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Anopheles gambiae Cadherin AgCad1 Binds the Cry4Ba Toxin of *Bacillus thuringiensis israelensis* and a Fragment of AgCad1 Synergizes Toxicity[†]

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ABSTRACT: A midgut cadherin AgCad1 cDNA was cloned from *Anopheles gambiae* larvae and analyzed for its possible role as a receptor for the Cry4Ba toxin of *Bacillus thuringiensis* strain *israelensis*. The AgCad1 cadherin encodes a putative 1735-residue protein organized into an extracellular region of 11 cadherin repeats (CR) and a membrane-proximal extracellular domain (MPED). AgCad1 mRNA was detected in midgut of larvae by polymerase chain reaction (PCR). The AgCad1 protein was localized, by immunohistochemistry of sectioned larvae, predominately to the microvilli in posterior midgut. The localization of Cry4Ba binding was determined by the same technique, and toxin bound microvilli in posterior midgut. The AgCad1 protein was present in brush border membrane fractions prepared from larvae, and Cry4Ba toxin bound the same-sized protein on blots of those fractions. The AgCad1 protein was expressed transiently in *Drosophila melanogaster* Schneider 2 (S2) cells. ¹²⁵I-Cry4Ba toxin bound AgCad1 from S2 cells in a competitive manner. Cry4Ba bound to beads extracted 200 kDa AgCad1 and a 29 kDa fragment of AgCad1 from S2 cells. A peptide containing the AgCad1 region proximal to the cell (CR11-MPED) was expressed in *Escherichia coli*. Although Cry4Ba showed limited binding to CR11-MPED, the peptide synergized the toxicity of Cry4Ba to larvae. AgCad1 in the larval brush border is a binding protein for Cry4Ba toxin. On the basis of binding results and CR11-MPED synergism of Cry4Ba toxicity, AgCad1 is probably a Cry4Ba receptor.

Mosquitoes in the genus *Anopheles* vector *plasmodia* that cause malaria, a major public health problem in the world. In some habitats, Anopheline species are controlled by nonchemical larvicides based on the bacterium *Bacillus thuringiensis* (Bt)¹ serovariety *israelensis* de Barjac. The specific toxicity of Bti to *Anopheles* and *Aedes* spp. is due to the protein components of the parasporal crystal (reviewed in ref 1).

The parasporal crystal of Bti is composed of three major insecticidal Cry proteins (Cry4Aa, Cry4Ba, and Cry11Aa) and cytolytic proteins (Cyt1 and Cyt2). The Cry4Ba insecticidal protein is highly toxic to *Anopheles* and *Aedes* larvae but not to *Culex* larvae (2, 3). The Cry4Ba toxin crystal structure shows that it is very similar to other Cry toxins with their three-domain structures (4, 5). Each domain has a unique role essential to the intoxication process. Although

details of Cry toxin action are best known for Cry1A toxins, their structural similarities suggest that Cry4Ba may function like the lepidopteran-active Cry1A toxins.

Studies on lepidopteran insects revealed several types of Cry toxin receptors: cadherin-like proteins (6–8), aminopeptidase N (APN) (9, 10), alkaline phosphatase (ALP) (11, 12), a glycoconjugate (13), and glycolipids (14). An emerging model suggests that these receptor molecules work in a stepwise fashion to mediate toxicity. After binding cadherin, Cry toxin forms a prepore oligomer that binds APN and ALP and inserts into membrane microdomains called lipid rafts (15). The insertion of the prepore complex into the membrane leads to the formation of ion channels/pores in the brush border membranes of the larval gut leading to cell lysis. Each of these molecules that mediate binding and pore formation has been implicated in resistance development against Cry toxins (16–18).

Recent evidence suggests that Cry toxins active against dipteran larvae bind, and probably use as receptors, the same classes of proteins that function as receptors for Cry1 toxins in lepidopteran larvae. For example, an aminopeptidase N (APN) from *Anopheles quadrimaculatus* binds Cry11Ba (19), and an alkaline phosphatase (ALP) from *Aedes aegypti* (20) was recently identified as a receptor for Cry11Aa.

Inhibition of toxicity is accepted evidence for function as a Cry toxin receptor. Typically, a peptide fragment of the receptor (21) or a phage mimic of the receptor (20) attenuates Cry *in vivo* toxicity to larvae. Recently, we reported an opposite effect in which a fragment of Bt-R₁ cadherin, the Cry1A receptor from *Manduca sexta* (21, 22), not only bound

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¹ Abbreviations: ALP, alkaline phosphatase; APN, aminopeptidase N; Bt, *Bacillus thuringiensis*; BSA, bovine serum albumin; CR, cadherin repeat; Cyto, cytoplasmic; TAMRA, 5(6)-carboxytetramethylrhodamine; S2, *Drosophila melanogaster* S2 cells; IPTG, isopropyl β-D-thiogalactopyranoside; MPED, membrane proximal extracellular domain; PCR, polymerase chain reaction; PCAP, putative cell adhesion protein; RACE, rapid amplification of cDNA ends; TM, transmembrane.

