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Localization of *Bacillus thuringiensis* Cry1A toxin-binding molecules in gypsy moth larval gut sections using fluorescence microscopy

Algimantas P. Valaitis *

USDA Forest Service, 359 Main Road, Delaware, OH 43015, United States

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ABSTRACT

The microbial insecticide *Bacillus thuringiensis* (Bt) produces Cry toxins, proteins that bind to the brush border membranes of gut epithelial cells of insects that ingest it, disrupting the integrity of the membranes, and leading to cell lysis and insect death. In gypsy moth, *Lymantria dispar*, two toxin-binding molecules for the Cry1A class of Bt toxins have been identified: an aminopeptidase N (APN-1) and a 270 kDa anionic glycoconjugate (BTR-270). Studies have shown that APN-1 has a relatively weak affinity and a very narrow specificity to Cry1Ac, the only Cry1A toxin that it binds. In contrast, BTR-270 binds all toxins that are active against *L. dispar* larvae, and the affinities for these toxins to BTR-270 correlate positively with their respective toxicities. In this study, an immunohistochemical approach was coupled with fluorescence microscopy to localize APN-1 and BTR-270 in paraffin embedded midgut sections of *L. dispar* larvae. The distribution of cadherin and alkaline phosphatase in the gut tissue was also examined. A strong reaction indicative of polyanionic material was detected with alcian blue staining over the entire epithelial brush border, suggesting the presence of acidic glycoconjugates in the microvillar matrix. The Cry1A toxin-binding sites were confined to the apical surface of the gut epithelial cells with intense labeling of the apical tips of the microvilli. APN-1, BTR-270, and alkaline phosphatase were found to be present exclusively along the brush border microvilli along the entire gut epithelium. In contrast, cadherin, detected only in older gypsy moth larvae, was present both in the apical brush border and in the basement membrane anchoring the midgut epithelial cells. The topographical relationship between the Bt Cry toxin-binding molecules BTR-270 and APN-1 and the Cry1A toxin-binding sites that were confined to the apical brush border of the midgut cells is consistent with findings implicating their involvement in the mechanism of the action of Bt Cry toxins.

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1. Introduction

The gypsy moth, *Lymantria dispar*, is one of the most damaging forest insect pests attacking oaks, maples, elms, and other deciduous trees in North America. *Bacillus thuringiensis* (Bt) is currently the most effective microbial pesticide used for gypsy moth suppression. Many strains of Bt produce parasporal crystals containing insecticidal proteins. These proteins, called Cry toxins, are proteolytically activated in the alkaline insect gut environment and bind to specific receptors localized on the brush border membrane of the midgut epithelium (Pigott and Ellar, 2007). The interaction of the activated toxins with their binding sites is followed by an irreversible insertion of the toxins into the cell membrane and then by either assembly of transmembrane pores or by activation of cation- and anion-selective channels. In addition, the insecticidal proteins elicit a powerful response from intoxicated gypsy moth larval gut cells: the selective shedding of GPI-anchored aminopeptidase N (APN-1) into the intestinal fluid (Valaitis, 2008). Because the amount of APN released by intoxicated larvae is directly related to the insecticidal potency of the insecticidal proteins, an APN assay of intestinal fluid can be used as a sentinel screen to discriminate between proteins that possess varying degrees of insecticidal activity toward Bt.

It is generally accepted that a high affinity interaction of Cry1A toxins with specific brush border receptors located on the apical surface of gut epithelial cells is a pivotal step in the mechanism of the action of Bt. Immunocytochemical localization of toxin-fed larvae has demonstrated that the insecticidal proteins accumulate in the microvilli of the gut epithelial cells (Bravo et al., 1992; Yi et al., 1996; Rausell et al., 2000). Histopathological studies have shown that one of the most rapid effects of Bt Cry intoxication is a striking change in the microvilli targeted by the Bt Cry toxins (Lane et al., 1989). A reduction of binding sites is a hallmark of development of resistance to Bt, because toxicity depends on the availability of specific high affinity receptors (Gahan et al., 2001).

