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DOMAIN III EXCHANGES OF *BACILLUS THURINGIENSIS* CRYIA TOXINS AFFECT BINDING TO DIFFERENT GYPSY MOTH MIDGUT RECEPTORS

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SUMMARY: Aminopeptidase-N, purified from gypsy moth (*Lymantria dispar* L.) brush border membrane vesicles, exhibited specific binding to CryIAc toxin but not to CryIAa toxin. CryIAa-CryIAc hybrid toxins were used to localize the aminopeptidase-N binding region on CryIAc. Slot blot assays and ligand blot experiments demonstrated that the hybrid toxins which have the residues 451 to 623, comprising essentially domain III, from CryIAc toxin exhibited strong binding to purified aminopeptidase- N and 120 kDa brush border membrane protein. In contrast, the hybrid toxins which have the residues 451 to 623 from CryIAa toxin failed to bind to aminopeptidase-N, but did bind to another receptor, a 210 kDa protein. This is the first direct evidence that domain III is involved in receptor binding and the first to demonstrate that domain III substitutions direct the binding of these toxins to different gypsy moth midgut receptors.

Bacillus thuringiensis (Bt) δ–endotoxins are potent to several orders of insect larvae. The mechanism of action consists of solubilization of Bt crystal, activation of protoxin to toxin by midgut proteases, binding of the toxin to receptors, oligomerization, insertion into the midgut membrane, and pore formation (1). Receptor binding of Bt toxins has been extensively studied for many years using radiolabeled toxins and brush border membrane vesicles (BBMV) (2-10). A direct correlation between toxicity and receptor binding has been observed in most studies (2-9, 11) although several exceptions have been also reported (12, 13).

A three-domain structure for the coleopteran specific CryIIIA toxin has been determined by X-ray crystallography (14). Domain I, a 7α–helix bundle composed of the N-terminal 250 amino acid residues, is believed to participate in ion channel formation. Experiments with

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Abbreviations

APN: Aminopeptidase-N, BBMV: Brush border membrane vesicle, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, Bt: *Bacillus thuringiensis*, PVDF: Polyvinyliden difluoride.

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synthetic peptides identical with certain of these helices support pore formation function of domain I (15, 16). Mutations in domain I exhibited alterations in toxicity (17) and irreversible binding (18). A previous study using homolog-scanning mutant hybrids demonstrated that the amino acid residues 332 to 450, within domain II, of CryIAa toxin were important for insect specificity and receptor binding to *B. mori* midguts (7, 19). Further studies reported that mutations in the loop regions in domain II of CryIAb toxin affected initial binding and irreversible binding (9, 10). The function of domain III, although less clear, has been suggested to be a toxin stabilizer (14, 20). However, mutations in a conserved block in domain III indicated a possible role in ion channel formation (21).

Toxin binding proteins (receptors) in insect midgut BBMVs have been identified by BBMV protein blot experiments. Recently, a 120 kDa BBMV protein, aminopeptidase-N (APN), has been purified from *Manduca sexta* (22, 23) and gypsy moth (*Lymantria dispar*) BBMV and identified as a CryIAc receptor (24). More recently, APN from *M. sexta* was cloned and characterized (25).

In this study, we performed slot blot assays and BBMV protein blot experiments to identify the APN binding region on CryIAc toxin by using series of hybrid toxins between CryIAa and CryIAc. These results clearly demonstrate that the residues located between amino acid 451 to 623, containing the end of domain II and the entire domain III of CryIAc toxin is responsible for binding to APN . This region is also important in recognizing the 210 kDa CryIAa binding protein.

MATERIALS AND METHODS

Purification and iodination of Bt toxins and APN

CryIAa, CryIAc, and hybrid mutant proteins (Fig. 1) were purified as described by Lee *et al.* (7). Construction of hybrid toxin gene is described in Ge, *et al.* (19). Purified crystal proteins were solubilized in 50mM sodium carbonate buffer, pH 9.5, containing 10mM dithiothreitol. Protoxins were activated to toxins by treatment with 2% trypsin (w/w). Purity of the toxins was examined in 9% SDS-PAGE (26). Purification of APN from gypsy moth BBMV was described in detail in Valaitis *et al.*(27).

For iodination, 25 μg of toxins and APN were labeled with 1 mCi ^{125}NaI (Amersham) and IODO-BEAD (Pierce) as described by Lee *et al.* (7). Specific activities of the Bt toxins and APN were in the range from 1.5 to 2.0 μCi/μg.

BBMV preparation

BBMV from last instar larvae of gypsy moth was prepared by the differential magnesium precipitation method of Wolfersberger *et al.* (28). The final BBMV pellet was resuspended in 8mM $NaHPO_4$, 2mM KH_2PO_4, 150mM NaCl, pH 7.4. The BBMV was used either immediately or frozen in liquid nitrogen until use.

