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

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AgCad2 cadherin in *Anopheles gambiae* larvae is a putative receptor of Cry11Ba toxin of *Bacillus thuringiensis* subsp. *jegathesan*

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ABSTRACT

In an effort to study the mode of action of Cry11Ba, we identified toxin binding proteins in *Anopheles gambiae* larval midgut and investigated their receptor roles. Previously, an aminopeptidase (AgAPN2) and an alkaline phosphatase (AgALP1) were identified as receptors for Cry11Ba toxin in *A. gambiae*. However, an *A. gambiae* cadherin (AgCad1) that bound Cry11Ba with low affinity (K_d = 766 nM) did not support a receptor role of AgCad1 for Cry11Ba. Here, we studied a second *A. gambiae* cadherin (AgCad2) that shares 14% identity to AgCad1. Immunohistochemical study showed that the protein is localized on *A. gambiae* larval midgut apical membranes. Its cDNA was cloned and the protein was analyzed as a transmembrane protein containing 14 cadherin repeats. An *Escherichia coli* expressed CR14MPED fragment of AgCad2 bound Cry11Ba with high affinity (K_d = 11.8 nM), blocked Cry11Ba binding to *A. gambiae* brush border vesicles and reduced Cry11Ba toxicity in bioassays. Its binding to Cry11Ba could be completely competed off by AgCad1, but only partially competed by AgALP1. The results are evidence that AgCad2 may function as a receptor for Cry11Ba in *A. gambiae* larvae.

1. Introduction

Bacillus thuringiensis (Bt) is widely used commercially as a nature-based bacterial insecticide. Bt is a soil inhabitant found all over the world and its insecticidal activity was discovered in the early 1900s. This biocontrol agent (Bt) has been used for decades due to its narrow toxin specificity against insect pests and its nontoxic property to human, animals and beneficial insects. To date, Bt bioinsecticides have been developed to control major agricultural insect pests such as moth/butterfly larvae and beetles, and to alleviate public health concerns such as from mosquitoes (Bravo et al., 2011; Federici et al., 2007; Lacey, 2007). As one of the most important public health problems in the world, great attention has been placed on developing strategies to prevent the occurrence of malaria. The *Anopheles gambiae* mosquito is a vector for *Plasmodium falciparum* which causes human malaria. Bti has been successfully used by researchers as an environmentally friendly agent for mosquito control.

Bt is a Gram-positive bacterium that is toxic to larval stages of certain insects through the action of its crystalline protein inclusions produced during sporulation. It is generally accepted that the crystalline proteins, known as Cry toxins, are released as protoxin and activated by gut proteases to be harmful to insect larvae. A model for Bt Cry1 toxin mode of action proposes that the active toxin first binds to cadherin protein as a primary receptor in the midgut microvilli; the binding allows cleavage of helix α-1 from domain I of the toxin that induces toxin oligomerization (Bravo et al., 2007). Some glycosylphosphatidyl inositol (GPI)-anchored proteins, such as aminopeptidase N (APN) or alkaline phosphatase (ALP) sorted to brush border membranes by GPI anchors, have been illustrated as the secondary receptors that contain higher binding affinity with the toxin oligomer, compared to monomeric toxin. The action of binding between oligomer and the secondary receptors leads to membrane penetration, followed by the formation of transmembrane pores or channels that cause osmotic lysis and death of larval midgut epithelial cells (Bravo et al., 2007, 2004).

Receptors for Cry toxins in the midgut of susceptible mosquito larvae include aminopeptidase, alkaline phosphatase and cadherin proteins (Bravo et al., 2011). The similarity in types of receptor

Abbreviations: alkaline phosphatase, ALP; aminopeptidase N, APN; *Bacillus thuringiensis*, Bt; bovine serum albumin, BSA; brush border membrane vesicles, BBMV; cadherin repeat, CR; cytoplasmic region, CYTO; differential interference contrast, DIC; membrane proximal extracellular domain, MPED; polymerase chain reaction, PCR; signal peptide, SP; transmembrane, TM.

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proteins between caterpillars and mosquitoes suggests a conserved mode of Cry toxin action. Cadherin AeCad was described as a Cry11Aa and Cry11Ba receptor in *Aedes aegypti* larvae (Chen et al., 2009; Likitvivatanavong et al., 2011). Cadherin AgCad1 was identified as a putative Cry4Ba receptor in *A. gambiae* larvae (Hua et al., 2008). Support for extending the oligomerization model of Cry action to mosquitocidal Cry toxins comes not only from the conservation of Cry receptor types, but also from recent data showing Cry11Aa oligomerization induced by *Aedes* cadherin (Rodríguez-Almazán et al., 2012).

To understand the interaction between Bt toxins and receptors, some Cry toxins have been mutated to study the toxin functional binding region to receptors. For example, a mutagenesis-based study of mosquitocidal Cry11Aa toxin shows that the loops in domain II are involved in receptor binding (Fernández et al., 2005). Mutagenesis of Bt Cry11Ba also shows that the loops in domain II are very important toxicity determinants to some mosquito species (Likitvivatanavong et al., 2009). Additionally, recent studies on Bt receptors showed that cadherin is not only important for toxin binding, but also cadherin domains could function as Bt toxin synergists (Chen et al., 2007). For example, AgCad1-CR11MPED enhances Bt Cry4Ba toxicity in *A. gambiae* larvae (Hua et al., 2008; Park et al., 2009) and AeCad CR regions enhance Cry4Ba and Cry11Aa toxicity to *A. aegypti* larvae (Rodríguez-Almazán et al., 2012). Research on the mode of action of Cry toxins provides the basis for developing Bt transgenic technology and improving insect resistance management.

The purpose of this work was to determine if a predicted *A. gambiae* cadherin-like protein, called AgCad2 in this study, is in the brush border membrane of larvae and examine the possibility that the protein has a function in the insecticidal activity of Cry11Ba. Our results support the role of AgCad2 as a receptor of Cry11Ba. Our results also support the hypothesis that different cadherins in *A. gambiae* have different functions in regulation of Bt toxicity *in vivo*.

2. Materials and methods

2.1. Insects

A. gambiae (UGAL strain) were maintained at 27 °C with a light–dark photoperiod of 16 h:8 h as described (Zhang et al., 2008). Fourth instar larvae were collected and used for RNA extraction and bioassays, or stored at −80 °C until needed for brush border membrane vesicle preparation.

2.2. Cloning of AgCad2

Total RNA was extracted from *A. gambiae* 4th instar larvae as described in a previous paper (Hua et al., 2008) and cDNA was synthesized from total RNA using reverse transcriptase (Gibco-BRL) and an oligo(dT)$_{17}$ primer. PCR was performed to amplify the AgCad2 transcript using cDNA as template. Two pairs of primers were designed according to the sequence of *A. gambiae* GenBank AJ439060 as follows: F1: 5′-CCG CAC TAG TAT GGA ACA GAA TCG CTC AAC CGA TA-3′ & R1: 5′-GTC CAC CGT CTC TCG GGC CGT GTT CGG TAC GCC GC-3′; and F2: 5′-GGT CAA CTG AGC TTC GAC ATT GTG GAC CCC TCG GG-3′ & R2: 5′-GCT ACC GCG GCA GTT CGG TCG TTT CCA AGT TCC GG-3′. PCR products from F1 & R1, or F2 & R2 are the overlapping 5′-end and 3′-ends of the AgCad2 cDNA, respectively. The PCR amplification conditions included 1 U Taq polymerase (Qiagen) with 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 4 min. The resulting two PCR products were cloned into pGEM-Teasy TA-cloning vector (Promega), named pGEM-AgCad2/5′end and pGEM-AgCad2/3′end. The DNA inserts were sequenced in both forward and reverse directions at the Molecular Genetics Instrumentation Facility at the University of Georgia. The sequencing data indicated that the obtained cDNA sequences were identical to the *A. gambiae* gene AJ439060 as a complete open reading frame. The 5′-fragment from pGEM-AgCad2/5′end plasmid with SpeI/SacII was subcloned to pIZT/V5-His vector (Invitrogen) to form pIZT-AgCad2/5′end, into which the SacII treated 3′-fragment from pGEM-AgCad2/3′end plasmid was inserted. The complete AdCad2 gene in pIZT-AgCad2 was confirmed by sequencing.

