Binding Properties of *Bacillus thuringiensis* Cry1C \(\delta\)-Endotoxin to the Midgut Epithelial Membranes of *Culex pipiens*

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The Cry1C \(\delta\)-endotoxin from *Bacillus thuringiensis* is toxic to both lepidopteran and dipteran insect larvae. To analyze the dipteran-specific insecticidal mechanisms, we investigated the properties of Cry1C binding to the epithelial cell membrane of the larval midgut from the mosquito *Culex pipiens* in comparison with dipteran-specific Cry4A. Immunohistochemical staining of the larval midgut sections from *Culex pipiens* showed that Cry1C and Cry4A bound to the microvilli of the epithelial cells. The Cry1C binding to brush border membrane vesicles from the mosquito larvae was specific and irreversible, and did not compete with Cry4A. By ligand blotting analyses, we detected several Cry1C-binding proteins, the Cry1C binding to which did compete with excess unlabeled Cry4A. These results suggested that Cry1C and Cry4A recognized the same binding site(s) on the epithelial cell surface but that their interaction with the target membrane differed.

**Key words:** *Bacillus thuringiensis*; Cry toxin; *Culex pipiens*; midgut; membrane binding

*Bacillus thuringiensis* is a Gram-positive bacterium that produces crystalline inclusions during sporulation. These inclusions consist of insecticidal proteins called \(\delta\)-endotoxins that are toxic to lepidopteran, dipteran, and coleopteran insect larvae when ingested.¹ Upon ingestion, the \(\delta\)-endotoxins are solubilized and proteolytically activated by gut proteases in susceptible larvae.² The activated toxin binds to a receptor in the apical microvilli of epithelial cells of the midgut.³⁻⁵ A conformational change in the toxin molecule triggers the insertion of its channel-forming domain into the membrane.⁶⁻⁷ Colloid-osmotic swelling and lysis of the epithelial cells result in the death of the larvae.⁸ Binding of toxins to specific receptors in the epithelial cell membrane is the key step in deciding insecticidal specificity.⁹⁻¹¹ Toxin-binding proteins have been purified, and genes encoding them have been cloned.⁹,¹²,¹³ Loss of the binding sites for toxins is associated with acquired resistance in several lepidopteran insects, although there are exceptions. Studies of toxin-receptor binding assume that the Cry toxin binds to the midgut epithelial membrane by a two-step interaction: the first step is of reversible binding to the receptor, and the second is of irreversible binding when a part of the toxin molecule is inserted into the membrane.¹⁴

The Cry1C genes have been cloned from *B. thuringiensis* subsp. *entomocidus*¹⁵ and *B. thuringiensis* subsp. *aizawai*.¹⁶,¹⁷ The genes encode 135-kDa protoxins, which are converted into activated toxins of about 60 kDa by proteolytic digestion.¹⁸,¹⁹ Cry1C \(\delta\)-endotoxins are potent insecticides toward several lepidopteran insect larvae including *Spodoptera* species.¹⁵,¹⁶,¹²⁰ Cry1C has weak activity toward dipteran insect larvae of *Aedes aegypti*, *Anopheles gambiae*, and *Culex quinquefasciatus*.²¹ There are many studies of the specificity of Cry1C toward lepidopteran insects, but little is known about its specificity toward dipteran insect larvae. The putative Cry1C receptor has been found in lepidopteran insect larvae only.²²

The dipteran-specific Cry4A \(\delta\)-endotoxin from *B. thuringiensis* subsp. *israelensis* is very toxic toward larvae of dipteran insect species.²³⁻²⁵ The 130-kDa protoxin of Cry4A is processed into 20- and 45-kDa fragments, which associate to form an active complex of 60 kDa.²⁶ Recently, we proposed a possible explanation of the binding of Cry4A to midgut epithelial membranes from *Culex pipiens*.²⁷ Cry4A may take two parallel pathways leading to the irreversible binding to the cell surface.

