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Loop residues of the receptor binding domain of *Bacillus thuringiensis* Cry11Ba toxin are important for mosquitocidal activity

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Abstract

Using a Cry11Ba toxin model, predicted loops in domain II were analyzed for their role in receptor binding and toxicity. Peptides corresponding to loops $\alpha 8$, 1 and 3, but not loop 2, competed with toxin binding to *Aedes* midgut membranes. Mutagenesis data reveal loops $\alpha 8$, 1 and 3 are involved in toxicity. Loop 1 and 3 are of greater significance in toxicity to *Aedes* and *Culex* larvae than to *Anopheles*. Cry11Ba binds the apical membrane of larval caecae and posterior midgut, and binding can be competed by loop 1 but not by loop 2 peptides. Cry11Ba binds the same regions to which anti-cadherin antibody binds, and this antibody competes with Cry11Ba binding suggesting a possible role of cadherin in toxication.

Keywords

Bacillus thuringiensis; Cry11Ba toxin; mutagenesis; larvicidal activity; receptor binding; loop regions

Introduction

In an effort to develop environmentally responsible methods of mosquito control, researchers have been evaluating microbiological control strategies involving mainly *Bacillus thuringiensis* subspecies *israelensis* or *B. sphaericus*. Both bacterial species produce insecticidal inclusions. Subspecies *israelensis* produces four major insecticidal proteins, classified as Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, all of which are toxic to mosquito larvae [1,2]. However, a number of other *B. thuringiensis* strains producing Cry toxins have been identified including *B. thuringiensis* subsp. *jegathesan* [3]. This *Bacillus* produces a protein called Cry11Ba, which to date is the most mosquitocidal single toxin having about 6–40 times more activity against mosquitoes (depending on the species of mosquito tested) than the most active toxin of *B. thuringiensis* subsp. *israelensis* [2,4]. Consequently, the Cry11Ba toxin provides an alternative to toxins used in current control programs thereby addressing risk of potential mosquito resistance development to *B. thuringiensis* subspecies *israelensis* or *B. sphaericus* toxins.

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Like other Cry toxins, mosquitocidal toxins when ingested by susceptible larvae are processed in the larval alkaline midgut releasing soluble proteins, which are then activated by digestive enzymes. For several Cry toxins, it has been shown that the activated toxins bind to specific receptors on the brush border of target insects. Many putative Cry toxin receptors have been reported and the best characterized are those in lepidopteran insects. These include cadherin-like proteins [5–7], glycosylphosphatidylinositol (GPI)-anchored aminopeptidases N (APN) [8,9], a GPI-anchored alkaline phosphatase (ALP) [10,11] and a 270 kDa glycoconjugate [12]. Recent work show a similar set of proteins also act as receptors for mosquitocidal toxins, with reports of Cry4Ba binding to *Anopheles gambiae* cadherin [13], Cry11Ba binding to APNs from *An. quadrimaculatus* and *An. gambiae* [14,15], and Cry11Aa binding to ALP from *Ae. aegypti* [16].

Presently two models of Cry protein toxicity have been proposed. In one, sequential binding to multiple receptors might be required to induce toxicity [17,18], while in the other binding to the cadherin alone initiates an intracellular cascade that leads to cell toxicity [19]. However, in both models binding to specific receptors is essential for initiating toxicity. This key binding step between active three-domain globular proteins and their receptors has been investigated with many Cry toxins. These reports demonstrate domain II is involved in receptor recognition and hence insect specificity [20–23]. Recent reports with mosquitocidal Cry toxins support the role of domain II in binding receptors in mosquito midgut [24,25].

Site-directed mutagenesis approaches have been used by several research groups to investigate the role of the domain II surface exposed loops in the toxicity of Cry proteins [23,26–28]. In Cry1Ac toxin, three putative surface-exposed loops (loops 1, 2 and 3) are involved in toxicity and at least two of them (loop 2 and loop 3) are involved in binding ability [23,29–31]. In the Cry3A toxin loop 1 and loop 3 of domain II are involved in receptor binding whereas loop 2 double mutations had no effect on binding or toxicity [32]. To show that the loop regions in domain II of the Cry11Ba are also involved in binding and toxicity we initially developed a homology model of Cry11Ba to identify these loop regions. Then using a combination of the four loop peptides (loop α 8, loop 1, loop 2 and loop 3) and site directed triple- and single-amino acid replacements in the all exposed loops we show loop α 8, loop 1 and loop 3 play a role in Cry11Ba mosquitocidal activity. By combining immunohistochemistry and competitive binding assays, we suggest Cry11Ba toxin binds the cadherin protein in *Ae. aegypti*.

Materials and Methods

Homology modeling structure

The secondary structure of Cry11Ba was predicted with Predict Protein to compare to the Cry2Aa structure [33] using default parameters. The exposed loop regions were then identified using both Cry2Aa and the predicted loops of Cry11Aa [24]. The Cry11Ba secondary-structure prediction was threaded against the whole backbone database using Phyre (web-based program). Top significant hits corresponded to Cry protein structures, with the top hit being Cry2Aa (1i5p.pdb) at 15% homology. The PDB file produced by EsyPred 3.1 (web-based program) was used to build a model with Swiss-pdb 3.51 [34]. Energy minimization levels were also analyzed to ensure that the run was taken to equilibrium.

