The Role of *Bacillus thuringiensis* Cry1C and Cry1E Separate Structural Domains in the Interaction with *Spodoptera littoralis* Gut Epithelial Cells*

Received for publication, November 18, 2003, and in revised form, February 2, 2004
Published, JBC Papers in Press, February 12, 2004, DOI 10.1074/jbc.M312597200

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The *Bacillus thuringiensis* δ-endotoxins Cry1C and Cry1E share toxicity against several important lepidopteran species. Their combined use to delay development of resistance in target insects depends on their differential interaction with the gut epithelial cells. The three structural domains and combinations of two consecutive domains of Cry1C and Cry1E were separately expressed in *Escherichia coli*, and their interactions with the brush border membrane vesicles (BBMV) of Cry1E-tolerant and -susceptible *Spodoptera littoralis* larvae were studied. About 80% reduction in binding of Cry1E and each of its separate domains to BBMV of Cry1E-tolerant larvae was observed, whereas Cry1C was toxic to all larvae and bound equally to BBMV derived from both Cry1E-tolerant and -susceptible larvae. These results suggest differential interactions of the two toxins with BBMV encompassing all three domains. Comparable binding assays performed with fluorescent Cry1C and Cry1C domain II showed that Cry1C has higher B<sub>max</sub> and lower K<sub>d</sub> than Cry1C domain II and further supported the existence of toxin multisite interactions. Competitive binding assays were used to estimate the sequence of interaction events. Cry1C domain II could compete with domain III binding, whereas domain III did not interfere with domain II binding, indicating sequential interactions of domain III and then domain II with the same membrane site. No competition between domain II of Cry1C and Cry1E was observed, confirming the existence of different domain II binding sites for the two toxins. Taken together, all three domains specifically interact with the epithelial cell membrane. The folding of the three-domain toxin probably dictates the sequence of interaction events.

Cry proteins are produced and assembled as heterologous crystalline bodies during sporulation of *Bacillus thuringiensis*. Usually Cry proteins display a narrow, specific spectrum of insecticidal activity. Cry1C is an effective insecticide against about 35–40 species and differs in its insecticidal host range from the three Cry1A toxins. Among the Cry1C-sensitive lepidopteran species, some are also sensitive to Cry1E, which has been considered as a potential alternative to avoid evolution of resistance due to intensively used Cry1C either as a component of bacterial formulations or as a transgenic plant protein (1). The N-terminal toxic parts of Cry1C and Cry1E are composed of three structural domains found to be highly similar in Cry1, Cry2, and Cry3 proteins (2–4). Domain I is composed of seven α-helices and is involved in ion channel formation. The β-sheets comprising domain II and domain III are involved in specific interaction(s) with membrane receptors of the larval midgut epithelium, leading to the insertion into the membrane of domain I amphipathic α<sub>3</sub> and α<sub>4</sub> and their connecting loop. It is assumed that α<sub>3</sub>–α<sub>4</sub> pairs from at least 4 toxin molecules are assembled to form a lethal nonelective ion channel (5–8). Insertion is also linked to the spreading of the other domain I α-helices over the membrane (6, 9, 10). Oligomer formation may occur prior to the insertion of α-helices assisted by an earlier interaction with a membrane receptor as was recently shown for the interaction of cadherin-like Bt-R1 receptor with Cry1Ab (11).

Domain II loops are the least homologous parts and extend beyond the β-sheet core surface (3). Introduced changes/mutations in these loops affect toxin binding and specificity (12–15). Domain III is considered to be involved in both correct folding of the whole active toxin and receptor recognition. A specific site, only present in Cry1Ac domain III (amino acid residues 503–525), is responsible for the initial interaction with the N-acetylgalactosamine moiety of the aminopeptidase N (APN)<sup>1</sup> receptor (16). The rest of the domain III amino acid sequence is highly homologous among Cry1 proteins (13). Mutations or differences in the structural loops of Cry1C domain II (C-II) as well as domain III (C-III) swapping combinations demonstrated their involvement in specific Cry1C-membrane receptor/complex interaction (12, 17–20).