It is worth finding how Cry1C interacts with the midgut epithelial membranes of both lepidopteran and dipteran insect larvae. Interpretation of the mode of interaction of Cry1C with the target cell membrane from mosquito larvae will contribute to understanding of dipteran-specific insecticidal mechanisms. In this study, we analyzed the binding properties of Cry1C in comparison with those of
Cry4A toward brush border membrane vesicles (BBMVs) from the larval midgut of *C. pipiens*.

**Materials and Methods**

**Toxin preparation and digoxigenin labeling.** Cry1C and Cry1Aa crystals were prepared from recombinant *Escherichia coli* DH5α strains expressing each toxin as described previously[20] (plasmids carrying Cry1C and Cry1Aa genes were the kind gifts of Dr. L. Masson and Dr. M. Himeno, respectively). The Cry4A crystals were prepared from the recombinant *B. thuringiensis* strain expressing the toxin as described before.[29] The activated toxin was purified as described elsewhere.[29] The solubilized and trypsin-activated toxins were purified by fast protein liquid chromatography on a MonoQ HR 5/5 anion-exchange column (Amersham Pharmacia Biotech). Digoxigenin labeling of the activated Cry toxin was done as directed by the manufacturer’s instructions (Boehringer Mannheim). In brief, the activated Cry toxin in phosphate-buffered saline (PBS) was diluted with digoxigenin-3-O-succinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester at the molar ratio of 1:25 and incubated for 2 h at room temperature. Reagent that had not reacted was removed by gel filtration on a Sephadex G-25 column.

**Preparation of BBMVs.** BBMVs were prepared from whole larvae of *C. pipiens* by magnesium precipitation described by Silva-Filha et al.[20] The BBMVs were suspended in PBS and stored in portions at ~80°C until use. The protein concentration was measured by the method of Bradford.[21]

**Immunohistochemical staining.** Preparation of mosquito larval sections and immunohistochemical staining were done as described previously.[27] In brief, *C. pipiens* larvae were exposed to 1 μg/ml Cry1C or Cry1Aa solution after having no food for 4 h. Immunohistochemical analysis of sections of the intoxicated larvae was done with the antibodies to *B. thuringiensis* subsp. *aizawai* crystal in combination with the anti-rabbit IgG conjugated with horseradish peroxidase and 3,3’-diaminobenzidine tetrahydrochloride (Nacalai Tesque) staining.

**Insertion assay.** Before the experiment, the digoxigenin-labeled toxin was centrifuged at 100,000 × g to remove insoluble material. Two hundred nanograms of digoxigenin-labeled toxin was incubated at room temperature for 1 h with 20 μg of BBMVs in PBS containing 1 mg/ml bovine serum albumin, 0.1% Tween 20, and protease inhibitor cocktail (Roche Diagnostic) in a volume of 200 μl. The sample was centrifuged at 100,000 × g for 30 min at 4°C, and the pellet of the membrane fraction was washed twice. The samples were put through SDS-PAGE (10% acrylamide), and transferred to nitrocellulose membranes (Schleicher and Schuell). Membranes were treated with anti-digoxigenin antibody conjugated with horseradish peroxidase (Boehringer Mannheim) with ECL detection reagents for western blotting (Amersham Pharmacia Biotech).

**Ligand blotting.** Twenty micrograms of BBMV proteins was separated by SDS-PAGE (14% acrylamide) and transferred to a nitrocellulose membrane. The subsequent procedure was done by the method of Krieger et al.[32] The digoxigenin-labeled toxin bound to the membrane protein from the BBMVs was made visible as described above.

**Results**

**In vivo binding of Cry1C to midgut epithelial cells of *C. pipiens* larvae.**

Cry1C bound to the epithelial cells of the gastric caeca (Fig. 1(A)) and the posterior midgut (Fig. 1(B)). Figure 1(C) shows the binding of Cry1C to the apical microvilli of epithelial cells of the posterior midgut. Cry1Aa, which was not toxic to *C. pipiens* larvae, did not accumulate at all on the apical microvilli of epithelial cells of the larval midgut (Figs. 1(D)–1(F)).

