mM NaCl, 100 mM $MgCl_2$, pH 7.4. APN activity assays with enriched total cellular membranes purified from vector alone and pIZT-110 transfected cells were conducted on two separate occasions. Three measurements were taken each time, yielding 6 values for both pIZT and pIZT-110 transfected cells.

2.7 Toxicity assays

S2 cells from a confluent culture (1.5×10^6) were seeded overnight in a 60 x 15 mm tissue culture dish (Falcon) containing 3 microscope cover slides (Fisher; 18 x 18 mm). The following day, cells were transfected with either pIZT or pIZT-110, as above, and cultured for 48 hours. A single microscope coverslip containing transfected cells was then placed in a 30 x 10 mm tissue culture dish (Falcon), and 30 μg toxin/ml of fresh media was added. After 2 hours incubation, 2μl of a 4 μM ethidium bromide (EtBr) homodimer stock (Molecular Probes) or 1 μl of propidium iodide (PI; 1 mg/ml stock) (Sigma) was added to the cells and allowed to remain at room temperature for 10 minutes. Cells were then excited with a krypton/argon laser and EtBr or PI fluorescence was visualized with a Leica confocal microscope using a 530 nm band pass filter. A similar cytotoxicity assay was also conducted with trypan blue dye and an inverted light microscope (Nikon). Cells were incubated with toxin as described above, then incubated with trypan blue dye for 10 minutes.

2.8 Binding of rhodamine-labeled Cry1Ac toxin to cells

For localization of rhodamine-labeled Cry1Ac toxin binding to cells, S2 cells were transfected as described above, except that at 48 hours post-transfection, the cells were washed three times in PBS, and fixed in PBS containing 2% paraformaldehyde 30 minutes. Cells were then washed three times in PBS, and blocked at 4°C overnight in 3% bovine serum albumin (BSA) (Sigma) in PBS containing 0.1% Tween (PBST) (Sigma). Cry1Ac was labeled with the rhodamine derivative, 5-(6)-TAMRA, SE (Molecular Probes). Toxin (1 mg/ml) was dialyzed overnight in 0.1M $NaHCO_3$, 150 mm NaCl, pH 8.5. One hundred μl of 5-(6)-TAMRA (10 mg/ml stock) in dimethylformamide was added to 2 ml of dialyzed toxin and the mixture was stirred at room temperature in the dark for 1 hour. Labeled toxin was separated from free label by size exclusion chromatography with Sephadex G-50 (Sigma) packed in a 25ml plastic disposable pipette. We were unable to label Cry1Fa with 5-(6)-TAMRA, SE. The following day, cells were incubated for 1 hour with 1.5 μg labeled Cry1Ac toxin/ ml PBST containing 0.1% BSA. The cells were then washed three times in PBST containing 0.1% BSA, and visualized with a Leica confocal microscope. Cells were examined by differential interference contrast microscopy. Cells were also examined for GFP expression by excitation with a krypton-argon laser, and emission was observed by a 530/30 nm band-pass filter. Cells were also observed for binding of 5-(6)-TAMRA-labeled Cry1Ac by excitation with a krypton-argon laser, and emission was observed by a 585/42 nm band-pass filter.

2.9 Flow cytometry

S2 cells were seeded, transfected with either pIZT or pIZT-110 and allowed to express the transgene for 48 hours as above. Approximately 1.0 x 10^6 cells were then resuspended in 1 ml fresh media containing 30 μg toxin. After incubation at room temperature for 1.5 hours, 1 μg of propidium iodide (PI) was added. After 10 minutes incubation with PI, the cells were analyzed on a Becton Dickinson FACSCalibur (Becton-Dickinson) flow cytometer. To exclude cellular debris, cells were first gated for size determination by comparing forward scattered and side scattered light dual parameters. For GFP fluorescence, cells were then excited with krypton-argon laser, and emission was monitored with a 530/30 nm band-pass filter. These GFP-gated cells were then examined for PI fluorescence by monitoring emission with a 585/42 nm band-pass filter.

3.0 RESULTS

3.1 110 kDa APN cDNA

We previously identified a *H. virescens* 110 kDa APN that binds Cry1Ac, the Cry1Ac mutant ^{509}QNR^{511}-AAA, and Cry1Fa toxins, and cloned an internal 0.9 kb region of the cDNA encoding the 110 kDa APN (Banks *et al*., 2001). Here we report the RACE-PCR cloning of a 3.0 kb cDNA encoding the complete 110 kDa APN. A missing nucleotide that caused a frame shift mutation and a stop codon appeared to have been introduced during PCR of the APN gene. Site-directed mutagenesis was conducted to introduce a cytosine corresponding to the homologous codon in *H. punctigera* APN 2. When translated, the amino acid

sequence of this mutagenized 110 kDa APN gene contained a 3.0 kb ORF with a start methionine, N-terminus signal sequence for retention in the endoplasmic reticulum, zinc metalloprotease binding site, and a putative C-terminal signal sequence for the GPI anchor site stop codon, polyadenylation signal and a poly-A tail (GENBANK accession no. AF378666) (Fig 2.1).

3.2 In vitro *transcription and translation*

Experiments to express the 110 kDa APN in *E. coli* yielded no apparent recombinant protein (data not shown). To confirm that the contruct contained a 3.0 kb ORF, the gene was transcribed and translated *in vitro* using the pCITE4a expression vector and STP 3 expression system. *In vitro* transcription and translation of the positive control pCITE4a-β-galactosidase yielded the expected 119 kDa protein (Fig 2.2, lane 1), while *in vitro* transcription and translation of pCITE4a-110 yielded a 111 kDa protein (Fig 2.2, lane 2). The pCITE4a-110 construct contains both N-terminal and C-terminal signal sequences (Fig. 2.1). Therefore, the molecular weight of the *in vitro* expressed protein should have been greater than the mature *in vivo* protein. Without the N-terminal and C-terminal signal sequences, and without glycosylation, the molecular weight of the mature protein is predicted to be 101.6 kDa (GCG, Wisconsin Package; peptidesort). The difference of 8.4 kDa in the molecular weight of the mature protein predicted using GCG and the mature protein in *H. virescens* is likely due to differences in glycosylation (Fig. 2.1).

3.3 Aminopeptidase N activity in total enriched membranes from transiently transfected Drosophila *S2 cells*

When *in vitro* transcription and translation confirmed that the 110 kDa APN gene contained a functional 3.0 kb ORF, the 110 kDa APN gene was cloned into the pIZT vector, yielding pIZT-110, for transient expression in S2 cells. APN enzymatic activity can be measured by the cleavage of leucine-p-nitroanilide substrate, which results in a colorimetric reaction monitored at OD_{405}. Enriched total membranes from transfected cells were assayed for APN activity 48 hours post-transfection, and membranes from *Drosophila* S2 cells transfected with the pIZT-110 (Fig. 2.3, B) had four-fold greater APN activity relative to enriched total cellular membranes from cells transfected with pIZT (Fig. 2.3, A). The standard error of the mean is plotted for each treatment, and indicates that the difference in APN activity for the two treatments was statistically significant. These results indicated that S2 cells transfected with the 110 kDa APN gene were expressing biologically active APN that was probably membrane bound.

3.4 Binding of rhodamine-labeled Cry1Ac to cells

Cells transfected with the 110 kDa APN construct were then examined for the ability to bind rhodamine-labeled Cry1Ac. Cells transfected with either pIZT (Fig. 2.4 A, B, C) or with pIZT-110 (Fig. 2.4 D, E, F) were viewed for differential interference contrast (Fig. 2.4 A, D), GFP fluorescence (Fig. 2.4 B, E), and binding of rhodamine-labeled Cry1Ac (Fig. 2.4 C, F). Results showed that rhodamine-labeled Cry1Ac did not bind pIZT transfected cells (Fig. 2.4 C),

whereas rhodamine-labeled Cry1Ac did bind some pIZT-110 transfected cells (Fig. 2.4 F). It should be noted that not all cells transfected with pIZT-110 that expressed GFP also bound rhodamine-labeled Cry1Ac, indicating that rhodamine-labeled Cry1Ac, at 1.5 μg/ml, only bound a subset population of APN transfected cells. Binding assays at high rhodamine-labeled Cry1Ac concentrations resulted in non-specific toxin binding (data not shown).

