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***Aedes aegypti* alkaline phosphatase ALP1 is a functional receptor of *Bacillus thuringiensis* Cry4Ba and Cry11Aa toxins**

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Abstract

Bacillus thuringiensis subs. *israelensis* produces at least three Cry toxins (Cry4Aa, Cry4Ba, and Cry11Aa) that are active against *Aedes aegypti* larvae. Previous work characterized a GPI-anchored alkaline phosphatase (ALP1) as a Cry11Aa binding molecule from the gut of *A. aegypti* larvae. We show here that Cry4Ba binds ALP1, and that the binding and toxicity of Cry4Ba mutants located in loop 2 of domain II is correlated. Also, we analyzed the contribution of ALP1 towards the toxicity of Cry4Ba and Cry11Aa toxins by silencing the expression of this protein through RNAi. Efficient silencing of ALP1 was demonstrated by real-time quantitative PCR (qPCR) and Western blot. ALP1 silenced larvae showed tolerance to both Cry4Ba and Cry11Aa although the silenced larvae were more tolerant to Cry11Aa in comparison to Cry4Ba. Our results demonstrate that ALP1 is a functional receptor that plays an important role in the toxicity of the Cry4Ba and Cry11Aa proteins.

Keywords

Bacillus thuringiensis; Cry toxins; Receptors; alkaline phosphatase; RNA silencing; *Aedes aegypti*

Introduction

Bacillus thuringiensis subs *israelensis* (Bti) is effective for controlling mosquito vectors of human diseases like certain *Aedes*, *Anopheles* and *Culex* species (Bravo et al., 2007; Bravo et al., 2011). During the sporulation phase, Bti produce crystal inclusions composed of a number of proteins, including the Cry11Aa, Cry10Aa, Cry4Aa and Cry4Ba Cry toxins that belong to the three-domain (3D) family of Cry proteins and two cytolytic Cyt toxin, Cyt1Aa and Cyt2Ba, all of which are active against mosquito larvae or are cytolytic (Bravo et al., 2007; Bravo et al., 2011; Nisnevitch et al., 2006).

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Different members of the 3D Cry toxin family show toxicity to a variety of insect species belonging to a number of insect orders. Domain I is a seven α -helix bundle involved in membrane insertion, toxin oligomerization and pore formation. Domain II is a β -prism of three anti-parallel β -sheets packed around a hydrophobic core with exposed loop regions, and domain III is a β -sandwich of two anti-parallel β -sheets; both domains are involved in midgut protein recognition (Bravo et al., 2007). In the case of Cry4Ba toxin, mutagenesis studies have shown that domain II loop 2 region is important for *A. aegypti* toxicity (Khaokhiew et al., 2009).

A number of Cry toxin binding molecules have been described in the insect midgut and these have been shown to be involved in Cry toxicity (Piggot and Ellar 2007). Among them are glycosylphosphatidylinositol (GPI)-anchored aminopeptidases (APN), alkaline phosphatases (ALP) and transmembrane cadherins (Gahan et al., 2001; Fernandez et al., 2006; Hua et al., 2008; Chen et al., 2009a; Chen et al., 2009b; Arenas et al., 2010; Fabrick et al., 2009). Recently Jurat-Fuentes et al., (2011) showed that lepidopteran colonies resistant to Cry1 toxins have diminished ALP activity and expression of ALP in their gut, indicating an important role of this Cry-binding protein in the mode of action of Cry1 toxins. It has been proposed that Cry toxins undergo a sequential binding mechanism with GPI-anchored proteins and cadherin with subsequent oligomerization of the Cry toxin to form pre-pore oligomers that insert into the membrane of the insect midgut epithelium cells bursting them by osmotic cell lysis (Bravo et al., 2004; Pacheco et al., 2009).