Ongoing research for over a decade has sought to identify the specific high-affinity toxin-binding molecules and to understand

* Fax: +1 740 368 0152.
E-mail address: avalaitis@fs.fed.us

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how these receptors facilitate the toxin to gain access to the apical membrane on the brush border of the gut cells. A number of putative Bt Cry toxin receptors have been identified in various insect species based on their specificity and strength of their interaction with various Bt Cry toxins and, indirectly, through the genetic analysis of their association with resistance to Bt (Pigott and Ellar, 2007). These include APNs, cadherins, and, in *L. dispar*, a 270 kDa glycoconjugate (BTR-270). Alkaline phosphatases, polycalin, and lipids have also been demonstrated to bind to Cry toxins. Several APNs and toxin-binding cadherins have been examined for their toxin-binding properties and also their ability to promote cytotoxicity in transfected insect and mammalian cell lines. The *in vivo* and *in vitro* testing of these proteins has provided some evidence that they are involved in the mechanism of Bt action, but efforts to demonstrate their ability to promote toxicity has only been partially successful. A growing body of data suggests that these proteins alone do not promote all of the activity associated with Bt action and implies that the action may be mediated by a multiple-component complex, which remains to be discerned. One hypothesis is that sequential binding of Cry toxins to multiple receptors gives rise to a pre-pore oligomeric toxin complex that precedes membrane insertion and pore formation (Bravo et al., 2004; Aimanova et al., 2006; Pacheco et al., 2009; Arenas et al., 2010). This model, based mainly on studies of the toxin-binding properties of *Manduca sexta* cadherin and the GPI-anchored APN and alkaline phosphatase *in vitro*, needs validation and may not be broadly applicable to other insects. Indeed, while gypsy moth APN-1 has been demonstrated to bind Cry1Ac toxin, efforts to demonstrate toxin binding to gypsy moth cadherin have been negative (Valaitis, 2009). In addition, gypsy moth APN and cadherin expressed in insect cells and transgenic flies failed to confer any toxic effects in response to Cry1A toxins (unpublished results). These findings highlight dramatic differences in the toxin binding properties and the prospective role of APNs and cadherins in the mechanism of Bt action in different insects and suggest that a different molecule or complex serves as the Bt Cry toxin receptor in gypsy moth and possibly in other insect species. It has been recently reported that the impact on the toxin binding to cadherins from *Heliothis virescens* and *Bombyx mori* as a result of modified Cry toxins generated through protein engineering do not correlate with insecticidal activity of these toxins (You et al., 2008; Pigott et al., 2008). Several studies have also shown that APNs that bind toxins do not promote toxicity (Jenkins et al., 1999; Banks et al., 2003). It is conceivable that some APNs and cadherins which bind toxins may serve to sequester toxins at the surface of the brush border and that binding to another receptor is required to deliver the toxin into the membrane. Additional data is needed to fully resolve the identity of the true receptor(s) and to decipher the underlying mechanism in Bt Cry intoxication before conclusions or a functional model can emerge.

A unique high molecular weight Cry1A toxin-binding molecule, BTR-270, displaying some very distinct properties among the toxin-binding molecules identified in insect larvae was identified in gypsy moth brush border membrane vesicle (BBMV) preparations in previous studies. The acidic glycoconjugate is not stained using the conventional protein staining procedures but can be visualized using cationic dyes such as Stains-all (Valaitis et al., 2001). The carbohydrate moiety of BTR-270 contains glucuronic acid and high levels of hexoamines, features associated with acidic mucopolysaccharides covalently attached to proteoglycans. Although studies have shown that BTR-270 is an intrinsic component of the brush border, the BTR-270 in the gypsy moth larval gut tissue has not previously been localized. The focus of this study was to identify the topological location of BTR-270 in gypsy moth gut tissue in order to determine whether BTR-270 was present on the brush border of the gut epithelial cells. The locations of APN-1, cadherin and alkaline phosphatase in the gypsy moth gut specimens were also investigated using specific antibodies directed towards these proteins in conjunction with fluorescence microscopy.

2. Materials and methods

2.1. Purification and activation of recombinant B. thuringiensis Cry1A toxins

Recombinant Cry1Aa, Cry1Ab and Cry1Ac toxins over expressed in *Escherichia coli* were prepared from purified crystals (Lee et al., 1992). The crystals were resuspended in 50 mM sodium carbonate, 3 mM DTT and 5 mM EDTA at pH 10.4 and incubated at 37 °C for 2 h. Insoluble material was removed by centrifugation at 16,000*g* for 15 min. The soluble protein was digested with TPCK-treated bovine trypsin for 30 min at 37 °C using a trypsin to toxin ration ratio of 1:20 followed by centrifugation to remove any insoluble material. The activated toxins were purified by ion-exchange chromatography using a Macro-Prep High Q anion-exchange support (Bio-Rad Laboratories) and concentrated by Amicon PM-30 ultrafiltration. The purity of the toxins was assessed by SDS–PAGE and Coomassie staining. Protein concentration was determined by the Bio-Rad protein dye assay. Toxins were stored at 6 °C in 50 mM carbonate, 0.5 M KCl and 5 mM EDTA at pH 9.6.