Table 1: Primers Used in This Study

Primer	Primer Sequence (5'-3')
Internal Gene Specific Primer	
AgCad/F1	5'-GGT GGC CGC TGG TCG ATC GTA ATC AAT CGC CG-3'
AgCad/R1	5'-CTT AAT TTT CAG TGT CCA CGT TCC GTA AAA TCC-3'
3' RACE Primers	
AgCad/F2	5'- CGT GTA TCG TTC ACG ATC AAC ATC AAC AAT GCG-3'
AgCad/F3	5'-ATC ATC GCT CAC GAC ATT GAC GGA CCA GG-3'
5' RACE Primers	
AgCad/R3	5'-GCA CCC TCG CTG GAG GTG TTC AGC AGC CGG TT-3'
AgCad/R2	5'-GGT CCC GCG CAC CGG CCA CAT CAC CGA TCT CG-3'
Primers for cloning 5' and 3' fragments of cDNA	
AgCad/F-Spe	5'-GTT ACC TAG TGTACC GGC TGC TGG CGG CCT TAA-3'
AgCad/R-Bam	5'-CGT TCG TCT CAG CGC CGG GAA GGC CCG C-3'
AgCad/F-BamH	5'-CCG TTT GCC GAG GAT CCG AAG AAC GCG GGC-3'
AgCad/R-Sac	5'-GAA TTC GCG GCC GCG GGA ATT TTT TTT TTT TTT TTT-3'
For cloning CR11-MPED	
CR11-MPED/F	5'-GAC CCA TAT GGA CGA AAC GCT GCA GAT CAT CCT GA-3'
CR11-MPED/R	5'-ACA CCT CGA GGA ACC GGT GGG ACA GCT CGT CGT CA-3'
For cloning TM-Cyto	
TM-Cyto/F	5'-GAC CCA TAT GGA CGA AAC GCT GCA GAT CAT CCT GA-3'
TM-Cyto/R	5'-ACA CCT CGA GGA ACC GGT GGG ACA GCT CGT CGT CA-3'
For PCAP PCR	
AgPCAP/F	5'-GGT ATC TCA ACG TCG TCG CTG-3'
AgPCAP/R	5'- CCTCCAGCACGGAGTTGTT-3'

toxin but enhanced Cry1A toxicity against lepidopteran larvae (23). If the binding residues within cadherin repeat 12 (CR) were removed, the resulting peptide lost the ability to bind toxin and lost its function as a toxin synergist.

In this work, we describe the cloning and identification of a cadherin-like protein from the gut of *Anopheles gambiae*. Bioassays, immunohistochemistry, and toxin binding studies are utilized to characterize this cadherin protein, the first reported function of a cadherin as a putative Bt toxin receptor in mosquito larvae.

EXPERIMENTAL PROCEDURES

Insects. *A. gambiae* (CDC G3 strain) were maintained at 27 °C with a photoperiod of 14 h light:10 h dark. Larvae (ca. 200/pan) were fed ground fish food (TetraMin) daily. Adults were fed with 10% sucrose solution, and females were fed on anesthetized mice until engorged. Freshly laid eggs were collected, washed with 0.1% bleach, and hatched in distilled water. Larvae were grown until fourth instar, collected, and stored at -80 °C until use.

Primers. The sequences of primers used for cloning in this study are listed in Table 1.

Synthesis of cDNA and Cloning *A. gambiae* Cadherin. RNA was extracted from *A. gambiae* fourth instar larvae (75 mg wet weight) using the total RNA mini kit (Bio-Rad, Richmond, CA). First strand cDNA was synthesized from total RNA with oligo(dT)₁₇ primer, dNTPs, and SuperScript reverse transcriptase II (Gibco-BRL) according to the manufacturer. A pair of primers, AgCad1/F1 and AgCad1/R1 (Table 1), was designed to match the ends of the partial sequence of *A. gambiae* cadherin (GenBank XM_312086). PCR products were amplified using synthesized cDNA as template and cloned into pGEM-T easy vector (Promega,

Madison, WI). The DNA inserts were sequenced in both forward and reverse directions at the Molecular Genetics Instrumentation Facility at the University of Georgia, confirming the cloned cDNA as identical to the *A. gambiae* cadherin sequence (XM_312086).

