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Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* Synergizes Cry11Aa Toxin Activity by Functioning as a Membrane-Bound Receptor

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The synergism between Cry11Aa and Cyt1Aa, two *Bacillus thuringiensis* subsp. *israelensis* (Bti) toxins, is an interesting molecular event with regard to the mode of action of Bti proteins. In this work, we demonstrate the in vitro interaction between these toxins and provide evidence that Cyt1Aa enhances the binding of Cry11Aa to brush border membrane vesicles of *Aedes aegypti* larvae. We mapped the Cyt1Aa and Cry11Aa binding epitopes involved in their interaction and identified, using site-directed mutagenesis, key residues that affect binding interaction and synergism in vivo. This data strongly indicates that the Cyt1Aa-Cry11Aa interaction is the determinant of synergism between the two toxins and that Cyt1Aa may function as a Cry11Aa receptor in the mosquito midgut.

Bacillus thuringiensis (Bt) is an ubiquitous Gram-positive, spore-forming bacterium that produces parasporal crystals with insecticidal activity against different insect orders such as Lepidoptera, Diptera and Coleoptera (1). Bioinsecticides manufactured with Bt spores and crystals are a useful alternative to synthetic chemical pesticide in agriculture and for mosquito control. Moreover, Bt is also a source of genes for transgenic expression in plants to protect them against pests (7). One of the most important species of Bt is Bt subsp. *israelensis* (Bti) that produces a parasporal crystal containing Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba proteins (6). Since 1980, it was demonstrated that Cry and Cyt toxins from Bti are not only individually toxic, but act also synergically when used in combination (2, 3, 10). Moreover, in *Culex quinquefasciatus* larvae, Cyt toxins are able to overcome resistance to Cry toxins. It was proposed that the lack of resistance to Bti in the field was actually due to the presence of Cyt proteins in the crystal (5, 9).

The molecular mechanism responsible for the interaction between Cyt and Cry toxins is not known. We propose that after solubilization and activation of these proteins in mosquito midguts, Cyt toxins insert into the membrane and expose specific regions that interact with some Cry toxins. Our hypothesis is that Cyt toxins function as membrane bound receptors of these particular Cry proteins

To probe this hypothesis, we decided to work with two of the most abundant protein produced by Bti, i.e., Cry11Aa and Cyt1Aa, which show synergic activity against *Aedes aegypti* larvae. To evaluate the pattern of binding of these two toxins, we performed a sequential binding assay. In this assay, the binding of biotinylated Cry11Aa to BBMVs pre-incubated or not with unlabelled Cyt1Aa was analyzed. We found that the presence of Cyt1Aa increased the binding of Cry11Aa to *A. aegypti* BBMVs, contrary to BBMVs of *M. sexta*, for which no binding of biotinylated Cry11Aa was observed, even in the presence of Cyt1Aa. Homologous competition of biotinylated Cry11Aa in the presence of increased concentrations of unlabelled Cry11Aa showed that the interaction was specific. In fact, we found that higher concentrations of unlabelled Cry11Aa were needed to displace labeled Cry11Aa with Cyt1Aa, suggesting a higher number of binding sites. We concluded that the binding of biotinylated Cry11Aa in BBMVs containing Cyt1Aa was saturable and specific.

To demonstrate further the interaction between Cry11Aa and Cyt1Aa, we performed three assays: ELISA, ligand blot and co-immunoprecipitation. We showed that Cry11Aa bound to Cyt1Aa in solution and to membrane-inserted Cyt1Aa. This interaction was specific, since Cry1Ab and Cry3A toxins did not interact with Cyt1Aa.

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TABLE 1. Synergism and binding affinities of mixtures of Cry11Aa and Cyt1Aa proteins at 1:0.2 ratio.