2.2. Antibody preparation and protein labeling

A mixture of purified Cry1Aa, Cry1Ab and Cry1Ac toxins were used to produce rabbit polyclonal antibodies specific for Cry1A toxins used in Western blots and detection of toxin binding sites in gut sections examined by fluorescent microscopy. Antibodies specific to the Cry1Ac toxin-binding APN-1 from gypsy moth were developed as previously described (Valaitis et al., 1995). Rabbit polyclonal antibodies to purified BTR-270 from gypsy moth were developed to the toxin-binding molecule purified by preparative gel electrophoresis (Valaitis et al., 2001). Rabbit antiserum against *Aedes gambiae* alkaline phosphatase was kindly provided by Dr. Michael Adang (Department of Entomology, University of Georgia, Athens, GA, USA). Antibodies specific for cadherin were produced in rabbits against a mixture of gypsy moth cadherin fragments expressed in *E. coli* using the pMAL Protein Fusion and Purification System (New England BioLabs). Alexa Fluor 546 goat anti-rabbit IgG, Alexa Fluor 488 wheat germ agglutinin, and Alexa Fluor 546 Protein Labeling Kit were obtained from Invitrogen. BSA and other reagents were obtained from Sigma–Aldrich unless noted otherwise.

2.3. Preparation of paraffin-embedded tissue sections for immunohistochemistry

Gypsy moth larvae were obtained from United States Department of Agriculture (Otis Methods Development Center), Otis ANGB, MA. Fourth instar larvae were decapitated and the guts were pulled out. The foregut was excised and the entire luminal content enveloped in the peritrophic membrane was removed. The midgut fragments were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 6 °C for 24 h. The fixed specimens were rinsed with PBS to remove the PFA and dehydrated through a graded ethanol series (35%, 50%, 70%, 95%, 100%, 100% ethanol) for 45 min each. The tissue was cleared in sequential incubation in 70/30% and 30/70% (ethanol/xylene) for 20 min and then transferred to a mixture of 100% xylene saturated with Gem Cut Opal paraffin (Polysciences) at incubated at 35 °C. After 4 h the samples were transferred to 100% paraffin and infiltrated at 58 °C with four changes at 12–14 h intervals. The specimens were embedded in

paraffin blocks, cooled overnight and sectioned at 6–7 μm thickness on a rotary microtome. The sections were floated on a 40 °C water bath containing de-ionized water and mounted poly-L-lysine glass slides. Slides were dried at 37 °C overnight. The paraffin was removed by two changes of xylene, transferred to 100% ethanol and rehydrated through serial ethanol washes (70%, 40% 20% and purified water) for 10 min each. The gut tissue specimens were permeabilized by treatment with 10 mM citrate and 0.05% Triton-X 100 at pH 6.0 for 10 min, and the blocked with 1% BSA in Tris-buffered saline at pH 8.4 (TBS) overnight.

2.4. Immunohistochemical staining

Immunostaining with various antibodies were performed using Tris-buffered saline (TBS) supplemented with 0.05% Tween-20 (TBST) at pH 7.6. Tissue sections on the slides were probed with BTR-270 antibody (1:30,000 dilution), anti-APN-1 antibody (1:25,000 dilution), anti-alkaline phosphatase (1:4000 dilution) and cadherin-specific antibodies (1:3000 dilution) for 1 h at room temperature. Unbound antibodies were washed off with four TBST rinses (5 min each) and, subsequently incubated with Alexa Fluor 546 labeled goat anti-rabbit IgG (1:1000 dilution) in TBST for an additional 1 h. The green fluorescent Alexa Fluor 488 wheat germ agglutinin (0.2 mg/ml) and 0.5 μg/ml of the blue fluorescent DAPI (4, 6-diamino-2-phenylindole) were added as counter stains to the secondary antibody solution to label N-acetylglucosaminyl residues and nuclei, respectively. To analyze tissue specimens for Cry1A toxin binding sites the slides were incubated with 10 ml of 0.4 μg/ml solution of toxin in TBST at 6 °C overnight on a rocker platform. After washing four time with TBST the sections on the slide were incubated for 1 h with antibodies specific for Cry1A toxins (1:3000 dilution), washed as described above, and then Alexa Fluor 546 labeled goat anti-rabbit IgG secondary antibody solution. DAPI and wheat germ agglutinin were added prior to the final washes to stain nuclei and N-acetyl glucosamine-rich peritrophic and basement membrane, respectively. After the slides were washed with TBST, cover slips were mounted using 90% glycerol and sealed with nail polish. The specimens were then examined using a Zeiss Axiophot microscope equipped with a ProgRes CapturePro digital camera (Jenoptik Laser). Fluorescent images acquired in the red, blue and green channels were merged using the ProgRes Capture Pro Camera Control Software.