Slot blot assays and BBMV protein blot assays

2.5 μg of Bt toxins were blotted onto PVDF (polyvinylidene difluoride) membrane (BioRad) by using a slot blot apparatus (BioRad) and blocked with 3% BSA. The membrane was incubated with 5 nM of [^{125}I] labeled APN for 2 h . The membrane was washed with TTBS (50mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, pH 7.5) and exposed to Fuji RX film for 2 days. In another experiment, 2.5μg of APN was blotted onto PVDF membrane and probed with [^{125}I] labeled Bt toxins.

For BBMV protein blot assays, 40 μg of gypsy moth BBMV proteins were separated onto 7.5% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 3% BSA and incubated with 5nM of [^{125}I] labeled CryIAa, CryIAc, and hybrid toxins for 3 hr. The membrane was washed extensively with TTBS buffer and exposed to Fuji-RX film for 2 to 3 days.

RESULTS AND DISCUSSION

Homolog-scanning is virtually equal to Domain-substitution between CryIAa and CryIAc

The alignment of the primary amino acid sequences of CryIA toxins (29) with the secondary structure of the coleopteran specific CryIIIA toxin, determined by X-ray crystallography (14), shows that domain II contains the residues from 291 to 463 and domain III has the residues from 464 to the C-terminal end of the toxin at residue 623 (30). Homolog-scanning between CryIAa and CryIAc has been previously described by Ge, *et al.* (19). Figure 1 illustrates the construction of the hybrid toxins. The 4100 series of hybrid toxins have a *cryIAa* gene background. The 4105, 4109, and 4110 toxins have the residues from 332 to 612, 451 to 612, and 332 to 450 of CryIAc toxin, respectively. The 4200 series have a *cryIAc* gene background. The 4205, 4209, and 4210 toxins have the residues from 332 to 612, 451 to 612, and 332 to 450 of CryIAa toxin, respectively. The hybrid mutants, 4109, 4110, 4209, and 4210 make an exchange between an SstI site (in codon 451) and an XhoI site (in codon 612). From the SstI site to the beginning of domain III, there are 13 amino acid residues, only two of which differ between CryIAa and CryIAc: threonine and glutamine at the residues 453 and 457 of CryIAa, respectively, are replaced with methionine and isoleucine on those position in CryIAc. Alignment of amino acid sequences of these genes with CryIIIA (29) and comparison with the

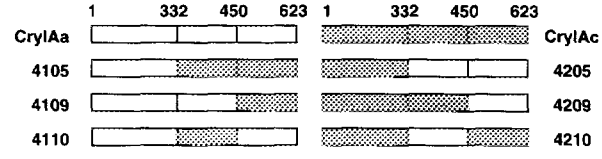

Fig1. Diagram of hybrid toxins between CryIAa and CryIAc. White background represents *cryIAa* gene while gray background represents *cryIAc* gene. The 4105, 4109, and 4110 hybrid toxins contain the residues from 332 to 623, 450 to 623, 332 to 450 of CryIAc, respectively. The 4205, 4209, and 4210 hybrid toxins contain the residues from 332 to 623, 450 to 623, 332 to 450 of CryIAa toxin, respectively.

structure of CryIIIA with the QUANTA molecule Graphics system (14) places these residues in β-sheet 11, which is centrally located in domain II (not on the surface) and is not expected to interact with the receptor. From the XhoI site (612) to the end of the toxin moiety (623) there are 11 amino acid residues and no differences between CryIAa and CryIAc (29). Considering this information, the homolog-scanning mutant pairs 4109-4209 and 4110-4210 essentially involve amino acid differences in Domain III.

Involvement of domain III in binding of toxins to purified gypsy moth APN

The data in Fig. 2 shows that CryIAc toxin strongly binds to APN, while the structurally related CryIAa toxin showed very weak binding. In order to localize the APN binding region on CryIAc toxin, hybrid toxins between CryIAa and CryIAc were used for slot blot assays. Iodine labeled APN was used as a probe. [^{125}I] labeled APN strongly bound to 4105, 4109, and 4210 toxins, which include residues from 451 to 623 of CryIAc. However, APN did not show measurable binding to 4205, 4209, and 4110 toxins, which include the corresponding region from CryIAa (Fig 2). These results demonstrate that the region from residues 451 to 623 of CryIAc (essentially domain III) is responsible for binding to APN.

In order to confirm these results, another experiment was performed. 2 μg of APN was first blotted onto the membrane and then probed with 5nM of [^{125}I] labeled hybrid toxins. The results were same as in the previous assays. The 4105, 4109, and 4210 toxins showed strong binding to APN but the 4205, 4209, and 4110 toxins did not bind to APN (Fig 3).

Domain substitutions and receptor selection

BBMV ligand blotting assays have been used to identify Bt toxin binding proteins in many studies (31-33). In a previous study, we have identified 210 kDa and 120 kDa proteins for CryIAa and CryIAc toxin binding proteins, respectively (24). Taking advantage of the fact that these two toxins recognize distinct BBMV proteins, we performed BBMV ligand blotting assays with [^{125}I] labeled hybrid toxins to localize the region responsible for binding to 120 kDa (APN) and 210 kDa protein. The 4105, 4109, and 4210 hybrid toxins recognize the 120kDa BBMV protein. On the other hand, the 4205, 4209, and 4110 hybrid toxins failed to bind to 120 kDa. Interestingly, these toxins bound to 210 kDa peptide (Fig 4). From these results, we further confirm that the residues between 451 to 623 are responsible for the binding to the 120 kDa CryIAc receptor, APN. Also we demonstrate that these residues from CryIAa are also important in recognizing 210 kDa CryIAa binding protein.

