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# Domain III Substitution in *Bacillus thuringiensis* Delta-Endotoxin CryIA(b) Results in Superior Toxicity for *Spodoptera exigua* and Altered Membrane Protein Recognition

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**To test our hypothesis that substitution of domain III of *Bacillus thuringiensis* delta-endotoxin (Cry) proteins might improve toxicity to pest insects, e.g., *Spodoptera exigua*, in vivo recombination was used to produce a number of *cryIA(b)-cryIC* hybrid genes. A rapid screening assay was subsequently exploited to select hybrid genes encoding soluble protoxins. Screening of 120 recombinants yielded two different hybrid genes encoding soluble proteins with domains I and II of CryIA(b) and domain III of CryIC. These proteins differed by only one amino acid residue. Both hybrid protoxins gave a protease-resistant toxin upon in vitro activation by trypsin. Bioassays showed that one of these CryIA(b)-CryIC hybrid proteins (H04) was highly toxic to *S. exigua* compared with the parental CryIA(b) protein and significantly more toxic than CryIC. In semiquantitative binding studies with biotin-labelled toxins and intact brush border membrane vesicles of *S. exigua*, this domain III substitution appeared not to affect binding-site specificity. However, binding to a 200-kDa protein by CryIA(b) in preparations of solubilized and blotted brush border membrane vesicle proteins was completely abolished by the domain III substitution. A reciprocal hybrid containing domains I and II of CryIC and domain III of CryIA(b) did bind to the 200-kDa protein, confirming that domain III of CryIA(b) was essential for this reaction. These results show that domain III of CryIC protein plays an important role in the level of toxicity to *S. exigua*, that substitution of domain III may be a powerful tool to increase the repertoire of available active toxins for pest insects, and that domain III is involved in binding to gut epithelium membrane proteins of *S. exigua*.**

An important feature of the gram-positive bacterium *Bacillus thuringiensis* is the production of large, proteinaceous, crystalline inclusions during sporulation. These crystals consist of one or more crystal proteins (Cry and Cyt proteins, with molecular masses of 60 to 130 and 20 kDa, respectively), which are responsible for the entomocidal properties of many *B. thuringiensis* strains (for a review, see reference 15). Because of these properties, formulations of *B. thuringiensis* spores have been produced for agricultural use for many years (2). More recently, genes encoding *B. thuringiensis* toxins have been used to transform crop plants in order to endow the plants with inheritable resistance to pest insects (34).

Purification of Cry proteins and cloning of the encoding genes have shown that the diverse specificity of *B. thuringiensis* isolates results from differences in toxin composition and the unique specificity spectra of individual toxins. This diversity has led to the definition of different toxin subclasses (15). The *cryI* gene-encoded lepidopteran-specific proteins are formed as 120- to 130-kDa protoxins which, upon ingestion by the insect, solubilize in the alkaline environment of the insect midgut and are subsequently processed by gut lumen proteases. This process results in the formation of an active, toxic N-terminal fragment of approximately 65 kDa. In susceptible insects, the activated toxin binds to receptors on the midgut epithelial brush border membrane and subsequently forms a pore in the membrane by a process that is not yet fully understood (19). Pore formation leads to colloid osmotic lysis of the epithelial target cells, cessation of feeding, and, finally, death. The specificity of a particular toxin depends to different extents on the efficiency of each of the steps mentioned above. Hence, the ability to solubilize and activate protoxins has been shown to influence toxicity (17). The activity of the protease-activated toxin is dependent on the type of gut protease involved (13) and on the presence of a specific receptor in the brush border membrane (14, 35, 36). Changes in both solubility and activation properties (28), as well as decreased affinity of receptor binding (9, 37), account for the development of resistance to *B. thuringiensis* toxins.

Hybrids derived from toxins with different specificities are powerful tools in determining the role of the primary amino acid sequences in specificity (6, 10, 11, 16, 20, 31). Moreover, the unraveling of the three-dimensional structure of two crystal proteins has greatly improved the possibilities of correlating structural features with specific functions (12, 22). Li et al. (22) have shown that the coleopteran-specific CryIIIA protein consists of three domains. The N-terminal domain I, consisting of seven α-helices, is thought to be involved in membrane penetration. Domain II, consisting mainly of β-sheets, is thought to be responsible for receptor recognition and binding through one or several surface-exposed loops and is therefore considered to be a major determinant of toxin specificity. This is, in retrospect, consistent with the conclusions of previous hybrid studies (10, 11, 16, 20) and is supported by the results of more recent mutagenesis studies (23, 32). The function of the C-terminal domain III is still largely unknown, although involvement in protection against extended proteolytic cleavage has

