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Different Domains of *Bacillus thuringiensis* δ-Endotoxins Can Bind to Insect Midgut Membrane Proteins on Ligand Blots

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We investigated the role of the constituent domains of the CryIA(b) and CryIA(c) δ-endotoxins in binding to midgut epithelial cell membrane proteins of *Spodoptera exigua* and *Manduca sexta* on ligand blots. A collection of wild-type and CryIC-CryIA hybrid toxins was used for this purpose. As demonstrated elsewhere (R. A. de Maagd, M. S. G. Kwa, H. van der Klei, T. Yamamoto, B. Schipper, J. M. Vlak, W. J. Stiekema, and D. Bosch, Appl. Environ. Microbiol. 62:1537–1543, 1996), CryIA(b) domain III recognized a 205-kDa protein on *S. exigua* blots, while no specific binding by domain I or II could be detected. In contrast, on ligand blots of *M. sexta* proteins CryIA(b) domain II recognized a 210-kDa protein and CryIA(b) domain III recognized a 250-kDa protein. Domain III is responsible for the interaction of CryIA(c) with 120-kDa major binding proteins of both *S. exigua* and *M. sexta*. In addition, in *M. sexta* CryIA(c) also reacts with a 210-kDa binding protein through its domain I and/or domain II. These results show that besides domain II, domain III of δ-endotoxins plays a major role in binding to putative receptors on ligand blots. However, for *S. exigua* there was no clear correlation between binding of toxins on ligand blots and the in vivo toxicity of the toxins. These and previous results suggest that interactions of insect membrane proteins with both domain II and domain III can occur and that detection of these interactions depends on the type of binding assay used.

Bacillus thuringiensis forms proteinaceous crystalline inclusions during sporulation. The crystals consist of one or more so-called δ-endotoxins, which are also called Bt toxins or insecticidal crystal proteins and are toxic to insects. Toxins of the CryI class, which are active against lepidopteran insects, are formed as 120- to 130-kDa protoxins, which are solubilized in the alkaline environment of the lepidopteran midgut. After solubilization, processing by midgut proteases results in a relatively stable mature 60- to 65-kDa toxin. In susceptible insects, the mature toxin binds to the midgut epithelium and forms membrane pores, which results in lysis of the epithelial cells and eventually in death of the insect (10, 14). A major factor determining the insecticidal specificity of the various individual δ-endotoxins, each of which is toxic to only a limited number of insect species, is the presence of specific receptors on the surface of the midgut epithelium of the susceptible insect (9, 25). The importance of these receptors became even clearer with the observation that insects which had become resistant to one or several toxins often had lost the specific binding capacity for these toxins by either losing or modifying the receptors on the midgut epithelium (5, 15, 26).

Recently, four different toxin-binding molecules of three different insects have been purified, and the genes encoding three of these molecules have been cloned and sequenced. For *Manduca sexta* (tobacco hornworm), the putative CryIA(c) receptor is a 120-kDa aminopeptidase N (12, 13). One CryIA(b) receptor of this insect is a 210-kDa protein with homology to the cadherin family of proteins (22, 23). The CryIA(c) receptors of *Heliothis virescens* (tobacco budworm) and *Lymantria dispar* (gypsy moth) are also a 120-kDa aminopeptidase N (7, 24). A common technique used for the identification of toxin-binding midgut membrane proteins used in the isolation of the receptors mentioned above, as well as for identification of other putative receptors in various insects, is the ligand blot or toxin overlay technique (3, 6, 16, 18, 20). In this procedure, midgut membrane proteins from brush border membrane vesicle (BBMV) samples are separated by electrophoresis and transferred to nitrocellulose filters. Subsequently, toxin-binding molecules are identified by incubation with toxins and detection by autoradiography or with toxin-specific antibodies.

On the toxin side, elucidation of the three-dimensional structure of two δ-endotoxins has contributed greatly to a better understanding of the structure-function relationship (8, 17). The mature toxins consist of three domains, to which putative functions have been ascribed. N-terminal domain I is thought to be involved in insertion into the epithelial cell membrane. Domain II is thought to be involved in receptor binding, while the function of C-terminal domain III has so far eluded understanding. These conclusions were primarily based on a combination of structural information, the results of studies performed with hybrid toxins in which homologous parts had been exchanged between genes encoding toxins with different specificities, and the results of mutagenesis studies.