Although several studies have demonstrated that domain II (in particular the loop regions) is involved in receptor binding in certain insect systems (8, 9, 10, 14), other reports have

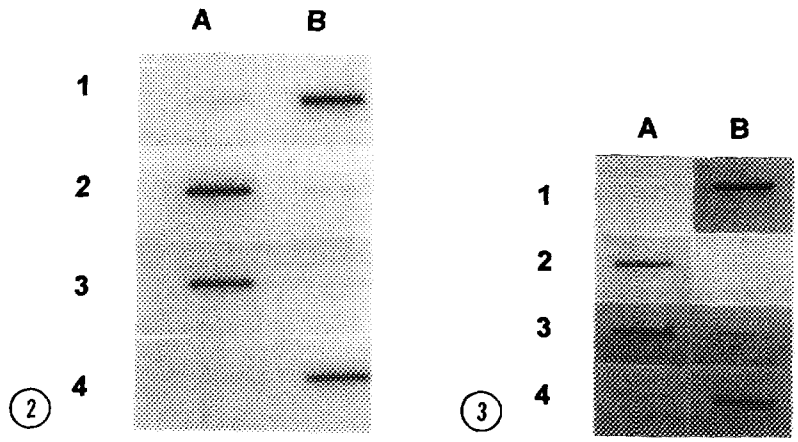

Fig.2. Binding of [^{125}I] labeled aminopeptidase N to Bt toxins. 2.5μg of Bt toxins (A1, CryIAa; B1, CryIAc; A2, 4105; B2, 4205; A3, 4109; B3, 4209; A4, 4110; B4, 4210) were blotted onto PVDF membrane by using a slot blot assay apparatus. The membrane was blocked and incubated with 5nM of labeled APN for 2hr. The membrane was washed and exposed to Fuji-RX film for 2 days.

Fig.3. Binding of [^{125}I] labeled CryIAa, CryIAc and hybrid toxins to APN. 2.5μg of APN was blotted onto the PVDF membrane by using a slot blot assay apparatus. The membrane was blocked and incubated with 5nM of labeled Bt toxins (A1, CryIAa; B1, CryIAc; A2, 4105; B2, 4205; A3, 4109; B3, 4209; A4, 4110; B4, 4210) for 2hr. The membrane was washed and exposed to Fuji-RX film for 2 days.

implicated that domain III of CryIA toxins might be involved in specificity and receptor binding to different insects. Ge, *et al.* (34) examined a larger set of homolog-scanning mutants employed in the present study for toxicity to *Trichoplusia ni* and *Heliothis virescens*. In the case of *H. virescens*, but not *T. ni*, insect specificity was determined by both domains II and III of CryIAc. The results for *H. virescens* were substantiated by Schnepf, *et al.* (35). In these cases it was

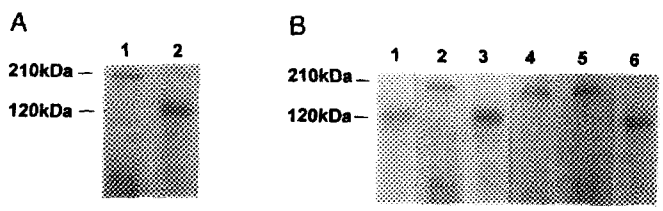

Fig.4. Gypsy moth BBMV ligand blotting with CryIAa, CryIAc, and hybrid toxins. 40μg of BBMV was separated onto 7.5% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked and probed with 5nM of [^{125}I] labeled CryIAa (A, lane1), CryIAc (A, lane 2) and hybrid toxins (B) (lane 1, 4105; lane 2, 4205; lane 3, 4109; lane 4, 4209; lane 5, 4110; lane 6, 4210) for 3 hr. The membrane was washed and exposed to Fuji-RX film for 2-3 days.

assumed that insect specificity was correlated with receptor binding. Domain III of CryIC was also observed to be associated with specificity to *Spodoptera exigua* but in this case receptor binding did not appear to be correlated to specificity (36). It would appear that these insect toxins are versatile in the utilization of their residues (and indeed in the use of different domains) in binding to diverse receptors in a variety of insects.

In the present study, we located the APN binding region and also the 210 kDa peptide binding region on CryIAc and CryIAa toxin, respectively, by using simple blot assays and BBMV ligand blotting assays with CryIAa/CryIAc hybrid toxins. We observe that the critical binding region for gypsy moth receptors is in domain III of these toxins. We also demonstrate that exchange of the domain III region results in differential receptor selection. These results indicate that the residues within domain III of CryIAa and CryIAc toxins play a major role in recognizing the receptors in gypsy moth. More detailed experiments are under progress to identify individual amino acids in this domain that are responsible for binding to gypsy moth receptors.

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