Preparation of Cry11Ba toxins from *Bacillus thuringiensis*

Cry11Ba was produced in *B. thuringiensis* harboring a pHT315 plasmid that has the *cry11Ba* gene [4]. The bacterial culture was grown in sporulation medium supplemented with erythromycin (25 μ g/ml) at 30°C for 3 days. Cultures expressing Cry11Ba wild-type or mutant toxins were harvested by centrifugation and resuspended in distilled water. Cells were then

washed with 1M NaCl, 10 mM EDTA 3–4 times. The inclusions were purified on NaBr gradients [35].

Toxin purification and toxin biotinylation

The purified inclusions were solubilized by incubation at 37°C for 4 h in 50 mM Na₂CO₃, pH 10 and then activated by digestion with trypsin (phenylalanyl chloromethyl ketone treated) at a ratio of 1:20 (w/w) enzyme: toxin in 50 mM Na₂CO₃, pH 10 for 16 h. The activated toxin was purified by ion-exchange chromatography using a MonoQ column (GE Health Care, USA) with a linear gradient of 50 mM Na₂CO₃, pH 10.0 and 50 mM Na₂CO₃, pH 10.0 with 1M NaCl at a flow rate of 0.4 ml/min. Eluted fractions were collected and concentrated to 1–2 mg/ml by ultrafiltration at 4°C using a Ultrafree-CL column (10-kDa cutoff, Millipore, Bedford, USA). The purified and concentrated activated Cry11Ba toxin was biotinylated using the protein biotinylation module kit (GE Health Care) and then purified using a Sephadex G25 column.

Preparation of brush border membrane vesicles (BBMV)

Midguts were dissected from early fourth instar *Ae. aegypti* larvae and kept in –80°C until use. BBMV were prepared by the differential magnesium precipitation method [36]. The BBMV were resuspended in the ice-cold buffer A (0.3 M Mannitol/0.5 M EGTA/20 mM Tri-Cl, pH 8), and the concentration of total protein was measured by a BCA kit (Pierce, USA). Alkaline phosphatase and leucine aminopeptidase activities were determined as previously described [11,36].

BBMV Binding assays

Binding of Cry11Ba toxin to *Aedes* BBMV was performed as described [24]. BBMV (20µg protein) were incubated with biotinylated Cry11Ba (10nM) in 100µl binding buffer (0.1% BSA, 0.1% Tween 20, PBS pH 7.4) in the presence or absence of cold-Cry11Ba toxin, loop peptides or purified mutant Cry11Ba toxins. After incubation for 1 h at room temperature, the mixture was centrifuged at 10000 g for 10 min, and the pellet then washed three times with binding buffer to remove unbound toxins. The washed pellet was boiled for 5 min in sample buffer and then separated by SDS-PAGE and electrotransferred to PVDF membranes (Immobilon, Amersham Biosciences). The membranes were incubated with streptavidin-peroxidase conjugate (1:2500 dilution, Amersham Biosciences) for 1 h and then visualized using luminal (ECLTM, Amersham Biosciences). For competitive assays with antibodies, BBMV were pre-incubated with polyclonal anti-cadherin or anti-*Aedes* NHE3 antibodies (1/10 or 1/1000 dilutions) for 1 h at room temperature before incubating with biotinylated-Cry11Ba as described above.

Binding assays to tissue sections

Tissue preparation—Early fourth instar *Ae. aegypti* larvae were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Then the larvae were washed in PBST (1× PBS/0.1% Triton X-100) for 30 min three times, dehydrated in 20, 40, 70, 96% ethanol series in 1×PBST for 30 min each and finally in 100% ethanol for 1 h at room temperature (RT). The samples were placed in ethanol/xylene mixture (70/30, then 30/70) for 3 h each and then in 100% xylene at room temperature overnight. Paraplast chips (Xtra, Fisher Scientific) were added at 20–50% of total 100 % xylene volume for 6–8 h at RT and then replaced with fresh Paraplast at 56°C over 2 days until tissues were completely infiltrated. The larvae were embedded in Paraplast blocks and then 8–10 µm thick sections were cut, placed on to poly-L-lysine (Sigma, St Louis, MO) slides with 1% gelatin (Becton Dickson, Franklin Lakes, NJ), and the sections dried for 1–2 days at 40°C.

Immunolocalization—The sections were dewaxed in 100% xylene for 15 min twice, rehydrated in 100, 70, 40 and 20% ethanol for 5 min each and rinsed in deionized water and 1xPBST. The tissues were blocked in 2% BSA/PBST (Bovine Serum Albumin) for 1 h at RT and then incubated with Cry11Ba toxin (10nM), in the absence or presence of competitor loop 1 and loop 2 peptides, in 1% BSA/PBST. The sections were washed in 0.1% BSA/PBST six times to eliminate unbound toxin. The tissues were incubated with primary anti-Cry 11Ba whole serum antibody diluted 1:100 or 1:250 in PBST/1% BSA at 4°C overnight and then washed in PBST/0.1% BSA/2% goat serum for 30 min twice. Finally, the sections were incubated for 1 h in the dark with secondary antibodies (Jackson ImmunoResearch Labs). For Cry11Ba toxin detection Cy3-conjugated goat-anti-rabbit was diluted 1:1,000 and Phalloidin-Alexa 488 diluted 1:100 was applied for actin F detection. After washing twice in 0.1% BSA/PBST the sections were mounted in Shur/Mount media (Electron Microscopy Science, Hatfield, PA). The images were obtained using laser-scanning confocal Zeiss Axioplan microscope (LSM Zeiss 510, Institute of Integrative Genome Biology, University of California, Riverside) at $\times 40$ and $\times 100$ magnifications. All images were imported in Adobe Photoshop (V6) for assembly and annotation.