In the case of the dipteran *Aedes aegypti*, similar Cry toxin-binding midgut proteins, including, cadherin, APN and ALP, have been identified (Likitvivatanavong et al., 2011). A GPI-anchored ALP isoform (ALP1) was shown to be functionally involved in Cry11Aa binding and toxicity to *A. aegypti* larvae (Fernández et al., 2006; Fernández et al., 2009). Additionally, it was recently reported that expression of a different *A. aegypti* GPI-anchored ALP isoform (mALP) in insect cells resulted in Cry4Ba binding to transfected cells and susceptibility to this toxin (Dechlar et al., 2011). Finally, three APN isoforms were shown to be involved in the toxicity of Cry4Ba in *A. aegypti* by gene silencing with dsRNA (Saengwiman et al., 2011). One of these APN isoforms was previously characterized as a Cry11Aa binding molecule (Chen et al., 2009b).

In this work we analyzed the role of ALP1 in Cry4Ba toxicity by characterizing Cry4Ba loop 2 mutants affected in ALP1 binding and also by analyzing the toxicity of Cry4Ba to *A. aegypti* larvae silenced of the ALP1 gene. Our data indicates that ALP1 is a functional receptor of Cry4Ba.

Materials and Methods

Growth of *Bacillus thuringiensis* (Bt), purification of Cry4Ba and Cry11Aa crystal inclusions and toxin activation

For the production of Cry4Ba and Cry11Aa crystal inclusions, acrySTALLIFEROUS Bti strain Q2-81 containing plasmid pHT618 (Delecluse et al., 1993) or pGC6 (Chang et al., 1993) respectively, were cultured for 3 days at 29°C and 200 rpm in nutrient broth sporulation medium supplemented with erythromycin (10 µg/ml) (Lereclus et al., 1995). Spores and crystals produced by the Bt strains were harvested and washed three times with 0.3 M NaCl, 0.01 M EDTA, pH 8.0. For Cry4Ba activation, the crystals from the spore crystal mixture were solubilized in carbonate buffer (sodium bicarbonate/carbonate 0.1M, pH 10.5) supplemented with 0.1% dithiothreitol, and activated with 1:20 w/w trypsin (Sigma- Aldrich Co.), for 16 h at 37 °C. Cry11Aa toxin was not solubilized since it was only used for determining insecticidal activity using spore crystals.

Site directed mutagenesis of the cry4Ba gene

Mutagenesis of the *cry4Ba* gene cloned in the pHT618 plasmid (Delecluse et al., 1993) was performed using a QuikChange XL-kit (Stratagene, La Jolla CA). Appropriate oligonucleotides were synthesized for each mutant construction. Mutants were sequenced and transformed into acrycystalliferous Bt strain 407 (Lereclus et al., 1989). Cry4Ba mutant toxins were purified and activated as described above.

Expression of ALP1

The *E. coli* strain ER2566 transformed with pET-32b plasmid containing *alp1* gene was previously described (Fernández et al., 2009). This strain produced ALP1 as a N-terminally His-tagged protein fused with thioredoxin. For protein expression, the ALP1 gene construct was induced by addition of 1 mM isopropyl β -D-thiogalactoside (IPTG) and protein was recovered as an inclusion body. The recombinant protein was purified using Ni-NTA resin (Qiagen) in the presence of 8 M Urea, dialyzed against PBS buffer and then analyzed by SDS-PAGE before use.

Preparation of Brush Border Membrane Vesicles (BBMV)

BBMV were prepared from dissected midguts of fourth instar *Ae. aegypti* larvae by differential precipitation using $MgCl_2$ as previously reported (Nielsen-LeRoux and Charles., 1992), and stored at $-70^\circ C$ until used.

Qualitative assays of Cry4Ba binding to BBMV

Binding of Cry4Ba or Cry4Ba mutants was performed in 100 μ l binding buffer (1 $\times$ PBS, 0.1% BSA, 0.1% Tween 20 pH 7.6). After incubation of 10 nM toxin with 10 μ g *A. aegypti* BBMV for 1 h at $25^\circ C$, unbound toxin was removed by centrifugation (10 min at $14\,000 \times g$). The membrane pellet with bound toxin was washed twice with 100 μ l binding buffer and suspended in 1 $\times$ PBS pH 7.6. An equal volume of 2 $\times$ sample loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) was added, samples were boiled for 3 min, separated by SDS-PAGE (10%), and electrotransferred to nitrocellulose membranes. The bound toxin was visualized Western blot using anti-Cry4Ba polyclonal antibody (1:100,000 dilution) followed goat anti-rabbit secondary antibody coupled with horseradish peroxidase (Amersham) (1:5000 dilution) for 1 h. Finally, the blot was revealed by incubation with luminol (Pierce, Rockford, IL) as described by the manufacturer. Binding was quantified by scanning optical density of the 46 kDa Cry4Ba band in the blot.

