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Anopheles gambiae Alkaline Phosphatase Is a Functional Receptor of *Bacillus thuringiensis jegathesan* Cry11Ba Toxin†

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ABSTRACT: Alkaline phosphatases (ALPs, EC 3.1.3.1) isolated from lepidopteran and dipteran species are identified as receptors for Cry1Ac and Cry11Aa toxins, respectively [Jurat-Fuentes, J. L., and Adang, M. J. (2004) *Eur. J. Biochem. 7*, 3127−3135; Fernandez, L. E., et al. (2006) *Biochem. J. 396*, 77−84]. In our study, an alkaline phosphatase cDNA (AgALP1) was cloned from the midgut of *Anopheles gambiae* larvae. The encoded 63 kDa protein has a predicted glycosylphosphatidylinositol (GPI) anchor ω-site (^{526}Asp), an N-glycosylation site (^{239}Asn-Leu-Thr), and an O-glycosylation site (^{312}Ser). AgALP1$_t$ was expressed in *Escherichia coli* and used to prepare antiserum and to analyze the interaction of AgALP with mosquitocidal Cry11Ba toxin. Anti-AgALP serum localized AgALP to the apical brush border in the anterior and posterior midgut of larvae and detected a 65 kDa species on a blot of brush border membrane vesicles (BBMVs) protein prepared from larvae. ALP activity was released from larval BBMVs prepared by phosphatidylinositol-specific phospholipase C (PIPLC) treatment, and after separation by two-dimensional gel electrophoresis and blotting, a chain of doublet spots at 65 kDa was detected by anti-AgALP. A subset of these doublet spots bound Cry11Ba on a reprobed blot. Heterologously expressed AgALP1$_t$ bound [^{125}I]Cry11Ba on dot blots and reduced the level of binding of [^{125}I]Cry11Ba to brush border membrane vesicles by 41%, a percentage comparable to that of unlabeled Cry11Ba and aminopeptidase AgAPN2$_{t1}$ peptide. AgALP1$_t$ binds Cry11Ba toxin with a high affinity (23.9 nM) and shares a binding site on Cry11Ba with AgAPN2$_{t1}$. In bioassays against *An. gambiae* larvae, the presence of AgALP1$_t$ reduced larval mortality from 78 to 8%. We conclude that AgALP1 is a binding protein and a functional receptor for Cry11Ba toxin.

Anopheles gambiae is a vector of *Plasmodium falciparum*, the causative agent of malaria. Because of its high potency against mosquito larvae, *Bacillus thuringiensis israelensis* (Bti) is becoming increasingly important for *Anopheles* control. The parasporal crystal of Bti is a complex aggregate of Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa major proteins (reviewed in ref *1*). The isolation of Bt[1] strains harboring novel *cry* genes has taken place for decades. The mosquitocidal *Bacillus thuringiensis jegathesan* produces Cry11Ba, which is 7−34-fold more toxic to mosquito larvae than the related Cry11Aa protein (*2*).

A mode of action for lepidopteran Cry1A toxins involves the sequential steps of protoxin activation, specific binding, and cell toxicity (*3*). A current model includes Cry1A toxins binding cadherin, oligomerizing, and then binding glycosylphosphatidylinositol (GPI)-anchored aminopeptidase and possibly GPI-anchored alkaline phosphatase (*4*). An alternative model proposes the activation of intracellular signaling pathways via binding of the toxin monomer to cadherin without the need of the toxin oligomerization step to cause cell death (*5*). Refer to ref *6* for a recent review of these models of Cry toxin action. Although these models do not preclude a direct role for Cry toxins in insect mortality, there is evidence that midgut lesions caused by Cry toxins lead to septicemia by midgut bacteria culminating in insect death (*7*).

The conserved structure of Cry proteins active against lepidopteran and dipteran larvae suggests a common mode of action, and recently identified Cry toxin receptors in dipteran larvae support this prediction. We identified a cadherin, AgCad1, in midgut of *An. gambiae* larvae as a Cry4Ba binding protein and putative receptor for Cry4Ba toxin (*8*). A GPI-anchored protein, a 100 kDa aminopeptidase (APN) in *Anopheles quadrimaculatus*, was determined to specifically bind Cry11Ba and was considered a potential toxin receptor (*9*). We cloned the *An. gambiae* orthologue, AgAPN2, of the *An. quadrimaculatus* APN and using a combination of approaches established AgAPN2 as a putative receptor for Cry11Ba toxin (*10*).

Alkaline phosphatases (ALPs, EC 3.1.3.1) constitute a family of dimeric metalloenzymes that catalyze the hydrolysis of phosphate monoesters (*11*). Each subunit typically has a catalytic site with two Zn^{2+} ions and one Mg^{2+} ion (*12*). ALPs play a role in phosphate uptake and transport mechanisms (*13*, *14*). ALPs may be important to the integrity of the brush border under environmental and pathogenic challenges. In mice, intestinal ALP detoxifies lipopolysaccharides of enteric bacteria and prevents

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[1]Abbreviations: ALP, alkaline phosphatase; APN, aminopeptidase; Bt, *Bacillus thuringiensis*; BBMVs, brush border membrane vesicles; Cry, crystal protein; GPI, glycosylphosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C; BSA, bovine serum albumin; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction.

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bacterial invasion of the gut (*15*). Midgut ALPs in the lepidopterans *Manduca sexta*, *Heliothis virescens*, and *Helicoverpa armigera* bind Bt Cry1Ac toxin (*16*−*19*). In *H. virescens*, ALP appears to function as a Cry1Ac receptor and reduced levels of ALP in midgut correlate with resistance to Bt toxins (*17*, *20*). Downregulation of ALP is reported in several strains of *H. virescens* resistant to Cry1Ac toxin (*17*, *21*).

Recently, we identified isoforms of ALP as Cry4Ba binding proteins in *Aedes aegypti* (*22*). A 65 kDa ALP is identified as a Cry11Aa receptor in *A. aegypti* larvae (*23*). Two BBMV peptide binding phages were shown to bind ALP, compete for Cry11Aa binding to BBMVs, and reduce Cry11Aa toxicity to larvae. These functional assays established a 65 kDa ALP as a receptor for Cry11Aa toxin in *A. aegypti* (*23*).

In this study, we cloned AgALP1 from *An. gambiae* and established AgALP1 as a high-affinity binding protein for Cry11Ba toxin. Heterologously expressed AgALP1$_t$ competes for Cry11Ba binding to BBMVs prepared from larvae and neutralizes Cry11Ba toxicity to larvae. We propose that AgALP1 is a functional receptor for Cry11Ba in *An. gambiae* midgut.