Two families of putative Cry protein receptors, APN and cadherin-like membrane protein (21), have been characterized. Interaction of Cry1A, Cry1C, Cry1F, and Cry1D toxins with APNs have been shown in different lepidopteran species (15, 22, 23). No interaction between Cry1E and any putative receptor or ligand has been reported. APN is an exopeptidase whose insect-specific C-terminal part is anchored in the membrane lipid rafts (24) by a glycosylphosphatidylinositol moiety (25).
Cry1C and Cry1E Separate Domains

**Table I**

Summary of Cry1C and Cry1E domains and the primers used for their amplification

| Name                | Abbreviation | Primers                                                                 | Base pairs | Amino acids | Mass (kDa) |
|---------------------|--------------|--------------------------------------------------------------------------|------------|-------------|------------|
| 1C domain I         | C-I          | GCCATGGGGAGGGAAAAATAAC (primer 1)                                      | 756        | 1–252       | 28.80      |
| 1C domain II        | C-II         | GAATTGGGATCTACACCTAGTT (primer 2)                                      | 621        | 253–459     | 23.35      |
| 1C domain III       | C-III        | CGCGGATCTCTGAAAGGAGTTGCACCT (primer 3)                                  | 513        | 460–630     | 19.15      |
| 1C domain [I + II]  | C-[I + II]   | Primers 1 and 4                                                         | 1377       | 1–459       | 52.14      |
| 1C domain [II + III]| C-[II + III]| Primers 3 and 6                                                        | 1134       | 253–630     | 42.48      |
| 1C domain [I + II + III] | C-[I + II + III] | Primers 1, 6 and 7                                                   | 1890       | 1–630       | 65.00      |
| Truncated Cry1C     | C-86         | Used as template                                                       | 2268       | 1–756       | 86.00      |
| 1E domain I         | E-I          | GGATCTCAAGAGCATGTGG (primer 7)                                          | 756        | 1–252       | 28.80      |
| 1E domain II        | E-II         | CGCGGATCTACTGATCCTAATT (primer 8)                                       | 612        | 253–456     | 22.86      |
| 1E domain III       | E-III        | CGCGGATCTCTTGTTCCTCTCTAAACT (primer 12)                                 | 477        | 457–616     | 17.87      |
| 1E domain [I + II]  | E-[I + II]   | Primers 9 and 12                                                       | 1368       | 1–456       | 51.74      |
| 1E domain [II + III]| E-[II + III]| Primers 12 and 9                                                      | 1089       | 253–616     | 40.72      |
| Truncated Cry1E     | E-86         | Used as template                                                       | 2226       | 1–742       | 86.00      |

* Described in Ref. 30.

Binding specificity has always been studied by using the complete toxic part of each Cry protein, carrying different point mutations or domain shuffling changes. However, the net contribution of each of the structural domains independent of the two other domains has not been examined.

Here we describe the expression and separate interaction of each of the structural Cry1C and Cry1E domains and combinations of two consecutive domains with *Spodoptera littoralis* brush border membrane vesicles (BBMV). In a natural *S. littoralis* population that reveals a split sensitivity to Cry1E and homogenous sensitivity to Cry1C, an 80% reduction in the interaction of all three Cry1E domains with BBMV of the Cry1E-tolerant larvae was observed. The interaction of Cry1C with BBMV of both Cry1E-tolerant and Cry1E-sensitive larvae was similar. This overall reduction in binding capacity correlates with a lack of larval sensitivity to Cry1E and indicates that all three domains, as independent entities, reveal specific interactions with the gut epithelial membrane.

**EXPERIMENTAL PROCEDURES**

**Identification of Cry1E-tolerant Larvae**—*S. littoralis* neonate larvae (100 larvae) from a population that had never been exposed to any Cry protein were fed on an artificial diet (26) containing 1 µg/ml Cry1E for 48 h and then rescued by feeding on fresh castor bean leaves until pupation. After 7 days of recovery, the larvae were separated according to their size into Cry1E-susceptible subpopulation exhibiting retarded development and Cry1E-tolerant subpopulation with normal larval size. This Cry1E-tolerant larval population was separately propagated for an additional five subsequent generations, repeating the same selection procedure. In each generation, the percentage of the Cry1E-“resistant” larvae remained constant and ranged between 10 and 20%. No typical genetic segregation was observed in three identical rearing experiments; therefore, the Cry1E recoverable larvae were termed as “Cry1E-tolerant” to discriminate them from Cry1C-resistant larvae that exhibit usual genetic segregation (27, 28).