Enzyme Linked Immunosorbent Assay (ELISA)

To determine the Cry4Ba binding to ALP1, ELISA 96-well plates (Costar, NY) were incubated for 12 h at $4^\circ C$ with 2.5 mg of ALP1 in $NaHCO_3$ pH 9.6, followed by five washes with PBS, 0.2% Tween 20. The plates were then blocked by incubation with PBS, 0.5% gelatin (BioRad) 0.2% Tween 20, for 1 h at $37^\circ C$ and washed five times with buffer A (PBS, 0.1% Tween 20). The ELISA plates were then incubated with different concentrations of Cry4Ba for 2 h at $37^\circ C$ and washed again with buffer A. Cry4Ba that bound ALP1 was revealed with a 1:10,000 dilution of polyclonal anti-Cry4Ba antibody for 1 hour and washed three times with buffer A followed by incubation with 1:10,000 dilution of anti-rabbit antibody (Santa Cruz) for one hour and washed three times. Finally, plates were incubated with 2 mM α -phenylene-diamine, 0.7% H_2O_2 in 0.1 M Na_2HPO_4 pH 5. The enzymatic reaction was stopped with 6N HCl and absorbance read at 490 nm with a microplate reader from Molecular Devices (Sunnyvale, CA). Alternatively the binding of ALP1 to Cry4Ba and mutants was analyzed by incubating ELISA 96-well plates with 2.5 μ g of Cry4Ba or Cry4Ba mutants as described above. The ELISA plates were then incubated with 50 nM of

ALP1 recombinant protein and ALP1 that bound Cry4Ba was revealed with a 1:5000 dilution of anti-His antibody coupled to HRP (Qiagen) and treated as described above. ELISA was revealed as described above. As controls, wells coated with ALP1 but incubated without Cry4Ba or coated with Cry4Ba but incubated without ALP1 and revealed with the corresponding antibody were included in every ELISA. The absorbance of control, which was never greater than 0.1 OD, was subtracted from the experimental absorbance values.

Insect bioassays

An *A. aegypti* colony originally from CINVESTAV-Irapuato (CINVESTAV strain) was kept under laboratory conditions (Ibarra et al., 2003). To determine the mean lethal concentration (LC₅₀) of Cry4Ba and loop 2 mutants, ten early fourth-instar *A. aegypti* larvae reared under 12:12 light:dark period were placed in 100 ml of de-chlorinated tap water (dechlorination was achieved by leaving tap water standing overnight). The effect of spore crystal suspension of Cry4Ba toxin and different mutants on mortality was analyzed after 24 h. Four replicates were used to obtain fiducial limits. The median lethal concentration (LC₅₀) was calculated by Probit analysis using statistical parameters after four independent assays (Polo-PC LeOra Software). For dsRNA treated larvae, bioassays were performed using 20 larvae in 200 ml de-chlorinated tap water, which contained individual spore crystal concentrations at the LC₅₀ dose (100 ng/ml for Cry4Ba and 250 ng/ml for Cry11Aa). Larval mortality was analyzed after 24 h.

RNA Interference (RNAi) assays

A 149 bp fragment of untranslated 3' end of the *A. aegypti* ALP1 gene was obtained using the sense and antisense primers and the cloned ALP1 gene as template (Fernández et al., 2009). 3' untranslated regions are highly divergent in conserved gene families. As control dsRNA lacZ fragment of 900 bp was amplified with appropriate primers. The fragment was cloned into pLitmus28i (HiScribe™, New England Biolabs, Beverly, MA) vector containing two T7 promoters flanking the multi-cloning site. These promoters enabled amplification of the cloned fragment by PCR using a T7 oligonucleotide. The PCR product was purified with QIAquick PCR purification kit protocol (Qiagen Valencia, CA). *In vitro* transcription of both DNA strands to yield dsRNA of the insert was performed with T7 RNA polymerase using the HiScribe RNAi Transcription Kit (New England Biolabs) as reported by the manufacturer.