2.3. Bioinformatic analysis

Bioinformatic analysis using PROSITE on ISREC ProfileScan server (http://hits.isb-sib.ch/cgi-bin/PFSCAN) was performed to analyze the full cadherin sequence. PROSITE analysis uses protein patterns from well characterized proteins in the database to predict functions of similar regions in uncharacterized proteins. Multiple cadherin amino acid sequences were aligned using ClustalW at http://www.genome.jp/tools/clustalw/.

2.4. Expression of a truncated AgCad2 fragment and preparation of anti-AgCad2 serum

An AgCad2 fragment peptide near the C-terminus of the extracellular domain (CR14MPED; residues 1517–1729) was expressed in *Escherichia coli* and purified for injection into rabbits as follows. One pair of primers was designed as forward: 5′-TTC ACC ATGGGT ATC TCA ACG TCG TCG CTG TTC GG-3′ and reverse: 5′-CAT ACT CGA GTG ACG GAC AGC TCG TCC ATC TCT GC-3′. The PCR fragment was purified with a Qiaex II gel extraction kit (Qiagen) and cleaved with Nco I and Xho I restriction enzymes. The fragment was inserted into the protein expression vector pET-30a (Novagen) yielding pET-AgCad2-CR14MPED that was sequenced in both directions to confirm correct insertion and fidelity of the AgCad2 reading frame. Plasmid pET-AgCad2-CR14MPED was transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL. The AgCad2 peptide with a C-terminal 6-His-tag was over expressed in *E. coli* by culturing 1 l LB broth with induction with 1 mM isopropyl β-D-thiogalactopyranoside when the OD_{600} value reached 0.5–0.6. The culture was harvested by centrifugation 4 h after induction and the *E. coli* pellet was suspended in 80 ml buffer (50 mM Tris–HCl, 10 mM EDTA, 15% w/v sucrose, pH 8.0) with 20–40 mg lysozyme (Sigma). The suspension was shaken (150–200 rpm) at 37 °C for 30 min followed by centrifugation at 7800 × g for 10 min to collect *E. coli* inclusions. Inclusions were processed and washed as follows. The pellet with inclusions was suspended in 80 ml crystal wash buffer [0.1 M NaCl, 2% Triton X-100, 20 mM Bis–Tris (pH 6.5), 2% sodium deoxycholate] and then sonicated on ice for two 3 min intervals. The sonicated suspension was resuspended in 80 ml crystal wash buffer and shaken vigorously for 30 s before centrifugation at 7800 × g for 10 min. The pellet was suspended in crystal wash buffer (without deoxycholate) and centrifuged as above. The pellet was washed in deionized H_2O, centrifuged, and the pellet was suspended in 10 ml deionized H_20. An aliquot was removed and dissolved in 6 M guanidine-HCl, 20 mM Na_2HPO_4, 0.5 M NaCl pH 7.6 for quantitation by Bio-Rad protein assay and analysis by SDS-PAGE. Inclusion bodies were stored on ice at 5 °C until used for bioassay or peptide purification. AgCad2 peptide was purified from inclusion bodies according to “protocol 7” in QIAexpressionist (2nd Edition, summer 1992, QIAGEN) with minor modifications. The soluble extract in 8 M Urea, 20 mM Na_2HPO_4, 0.5 M NaCl pH 7.6 was loaded on a nickel-chelating Sepharose column (GE Healthcare) and eluted with imidazole. The AgCad2 peptide was dialyzed against 20 mM sodium carbonate pH 9.6, and the peptide purity was confirmed by separation on SDS-15% PAGE. Polyclonal anti-

AgCad2 serum was produced in New Zealand White rabbits at the Animal Resources Facility at the University of Georgia using purified CR14MPED peptide as immunogen. AgCad1-CR11MPED (Hua et al., 2008) was produced by the same method as AgCad2-CR14MPED.

2.5. Preparation of brush border membrane vesicles (BBMV) from A. gambiae larvae

BBMV were prepared from whole 4th instar larvae according to Zhang et al. (2008) and protein quantified by the BioRad protein assay with BSA as standard. Aminopeptidase N activity (Garczynski and Adang, 1995), a marker for brush border membranes, was enriched about 6-fold for the final BBMV preparation compared to the initial crude larval homogenate (data not shown).

2.6. Immuno, toxin blots and immunohistochemistry

A. gambiae BBMV proteins were separated by SDS-PAGE and proteins electroblotted to PVDF filters. Filters were blocked with 3% bovine serum albumin (BSA) in PBST (PBS + 0.1% Tween 20) for 1 h at room temperature, and then probed with anti-AgCad2 serum (1:500 dilution) in PBST containing 0.1% BSA for 2 h. After washing, the filters were incubated with horse radish peroxidase-conjugated anti-rabbit IgG (1:25,000 dilution, Pierce) in the same buffer for 1 h at room temperature. Finally, the filters were developed with the ECL kit (GE Healthcare) and exposed to X-ray film. AgCad1 and AgCad2 were expressed in *Drosophila* S2 cells transfected with pIZT-AgCad1 and pIZT-AgCad2 as described (Hua et al., 2008) and cadherin was detected on blots probed with anti-AgCad2 or anti-AgCad1 serum (Hua et al., 2008). To investigate AgCad2 localization on the larval mosquito gut with reference to AgCad1 and AgALP1, their locations were analyzed with immunohistochemistry. All the procedures for immunohistochemistry were as previously described (Linser et al., 2012). For histological sections of mosquito larvae, early 4th instar larvae were injected with primary fixative (4% *p*-formaldehyde in 0.1 M Na Cacodylate buffer pH 7.4) and then immersed in the fixative solution for 12 h to several days at 4 °C. Then the larvae were moved to fresh, ice-cold Carnoy's and then incubated in the secondary fixative for 90 min on ice. Fixed larvae were taken through alcohol and methyl salicylate transition solvents and embedded in paraffin as described (Smith et al., 2007). Longitudinal histological sections were cut at 6–12 microns and mounted on SuperFrost charged slides (Thermo/Fisher USA). After rehydration, slides with sections were placed in 0.1 M Na-citrate pH 6.0 and heated in an autoclave to sterilization temperature for 15 min as an "antigen recovery" technique. Then slides with sections were transferred to TBS followed by PreInc (2% normal goat serum, 1% bovine serum albumin, 0.1% Triton-X100 in TBS). Antibodies were diluted in PreInc and applied to the sections mounted on slides overnight at 4 °C. Slides were subsequently washed extensively in TBS followed by incubation with diluted secondary antibodies of appropriate specificity and with fluorescent tags (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at 37 °C. Sections on slides were washed with TBS extensively and then mounted in 60% glycerol/TBS with *p*-phenylenediamine (0.001%) to inhibit fluorescence quench and viewed with a Leica SP5 LSCM confocal microscope. As a basal membrane reference, mouse monoclonal antibody to the alpha subunit of NaK-ATPase was used for double-labeling (The Developmental Studies Hybridoma Bank, University of Iowa). Digital images were assembled into figures using CorelDraw12 software. Shown in Fig. 2 are images of the posterior midgut.