3.5 Toxicity assays: microscopy

Control cells transfected with pIZT should not experience an increase in cytotoxicity when exposed to Cry1Ac or Cry1Fa toxin, since *Drosophila* cells have innate resistance against these toxins at 30 μg/ml (data not shown). Conversely, we predicted that S2 cells transfected with pIZT-110 should experience an increase in cytotoxicity when exposed to toxin, if the 110 kDa APN mediates Cry1Ac or Cry1Fa toxicity. Cytotoxicity should be indicated by an increase in EtBr infiltration and fluorescence, or by trypan blue infiltration assays. There was no difference in cytotoxicity assays between control cells transfected with pIZT and experimental cells transfected with pIZT-110 (data not shown). This indicates that the 110 kDa APN transfected cells remained resistant to Cry1Ac and Cry1Fa toxin, and suggested that the 110 kDa APN does not mediate cytotoxicity *in vivo*.

3.6 Toxicity assays: flow cytometry

Flow cytometry enabled the examination of 1 X 10^6 cells per cytotoxicity assay. After incubation with toxin, cells were gated for GFP expression, as a marker for transfection, and were examined for PI infiltration and fluorescence. Relative to cells transfected with pIZT, cells transfected with pIZT-110 experienced a 3% increase in PI fluorescence when incubated with Cry1Fa or Cry1Ac. These values suggest that S2 cells transfected with the 110 kDa APN gene remain viable after toxin treatment when.

4.0 DISCUSSION

The 110 kDa APN gene was cloned with the 5' and 3' rapid amplification of cDNA ends (RACE) technique (Frohman *et al*., 1989). The 110 kDa APN shares 89% sequence identity to the *Helicoverpa punctigera* APN 2 (Emmerling *et al*., 2001), but these two genes share only 27% sequence identity to the nearest branch of APNs (Emmerling *et al*., 2001). The pIZT vector was used for expression of the 110 kDa APN gene in S2 cells since this vector has one cassette that contains the constitutive baculovirus IE-2 promoter for expression of the gene of interest and a second cassette directed for expression of GFP as a marker protein. We chose an early baculovirus constitutive promoter for directed expression of the 110 kDa APN for four reasons: *i*) previous research has suggested that baculovirus-infected insect cells are unable to convert N-linked side chains to complex carbohydrate structures (Jarvis and Finn, 1995; van Die *et al*., 1996); *ii*) Denolf et al (1997) observed only non-specific binding of Cry

toxins to *Plutella xylostella* APN expressed in baculovirus-infected Sf9 cells, and suggest that glycosylation patterns of native APN and recombinant APN were different; *iii*) it was recently discovered that baculovirus-mediated expression of glycosylphosphatidyl inositol (GPI)-anchored proteins in insect cells results in a deficiency of the de-acylation step of GalNAc during synthesis of the GPI anchor in the endoplasmic reticulum (Azzous *et al*., 2000); *iv*) early baculovirus promoters have been demonstrated to be efficient for expressing recombinant proteins in insect cells independent of baculovirus infection (Jarvis *et al*., 1990). When considered together, these results suggest that baculovirus-mediated expression of recombinant APN could adversely affect both GPI-anchored protein structure and function as well as the synthesis of complex oligosaccharides. Additionally, these results suggest that utilization of an early baculovirus promoter for its ability to express foreign genes in insect cells independent of baculovirus infection could reduce these likelihood of these phenomena. Whether or not baculovirus-mediated expression of APNs in insect cells indeed effects APN biological activity or interaction with ligands, such as Cry toxins, is unknown.

Enzyme activity assays indicated that APN activity was four-fold greater in enriched membranes from 110 kDa APN transfected cells, relative to vector-alone transfected cells, indicating that S2 cells expressed biologically active APN. Additionally, toxin binding assays showed that rhodamine-labeled Cry1Ac toxin bound to some 110 kDa APN transfected S2 cells, but not to vector-alone transfected S2 cells. This is the first report of Bt toxin binding to whole cells

transfected with an APN. However, not all cells expressing GFP marker protein also bound rhodamine-labeled toxin. This might suggest that proper expression of recombinant APN occurs only in a subset population of transfected cells, or that expression levels are variable throughout transfected cells.

Microscopy and flow cytometry assays detected no difference in cytotoxicity between S2 cells transfected with the pIZT vector-alone or with pIZT-110, measured as a function of membrane integrity. These results do not support the role of the 110 kDa APN as mediating Cry1Ac and Cry1Fa cytotoxicity, at least in transfected S2 cells. Alternatively, it remains possible that Bt toxin mode of action in the cellular model utilized herein may not be dependent on pore formation, but rather on a different mechanism, such as channel formation (Schwartz *et al*., 1993; Schwartz *et al*., 1997) or the release of internal calcium stores (Potvin *et al*., 1998). It also remains possible that a second membrane bound factor is required in conjunction with the 110 kDa APN in order for cytotoxicity to occur. For example, we suspect that since GPI-anchored proteins are known to be sequestered in cholesterol-rich rafts (Varma and Mayor, 1998), different species of GPI-anchored APNs may also be sequestered in cholesterol-rich rafts in the insect midgut epithelial cell brush border. Thus, certain APNs, such as the *H. virescens* 110 kDa APN, may function as toxin binding proteins sequestered rafts. Further, these binding proteins may promote toxin interaction with receptors that mediate cytotoxicity.

Our results raise the following question: why do the 110 kDa APN from BBMV (Banks *et al*., 2001) and S2 cells transfected with the 110 kDa APN both

bind Cry1Ac toxin, yet S2 cells transfected with the 110 kDa APN remain resistant to toxin while *H. virescens* is susceptible Cry1Ac? Other evidence indicates that specific and saturable toxin binding to BBMV (Luo *et al*., 1999) and to APN in ligand blots (Garczynski *et al*., 1991) are sometimes independent of toxicity. That is, toxin binding is not necessarily synonymous with susceptibility. Rather, irreversible binding, or toxin insertion into the membrane is a more accurate indicator of toxicity (Ihara *et al*., 1993). We suspect that while Cry1Ac toxin is binding recombinant APN expressed on S2 cells, toxin apparently is not oligomerizing or inserting into the membrane. Thus, cells remain resistant to toxin, and the *H. virescens* 110 kDa APN does not function as true Bt toxin receptor, but rather functions as a Bt binding protein. It should be noted that the *Bombyx mori* 120 kDa APN is a functional Cry1Aa receptor when expressed in S2 cells, in that this APN confers Bt cytotoxicity to transfected cells (Hua *et al*., unpublished). This further supports the conclusion that the *H. virescens* 110 kDa APN does not mediate cytotoxicity. Previously, we predicted either the *H. virescens* 110 kDa APN, 170 kDa APN, or an unidentified >210 kDa molecule were likely candidates as mediators of Cry1Ac and Cry1Fa cytotoxicity *in vivo* (Banks *et al*., 2001). Evidence herein suggests the 110 kDa APN does not mediate cytotoxicity *in vivo*, while the *H. virescens* 170 kDa APN and the >210 kDa molecules remain good candidates as molecules that may mediate cytotoxicity *in vivo*. Further research concerning *H. virescens* molecules that mediate cytotoxicity should concentrate on 170 kDa APN and the >210 kDa molecule.