EXPERIMENTAL PROCEDURES

Purification and Biotinylation of Cry11Ba Toxin. Bt strain 407 (*2*) producing Cry11Ba protein was grown in complex sporulation medium (*24*) supplemented with erythromycin antibiotic, a selectable marker for the plasmid we used. The spore crystal mixture was washed; crystals were separated on NaBr step gradients, and protoxin was prepared as previously described (*10*). Cry11Ba protoxin was activated with bovine pancreatic trypsin (Sigma) at a mass ratio of 50:1 (protoxin:trypsin) for 2 h at 37 °C. Activated Cry11Ba was purified by fast protein liquid chromatography (FPLC), using a Bio-Scale mini High Q cartridge (Bio-Rad, Richmond, CA). Cry11Ba toxin was biotinylated using a 50-fold molar excess of sulfo-NHS (*N*-hydroxysuccinimide)-PC-biotin according to the manufacturer's (Pierce, Rockford, IL) instructions. The reaction mixture was dialyzed against 20 mM Na_2CO_3 (pH 9.6) at 4 °C to remove free biotin.

Insects and Cloning of AgALP cDNA. An. gambiae (CDC G3 strain) were maintained as described previously (*10*). Midguts were dissected from fourth instar larvae, and RNA was extracted with a total RNA mini kit (Bio-Rad). Complementary DNA was synthesized using reverse transcriptase (Gibco-BRL) and oligo-$(dT)_{17}$ primer. On the basis of the similarity to *Bombyx mori* membrane-bound mALP (GenBank entry NP 001037536), we designed primers to amplify the cDNA of *An. gambiae* alkaline phosphatase (GenBank entry XM_313890). PCR was performed using midgut cDNA as a template and the specific primers AgALP1-F1 (5′-GAAAAGGACCGCGAATATTGGTTGA-3′) and AgALP1-R1 (5′-GTATACAATCACCATAAGCATTGC-TAG-3′). The positions of primers are shown in Figure 1A; primers were synthesized by Integrated DNA Technologies, Inc. The PCR amplification reaction included 1 unit of Taq polymerase (Eppendorf) with 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. The resulting PCR band was extracted from an agarose gel with a Qiaex II gel extraction kit (Qiagen) and then cloned into a pGEM-T Easy TA vector (Promega) to construct the pGEM-AgALP plasmid. The DNA inserts from several clones were sequenced in both forward and reverse directions at the Molecular Genetics Instrumentation Facility at the University of Georgia. The cloned fragment had a continuous open reading

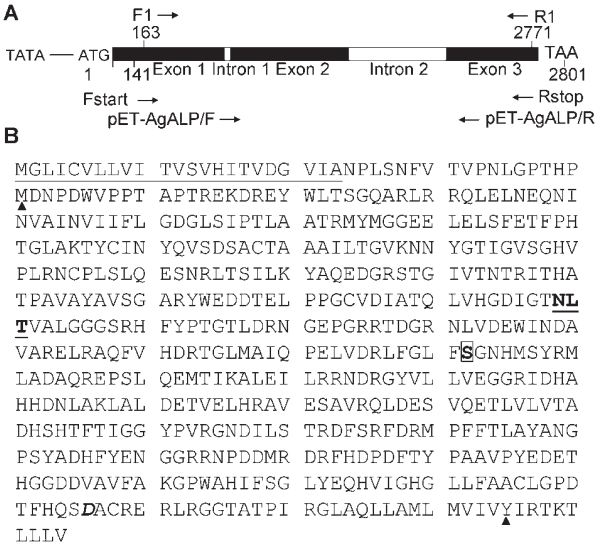

FIGURE 1: (A) Diagram of the AgALP1 cloning and expressing primer locations. The exon and intron are indicated with black and white boxes, respectively. (B) Deduced amino acid sequence of AgALP1. The putative N-terminal signal leading peptide is underlined. The putative C-terminal GPI anchor site is in boldface and italics. The putative O-glycosylation site is in a boldface box. The putative N-glycosylation site is in boldface and underlined. The initiation methionine and C-terminal residue of AgALP1$_t$ are denoted by black triangles.

frame, but the translated protein lacked an apparent leader peptide and stop codon. We analyzed the genomic sequence (AGAP004578-RA) and designed primers AgALP1-Fstart (5′-ATGGGTCTCATTTGTGTGCTGTT-3′) and AgALP1-Rstop (5′-TTACACTAACAACAACGTTTTTGTGCGGA-TGTA-3′). The PCR product derived from amplification with this pair of primers was cloned, sequenced, and called AgALP1.

Bioinformatic Analyses of AgALP1. AgALP1 was analyzed for a predicted signal peptide using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Possible sites for posttranslational modification were identified using big-PI predictor (http://mendel.imp.ac.at/sat/gpi/gpi_server.html), Center for Biological Sequence Analysis prediction servers (http://www.cbs.dtu.dk/services/), and the O-glycosylation prediction electronic tool (OGPET version 1.0, http://129.108.112.23/OGPET).

Preparation of Rabbit Polyclonal Serum against Escherichia coli-Expressed AgALP1$_t$ Peptide. A region of AgALP1 encoding amino acid residues 41−555 of AgALP1 with a C-terminal Glu-Leu and a six-His tag was amplified using pGEM-AgALP1 as a template in a PCR with primers pET-AgALP1/F (5′-AACCCATATGATGGATAACCCTGACTG-GGTACC-3′) and pET-AgALP1/R [5′-TTGTCTCGAGGTA-TACAATCACCATAAGCATTG-3′ (Figure 1A)]. The PCR fragment was purified with a Qiaex II gel extraction kit (Qiagen), cleaved with NdeI and XhoI, and cloned into the pET-30a vector (Novagen). After sequencing had been conducted, pET-AgALP1$_t$ was transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL. The AgALP1$_t$ protein was overexpressed and purified on a HiTrap Ni^{2+} chelating HP column (GE Healthcare, Piscataway, NJ) as previously described (*25*). AgALP1$_t$ protein eluted with imidazole was dialyzed against 20 mM Na_2CO_3 (pH 9.6) and 150 mM NaCl, and the peptide purity was confirmed by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Purified AgALP1$_t$ peptide was used to generate rabbit anti-AgALP1

serum at the Animal Resources Facility of the University of Georgia.

Preparation of Brush Border Membrane Vesicles (BBMVs) from An. gambiae Larvae. Frozen *An. gambiae* fourth instar larvae were kindly provided by the Malaria Research and Reference Reagent Resource Center (MR4) and stored at −80 °C until they were used. BBMVs were prepared from fourth instar larvae by $MgCl_2$ precipitation according to the method of Silva-Filha et al. (*26*) and stored in aliquots at −80 °C. The amount of protein was measured by a Bio-Rad protein assay using bovine serum albumin (BSA) as a standard (*27*). APN activity (*28*), a marker for brush border membranes, was enriched ~5-fold for the final BBMV preparation compared to the initial crude larval homogenate.