In recent studies in which hybrids between CryIC and either CryIE (1) or CryIA(b) (4) were used, we have shown that domain III plays an important role in determining activity against at least one insect, *Spodoptera exigua* (beet armyworm). Both CryIE and CryIA(b) became much more toxic to this insect when their domain III was exchanged for domain III of CryIC, even to the extent of producing a hybrid toxin with the highest activity against this insect reported so far (4). Although exchange of domain III apparently did not affect specificity of binding to intact *S. exigua* BBMVs, as would be expected when mainly domain II determines receptor binding, we showed that it did affect binding to BBMV proteins on ligand blots (4). CryIA(b) recognized a 205-kDa *S. exigua* protein on ligand

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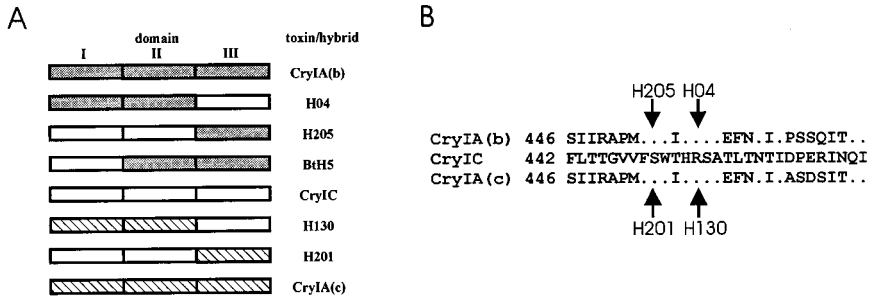

FIG. 1. (A) Schematic representation of the domain compositions of the wild-type and hybrid δ-endotoxins used in this study. (B) Alignment of the amino acid sequences of CryIA(b), CryIC, and CryIA(c) around the border between domains II and III, showing the locations of the crossover sites in the hybrid toxins used in this study.

blots, whereas a derivative with domain III of CryIC did not. The reciprocal hybrid consisting of CryIC and domain III of CryIA(b) recognized the same protein as CryIA(b), proving that domain III of CryIA(b) was sufficient for binding to the 205-kDa protein (4).

In this study we used an extended collection of wild-type and hybrid toxins to study the roles of CryIA(b) and CryIA(c) domains in recognizing membrane proteins on ligand blots for two different insects, *M. sexta* and *S. exigua*. We demonstrated that depending on the type of toxin and insect species used, either domain III alone or both domain II and domain III are involved in binding to target insect midgut membrane proteins. For *S. exigua* there was no clear correlation between binding of toxins on ligand blots and the in vivo toxicity of the toxins.

MATERIALS AND METHODS

Plasmids and hybrid toxin gene construction. CryIA(b) expression vector pBD140 and CryIC expression vector pBD150 have been described previously (1, 2). Isolation of CryIA(b)-CryIC hybrid H04 (plasmid pRM8) and isolation of CryIC-CryIA(b) hybrid H205 (plasmid pHK6) have also been described previously (4), as has isolation of CryIC-CryIA(b) hybrid protein BtH5 (11). Plasmid pSB645-2, a pBluescript KS$^+$-derived vector containing the entire *cryIA(c)* (holotype) gene, was obtained from T. Yamamoto (Sandoz Agro, Inc., Palo Alto, Calif.). A *cryIA(c)-cryIC* hybrid gene was constructed by replacing an *Nco*I-*Sac*I fragment of pRM8 containing the first 1,350 bp of *cryIA(b)* (encoding domains I and II) with the corresponding fragment of *cryIA(c)* from pSB645-2, which resulted in plasmid pHK13 (encoding hybrid toxin H130). To select a CryIC-CryIA(c) hybrid, an expression vector containing parts of the *cryIC* and *cryIA(c)* genes was constructed. In *cryIC-cryIA(b)* tandem plasmid pRM23, which was used previously for construction and selection of pHK6 (4), the 2,020-bp *Spe*I-*Kpn*I fragment encoding most of the mature toxin part of CryIA(b) was replaced by the corresponding fragment of *cryIA(c)* from pSB645-2. This resulted in *cryIC-cryIA(c)* tandem plasmid pHK12, which was transformed into *Escherichia coli* JM101 (*recA*$^+$). Plasmid DNA was isolated from five individual transformants, cut simultaneously with *Not*I and *Sac*I, and retransformed into *E. coli* XL-1 Blue to select for recombination events. Plasmids of individual transformants were isolated and analyzed by performing a restriction analysis and then DNA sequencing to determine the exact site of crossover. Selected recombinants were subsequently tested for production of a soluble protoxin.