2.5. Histological staining acidic glyconjugates using alcian blue

Alcian blue staining was used for detection of polyanionic macromolecules in the paraffin-embedded tissue sections according to a modified procedure of Buee et al. (1991). Staining with alcian blue, which binds to negative charges on macromolecules, results in a predictable color based on the acid–base characteristics of the tissue. After de-waxing and rehydration, the tissue specimens on the slides were equilibrated with a 50 mM sodium acetate buffer containing 50 mM $MgCl_2$ at pH 5.7 and then stained with a 0.2% alcian blue solution in the pH 5.7 acetate buffer. After staining for 60 s the slides were destained by rinsing with the sodium acetate buffer followed with de-ionized water before mounting a coverslip.

3. Results and discussion

3.1. Cytochemical features of the brush border epithelium

Fig. 1 shows a phase contrast micrograph of a 4th instar gypsy moth midgut tissue section. The gypsy moth larval midgut tissue consists of a simple epithelium anchored to a basement membrane. The major functions of the epithelial cells include absorption of nutrients, secretion of digestive enzymes, and mediation of transmembrane signaling via membrane embedded receptors (Chang and Roth, 2003). The epithelium is comprised primarily of columnar cells bearing a border of tightly packed 4–5 μm-long apical membrane protrusions (microvilli). The microvilli are present along the entire apical surface of the polarized epithelium. Interdispersed among the columnar cells are goblet cells, whose apical surface is invaginated to form a cavity. The brush border microvilli of the epithelial cells are covered with an electronegative surface coat, the glycocalyx. The glycocalyx surrounds the tip and the lateral borders of the microvilli and is believed to be composed of acidic, carbohydrate-rich proteoglycans and mucopolysaccharides. The morphological characteristics of the midgut in the gypsy moth do not change during development.

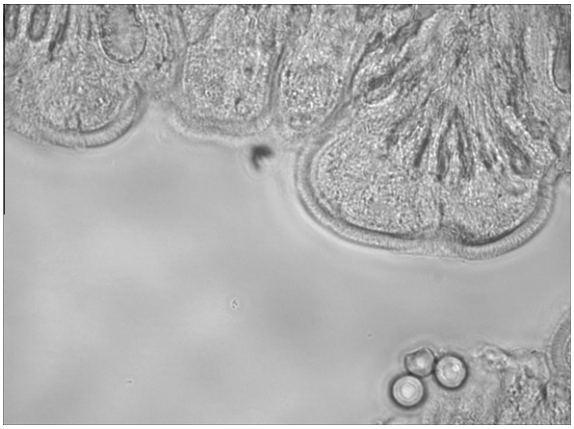

Fig. 1. A high magnification phase contrast view of the gypsy moth 4th instar midgut tissue section. A brush border of tightly packed microvilli can be seen along the entire apical surface of the insect midgut epithelial cells.

Staining of the gypsy moth midgut tissue section using the cationic dye alcian blue showed a strong reaction with the microvilli covering the luminal surface of the epithelial cells (Fig. 2). Since alcian blue is known for its selective interaction with acidic molecules, these results indicate that the microvilli contain relatively high amounts of an acidic component containing either reactive sulfate or carboxyl groups. Alcian blue forms very strong complexes with sulfated glycosaminoglycans attached to heparan sulfate and chondroitin sulfate proteoglycans. The alcinophilia observed in tissue sections may also be accounted for by the acidic,

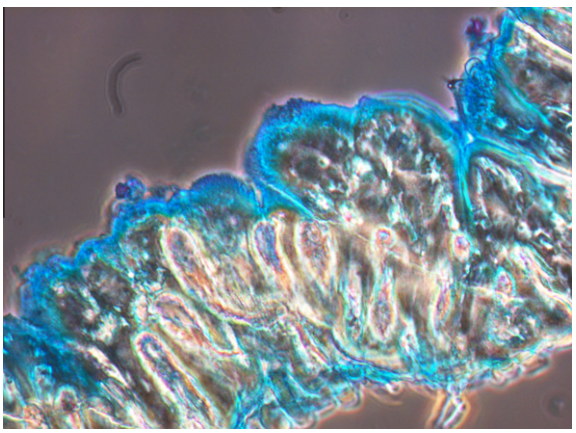

Fig. 2. Alcian blue staining of the gypsy moth midgut shows strong labeling of the microvilli and the basement membrane indicating the presence of relatively high amounts of an acidic component containing either sulfate or carboxyl reactive groups.

non-sulfated glucuronic acid residues in the carbohydrate moiety of insect glycoproteins and glycosphingolipids (Dennis et al., 1991).