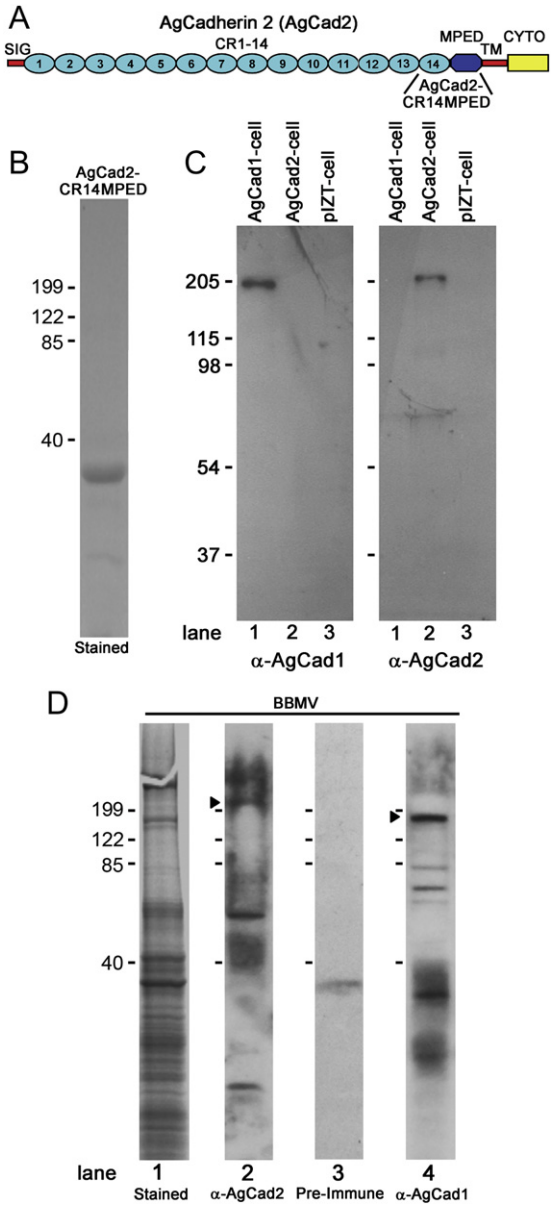

Fig. 1. AgCad2 structure showing CR14MPED region produced in *E. coli* for antiserum production and experimentation (A). Panel B shows a Coomassie Blue stained SDS-gel strip with purified 25-kDa AgCad2-CR14MPED peptide (5 μg). Rabbit antiserum was prepared against CR14MPED peptide. Panel C shows expression of AgCad2 or AgCad1 in Dm-S2 cells transfected with pIZT-AgCad1, pIZT-AgCad2 or pIZT vector. Cell proteins were separated by SDS-PAGE, blotted to PVDF membrane, blocked and probed with anti-AgCad1 or anti-AgCad2 serum. Panel D shows the expression of AgCad2 in BBMV. *A. gambiae* BBMV proteins (20 μg per lane) were separated by SDS-PAGE and the gel strip for lane 1 was stained with Coomassie Blue while the gel region for lanes 2–4 was blotted to PVDF membrane. After blocking, filters were probed with anti-AgCad2 serum (lane 2), preimmune-serum (lane 3) or anti-AgCad1 serum (lane 4). Anti-AgCad2 serum detected the expected size band at 208-kDa (lane 2, marked with a triangle) plus several additional bands. No 208 kDa band was detected with pre-immune serum (lane 3). Lane 4 shows the smaller-sized AgCad1 protein (marked with a triangle) that was detected with anti-Cad1 serum.

2.7. Purification of Cry11Ba protoxin and toxin

The Bt strain 407, harboring plasmid pJEG80.1 encoding Cry11Ba (Delécluse et al., 1995), was kindly provided by Dr. D.H. Dean (Ohio State University). The strain was grown at 30 °C in sporulation medium (Schaeffer et al., 1965) with erythromycin as described previously (Zhang et al., 2008). When sporulation was complete, spores and crystals were harvested by centrifugation and washed