Toxin isolation and purification. All wild-type toxins and hybrid toxins except CryIA(c) were produced by expression in *E. coli* XL-1 Blue, solubilization, trypsin activation of the protoxin, and fast protein liquid chromatography purification of the mature toxin as described elsewhere (1), except that 0.5 mM phenylmethylsulfonyl fluoride was added after incubation of protoxins with trypsin to prevent further degradation. Purified, trypsinized CryIA(c) was a gift from J. van Rie (Plant Genetic Systems, Ghent, Belgium).

Isolation of BBMVs. BBMVs were isolated essentially as described by Wolfersberger et al. (27) from dissected midguts of 5-day-old *M. sexta* and *S. exigua* larvae, except that 1 mM phenylmethylsulfonyl fluoride and 100 μM chymostatin in MET buffer [300 mM mannitol, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 17 mM Tris-HCl; pH 7.5] were added during the initial homogenization step. Isolated BBMVs were resuspended in 0.5× MET buffer and stored at −80°C.

Ligand blotting. Proteins from isolated BBMVs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For ligand blotting 50 μg of BBMV protein was mixed with SDS-PAGE sample buffer, heated for 5 min at 100°C, and loaded onto a 7.5% acrylamide gel in a continuous, 7.4-cm-wide sample slot. Prestained molecular mass marker proteins (Bio-Rad) were run alongside in a separate single slot. After electrophoresis, separated proteins were electrophoretically transferred to nitrocellulose filters (pore size, 0.22 μm; Schleicher and Schuell). Then 3-mm-wide strips were cut from the blots for incubation with toxins, and this was followed by incubation with a CryI antiserum and detection with ECL reagent as described previously (4).

RESULTS

Construction of hybrid toxins. In a previous study, we showed that domain III of CryIA(b) is involved in binding to a 205-kDa membrane protein of *S. exigua* on a ligand blot. This observation prompted us to use an extended collection of wild-type and hybrid toxins to study the role of the domains of CryIA(b) and CryIA(c) in binding to gut epithelial membrane proteins of two different insects, *S. exigua* and *M. sexta*. Figure 1A is a schematic representation of the domain compositions of wild-type and hybrid toxins used in this study. Construction of all of the hybrids except hybrids H130 and H201 has been described previously (see Materials and Methods).

CryIA(c)-CryIC hybrid H130, which has domains I and II of CryIA(c) and domain III of CryIC, was made by replacing domains I and II of CryIA(b)-CryIC hybrid H04, which were derived from CryIA(b), with the homologous fragment of CryIA(c) (see Materials and Methods). The reciprocal hybrid, CryIC-CryIA(c) hybrid H201, was selected after recombination of *cryIC-cryIA(c)* tandem plasmid pHK12. One of a group of several recombinants, which had a crossover site close to the border between domains II and III that gave a soluble protoxin, was used for further work. The crossover sites of the new hybrids, as well as those of the hybrids that were made previously, are shown in Fig. 1B.

Ligand blotting. To investigate the binding of the various wild-type and hybrid toxins to target midgut epithelial membrane proteins, proteins from isolated BBMVs of *S. exigua* and *M. sexta* were separated by SDS-PAGE under denaturing conditions and blotted electrophoretically onto nitrocellulose filters. Strips cut out of the resulting blots were incubated with purified toxins, and the bound toxins were detected by using a CryI antiserum. Figures 2A and B show the results obtained for *S. exigua* and *M. sexta*, respectively. For clarity, the domain composition of each of the toxins used is shown above its lane.