**Binding of Cry1C to BBMVs from *C. pipiens* larvae.**

Digoxigenin-labeled Cry1C bound to BBMVs from *C. pipiens* larvae in a dose-dependent way (not shown). The binding of digoxigenin-labeled Cry1C was reduced in the presence of an excess of unlabeled Cry1C as competitor (Fig. 2(A)). The binding of digoxigenin-labeled Cry1C was not reduced even when unlabeled Cry1C was added to a 200-fold concentration to displace the membrane-bound digoxigenin-labeled Cry1C (Fig. 2(B)).

**Competitive binding analysis of Cry1C and Cry4A**

The excess of unlabeled Cry4A did not interfere with digoxigenin-labeled Cry1C binding to the BBMVs from mosquito larvae (Fig. 3(A)). Excess unlabeled Cry1C did not interfere with binding of digoxigenin-labeled Cry4A to the BBMVs (Fig. 3(B)).

**Cry1C-binding proteins**

Cry1C-binding proteins were detected by ligand blotting; the major species of protein had a molecular mass of 40 kDa (Fig. 4(A), lanes 3 and 6). The association between digoxigenin-labeled Cry1C and the binding proteins was abolished in the presence of a 500-fold amount of unlabeled Cry1C as competitor (lane 4). The major band of the Cry1C-binding protein disappeared in the presence of 100-fold and 500-fold amounts of unlabeled Cry4A as competitor (lanes 2 and 5). We also found some Cry4A-binding...
C. pipiens larvae were exposed to purified Cry1C (A to C) or Cry1Aa (D to F) crystals at the concentration of 1 μg/ml. The intoxicated larvae were washed with distilled water, and fixed in 4% paraformaldehyde in PBS. The bound Cry1C or Cry1Aa in the sections of the intoxicated larvae was immunologically detected with antibodies to B. thuringiensis subsp. aizawai crystal in combination with anti-rabbit IgG conjugated with horseradish peroxidase. Stained larval midgut sections were observed under a light microscope after treatment with 3,3′-diaminobenzidine tetrahydrochloride solution. A and D, gastric caecae and anterior midgut; B and E, posterior midgut; C and F, epithelial cells of the posterior midgut. The length of the bar in panel A corresponds to 100 μm. Panels B, D, and E are at the same magnification as panel A. The length of the bar in panel C corresponds to 10 μm. Panel F is at the same magnification as panel C. GC, gastric caecae; AM, anterior midgut; PM, posterior midgut; L, lumen.

proteins, the major molecular species of which had a molecular mass of 40 kDa (Fig. 4(B), lanes 3 and 6). The association between digoxigenin-labeled Cry4A and the binding protein was decreased by the presence of a 500-fold amount of unlabeled Cry1C (lane 5). The band disappeared in the presence of 100-fold and 500-fold amounts of unlabeled Cry4A (lanes 1 and 4). These results strongly suggested that the BBMV contained a Cry4A-specific binding protein of 40 kDa.
Fig. 4. Ligand Blotting of *C. pipiens* BBMV Proteins Separated by SDS-PAGE.

BBMV proteins blotted onto nitrocellulose membranes were probed with digoxigenin-labeled toxin in the presence of unlabeled toxin or bovine serum albumin as the competitor. Detection of the digoxigenin-labeled toxins was done as described in the legend of Fig. 2. (A) Binding of digoxigenin-labeled Cry1C in the presence of a 100-fold excess (lanes 1–3) or 500-fold excess (lanes 4–6) of unlabeled Cry1C (lanes 1 and 4), Cry4A (lanes 2 and 5), or bovine serum albumin (lanes 3 and 6). Molecular mass markers (in kilodaltons) are shown on the left of the panels.

(B) Binding of digoxigenin-labeled Cry4A in the presence of a 100-fold excess (lanes 1–3) or 500-fold excess (lanes 4–6) of unlabeled Cry4A (lanes 1 and 4), Cry1C (lanes 2 and 5), or bovine serum albumin (lanes 3 and 6). Molecular mass markers (in kilodaltons) are shown on the left of the panels.