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6.0 FIGURES

MGAKMLLPTVFCILLGSIAAIPQEDFRSNLEWADYSTNLDEPAYRLRDVVYPTDVNLDLD 60

VYLDELRFNGLVQIDVEVRENDLRQIVLHQKVVSINAVNVVGPNGPVGLQFPYPYTTDDY 120

YEILLINLAEPINIG***NYS***ITIRYNGQINDNPIDRGFYKGYYYLNNELRLYATTQFQPYHA 180

RKAFPCFDEPQFKSRFTISITRASSLSPSYSNMAISNTQILGARTRETFHPTPIISAYLV 240

AFHVSDFVATEYTSTDAKPFSIISRQGVTDQHEYAAEIGLKITNELDDYLGIQYHEMGQG 300

TLMKNDHIALPDFPS**GAMENWG**MVNYREAYLLYDA***NNT***NLNNKIFIATIMAHELGHKWFG 360

NLVTCFWWSNLWL***NES***FASFFEYLGAHWADPALELDDQFVVDYVHSALNSDASQFATPMN 420

HVDVVD***NDS***ITAHFSVTSYAKGASVLRMMEHFVGSRTFRNALRYYLRNNEYSIGFPVDMY 480

AAFKQAVSEDFTFERDFPGIDVGAVFDTWVQNRGSPVLNVARNSNTGVISVSQERYVLSG 540

AVAPALWQIPLTLTQ***NGS***LNFENTRPSLVLTTQSQNINGASGDNFVIFNNAQSGLYRVNY 600

DTNNWQLLASYLKSNNRENIHKLNRAQIVNDVLNFVRSNSI***NRT***LAFEVLDFLRDETDYY 660

VWNGALTQIDWILRRLEHLPAAHAAFSEYILDLMSTVINHLGYNEQSTDSTSTILNRMQI 720

MNYACNLGHSGCIADSLDKWRQHRENPNNLVPVNLRRYVYCVGLREG***NET***DYSYLFSVY***N*** 780

SSENTADMVVILRALACTKHQPSLEHYLQESMYNDKIRIHDRTNAFSFALQGNPENLPIV 840

LNFLYNNFAAIRETYGGVARLNLCINAIPAFLTDYQTITQFQSWVYANQLALVGSFNNGV 900

SVVNTALDNLTWGNGAAVEIVNFLNYK**SAS**PSILASSILILAAMLVQMFR 950

Fig. 2.1. Amino acid sequence of the *H. virescens* 110 kDa APN. The start methionine is in bold. The N-terminus of the mature protein is underlined. The conserved aminopeptidase Zn^{+} metalloproteinase binding site is boxed. The conserved GAMEN motif is in boldface and underlined. The putative C-terminal GPI anchor site is in bold face. Potential glycosylation sites are in boldface and italics.

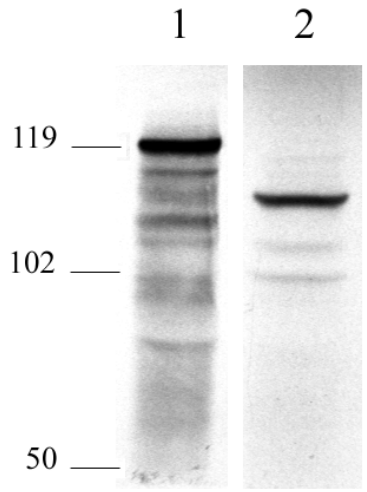

Fig. 2.2. *In vitro* transcription and translation of the *H. virescens* 110 kDa APN. The 110 kDa APN was cloned into pCITE4a (Novagen). During translation, ^{35}S was incorporated into expressed protein. Total protein was then 8% SDS-PAGE separated, exposed to film overnight, and the film was developed. Lane 1 shows *in vitro* transcription and translation of the positive control β-galactosidase gene in pCITE4a, which correspods to a 119 kDa protein. Lane 2 shows *in vitro* transcription and translation of pCITE4a-110, which contains the *H. virescens* 110 kDa APN gene.

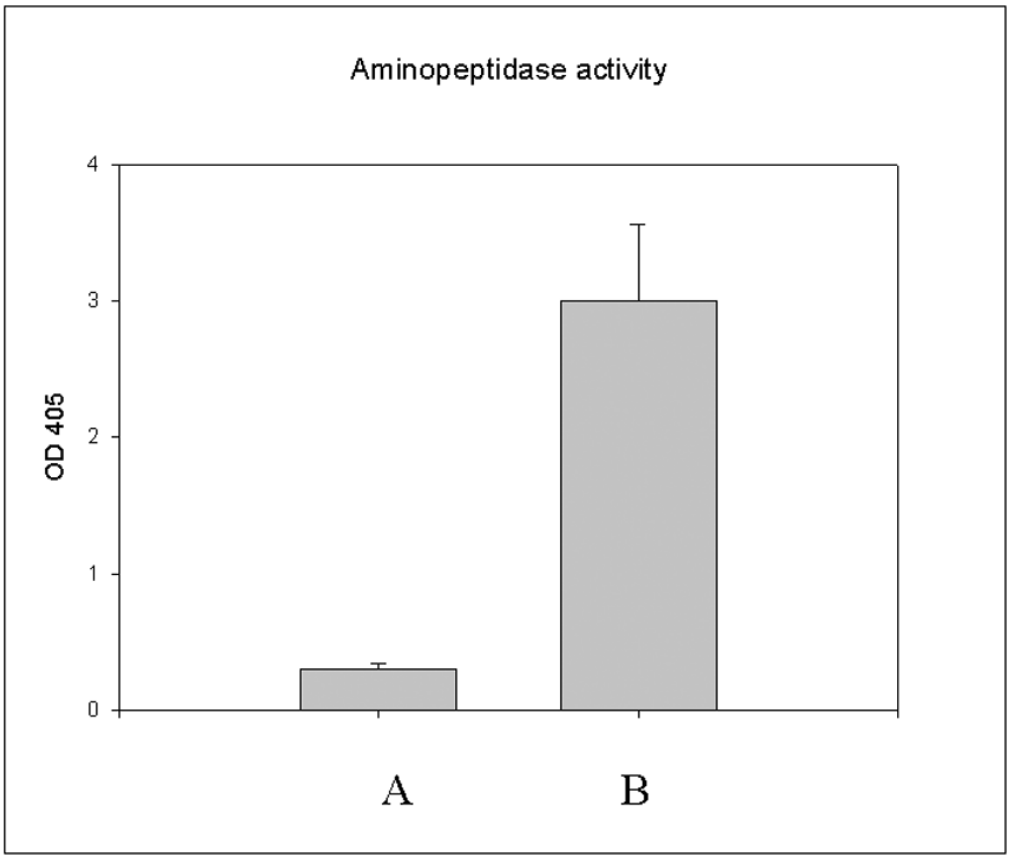

Fig. 2.3. Aminopeptidase N activity in membranes purified from transfected S2 cells. *Drosophila* S2 cells were transiently transfected with pIZT or with pIZT-110, and allowed to express for 48 hours. Cellular membranes were then purified from vector alone and *H. virescens* 110 kDa APN transfected cells. 150 μg of total membrane protein from vector alone transfected cells (A) and from *H. virescens* 110 kDa APN transfected cells (B) were then incubated with the APN substrate leucine-p-nitroanilide, and enzyme activity was measured as absorbance at 405 nm.