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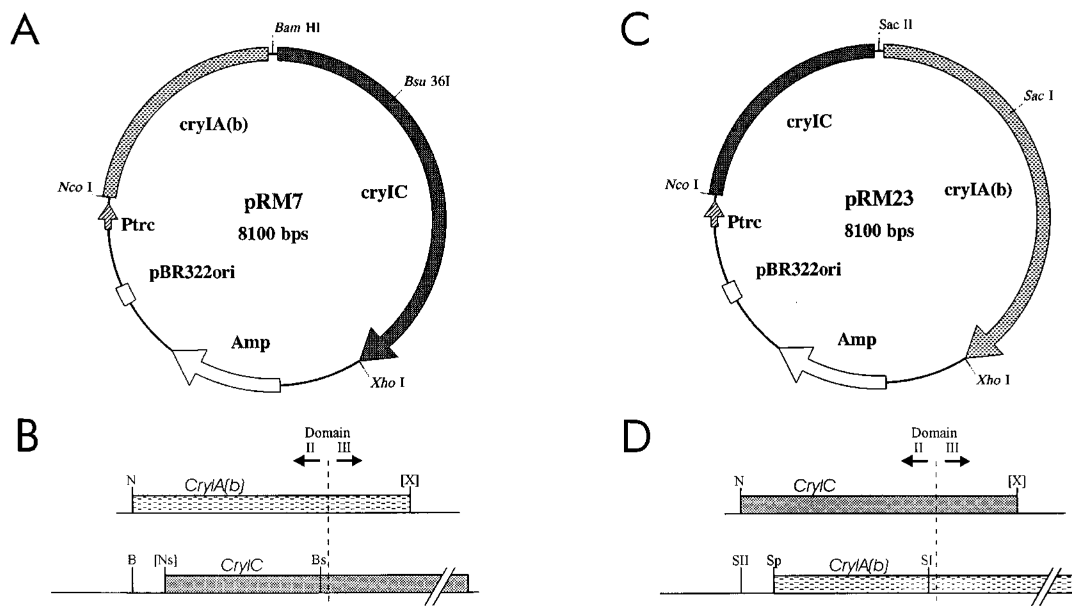


FIG. 1. Plasmids used for generation of hybrids through in vivo recombination. (A and B) *cryIA*(*b*)-*cryIC* tandem plasmid pRM7. (C and D) *cryIC*-*cryIA*(*b*) tandem plasmid pRM23. (A and C) Circular representations showing the relative orientations of the truncated *cryIA*(*b*) and *cryIC* genes. (B and D) Schematic alignments of the two genes showing the area of overlap. Abbreviations: N, *Nco*I; X, *Xmn*I; B, *Not*I; Ns, *Nsi*I; Bs, *Bsu*36I; SI, *Sac*I; SII, *Sac*II; Sp, *Spe*I.

been suggested (22), as well as a possible role in pore function (8).

In a study of hybrids derived from CryIC and CryIE, we could show that domain III is a major determinant of toxicity to *Spodoptera exigua* (beet armyworm) and *Mamestra brassicae* (cabbage moth) (4). Whereas CryIC is toxic for these two insects and CryIE is not, a hybrid consisting of domains I and II of CryIE and domain III of CryIC is also toxic. This suggests that domain III, through an as yet unknown mechanism, can play an important role in determining specific toxicity against insects. In retrospect, some of the earlier studies with hybrid toxins resulted in toxins with exchanged domain III. These results showed that exchange of domain III can also increase toxicity to *Heliothis virescens* (10), to *Trichoplusia ni* (6), and to *Bombyx mori* (26) (see also Discussion). On the basis of these observations, the toxicity of any existing toxin to an insect could be improved by substituting its domain III. Moreover, such improved toxins recognizing different receptors could be used in resistance management strategies as alternatives for toxins to which insects have become resistant by losing or changing a receptor.

In this study, we determined whether toxin improvement by domain III substitution is more generally applicable. We created and studied CryIA(b)-CryIC hybrids to determine whether domain III of CryIC would be able to improve the moderate toxicity of CryIA(b) to *S. exigua*. Furthermore, using two different techniques, we investigated if and how domain III substitution alters binding of toxins to putative insect gut membrane receptors.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Escherichia coli* XL1-blue (Stratagene Inc.) was used as the plasmid host except when JM101 was used to provide a recombination-proficient background. Plasmids pBD140, pBD1400, pBD150, pBD560, and pBD650 are all derivatives of pBD10. pBD10 is a modification of the expression vector pKK233-2 (Pharmacia LKB Biotechnology), made by deletion of a 1,690-bp *Eco*RI-*Pvu*II fragment and insertion of an *Xho*I linker in the unique *Hin*dIII site (4). Construction of the *cryIC* expression vector pBD150 and the *cryIE*-*cryIC* tandem plasmid pBD650 and selection of the *cryIE*-*cryIC* hybrid G27 have been described previously (4). pBD140 contains the *cryIA*(*b*) gene as a 3.5-kb *Nco*I-*Bgl*II fragment in pBD10, followed by the pBluescript SK$^+$ polylinker sites from *Bst*XI to *Xho*I (5). Plasmid pBD141 contains a *cryIA*(*b*) gene C-terminally truncated at the *Xmn*I site at position 1811. It was constructed by replacing the C-terminal 2.1-kb *Sac*I-*Xho*I fragment of pBD140 by the truncated 470-bp *Sac*I-*Xho*I fragment of pJM21Bg as described elsewhere (24). A *cryIA*(*b*)-*cryIC* tandem plasmid was constructed by ligating a *Not*I-*Xho*I fragment of pBD650 containing *cryIC* (bases 220 to 3567) into *Not*I-*Xho*I-digested pBD141. The resulting plasmid was designated pRM7 (Fig. 1A and B). pBD560 is a *cryIC*-*cryIE* tandem plasmid described previously (4). A *cryIC*-*cryIA*(*b*) tandem plasmid, pRM23 (Fig. 1C and D), was constructed by replacement of the 3.5-kb *Spe*I-*Xho*I fragment of pBD560, containing the *cryIE* part, by the corresponding fragment of *cryIA*(*b*) (bases 178 to 3468).