For 3' rapid amplification of cDNA ends (RACE), *A. gambiae* cDNA was synthesized from total RNA using *NotI*-d(T)₁₇ primer and SuperScript reverse transcriptase (Gibco-BRL). The cDNA was amplified by PCR with AgCad1/F1 and *NotI*-d(T)₁₇ as primers. The PCR product was further amplified with first round primers AgCad1/F2 and *NotI*-d(T)₁₇. The resultant PCR product was then subjected to a second round amplification with the nested primer AgCad1/F3 and *NotI*-d(T)₁₇. The product was purified, cloned into pGEM-T easy vector (Promega, Madison, WI), and then sequenced.

The 5' end of the cadherin region was amplified with the Gibco-BRL 5' RACE kit and two gene-specific primers (GSPs), AgCad1/R2 and AgCad1/R3. SuperScript reverse transcriptase was used to synthesize first strand cDNA with GSP1 (AgCad1/R1). The resultant cDNA was then used as template for amplification with GSP2 (AgCad1/R2) and oligo(dG) abridged anchor primer tailed with dCTP (Gibco-BRL). Nested PCR with the oligo(dG) abridged anchor primer and AgCad1/R3 confirmed the above PCR product. The PCR product from the AgCad1/R2 reaction was purified, cloned into plasmid pGEM-Teasy (Promega, Madison, WI), and sequenced.

Bioinformatic Analysis. Bioinformatic analysis using the ISREC ProfileScan server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) was performed to analyze the full cadherin sequence. The software basically performs computational predictions using protein sequence patterns (or motifs) from known, well-characterized proteins in the database to elucidate the potential function(s) of uncharacterized proteins (24).

PCR Detection of AgCad1 mRNA in Larval Gut Tissue. Twenty-fourth instar larvae were placed in RNAlater (Sigma, St. Louis, MO) for fixation and dissection. While observing under a dissecting scope, the whole intestine was gently pulled out using fine forceps. After removing Malpighian tubules, dissected guts were immediately used for cDNA synthesis. Methods for cDNA synthesis were the same as described above for larval cDNA synthesis. Plasmids pIZT-AgCad1, pIZT-AgPCAP, and gut cDNA served as templates for PCR. Primers CR11-MPED/F and CR11-MPED/R served as AgCad1-specific primers. As a control, primers AgPCAP/F and AgPCAP/R were designed to amplify a region from a second cadherin-like gene in *A. gambiae* [putative cell adhesion protein (PCAP); GenBank AJ439060]. PCR was performed with 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, and the products were separated on a 1% agarose gel.

Assembling and Cloning the Cadherin Gene into the pIZT/V5 Insect Cell Expression Vector. PCR was conducted using *NotI*-d(T) cDNA as a template with primers AgCad1/F-Spe and AgCad1/R-BamH. Long-template polymerase (Roche Applied Science, Indianapolis, IN) was used in a PCR with 30 cycles of 94 °C for 2 min and 68 °C for 4 min. The resultant PCR fragment was purified and then cleaved with *SpeI* and *BamHI* followed by cloning into the pMECA plasmid vector (GenBank AF017063) (25), yielding the 5' cadherin clone called pMECA-AgCad1-5'. Oligonucleotide

primers AgCad1/F-BamH and AgCad1/R-Sac were used to amplify the 3' end of the *AgCad1* coding region using the cDNA template, using an Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) with 30 cycles of 94 °C for 2 min and 68 °C for 2 min. The PCR fragment was extracted from an agarose gel, digested with *Bam*H and *Sac*II, and then cloned into the pMECA vector to obtain pMECA-AgCad1-3'. Both 5' and 3' clones were sequenced in forward and reverse directions.

The DNA insert in pMECA-AgCad1-3' was excised by digestion with *Bam*H and *Sac*II and cloned into pMECA-AgCad1-5' treated with the same two restriction enzymes, yielding pMECA-AgCad1. The full-length cadherin coding region was excised from pMECA-AgCad1 with *Spe*I and *Sac*II, purified, and cloned into plasmid pIZT (Invitrogen, Carlsbad, CA) previously digested with the same enzymes. Fidelity of the full-length cadherin in plasmid pIZT was confirmed by DNA sequencing, and the plasmid was named pIZT-AgCad1.