**BBMV Preparation**—BBMV were produced from isolated midguts of fifth-instar larvae of *S. littoralis*, according to Wolfersberger et al. (29). Cloning and Expression of Cry1C and Cry1E Domains—All proteins were expressed using the pET expression system (Novagen) in the *E. coli* strain BL21(DE3). Cloning of the C-terminally truncated 86-kDa Cry1C and Cry1E was described by Strizhov et al. (30). The size, molecular weight, and primers used for the amplification of separate domains or combinations of two consecutive domains of Cry1C and Cry1E are described in Table I. All domains were amplified by PCR, using the corresponding listed primers and were cloned in pET11a expression vector. Overexpression, induced by isopropyl-1-thio-β-D-galactopyranoside, was carried out at 18 °C for 6 h. Cells were collected by centrifugation and resuspended in 10 ml 50 mM sodium phosphate buffer, pH 8. Total proteins were obtained by three cycles of French Press extraction. After centrifugation at 20,000 × g, for 10 min at 4 °C, the pellet was dissolved in 5 ml of 50 mM Na2CO3, pH 10, 20 mM dithiothreitol and incubated with gentle shaking at 37 °C for 12 h. After an additional spin, the supernatant was loaded on a desalting column (HiTrap; Amersham Biosciences) and eluted with 6 ml of 50 mM Na2CO3, pH 10.0. Cry1C (65 kDa) and Cry1C domain II proteins were further purified by an FPLC-Mono Q column (Amersham Biosciences), using a 0–600 mM NaCl gradient. Cry1C was eluted at 200 mM and Cry1C domain II at 400 mM NaCl, as verified by SDS-PAGE.

**Fourier Transform Infrared Spectroscopy**—Purified Cry1C domain II was subjected to Fourier transform infrared spectroscopy analysis. Infrared spectra were recorded using a Nicolet Nexus 470 Fourier transform infrared spectrometer with a deuterated triglycine sulfate detector. The measurements were recorded using 2 cm⁻¹ resolution and 2000 scans averaging. The transmittance minimal value was determined by the OMNIC analysis program (Nicolet). Purified Cry1C domain II (1 µg) in 100 µl of 50 mM Na2CO3 buffer, pH 10.5, was lyophilized and then resuspended in 50 µl of D2O. The sample was loaded between two CaF2 windows using a 0.05-mm spacer (31).

**FITC Labeling**—FITC-labeled Cry1C or Cry1C domain II was dissolved in 50 mM Na2CO3 buffer, pH 10, and incubated with fluorescein 5-isothiocyanate (FITC) (Sigma) in a 1:5 molar ratio, respectively, for 48 h, at 25 °C. The labeled proteins were separated from the nonbound FITC by using a G-25 desalting column (Amersham Biosciences) and verified at 310 nm after SDS-PAGE separation. The fluorescence was measured in a PerkinElmer Life Sciences 50B luminescence spectrometer (excitation light at 470 nm and emission at 530 nm).

**Binding Assays**—Binding experiments with FITC-labeled Cry1C or Cry1C domain II were performed in triplicates in a total volume of 100 µl of 50 mM sodium phosphate buffer, pH 8, containing 50 µg of BBMV proteins. Various concentrations (0–50 nM) of FITC-labeled Cry1C or Cry1C domain II were tested in the absence (total binding) or presence of specific binding of 1000 nM unlabeled competitor. After incubation for 30 min at room temperature, the BBMV-bound proteins were separated from free proteins by centrifugation at 20,000 × g for 5 min at room temperature. The pellet, containing BBMV and bound proteins, was washed twice with 1 ml of 50 mM sodium phosphate buffer, pH 8. After washing, the pellet was dissolved in 2 ml of 50 mM Na2CO3 buffer, pH 10, containing 5% SDS, and boiled for 5 min to denature and release the bound labeled proteins. Luminescence of the bound proteins was then measured in a luminescence spectrometer (as above). Binding data were analyzed using the LIGAND computer program (32) adapted for Macintosh (Biosoft/Elsevier).