RNAi experiments were performed as reported previously (Cancino et al., 2010). Two hundred neonate *A. aegypti* larvae in 10 ml of dechlorinated water were fed for 16 h with 200 µg of dsRNA previously encapsulated with Effectene transfection reagent as described by the manufacturer (Qiagen). The encapsulated dsRNA sample was diluted with dechlorinated water to a final volume of 10 ml. After dsRNA feeding the mosquito larvae were transferred to clean water and fed with regular diet until they reached early fourth instar for bioassays or for dissection of the gut. To quantify ALP1 transcript levels in dsRNA-treated and untreated larvae, total RNA was isolated from the dissected midguts and then reverse transcribed using oligo dT and SuperScript II (Invitrogen). For qPCR, we obtained the cDNA coding sequences of all *A. aegypti* ALP from GenBank (<http://www.ncbi.nlm.nih.gov>) and made a multiple sequence alignment using Bioedit program (www.mbio.ncsu.edu/bioedit/bioedit.html). Two primers 969F20 (5'-CGATCTCACTTACGCCGCTA-3') and 1069R20 (5'-TCCAGGGATTTCCGAGCTTT-3') that amplified a 120 bp ALP1 region were used, while for mALP we designed two specific primers dsALPm-F (5'-GCGCACAAGGAGAACAAACCGATCAAGG-3') and dsALPm-R (5'-ATGTCTGCTCCTTCTGCGTCGGAGTGCT-3') to amplify a 328 pb. RT-PCR was performed using total RNAs extracted from *A. aegypti* larval midgut using the following

profile: 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s with a final extension of 7 min at 72°C. The resulting PCR products were cloned and sequenced showing the designed primers specifically amplified the ALP1 or mALP gene sequences indicating that the primers used were specific for each gene sequence. The level of rps3 mRNA (AAEL008192-RB), were used as internal control to normalize the results obtained for the ALP1 mRNA among the tested groups using primers Rps3 forward (5'-GGCATGTTCCGTGCTGAATTGAACG-3') and Rps3 reverse (5'-TTCTCGGCG TACAGCTCGACG 3'). qPCR was performed on a Light Cycler 480 II Instrument (Roche) using SYBER Green/Rox Master Mix (Fermentas). Amplification conditions were 10 min at 95°C for 15s and 60°C for 60s for a total of 40 cycles. The final primer concentration was 250nM. All qPCR reactions were performed in triplicate for ALP1 and twice for mALP genes of each experimental group. A nontemplate control of each reaction was included in each reaction. The quantification technique used to analyze data was as reported (Livak and Shmittgen., 2001).

Western blot

For ALP detection 40 midguts from larvae treated with ALP1 dsRNA or LacZ dsRNA were dissected and homogenized in PBS buffer containing 2mM PMSF. The homogenate was centrifuged (5,000 g) and the supernatant recovered to load gels. For two dimensional electrophoresis, midgut protein lysates were precipitated and cleaned using 2-D Clean up kit (Amersham, Biosciences) as described by the manufacturer and resuspended in rehydration electrofocusing buffer (0.8 M urea, 2% Chaps, 0.5% IPG buffer v/v pH 3–10, 0.002% bromophenol blue m/v). The protein concentration was determined by 2-D Quant (Amersham Biosciences). Seventy µg of protein in 125 µl of rehydration electrofocusing buffer were used to rehydrate overnight 18 cm immobilized pH gradient (IPG) strip (pH 3 to 11 non-linear, GE Life Sciences). First dimension electrofocusing was run on the IPGphor III (GE Life Sciences) with the following settings: step 1, 500 V, 30 min; step 2, 1,000 V, 30 min; step 3, 5,000 V, 1.5 h. Before the SDS-PAGE (second dimension), the IPG strip was reduced for 10 min with 65 mM of dithiothreitol in sodium dodecyl sulfate (SDS) equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue), and then alkylated for 15 min with 135mM of iodoacetamide in the same equilibration buffer. The second dimension SDS-PAGE was performed in an Ettan DALT Six system (GE Life Sciences), using 15% homogeneous acrylamide gels. Electrophoresis was performed using an initial step of 2 W/gel for 25 min followed by 17 W/gel until the dye front reached the bottom of the gel. Gels from two-dimensional electrophoresis were transferred onto nitrocellulose membranes. Proteins were detected with polyclonal anti-ALP1 (1/5,000; 2 h) and a goat anti-rabbit secondary antibody coupled with horseradish peroxidase (Amersham) (1/10,000; 1 h), followed by Super Signal chemiluminescence substrate (Pierce) as indicated by the manufacturer. The anti-ALP1 antibody was previously obtained by producing a recombinant ALP1 protein in *E. coli* to use it to immunize rabbits (Fernandez et al., 2009).