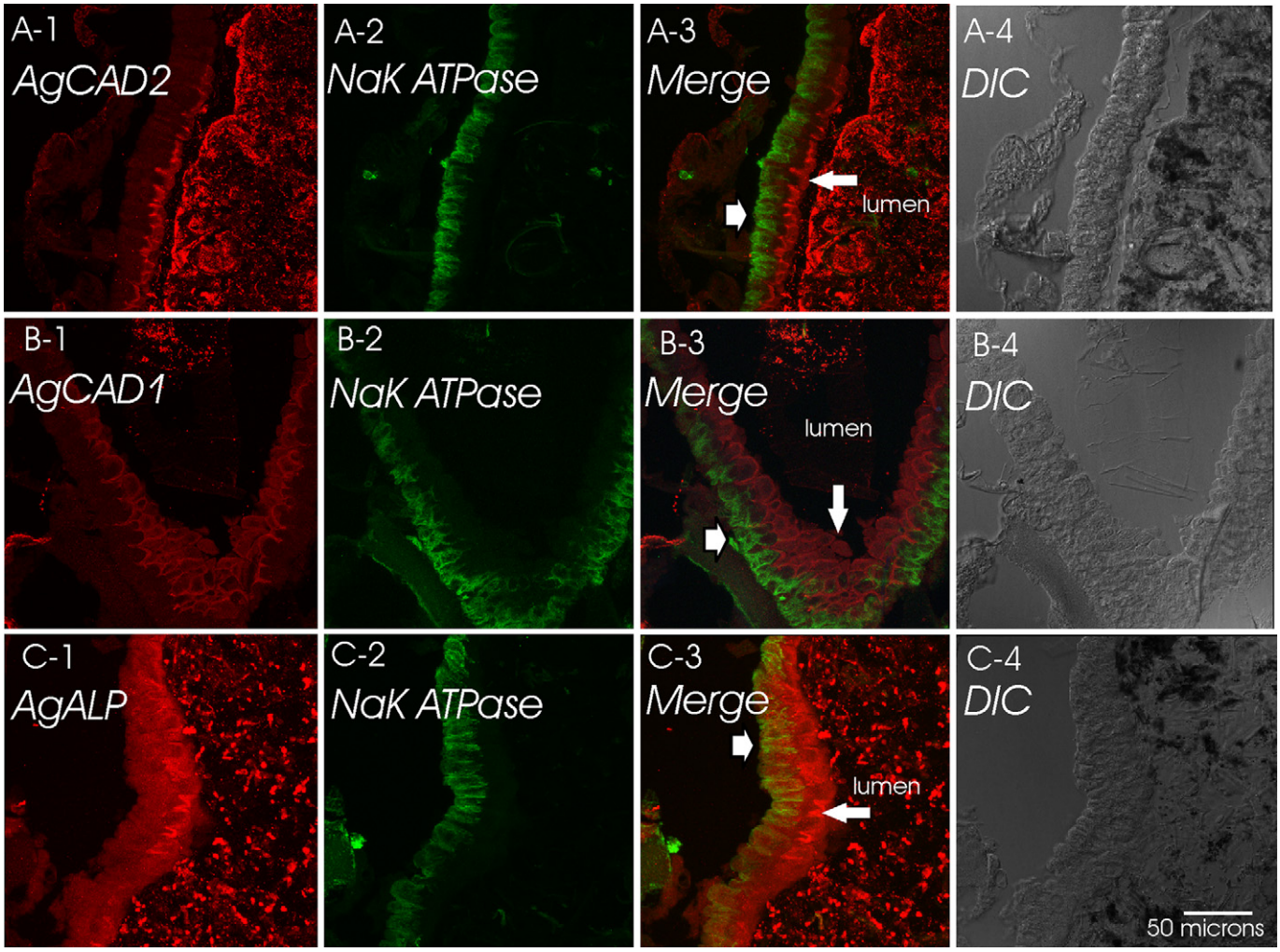

Fig. 2. Localization of expression of BBM/apical AgCAD2 (A-1, red), AgCAD1 (B-1, red) and AgALP (C-1, red) in the posterior midgut of 4th instar *A. gambiae* larvae. Contrasted to the apical Bti-binding proteins adjacent to the gut lumen is the basal membrane marker NaK-ATPase (A-2, B-2 and C-2, green). Merge of the two marker patterns is shown in A-3, B-3 and C-3. Longer arrows indicate location of the BBM/apical membrane labeling with the Bti-binding protein antibodies and the shorter arrow indicates the basal membrane labeling with NaK-ATPase. A-4, B-4 and C-4 show DIC images of the sections. The magnification bar indicates 50 microns.

according to Zhang et al. (2008). Crystals were purified from spores by centrifugation through a 30–60% (w/v) NaBr step gradient at 47,000 × g for 2 h. Purified crystals were washed twice with deionized H_2O and then dissolved in 25 ml 100 mM 3-(cyclohexylamino) propanesulfonic acid (CAPS), pH 10.6, supplemented with 0.05% β-mercaptoethanol to release protoxin. The protoxin solution was clarified by centrifugation at 10,000 × g for 10 min and filtration through a 0.45 μM BA85 membrane filter (Whatman). The solution was dialyzed overnight at 5 °C against 20 mM sodium carbonate pH 9.6. Protoxin was used directly in bioassays or activated into toxin using TPCK-treated bovine pancreatic trypsin (Sigma) at a mass ratio of 1:10 (trypsin:protoxin) for 2 h at 37 °C. Toxin was purified by fast protein liquid chromatography (Akta Explorer; GE Healthcare) using a Bio-Scale mini High Q cartridge column (Bio-Rad) with a linear gradient of 0–0.5 M NaCl in 100 mM CAPS (10.6). Toxin was stored in aliquots at −80 °C for use in binding assays.

2.8. Microtiter plate binding assays

The preparation of biotinylated AgCad peptides was as described previously (Zhang et al., 2008). Microtiter plates (high binding 96-well, Immulon® 2HB, Thermo Fisher Scientific Inc., Waltham, MA) were coated overnight at 4 °C with 1.0 μg Cry11Ba toxin/well in 100 μl coating buffer (100 mM Na_2CO_3, pH 9.6) and blocked as described previously (Zhang et al., 2008). In the saturation binding assay, biotin-AgCad2 or biotin-AgCad1 peptides were diluted to the desired concentrations (0.5 nM–100 nM) in 100 μl coating buffer with or without 1000-fold molar excess unlabeled peptides. Data from saturation binding assays were analyzed using SigmaPlot software (version 11; Systat Software, Inc., San Jose, CA) and 'best fit' for the curves was to a one-site saturation binding equation. For competition binding assays, plates were coated with Cry11Ba and blocked as described above and then incubated with 10 nM biotinylated AgCad2-CR14MPED, or AgCad1-CR11MPED in the presence of increasing nM concentrations of unlabeled homologous or heterologous peptide. $AgALP1_t$ was produced as purified as previously described (Hua et al., 2009).

2.9. Brush border membrane vesicles (BBMVs) binding assay

Trypsinized Cry11Ba toxin (10 μg) was labeled with 0.5 mCi of $Na^{125}I$ (PerkinElmer) using the chloramine-T method (Garczynski et al., 1991) and the specific activity obtained was 16.6 μCi/μg of input toxin. BBMV binding assays were performed according to Garczynski et al. (1991). For homologous and heterologous

competition assays, duplicate samples of ^{125}I-Cry11Ba toxin (10 nM) were mixed with increasing amounts of unlabeled Cry11Ba, AgCad2-CR14MPED or AgCad1-CR11MPED peptides and the mixture added to 8 μg of *A. gambiae* BBMV in binding buffer (20 mM Na_2CO_3, 1.5% BSA, 0.15 M NaCl, 0.1% Tween-20, pH 9.6). Binding reactions were placed at 8 °C for 18 h with agitation. Binding reactions were stopped by centrifugation and each pellet was washed once with 1 ml of ice-cold binding buffer. Radioactivity of the final pellets was measured with a Beckman model Gamma 4000 detector. Each binding assay was repeated. Data from independent experiments conducted in duplicate were combined and then analyzed and plotted with SigmaPlot version 11.0 using 'multiple line and scatter error bars.'

2.10. Bioassay

Early 4th instar *A. gambiae* (CDC G3 strain) larvae were used for bioassays. Cry11Ba protoxin was prepared as described above. Production of Cry4Ba mutant Cry4BRA in *E. coli* and extraction of soluble protoxin from washed crystals was as described previously (Abdullah et al., 2003). Cleaned inclusions of AgCad2-CR14MPED, AgCad1-CR11MPED and MsCad-CR12MPED (Chen et al., 2007) peptides were mixed with low or high concentrations of soluble Cry11Ba or Cry4Ba in a mass ratio of 1:100 (Cry:peptide) in deionized water and transferred to a six-well Costar culture plate (Corning). The low and high concentrations of soluble Cry11Ba or Cry4Ba were selected to cause about 20% and 80% larval mortality, respectively. Ten larvae were added to each well in a total 2 ml volume and the plates incubated at 27 °C. Bioassays were also performed with the following controls: toxin alone, peptide alone, and buffer alone. Bioassays were performed three times for each treatment, and mortality was scored after 24 h. Data were analyzed and plotted with SigmaPlot version 11.0 using 'multiple line and scatter error bars.'

3. Results

3.1. Cloning and analysis of A. gambiae AgCad2

A. gambiae AgCad2 cadherin (GenBank AJ439060) was retrieved by BLAST search with the Cry receptor Bt-R_{1a} (*Manduca sexta* cadherin). We cloned AgCad2 from larval midgut cDNA, and the sequence, which is identical to GenBank AJ439060 and 97% identical at the amino acid level to AGAP001591 in Vectorbase, encodes a 208-kDa protein composed of 1881 amino acids (Fig. S1). Computational analyses of the amino acid sequence by the program PROSITE on ISREC ProfileScan server (http://hits.isb-sib.ch/cgi-bin/PFSCAN) predicts a structure typical of a cadherin. The full-length AgCad2 molecule (Fig. 1A; Fig. S1) includes two hydrophobic regions: one is located at the N-terminus as a signal peptide (SIG) and the other is close to the C-terminus as a transmembrane domain (TM). The extracellular domain consists of 14 cadherin repeats (CR) and a membrane-proximal extracellular domain (MPED) that links the last CR to the TM. As predicted by PROSITE analysis, the AgCad2 protein contains seven putative calcium-binding sequences distributed throughout the extracellular domain. Integrin recognition sequences, LDV (Komoriya et al., 1991; Tselepis et al., 1997; Wayner et al., 1989) and RGD (Pierschbacher and Ruoslahti, 1984) are located within CR5 and CR8, respectively. Overall, the mosquito AgCad2 protein is structurally similar to other members of the insect cadherin family (Bel and Escriche, 2006).