CryIA-binding proteins of *S. exigua*. As we demonstrated previously, in *S. exigua* CryIA(b) recognizes a 205-kDa protein and to a lesser extent an approximately 140-kDa protein (Fig. 2A, lane 1). These proteins were not recognized by CryIA(b)-CryIC hybrid H04 (Fig. 2A, lane 2), whereas they were bound by CryIC-CryIA(b) hybrid H205 (Fig. 2A, lane 3), showing that domain III of CryIA(b) is necessary and sufficient for recognition of these proteins. However, since the reaction of H205 with the 140-kDa protein was relatively weak, domain II prob-

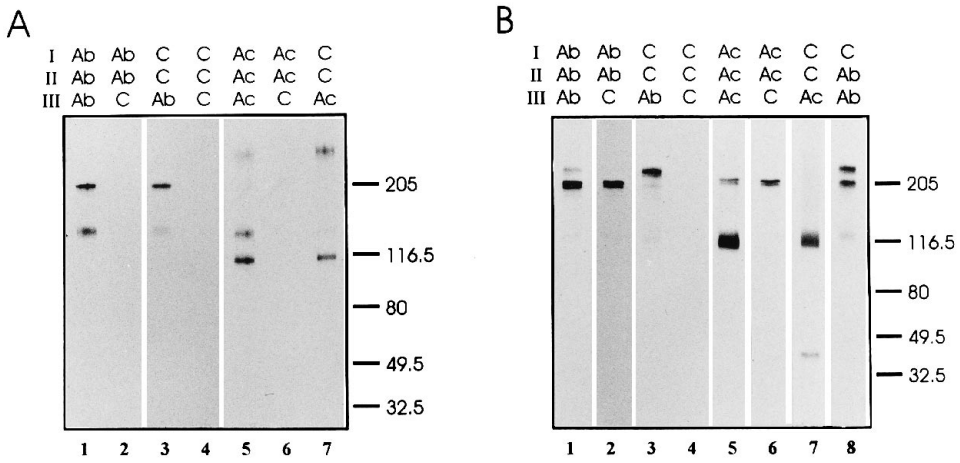

FIG. 2. Ligand blots of BBMV proteins from *S. exigua* (A) and *M. sexta* (B). Lanes 1, CryIA(b); lanes 2, hybrid H04; lanes 3, hybrid H205; lanes 4, CryIC; lanes 5, CryIA(c); lanes 6, hybrid H130; lanes 7, hybrid H201; lanes 8, hybrid BtH5. For clarity, the domain compositions of the toxins used for detection are shown above the lanes [Ab, CryIA(b); Ac, CryIA(c); C, CryIC]. The positions of molecular mass marker proteins are indicated on the right.

ably plays a role in this reaction. Under the conditions used for these experiments, we could not demonstrate any specific binding to membrane proteins by CryIC. This negative result was obtained with a number of different fresh preparations of toxin, as well as a number of BBMV protein preparations. The anti-CryI serum had been shown previously to react well with all of the proteins used in this study, including CryIC (data not shown).

We used a similar set of hybrid toxins to determine the role of CryIA(c) domains in recognition of midgut membrane proteins. In *S. exigua*, CryIA(c) bound mainly to an approximately 120-kDa protein and to a much lesser extent to larger proteins (140 and 250 kDa) (Fig. 2A, lane 5). Hybrid H130, in which domains I and II of CryIA(c) were combined with domain III of CryIC, did not bind to these proteins (Fig. 2A, lane 6). On the other hand, the inverse hybrid, hybrid H201, which had domain III of CryIA(c), bound to all three proteins mentioned above for CryIA(c) (Fig. 2A, lane 7). This shows that in *S. exigua* mainly domain III of CryIA(c) is responsible for recognition of midgut membrane proteins. The observation that binding of H201 to the 140-kDa minor protein was relatively weak compared with the binding of CryIA(c) (Fig. 2A, lanes 5 and 7) indicates that domain I and/or domain II of CryIA(c) probably plays a role in binding to this protein.