Binding assays and heterologous competitions with nonlabeled domains were carried out in a 0.5-ml total reaction volume containing 50 µg of BBMV and 250 µM Cry protein domains of otherwise stated under “Results.” The binding reactions were incubated for 5 min at 25 °C. Initial calibration of the binding reaction showed that saturated binding is reached in less than 5 min. The washed pellets were suspended in 30 µl of H2O and boiled for 5 min after adding 10 µl of sample.
Expression of the Cry1C and Cry1E Domains and Assessment of Binding—Binding ability of Cry proteins to the plasma membrane of lepidopteran larval epithelial cells has been linked to the correct folding of the three-domain entity. Further information was gained when isolated Cry1Ab domain (II + III) polypeptide was shown to specifically interact with Manduca sexta and Trichoplusia ni BBMV, suggesting that interaction of these two domains may maintain toxin specificity (33) and raised the question that perhaps specificity is domain-dependent. To further characterize the interaction of each of the three Cry protein structural domains with the plasma membrane of the epithelial cells, all three domains of two closely related Cry1C and Cry1E toxins were separately expressed, and their interaction with BBMV prepared from S. littoralis BBMV was estimated.

The borders of each domain were determined by multiple amino acid sequence alignment of Cry1Aa (3) with Cry1C and Cry1E. All domains and combinations of two consecutive domains were PCR-amplified by using the corresponding primers (Table I). pET11a vectors harboring the 3′-truncated Cry1C (C-86) or Cry1E (E-86) genes encoding 86-kDa toxins (30) served as templates. Most of the domains and domain combinations were sufficiently expressed in E. coli, except for Cry1E domain III and domain [II + III], which were insoluble and therefore not tested in binding assays.

The interaction of separate domains and combinations of two consecutive domains with S. littoralis fifth instar larval BBMV was estimated by binding assays. The bound fractions were quantified by Western blot analysis using anti-Cry1C polyclonal antibodies that also recognize all three Cry1E domains. All three Cry1C domains as well as combinations of two consecutive domains bound significantly to the BBMV (Fig. 1).

Estimation of binding parameters, using increasing BBMV concentrations in the binding assays, showed that in the range of 0.1–0.6 μg/ml total BBMV proteins, constant amounts of Cry1C-86, Cry1C-65, and individual Cry1C domains interacted with the membrane, reaching binding saturation within 5 min (data not shown). Longer incubation periods up to 30 min did not increase binding, not even to the full-length C-65 or its shorter version C-85. Thus, the whole binding process (34) occurred rapidly, within 5 min of incubation.

The binding capacity of Cry1E domains was estimated by using two types of BBMV prepared from Cry1E-tolerant and Cry1E-susceptible subpopulations of S. littoralis. These two subpopulations were obtained by feeding a laboratory reared S. littoralis population, which has never been exposed to any Cry protein, a Cry1E-containing diet. About 10–20% of the larvae could tolerate a 48-h exposure to 1 μg/ml Cry1E and then reached normal larval size when fed on castor bean leaves (Fig. 2A). The rest of the larval population (80–90%) could not tolerate the Cry1E treatment and showed significantly retarded development when being rescued by feeding on castor bean leaves (Fig. 2A). No changes in Cry1C susceptibility were observed in the examined population, which remained consistently sensitive to Cry1C.