Transient Expression of A. gambiae Cadherin in Drosophila S2 Cells. *Drosophila melanogaster*–*Drosophila melanogaster* (Dm) S2 cells (Invitrogen) were cultured in serum-free insect cell medium (HyClone, Logan, UT). For plasmid transfection, fresh S2 cells (1.5×10^6) were seeded into a 60 mm² polystyrene culture dish and allowed to adhere overnight. Plasmid transfection mixtures consisted of pIZT (5 µg) or pIZT-AgCad1 (10 µg) in 1 mL of culture medium plus 10 µL of Cellfectin reagent (Invitrogen, Carlsbad, CA). Each transfection mixture was preincubated at room temperature for 30 min and transferred to a dish containing S2 cells, and the dishes were incubated with gentle shaking for 4 h. Fresh medium (5 mL) was added to the dish after removal of the transfection mixtures, and S2 cells were incubated at 25 °C for 3 days.

Preparation of Brush Border Membrane Vesicles (BBMV) from A. gambiae Larvae. BBMV were prepared from whole fourth instar larvae according to ref 26 with slight modifications. Eight grams of larvae was homogenized in 100 mL of ice-cold MET buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris, pH 7.5) containing Complete cocktail protease inhibitor (Roche Applied Science, Indianapolis, IN) for 1 min using a tissue homogenizer (Kinematica GmbH) set at the highest speed, followed by further homogenization with 15–20 strokes of a Dounce homogenizer. An equal volume of ice-cold 24 mM MgCl₂ was mixed with the homogenate and the mixture placed on ice for 15 min. The mixture was centrifuged at 1900g for 15 min at 4 °C and the supernatant further centrifuged at 27000g for 30 min. The resulting pellet was homogenized in MET buffer, mixed with an equal volume of 24 mM MgCl₂, and centrifuged at low and high speed as above. The final pellet was resuspended in 3 mL of ice-cold MET buffer with protease inhibitors. Protein amount was determined by the Bio-Rad protein assay with BSA as standard. Aminopeptidase N activity (27), 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).

Cloning and Expression of A. gambiae CR11-MPED and TM-Cyto Peptides. A partial cadherin peptide (amino acids 1358G to 1569A) spanning domains CR11-MPED was overexpressed and expressed in *Escherichia coli* as inclusion bodies. The plasmid pMECA-AgCad1-3' was used as a

template to amplify the region encoding CR11-MPED by PCR with CR11-MPED/F and CR11-MPED/R primers. The resulting PCR fragment was cloned into the pET-30a(+) vector (Novagen, Madison, WI) to yield plasmid pET-AgCad1/CR11-MPED. After confirmation by sequencing, plasmid pET-AgCad1/CR11-MPED was transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL (Stratagene, La Jolla, CA). The CR11-MPED region was overexpressed by induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the culture OD₆₀₀ reached 0.5–0.6. The expression and purification protocols are described in a previous paper (23). Polyclonal antiserum against purified CR11-MPED, referred to as α-AgCad1 serum, was produced in New Zealand White rabbits at the Animal Resources Facility at the University of Georgia.

A cadherin truncation (amino acids 1570D to 1735F) containing predicted transmembrane (TM) and cytoplasmic (Cyto) domains was also subcloned to the pET-30a(+) vector to yield pET-AgCad1/TM-Cyto by PCR with primers TM-Cyto/F and TM-Cyto/R.

Immunohistochemistry and Cry4Ba Binding Localization. Dissected guts of early fourth instar larvae of *A. gambiae* were fixed with 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) for 2 h on ice. Fixed tissues were soaked in 30% sucrose solution overnight at 4 °C and embedded in a capsule containing tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). The capsule was snap-frozen in liquid nitrogen and the block transferred immediately into the chamber of a cryostat (Reichert-JuAg 2800 Frigocut-E cryostat). Sections (10 µm) of embedded guts were cut serially and mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA).

Slides containing air-dried tissue sections were washed with PBS for 20 min, blocked with 1 mL of PBST–5% BSA (PBS with 0.2% Tween-20 and 5% BSA) for 1 h at room temperature. Subsequent steps of immunodetection, toxin binding, and observation were according to Chen et al. (28). Cadherin was detected by α-AgCad1 serum diluted 1:500 in blocking solution; preimmune serum diluted in blocking solution served as a negative control. To detect Cry4Ba binding, tissue sections were treated with 5 µg/mL rhodamine-labeled [rhodamine derivative, 5(6)-carboxytetramethylrhodamine (TAMRA)] Cry4Ba. Rhodamine-labeled BSA (5 µg/mL) was used as a control.