AgCad2-CR14MPED peptide with a C-terminal 6-His tag was expressed in *E. coli* where it was deposited in inclusion bodies. Fig. 1B shows a lane from a stained SDS-gel with purified CR14MPED migrating at the expected 25-kDa size. Rabbit antiserum was prepared against purified CR14MPED peptide. The specificity of anti-AgCad2 serum for AgCad2 and not AgCad1 was established by transfecting S2 cells with pIZT, pIZT-AgCad1 or pIZT-AgCad2 and probing blots of S2 cells with either anti-AgCad1 or anti-AgCad2 serum. A 200-kDa AgCad1 was expressed in pIZT-AgCad1 transfected cells and a 208-kDa AgCad2 was expressed in pIZT-AgCad2 cells as detected by the respective antisera (Fig. 1C). Antiserum was then used to detect AgCad2 in BBMV prepared from larvae and localize AgCad2 on sectioned larval guts.

3.2. Localization of A. gambiae AgCad2 on gut tissue

Brush border cadherins in mosquito larvae are present in BBMV prepared from whole larvae (Hua et al., 2008) and dissected guts (Chen et al., 2009). BBMV from whole 4th instar larvae of *A. gambiae* proteins were separated by SDS-PAGE and blotted onto a membrane filter and lanes of BBMV protein cut into strips. The filter strips were probed with anti-AgCad2, pre-immune or anti-AgCad1 serum. Fig. 1D (lane 2) shows the bands detected by anti-AgCad2 serum, including the expected 208-kDa band. Pre-immune-serum had no reaction with the 208 kDa band, but did show cross-reactivity to a band of about 35-kDa. In comparison anti-AgCad1 serum detected a slightly smaller-sized protein at about 195-kDa (Fig. 1D, lane 4), a result consistent with the smaller-sized AgCad1 protein. These results are evidence that AgCad2 protein is present in BBMV and that anti-AgCad2 serum detects AgCad2, but not AgCad1 protein in *A. gambiae* larvae.

To further characterize the expression of AgCad2 in the larval alimentary canal of *A. gambiae*, we employed immunohistochemistry and confocal microscopy of sectioned tissues similar to previous studies. Fig. 2 shows analyses of larval gut labeled for AgCad2 localization in comparison to that of AgCad1 and of AgALP1. Evidence from analysis of BBMV via immunoblotting predicts that all three of these putative Bt Cry11Ba receptors should be detectable on the apical microvilli (BBM) of gut cells. To contrast with this hypothetical apical labeling, we also contrasted the staining with the established basal membrane marker for the posterior midgut and the gastric caeca, NaK-ATPase (Linser et al., 2012). Fig. 2 shows that all three of the putative Bt receptors are detectable on the BBM of the posterior midgut which is a major source of the material purified in the BBMV preparations (Zhang et al., 2008). The apical BBM localization is confirmed by contrasting with the basal membrane maker NaK-ATPase. Other regions of the alimentary canal show differential patterns of expression of these markers (data not shown). Preimmune serum did not label BBM (Fig. S2). The analyses of the posterior midgut clearly show that AgCad2 is a constituent of the BBM of these epithelial cells and hence positioned as a possible target for Bt action. As observed before, AgALP1 was sorting on the same region of both *A. gambiae* cadherin proteins.

3.3. Binding affinity of AgCad2 to Cry11Ba

The binding affinity of AgCad2 to Cry11Ba was determined with a microplate binding assay using a saturation format as described before (Zhang et al., 2008). The biotin-labeled AgCad2-CR14MPED specifically and saturably bound to Cry11Ba toxin as shown in Fig. 3A. Using a one-site saturation binding model, a K_d of 11.8 ± 1.9 nM was calculated for AgCad2-CR14MPED binding to Cry11Ba (Fig. 3A). However, a much lower binding affinity was observed for biotin-labeled AgCad1-CR11MPED binding to Cry11Ba (K_d = 766 ± 21 nM). Although most of the AgCad1-CR11MPED binding to Cry11Ba was specific, the cadherin peptide did not reach saturation binding to Cry11Ba at the highest concentration of input peptide (200 nM) (Fig. 3B). To determine if these two cadherin