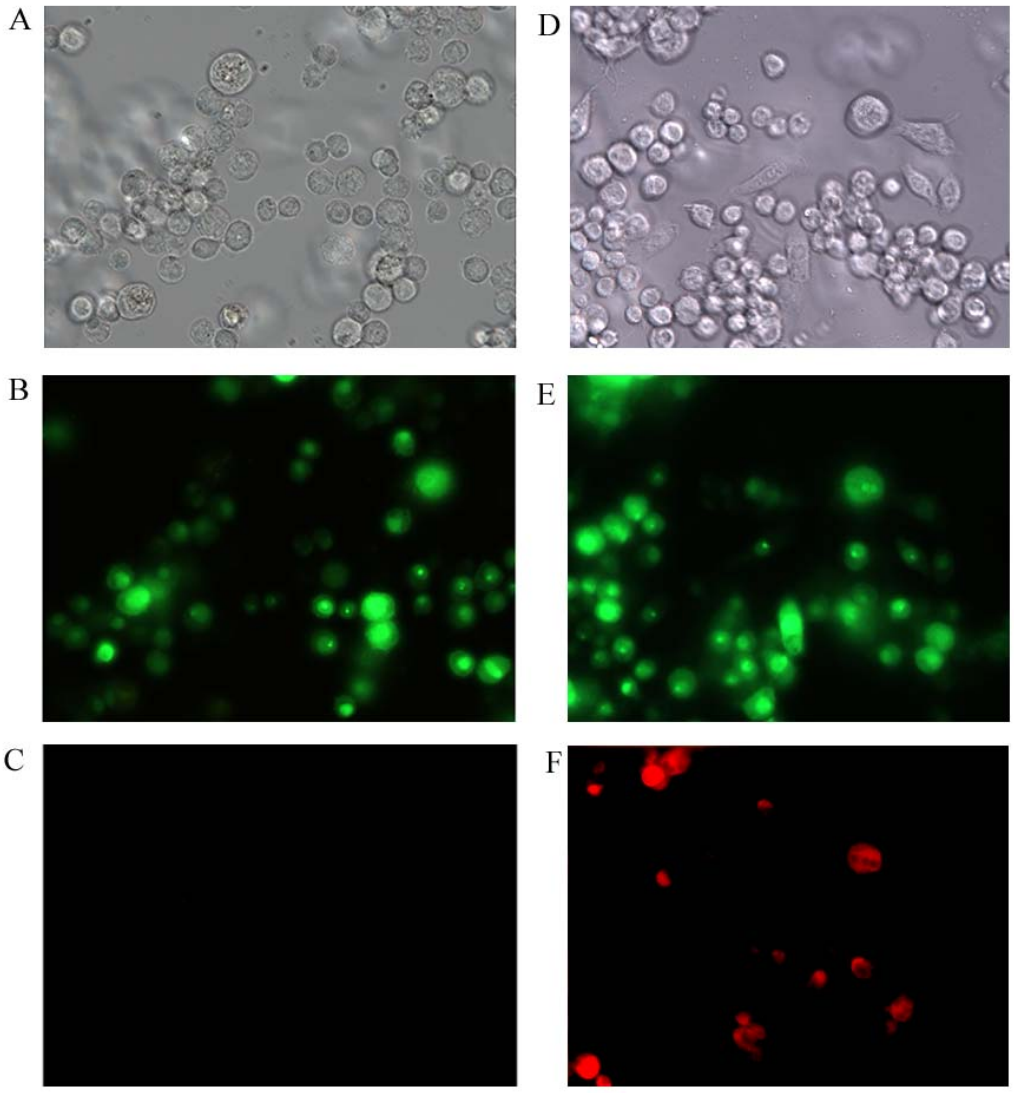

Fig. 2.4. S2 cells were seeded on microscope coverslips and transfected with either pIZT (A, B, C) or pIZT-110 (D, E, F). Cells were fixed in 2% paraformaldehyde, blocked in 3% BSA in PBST, incubated with 1.5 μg/ml rhodamine-labeled Cry1Ac in 0.1 % BSA in PBST, and then washed. Cells were examined for differential interference contrast (A, D), GFP expression (B, E) and binding of rhodamine-labeled Cry1Ac (C, F).

7.0 REFERENCES

Aranda, E., Sanchez, J., Peferoen, M., Guereca, L., Bravo, A. 1996. Interactions of *Bacillus thuringiensis* crystal proteins with the midgut epithelial cells of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 68, 203-212.

Azzouz, N., Kedees, M.H., Gerold, P., Becker, S., Dubremetz, J. Klenk, H., Eckert, V., Schwarz R.T. 2000. Glycobiology. An early step of glycosylphosphatidyl-inositol anchor biosynthesis is abolished in lepidopteran insect cells following baculovirus infection. 10(2) 177-183.

Banks, D.J., Jurat-Fuentes, J. L., Dean, D.H., Adang, M.J. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ–endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* in not N-acetylgalactosamine mediated. Insect Biochem. Molec. Biol. 31, 909-918.

De Maagd, R.A., Bakker, P.L., Masson, L., Adang, M.J., Sangadala, S., Stiekema, W., Bosch, D. 1999. Domain III of the *Bacillus thuringiensis* δ–endotoxin Cry1Ac in involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Molec. Microbiol. 31(2), 463-471.

Denolf, P., Jansens, S., Van Houdt, S., Peferoen, M., Degheele, D., Van Rie, J., 1993. Biotinylation of *Bacillus thuringiensis* insecticidal crystal proteins. Appl. Environ. Microbiol. 59,1821-1827.

Emmerling, M., Chandler, D., Sandeman, M. 2001. Molecular cloning of three cDNAs encoding aminopeptidases from the midgut of *Helicoverpa punctigera*, the Australian native budworm. Insect Biochem. Molec. Biol. 31, 899-907.

Frohman, M.A., Dush, M.K., Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. 85, 8998-9002.

Garczynski, S. F., Crim, J.W., Adang, M. J., 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ–endotoxin by protein blot analysis. Appl. Environ. Microbiol. 57, 2816-2820.

Gill, S. S., Cowles, E. A, Francis, V., 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J. Biol. Chem. 270, 27277-27282.

Hofmann, C., Vanderbruggen, H., Hofte, H., Van Rie, J., Jasens, S., Van Mellaert, H., 1988. Specificity of *Bacillus thuringiensis* δ–endotoxin is correlated with the presence of high affinity binding sites in the brush border membrane of target insect midgets. Proc. Natl. Acad. Sci. 85, 7844-7848.

Hua, G., Banks, D.J., Adang, M.J. 2002. The *Bombyx mori* 120 kDa APN is the functional Cry1Aa receptor. In preparation.

Jarvis, D., L., Fleming, J-A., G., W., Kovacs, G. R., Summers, M. D., Gaurino, L. A. 1990. Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. Biotechnology. 8, 950-955.

Jarvis, D. L., Finn, E. E. 1995. Biochemical analysis of the N-glycosylation pathway in baculovirus-infected lepidopteran insect cells. Virology 212 (2), 550-511.

Jarvis, D. L., Kawar, Z, S., Hollister, J. R. 1998. Engineering N- glycosylation pathways in the baculovirus-insect cell system. Curr. Opinion in Biotech. 9, 528-533.

Jenkins, J. L., Lee, M. K., Sangadala, S., Adang, M. J., Dean, D. H., 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Letters 462, 373-376.

Jurat-Fuentes, J.L., Adang, M.J. 2001. Importance of Cry1 δ–endotoxin domain II loops for binding specificity in *Heliothis virescens*. Appl. Environ. Microbiol. 67, 323-329.

Keeton, T.P., Bulla, L.A.1997. Ligand specificity and affinity of BT-R1, the *Bacillus thuringiensis* Cry1A toxin receptor from *Manduca Sexta*, expressed in mammalian and insect cell cultures. Appl. Environ. Microbiol. 63, 3419-3425.

Knight, P. J. K., Crickmore, N., Ellar, D. J., 1994. The receptor for *Bacillus thuringiensis* Cry1Ac delta-endotoxin in the brush border of the lepidopteran *Manduca sexta* is aminopeptidase J. Molec. Microbiol. 11, 429-436.

Knowles, B. H., Knight, P. J. K., Ellar, D. J., 1991. N-acetylgalactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. Proc. R. Soc. Lond. B 245, 31-35.

Knowles, B. H., Dow, J. A. T., 1993. The crystal δ–endotoxin of *Bacillus thuringiensis*: models for their mechanisms of action on the insect gut. Bioessays 15, 469-476.

Lee, M. K., You, T. H., Gould, F. L., Dean, D. H., 1999. Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. Appl. Environ. Microbiol. 65, 4513-4520.

Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R., Adang, M. J., 1997. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* δ–endotoxin binding and pore formation. Insect Biochem. Molec. Biol. 27, 735-743.

Luo, K., McLachlin, J. R., Brown, M. R., Adang, M. J., 1999b. Expression of a glycosylphosphatidylinositol-linked *Manduca sexta* Aminopeptidase N in insect cells. Protein Expression and Purification 17, 113-122.

Masson, L., Lu, Y-J., Mazza, A., Brousseau, R., Adang, M. J. 1995. The Cry1Ac receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. 270, 20309-20315.

Meng, J., Candas, M., Keeton, T.P., Bulla, L.A. 2001. Expression in *Spodoptera frugiperda* (Sf21) insect cells of BT-R_1, a cadherin-related receptor for *Manduca sexta* for *Bacillus thuringiensis* Cry1Ab toxin. Prot. Expr. Purif. 22, 141-147.

Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S., Kogure, M., 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal Cry1Aa toxin. Biosci. Biotechnol. Biochem. 62, 727-734.

Oltean, D. I., Pullikuth, A.K., Lee, H-K., Gill, S. S. 1999. Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. Appl. Environ. Microbiol. 65, 4760-4766.

Peferoen, M., 1997. Progress and prospects for field use of Bt genes in crops. Trends in Biotechnology 15, 173-177.

Potvin, L., Laprade, R., Schwartz, J.L. 1998. Cry1Ac, a *Bacillus thuringiensis* toxin, triggers extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores in Cf1 cells (*Choristoneura fumiferana*, Lepidoptera). The J. of Exp. Biol. 201, 1851-1858.