Results

Cry4Ba domain II loop 2 is involved in ALP1 binding

Since previous work showed that Cry11Aa and Cry4Ba bound ALP1, and that Cry11Aa mutants in domain II loop α8 or domain III β18-β19 region affected ALP1 binding and toxicity to *A. aegypti* (Fernández et al., 2009) we decided to identify the Cry4Ba regions involved in ALP1 binding. Site directed mutagenesis of Cry4Ba domain II loop 2 revealed that this toxin region is important for toxicity to *A. aegypti* (Khaothiew et al., 2009). The loop 2 region of domain II corresponds to residues 387SSPS390 (Boonserm et al., 2005). Four mutants were constructed in the domain II including loop 2 residues and some residues of β6 and β7 strands. These mutants were named B6M1 (ITD383-385AAA), L2M2

(TSS386-388AAA), L2M3 (PSN389-391AAA) and B7M4 (RVTK392-395AAAA). The four mutants were structurally stable since two bands of 46 and 18 kDa were obtained when they were proteolytically activated with trypsin as observed with wild-type Cry4Ba (Fig. S1). Bioassays with fourth instar *A. aegypti* larvae were performed to determine the median lethal concentration (LC50) of Cry4Ba and loop 2 mutants. The mutants L2M2, L2M3 and B7M4 showed two-, six- and ten-fold lower toxicity than Cry4Ba, respectively (Table 1), while for mutant B6M1, which had two-fold lower toxicity than Cry4Ba, the fiducial limits of the corresponding LC50 values overlap.

To analyze if these differences in toxicity result from changes in toxin binding qualitative binding analyses of activated Cry4Ba and domain II loop 2 mutants to *A. aegypti* BBMVs were performed. Indeed the four Cry4Ba domain II loop 2 mutants showed 45 to 70 % reduction in binding of the 46 kDa protein to BBMVs in comparison to Cry4Ba (Figure 1). To analyze the binding of the Cry4Ba loop 2 mutants to ALP1 we first performed a binding saturation curve of Cry4Ba to purified ALP1 produced in *E. coli* cells by ELISA binding assay. Figure 2A shows that Cry4Ba binds to ALP1 in a saturable way with apparent relative low binding affinity. Binding analysis of ALP1 to the Cry4Ba was performed by ELISA assays under non-saturating conditions confirmed that ALP1 has reduced binding to Cry4Ba mutants (Fig. 2B). In conditions where binding is analyzed under non saturated conditions a reduction in binding is likely to reflect a decreased in binding affinity. Moreover, the binding of ALP1 to Cry4Ba correlates with their toxicity levels, that is, B7M4 mutant that showed tenfold lower toxicity (Table 1) had ten-fold less binding to ALP1 (Fig 2B). However, B6M1 mutant showed reduced toxicity and reduced binding to *A. aegypti* BBMVs but had no significant effect on ALP1 binding.