CryIA(b)-binding proteins of *M. sexta*. In contrast to most previous reports, in our experiments CryIA(b) recognized not only a major 210-kDa protein but also (to a lesser extent) an approximately 250-kDa protein which has not been reported previously (Fig. 2B, lane 1). Closer inspection with shorter exposure times of the blot during detection showed that the 210-kDa protein was actually a closely spaced doublet (data not shown). The CryIA(b)-CryIC hybrids mentioned above were also used to determine the role of the toxin domains in recognition of these proteins. Hybrid H04, with domains I and II of CryIA(b), was able to bind to the 210-kDa doublet but not to the minor 250-kDa protein (Fig. 2B, lane 2). On the other hand, hybrid H205, with domain III of CryIA(b), bound to a 250-kDa protein but not to the 210-kDa doublet (Fig. 2B, lane 3). Again, under the conditions used in this study we could not detect any binding of CryIC to *M. sexta* membrane proteins (Fig. 2B, lane 4). These results show that for CryIA(b) in *M. sexta*, domain I and/or domain II is responsible for binding to the 210-kDa membrane protein, while domain III is involved in binding to the newly identified 250-kDa protein. To determine whether it is domain I or domain II of CryIA(b) or both that are responsible for specific binding to the 210-kDa *M. sexta* protein, we used a previously constructed CryIC-CryIA(b) hybrid, hybrid BtH5, which has domain I of CryIC and domains II and III of CryIA(b) (11). This hybrid recognized the same two proteins of *M. sexta* that CryIA(b) recognized (Fig. 2B, lanes 1 and 8), showing that domain II of CryIA(b) is probably sufficient for recognition of the 210-kDa protein. For BtH5, reaction with the 250-kDa protein was stronger than binding to the 210-kDa protein compared with CryIA(b) protein binding. The same observation was made for H205, although the results maybe less convincing because there is no binding to the 210-kDa protein to compare with (Fig. 2B, lane 3). This suggests that although domain III is the major binding component for the 250-kDa protein, the other domains can play a role in binding by either taking part in binding directly or by taking part indirectly by influencing the overall structure of the molecule and thereby the binding affinity of other domains.

CryIA(c)-binding proteins of *M. sexta*. In *M. sexta*, CryIA(c) recognized an approximately 120-kDa protein (Fig. 2B, lane 5) and (with much lower intensity) a 210-kDa doublet that comigrated with the major protein recognized by CryIA(b) (Fig. 2B, lane 1). Hybrid H130, with domains I and II of CryIA(c), recognized the minor band at 210 kDa, but not the major band at 120 kDa (Fig. 2B, lane 6). On the other hand, H201, with domain III of CryIA(c), was able to bind to the 120-kDa protein, but not to the minor 210-kDa protein (Fig. 2B, lane 7). These results show that for CryIA(c), as for CryIA(b) in *M. sexta*, domain I and/or domain II recognizes a 210-kDa protein doublet. These proteins may well be identical, which would not be surprising considering that in domains I and II CryIA(b) and CryIA(c) differ only by six amino acids. In contrast to the situation for CryIA(b), recognition of the major binding protein by CryIA(c) in *M. sexta* is through its domain III.

In conclusion, our results show that depending on the host insect, both domain II and domain III can play a role in recognition of membrane proteins on ligand blots.

DISCUSSION

Ligand blotting has been shown to be a useful technique for identifying and isolating putative δ-endotoxin receptors of in-

sect midgut epithelial cell membranes. All three lepidopteran δ-endotoxin receptors that have been purified and cloned could initially be detected by this technique (3, 6, 12, 22). Study of hybrid toxins has been shown to be a powerful approach for determining which parts or domains of the toxin molecules are involved in toxicity and insect specificity. In this study we combined these approaches to assess the roles of the individual domains of two toxins, CryIA(b) and CryIA(c), in two different target insects. Our results show that in both *S. exigua* and *M. sexta*, domain III plays a major role in recognition of membrane proteins on ligand blots, while only in *M. sexta* could a role for domain II be demonstrated. In *Lymantria dispar* domain III has also been shown to be responsible for the recognition of the 120-kDa protein aminopeptidase N by CryIA(c) on ligand blots (16).

In *S. exigua* we could demonstrate a role in binding only for domain III of both CryIA(b) and CryIA(c). Whether the proteins recognized by domain III are true receptors or parts of receptors remains to be determined. Our previous results (4) indicate that hybrid H04 competes for binding to the same sites on intact *S. exigua* BBMVs as CryIA(b), suggesting that there is a receptor for CryIA(b) that could not be detected by ligand blotting. One likely possibility is that some binding proteins or putative receptors, in this case proteins or receptors recognized by domain II of the various toxins, are very sensitive to denaturation or to the action of gut proteases, so that some of the physiologically important binding sites remain undetected on ligand blots. For the same reasons proteins recognized by domain III of CryIC could go undetected. Both the 210-kDa CryIA(b)-binding protein and the 120-kDa CryIA(c)-binding protein are similar if not identical to binding proteins for the respective toxins described by Oddou et al. (20).