**Discussion**

δ-Endotoxins from *B. thuringiensis* have been used as biological pesticides. However, the molecular mechanism of the insecticidal activity and specificity is not understood. Cry1C δ-endotoxins showed a potent insecticidal activity toward several kinds of lepidopteran insect larvae, and also showed a weak insecticidal activity toward some dipteran insect larvae. The immunohistochemical staining showed that Cry1C bound to the microvilli of epithelial cells of the gastric caeca and posterior midgut, although Cry1Aa did not. The binding of digoxigenin-labeled Cry1C and unlabeled Cry1C suggested that the binding of Cry1C was specific. The results of the displacement assay showed that the binding of Cry1C to the membrane was irreversible. The specific and irreversible binding of Cry1C observed above may account for its toxicity toward *C. pipiens* larvae. A putative receptor for Cry1C, aminopeptidase N with the molecular mass of 106 kDa, has been found in *Manduca sexta.* A 40-kDa Cry1C-binding protein has been found in species of the order *Spodoptera.* We detected proteins with an apparent molecular mass of 40 kDa that bound specifically with digoxigenin-labeled Cry1C. The digoxigenin-labeled toxin competed with the unlabeled one for binding to the 40-kDa protein. How does Cry1C recognize both lepidopteran and dipteran receptors? The three-dimensional structure of the Cry2Aa molecule, which is toxic toward both lepidopteran and dipteran insect larvae, has been identified at 2.2 Å resolution. Its molecular structure is similar to the structures of Cry1Aa and Cry3A, despite the amino acid sequences being quite different. On the basis of the structure of the Cry2Aa molecule, Morse et al. speculated that the same area of surface of the toxin molecule was involved in binding to both lepidopteran and dipteran receptors. They suggested that the residues inaccessible to the solvent behind the putative receptor-binding surface of the toxin molecule contribute to the specificity by directing the orientation of the hydrophilic residues in the binding loop. We do not know if a single area of the surface of the Cry1C molecule binds to both lepidopteran and dipteran.

We found, in the immunohistochemical analysis of sections of the larval midgut, that Cry4A binds specifically to the apical microvilli of midgut epithelial cells in the posterior midgut of *C. pipiens* larvae. A plausible interpretation is that the specific binding of Cry4A to the epithelial cell membrane is important in deciding the insecticidal specificity toward *C. pipiens.* Binding of a Cry toxin to a specific receptor in the epithelial cell membrane is a key step in deciding the insecticidal specificity. Digoxigenin-labeled Cry4A specifically binds to several kinds of proteins with molecular masses from 18 to 30 kDa. In this report, we also found that digoxigenin-labeled Cry4A bound to the 40-kDa major protein of BBMVs from *C. pipiens.* The binding of digoxigenin-labeled Cry1C with proteins from BBMVs was prevented by unlabeled Cry4A, and the same was found when the Cry4A was labeled, suggesting that the two toxins had binding sites in common in the binding protein molecule from epithelial cell membranes of the mosquito larval midgut. In contrast, by the membrane insertion assay, membrane binding between Cry1C and Cry4A was not competitive. This discrepancy suggested that the membrane binding properties of Cry4A were different in some aspect from those of Cry1C. As mentioned above, we have proposed that Cry4A has two pathways that lead to irreversible binding to a cell surface. The Cry4A membrane binding through one of these two pathways seems to depend on specific binding proteins (receptors) and, therefore, follows the two-step mode.
of interaction. This first pathway may result in the correct orientation of insertion of the toxin molecule into the membrane (irreversible binding) leading to the formation of functional channels. The Cry4A membrane binding through the other one may be nonspecific and involve direct association with membrane. The Cry4A molecules that are nonspecifically and directly bound to the membrane in this second mode of interaction may not be involved in deciding the toxicity toward Spodoptera species.

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