Five consecutive generations of Cry1E-tolerant larvae were
equally treated with Cry1E. Similar ratios of tolerant to susceptible larvae were recorded in all five generations of inbreeding, without any increase in the number of Cry1E-tolerant larvae. When comparable binding assays were performed with BBMV prepared from Cry1E-tolerant and Cry1E-susceptible larvae, the two types demonstrated differential binding capacity of Cry1E-86, whereas the ability to bind Cry1C-86 was fully maintained by the whole population (Fig. 2B). The binding capacity of BBMV derived from Cry1E-tolerant larvae was 20% of that of the susceptible larvae (Fig. 2B). The binding capacity of BBMV derived from Cry1E-tolerant larvae was 20% of that of the susceptible larvae (Fig. 2B). All Cry1E domains, including domain I, showed a similar reduction in binding capacity (Fig. 2C), suggesting a general change in the interaction between the plasma membrane of the epithelial cells and Cry1E. The differential interaction of Cry1C and Cry1E domains indicated that these two Cry proteins interact with different membrane sites/receptors and that all three structural domains are involved in the interaction.

Characterization of Specific Domain Membrane Interactions—To further evaluate the specificity of individual domain interactions with BBMV, kinetic parameters of initial binding of Cry1C-65 and Cry1C domain II (C-II) were determined using FITC-labeled proteins (Fig. 3A). Larval feeding bioassays confirmed that FITC-labeled Cry1C was as toxic as the unlabeled toxin. Binding assays were carried out using labeled Cry1C or C-II at a concentration range of 5–50 nM. Nonspecific binding was determined in the presence of 1000 nM unlabeled protein (Fig. 3B), and the binding values were estimated by the LIGAND program based on the Scatchard equation. The $K_d$ value of C-II was 19-fold higher than that of Cry1C. Also, the higher $B_{max}$ value of Cry1C-65 binding compared with that of C-II indicated that there may be additional interacting sites within the whole toxin. FTIR analysis of purified C-II (Fig. 3C) and second derivative analysis of the absorbance spectrum using the OMNIC analysis program (Nicolet) clearly revealed a sin-

![Figure 3: Comparison of Cry1C and Cry1C domain II binding to BBMV.](https://example.com/figure3)

**A**

![Graph showing binding data](https://example.com/graph1)

**B**

|       | $K_d$(nM) | $B_{max}$(pmol/mg) | Hill co. |
|-------|-----------|--------------------|----------|
| C-65  | 1.2±0.71  | 14.8±0.98          | 1.04     |
| C-II  | 22.8±0.79 | 2.02±0.98          | 0.97     |

**C**

![Fourier transform infrared spectroscopy analysis](https://example.com/spectrogram)
gle absorbance peak at 1626 cm$^{-1}$, which is consistent with a $\beta$-sheet secondary structure (31). No peaks corresponding to other secondary structure elements were observed. This suggests that the isolated domain maintains the predominant $\beta$-sheet structure, and thus the loops extending from the domain core structure may exert similar interaction when they are a part of the Cry1C-65 toxin or a separately expressed C-II polypeptide.

The observed low $K_d$ (1.2 nM) of Cry1C-65 binding is in the affinity range measured for Cry1C binding to Spodoptera exigua BBMV (1.5 nM) (37) and for Cry1Ab binding to M. sexta cadherin-like protein (Bt-R1) (35) that interacts with Cry1Ab domain II loops $\alpha_6$ and 2. Under native binding conditions, a higher affinity of Cry1Aa to cadherin-like ($K_d = 2.6$ nM) than to the APN (75 nM) receptor, both derived Bombyx mori, was found (34). As yet, a cadherin-like protein has not been identified as a Cry1C receptor, whereas a 108-kDa APN capable of interacting with Cry1C has recently been isolated from S. littura (23), and a 106-kDa Cry1C binding APN was isolated from M. sexta (22). APN demonstrated to interact with domain II loops (15, 16). However, only in Cry1Ac domain III a specific binding site was identified, responsible for its primary interaction with the APN N-acetylgalactosamine moiety (16). Since all three domains of both Cry1C and Cry1E were able to interact with S. littoralis BBMV (Fig. 1 and 2), homologous and heterologous competitive binding assays of domains II and III were performed to verify their role in the binding process.