Toxicity of Cry11Aa and Cry4Ba to *A. aegypti* ALP1-silenced larvae

The reduction in binding of Cry4Ba loop 2 mutants to ALP1 and in the toxicity of these mutants suggests that ALP1 is a functional receptor of this toxin. To validate the role of ALP1 in mediating Cry4Ba and Cry11Aa toxicity to *Aedes* larvae we decided to silence ALP1 expression using dsRNA interference. Screening of the *A. aegypti* genome using the mALP gene sequence, revealed 13 different ALP isoforms including ALP1 (Dechlar et al., 2011). Of these 13 ALP sequences at least eight are expressed in the insect midgut (Fernández et al., 2009; Dechlar et al., 2011; Bayyareddy et al., 2009; <http://funcgen.vectorbase.org/ExpressionData/>). In order to specifically silence the ALP1 isoform, a DNA fragment of 149 bp from the untranslated 3' region of the ALP1 transcript was cloned into a suitable vector for dsRNA synthesis as described in Materials and Methods. Unlike the open reading frames 3' untranslated regions are not conserved even for homologous transcripts.

Because efficient gene silencing in *A. aegypti* larvae can be obtained by feeding dsRNA molecules encapsulated in Effectene liposomes (Cancino et al., 2010), encapsulated ALP1 dsRNA was fed to first instar *A. aegypti* larvae. Figure 3 shows that the ALP1 transcript levels were reduced three-fold in ALP1-silenced larvae in comparison to control larvae. We also analyzed the effect of ALP1 dsRNA on mALP transcript levels and show that the ALP1 RNAi did not affect the expression of mALP RNA (Fig. 3). However, Western blot did not reveal any effect of feeding ALP1 dsRNA at the protein level (data not shown). Since at least eight ALP isoforms have been shown to be expressed in the *A. aegypti* gut, we performed a Western blot from a two-dimension (2-D) gel electrophoresis of *A. aegypti* midguts (Fig. 2S). Figure 4A shows that the anti-ALP1 antibody recognized at least four proteins with similar molecular weights (65 kDa) but with different isoelectric points. Western blot analysis of 2-D gels of ALP1-silenced larvae showed that the less acidic isoform was reduced in comparison to the other three cross-reactive anti-ALP proteins (Fig. 4B). This agrees with the predicted PI for four ALP's, ALP1 (pH 6.8), ALP2 (pH 5.2), ALP3

(pH 6.1) and mALP (pH 5.8), although posttranslational modifications can contribute to the pIs of ALP isoforms. These results suggest that ALP1 was specifically silenced without affecting the expression of other ALP midgut isoforms.

ALP1-silenced larvae were exposed to an LC₅₀ dose of either Cry11Aa or Cry4Ba and the toxicity was compared to that of control lacZ silenced or non-silenced larvae. Figure 5 shows that ALP1-silenced larvae were more tolerant to both Cry11Aa and Cry4Ba toxins indicating that ALP1 has a functional role in the toxicity of both Cry toxins. However, ALP1-silenced larvae were more tolerant to Cry11Aa in comparison to Cry4Ba, since the silencing of ALP1 reduced the toxicity of Cry4Ba less than two-fold while a 4-fold reduction in Cry11Aa toxicity was observed (Fig. 6).

Discussion

We previously showed that ALP1 and a cadherin act as receptor proteins for the Cry11Aa toxin in *Aedes aegypti* larvae (Fernández et al., 2006, 2009; Chen et al., 2009a). More recently we also showed that Cry4Ba toxicity in this mosquito species is apparently not mediated by the cadherin molecule (Rodriguez-Almazan et al., 2012). Further our previous work (Fernandez et al., 2009) provided preliminary evidence that ALP1 also binds Cry4Ba. Here we provide further evidence ALP1 indeed has as an important a role in mediating the toxicity of Cry4Ba to *Aedes* larvae.

In mosquito larvae in which ALP1 gene expression was attenuated using dsRNA-mediated gene silencing, the toxicity of Cry4Ba was two-fold lower than in controls (Fig. 5). As expected in these silenced mosquitoes Cry11A toxicity was decreased four-fold since previous data showed that Cry11Aa relies on ALP1 binding for toxicity (Fernandez et al., 2006). Interestingly ALP1-silenced larvae become more tolerant to Cry11Aa than to Cry4Ba, suggesting that Cry11Aa relies more on ALP1 binding than does Cry4Ba. Further, this decrease in toxicity was correlated with a decrease in ALP1 transcript levels but not that of a homologous ALP, mALP (Dechlar et al., 2011). Moreover, protein levels of ALP1 were also observed to decrease in ALP1 silenced mosquitoes.