Sambrook, J., Fritsch, E. F., Maniatis, T. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press.

Sangadala, S., Walters, F. S., English, L. H., Adang, M. J., 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal Cry1Ac toxin binding and 86Rb+-K+ efflux in vitro. J. Biol. Chem. 269, 10088-10092.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. And Molec. Biol. Rev. 62(3), 775-806.

Schwartz, J.L., Garneau, L., Savaria, D., Masson, L., Brousseau, R., Rousseau, E. 1993. Lepidopteran specific crystal toxins from *Bacillus thuringiensis* form cation and anion selective channels in planar lipid bilayers. J. Membr. Biol. 132, 53-62.

Schwartz, J.L., Yiang-Jiang, L., Sohnlein, P., Brousseau, R., Laprade, R., Masson, L., Adang, M.J. 1997. Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. FEBS Letters 412, 270-276.

Simpson, R.M., Newcomb, R.D. 2000. Binding of *Bacillus thuringiensis* δ–endotoxins Cry1Ac and Cry1Ba to a 120 kDa aminopeptidase N of *Epiphyas postvittana* purified from both brush border membrane vesicles and baculovirus infected Sf9 cells. Insect Biochem. Molec. Biol. 30, 1069-1078.

Terra, W.R., Ferreira, C. 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109B, 1-62.

Vadlamudi, R. K., Weber, E., Inhae, J., Tae, H. J., Bulla, L. A ., 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270, 5490-5494.

Van Die, I., Van Tetering, A., Bakker, H., Van den Eijnden, D. H., Joziasse, D. H., 1996. Glycosylation in lepidopteran insect cells: identification of a β1 4-N-acetylgalactosaminyltransferase involved in the synthesis of complex-type oligosaccharide chains. Glycobiology 6, 157-164.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D., Van Mellaert, H., 1989. Specificity of *Bacillus thuringiensis* δ–endotoxins: importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. 186, 239-247.

Wolfersberger, M. G., Luthy, P., Maurer, A., Parenti, P., Sacchi, V. F., Giordana, B., Hanozet, G. M., 1987. Preparation and partial characterization of

amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol.86, 301-308.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

Bacillus thuringiensis toxins are highly effective biological control agents of agricultural insect pests, particularly Lepidopterans. Not only do Bt toxins have a high level of target insect specificity, but they also have a very low LC_{50} value. Perhaps the most attractive features of Bt toxins are that they do not affect mammalian tissues, and their proteinaceous composition prevents biological amplification in the food chain, unlike synthetic pesticides. In order for Bt to maintain its current status as an effective alternative to synthetic pesticides, the target pest species must remain susceptible to Bt toxins. Hundreds of insect pest species have evolved resistance to synthetic pesticides, rendering these chemicals obsolete. Therefore, a legitimate concern for farmers, as well as producers of Bt sprays and transgenic Bt crops, is that target pest species may also evolve resistance against Bt toxins. One current proposal to circumvent the development of resistance is to utilize different Bt toxins that target different receptors (Peferoen, 1997). The strategy of this approach is that an insect is much less likely to develop resistance to two or three toxins that target different receptors, than to develop resistance to two or three toxins that target a single receptor. This resistance-prevention strategy assumes that the source of Bt resistance is related to receptor modification. Indeed, Zhu et al (2000) provided evidence that a mutation in an APN that binds Cry1Aa toxin may indeed be the source for Bt resistance in the Indian meal moth, *Plodia interpunctella*. However, the same authors also provide evidence that differential midgut protease activation of toxins may also be related to Bt resistance (Oppert *et al*., 1996). More recently, genetic evidence indicated that disruption of a cadherin-like gene by retrotransposon-ediated insertion was linked to high levels of resistance to Cry1Ac in *H. virescens* (Gahan *et al*., 2001). While my research did not examine the possible causes of

Bt resistance in *H. virescens*, my initial objective was to address a possible source of cross-resistance. Elucidation of the mechanisms of Bt cross resistance is important as it may provide insight into the closely related area of resistance.

Based on two lines of evidence, we predicted that Cry1Ac and Cry1Fa would share binding proteins in *H. virescens*. First, the Cry1A and Cry1Fa toxins share high sequence homology in domain II loops (Tabashnik *et al.*, 1996), which are involved in toxin binding and activity (Rajamohan *et al.,* 1996a, 1996b). Second, cross-resistance between Cry1Ac and Cry1Fa has been described in the *H. virescens* Cry1Ac resistant YHD2 strain (Gould *et al.,* 1995). This means that the insect line YHD2 was selected for Cry1Ac resistance, but was not exposed to Cry1Fa during the selection process. After Cry1Ac resistance was acquired, it was subsequently discovered that YHD2 was also resistant to Cry1Fa. A mechanistic explanation of Cry1Fa cross-resistance by the YHD2 strain is that Cry1Ac and Cry1Fa toxins recognize the same binding proteins. That is, if resistance in *H. virescens* to Cry toxins is due receptor alteration and subsequent loss of toxin binding, then cross-resistance to Cry1Fa may also be related to loss of binding. Indeed, Lee et al (1995) showed that Cry1A resistance in *H. virescens* is related to the loss of binding. Further, heterologous competition binding experiments have shown that Cry1Ab and Cry1Fa toxins share a high-affinity binding site in the diamondback moth *Plutella xylostella* (Granero *et al*., 1996; Ballester *et al*., 1999). Additionally, binding assays have shown that Cry1Fa competes with low affinity for Cry1Ac binding sites in *H. virescens* brush border membrane vesicles (BBMV) (Jurat-Fuentes and Adang, 2001). These lines of evidence suggested to us that Cry1Ac and Cry1Fa should share binding proteins in *H. virescens*. This prediction was tested with an affinity chromatography

method that utilizes non-denaturing conditions to identify protein-protein interactions, which had been used to identify Cry1C binding proteins in *M. sexta* (Luo *et al*., 1997). Indeed, Cry1Ac and Cry1Fa share 110, 120, 170 and 210 kDa binding proteins, which supports the hypothesis that shared binding proteins are likely the source of Cry1Fa cross resistance in the Cry1Ac resistant YHD2 *H. virescens* strain (Banks *et al*., 2001).

Since the 120 and 170 kDa binding proteins had been identified as APNs (Gill *et al*., 1995; Luo *et al*., 1997), my work then shifted to identification, characterization, cloning and expression of the 110 kDa binding protein. The 110 kDa binding protein was recognized by anti-APN antibody (Chapter 2, Fig. 1). Western blot analysis with the GalNAc-recognizing lectin, soybean agglutinin, and ligand blot analysis with the Cry1Ac domain III mutant ^{509}QNR^{511}-AAA further indicated that Cry1Ac and Cry1Fa recognition of the 110 kDa binding protein was GalNAc-independent (Chapter 2, Fig. 2). This was important since several independent studies indicated that Cry1Ac toxicity was not necessarily dependent on toxin recognition of GalNAc (Jenkins *et al*., 1999; Lee *et al*., 1999; Burton *et al*., 1999). This suggested that the 110 kDa APN was a likely candidate as a receptor that mediated Cry1Ac and Cry1Fa toxicity. The 110 kDa APN gene was then cloned with the intention of determining whether or not it actually mediated Bt cytotoxicity *in vivo*. PCR amplification was conducted using degenerate primers based on the N-terminal sequence derived from the 110 kDa binding protein and a conserved APN motif. *H. virescens* first strand cDNA was used as template, yielding a 0.9 kb internal sequence of the 110 kDa molecule (Chapter 2, Fig. 3). BLAST searching identified the 110 kDa APN as a novel APN that shared 89% sequence similarity to *Helicoverpa punctigera* APN 2 (Emmerling *et al*., 2001). Based on the sequence derived