Expression and Purification of PIPLC. Plasmid pHN1403-PLC, harboring a gene encoding Bt PIPLC, was kindly provided by F. J. Sharom (University of Guelph, Guelph, ON). The plasmid was transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL. The *E. coli* pHN1403-PLC culture was grown overnight at 30 °C, and then Bt PIPLC with a C-terminal six-His tag was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested, suspended in ice-cold column binding buffer [20 mM Na_2HPO_4 (pH 7.4), 0.5 M NaCl, and 5 mM imidazole] supplemented with complete protease inhibitor cocktail (Roche, EDTA free), and sonicated on ice two times for 3 min with a Braun (Melsungen, Germany) Sonic 1510 sonicator set at 200 W. Bt PIPLC recovered from the supernatant was purified on a HiTrap Ni^{2+} chelating HP column (GE Healthcare) according to the manufacturer's instructions and stored in 10 mM Tris (pH 8.0), 10 mM EDTA, and 60% glycerol (v/v) as aliquots at −80 °C.

Western and Toxin Blots. *An. gambiae* BBMVs (25 μg of protein) were separated by SDS−PAGE and stained or transferred to polyvinylidene fluoride (PVDF) (Millipore) filters. Conditions and solutions used for probing blots were recently described (*8*). Anti-AgALP1 antibody (1:5000 dilution) was the primary antibody, and an anti-rabbit IgG−peroxidase conjugate (1:25000 dilution) was the secondary antibody used. The filter was developed with an ECL kit (GE Healthcare) and exposed to X-ray film.

For two-dimensional (2-DE) gel separation, *An. gambiae* BBMVs were treated with PIPLC according to the method in ref *10* and the suspension was centrifuged at 16000*g* for 10 min at 5 °C, and released proteins were recovered in the supernatant. Details for sample preparation and 2-DE separation of BBMV proteins were recently described (*22*). Briefly, *An. gambiae* BBMV proteins (100 μg) in the supernatant were precipitated using a Plus-One cleanup kit (GE Healthcare) according to the manufacturer's instructions. The final precipitant was solubilized and separated on a pH gradient (IPG) strip (pH 4−7, GE Healthcare) using a Multiphor-II flatbed system. SDS−PAGE was performed on an Ettan DALT*six* large vertical electrophoresis system (GE Healthcare) using 12% SDS−PAGE gels. Proteins were stained with silver or transferred overnight onto a PVDF membrane. The PVDF filter was blocked and probed with anti-AgALP serum as described above. After chemiluminescent imaging on film, the same PVDF filter was stripped in 50 mL of erasure buffer (*29*) [62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol] at 50 °C for 30 min and then rinsed for 10 min in PBST and 0.1% BSA . The filter was incubated for 5 min in ECL solution and exposed to film for 10 and 30 min to ensure removal of the anti-AgALP antibody signal. Subsequently, the membrane was washed four times for 5 min each in PBST and 0.1% BSA, blocked as described above, and reprobed with Cry11Ba (5 μg of toxin/mL). Bound toxin was detected with rabbit anti-Cry11Aa antibody (1:5000 dilution) and secondary anti-rabbit HRP conjugate using the conditions described above for Western blotting.

Immunohistochemistry. Whole *An. gambiae* fourth instar larvae were fixed with paraformaldehyde, embedded, and sectioned using the method described in ref *30* as modified for mosquito larvae (*10*). The localization of AgALP was detected with anti-AgALP serum diluted 1:500 in blocking solution [5% (w/v) BSA and 0.2% (v/v) Tween 20 in PBS], whereas a control section was probed by preimmune serum diluted in the same solution.

Dot Blot Binding Assays. $AgALP1_t$ inclusion bodies were solubilized in 8 M urea, 20 mM Na_2HPO_4 (pH 7.6), and 0.5 M NaCl, purified on a HiTrap Ni^{2+} chelating HP column (GE Helathcare), eluted with imadazole, and dialyzed against 20 mM Na_2CO_3 (pH 9.6) and 150 mM NaCl. CR11-MPED peptide from *An. gambiae* cadherin (AgCad1) purification has been described previously (*8*). Each peptide (1 μg) was dotted onto a PVDF filter and then directly probed with 0.125 nM [^{125}I]Cry11Ba (specific activity of 7.4 μCi/μg of input toxin). The filters were exposed to X-ray film at −80 °C for autoradiography.

Microplate Assay for Binding of $AgALP1_t$ to Cry11Ba. The quantitative interaction between Cry11Ba and $AgALP1_t$ was assessed with a microtiter plate enzyme-linked immunosorbent assay (ELISA) (*10*). Purified $AgALP1_t$ was biotinylated as described above. Microtiter plates (high binding 96-well, Immulon 2HB, Thermo Fisher Scientific Inc., Waltham, MA) were coated with 0.5 μg of Cry11Ba per well in 100 μL of coating buffer [100 mM Na_2CO_3 (pH 9.6)] overnight at 4 °C. The toxin solution was removed, and the plates were washed three times with wash buffer (PBS with 0.05% Tween 20), blocked with 0.5% BSA in wash buffer for 30 min, and then incubated for 2 h with increasing concentrations of biotinylated $AgALP1_t$ (from 0.001 to 20 nM). To determine nonspecific binding, a 1000-fold molar excess of unlabeled $AgALP1_t$ was added to a solution containing biotinylated $AgALP1_t$. The ability of $AgAPN2_{t1}$ (*10*) to compete with binding of biotin-$AgALP1_t$ to Cry11Ba was tested by the addition of a 1000-fold molar excess of $AgAPN2_{t1}$ to the binding reaction mixture. After being washed, plates were incubated with SA-HRP (Pierce) (1:10000 dilution) in wash buffer for 30 min, then washed, and incubated with HRP chromogenic substrate (1-Step Ultra TMB-ELISA, Thermo Fisher Scientific Inc.) to detect bound SA-HRP. Color development was stopped via addition of 2 M sulfuric acid, and absorbance was measured at 450 nm using a microplate reader (MDS Analytical Technologies, Sunnyvale, CA). Specific binding was determined by subtracting nonspecific binding from total binding. Data were analyzed using SigmaPlot version 9 (Systat Software Inc., San Jose, CA), and the curves were fitted on the basis of a best fit of the data to a one-site saturation binding equation.

Homologous and heterologous competition binding were also assessed in the microplate binding assay using a 10 nM concentration of biotin $AgALP1_t$ or $AgAPN2_{t1}$ with increasing concentrations of unlabeled $AgALP1_t$ or $AgAPN2_{t1}$ peptide. Cry11Ba coating and other reaction conditions were the same as described above.

Brush Border Membrane Vesicle Binding Assays. Saturation binding assays were performed according to the method of

ref *10*. Duplicate samples of 8 μg of BBMVs were incubated with increasing amounts of [^{125}I]Cry11Ba toxin in 100 μL of binding buffer [20 mM Na_2CO_3 (pH 9.6), 0.15 M NaCl, 0.1% Tween 20, and 1.5% BSA] at 4 °C for 18 h. Binding reactions were stopped by centrifugation and pellets washed twice with 1 mL of ice-cold binding buffer. The radioactivity of the final pellets was measured with a Beckman model Gamma 4000 detector. The addition of 10 μM unlabeled Cry11Ba or peptide to the binding reaction was used to calculate specific binding and peptide competition, respectively.