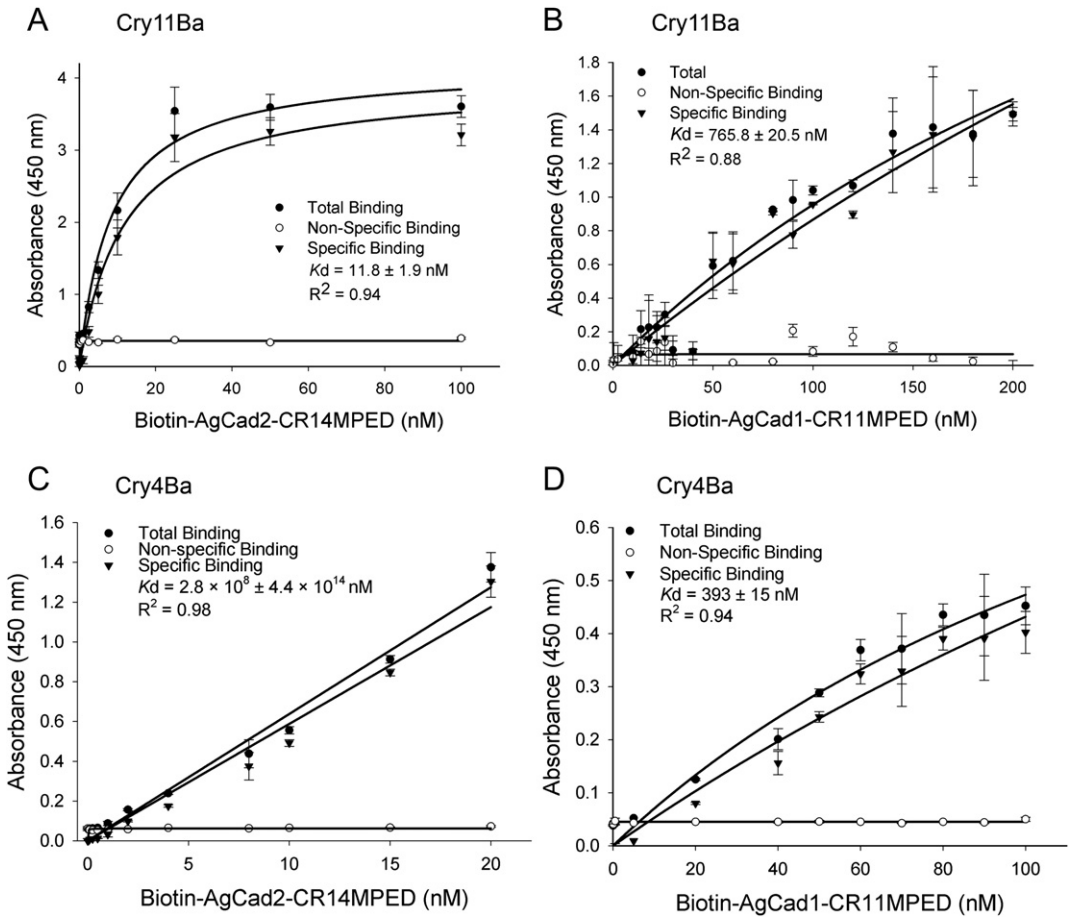

Fig. 3. Binding affinities of Cry11Ba or Cry4Ba for AgCad2-CR14MPED or AgCad1-CR11MPED peptide. Microtiter plates coated with 0.5 μg of trypsinized Cry11Ba (A and B) or Cry4Ba (C and D) per well were incubated with increasing concentrations of biotin-AgCad2-CR14MPED (A and C) or biotin-AgCad1-CR11MPED (B and D) alone, or with 1000-fold molar excess of unlabeled homologous peptide to determine specific binding. Experiments were replicated three times. Vertical lines indicate SEMs.

peptides could also bind to Cry4Ba, the saturation binding assay was performed with biotinlyated cadherin peptides (Fig. 3C & D). The results clearly showed that while AgCad2-CR14-MPED did bind Cry4Ba, the calculated binding affinity was very low ($K_d = 280 \pm 4.4 \times 10^8$ nM). In contrast, AgCad1-CR11MPED bound to Cry4Ba with a $K_d = 393 \pm 15$ nM affinity.

Competition between AgCad2-CR14MPED and AgCad1-CR11MPED peptides for binding sites on Cry11Ba was tested using biotin-labeled and non-labeled cadherin peptides in the microplate binding assay. Since AgALP1 is a receptor of Cry11Ba (Hua et al., 2009), AgALP1 was included as a competitor to determine if AgCad2 shared binding sites with AgALP1 on Cry11Ba. It was interesting to note that AgCad1 peptide showed the same binding pattern as AgCad2 (Fig. 4A & B) and that both cadherin fragments could compete with each other for binding to Cry11Ba. In contrast the results showed that AgALP only partially displaced binding of AgCad2 or AgCad1 to Cry11Ba.

The ability of AgCad2 to inhibit ^{125}I-Cry11Ba binding to *A. gambiae* brush border membrane vesicles was measured. It was interesting that the AgCad2-CR14MPED peptide more efficiently reduced the binding of ^{125}I-Cry11Ba to BBMV at low concentrations than unlabeled Cry11Ba, although Cry11Ba competed more than AgCad2-CR14MPED at the highest concentration in this experiment (Fig. 5A). The highest concentration of unlabeled Cry11Ba (10 μM) competed 65% of the ^{125}I-Cry11Ba binding to BBMV (Fig. 5A). The addition of AgCad2-CR14MPED at 10 μM concentration competed 56% of the ^{125}I-Cry11Ba binding (10 nM input), whereas AgCad1-CR11MPED only competed 31% of the binding (Fig. 5A). Overall, AgCad2-CR14MPED displaced ^{125}I-Cry11Ba binding to a level similar to Cry11Ba, but the binding was only slightly reduced by AgCad1-CR11MPED peptide.

3.4. Bioassay of AgCad2-CR14MPED with Cry11Ba to A. gambiae larvae

Because of the high binding affinity of AgCad2-CR14MPED to Cry11Ba and the competitive effect to the toxin against BBMV, bioassays were performed to determine whether AgCad2-CR14MPED peptide could neutralize Cry11Ba toxicity to *A. gambiae* larvae. We fed 4th instar *A. gambiae* larvae with Cry11Ba at high and low concentrations (4 μg/ml and 0.5 μg/ml) alone or with AgCad2-CR14MPED peptide inclusion bodies. The toxicity of Cry11Ba was reduced from 78 ± 3.5% to 41.9 ± 1.8% when larvae were fed with toxin supplemented with AgCad2-CR14MPED inclusion body in high dosage of Cry11Ba; a statistically significant difference at $P < 0.005$ (Fig. 5B). There was no significant difference between Cry11Ba only and Cry11Ba plus AgCad1-CR11MPED or MsCad-CR12MPED inclusion body (Fig. 5B). The toxicity of Cry11Ba at low dosage to the larvae was also reduced when AgCad2-CR14MPED was added, but no differences were observed with AgCad1-CR11MPED or MsCad-CR12-MPED. Since AgCad1-CR11MPED synergizes Cry4Ba toxicity to mosquito larvae (Hua et al., 2008; Park et al., 2009), we tested the two AgCad cadherin fragments with Cry4Ba at high and low concentrations of Cry4Ba in

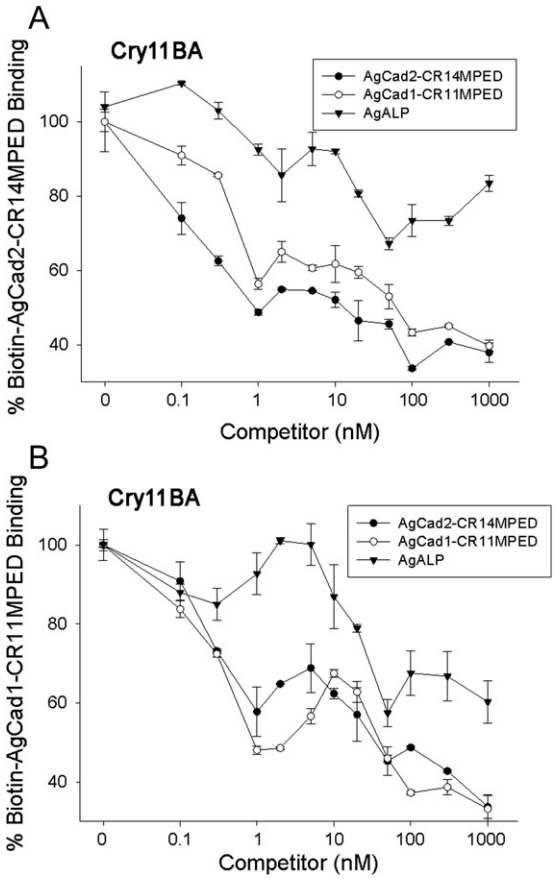

Fig. 4. Competition assays to reveal binding sites on Cry11Ba shared between AgCad2-CR14MPED and AgCad1-CR11MPED cadherin peptides and the Cry11Ba receptor, AgALP1. Microplates coated with 0.5 μg of trypsinized Cry11Ba were incubated with 10 nM biotin-AgCad2-CR14MPED (A) or biotin-AgCad1-CR11MPED (B) peptide in the presence of increasing molar concentrations of unlabeled peptides to determine the extent of binding in the presence of homologous or heterologous cadherin peptides or $AgALP1_t$ protein. $AgALP1_t$ peptide was produced and purified as previously described (Hua et al., 2009). Experiments were replicated three times. Vertical lines indicate SEMs.