A shuttle vector which is capable of replicating in *E. coli* as well as in *B. thuringiensis* was constructed with pBluescript KS(+) and pBC16.1 (3). At the *Kpn*I site of pBluescript KS(+), the *Pac*I and *Ase*I recognition sequences were inserted. In this process, the *Kpn*I site was destroyed. The pBC16.1 plasmid, a 2.4-kb *Eco*RI fragment of pBC16, was treated with Klenow fragment and inserted at the *Pac*I site of the modified pBluescript KS(+) in such a way that both antibiotic resistance genes were oriented in the same direction.

**DNA manipulations.** All recombinant DNA techniques were as described by Sambrook et al. (29). DNA sequencing was performed by the dideoxytriphosphate method with fluorescent dyes attached to the dideoxynucleotides. Analysis was automated by using an Applied Biosystems 370A nucleotide sequence analyzer. To generate in vivo recombinants, pRM7 and pRM23 were transferred to JM101. Plasmid DNA from JM101 (5 μg) was digested with *Not*I and *Bsu*36I (pRM7) or *Sac*II and *Sac*I (pRM23) and subsequently transformed to *E. coli* XL1-blue. All restriction enzyme digestions were performed as specified by the manufacturers.

**Screening of hybrids.** *E. coli* XL1-blue transformants of double-digested, JM101-derived pRM7 or pRM23 were inoculated in 1 ml of TB medium (1.2% [wt/vol] Bacto-tryptone, 2.4% [wt/vol] Bacto-yeast extract, 0.4% [vol/vol] glycerol, 17 mM $KH_2PO_4$, 72 mM $K_2HPO_4$) and grown overnight at 30°C. Cells were pelleted by centrifugation, resuspended in 100 μl of lysis buffer (50 mM Tris HCl, 5 mM EDTA, 100 mM NaCl [pH 8]) containing 1 mg of lysozyme per ml, and incubated at room temperature for 30 min. Lysis was completed by brief sonication, and soluble and insoluble cell components were separated by centrifugation. The supernatant was discarded, and the pellet was resuspended in 100 μl of solubilization buffer (50 mM $NaHCO_3$, 100 mM NaCl, 10 mM dithiothreitol [pH 10]) and incubated for 2 h at 37°C. After solubilization, the suspension was centrifuged and the supernatant was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Production of stable, trypsin-resistant mature toxin was tested by treatment of solubilized protoxin with trypsin (10% of protoxin weight) for 1 h at 37°C. Large-scale isolation of Cry protein from *E. coli* was performed as described earlier (4).

**Expression, preparation, and purification of protoxins in *B. thuringiensis*.** The H04 gene was cloned in the shuttle vector made with pBluescript KS(+) and

pBC16.1 as follows. First, the *cryIC* gene was cloned in pBluescript KS(+) with its own promoter [260-nucleotide upstream sequence of *cryIC* and the *cryIA*(*c*) terminator] (7). An *Nco*I site was engineered at the translation start site of *cryIC*. This *cryIC* clone was designated pSB629. The *Nco*I-*Kpn*I fragment of the H04 gene containing the *cryIA*(*b*) part and a portion of *cryIC* replaced the corresponding portion of *cryIC* in pSB629. This plasmid containing H04 in pBluescriptKS(+) was designated pSB647. The H04 gene was then removed from pSB647 by digesting the plasmid with *Apa*I and *Not*I and finally cloned in the shuttle vector with the same restriction sites. The H04 gene in the shuttle vector was designated pSB648.