Site-directed mutagenesis of Cry11Ba

A 2175-bp fragment, carrying the Cry11Ba gene, was PCR-amplified from a recombinant *Bacillus* strain that contained pJeg80 [4] and cloned into pQE30 an *E. coli* expression vector (Qiagen Inc). The recombinant plasmid encoding the 80-kDa Cry11Ba toxin was analyzed to show toxin expression and toxicity against mosquito larvae, and this construct was then used as a template for site-directed mutagenesis. Thirty-six pairs of complementary mutagenic oligonucleotides primers (Supplementary Table 1) were designed and synthesized (Sigma, St. Louis). Triple and single alanine mutations in loop $\alpha 8$, loop 1, loop 2, and loop 3 were generated by PCR using a high fidelity *Pfu* DNA polymerase, following the procedure of the QuickChangeTM Mutagenesis Kit (Stratagene, La Jolla). All mutant plasmids were verified by DNA sequencing (Institute of Integrative Genome Biology, UC Riverside).

Toxin expression in *E. coli*, *B. thuringiensis* and mosquito larvae toxicity assays

Wild-type and mutant toxins in pQE30 were transformed into M15 *E. coli* competent cells for protein expression. Bacterial clones harboring the wild-type plasmid or mutants were grown at 37°C in Luria–Bertani medium containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin until the OD₆₀₀ of the culture reached 0.3–0.5. Protein expression was induced with isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 4 h and subsequently analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) at 10% w/v. *B. thuringiensis* cultures expressing Cry11Ba wild-type and mutant toxins in pHT315 were harvested and the inclusions were purified by NaBr gradients as described above.

Bioassays for mosquito-larvicidal activity were performed with fourth instars of *Ae. aegypti*, *An. stephensi*, and *Culex quinquefasciatus* larvae (University of California Riverside). The bioassays were performed 3–5 times. Briefly, 20 early fourth instar larvae were placed in water in a 6-oz plastic cup (5 cm cup diameter, Costar, USA) to which an *E. coli* suspension of approximately 1×10^8 cells suspended in distilled water was added to a total volume of 200 ml. *E. coli* M15 cells were used as a negative control. Mortality was recorded after 24-h incubation at room temperature. All bioassays were examined and repeated at least three times. Data obtained for LC₅₀ and LC₉₅ determinations were statistically evaluated using Finney's Probit Analysis.

Purification of the *E. coli* expressed Cry11Ba and mutant toxins

E. coli cultures over expressing the toxins as cytoplasmic inclusions were harvested by centrifugation and resuspended in cold distilled water. Cells were then disrupted by sonication

for 10 min (Sonifier 450, USA). After centrifugation at 10,000 x g, 4°C for 10 min, the pellets were washed 3 times in 1% Triton X-100 and suspended by sonication. Protein concentrations of the partially purified inclusions were determined by using a BCA kit (Pierce, USA). Inclusions (1–2 mg/ml) were solubilized by incubation at 37°C for 2–3 h in 50 mM Na₂CO₃, pH 10 and then activated as described above using trypsin. The activated toxins were purified by FPLC as described above.

Analysis of toxin structure by circular dichroism (CD)

CD spectra of activated toxins were obtained using a Jasco J-715 CD spectropolarimeter (Core Analytical Chemistry Instrumentation Facility, UC Riverside) in the far UV region (190–260 nm) using a rectangular quartz cuvette. Samples were prepared in 50 mM Na₂CO₃, pH 10.0, with protein concentrations of 0.10 mg/ml, as determined by far UV absorbance. CD spectra were recorded at a scanning rate of 100 nm/min with a spectral bandwidth of 1 nm and response times of 1 msec. Eight data accumulations were taken and the results averaged. After background subtraction, all the CD data were converted from CD signal (mdegree) into mean residue ellipticity (deg cm²/dmole).

Results

Cry11Ba structure prediction

Because there was no structural information of the Cry11Ba toxin, we used available Cry toxin structures to predict its structure. The structures of Cry1Aa [20], Cry2Aa [33], Cry3Ba [37], Cry4Aa [38] and Cry4Ba [39] have been reported since the Cry3Aa toxin crystal structure was first elucidated [21]. Of these structures Cry2Aa has the highest amino acid sequence similarity to Cry11Ba, and based on this homology, a Cry11Ba structure was built with three domains. To more precisely predict the loop regions within domain II we compared the Cry11Ba protein with the secondary structure of Cry2Aa and that of the predicted Cry11Aa [24] using Phyre and PredictProtein [40,41]. The predicted loops α 8, 1, 2 and 3 are shown in Fig 1A. Together with these variable loops the predicted Cry11Ba structure is shown in Fig. 1B.

Binding of Cry11Ba toxin to BBMV

Cry11Ba inclusions were purified from the pJeg80 containing *B. thuringiensis* strain using NaBr gradients [4,42]. Upon activation the 65-kDa Cry11Ba toxin generated 30- and 35-kDa fragments. The activated toxin 30- and 35-kDa proteins were labeled with biotin and then analyzed for toxin binding to *Ae. aegypti* BBMV. Figure 2 shows the binding of biotinylated-Cry11Ba toxin can be competed by excess unlabeled Cry11Ba. At a molar ratio of 1:1 of labeled to unlabeled, the unlabeled toxin initially competed with binding of the 35-kDa fragment followed by competition with the 30 kDa fragment. At a ratio of 1:50 and higher, binding to both fragments of the biotinylated-Cry11Ba was nearly completely competed (data not shown).