from the 0.9 kb internal region, gene specific primers were then designed, and the entire 110 kDa APN gene encoded by a 3.0 kb ORF (Chapter 3, Fig. 1) was then cloned with 5' and 3' RACE (Frohman *et al*., 1989),. This gene was first expressed *in vitro* in order to confirm that the gene contained a 3.0 kb ORF. Transcription and translation *in vitro* confirmed that the 110 kDa APN gene contained a 3.0 kb ORF (Chapter 3, Fig. 3). The 110 kDa APN gene was then cloned into the pIZT vector (Novagen), yielding pIZT-110 for transient expression in *Drosophilla* S2. This vector contains one cassette for the gene of interest expression driven off the baculovirus IE1 promoter, and a separate cassette for GFP expression driven off the baculovirus IE2 promoter. Total membranes purified from pIZT-110 transfected cells displayed four-fold enrichment in APN enzymatic activity, relative to total membranes purified from vector-alone transfected cells (Chapter 3, Fig. 4). This indicated that biologically active, membrane bound APN was expressed transiently in S2 cells. S2 cells transfected with either pIZT-110 or pIZT were then examined for the ability to bind rhodamine-labeled Cry1Ac. Toxin bound to S2 cells transfected with the 110 kDa APN gene, but not to S2 cells transfected with vector-alone (Chapter 2, Fig. 5). This indicates that toxin binding to S2 cells is mediated by 110 kDa APN expression. However, microscopy and flow cytometry assays both indicated that 110 kDa APN transfected cells remained resistant to Cry1Ac and Cry1Fa toxins (data not shown). These results indicate that while the 110 kDa APN is a binding protein, it does not mediate cytotoxicity when expressed in S2 cells. Therefore, the 110 kDa APN is not considered a 'true' receptor. We suspect that since GPI-anchored proteins are known to be sequestered in membrane rafts, and are in close proximity with one another (Varma and Mayor, 1998), that different APNs interact with each other in the midgut epithelial

cell membrane. Further, we propose that some of these APNs may function as Bt binding proteins that promote the interaction of Bt toxins with APNs that actually function to mediate cytotoxicity.

We coincidentally discovered that 110 kDa APN transfection promotes S2 cell elongation phenotype, relative to vector alone transfection. These results are interesting in light of the role that human CD13/APN plays in endothelial cell morphogenesis and recruitment during angiogenesis (Pasqualini *et al*., 2000; Bhagwat *et al*., 2001). Additionally, other research indicates that methionine aminopeptidases function in cell growth and patterning in *Drosophila* (Cutforth and Tyler, 1999). Perhaps the *H. virescens* 110 kDa APN that binds Bt toxins may also be involved in cellular phenomena other than digestion.

2.0 REFERENCES

Ballester, V., Granero, F., Tabashnik, B. E., Malvar, T., Ferre, J. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamond back moth (*Plutella xylostella*). Appl. Environ. Microbiol. 65, 1413-1419.

Banks, D.J., Jurat-Fuentes, J. L., Dean, D.H., Adang, M.J. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ–endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* in not N-acetylgalactosamine mediated. Insect Biochem. Molec. Biol. 31, 909-918.

Bhagwat, S.V., Lahdenranta, J., Giordano, R., Arap, W., Pasqualini, R., Shapiro, L.H. 2001.CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. Blood, 97 No. 3, 652-659.

Burton, S. L., Ellar, D. J., Li, J., Derbyshire, D. J., 1999. N- acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Molec.. Biol. 287, 1011-1022.

Chang, W. X. Z., Gahan, L. J., Tabashnik, B. E., Heckel, D. G., 1999. A new aminopeptidase from diamondback moth provides evidence for a gene duplication event in Lepidoptera. Insect Molec. Biol. 8, 171-177.

Cutforth, T., Gaul, U. 1999. A methionine aminopeptidase and putative regulator of translation initiation is required for cell growth and patterning in *Drosophila*. Mech. Devel. 82, 23-28.

Emmerling, M., Chandler, D., Sandeman, M. 2001. Molecular cloning of three cDNAs encoding aminopeptidases from the midgut of *Helicoverpa punctigera*, the Australian native budworm. Insect Biochem. Molec. Biol. 31, 899-907.

Frohman, M.A., Dush, M.K., Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. 85, 8998-9002.

Gahan, L.J., Gould, F., Heckel, D G. 2001. Identification of a gene associated with Bt resistence in *Heliothis virescens*. Science. 293, 857-860.

Gill, S. S., Cowles, E. A, Francis, V., 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J. Biol. Chem. 270, 27277-27282.

Gould, F., Anderson, A., Reynolds, A., Bumgarner, L., Moar, W., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545-1559.

Granero, F., Ballester, V., Ferre, J. 1996. *Bacillus thuringiensis* crystal protein Cry1Ab and Cry1Fa share a high affinity binding site in *Plutella xylostella* (L.). Biochem. Biophys. Res. Commun. 224, 779-783.

Griffiths, J.S., Whitacre, J.L., Stevens, D.E., Aroian, R.V. 2001. Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science 293, 860-864.

Jenkins, J. L., Lee, M. K., Sangadala, S., Adang, M. J., Dean, D. H., 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Letters 462, 373-376.

Jurat-Fuentes, J.L., Adang, M.J. 2001. Importance of Cry1 δ–endotoxin domain II loops for binding specificity in *Heliothis virescens*. Appl. Environ. Microbiol. 67, 323-329.

Knight, P. J. K., Crickmore, N., Ellar, D. J., 1994. The receptor for *Bacillus thuringiensis* Cry1Ac delta-endotoxin in the brush border of the lepidopteran *Manduca sexta* is aminopeptidase J. Molec. Microbiol. 11, 429-436.

Lee, M. K., Rajamohan, F., Gould, F., Dean, D.H. 1995. Resistance to *Bacillus thuringiensis* Cry1A δ–endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. 61, 3836-3842.

Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R., Adang, M. J., 1997. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* δ–endotoxin binding and pore formation. Insect Biochem. Molec. Biol. 27, 735-743.

Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S., Kogure, M., 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus*

thuringiensis insecticidal Cry1Aa toxin. Biosci. Biotechnol. Biochem. 62, 727-734.

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H. 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J. Biol. Chem. 272, 23473-23476.

Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Shapiro, L.H., Arap, W. Ruoslahti, E. Aminopeptidase N is a receptor for tumor-homing peptides and a target inhibiting angiogenesis. Cancer Res. 60(3), 722-727.

Peferoen, M., 1997. Progress and prospects for field use of Bt genes in crops. Trends in Biotechnology 15, 173-177.

Rajamohan, F., Cotrill, J. A., Gould, F., Dean, D. H., 1996a. Role of domain II, loop 2 residues of *Bacillus thuringiensis* Cry1Ab δ–endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J. Biol. Chem. 271, 2390-2396.

Rajamohan, F., Alzate, O., Cotrill, J. A., Curtiss, A., Dean, D. H., 1996b. Protein engineering of *Bacillus thuringiensis* δ–endotoxin: mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. Proc. Natl. Acad. Sci. USA 93, 14338-14343.

Sangadala, S., Walters, F. S., English, L. H., Adang, M. J., 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal Cry1Ac toxin binding and 86Rb+-K+ efflux in vitro. J. Biol. Chem. 269, 10088-10092.

Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol.39, 47-79.

Vadlamudi, R. K., Weber, E., Inhae, J., Tae, H. J., Bulla, L. A ., 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270, 5490-5494.

Varma, R., Mayor, S. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 394, 798-801.

Zhu, Y-C., Kramer, K.J., Oppert, B., Dowdy, A.K. 2000. cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins. Insect Biochem. Molec. Biol. 30, 215-224.

APPENDIX I

1.0 INTRODUCTION

During the course of cytotoxicity assays with *H. virescens* 110 kDa APN transfected *Drosophila* S2 cells, it was incidentally discovered that cells displayed an elongate phenotype. This phenotype was specific for cells transfected with the *H. virescens* 110 kDa gene. Namely, neither cells transfected with vector-alone nor a pIZT-Bm100, a construct containing a *Bombyx mori* 100 kDa APN that shares 65% sequence identity with the *H. virescens* 110 kDa APN, displayed an elongate phenotype. Moreover, expression of this phenotype was sensitve to the APN antagonist bestatin, suggesting that APN enzymatic activity may be a source of an elongate phenotype. These results indicate that the 110 kDa APN may be pleiotropic, in that this protein facilitates not only toxin binding, but perhaps other phenomena such as cell phenotype determination as well.