To produce the H04 protein in *B. thuringiensis* BT51, a plasmid-cured, *cry*-negative derivative of *B. thuringiensis* subsp. *kurstaki* HD1 was transformed with pSB648 by electroporation. The plasmid used for the transformation was obtained from the methylation-negative *E. coli* GM2163. BT51(pSB648) was grown in 2-liter flasks containing 500 ml of CYS medium (40) at 30°C. When the cells produced spores and crystals and completely lysed, the crystals were harvested along with the spores by centrifugation. The crystals were repeatedly washed in 0.5 M NaCl containing 10 mM Tris HCl (pH 8) and 1 mM EDTA to remove proteinases. The washed crystals were dissolved in 2% mercaptoethanol–NaOH (pH 10.5), and the solubilized protein was further purified by Sephacryl S300HR column chromatography (40). Expression and purification of other protoxins were performed in a similar way.

The insecticidal activity of protoxins was determined with third-instar *S. exigua* larvae. Protoxins were mixed with an artificial diet, and the larvae were allowed to feed on the mixture at 30°C. The insecticidal activity, expressed as 50% and 90% lethal concentrations ($LC_{50}$ and $LC_{90}$), was determined from the mortality of larvae given different doses after a 4-day incubation.

**Semiquantitative binding assays on intact BBMVs.** Brush border membrane vesicles (BBMVs) of 5-day-old *S. exigua* larvae were isolated as described by Wolfersberger et al. (39), with modifications as described earlier (4). Binding of biotinylated crystal proteins to BBMVs was performed, essentially as described earlier (4), in 100 μl of borate-buffered saline (BBS; 10 mM $Na_3BO_3$, 150 mM NaCl [pH 9]) containing 0.1% Tween 20 and 0.1% bovine serum albumin.

**Ligand blotting.** *S. exigua* BBMVs were dissolved in concentrated SDS-polyacrylamide gel electrophoresis sample buffer and heated to 100°C for 5 min before being loaded on a 10% acrylamide gel. After electrophoretic separation, BBMV proteins (6 μg per lane) were transferred to nitrocellulose by electroblotting. Duplicate strips were cut from the filter and washed in deionized water and subsequently in Tris-buffered saline (TBS; 10 mM Tris HCl [pH 8], 150 mM NaCl) containing 0.5% Tween 20. Next, the strips were incubated for 2 h in blocking solution (TBS containing 1% dried nonfat milk, 0.1% bovine serum albumin, and 0.5% Tween 20). They were then incubated for 3 h in 1 to 2 μg of purified Cry protein per ml in blocking solution, washed three times for 5 min each in TBS containing 0.5% Tween 20, and incubated for 1.5 h in rabbit anti-CryI serum (37) diluted 1:1,000 in blocking solution. After three more washes for 5 min each in TBS–0.5% Tween 20, bound toxin was detected by incubation for 45 min in goat anti-rabbit–horseradish peroxidase conjugate diluted 1:3,000 in blocking buffer, followed by three washes for 5 min each in TBS–0.5% Tween 20 and visualization with the enhanced chemiluminescence kit (Amersham).

## RESULTS

**Production and selection of CryIA(b)-CryIC hybrid toxins.** To obtain CryIA(b)-CryIC hybrid toxins by in vivo recombination, we constructed an expression vector with a C-terminally truncated *cryIA*(*b*) gene and a N-terminally truncated *cryIC* gene cloned in tandem (Fig. 1). The pRM7 plasmid contains the *trc* promoter followed by bases 1 to 1811 of *cryIA*(*b*), part of the pBluescript SK+ polylinker, and bases 266 to 3570 of *cryIC*. After allowing intramolecular recombination in *E. coli* JM101, plasmid DNA was isolated and digested with *Not*I and *Bsu*36I to linearize nonrecombinant plasmids. In our experience, linearization with two uniquely cutting enzymes is necessary to efficiently lower the transformation frequency of nonrecombinant plasmids. Both *Not*I and *Bsu*36I have unique recognition sites in pRM7, in the polylinker and at position 1240 of *cryIC*, respectively. The overlap between the two truncated *cry* genes in pRM7, which allows recombination, extends approximately 1,600 bp, yet we were interested primarily in recombination in or close to domain III. We therefore chose to use *Bsu*36I rather than a second enzyme with a recognition site in the polylinker. This strategy allowed linearization of recombinants with crossovers in front of the *Bsu*36I site, thereby effectively selecting for recombinants with crossovers in or near the domain III-encoding sequences in the subsequent transformation step.

Digested plasmids were transferred to *E. coli* XL1-blue cells by transformation, and plasmids from transformants were subsequently analyzed by restriction enzyme digestion and DNA electrophoresis. Over 95% of the transformants contained a plasmid with an insert size corresponding to a single, intact *cry* gene (results not shown), indicating that selection for homologous recombination events had been efficient. A total of 120 colonies were grown in TB medium and assayed for production of an alkali-soluble protoxin-like protein. This screening method yielded five colonies that produced a soluble protoxin of the expected size (120 to 130 kDa). The location of the crossovers in the hybrid genes was determined first by restriction analysis and then by nucleotide sequencing. Only two different crossover sites occurred in the five hybrid genes thus tested. The locations of the two different crossover sites in these hybrid genes, designated H04 and H100, are shown in Fig. 2A. Both crossovers are located very close to or on the border between domains II and III, with the hybrid toxins differing by only one amino acid. A schematic representation of these and other toxins used in this study is shown in Fig. 2B.