Competitive binding assays in the presence of loop peptides

To determine whether the predicted loop regions are involved in binding ability, four synthetic peptides (α 8, L1, L1 and L3) corresponding to the putative loop regions (loop α 8, loop 1, loop 2 and loop 3) of Cry11Ba (see Fig. 1A) were synthesized and used to compete with binding of biotinylated Cry11Ba to larval midgut BBMV. As shown in Figure 2, three peptides corresponding to loop α 8, loop 1 and loop 3 were found to compete the binding of Cry11Ba to BBMV. Whereas peptide corresponding to loop 2 (Fig. 2, lane 6) as well as the negative control peptide NHE8 (Fig. 2, lane 12) derived from *Aedes* NHE ion exchanger protein [43] did not compete with Cry11Ba binding. The data suggests three domain II loops, loop α 8, loop 1 and loop 3, are involved in Cry11Ba toxin binding to *Aedes* BBMV.

We also analyzed the percentage of toxin binding to BBMV of both the 35- and 30-kDa Cry11Ba fragments in the presence of different competitors. Table 1 shows the 35-kDa fragment of biotinylated Cry11Ba toxin was more readily competed off than the 30-kDa fragment with almost all competitors. Of the competitors used, loop 3 peptide seemed to compete best with Cry11Ba binding to BBMV (Fig. 2, Table 1).

Effect of loop mutations on mosquitocidal toxicity

Since at least three loop region peptides competed with Cry11Ba binding to midgut BBMV from *Ae. aegypti* larvae, we then performed mutations in the loops to identify specific residues involved in toxicity. All residues in the predicted loop α 8, loop 1, loop 2, and loop 3 were changed to alanine via PCR-based site-directed mutagenesis. In an initial screen three residues were mutated at a time. The mutant protoxins were highly expressed in *E. coli* M15 cells upon IPTG induction, with yields similar to the wild-type Cry11Ba toxin, except the loop 1 N305A mutant showed no protein expression (data not shown). To determine mutational effects on toxicity, fourth instar larvae of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus*, were fed *E. coli* cells expressing each mutant toxin. The mortality recorded after 24-h incubation showed that the toxicity of three triple mutants (V256A/G257A/E258A, N262A/I263A/S264A, and W268A/K269A/G270A) on loop α 8 and a few from loop 1 (R303A/E304A/N305A and I306A/H307A/G308A) and loop 3 (L451A/T452A/Y453 and N454A/K455A/L456A) were reduced by 80–100% against *Ae. aegypti* (Table 2). On the other hand, it was interestingly to note that only two mutants (V256A/G257A/E258A and W268A/K269A/G270A) on loop α 8 exhibited substantially reduced larvicidal activity against *An. stephensi*, whereas the five other mutants had significant toxicity. In contrast results from bioassays performed on *Culex* are more similar to those against *Aedes*, showing toxin mutants in loop α 8, loop 1 and loop 3 were also reduced in toxicity whereas loop 2 mutants had no effect on toxicity. Single mutations then were made to identify the critical amino acid residues involved. We found that residues G257, I263, S246 and K269 on loop α 8; residues N305 and I306 on loop 1 are important for Cry11Ba toxicity. Interestingly, single mutations on loop 3 had no effect on toxicity to all three mosquito species even though the triple mutants were clearly affected in toxicity as shown in Table 2.

To determine the LC₅₀ and LC₉₅ values of the mutant toxins we expressed one mutant strain (R303A/E304A/N305) in *Bacillus thuringiensis* (4Q7). Inclusions from this strain and the pJeg80 strain were used to assess toxicity to larvae of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus*. The wild-type Cry11Ba conferred the highest toxicity against *An. stephensi*, with the toxicity being 3.5 times and 1.5 times greater than to *Ae. aegypti* and *C. quinquefasciatus*, respectively. The R303A/E304A/N305 mutant on the other hand was severely affected in its toxicity against all three species of larvae, being 1,500 to 3,000 times less toxic than that of the wild-type Cry11Ba toxin (Table 3). In contrast this same mutant when expressed in *E. coli* showed 4 to 10 times decrease in toxicity than wild-type Cry11Ba toxin against *Ae. aegypti* and *C. quinquefasciatus*, respectively (Table 2). It is interesting to note the *E. coli* expressed R303A/E304A/N305 mutant showed high toxicity to *An. stephensi* larvae. However, by changing toxin concentrations in bioassays we could show the R303A/E304A/N305 mutant was less toxic than the wild-type Cry11Ba (data not shown). Differences in toxicity observed between *B. thuringiensis* and *E. coli* expressed proteins are likely due to the use of protein mixtures in bioassays in the latter, and toxin levels could not be quantified in a crude *E. coli* mixture. Hence, only mutants which are severely affected in toxicity are identified in Table 2.