2.0 METHODS

2.1 Microscopy analysis of cell phenotype

During toxicity assays, it was incidentally noticed that 110 kDa APN transfected cells displayed an elongate cell phenotype. To determine if this phenotype was dependent on transfection with 110 kDa APN, cells were transfected with either pIZT, pIZT-110, or pIZT-Bm100, and cell phenotype was compared with differential interference contrast. The construct pIZT-Bm100 contains a *Bombyx mori* 100 kDa APN (GENBANK accession # AB013400) that shares 65% sequence identity with the *H. virescens* 110 kDa APN (G. Hua,

unpublished). Additionally, S2 cells transfected with pIZT-110 were incubated with 100 μM of the APN inhibitor bestatin (Sigma; solubilized in insect cell media) to determine if the elongate phenotype was due to APN enzymatic activity. Cells were also examined for differential interference contrast as described in Chapter 3.

3.0 RESULTS

3.1 Phenotype patterns in S2 cells transfected with pIZT, pIZT-110, and pIZT-Bm100. S2 cells transfected with pIZT-110, and treated with bestatin

During the course of cytotoxicity and binding assays, it was noticed that cells transfected with the 110 kDa APN displayed an elongate phenotype, which is indicative of cell migration and motility (Alberts *et al*., 1994). To determine if this phenotype was specific for expression of *H. virescens* 110 kDa APN, and did not did not occur when S2 cells were transfected with other GPI-anchored APNs, cells were also transfected with pIZT-Bm100. Cells transfected with pIZT vector-alone were rounded (Fig. 4.1 A, B), many cells transfected with pIZT-110 were elongate (Fig. 4.1 C, D), and cells transfected with pIZT-Bm 100 were rounded (Fig. 4.1 E, F). These results indicate the elongate cell phenotype was dependent on transfection with the *H. virescens* 110 kDa APN.

To determine whether the elongate cell phenotype was dependent on APN enzymatic activity, cells were transfected with pIZT-110 and incubated with the APN inhibitor bestatin. It was predicted that if the elongate phenotype was directly related to APN activity, that 110 kDa APN transfected cells incubated

with bestatin would display a rounded cell phenotype, typical of control cells. Results indicate that fewer elongate cells were visible after bestatin treatment (Fig. 4.2 A, B). However, the elongate cell phenotype was not completely abolished with bestatin treatment (Fig. 4.2 C, D). Additionally, high cell density was observed near the edges of cover slips that were treated with bestatin, and elongate cells were observed in this region (Fig. 4.2 E, F). It should be noted that the elongate cell phenotype in pIZT-110 transfected cells was uniform throughout populations of transfected cells, and did not appear to display location or density dependence. These results suggest that APN enzymatic activity promotes the elongate cell phenotype.

4.0 DISCUSSION

The expressionof the 110 kDa APN in S2 cells may be pleiotropic. S2 cells transfected with this gene displayed an elongate phenotype, while S2 cells transfected with either vector-alone or with a *B. mori* 110 kDa APN did not. Furthermore, this phenotype was reduced in 110 kDa APN transfected cells treated with the APN inhibitor bestatin. However, bestatin treatment did not completely eliminate the elongate phenotype. Similar to this incomplete inhibition, the inhibition of human CD13/APN mediated angiogenesis (formation of new blood vessels) by bestatin was not absolute (Pasqualini *et al*., 2000). In three separate assays, bestatin inhibition of angiogenesis was approximately 25%, 13% and 24% of experimental values. Human CD13/APN activation during the formation of new blood vessels is the rate-limiting step of tumor growth

(Pasqualini *et al*., 2000; Rubanyi 2000), and is essential for capillary tube formation that promotes and sustains tumor growth (Pasqualini *et al*., 2000). Additionally, tumor vasculature consistently overexpresses CD13/APN where angiogenesis is occurring (Pasqualini *et al*., 2000). Therefore, CD13/APN expression correlates with angiogenesis. Furthermore, a critical step in angiogenesis is endothelial cell migration and motility (Rubanyi 2000), and may be related to APN expression and activity. For example, CD13/APN antagonisits interfere with capillary tube formation, which suggests that CD13/APN functions in the control of endothelial cell morphogenesis (Bhagwat *et al*., 2001).

Since cell elongation is indicative of cell motility and migration, cell elongation of S2 cells transfectred with the 110 kDa APN may be analogous to the CD13/APN-mediated increase in endothelial cell recruitment during angiogenesis. Fujii et al (1995) demonstrated that cell invasion and migration of parental melanoma cells was significantly greater when transfected with CD13/APN relative to vector-alone transfected cells; however, these authors indicate that increased APN activity in invasive melanoma cells is primarily related to degredation of extracellular matrix (which allows cancerous cells to metastasize) rather than an altered phenotype.

Since APNs derived from the brush border of midgut epithelial cells typically function as digestive enzymes (Terra and Ferreira, 1994), why would the 110 kDa APN function to promote an elongate cell phenotype? We hypothesize that when expressed in the brush border of an insect midgut epithelial cell, the main function of the 110 kDa APN is likely digestion. However, when expressed

in either S2 cells or within cells of a developing insect embryo, a molting larvae, or a pupae, the 110 kDa APN may modulate cell elongation during tissue differentiation. It should be noted that *Drosophila* is susceptible to malignant neoplasms (Gateff, 1982). In the insect embryo, mixed tumors are found, and in the insect larva, malignant neuroblastomas, intermediate imaginal disc neoplasms with an invasive mode of growth, and malignant blood neoplasms occur (Gateff, 1982). Furthermore, the genome of *Drosophila* contains DNA sequences that are homologous to known vertebrate oncogenes (Baek, 1999). Therefore, insects as well as vertebrates are susceptible to tumorigenesis, and it is conceivable that the 110 kDa APN function could be subverted in insect tumorigenesis.

110 kDa APN-mediated cell elongation in transfected S2 cells could be related to the activation of a signal cascade involved in tissue differentiation. In essence, an APN mediated signal could activate S2 cells to change phenotype similar to that of endothelial cells during angiogenesis. Indeed, growth factors are present in conditioned medium from cultured insect cells (Kawamura *et al*., 1999), and APNs are known to activate several different bioactive peptides (Taylor, 1996). Therefore, expression of APN in transfected cells could process signal peptides in cultured medium, thus activating a signal cascade involved in cell phenotype. Since not all cells transfected with pIZT-110 were also elongated, induction of this morphology in a subset of APN transfected cells could be related to an APN activated signal peptide whose effect is dependent on differential cell cycling. That is, cells may be sensitive to activated mitogens during a specific phase of the cell cycle. Indeed, growth factor receptor regulation of cell

proliferation and migration is known to be related to cell cycling in different cell types (Tsang and Crowe, 1999).

In conclusion, the *H. virescens* 110 kDa APN gene has interesting effects on *Drosophilla* S2 cell phenotype, and these effects could be similar to the effects of human CD13/APN on endothelial cell morphogenesis during angiogenisis. Other research indicates that methionine aminopeptidases function as transcription factors involved in cell growth and patterning in *Drosophila* (Cutforth and Tyler, 1999). Likewise, results presented here suggest that perhaps lepidopteran APN molecules that bind Bt toxins may also be involved in cellular phenomena other than digestion.