Alcian blue staining is very specific for proteoglycans (Wall and Gyi, 1988). The basement membrane in the gypsy moth gut tissue section was stained blue with the alcian blue solution, presumably due to the presence of acidic proteoglycans, which have been localized in the basement membrane in other insect species (Campbell et al., 1987; Martin, 1987). An intense reaction of alcian blue with epithelial cell surfaces due to the presence of electronegative groups has been reported for both vertebrate and invertebrate cells (Tito, 1965; Trimble and Thompson, 1980). In addition, altering its ionic strength and pH, alcian blue can be used to discriminate between different classes of proteoglycans.

In the *Anopheles* mosquito, a sulfated proteoglycan distributed along the epithelial microvilli midgut has been implicated to play a vital role in midgut cell invasion by the *Plasmodium* parasite (Dinglasan et al., 2007). The interaction of cell surface heparin sulfate proteoglycans with pathogens can trigger a signaling cascade promoting their successful infection. Much evidence suggests that proteins that interact with proteoglycans bind to the negatively charged groups via basic residues present on distinct protein domains. A recent study of the *Clostridium perfringens* toxin shows that an electrostatic interaction is essential for the toxin binding to its receptor on mammalian cells (Kimura et al., 2010).

Specific receptor binding, essential for toxicity, is mediated by surface loops on domain II, one of the three structural domains of Bt Cry toxins. Analyses of the surface loops in toxin derivatives constructed in protein engineering experiments have shown that the extended loops are responsible for insect specificity of several Cry1A toxins (Rajamohan et al., 1996). A surface charge model of a Cry1A toxin highlights a strong positive field around the domain II loops (Jenkins et al., 2000). Hypothetically, the positively charged residues in domain II may bind a negatively charged target receptor located on the microvilli of the insect midgut epithelium. Since BTR-270 has been demonstrated to be an acidic component of the gypsy moth midgut epithelium brush border membrane, its negative charge may attract it to specific positively charged residues on the surface loops of the toxin, resulting in toxin insertion into the membrane.

3.2. Localization of Cry1A toxin binding sites

In Fig. 3 Cry1Aa toxin binding sites are shown stained orange with Alexa Fluor 546 fluorophore-conjugated antibody. The toxin binding sites were observed along the entire apical surface of the midgut epithelial cells using fluorescence microscopy. Nuclei were stained blue with DAPI and the basement membrane was stained green using an Alexa Fluor 488 wheat germ agglutinin (WGA). All three of the Cry1A toxins (Cry1Aa, Cry1Ab and Cry1Ac) bound to the apical microvillus surface. The intensity of the orange Cry1Ac fluorescence was stronger than that of Cry1Aa or Cry1Ab (data not shown). These observations are consistent the data reported by Wolfersberger (1990), that Cry1Ac displays a stronger binding affinity with gypsy moth brush border membrane vesicles (BBMV) than Cry1Ab, despite that Cry1Ac is about 400 times less toxic to gypsy moth larvae. These results clearly show that the initial binding sites for the Cry1A toxins are concentrated on the insect cell microvilli but very likely do not reflect irreversible binding associated with the insertion of the toxin into the membranes (Liang et al., 1995). Localization of the Bt Cry toxin binding sites in gypsy moth on the microvilli is consistent with similar observations in other insect species using immunochemical approaches and fluorescence microscopy (Chen et al., 2005; Fernandez et al., 2006; Aimanova et al., 2006).