For Cry11Ba competition binding assays, duplicate samples of BBMVs were incubated with 0.5 nM [^{125}I]Cry11Ba as described above. Increasing amounts of unlabeled Cry11Ba or peptide were used as binding competitors. All other reaction conditions were as described above. Samples were created in duplicate, and assays were repeated two or three times.

Toxicity Bioassays. Soluble Cry11Ba protoxin (4 μg/mL) was prepared from purified crystals as previously described (*10*). Washed inclusion bodies of AgALP1t or AgCad1-CR11-MPED produced in *E. coli* were solubilized in 6 M guanidine HCl, and the total amount of protein was determined with a Bio-Rad protein assay using BSA as a standard (*27*). One microgram of each peptide was separated by SDS−PAGE with Coomassie brilliant blue R-250 staining. The specific concentration of each peptide in the total protein was calculated on the basis of band density in comparison to a BSA standard curve on the Coomassie-stained gel with a gel image analyzer (Alpha Innotech, San Leandro, CA). Soluble Cry11Ba protoxin (4 μg/mL) was mixed with $AgALP1_t$ or AgCad-CR11-MPED inclusion bodies at a 1:100 toxin:peptide mass ratio. Cry11Ba alone, peptide alone, or toxin/peptide mixtures were transferred to wells of a six-well Costar culture plate (Corning). Ten early fourth instar larvae were added to each well and the plates kept in the 27 °C mosquito rearing room. Each treatment was conducted in at least triplicate, and the bioassays were conducted three times. Larval mortality was recorded after 48 h. Mortality values were statistically analyzed by one-way ANOVA using PROC GLM and PROC UNIVARIATE of the Statistical Analysis System (SAS 2002−2003, version 9.1, SAS Institute, Cary, NC). The means were compared with a least-squares difference (LSD) test at $\alpha = 0.05$. Different letters or asterisk symbols above the error bars indicate a significant difference between means.

RESULTS

Using *B. mori* membrane-bound mALP (GenBank entry NP 001037536), we searched *An. gambiae* sequences in GenBank using BLAST and identified AgALP proteins (XP_306068 and XM_313890) as being 37.9 and 29.9% identical, respectively, to *Bombyx* mALP and having a predicted GPI anchor. Three pairs of primers were designed on the basis of the DNA sequence of XP_306068; however, these primers, when tested in different combinations with *An. gambiae* fourth instar larval midgut cDNA as a template, did not yield visible PCR products (data not shown). A pair of primers designed for XM_313890 (Experimental Procedures and Figure 1) produced a PCR product with midgut cDNA as a template, and the product was cloned and sequenced. The cloned coding region was nearly identical to that of XM_313890, except the open reading frame in the AgALP cDNA lacked an expected N-terminal signal peptide and stop codon. Inspection of the *An. gambiae* genome sequence revealed the most likely initiation methionine based on a Kozak

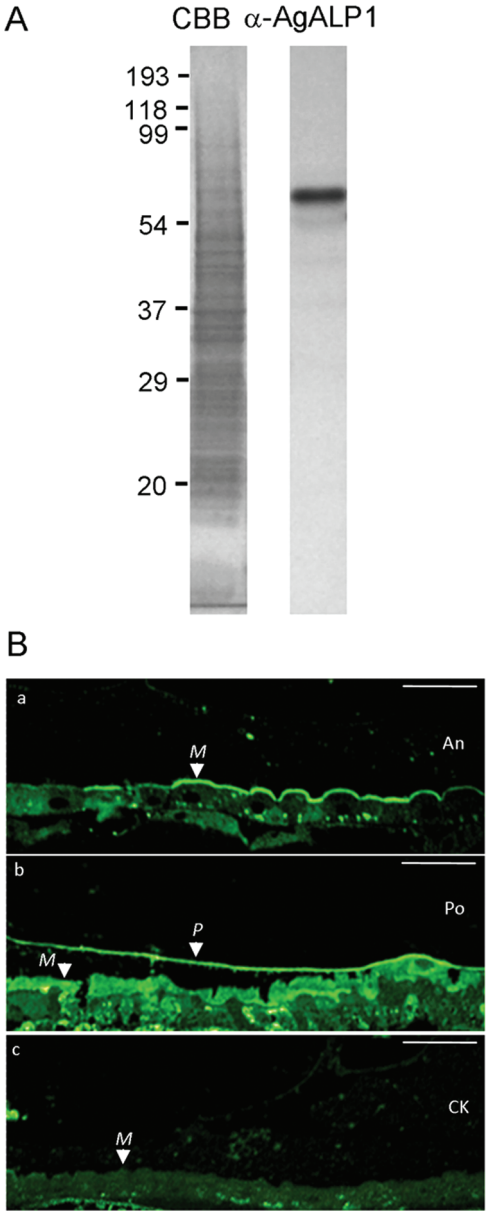

FIGURE 2: (A) *An. gambiae* BBMVs (20 μg) were separated via 12.5% SDS−PAGE. One gel was stained with Coomassie blue. Another was transferred to a PVDF filter and probed with anti-AgALP1 serum. (B) Immunolocalization of AgALP1 on the microvilli of anterior (a) and posterior (b) midgut of *An. gambiae* larvae. Note the detection of AgALP1, or a cross-reactive protein on the peritrophic matrix. Sectioned midgut was probed with anti-AgALP serum and detected with Alexa Fluor-488-conjugated goat anti-rabbit IgG. The control section was probed with preimmune serum (c). Abbreviations: M, microvilli; An, anterior; Po, posterior; P, peritropic membrane. The scale bar is 50 μm. Midgut sections were arranged with the anterior to the left.

consensus sequence [AATAATGG (*31*)] and an upstream TATA sequence. The predicted AgALP open reading frame, shown in Figure 1, includes three exons terminated by a stop codon and followed by a polyadenylation signal sequence (AATAAA). The Fstart primer with an initiation codon and the Rstop primer with a TAA stop codon resulted in a PCR product with midgut cDNA, and the resulting cDNA clone was called AgALP1. Annotation of the AgALP1 cDNA with the ALP genomic sequence indicated two introns and three exons within the genomic sequence (ENSANGG00000001826, currently AgaP_AGAP004578-PA). Recent annotation of the predicted expressed