Binding of Cry1C domain II was evaluated in the presence of increasing concentrations of Cry1E domain II (Fig. 4). Three-dimensional structure comparison and amino acid sequence alignment of C-II and E-II (Fig. 4, A and B, respectively) demonstrated that their antiparallel $\beta$-sheets overlap, revealing a high degree of sequence identity. Conversely, C-II and E-II loop regions diverge in their stereo-orientation, reflecting the differences in the amino acid sequences. In BBMV binding assays, increasing concentrations of E-II up to 10-fold did not change the amount of bound Cry1C domain II, which remained constant (Fig. 4C), indicating that there is no competition between the two domains. This agrees with the unimpaired Cry1C interaction with BBMV derived from Cry1E-tolerant larvae (Fig. 2).

**Cry1C Domain III Is the First to Interact with BBMV**—Both domain II and III have been implicated in binding of various Cry1 proteins. The presence of C-III specified the interaction of S. exigua BBMV with chimeric toxins consisting of various combinations of Cry1A domain I and II (20). Thus, an initial interaction of the membrane with C-III was suggested (19, 20). To verify this assumption, competitive binding assays between individual C-II and C-III domains were performed. These assays showed that C-III binding to BBMV gradually decreased upon increasing C-II concentration (Fig. 5), indicating that C-II has higher binding affinity and also that both of them interact with the same binding site. Hence, C-III is probably the first to interact with the membrane, and then it is most likely substituted by C-II. It also showed that the individual domains dictate the interaction, since no domain neighboring effect might be assumed when independent single domains are assayed.

Domain III of Cry1C and Cry1E are almost identical in amino acid sequence and in three-dimensional structure (Fig. 6, A and B), except for the presence of two loops in C-III that are missing from E-III. The Cry1Ac domain III lectin-like
specific site that interacts with APN (16, 36, 37) is missing in all other Cry1 proteins analyzed thus far, including Cry1C and Cry1E. However, C-III and E-III could specifically interact with S. littoralis BBMV (Fig. 1 and 2), whereas C-II efficiently competed with C-III on BBMV binding sites (Fig. 5). It might be suggested that similarly to C-II, E-II may compete with C-III on the same binding sites. Binding assays (Fig. 6) showed that these domains interact independently with BBMV, and the presence of one did not affect the interaction of the other with the membrane. These data further indicate that Cry1C and Cry1E interact with different and very specific BBMV sites.

**DISCUSSION**

Most of the Cry protein-membrane interaction studies have been performed with the full-length toxic parts comprising all three domains. This approach is based on the high structural similarity of Cry1, Cry2, and Cry3 domains (2–4) and the existing interdomain interactions, suggested to be essential for correct folding and even pore formation (3, 38). Van der Waals bonds, hydrogen bonds, and electrostatic (salt bridges) interactions were identified, contributing to the formation of a closely packed structure (3). Changes in salt bridges that interconnect domains I and II in the α7 vicinity, facilitated pore formation, probably by increasing molecular flexibility (38). In contrast, locking domain I to domain II by disulfide bonds of engineered cysteines in a mutated Cry protein abolished ion channel formation and indicated that unfolding of the Cry protein around the hinge region linking domain I and II is an essential step for subsequent pore formation (5). Thus, the accumulating data thus far support the existence of dynamic interdomain cross-interactions that occur during the progression of all steps of toxin-membrane interaction.