Structural characterization of the purified mutant toxins

We then selected two mutants, one each from loop α 8 (V256A/G257A/E258A) and loop 3 (N454A/K455A/L456A), which had lower toxicity against the mosquito larvae to analyze if these toxins are processed as the wild type Cry11Ba. Upon solubilization in 50 mM Na₂CO₃,

pH 10.0, the mutant protoxins expressed in *E. coli* were purified by FPLC using ion-exchange chromatography. The purified fractions of both wild-type and mutant strains were concentrated (Fig. 3A, lane 1) and cleaved into 30- and 35-kDa fragments (Fig. 3A, lanes 2 and 3). This similarity in protein cleavage indicates the mutations did not cause protein significant misfolding and also had no apparent effect on proteolytic processing. The purified mutant toxins (V256A/G257A/E258A and N454A/K455A/L456A) were then examined for its secondary structural by far-UV CD spectroscopy. The purified wild-type toxin and V256A/G257A/E258A mutant exhibited similar CD spectra as the wild-type toxin (Fig. 3B). Although, the N454A/K455A/L456A mutant gave subtle differences in CD spectrum, it should be noted that this mutant causes nearly 100% mortality against *Anopheles* larvae (Table 2), suggesting a negligible improper folding and structure. Thus far, the evidence suggests that the reduction in toxicity of the Cry11Ba mutants is unlikely due to changes in protein folding after alanine substitutions.

Effect of loop mutations on binding to BBMV

We then used two purified loop 1 mutants (R303A/E304A/N305 and H307A) on loop 1 that were expressed in *Bacillus thuringiensis* to assess if they could compete with binding of biotinylated-Cry11Ba to BBMV. Even though both mutants could compete with binding of Cry11Ba (Fig. 2, lanes 10 and 11), one (H307A) retained toxicity (data not shown), while the other (R303A/E304A/N305) lost toxicity (see Table 3)

In situ binding analysis and competitive binding assays in the presence of anti-cadherin antibody

For in situ competitive assays, we determined Cry11Ba binding to dissected *Ae. aegypti* midgut in the absence or presence of synthetic peptide competitors (L1 and L2). The results show that lower Cry11Ba binding was observed on the microvilli regions when competed with the L1 loop peptide (Fig. 4A, panels C1–2 and Fig. 4B, and panels C1–2). In contrast, the L2 peptide showed lower levels of competition with Cry11Ba (Fig. 4A panel D and Fig. 4B panel B). These data directly corroborate data above that show loop 1 peptides are capable of competing Cry11Ba toxin binding to larval midgut BBMV while the loop 2 peptide does not compete.

We also analyzed the Cry11Ba binding pattern in *Aedes* larval midgut. Using a similar approach as above we analyzed Cry11Ba binding regions in tissue sections of larval midgut which were incubated with purified Cry11Ba. Intense immunofluorescence was observed in the apical microvilli of posterior midgut epithelial cells (Fig. 4A) as well as in caecae (Fig. 4B). No Cry11Ba binding was observed in the anterior midgut (Fig. 4A, panel B) or hindgut cells (data not shown). Control larval midgut sections in which the activated Cry11Ba toxin was omitted showed no immunofluorescence on the apical microvilli membrane of caecae (Fig. 4B, panel A). A similar expression profile is also observed when midgut tissue sections are analyzed with anti-*Aedes* cadherin antibodies (Chen, J., K. Aimanova and S. S. Gill, unpublished data). From the data we expect *Aedes* cadherin is a likely candidate for Cry11Ba toxin binding. To strengthen this notion, we performed competitive binding of the Cry11Ba toxin to *Aedes* BBMV in the presence of anti-cadherin antibody and a control anti-NHE antibody. Fig. 5 shows the anti-cadherin antibody competes with Cry11Ba toxin binding to BBMV even in the presence of a 1:1000 dilution whereas anti-NHE8 antibody had little ability to compete with the Cry11Ba toxin binding.

Discussion

Cry11Ba toxin is one of the most toxic single *Bacillus* proteins to mosquito larvae. However, little is known about the structure of this toxin or its loop regions. Based on information gathered from the resolved Cry toxin structures and site directed mutagenesis studies [21,23,44] domain

II has been identified as primarily responsible for toxin selectivity to particular insects. Using the Cry2Aa structure [33] we made a homology structure of Cry11Ba toxin including the predicted loop regions of domain II.

Upon proteolytic activation, the 65-kDa Cry11Ba toxin generates 30- and 35-kDa fragments. A previous study with Cry11Bb1 proteins [45] shows the N-terminal fragment is the smaller of the two proteolytic fragments. Although not confirmed here with Cry11Ba the 35-kDa protein is likely the C-terminal fragment, which is composed of most of domain II and domain III, since in this work it preferably competes in binding on BBMV of *Ae. aegypti* (Fig. 2,5 and Table 1). Moreover it has been reported that domain III of several Cry toxins is also involved in receptor binding [46–48]. For example, replacement of domain III in Cry1Ab and Cry1Ac with domain III of Cry1Ca, resulted in increased toxicity towards *Spodoptera exigua* and altered membrane protein recognition [49]. In addition, the 21-kDa Cry4Ba-domain III fragment binds the apical midgut microvilli of *Ae. aegypti* but it has lower-binding capability when compared to the 43-kDa Cry4Ba-domain II–III fragment [25,50]. Therefore, the receptor-binding capability of Cry toxins is contributed mostly by domain II rather than domain III, even though the latter can be involved in binding.