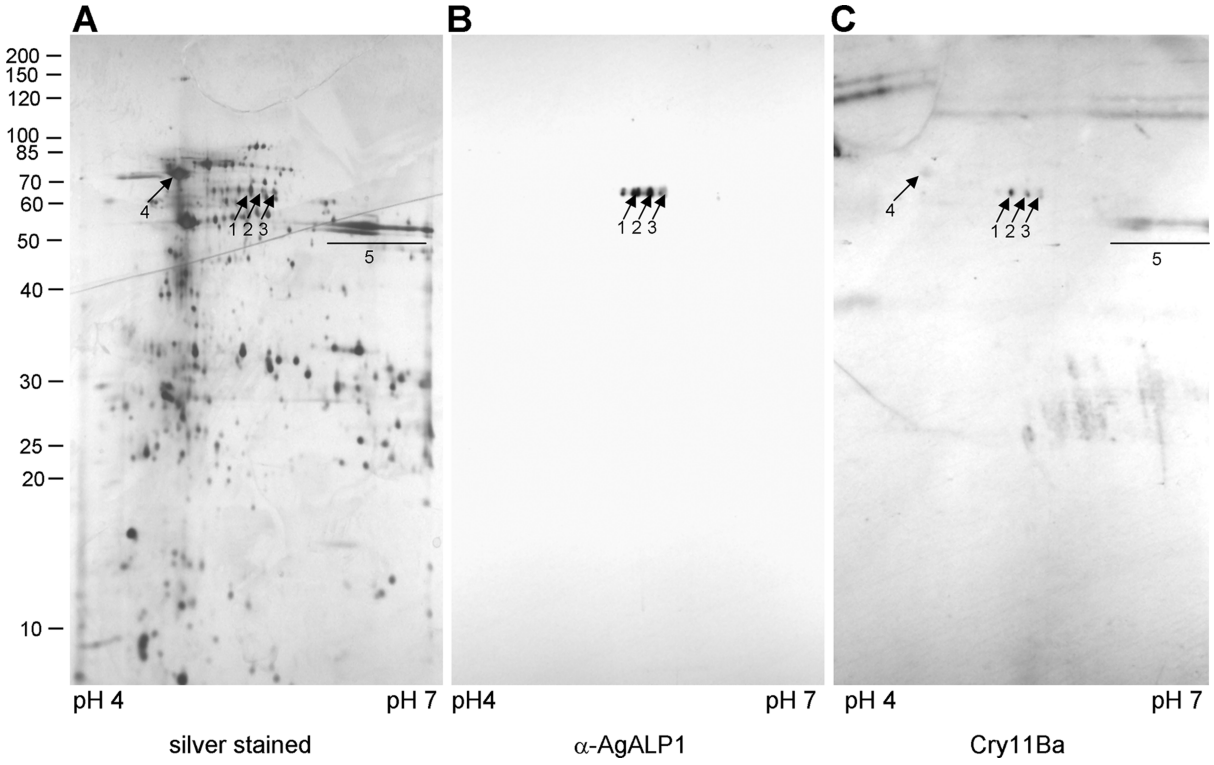

FIGURE 3: Membrane alkaline phosphatase and Cry11Ba blots of 2-DE-separated GPI-anchored *An. gambiae* brush border proteins. PIPLC-released proteins were resolved by iso-electric focusing using pH 4−7 strips and separated via SDS−12% PAGE. Panel A is a silver-stained 2-DE gel. A rabbit polyclonal antibody against $AgALP1_t$ was used to probe a blot (B) of a 2-DE gel run in parallel with the stained gel. The blot was stripped and reprobed with Cry11Ba and then anti-Cry11Aa antibody. Detection of the original and reprobed blots was achieved with goat anti-rabbit conjugated HRP followed by detection of chemiluminescence. Positions of molecular mass markers (kilodaltons) are indicated on the side of the stained gel.

sequence (XM_313890.4) is in agreement with our cloned AgALP1 coding sequence.

The nucleotide sequence of AgALP1 (from the G3 strain) differs from that of XM_313890.4 (from the PEST strain) by eight nucleotides, resulting in three amino acid changes. AgALP1 encodes a predicted 62.3 kDa protein of 564 amino acids. Sequence analysis identified a signal N-terminal peptide and a predicted GPI anchor attachment site at ^{526}Asp followed by a hydrophobic C-terminal region (Figure 1B). AgALP1 has a putative N-glycosylation site (^{239}Asn-Leu-Thr) and an O-glycosylation site (^{312}Ser) (Figure 1B). For expression in *E. coli*, we cloned and expressed $AgALP1_t$, a 57 kDa protein that lacks the predicted signal peptide and part of the hydrophobic C-terminus of AgALP1 while including 91% of the coding region. $AgALP1_t$ was overexpressed in *E. coli* as an inclusion body and purified via a six-His C-terminal tag. After extensive dialysis, $AgALP1_t$ regained detectable enzymatic activity (data not shown).

AgALPs in BBMVs prepared from *An. gambiae* larvae were detected on a one-dimensional (1-DE) SDS gel blot by anti-AgALP1 serum as a dark band at 65 kDa and a faint band at 59 kDa (Figure 2A). We localized AgALPs to the microvilli of anterior and posterior midgut (Figure 2B, a and b) by probing sections of whole *An. gambiae* larvae with anti-AgALP antibody. A strong signal to anti-AgALP antibody was also detected on the peritrophic matrix (Figure 2B, b). A larval section probed with preimmune serum was not stained in the midgut region (Figure 2B, c).

Since the brush border membrane of *A. aegypti* larvae has multiple forms of ALP that are detected with anti-AgALP1 antibody and bind Cry4Ba toxin on blots (*22*), we used the same 2-DE separation and blotting approach to analyze AgALP in BBMVs from *An. gambiae*. We treated BBMVs with PIPLC to release GPI-anchored proteins, separated solubilized proteins by 2-DE, silver-stained one gel, and blotted a second gel to a membrane filter. The blot was probed with anti-AgALP1, stripped, and reprobed with Cry11Ba. As shown in Figure 3A, 2-DE resolved proteins with molecular masses from 10 to 100 kDa. Anti-AgALP1 antibody detected two parallel chains of doublet spots at 63 and 65 kDa (Figure 3B). Cry11Ba detected three of the four pairs of spots detected by anti-AgALP1 antibody (Figure 3C, spots 1−3), and a faint Cry11Ba signal was detected for the fourth pair of spots. Apparently, the *An. gambiae* brush border membrane has 63 and 65 kDa forms of GPI-anchored AgALP, and Cry11Ba binds some but possibly not all putative forms of AgALP in the brush border membrane. Cry11Ba also recognized a protein chain at ~53 kDa (Figure 3C, group 5 spots) and showed a faint signal for a 75 kDa protein (Figure 3C, spot 4).

The 57 kDa $AgALP1_t$ protein was used to establish the qualitative and quantitative features of Cry11Ba binding. The specificity of binding of Cry11Ba to the 57 kDa $AgALP1_t$ peptide was established using dot blot and microplate binding assays. Purified $AgALP1_t$ (Figure 4A) and AgCad1 CR11-MPED peptides were each spotted directly onto a PVDF filter and probed with [^{125}I]Cry11Ba alone or with excess unlabeled Cry11Ba and Cry4Ba. AgCad1 CR11-MPED peptide binds mosquitocidal Cry4Ba toxin (*8*). As shown in Figure 4B, [^{125}I]Cry11Ba bound $AgALP1_t$, but not AgCad1 CR11-MPED peptide, and unlabeled Cry11Ba toxin, but not Cry4Ba toxin, competed for binding, evidence of specific and competitive binding to $AgALP1_t$.