Solubilized protoxins of one of each of the two different groups of *cryIA*(*b*)-*cryIC* hybrids (hybrids H04 and H100 [Fig. 2]) were subjected to trypsin treatment. Figure 3 shows each hybrid protein before and after trypsin treatment, compared with equivalent samples of the parent toxins, CryIA(b) and CryIC. Each of the protoxins gave a trypsin-resistant toxin of approximately 65 kDa after trypsin treatment. Both the solubility of the hybrid protoxins and the occurrence of a trypsin-resistant product of the expected size suggested that these hybrids proteins were properly folded and might have biological activity. Hybrid H04, with the crossover closest to what we considered to be the border between domains II and III, was selected for further study.

**Toxicity to *S. exigua*.** The *cryIC*, *cryIA*(*b*), and *cryIE*-*cryIC* hybrid G27 (4) and the newly isolated *cryIA*(*b*)-*cryIC* hybrid H04 genes were cloned in shuttle vector pSB634 and transferred into *B. thuringiensis* Bt51 by electroporation. When cultures containing these genes were grown, they sporulated normally and produced a bipyramidal inclusion body (results not shown). Protoxins were isolated and assayed for their toxicity to *S. exigua* larvae, and the results are shown in Table 1.

As reported above, the CryIA(b) protoxin is much less toxic to *S. exigua* than is CryIC. The hybrids containing domain III of CryIC, both G27 (4) and CryIA(b)-CryIC hybrid H04, have an $LC_{50}$ that is comparable to or lower than that of CryIC. Similar results were obtained in bioassays with trypsin-activated toxins from protoxins produced in *E. coli* (results not shown). These results indicate that, as was demonstrated previously for CryIE (4), CryIA(b) can be made considerably more toxic to *S. exigua* by substitution of its domain III with that of CryIC.

**Binding studies with intact BBMVs.** In a previous study (4), we showed that for CryIC and CryIE, exchanging domain III did not affect the binding-site specificity, which is determined by the N-terminal part of the protein, presumably domain II. To test whether this holds true for CryIA(b) and its domain III-substituted hybrid and to compare the binding-site specificity of CryIA(b) with that of CryIC, we studied the binding of biotin-labelled toxins to *S. exigua* BBMVs by the semiquantitative binding assay (4). Figure 4 shows the binding of biotin-labelled toxins both in the absence and in the presence of an excess amount of an unlabelled homologous or heterologous competitor toxin. Binding of labelled CryIA(b) (Fig. 4A) was inhibited only by CryIA(b) or H04 but not by CryIC. Con-