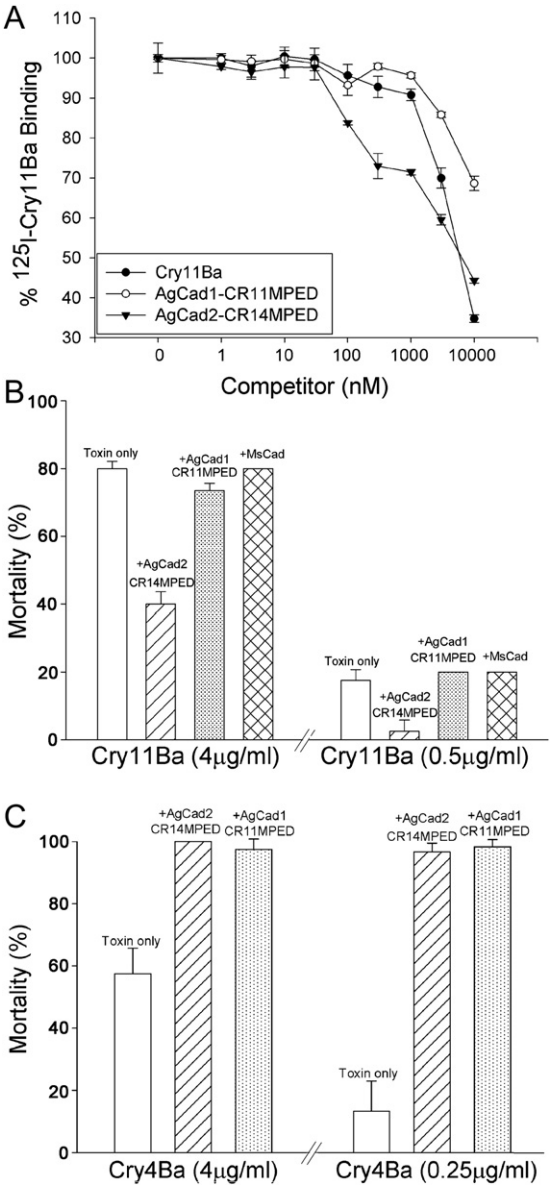

Fig. 5. (A) Competition assays for ^{125}I-Cry11Ba binding to *A. gambiae* BBMVs. Increasing amounts of unlabeled Cry11Ba, AgCad2-CR14MPED or AgCad1-CR11MPED peptides were incubated with a mixture of ^{125}I-Cry11Ba (10 nM) and BBMVs (8 μg). Each data point is a mean value based on the results from two independent experiments using duplicate samples. Standard errors among samples are shown by error bars. (B) Toxicity inhibition bioassays. Soluble Cry11Ba at high (4 μg/ml) or low (0.5 μg/ml) concentration alone or with AgCad2-CR14MPED or AgCad1-CR11MPED or MsCad-CR12MPED inclusions in a toxin:peptide molar ratio of 1:100 were tested against ten early fourth instar larvae of *A. gambiae*. (C) Toxicity synergistic bioassays. Soluble Cry4Ba at high (4 μg/ml) or low (0.25 μg/ml) concentration alone or with AgCad2-CR14MPED or AgCad1-CR11MPED inclusions in a toxin:peptide molar ratio of 1:100 were tested against ten early fourth instar larvae of *A. gambiae*. In both (B) and (C), each treatment was in triplicate, and the bioassays were conducted three times. Larval mortality was recorded after 24 h. Mean percent mortality (±SE) of larval mosquitoes treated with Cry11Ba or Cry4Ba when AgCad2-CR14MPED or AgCad1-CR11MPED or MsCad-CR12MPED peptide inclusion bodies were absent or present.

larval bioassays. Although AgCad1 could not significantly inhibit Cry11Ba toxicity, both cadherin fragments showed synergist function on Cry4Ba toxicity to *A. gambiae* larvae at both high and low dosage of the toxin (Fig. 5C).

4. Discussion

Cadherin-like proteins are receptors of Bt Cry toxins in different insect species. In previous work (Hua et al., 2008), BLAST search of *Anopheles* databases with the Cry1 receptor cadherin Bt-R_{1a} from *M. sexta* yielded AgCad1 (AGAP002828) and AgCad2 (AJ439060) as the two top matches. Computational analysis predicts AgCad1 and AgCad2 proteins to have 11 and 14 CR repeats, respectively, and only 14% amino acid identity. The mRNAs of both cadherins were detected in larval midgut supporting their evaluation as Cry receptors (Hua et al., 2008). AgCad1 protein was localized in the BBM of posterior midgut in larvae and peptide fragment of AgCad1 bound Cry4Ba with specificity (Hua et al., 2008) and high affinity (CR9-11 binding with a K_d = 13 nM and CR11MPED binding with a K_d = 23 nM) (Park et al., 2009). The low affinity of Cry4Ba binding to AgCad1-CR11MPED (K_d = 393 nM) measured in this study not only differs from our previous study (Park et al., 2009) but also calls into question the role of AgCad1 in Cry4Ba action. The recent finding that AeCad cadherin in *Aedes* is not required for Cry4Ba toxicity (Rodríguez-Almazán et al., 2012) further raises the question as to whether cadherins are necessary for Cry4Ba toxicity to mosquito larvae.

Cry11Ba is a unique toxin due to its high toxicity to *Aedes*, *Anopheles* and *Culex* larvae (Delecluse et al., 1995). Interactions between AgCad1 and Cry11Ba were analyzed in this study using the CR11MPED region which is located proximal to the BBM. Cry11Ba

bound CR11MPED with specificity, but the affinity of toxin binding to peptide was low (K_d = 766 nm, Fig. 3B). Additionally, AgCad1-CR11MPED was ineffective as a competitor of ^{125}I-Cry11Ba binding to BBMV and Cry11Ba toxicity to larvae (Fig. 5). Our results do not support a Cry11Ba receptor role for AgCad1 in *A. gambiae* larvae.

AgCad2 was examined as a possible receptor of Cry11Ba in larvae. AgCad2 was detected in BBMV (Fig. 1) and localized to the posterior midgut in BBM of sectioned larval midgut (Fig. 2). AgCad2-CR14MPED fragment bound Cry11Ba with high affinity (K_d = 11.8 nM; Fig. 3A) displaced ^{125}I-Cry11Ba binding to BBMV and when CR14MPED was fed with Cry11Ba to larvae, mortality was reduced by 40% (Fig. 5). The inhibitory effect of AgCad2-CR14MPED on Cry11Ba toxicity to *Anopheles* larvae is in contrast with the synergistic effect observed with the combination of CR14MPED and Cry4Ba (Fig. 5). Cry11Aa was also more toxic with the addition of AeCad toxin-binding peptides (Rodríguez-Almazán et al., 2012). The inhibitory effect of AgCad2-CR14MPED on Cry11Ba is similar to that of toxin-binding fragments of AgAPN2 and AgALP1 (Hua et al., 2009; Zhang et al., 2008). Fragments of those receptor proteins competed Cry11Ba binding to BBMV and were more inhibitory to Cry11Ba toxicity than AgCad2-CR14MPED when fed with toxin to larvae. AgAPN2 and AgALP1 peptides fed with Cry11Ba to larvae reduced toxicity 98% and 90%, respectively (Hua et al., 2009; Zhang et al., 2008). We propose that AgCad2, like AgAPN2 and AgALP1, is a key regulator and a receptor for Cry11Ba binding and toxicity to *A. gambiae* larvae.