In this report we demonstrate that three of the predicted loop regions, loop $\alpha 8$, loop 1 and loop 3, are involved in binding ability to *Aedes* BBMV. Loop $\alpha 8$ and loop 3 of Cry11Aa toxin are similarly involved in binding to toxin receptors in *Aedes* BBMV [24]. Loop $\alpha 8$, loop 2 and loop 3 of Cry1Ab have been suggested to play a role in toxin binding to *Manduca sexta* Bt-R1 [26,51,52] whereas only loop 2 and loop 3 of Cry1Ac are involved in binding to the cadherin receptor [53]. In addition, loop 1 and loop 3 of Cry3A have been reported in binding *Tenebrio molitor* BBMV [32]. The difference in binding ability of loops in different Cry toxins might be due to the fact that domain II is the most variable region of the toxin domains, and it is especially true for the apex loops [20,21,38,39]. Thus, differences in sequence, length, and conformation of the loops are believed to be key determinants of toxin selectivity.

We then show that the mutations of amino acid residues of loop $\alpha 8$, loop 1 and loop 3 affect the toxicity of Cry11Ba towards *Aedes* and *Culex* mosquito larvae whereas loop $\alpha 8$ appears to be the primary determinant for toxicity against *Anopheles* sp. These data are supported by competition experiments using loop peptides. For example, loop $\alpha 8$, loop 1 and loop 3 peptides all compete well with Cry11Ba binding to BBMV (Table 1, Fig 2). Thus not surprisingly mutations in these loops also demonstrated a loss of mosquitocidal activity.

When the binding ability was tested with the two purified loop 1 mutants expressed in *Bacillus*, we show that they could compete with the Cry11Ba binding. The ability of the mutant H307A to compete is not surprising since it retained toxicity. But the ability of the R303A/E304A/N305 mutant to compete with Cry11Ba binding to *Aedes* BBMV was unexpected since this mutant had much lower toxicity. The data implies that amino acid residues 303–305 of loop 1, which are important for toxicity, are not as affected in receptor binding ability. However, these residues might be involved in subsequent steps such as membrane insertion and/or pore-forming activity. As previous reported, residue G312 of loop 1 of Cry1Ac had no significantly effect on BBMV binding ability but was affected in toxicity [31]. It is conceivable that residues in domain II are not always involved in receptor binding. For example, the Cry1Ab F371 loop mutant lost its ion channel activity instead of binding activity suggesting a role for this residue in membrane insertion [54,55].

In addition we determined Cry11Ba binding to dissected *Ae. aegypti* midgut in the absence or presence of synthetic loop peptide competitors (L1 and L2). The results are similar to competitive toxin binding assays in which loop peptides $\alpha 8$, L1 and L3 compete with Cry11Ba binding to BBMV whereas loop peptide L2 does not compete. We also show the Cry11Ba

binding pattern in tissue sections of *Aedes* larval midgut is similar to previous tissue binding studies of several Cry toxins (i.e. Cry4Aa, Cry4Ba, Cry11Aa), which show toxin binding is located to the apical microvilli of the larval midguts [56]. The binding pattern is also similar to that observed with Cry11Aa and Cry11Bb toxins [16,57]. Previous work has demonstrated that an alkaline phosphatase (ALP) is localized on the apical side of distal and proximal caecae and apical regions of posterior midgut [16]. A similar expression profile is also observed when midgut tissue sections are analyzed with anti-*Aedes* cadherin antibodies (Chen, J., K. Aimanova and S. S. Gill, unpublished data). The binding pattern of ALP and cadherin antibodies is consistent with Cry11Ba toxin binding in *Aedes* larval guts. From this data we can conclude that either ALP and cadherin, or more likely both, could bind Cry11Ba toxin and they might be involved in the mechanism of Cry11Ba toxification.

In this report we also provide evidence of *Aedes* cadherin antibody inhibiting the binding of Cry11Ba toxin. The ability of the Cry11Ba to bind *Aedes* cadherin is not surprising since cadherins are important receptors for Cry1A toxins in many lepidopteran insects [5,58,59]. Recent studies shown the cadherin protein is also important for binding Cry4B in *An. gambiae* [13] and the Cry11Aa in *Ae. aegypti* (Chen, J., K. Aimanova and S. S. Gill, unpublished data). Since the majority of Cry toxins appear to bind cadherin-like protein, the cadherin protein is also a likely receptor protein for the Cry11Ba toxin.