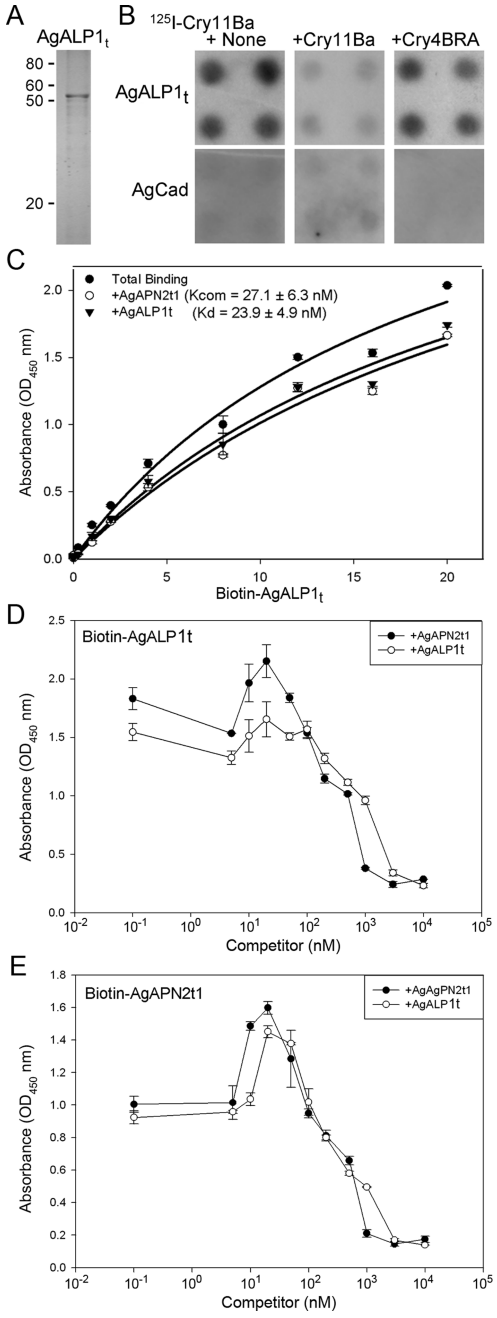

FIGURE 4: Analysis of the interaction between Cry11Ba and AgALP1 protein. (A) Truncated $AgALP1_t$ was expressed in *E. coli* and purified using a HiTrap Ni^{2+} chelating HP column. Partially purified recombinant ALP (1 μg) was resolved via SDS−PAGE and stained with Coomassie blue. (B) Purified $AgALP1_t$ and AgCad CR11-MPED peptides (1 μg/dot) were dotted on a PVDF filter directly and probed with 0.125 nM [^{125}I]Cry11Ba with or without 10 μM unlabeled Cry11Ba or Cry4Ba. The filters were exposed to X-ray film at −80 °C for autoradiography. (C) Binding affinity of Cry11Ba for $AgALP1_t$. Ninety-six-well microtiter plates coated with 0.5 μg of trypsinized Cry11Ba were incubated with increasing nanomolar concentrations of biotinylated $AgALP1_t$ peptide alone or with a 1000-fold molar excess of unlabeled $AgALP1_t$ peptide to determine specific binding or with a 1000-fold molar excess of $AgAPN2_{t1}$ to determine shared binding of Cry11Ba. (D and E) Competition microplate binding assays using microplates coated with Cry11Ba and 10 nM biotinylated $AgALP1_t$ or $AgAPN2_{t1}$ peptides as probes with increasing concentrations of nonlabeled $AgALP1_t$ or $AgAPN2_{t1}$ peptides.

Quantitative binding of $AgALP1_t$ to Cry11Ba was assessed using a microplate binding assay (*10*). Biotin-$AgALP1_t$ bound Cry11Ba toxin specifically and saturably and with high affinity (Figure 4C). Using a one-site saturation model, a K_d of 23.9 ± 4.9 nM was calculated for binding of $AgALP1_t$ to Cry11Ba. Since *E. coli*-expressed $AgAPN2_{t1}$ peptide binds Cry11Ba with high affinity (*10*), we tested $AgAPN2_{t1}$ for its ability to compete with binding of biotin-$AgALP1_t$ to Cry11Ba. AgAPN2t1 effectively competed with biotin-AgALPt1 binding with a K_{com} of 27.1 ± 6.3 nM (Figure 4C).

Homologous and heterologous competitions were further tested using 10 nM biotin-$AgALP1_t$ or $AgAPN2_{t1}$ and unlabeled peptides in the microplate binding assay. As seen in panels D and E of Figure 4, both peptides exhibited evidence of cooperative binding in the 10−100 nM concentration range.

We examined the ability of $AgALP1_t$ to compete off [^{125}I]Cry11Ba binding to *An. gambiae* brush border membranes. At the highest concentration tested, 10 μM unlabeled Cry11Ba competed off 41.8% of the [^{125}I]Cry11Ba binding to BBMVs, reducing the amount of bound toxin from 17.24 ± 0.06 to 9.8 ± 0.10 fmol (Figure 5A). Cry11Ba showed specific binding of 0.93 pmol/mg of BBMVs. To evaluate the capacity of the toxin to bind to AgALP1, we performed heterologous competition assays using 10 μM $AgALP1_t$ or AgCad1 CR11-MPED peptides as competitors. The addition of $AgALP1_t$ competed off 40.3% of the [^{125}I]Cry11Ba binding at 0.625 nM input toxin, whereas the AgCad1 CR11-MPED peptide competed off only 11.0% of the binding (Figure 5A). The amount of bound ^{125}I-labeled toxin was reduced from 17.24 ± 0.06 to 10.32 ± 0.12 fmol by 57 kDa AgALP1 and to 15.35 ± 0.28 fmol in the presence of AgCad1 CR11-MPED peptide. While $AgALP1_t$ displaced [^{125}I]Cry11Ba binding to a level similar to that of Cry11Ba, there was only a slight reduction in the level of binding by AgCad1 CR11-MPED peptide.

In the BBMV competition binding assay, Cry11Ba, $AgALP1_t$, and $AgAPN2_{t1}$ competed with binding of [^{125}I]Cry11Ba (0.5 nM input) to the brush border membrane preparation. $AgALP1_t$ and $AgAPN2_{t1}$ reduced the level of binding of [^{125}I]Cry11Ba to 35.5 and 23.5% of the maximal level, respectively. In contrast, the AgCad1 CR11-MPED peptide reduced the level of binding of [^{125}I]Cry11Ba to 71% of the maximal level.