5.0 FIGURES

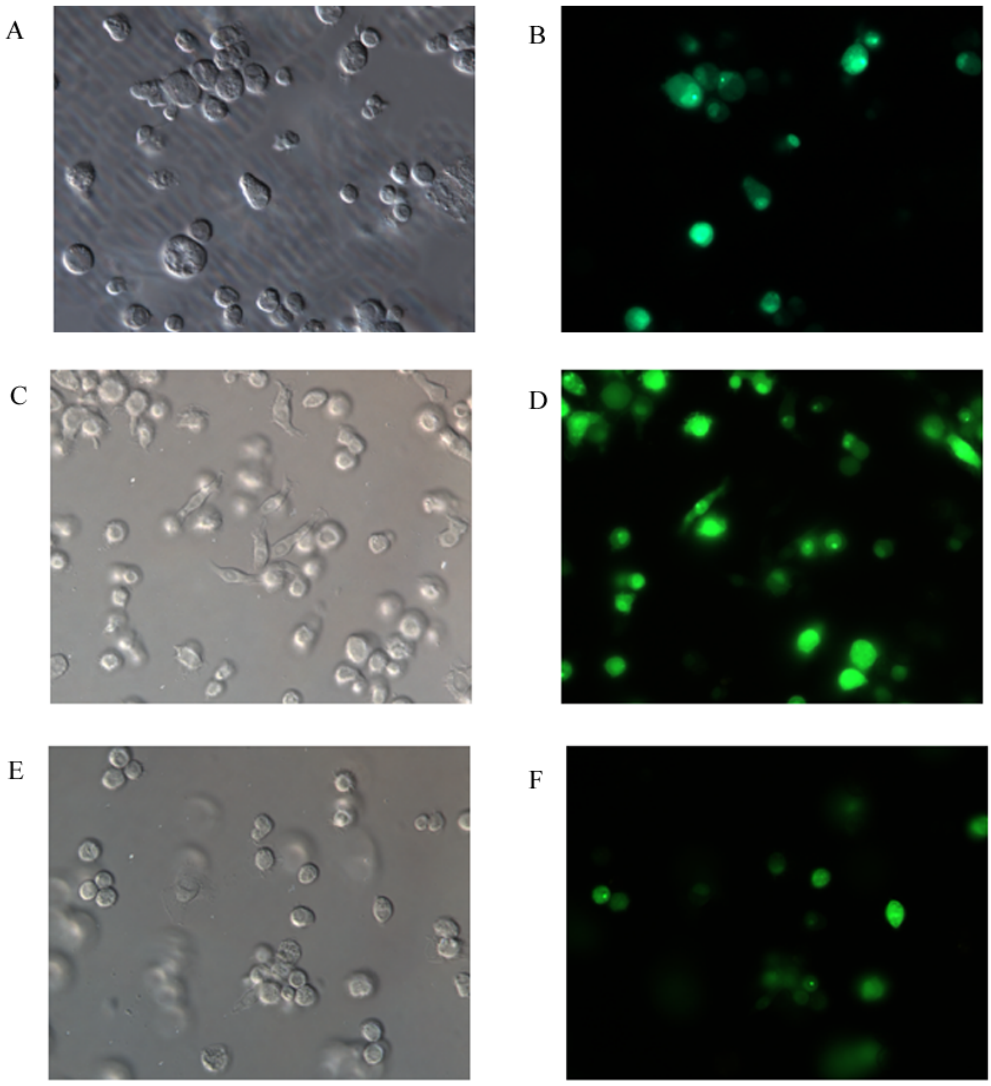

Fig. 4.1. Microscopy analyses of S2 cell phenotype. S2 cells were transfected with pIZT (A, B), pIZT-110 (C, D) or pIZT- Bm 100 (E, F). Cells were examined for differential interference contrast (A, C, E) and for GFP fluorescence (B, D, F).

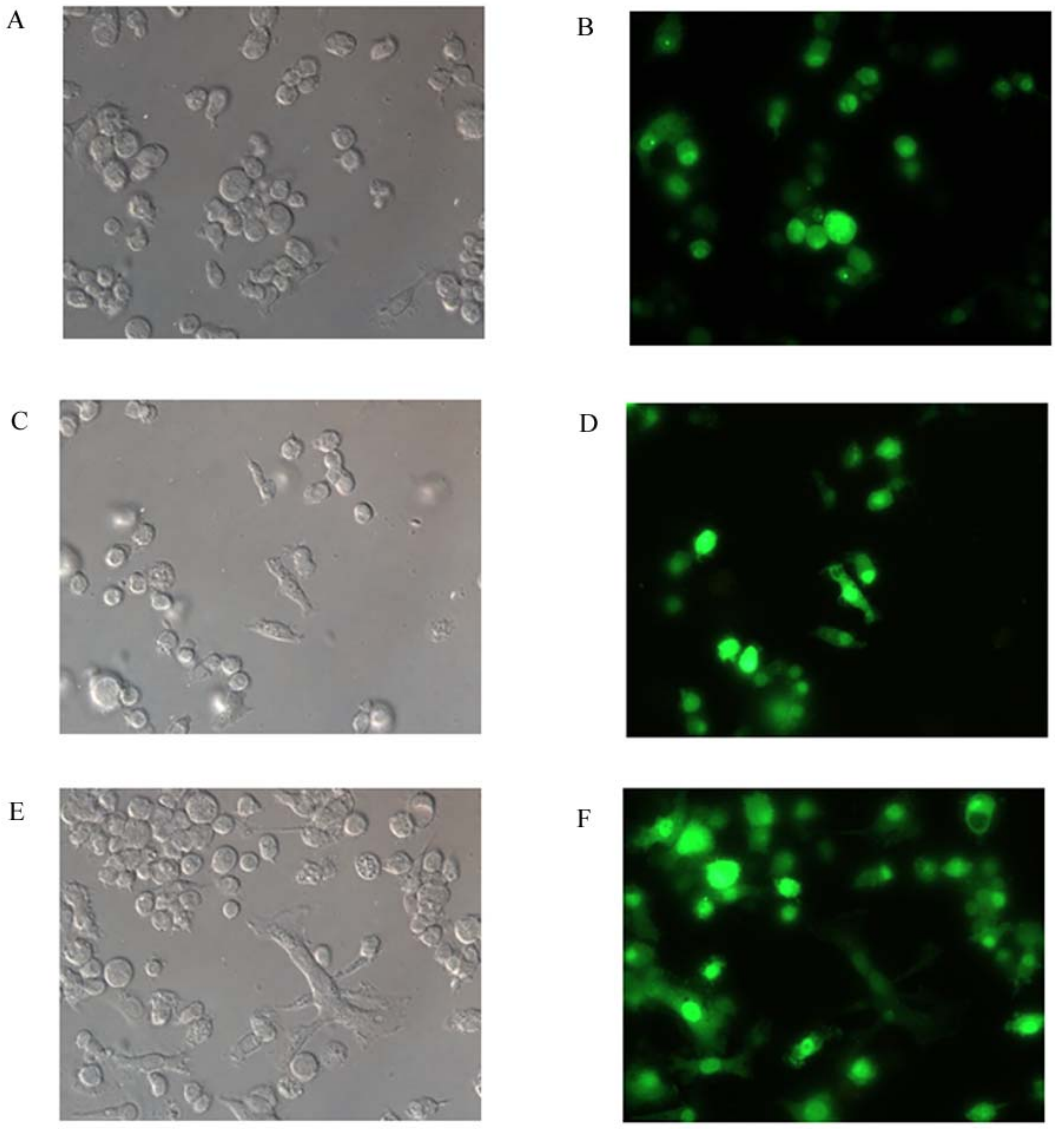

Fig. 4.2. Cells were transfected with pIZT-110 and incubated with 100 μM bestatin. Cells were examined at the center (A, B), middle (C, D), and edge (E, F) of the coverslip for differential interference contrast (A, C, E) and GFP fluorescence (B, D, F).

6.0 REFERENCES

Baek, K-H. 1999. The first oncogene in *Drosophila melanogaster*. Mutation Res. 436, 131-136.

Bhagwat, S.V., Lahdenranta, J., Giordano, R., Arap, W., Pasqualini, R., Shapiro, L.H. 2001. CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. Hemostasis, Thrombosis and Vascular Biol. 97, 652-659.

Cutforth, T., Gaul, U. 1999. A methionine aminopeptidase and putative regulator of translation initiation is required for cell growth and patterning in *Drosophila*. Mech. Devel. 82, 23-28.

Fujii, H., Nakajima, M., Saiki, I., Yoneda, J., Azuma, I., Tsuruo, T. 1995. Human melanoma invasion and metastasis enhancement by high expression of aminopeptidase N/CD13. Clin. Exp. Metastasis. 13, 337-344.

Gateff, E. 1982. Cancer, genes and development: the *Drosophila* case. Advances in Cancer Res. 37, 33-74.

Kawamura, K. Shibata, T., Saget, O., Peel, D., Bryant, P.J. 1999. A new family of growth factors produced by the fat body and active on imaginal discs. Development. 126, 211-219.

Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Shapiro, L.H., Arap, W. Ruoslahti, E. Aminopeptidase N is a receptor for tumor-homing peptides and a target inhibiting angiogenesis. Cancer Res. 60(3), 722-727.

Rubanyi, G. M. 2000. Angiogenesis in Health and Disease. Marcel Dekker, Inc.

Tsang, D.K., Crowe, D.L. 1999. The mitogen activated protein kinase pathway is required for proliferation but not invasion of human squamous cell carcinoma cell lines. Int. J. Oncol. 15(3), 519-523.

Varma, R., Mayor, S. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 394, 798-801.