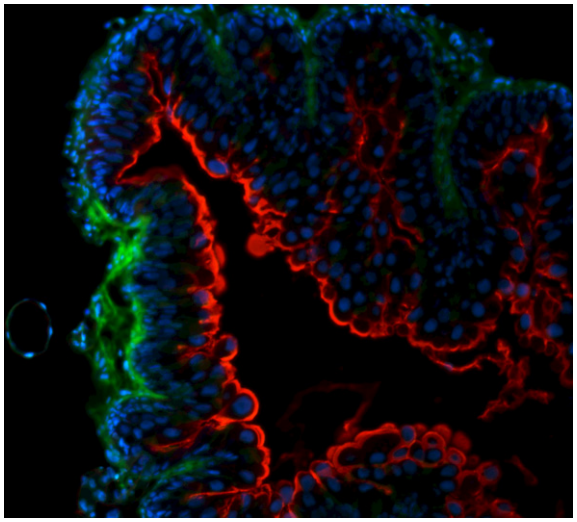

Fig. 3. Immunolocalization of *Bacillus thuringiensis* Cry1Aa toxin binding sites in the gypsy moth larval midgut tissue section with Alexa Fluor 546-conjugated secondary antibodies (orange) using fluorescence microscopy. Nuclei were stained blue with DAPI and the basement membrane was stained with the green fluorescent Alexa Fluor 488-conjugated wheat germ lectin (WGA).

3.3. Immunolocalization of gypsy moth aminopeptidase N (APN-1), alkaline phosphatase (ALP), cadherin (LdCad), and BTR-270

The gut of the gypsy moth larvae can be easily divided into three distinct sections: the foregut, the midgut, and the hindgut. Since previous studies of the gypsy moth and other lepidoptera have demonstrated that the midgut region of the gut is the site where the lepidopteran-specific toxins accumulate in intoxicated insects (Bravo et al., 1992; Yi et al., 1996), the current studies focused on sections of the anterior to the posterior region gypsy moth midgut. Fig. 4 shows that APN-1 is present in the brush border microvilli. The intensity of the fluorescence suggests APN-1 is abundant in the microvillar brush border, consistent with the observation that APN-1 is a prominent Coomassie-blue staining protein in brush border membrane vesicles (BBMV) prepared from larval insect gut samples. Controls in which the tissue sections

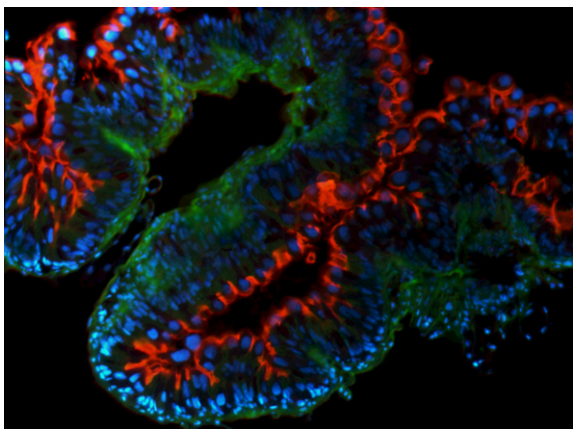

Fig. 4. Fluorescent micrograph of gypsy moth APN-1 localized in a paraffin embedded 4th instar midgut section. APN-1 was stained fluorescent orange with using Alexa Fluor 546 secondary antibodies, nuclei were stained blue with DAPI and N-acetyl glucosamine residues in the basement membrane were labeled green with Alexa Fluor 488 WGA.

were incubated with only Alexa Fluor 546-conjugated secondary antibody were negative. In a study of the distribution of *M. sexta* APN1 (MsAPN1) in different regions of the insect midgut, relative intensity of the fluorescence showed that MsAPN1 was enriched in the posterior region more than in the anterior (Chen et al., 2005). In contrast, fluorescence microscopy shows that gypsy moth APN-1 is relatively evenly distributed along the entire larval midgut, similar to findings reported for *B. mori* APN1 (Yaoi et al., 1997).

Like APN-1, gypsy moth alkaline phosphatase (ALP) was localized along the brush border of the midgut epithelium and appeared to be restricted to the microvilli (Fig. 5). In comparison to APN-1, the intensity of the fluorescence of ALP was weaker, suggesting that less ALP is present than APN-1. A recent study of *M. sexta* larval ALP enzyme activities in BBMV prepared from 1st through 5th instar larvae revealed that the amount of ALP changes during development, and that younger *M. sexta* larvae have more ALP than older larvae (Arenas et al., 2010). Whether similar developmental changes occur in gypsy moth is presently unknown.