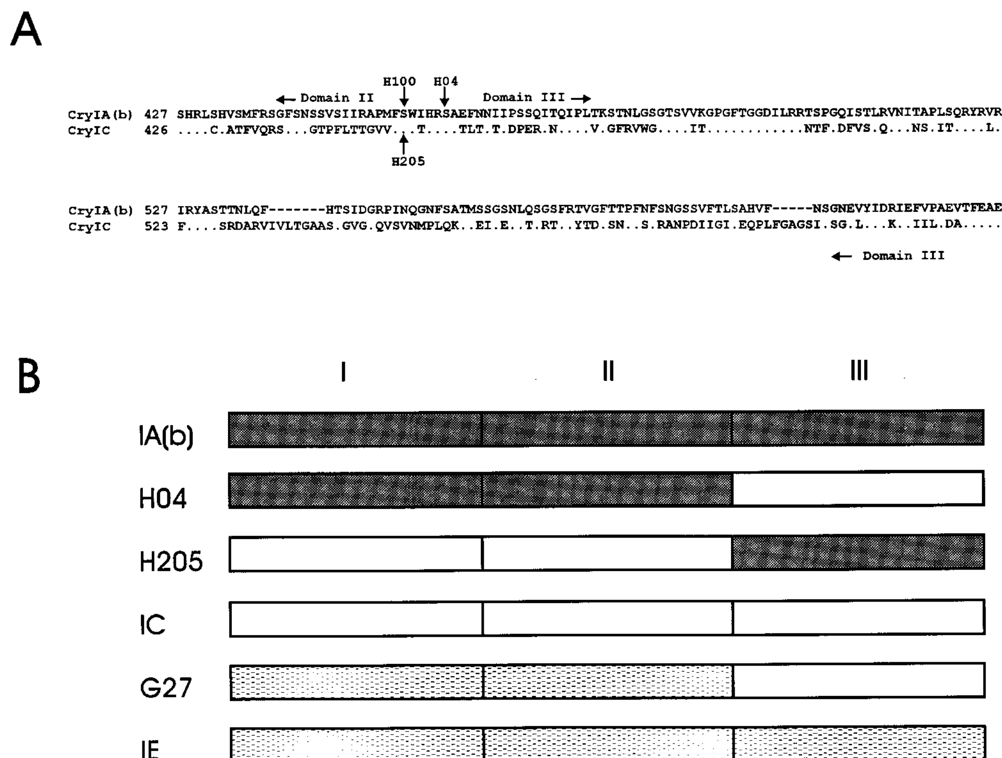


FIG. 2. (A) Alignment of the (partial) amino acid sequences of CryIA(b) and CryIC. The locations of the crossover sites in hybrids H04, H100, and H205 are indicated by arrows. (B) Schematic representation of the structures of the wild-type and hybrid toxins used in this study. Only the active toxin part with domains I to III is shown.

versely, binding of labelled H04 (Fig. 4C) was inhibited only by H04 or CryIA(b) but not by CryIC, showing that H04 indeed recognizes the same binding site as CryIA(b). The binding site of CryIA(b) and H04 appeared to be different from that of CryIC. This was confirmed in the binding experiments with biotin-labelled CryIC. Neither CryIA(b) nor H04 was able to inhibit binding of CryIC (Fig. 4B). Binding of CryIC is somewhat diminished in the presence of excess H04 (Fig. 4B, lane 3).

In conclusion, on intact BBMVs, CryIA(b) recognizes *S. exigua* binding sites which are different from those recognized by CryIC. Substitution of domain III of CryIA(b) does not change the binding-site specificity of this protein in these experiments.

**Domain III substitution affects recognition of gut epithelial membrane proteins in ligand blot experiments.** To identify toxin-binding components in *S. exigua* BBMV preparations, we performed ligand blot experiments. BBMV proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, incubated with various toxins, and subsequently detected with a rabbit anti-CryI antiserum. This antiserum reacted efficiently with CryIA(b), CryIC, and the hybrids used in this study (results not shown). In agreement with the results of an earlier report (27), we found that CryIA(b) protein binds to an *S. exigua* BBMV protein with a molecular mass of approximately 200 kDa and to a lesser extent to two smaller proteins of ca. 150 and 60 kDa (Fig. 5, lane 1) whereas CryIC binds to a single protein of ca. 40 kDa (lane 2). In our hands, this reaction was very weak and difficult to reproduce consistently, regardless of whether different freshly prepared BBMVs or freshly purified CryIC protein was used in the experiments. Surprisingly, despite being composed of domains I and II of CryIA(b), H04 no longer bound the same proteins as did CryIA(b) (lane 4), suggesting that domain III of CryIA(b) is essential for that reaction.

To directly test whether binding to *S. exigua* BBMV proteins by CryIA(b) is mediated by its domain III, we decided to construct a hybrid toxin in which domain III of CryIC is re-

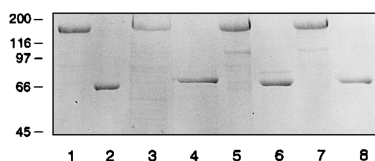


FIG. 3. SDS-polyacrylamide gel electrophoresis of solubilized protoxins of CryIA(b) (lanes 1 and 2), CryIC (lanes 3 and 4), H04 (lanes 5 and 6), and H100 (lanes 7 and 8) before (lanes 1, 3, 5, and 7) and after (lanes 2, 4, 6, and 8) digestion with trypsin. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

TABLE 1. Toxicity of parent and hybrid protoxins to *S. exigua* larvae

| Protoxin | $LC_{50}$ (mean ± SD)[a] | $LC_{90}$ (mean ± SD)[a] | n[b] |
|---|---|---|---|
| CryIA(b) | >100 | >100 | |
| CryIC | 11.0 ± 1.13 | 39.7 ± 12.1 | 2 |
| G27 | 7.37 ± 1.71 | 25.1 ± 12.7 | 5 |
| H04 | 1.66 ± 0.72 | 4.45 ± 2.26 | 8 |

[a] $LC_{50}$ and $LC_{90}$ are expressed in micrograms of protoxin per gram of artificial diet. SD, standard deviation.
[b] Number of samples tested.