To further analyze the role of ALP1 as a receptor of the Cry4Ba toxin, we characterized four Cry4Ba mutants located near or in loop 2 of domain II, since loop 2 was previously shown to be important for *A. aegypti* toxicity (Khaokhiew et al., 2009). All the mutants analyzed showed a decreased in toxicity. Our data also showed a correlation between the binding of Cry4Ba loop 2 mutants to the ALP1 receptor and toxicity (Fig. 3). The binding of loop 2 mutants to *A. aegypti* BBMV was also affected. These results suggest that the Cry4Ba protein relies on ALP1 binding to exert its toxic effect. Nevertheless, B6M1 mutant did not fit in this correlative effect since it showed reduced binding to BBMV, reduced toxicity but no significant effect on ALP1 binding. Also, since ALP1 silencing showed a moderate effect on Cry4Ba toxicity, it is possible that Cry4Ba mutations described here in addition affected Cry4Ba binding to other receptor proteins such as mALP. This remains to be determined.

Our results show that the GPI-anchored ALP1 is an important Cry toxin-binding molecule that is involved in Cry11Aa and Cry4Ba toxicity. In addition, it was previously shown that expression of a homologous ALP, mALP, in insect cells conferred susceptibility to Cry4Ba (Dechklar et al., 2011). Both results support the view that ALP's are functional receptors for the Cry4Ba toxin.

Several other GPI-anchored proteins may also be involved in the mode of action of these toxins suggesting a complex binding mechanism of these toxins with GPI-anchored receptors to exert their toxicity to *A. aegypti* larvae. For example three *A. aegypti* APN isoforms were silenced and also resulted in a tolerant phenotype to Cry4Ba (Saengwiman et

al., 2011). Nevertheless, it was shown that silencing one particular APN isoform (APN12783) conferred a stronger tolerant phenotype to Cry4Ba than the silencing of the two other APN isoforms, indicating that APN12783 has a predominant role in Cry4Ba toxicity (Saengwiman et al., 2011). It is surprising that three APN and two ALP isoforms are involved in Cry4Ba toxicity to *A. aegypti*. If all these GPI-anchored proteins fulfill the same role in Cry4Ba toxicity it should be expected that silencing one molecule should not result on a tolerant phenotype since the other GPI-anchored proteins should compensate for the absence of the silenced molecule. This could explain the moderate effect of silencing ALP1 on Cry4Ba toxicity. Nevertheless, it is possible that the different GPI-anchored molecules involved in Cry4Ba toxicity could be involved in different steps on the mode of action of this toxin.

It has been proposed that GPI-anchored proteins fulfill two roles – binding the monomeric toxin with low binding affinity to concentrate it in the microvilli membrane before the toxin binds to cadherin and also to bind with high affinity the pre-pore oligomer facilitating its insertion into the membrane (Pardo-López et al., 2006; Pacheco et al., 2009). Thus, it is possible that different GPI-anchored proteins identified could have differential roles on the mode of action of Cry4Ba. This remains to be determined by analyzing the binding affinities of the monomeric and oligomeric forms of Cry4Ba to pure GPI-anchored receptors.

Alternatively, it is also possible that different GPI-anchored proteins could form a protein complex that functions as a Cry toxin receptor, thus silencing one GPI-anchored protein could affect the binding of Cry4Ba to this complex receptor. It is interesting to mention that GPI-anchored proteins are specifically localized in lipid rafts and disruption of lipid rafts by sequestering cholesterol with methyl- β -cyclodextrin was shown to inhibit Cry1A pore-formation (Zhuang et al., 2002). Thus the integrity of lipid rafts could be important to maintain a set of GPI-anchored proteins together as complex of proteins that function as receptor of Cry4Ba. It will be interesting to compare the effect of silencing of the three APN isoforms on Cry11Aa and Cry4Ba toxicity to *A. aegypti*.