Two distinct midgut ALP isozymes have been characterized in *B. mori* larvae (Eguchi, 1995): a 58 kDa membrane-bound form (m-ALP) and a 61 kDa soluble form (s-ALP). The m-ALP localized in the midgut was found to be distributed roughly evenly along the brush border of the entire length of the midgut. Gypsy moth BBMV probed with anti-ALP antibodies in Western blots showed that ALP in gypsy moth has an apparent size of 58 kDa. This size is consistent with the apparent size of 58 kDa determined for the m-ALPs in both *B. mori* and *Aedes aegypti* (Dechklar et al., 2011). A growing body of data suggests that ALPs in some lepidopteran and dipteran insect larvae are capable of binding Bt Cry toxins similarly to toxin-binding APNs (Hua et al., 2009; Likitvivatanavong et al., 2011). Both of the brush border membrane-anchored proteins are believed to be clustered in lipid membrane rafts that may function to promote focal accumulation of toxins at the toxin-insertion sites. It has been proposed that raft membrane microdomains serve to accelerate the oligomerization of the Bt Cry toxins prior to insertion into the apical brush border membranes of the insect cells (Zhuang et al., 2002).

The results obtained by probing 5th instar gypsy moth gut sections with antibodies specific to gypsy moth cadherin (LdCad) and then with orange Alexa Fluor 546-conjugated secondary antibodies are shown in Fig. 6. Nuclei were stained with the blue fluorescent DAPI reagent. LdCad was detected along both the basement membrane and the apical brush border membrane. The localization of LdCad in the gypsy moth gut tissue differs from the restricted binding profile observed for Cry1A toxin binding sites, which were localized exclusively to the apical brush border membrane of the cells. The expression of LdCad in 1st through 3rd instar gypsy moth larval gut sections was not detectable with fluorescence microscopy. These results are consistent with immunoblot analysis of LdCad in BBMV prepared from 2nd to 5th instar gypsy moth larvae, which showed that LdCad was below detection levels in the early instars and induced only after the third instar (results not shown). LdCad antibodies are highly specific for the 185 kDa LdCad and display no cross-reaction with BTR-270, APN, or ALP in gypsy moth BBMV Western blots. The detection of LdCad along the basement membrane was unexpected since the homologous cadherin-like protein in *B. mori* larvae (BtR175) was specifically localized by immunostaining only on the brush border of the insect cells (Hara et al., 2003). In contrast, the localization of gypsy moth LdCad in the gut sections was comparable to the localization of the cadherin-like protein in *M. sexta*, (BT-R_1), which was also found along both the basement membrane and the brush border microvilli (Chen et al., 2005). A substantial amount of evidence suggests that toxin-binding cadherins found in various insect larvae play a role as receptors or co-receptors for Bt. However, all efforts to demonstrate either the toxin binding or receptor function of LdCad have failed, including studies of the expression of the full-length LdCad in transgenic flies and cell cultures, toxin binding studies with native and recombinant LdCad fragments, and analyses by surface plasmon resonance (unpublished results). The presence of LdCad in the basement membrane, the absence of toxin binding sites in the basement membrane, the lack of expression of LdCad in early larval instars, and no evidence for a capacity to bind Cry1A toxins, all suggest that LdCad is not involved in the recognition and binding interactions with the Bt Cry toxins that are active towards gypsy moth larvae.

A unique toxin binding molecule, BTR-270, in the gypsy moth brush border has been identified using several experimental approaches: toxin-binding analysis in ligand blots of BBMV proteins fractionated by SDS–PAGE, toxin-antibody pull-down assays, and affinity chromatography with immobilized toxin. Toxin-binding kinetics obtained using surface plasmon resonance analysis have provided evidence that BTR-270 may be the Bt Cry toxin receptor in the gypsy moth brush border membranes. BTR-270 is distinctive in that it is refractive to Coomassie staining and is stained blue

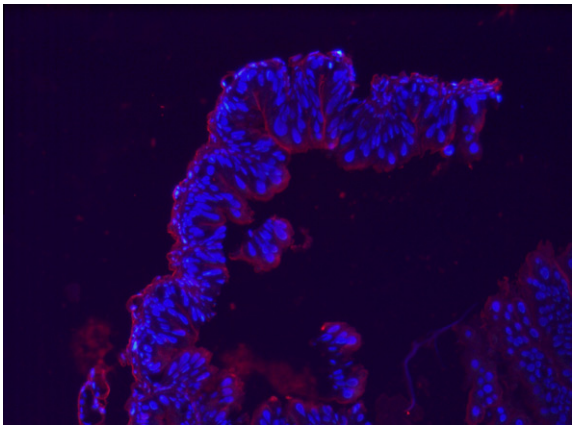

Fig. 6. Cadherin visualized with orange fluorescent secondary antibodies is observed both at the brush border and the basement membrane in the gypsy moth larval midgut tissue section by immunofluorescence microscopy. Nuclei were stained with the blue fluorescent DAPI reagent.