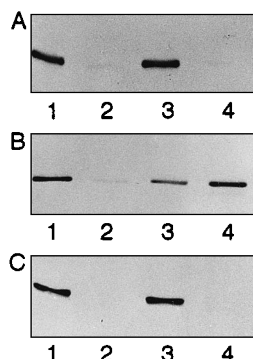


FIG. 4. Binding of biotin-labelled toxins and hybrids to *S. exigua* BBMVs. (A) Binding of labelled CryIA(b) in the absence of competitor (lane 1) and in the presence of a 1,000-fold excess of CryIA(b) (lane 2), CryIC (lane 3), or H04 (lane 4). (B) Binding of labelled CryIC in the absence of competitor (lane 1) and in the presence of a 1,000-fold excess of CryIC (lane 2), H04 (lane 3), or CryIA(b) (lane 4). (C) Binding of labelled H04 in the absence of competitor (lane 1) and in the presence of a 1,000-fold excess of H04 (lane 2), CryIC (lane 3), or CryIA(b) (lane 4).

placed by that of CryIA(b), i.e., the inverse of H04 described above. For this purpose, we constructed *cryIC-cryIA*(*b*) tandem plasmid pRM23 (Fig. 1C and D). As for isolation of H04, selection for recombinant plasmids with crossover sites in or close to domain III was applied by cutting isolated pRM23 plasmid with two restriction enzymes, one in the linker between the two genes (*Sac*II) and one close to the border between domains II and III in *cryIA*(*b*) (*Sac*I) (Fig. 1D). Screening of recombinant colonies for the production of soluble protoxins giving a stable product upon in vitro trypsin activation yielded CryIC-CryIA(b) hybrid H205, which had a crossover site close to the border between domains II and III and therefore was an almost perfect inverse of CryIA(b)-CryIC hybrid H04 (Fig. 2). H205 protein isolated from *E. coli* had very low toxicity against both *S. exigua* and *Manduca sexta* (results not shown) and therefore was not produced in *B. thuringiensis* for large-scale production and bioassays.

H205 bound to the same three *S. exigua* proteins on ligand blots as CryIAb did (Fig. 5, lane 3), showing that domain III of CryIA(b) is involved in and essential for this reaction. In fact, when the same concentration as that of CryIA(b) was used for incubation, H205 reacted much more strongly with all components recognized by CryIA(b). Moreover, H205 also reacted with the same 40-kDa protein as CryIC did, albeit to a lesser extent, showing that domain III of CryIC is not essential for this reaction (compare lanes 2 and 3).

## DISCUSSION

In this study, we have demonstrated that substitution of domain III of a moderately *S. exigua*-toxic *B. thuringiensis* toxin, CryIA(b), can greatly increase the toxicity of the protein to this target insect. By exchanging domain III of CryIA(b) with that of the more active CryIC protein, the toxicity of CryIA(b) was increased to a level that was even higher than that of CryIC without changing the binding specificity on intact BBMVs. Together with our earlier results with CryIE-CryIC hybrids (4), the present results strongly indicate that domain III plays a major role in determining the level of toxicity to a target insect.

Our present and earlier results show that exchanging domain III of Cry proteins may improve toxicity to a particular target insect. This suggests that domain swapping could become a valuable tool in the modification of existing toxins, thereby increasing the repertoire of available toxins for use in agriculture. So far, this phenomenon has been demonstrated only in terms of the toxicity to *S. exigua* and *M. brassicae*. However, closer inspection of the literature reveals that a similar phenomenon may occur with toxicity to other insects, i.e., *Heliothis virescens*, *Trichoplusia ni*, and *Bombyx mori*. Ge et al. (10) showed that by replacing amino acid residues 450 to 612 (domain III) of CryIA(a) by those of CryIA(c), toxicity to *H. virescens* was increased 300-fold. Caramori et al. (6) demonstrated that similar CryIA(a)-CryIA(c) hybrids had toxicity levels for *T. ni* similar to that of CryIA(c), which was ninefold higher than that of CryIA(a), with its own domain III. An almost identical CryIA(a)-CryIA(c) hybrid was approximately 100 times more toxic to *Bombyx mori* than was CryIA(a) itself (26). Whereas CryIC is relatively more toxic to *S. exigua*, CryIA(b) is relatively more toxic to *H. virescens* and the toxicity of CryIA(b)-CryIC hybrid H04 for the latter species is comparable to that of CryIC (8a). Formation of hybrids by substituting domain III is possibly also an evolutionary strategy for *B. thuringiensis* itself to modify toxicity: CryIA(b) can be viewed as a hybrid consisting of domains I and II of CryIA(c) and domain III of CryIA(a) and may have arisen as a product of recombination between the two encoding genes.

Recognition of a specific receptor on the midgut epithelium is likely to be a prerequisite for the effectiveness of a particular toxin against a target insect. However, our results show that weak or inactive toxins, e.g., CryIA(b), that do recognize a receptor in the target insect can be made more active than the most effective toxin (e.g., CryIC) by replacing their domain III. Both CryIA(b) and CryIC were shown to recognize different specific binding sites on intact BBMVs of *S. exigua*. We have also demonstrated that at least most of the *S. exigua* binding sites for CryIA(b) are different from those for CryIC. This suggests that if *S. exigua* became resistant to CryIC by losing this specific binding site, it might still express the CryIA(b)-binding site and would therefore remain sensitive to the CryIA(b)-CryIC hybrid H04. Therefore, H04 may become a valuable alternative to CryIC in integrated pest management strategies.