Since 57 kDa $AgALP1_t$ binds Cry11Ba with high affinity and effectively competed for binding to BBMVs, we tested the ability of the $AgALP1_t$ peptide to neutralize Cry11Ba toxicity to larvae. We fed fourth instar *An. gambiae* larvae Cry11Ba alone or in combination with $AgALP1_t$ peptide inclusion bodies. Since Cry11Ba shows limited interaction with AgCad1 CR11-MPED peptide, we also fed larvae Cry11Ba with AgCad1 CR11-MPED inclusion bodies. Bioassays tested the effect of $AgALP1_t$ on Cry11Ba toxicity against the larvae. The toxicity of Cry11Ba was reduced from 78 ± 3.5 to 10 ± 1.8% when larvae were fed toxin with 57 kDa AgALP1 inclusion bodies, a statistically significant difference at $P < 0.0001$. There was no significant difference between Cry11Ba alone and Cry11Ba with AgCad1 CR11-MPED inclusion bodies (Figure 5B). The mortality of larvae fed $AgALP1_t$ alone was 4.4%, a mortality level comparable to that of untreated larvae.

DISCUSSION

The AgALP1 cDNA we cloned belongs to a family of insect ALPs that includes at least seven *An. gambiae* ALPs. AgALP1 shares 30−49% amino acid identity with six other putative AgALPs (Figure 6). A phylogenetic tree was constructed by

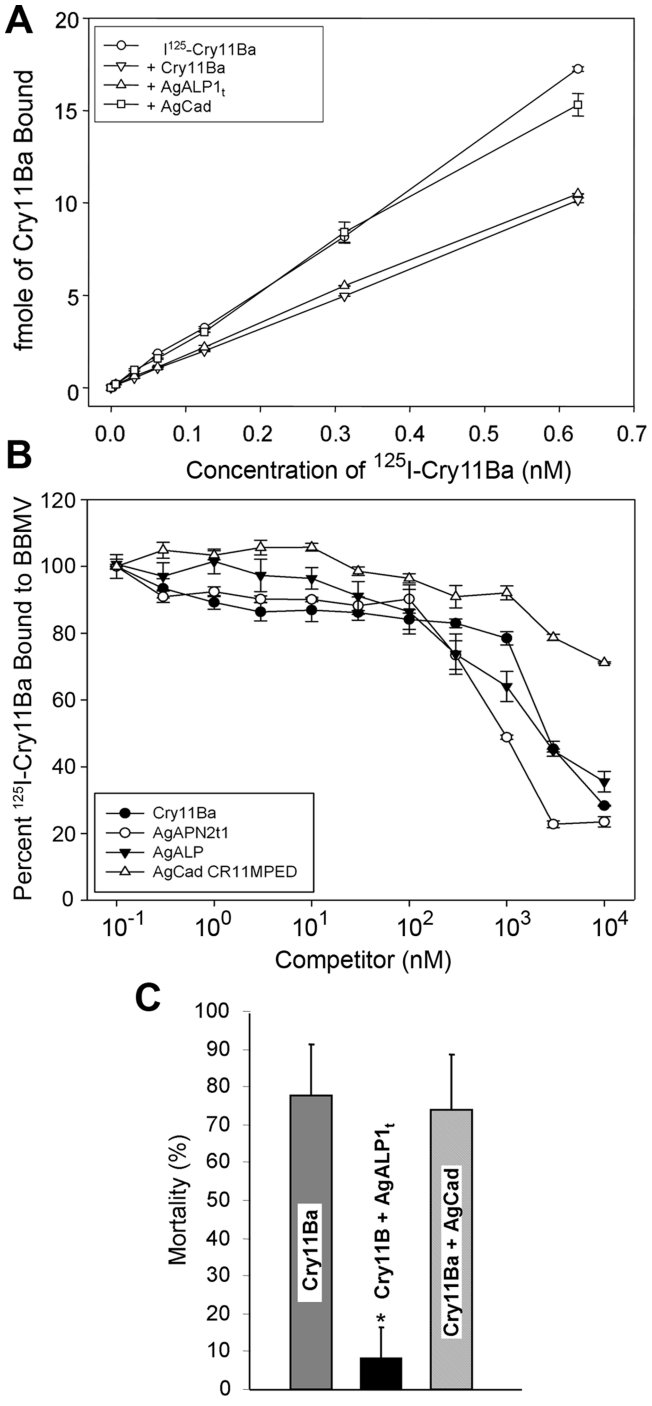

FIGURE 5: (A) Increasing amounts of [^{125}I]Cry11Ba were incubated with *An. gambiae* BBMVs (8 μg) with or without each competitor at 10 μM in binding buffer. (B) Cry11Ba competition binding assay. BBMVs were incubated with 0.5 nM [^{125}I]Cry11Ba alone or with increasing amounts of AgALP1$_t$ or AgAPN2$_{t1}$ to test the abilities of peptides to compete for Cry11Ba binding sites relative to nonlabeled toxin. Samples were tested in duplicate, and assays were repeated two or three times. (C) The truncated AgALP1$_t$ inclusion body was used for the toxicity inhibition assay. Cry11Ba toxin alone or a mixture of Cry11Ba with AgALP1$_t$ or AgCad CR11-MPED (1:100, w/w) was diluted in plastic plates containing deionized water and tested against 10 early fourth instar larvae of *An. gambiae*. Each treatment was conducted in at least triplicate, and the bioassays were conducted three times. Larval mortality was recorded after 48 h. Statistics was performed with one-way ANOVA using PROC GLM. The asterisk above the standard error bars indicates a very significant difference ($P < 0.0001$).

the maximum likelihood method to determine the relationship between AgALP1 and insect ALPs. The 25 ALPs analyzed are of