High sensitivity to denaturation may also explain the poor reproducibility of the detection of a putative 40-kDa CryIC receptor in *S. exigua*. We could find only one report of this protein (20) besides our own (4) and one report of a similar protein in *Spodoptera littoralis* (21). Repeated experiments were performed with different preparations of purified CryIC and freshly isolated BBMVs (in the presence of protease inhibitors), and no clear correlation with detectability of CryIC-binding proteins could be deduced. In this study, the lack of detectable binding by CryIC actually helped define the role of CryIA(b) and CryIA(c) domains in binding.

For *S. exigua*, there is no correlation between binding on the ligand blot and toxicity; both CryIC and hybrid H04 are toxic but exhibit no detectable binding on a ligand blot, whereas hybrid H205, which is hardly toxic (unpublished data), does exhibit binding. Thus, these results strongly suggest that some important toxin-membrane protein interactions go undetected in this type of experiment, whereas some types of binding do not result in toxicity (15).

For *M. sexta*, both domain II and domain III were shown to be involved in binding, albeit to different proteins. A 210-kDa CryIA(b)-binding protein which was recognized by domain II may well be identical to the CryIA(b) receptor that has been purified and cloned previously (22, 23). This protein has been shown to allow cell surface binding of CryIA(b) when it is expressed in transfected human cells, suggesting that it is a receptor. Other studies have shown that the same protein is also recognized by CryIA(a), as well as by CryIA(c), on ligand blots (18), while binding studies with intact BBMVs have identified a common receptor for all three CryIA type toxins (25). Our results confirm the former observation for CryIA(c). Both CryIA(b) and CryIA(c) bind to presumably the same 210-kDa protein. However, the major CryIA(c)-binding protein in *M. sexta* is a 120-kDa protein, which is recognized by domain III. This protein may well be identical to the CryIA(c)-binding protein that has been purified and cloned previously (12, 13). In surface plasmon resonance experiments, this purified CryIA(c) receptor also exhibits binding with CryIA(b) and CryIA(a) (19), although it does not appear to bind CryIA(b) on a ligand blot of BBMV proteins (this study). Again, higher instability of the CryIA(b) binding site may explain this observation. These inconsistencies clearly indicate that the picture is far from complete and that any single type of binding assay is probably not sufficient to provide a complete picture. At present, we have no way to definitively prove that the CryIA(b)- and CryIA(c)-binding proteins of *M. sexta* detected by us are indeed identical to the previously purified receptors for these toxins. However, in our opinion both the observation that the molecular masses of the binding proteins are very similar to the molecular masses reported for the purified receptors and the fact that both receptors were first detected and later purified by using a detection method similar to our method suggest that these proteins are identical. Ligand blotting experiments in which our hybrid toxins are used in combination with the purified receptors may reveal whether these receptors behave like the binding proteins detected in this study, making it even more likely that they are identical. Thus, it is very possible that the cloned receptors are two different types of receptors, one which is recognized by domain II of a toxin and one which is recognized by domain III, and that these proteins are representatives of two separate classes of receptors present in other insects as well. Additional studies of the toxin-receptor interaction (e.g., studies in which toxin mutants are used) would have to take this interesting possibility into account.

In addition to the major CryIA(b)-binding protein in *M. sexta*, we identified a previously undetected approximately 250-kDa protein which is specifically recognized by domain III of CryIA(b). Additional studies will have to be performed to determine the possible function of this protein in initial binding (which is the function presumably measured by equilibrium binding studies with intact BBMVs) or in another stage of toxin action. Binding studies performed with intact BBMVs of *M. sexta* have shown that CryIA(b) and CryIA(c) have a single, common receptor (25), while the results described in this paper show that CryIA(b) and CryIA(c), through their very different domains III, recognize other unique binding proteins. Apart from the possibility that the domain III-binding proteins are not involved in the initial binding, as measured with intact BBMVs, both CryIA(b) and CryIA(c) may bind in vivo to different heterodimers consisting of the common 210-kDa domain II-binding protein and either the 250-kDa CryIA(b) domain III-binding protein or the 120-kDa CryIA(c) domain III-binding protein. They could even bind to a single heterotrimer consisting of all three binding proteins. These possibilities are also consistent with the apparent competition for binding sites between CryIA(b) and CryIA(c) in *M. sexta* (23).

In conclusion, the results presented above suggest that binding by δ-endotoxins to target midgut eptihelial membrane proteins may involve both domain II and domain III of the toxin molecule and that at least two distinct classes of δ-endotoxin-binding proteins are present in the target insects.

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