Surprisingly, we showed that exchange of domain III does alter binding to gut membrane proteins on ligand blots. Domain III of CryIA(b) appears to be essential for binding to the 200-kDa BBMV protein of *S. exigua* in these experiments. Substitution of this domain, as in H04, abolishes binding to this protein. On the other hand, H205, which has low activity against *S. exigua* as well as against *M. sexta*, does bind to this protein, apparently because it contains domain III of CryIA(b). We do not know why H205 seems to bind more strongly than

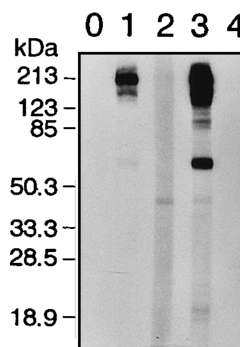


FIG. 5. Ligand blot detection of binding to *S. exigua* BBMV proteins by different toxins. Lanes: 0, no toxin added; 1, CryIA(b); 2, CryIC; 3, CryIC-CryIA(b) hybrid H205; 4, CryIA(b)-CryIC hybrid H04. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

does CryIA(b) itself to the BBMV proteins that are recognized by CryIA(b) (Fig. 5). This observation suggests that domain I and/or II may indirectly affect the affinity of domain III binding to membrane proteins, but more experimentation is clearly needed. These results add to the growing evidence suggesting that domain III of Cry proteins is involved in receptor recognition and/or binding to gut membrane proteins. Aronson et al. (1) demonstrated that mutations in domain III of CryIA(c) affect the affinity for membrane proteins in ligand blots of *Manduca sexta* and *H. virescens* BBMVs. Lee et al., using CryIA(c)-CryIA(a) hybrids, have shown that the specificity of binding to *Lymantria dispar* BBMV proteins and to the purified CryIA(c) receptor on ligand blots is determined by the origin of the domain III part of the hybrid protein (21). Domain III may very well be involved in binding to membrane proteins in many other cases too. Oddou et al. (27) showed for two *Heliothis* spp. and three *Spodoptera* spp. that CryIA(b) and CryIA(a) recognize apparently identical protein sets in ligand blots of all these insects whereas CryIA(c) recognizes a different set. Since CryIA(b) and CryIA(a) have identical domains III but different domains I and II whereas domains I and II of CryIA(b) and CryIA(c) are almost identical, it seems likely that in these cases also, binding specificity is determined by domain III.

It remains unclear why in this study binding specificity seems to be determined by domain I or II in experiments with intact BBMVs but seems to be determined by domain III in ligand blot experiments. Possibly, different techniques detect or emphasize different aspects of a complex binding mechanism. This is best demonstrated with the example of binding studies with *Manduca sexta*. Using iodinated toxins binding to isolated BBMVs, Van Rie et al. (36) determined that all three CryI proteins have a common binding site in this insect, with only CryIA(a) having a second, specific receptor. In contrast, the recently isolated receptors for CryIA(c) (18, 30) and CryIA(b) (33) (both isolated by using ligand blotting for detection) are distinct proteins with molecular masses of 120 and 210 kDa, respectively. Finally, by using surface plasmon resonance for monitoring binding, the purified 120-kDa CryIA(c)-receptor was shown to contain (putatively common) binding sites for all three CryI proteins as well as a unique binding site for CryIA(c) only (25). Clearly, binding of toxins is a complex process in which both domain II and domain III seem to play a role, either by being involved in binding to receptors together or by having distinct functions in different binding steps such as initial binding, membrane insertion, oligomerization, or pore formation. Moreover, the relative contribution of each domain to the binding process may differ from one insect to another as well as from one receptor to another in the same insect species. Future studies will have to determine the relative importance of the observed types of binding and their relevance for in vivo binding and toxicity.

Although in this study we found no direct correlation between protein recognition on ligand blots and toxicity in vivo, the results suggest that binding to the proper membrane protein is necessary for the toxicity of a crystal protein in a particular insect or, alternatively, that binding to the wrong protein [for example, binding of CryIA(b) to the 200-kDa protein] has a negative effect on toxicity. Simultaneous or sequential binding to several membrane proteins might be necessary to bring about the conformational changes thought to take place during membrane insertion and pore formation. Thus, a more optimal combination of binding proteins recognized by a domain III hybrid might improve toxicity by increasing the efficiency of membrane insertion and/or pore formation.

In conclusion, we have shown that a combination of in vivo recombination between two *cry* genes with limited stretches of homology and subsequent screening for the production of soluble protoxins can be a powerful tool to modify toxicity and to increase the available repertoire of active toxins against pest insects. Furthermore, we have presented evidence that domain III of delta-endotoxins is involved in binding to gut membrane proteins in *S. exigua*, revealing that the binding process has an additional level of complexity that may underlie the strongly increased efficacy of some hybrid toxins.

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