Toxin combination	LC_{50} (ng/ml) predicted	LC_{50} (ng/ml) experimental	SF[a]	K_d[b]
Cry11Aawt :Cyt1Aawt	227.3	12.1 (0.4-30.8)[c]	18.7	0.4
Cry11Aawt:Cyt1Aa-K198A	227.7	5.7 (0.3-14.2)	40.0	0.3
Cry11Aawt:Cyt1Aa-E204A	202.6	25.8 (11.4- 42.0)	7.9	1.5
Cry11Aawt:Cyt1Aa-K225A	222.5	66.4 (42.3-111.4)	3.4	4.0
Cry11Aa-E266A :Cyt1Aawt	650.3	286.0 (164.9-521.7)	2.3	1.2
Cry11Aa-S259A :Cyt1Aawt	179.4	70.9 (35.6-119.6)	6.6	4.0
Cry11Aa-S259A :Cyt1Aa-K225A	176.5	445.9 (375.9- 515.9)	0.4	30.0

[a]Synergism factor (Predicted LC_{50}/Experimental LC_{50})
[b]Apparent dissociation constant obtained from ELISA competition assays
[c]95% fiducial limits

In order to identify the specific region of Cry11Aa that interacts with Cyt1Aa, we performed competitive binding assays (by ELISA and ligand blot) using synthetic peptides corresponding to different exposed regions of domain II of Cry11Aa (4). These experiments demonstrated that loop-α8, loop-2 and β4 synthetic peptides inhibited the interaction between fixed Cyt1Aa and soluble Cry11Aa toxins.

To identify the region of Cyt1Aa that interacts with Cry11Aa, we used the yeast two-hybrid system. A domain II and III region of Cry11Aa was cloned in a bait plasmid pHybLex/Zeo and different fragments of Cyt1Aa were cloned in a prey plasmid pYESTrp2. Both plasmids were co-transformed in a yeast strain auxotroph for histidine and tryptophan. Cry-Cyt protein-protein interaction was monitored by the reporter genes *his3* and *lacZ*. It was shown that the domain II and III region of Cry11Aa interacted with the F2 and F6 fragments of Cyt1Aa, which correspond to the region from αC to β7.

To narrow down the identification of the interaction sites, we analyzed the binding of biotinylated Cry11Aa to 30 overlapping peptides of the Cyt1Aa αC-β7 region immobilized in nitrocellulose membranes. Two regions were identified: P1 and P2. Competitive binding assays (by ELISA and ligand blot) using synthetic peptides that corresponded to P1 and P2 established that the β6-αE region (P1) and β7 region (P2) of Cyt1Aa inhibited the interaction between Cry11Aa and Cyt1Aa toxins.

Finally we introduced mutations in the identified regions of Cry11Aa and Cyt1Aa by site-directed mutagenesis. The mutants were tested in bioassays against 4th instar *A. aegypti* larvae. Cyt1Aa mutants in the β6-αE and β7 regions were toxic and bound normally to BBMVs. For Cry11Aa, we used the loop-α8 mutants previously reported (4), since it was shown that this loop is important for the interaction between Cry11Aa and its natural receptor in *A. aegypti* BBMVs, and that a loop-α8 synthetic peptide inhibited the interaction between Cry11Aa and Cyt1Aa. While the Cry11Aa-S259A mutant displayed wild-type activity, toxicity and binding of the Cry11Aa-E266A protein was altered (4).

In vivo synergism was evaluated using the Tabashnik equation (8). The synergy factors (SF) of the different combinations of wild-type and mutant proteins are shown in Table 1. All mutant toxins showed a decrease in SF compared with the wild-type proteins, except for the mutant Cyt1Aa-K198A that showed an SF increase. The decrease in synergism correlated with decreased binding (Table 1). In fact, the K_d of the mutants with reduced synergism were larger than that of the wild-type proteins.

Therefore, our data support the fact that Cry11Aa uses similar regions for the interaction with its natural receptor on *A. aegypti* BBMVs and with Cyt1Aa, which implies that Cyt1Aa toxin acts as a receptor of Cry11Aa toxin.

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