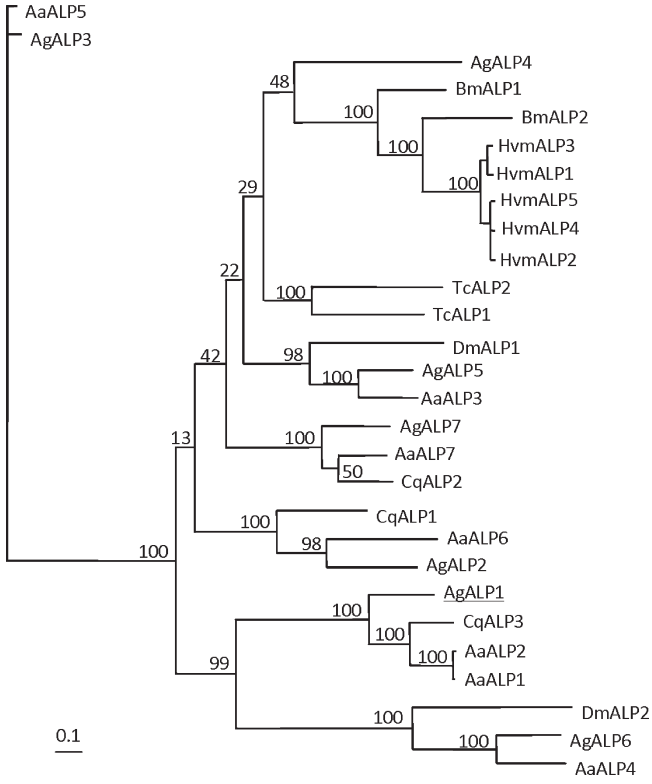

FIGURE 6: Phylogenetic tree derived from ClustalX alignment of insect ALPs. The putative signal peptides were removed for analysis. The tree was constructed by the maximum likelihood method. GenBank accession numbers: AaALP1 (XP_001663478), AaALP2 (XP_001649092), AaALP3 (XP_001648006), AaALP4 (XM_001657478), AaALP5 (XP_001650469), AaALP6 (XP_001663538), AaALP7 (XP_001663535), AgALP1 (XP_313890), AgALP2 (XP_001688180), AgALP3 (XP_316433), AgALP4 (XP_308522), AgALP5 (XP_321411), AgALP6 (XP_314561), AgALP7 (XP_309345), BmALP1 (NP_001036856), BmALP2 (NP_001037536), CqALP1 (XP_001842934), CqALP2 (XP_001842932), CqALP3 (XP_001848981), DmALP1 (NP_001034040), DmALP2 (NP_524601), HvmALP1, HvmALP2, HvmALP3, HvmALP4 (J. L. Jurat-Fuentes, personal communication), HvmALP5 (ABR88230), TcALP1 (XP_971418), and TcALP2 (XP_971358). Species name abbreviations are as follows: Aa, *A. aegypti*; Ag, *An. gambiae*; Bm, *B. mori*; Cq, *Culex quinquefasciatus*; Dm, *Drosophila melanogaster*; Hv, *H. virescens*; Tc, *Tribolium castaneum*. Bootstrap values represent the percentage frequency of which sequences resample in 100 replicates.

primarily dipteran origin, with two cloned BmALP sequences, five cloned HvmALP cDNAs (*32*), and two predicted *Tribolium castaneum* ALPs. According to the constructed phylogenetic tree (Figure 6), AgALP1 and -6 are the most closely related AgALPs. AgALP2, -3, and -7 are each in a cluster with *Aedes* ALPs identified as Cry4Ba binding proteins (AaALP5, AaALP6, and AaALP7). AgALP4 was assigned to a phylogenetic cluster with the five HvmALPs. These HvmALPs, which share more than 94% sequence identity with each other, are considered synonymous with the Cry1Ac-binding HvALP in *H. virescens* midgut (*32*). AgALP5 was assigned to a separate cluster with a *Aedes* ALP and a *Drosophila* ALP. AgALP3 and AaALP5 are the most divergent of the ALPs included in the phylogenetic analysis.

Alkaline phosphatase in BBMVs prepared from *An. gambiae* (Figure 2) and *A. aegypti* BBMVs has an apparent size of ~65 kDa on 1- and 2-DE gels (*22*, *23*). Separation of PIPLC-released *An. gambiae* BBMV proteins by 2-DE resolved forms of AgALP detected by the anti-AgALP1 antibody into chains of spots at ~65 kDa (Figure 3B). In *Aedes*, the anti-AgALP1

antibody detected spots at 64 kDa that were identified by mass spectrometry as the products of two ALP genes (*22*), suggesting the possibility that a similar chain of spots in *An. gambiae* BBMVs arises from different AgALP genes. Another non-exclusive possibility is that differential glycosylation of one or more AgALPs contributes to altered charge and mobility on 2-DE gels. In Figure 3C, Cry11Ba does not bind to all the spots that are detected by the anti-AgALP1 antibody, suggesting the possibility that not all the alkaline phosphatases in *An. gambiae* midgut BBMVs are involved in Cry11Ba toxin binding or intoxication.

Midgut ALPs are localized to the brush border in the dipterans *A. aegypti* (*23*) and *An. gambiae* (Figure 2) and the lepidopterans *M. sexta* and *H. virescens* (*30*, *32*). Our results differed from the reported ALP localization in *Aedes*, in that we detected AgALP in the anterior and posterior midgut brush border (Figure 2B), whereas ALP was localized only to the posterior midgut in *Aedes* (*23*). We also localized AgALP, or at least a cross-reactive protein, to the peritrophic matrix of larvae (Figure 2B). While the two mosquito genera may differ in ALP localization, it is quite possible that the difference is due to the probes used to detect ALPs. The polyclonal antibody used in this study detects multiple ALPs in mosquitoes, whereas the phage-displayed peptide used to detect ALP in *Aedes* midgut (*23*) probably recognizes a single epitope that might or might not be conserved across different ALP isoforms.

Cry11Ba bound multiple GPI-anchored AgALPs on blots of 2-DE gels (Figure 3B). Since Cry11Ba binds *E. coli*-expressed $AgALP1_t$ on dot blots and microtiter plates, the toxin must recognize a peptide sequence as a binding site(s) as glycosylation very rarely occurs in *E. coli* (*33*). This contrasts to binding of Cry1Ac to ALP in the heliothines *H. virescens* and *H. armigera*, where a GalNAc moiety is required for binding (*17*, *19*, *32*). Cry11Ba also binds to a peptide region of an aminopeptidase N ($AgAPN2_{t1}$) in *An. gambiae* larvae (*10*). The binding affinity of Cry11Ba for $AgALP1_t$ (K_d = 23.9 nM) is lower than the binding affinity of Cry11Ba for the $AgAPN2_{t1}$ peptide [K_d = 6.4 nM (data not shown and ref *10*)]. Both $AgALP1_t$ (Figure 5B) and $AgAPN2_{t1}$ inhibit binding of Cry11Ba to BBMVs from *An. gambiae*, evidence that the native forms of both proteins bind toxin to the brush border membrane.

An in vivo approach to correlating binding of toxin to the intoxication process is to feed larvae a mixture of Cry protein and binding peptide. Previously, $AgAPN2_{t1}$ peptide fed with Cry11-Ba to larvae was shown to inhibit 98% of Cry11Ba toxicity (*10*). In this study, $AgALP1_t$ fed with Cry11Ba inhibited 90% of the Cry11Ba toxicity to larvae. The ability of $AgALP1_t$ and $AgAPN2_{t1}$ to inhibit Cry11Ba binding to BBMVs and inhibit toxicity is evidence that both proteins are functional receptors and play a critical role in the intoxication process.

The binding of Cry11Ba to alkaline phosphatase (AgALP1) and aminopeptidase (AgALP2) presents an intriguing contrast to the binding of Cry1Ab to aminopeptidase MsAPN1 from *M. sexta*. While Cry11Ba binds $AgALP1_t$ and $AgAPN2_{t1}$ with affinities of 23 and 6 nM, Cry1Ab binds MsAPN1 with a significantly lower affinity of 100 nM (*34*). It is only after Cry1Ab binds cadherin and forms a prepore oligomer that MsAPN1 is bound with a high affinity (*4*), leading to membrane insertion. Whether Cry11Ba oligomerizes is unknown; however, Cry11Aa forms a prepore oligomeric structure after contact with Cyt toxin (*35*). Additional research is needed to define the interaction of Cry11Ba with GPI-anchored proteins such as AgALP1 and AgAPN2 in the context of the overall mechanism of Cry11Ba action and mosquito intoxication.

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