A single cadherin AeCad in *Aedes* larvae has been identified and receptor function established for Cry11Aa and Cry11Ba toxins (Chen et al., 2009; Likitvivatanavong et al., 2011). AeCad binds Cry11Ba with a K_d = 8.2 nM (Likitvivatanavong et al., 2011), a value equivalent to the calculated association constant for Cry11Ba binding to AgCad2. Of the two cadherins identified in *A. gambiae* larval BBM, AgCad1 at 40% identity to AeCad is the low affinity Cry11Ba binder, while AgCad2 at 13% identity to AeCad by Clustal analysis is the high affinity binder and putative receptor. Ikawa et al. propose that the multiple cadherin variants present in an insect might have the same kind of genetic polymorphism as Cry-toxin receptors (Ikawa et al., 2000). We agree and, based on the results of this study and (Hua et al., 2008), propose that the two cadherin-like proteins in *A. gambiae* midgut BBM interact with Cry toxin in various modes such as with high or low affinity binding and may cause toxin inhibition or enhancement or no effect at all.

Aminopeptidase (APN) and alkaline phosphatase (ALP) work in concert with cadherin as Cry receptors in insects (Bravo et al., 2011; Pigott and Ellar, 2007). With the identification of AgCad2 in this study, each of these midgut proteins is established as a receptor, or at least a putative receptor of Cry11Ba, in *Aedes* and *Anopheles* (Hua et al., 2009; Likitvivatanavong et al., 2011; Zhang et al., 2008). In *M. sexta* larvae Cry1Ab binding with Bt-R_1 cadherin facilitates proteolytic cleavage of helix alpha 1, toxin oligomerization and subsequent high affinity binding to GPI-anchored APN or ALP (Bravo et al., 2004; Gómez et al., 2002; Jurat-Fuentes et al., 2004, 2011). Recent evidence supports a similar critical role for *Aedes* cadherin in Cry11Aa toxin action (Rodríguez-Almazán et al., 2012). High affinity interaction between Cry11 toxins and midgut BBM proteins may be a necessary feature for toxin efficacy. We empirically determined the affinity of AgCad2-CR14-MPED and AgCad1-CR11-MPED and whether or not AgCad2, AgCad1, and AgALP1 share binding sites on Cry11Ba toxin. The high affinity-binding AgCad2-CR14MPED (K_d = 11.8 nM) and low affinity-binding AgCad1-CR11MPED (K_d = 766 nM) share a binding site(s) on Cry11Ba (Fig. 4). AgALP1 had limited competition for AgCad2 or AgCad1 peptide binding to Cry11Ba. Since AgALP1 binds Cry11Ba with high affinity (K_d = 23.0 nM) it is likely that its high binding site is not recognized by the AgCads. In the case of *Aedes* AeALP1 which functions as a receptor of Cry11Ba and Cry11Aa, AeALP1 has two Cry binding regions that bind separate regions on Cry11Aa (Fernández et al., 2009). Region R59-G102 of AeALP1 binds to Cry11Aa domain II loop alpha-8, while N257-I296 region binds to domain III 561RVQSQNSGNN570. With respect to APNs the other major class of Cry receptors, AgAPN2 and AgALP1 bind Cry11Ba with high affinity (K_d = 6.4 nM and 23.9 nM, respectively) and share binding sites (Hua et al., 2009; Zhang et al., 2008). The properties of Cry11Ba interactions with AgCad2, AgAPN2 and AgALP1 are consistent with the model of Cry toxin action requiring both cadherin and GPI-anchored midgut proteins for toxicity to insects (Fig. 6) (reviewed in (Bravo et al., 2011)).

With regard to the ability of the cadherin peptides to enhance Bt toxicity, fragments of AgCad2 and AgCad1 are able to enhance Cry4Ba toxicity against *A. gambiae* larvae (Fig. 5C). While the ability of AgCad1-CR11MPED to enhance Cry4Ba toxicity was expected from previous results (Park et al., 2009), AgCad2-CR14MPED enhancement of Cry4Ba toxicity was unexpected due to their very low binding affinity (K_d = 280 mM). However, the enhancing ability was not maintained with Cry11Ba in bioassays against *A. gambiae* larvae; AgCad2 inhibited toxicity while AgCad1 had no effect on Cry11Ba toxicity at both high and low dosages of Cry11Ba (Fig. 5B). The inhibitory effect of cadherin to Bt toxin has been observed previously by other researchers (Dorsch et al., 2002). In contrast, the AeCad-CR7-11 peptide of *Aedes* enhanced Cry11Aa toxicity to larvae (Rodríguez-Almazán et al., 2012) and the enhancement effects of AeCad-CR7-11 peptide correlated with the facilitated protease removal of α-helix 1 and induction of toxin

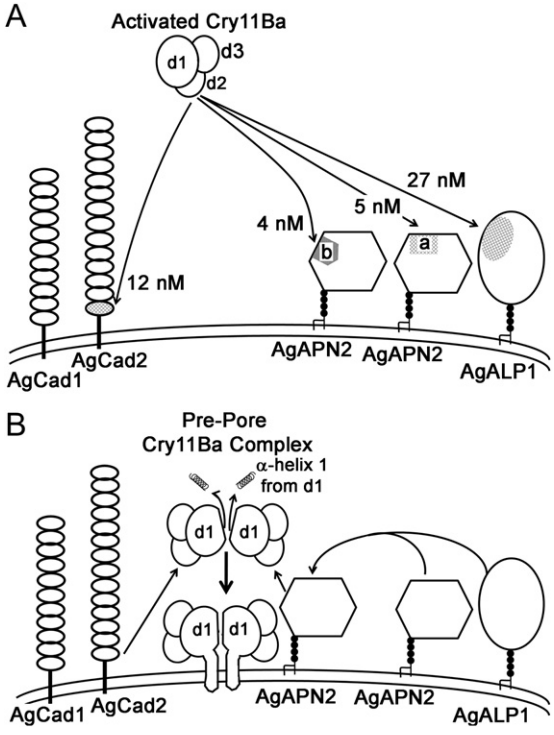

Fig. 6. Model of Cry11Ba action in *A. gambiae* larvae. A. High-affinity binding of activated Cry11Ba to AgCad2, AgAPN2 and AgALP2 (K_d values in nM; (Hua et al., 2009; Zhang et al., 2008; Zhang et al., 2010) and this study). Peptide fragments of each binding region (hatched pattern), except for AgAPN2 region b, are inhibitory to Cry11Ba binding to BBMV and Cry11Ba toxicity to larvae. Peptide AgAPN2b synergizes Cry11Ba binding and toxicity to larvae (Zhang et al., 2010). B. Diagram of Cry11Ba pre-pore formation and membrane insertion according to the Bravo–Soberon model (Bravo et al., 2011).

oligomerization (Chen et al., 2009). The inhibitory effects of AgCad2 on Cry11Ba and the synergist effects of AeCad on Cry11Aa suggest that these two cadherins have different roles in the action of the respective toxins. The synergist effect of AeCad on Cry11Aa toxin in *Aedes*, parallels the synergist effect we observed with a small AgAPN2 peptide having on Cry11Ba toxicity to larvae (Zhang et al., 2010) (Fig. 6). While the overall synergistic effects of the cadherin and APN peptide are similar, the mechanism for how an AgAPN2 peptide synergizes Cry11Ba toxicity is unknown.

All the data observed in this study demonstrate that *A. gambiae* midgut AgCad2 is a likely receptor/binding protein of Cry11Ba toxin. Understanding how the putative receptors AgCad2, AgALP1 and AgAPN2 function together in regulating Cry11Ba toxicity on mosquito larvae is worthy of further investigation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2012.11.007.

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