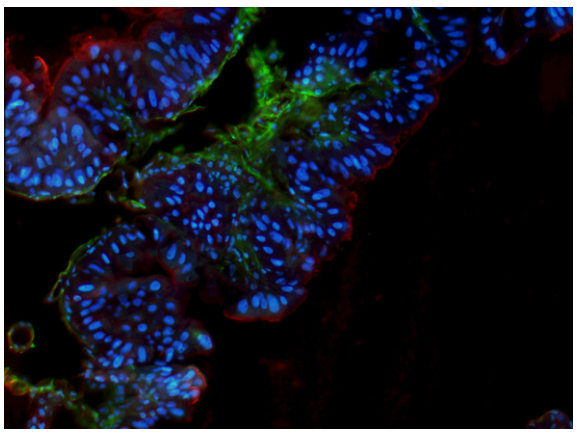

Fig. 5. A fluorescent micrograph of the immunolocalization of alkaline phosphatase (ALP) in the gypsy moth larval midgut shows that expression of APN-1 (orange) in the tissue is restricted to the brush border of the midgut epithelial cells. Nuclei were stained blue with DAPI and the basement membrane was counterstained with the green fluorescent Alexa Fluor 488-conjugated WGA.

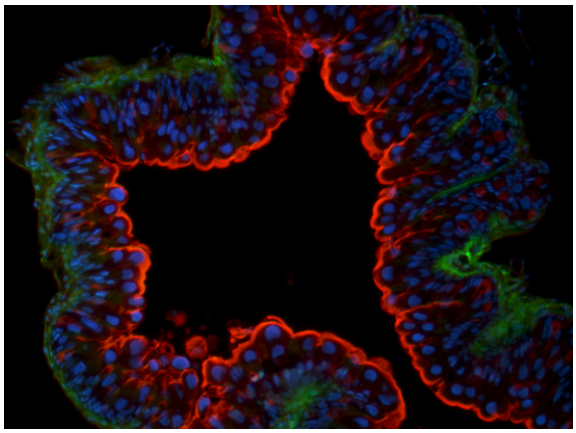

Fig. 7. Immunolocalization of BTR-270 in the gypsy moth midgut tissue. BTR-270 (orange) was localized exclusively in the microvillar brush border of the midgut known to be the target site of *Bacillus thuringiensis* Cry1A toxins. Nuclei were stained blue with DAPI and the basement membrane was counterstained with the green fluorescent WGA lectin.

with the metachromatic dye Stains-all, while other brush border membrane proteins stain red or pink. Although this acidic glycoconjugate has been demonstrated to be a component of brush border membrane vesicles (BBMV) which are derived from gypsy moth larval gut tissue, its localization in the gut tissue has not been reported. The immunohistochemical localization of BTR-270 in the midgut sections, determined using anti-270 antibodies followed by Alexa Fluor 546-conjugated secondary antibodies, is shown in Fig. 7. The orange fluorescence in the gut section correlates with the immunohistochemical staining of BTR-270. Nuclei were stained with the blue fluorescent dye DAPI, and N-acetyl glucosamine sugar residues associated with basement membrane components were stained with the green fluorescent Alexa Fluor 488-conjugated WGA lectin. BTR-270 was not detected in the basement membrane and appeared to be exclusively localized to the microvilli at the apical surface of the midgut epithelial cells. BTR-270, APN-1, and *L. dispar* ALP are located in the brush border at the cell surface, demonstrated to be the target site for toxin binding interactions, which trigger toxin conformational change and insertion. Gypsy moth APN-1 is a specific Cry1Ac toxin binding protein. Whether gypsy moth ALP interacts with Bt Cry toxins and functions as a midgut microvilli receptor remains to be assessed. Both new and previously established data indicate that there are specific receptor elements lining the electronegative surface of the midgut microvilli that regulate toxin binding, and the insecticidal activity of Bt is largely determined by toxin affinity for these binding sites. Investigation of the anionic chemistry of the brush border matrix is needed to determine how the negatively charged surface serves as a mechanism by which slight differences in the structure of the toxins may affect focal binding capacity and toxin affinity for the microvillar cell surface. The acidic glyconjugate BTR-270 has the capacity for high affinity interactions with Bt Cry toxins which have different interaction affinities, and, in this study is confirmed to be strategically located at the brush border membrane targeted by the Bt pore-forming toxins.

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