Evaluating separate domain interactions with the epithelial cells provides insights into the initial interaction of toxin components with the membrane and distinguishes between the whole toxic process and the initial interactions with the membrane. In this study, individual Cry1C and Cry1E structural domains were expressed and found to interact with S. littoralis BBMV. It was also shown by FTIR analysis that domain II primary structure is sufficient for maintaining its β-sheet structure. The observed 80% reduction in binding to BBMV prepared from Cry1E-tolerant larvae of all Cry1E domains correlated with a similar decrease in binding of the whole Cry1E (Cry1E-86) toxin and indicated the specificity of the interaction of individual domains, including domain I. The ratio of tolerant to susceptible larvae was constant in five generations of inbreeding progeny. Although the genetic inheritance of Cry1E tolerance is not explainable, correlative reduction in Cry1E binding was always observed. The possible existence of a series of events strengthens the involvement of more than one specific receptor and single loop determinants in the overall interaction. Domain II of Cry1 proteins was identified by extensive mutagenesis as the one containing the interacting sites of both APN and cadherin-like receptors. APN is supposed to interact with domain II, loop 2, and loop 3 (16) and the cadherin-like receptor first with domain II loop 2 and then with domain II loop α7 (11). Variations in the affinity of APN and cadherin-like receptors to the whole Cry1A toxins or to single loop determinants were described based on analyses of different lepidopteran species (11, 39), leading to the suggestion that the interaction with a cadherin-like protein is playing a major role (21, 40) and is the first to occur (35). In lepidopteran larvae susceptible to Cry1C, as yet only putative APN receptors have been defined (22, 23, 41). Furthermore, in Cry1C-resistant S. exigua, a 5-fold decrease in Cry1C binding affinity, and no change in Cry1C binding sites was observed (42), whereas in Cry1C-resistant Plutella xylostella, an increase in binding sites was reported linked to a decrease in affinity (27). The estimated $K_f$ value for Cry1C binding to S. littoralis BBMV, evaluated in the present study, is in the range reported for P. xylostella BBMV (about 7 nM) and S. exigua (1.5 nM) (27, 42), whereas the higher $K_f$ and lower $B_{max}$ values that were estimated for Cry1C domain II binding indicate that the interaction with the membrane might encompass more sites or and membrane components, as was evident by the binding of all three domains. A similar conclusion was reached upon analyzing Cry1C-resistant populations of P. xylostella (27, 28). It might also hold for other Cry1A interactions, since attempts to

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**Fig. 5.** Binding competition between C-II and C-III. A, immunoblot of bound C-II and C-III resulting from BBMV (25 μg) incubation with 260 nM C-III and increasing concentrations of C-II. B, gradual decrease in C-III binding correlates with the increase in C-II concentration in the assay and with bound C-II (23-kDa band in A). C-III-bound values were quantified from A.
gain susceptibility to Cry proteins by expressing APN receptors on the cell membrane of insect cells grown in culture indicate that additional as yet unknown components are required for acquiring cytoxicity (43–45).

Several studies have clearly shown that it is impossible to gain comparable Cry1C-like toxic activity by using chimeric proteins containing Cry1C domain III linked to domain I + II of other Cry1 proteins including Cry1E (18, 19). All hybrid toxins containing various Cry1 domains I and II linked to Cry1C domain III were more active against S. exigua than the original Cry1 toxins but less toxic than Cry1C. By using individual domains, our study shows that indeed Cry1C domain III interacts specifically with S. littoralis BBMV and that domain II can compete with domain III binding, suggesting that the initial specific interaction is mediated by domain III as was demonstrated by domain swapping studies (19, 20). It also suggests that this specific binding site is common to domains II and III. Despite the close homology between domain III of Cry1C and Cry1E (Fig. 6), sharing a close evolutionary origin (20), Cry1E-domain III could not functionally substitute Cry1C domain III in the domain shuffling experiments (19, 37). Our results show that there is no competition for binding between domain II of Cry1C and Cry1E, and E-II is not competing with C-III.

Taken together, in Cry1C- and Cry1E-susceptible S. littoralis larvae, different receptors for these toxins exist and dictate specific domain interactions, whereas the whole initial interaction of the toxin with the membrane is broader, probably encompassing all three domains and as yet additional unknown larval midgut epithelial cell membrane components. It may also differ among Cry toxins as has been recently shown by comparing Cry1Ac- and Cry1C-resistant P. xylostella larval populations (28). Cry1C resistance was attributed to two separate linkage groups, whereas that of Cry1Ac was found to be controlled by a single linkage group (28). As yet, the direct correlation between the genetic mapping and specific genes remains to be elucidated.

Acknowledgment—We thank J. Gressel for critically reading the manuscript.

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The Role of *Bacillus thuringiensis* Cry1C and Cry1E Separate Structural Domains in the Interaction with *Spodoptera littoralis* Gut Epithelial Cells

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*J. Biol. Chem. 2004, 279:15779-15786.*
doi: 10.1074/jbc.M312597200 originally published online February 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312597200

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