Although we identify here an important role for ALP1 in Cry4Ba toxicity in *Aedes aegypti* it is important to cognizant of the fact that in other mosquito species other proteins may be involved in toxicity. Clearly in *An. gambiae* the Cry4Ba toxin targets the cadherin-like protein, which also synergizes its toxicity (Hua et al., 2008), and in *An. albimanus* an α -amylase acts as a Cry4Ba receptor (Fernandez-Luna, et al., 2010).

Finally, it is essential to note that there is an even greater diversity of proteins involved in binding mosquitocidal toxins. The diversity is too extensive for a discussion here but can be assessed in recent reviews (Bravo et al., 2011; Likitvivatanavong et al., 2011)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Bacillus thuringiensis* Cry4Ba domain II loop 2 mutants show affected correlative effect on ALP1 binding and toxicity to *A. aegypti*.
- ALP1 silenced larvae showed tolerance to both Cry4Ba and Cry11Aa toxins.
- ALP1 silenced larvae were more tolerant to Cry11Aa in comparison to Cry4Ba.
- ALP1 is a functional receptor that plays an important role in the toxicity of Cry4Ba and Cry11Aa toxins to *A. aegypti*.

Figure 1. Cry4Ba loop 2 mutants are affected on *A. aegypti* BBMVs binding

Qualitative binding analysis of Cry4Ba (lane 1) and Cry4Ba mutants B6M1 (lane 2), L2M2 (lane 3), L2M3 (lane 4), and B7M4 (lane 5): Numbers below bands show the percentage of binding (normalized to Cry4Ba) obtained by scanning optical density of the 46 kDa toxin band.

Figure 2. Cry4Ba domain II loop 2 is involved in binding to ALP1

A. Cry4Ba binding to ALP1 analyzed by ELISA saturation binding assays. ELISA binding assay was performed by fixing 2.5 μ g of ALP1 in ELISA plates and incubating with different concentrations of Cry4Ba and revealing bound Cry4Ba with polyclonal anti-Cry4Ba antibody. B. ELISA binding assays of 2.5 μ g of Cry4Ba or loop 2 mutants to 50 nM ALP1. ELISA was revealed with anti-His antibody.

Figure 3. ALP1 silenced *A. aegypti* larvae have reduced ALP1 transcript levels

qPCR analysis of ALP1 transcript (lane 1) or mALP (lane 2). ALP1 and mALP qPCR was performed from total RNA extracted from control (Effectene treated larvae) or ALP1 silenced larvae. ALP1 transcript levels are the average of three independent experiments, while those of mALP are from two independent experiments.

Figure 4. ALP1 silenced larvae show reduced levels of one ALP isoform

Western blot analysis of ALP protein in two-dimensional electrophoresis from Seventy μ g of midgut protein from *A. aegypti* larvae. Arrows show four anti-ALP1 cross-reacting proteins, the asterisk show the ALP isoform that with reduced expression in ALP1 silenced larvae.

Figure 5. ALP1 silenced larvae are more tolerant to Cry4Ba and Cry11Aa

Two hundred control, or ALP1 silenced (dsALP) or lacZ dsRNA treated (dslacZ) larvae were exposed to Cry4Ba or Cry11Aa at the LC50 dose and mortality was recorded. Bars show average of three independent repetitions with standard deviations.

Table 1Toxicity of Cry4Ba and its loop 2 mutants to *A. aegypti* fourth instar larvae

Toxin	LC ₅₀ ^a (ng/ml)	LC _{50mut} /LC _{50wt}
Cry4Ba	79.4 (61.0–102.4) ^b	
B6M1 (ITD383-385AAA) ^d	114.9 (81.6–160.4)	1.4 ^c
L2M2 (TSS386-388AAA)	173.1 (105.1–282.9)	2.1
L2M3 (PSN389-391AAA)	455.9 (355.2–596.6)	5.7
B7M4 (RVTK392-395AAAA)	767.5 (678.2–861.8)	9.6

^aFifty percent lethal concentration (LC₅₀) of spore/crystal inclusions.^b95% fiducial limits.^cfiducial limits overlap.^dResidues that were changed to alanine in each mutant.