Overall, the data reveal three loops (loop α 8, loop 1 and loop 3) in domain II of Cry11Ba are involved in toxicity against mosquito larvae, with loop 1 and loop 3 being of greater significance to *Aedes* and *Culex* larvae than to *Anopheles* larvae. Considering the loop regions of domain II are the most variable of the toxin domains, these regions are important determinants in toxin selectivity. Three loop peptides were observed to compete with Cry11Ba binding activity on *Aedes* BBMV. As for *Aedes*, the loss of toxicity observed in loop α 8, loop 1 and loop 3 mutants might correspond to the loss of toxin binding ability as observed in competitive assays in which loop peptides α 8, L1 and L3 compete with Cry11Ba binding to BBMV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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		loopα8	
Cry2Aa	F-RGLNTRLHDMLEFRTYMFLNVFEYVSIWSLFKYQSLMVSSGANLYASGSGPQQTQSEF		289
Cry11Aa	FGRLCKVSLKDGLTFRNMCNLYVFPFAEAWSLMRYEGLKLQSSLSLWDYVG--VSI-PVN		263
Cry11Ba	FGRLAKNLNEALNFRNMCNLYVFPFSEAWSLRYEGTKLENTLSLWNFVG--ESINNIS		264
		loop1	
Cry2Aa	AQNWP-FLYSLFQVNSNYILSGI-----SGTRLSITFPNIGGL-----PGSTTTHSLN		336
Cry11Aa	<u>Y</u> NEWGGLVYKLLMGEVNQRLTTVKFNYSFTNEPADIPARENIRGVHPIYDPSSGLTGWIG		323
Cry11Ba	<u>P</u> NDWKGALYKLLMGAPNQRLNNVKFNYSYFSDTQATI <u>H</u> RENI <u>H</u> GVLPPTYNGGPTITGWIG		324
Cry2Aa	SARVN-----YSGGVSSGLIGATNLNHNH--NCSTVLP-----PLSTPFVR		375
Cry11Aa	NGRTNNFNFADNNGNEIMEVRTQTFYQNP--NNEPIAPRDIINQILTA--PAPADLFFKN		379
Cry11Ba	NGRFSGLSFPCSNELEITKIKQEITYNDKGGNFNSIVPAATRNEILTATVPTSADPFFKT		384
		loop2	
Cry2Aa	SWLDSGTDREGVATSTNWQTE-SFQTTLRLRC-GAFSARGNSNYFPDYFIRNISGVPLVI		433
Cry11Aa	ADINVKFTQWFQSTLYGWNIGLGTQTVLSSRT-GTIPP--NYLAYDGYIIRAIACPRGV		436
Cry11Ba	ADINWK-- <u>Y</u> FSPGLYSGWNIGKFDFTVTLKSRVPSIIPS--NILKYDDYYIRAVSACPKGV		440
		loop3	
Cry2Aa	R---NEDLTRPLHYNQIRNIESPSGTPGGARAYLVSVHNRKNNIYAANENGTMHILAPED		490
Cry11Aa	SLAYNHDLT-TLTYNRIE-----YDSPTTENIIIVGFAPDNTKDFYSKKS-----HYLSET		485
Cry11Ba	SLAYNHDFL-TLTYNKLE-----YDAPTTQNIIVGFSPDNTKSFYRSNS-----HYLSTT		489

Figure 1. Modeling of Cry11Ba and the predicted loop regions from multiple alignment of loop regions with several cry toxins

(A) Amino acid alignment of domain II of Cry11Aa, Cry11Ba and Cry2Aa toxins (generated by using MAFFT V. 5.743). Underlined regions correspond to loop residues in the Cry toxins. (B) 3D-structural model of Cry11Ba, which was obtained based on alignment in the structure of Cry2Aa toxin. The predicted loop regions are indicated as blue loop α 8; red, loop 1; purple, loop 2; and yellow, loop 3.

Figure 2. Unlabeled Cry11Ba, loop peptides and a mutant Cry11Ba compete with biotinylated Cry11Ba toxin binding to *Aedes* larval midgut BBMVs

Competition of varying molar excess of unlabeled Cry11Ba (lanes 2, 3 and 9), four loop peptides (lanes 4–7), mutant Cry11Ba toxin (lanes 10 and 11) and with a control NHE8 peptide (lane 12) with biotinylated Cry11Ba binding to BBMVs. Synthetic loop peptides, α 8, L1, L2 and L3, were used in lanes 4–7 respectively. Purified mutant toxins L1-H307A and R303A/E304A/N305 were used in lanes 10 and 11, respectively. In lane 12 an unrelated peptide, NHE8, used as a control does not compete with biotinylated Cry11Ba binding to BBMVs.

Figure 3. Processing of the purified mutants toxins and its CD spectra show the structures of mutant toxins are not substantially different from the wild-type Cry11Ba

A. SDS-PAGE of ion exchange purified mutant toxin (α 8-V256A/G257A/E258A) from *E. coli*, lane 1. Lanes 2 and 3 are trypsinized purified toxins for α 8-V256A/G257A/E258A and L3-N454A/K455A/L456A, respectively, showing formation of 35- and 30-kDa fragments.

B. CD spectra of the purified and activated Cry11Ba toxin (■) in comparison with that of the mutant V256A/G257A/E258A (●) and L3-N454A/K455A/L456A (▲).

Figure 4. The Cry11Ba toxin binds the apical membrane in caeca and posterior midgut of larval *Ae. aegypti* and a loop peptide competes with this binding

A. Merged midgut sections of 4th instar larvae showing anti-Cry11Ba immunoreactivity (Cy3, red) and Phalloidin (Alexa-488, green). A1,A2: Anti-Cry11Ba reactivity in the apical membrane of posterior midgut; B: no immunoreactivity was observed in the apical membrane of anterior midgut. C1 and C2: competition binding assay of Cry11Ba to the posterior midgut

region using synthetic peptide L1 (C1, C2) and L2 (D) as competitors. Scale bar 10 μm . **B.** Competition binding assay in *Aedes aegypti* gastric caecae cells using Cry 11Ba toxin and the L1 and L2 peptides as competitors. Anti-Cry11Ba immunoreactivity was detected in the apical membrane of gastric caeca cells (B, C1, C2). B: competitor peptide L2; C1 and C2: competitor peptide L1. A: no immunoreactivity in the apical membrane of gastric caeca epithelial cells when the tissues were probed with only the preimmune serum. Scale bar 50 μm .

Figure 5. The anti-Aedes cadherin antibody competes with Cry11Ba binding to BBMV

Lane 1, biotinylated Cry11Ba toxin was incubated with BBMV in the presence of unlabeled Cry11Ba; lanes 2 and 3 biotinylated Cry11Ba toxin incubated with BBMV in the presence of different dilutions of anti-*Aedes* cadherin antibody (1:1000, 1:10, respectively); lanes 4 and 5, similar experiments in the presence of anti-*Aedes* NHE8 antibody (1:1000, 1:10, respectively).

Loop region peptides and mutant toxins preferentially compete with the domain II and III of Cry11Ba binding to BBMV

Table 1

Competitor peptide	Percentage of toxin binding of Cry11Ba						
	Domain II–III 35-kDa fragment			Domain I 30-kDa fragment			
	1:1	1:100	1:250	1:1	1:100	1:250	
Cry11Ba toxin	21	9	-	87	11	-	
Peptide $\alpha 8$	-	-	14	-	-	39	
Peptide L1	-	-	8	-	-	7	
Peptide L2	-	-	77	-	-	92	
Peptide L3	-	-	2	-	-	15	
Peptide NHE8	-	-	95	-	-	86	
H307A	-	5	-	-	36	-	
R303A/E304A/N305A	-	10	-	-	43	-	

Competitor peptides were used at 1:1, 1:100 or 1:250 molar ratio excess of Cry11Ba. Values are percent of Cry11Ba binding to BBMV in the presence of competitor peptides.

Table 2

Toxicity of wild-type and Cry11Ba mutant proteins expressed from *E. coli* against fourth instar larvae of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus*.

Toxins	Mortality (%)		
	<i>Ae. aegypti</i>	<i>An. stephensi</i>	<i>C. quinquefasciatus</i>
M15	0±0	0±0	0
Cry11Ba wild-type	88±6.01	98±1.67	98±2.88
α8-V256A/G257A/E258A	0±0	3±1.67	0±0
α8-S259A/I260A/N261A	90±2.89	100±0	95±5
α8-N262A/I263A/S264	27±4.41	100±0	72±17.5
α8-P265A/N266A/D267A	82±4.41	100±0	98±2.88
α8-W268A/K269A/G270A	0±0	17±6.61	6±2.88
L1-R303A/E304A/N305A	22±11.67	93±4.41	8±2.88
L1-I306A/H307A/G308A	3±1.67	100±0	86±15.27
L1-V309A/L310A/P311A	92±3.33	100±0	98±2.88
L2-Y291A/F392A/S393A	97±3.33	100±0	96±5.8
L2-P394A/G395A/L396A	72±6.01	100±0	96±5.8
L3-L451A/T452A/Y453A	27±8.82	100±0	23±7.64
L3-N454A/K455A/L456A	23±7.26	98±1.67	33±17.56
L3-E457A/Y458A/D459A	87±1.67	100±0	96±5.8
L3-A460A/P461A/T462A	97±3.33	100±0	95±5
L3-T463A/Q464A/N465A	82±8.82	100±0	93±2.88
α8-V256A	83±1.67	100±0	96±2.88
α8-G257A	2±1.67	33±6.01	3±2.88
α8-E258A	78±3.33	100±0	96±5.77
α8-N262A	90±2.89	100±0	95±5
α8-I263A	0±0	0±0	6±2.88
α8-S264A	0±0	100±0	38±25.2
α8-W268A	88±1.67	98±1.67	96±2.88
α8-K269A	5±2.89	100±0	8±2.88
α8-G270A	83±1.67	98±1.67	96±2.88
L1-R303A	88±6.61	100±0	98±2.88
L1-E304A	75±2.89	92±4.41	96±5.77
L1-N305A	NE	NE	NE
L1-I306A	0±0	100±0	12±5.77
L1-H307A	87±1.67	100±0	96±2.88
L1-G308A	75±2.89	100±0	96±2.88
L3-L451A	90±0	97±3.33	80±5
L3-T452A	92±1.67	98±1.67	92±5.77
L3-Y453A	75±2.89	100±0	95±5
L3-N454A	87±1.67	100±0	92±7.6
L3-K455A	83±1.67	100±0	96±5.77
L3-L456A	87±1.67	100±0	92±7.6

Toxins	Mortality (%)		
	Ae. aegypti	An. stephensi	C. quinquefasciatus

Table 3

Loop 1 Cry11Ba mutant toxin expressed in *B. thuringiensis* shows decreased mortality to early fourth instar larvae of *A. aegypti*, *An. stephensi* and *C. quinquefasciatus*.

Mosquito larvae	Wild-type Cry11Ba		L1-R303A/E304A/N305A	
	LC ₅₀ (ng/ml)	LC ₉₅ (ng/ml)	LC ₅₀ (ng/ml)	LC ₉₅ (ng/ml)
<i>Ae. aegypti</i>	7.86 (5.1–11.1)	85.2 (48.4–234.5)	2176 (1389–3432)	29210 (15111–79822)
<i>An. stephensi</i>	2.2 (1.02–2.9)	5.8 (4.3–14.5)	3128 (2028–4841)	33382 (17850–89189)
<i>C. quinquefasciatus</i>	3.3 (2.1–4.4)	12.3 (8.8–23.9)	9249 (6138–13989)	73702 (40956–194201)

() represents upper and lower 95% confidence limits