Immunoblots and Toxin Blots. *A. gambiae* BBMV proteins were separated by SDS–PAGE and electroblotted to polyvinylidene fluoride (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 α-AgCad1 serum (1:5000 dilution) in PBST–0.1% BSA for 2 h. After washing, the filters were incubated with α-rabbit IgG–peroxidase conjugate (1:25000 dilution) in the same buffer for 1 h at room temperature. Finally, the filters were developed with an ECL kit (GE Healthcare, Piscataway, NJ) and exposed to X-ray film. To detect cadherin expression in S2 cells, 1×10^7 cells were harvested by centrifugation at 400g for 2 min followed by three washes with PBS. Whole cells were suspended in SDS–PAGE sample buffer and boiled for 10 min. Expressed cadherin on S2 cells was detected on Western blots using anti-AgCad1 serum.

A. gambiae BBMV were treated with the Plus-One 2-D clean-up kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions, and 20 µg of protein was separated by SDS-PAGE. After electrophoresis, separated proteins were transferred to a PVDF filter and blocked with 3% BSA in PBST. The filter was incubated with Cry4Ba (5 µg/mL final concentration) in PBST for 1 h at room temperature. Toxin binding proteins were detected with rabbit α-Cry4Ba serum and developed by an ECL kit (GE Healthcare, Piscataway, NJ).

Cry4Ba–Bead Extraction of AgCad1 Expressed in S2 Cells. S2 cells expressing cadherin were harvested and washed as described above. The cells were suspended and solubilized in PBS containing 1% CHAPS with cocktail protease inhibitor (Roche) with rotation at room temperature for 1 h. Solubilized proteins were clarified by centrifugation at 16200g for 30 min. The proteins were mixed with a Cry4Ba–α-Cry4Ba serum conjugated protein A bead column. Protein A Sepharose 6MB (GE Healthcare) beads were washed with PBS three times and then incubated with α-Cry4Ba serum plus varying amounts of Cry4Ba with rotation for 1 h at room temperature. Beads without the Cry4Ba–α-Cry4Ba serum conjugate were used as background control.

The solubilized S2 cell proteins were incubated with the Cry4Ba–anti-Cry4Ba/protein A bead complex for 2 h at room temperature with rotation. The beads were pelleted by centrifugation at 100g for 1 min and vigorously washed three times with PBS. The sample was then heated in a 100 °C bath for 10 min with SDS sample buffer to extract the bound proteins. Proteins released from the Cry4Ba–anti-Cry4Ba/protein A beads were separated by SDS–10% PAGE gel (Bio-Rad) and blotted to PVDF filter. The filter was blocked in 3% BSA–PBST for 1 h and then probed with α-V5 antibody (Invitrogen, Carlsbad, CA) for 2 h at room temperature. After washing, the filter was developed by the ECL kit (GE Healthcare, Piscataway, NJ) and exposed to X-ray film.

Preparation of Cry4Ba Toxin and α-Cry4Ba Serum. A Cry4Ba mutant, Cry4BRA, was used in all experiments and will be referred to herein as Cry4Ba. The mutated Cry4Ba has a trypsin cleavage site removed by the replacement of R203 with an A residue (2, 29). The R203 to A change is in the loop between α-helices. The Cry4BRA toxin is equal to wild-type Cry4Ba in toxicity to *A. quadrimaculatus*, *Ae. aegypti* and *Culex pipiens* (2). We used Cry4BRA as a surrogate for Cry4Ba due to trypsin stability of the active toxin. Production of Cry4BRA (i.e., Cry4Ba) crystals and purification of toxin were as described previously (2). Trypsin digestion of Cry4Ba protoxin according to Abdullah et al. (2) produced an ~66 kDa toxin mosquitocidal fragment. Antiserum against Cry4Ba toxin was prepared in New Zealand White rabbits at the Animal Resources Facility at the University of Georgia.

Dot Blots. S2 cells expressing *A. gambiae* cadherin were solubilized in PBS containing 1% CHAPS and Complete protease inhibitor (Roche, Indianapolis, IN), and the sample tube was rotated at room temperature for 1 h. The soluble proteins were applied to a HiTrap Ni²⁺-chelating HP column (GE Healthcare, Piscataway, NJ) and eluted with imidazole. The partially purified proteins were separated by SDS-PAGE and transferred to a PVDF filter for Western blotting. The

partially purified proteins were also dotted onto a PVDF filter directly and probed with ¹²⁵I-Cry4Ba or with ¹²⁵I-Cry4Ba plus unlabeled Cry4Ba (1000-fold) or unlabeled Cry1Ab (1000-fold). The toxin was labeled with Na¹²⁵I (GE Healthcare, Piscataway, NJ) as described previously (6). The filters were exposed to X-ray film at –80 °C for autoradiography.

The truncated cadherin peptides, CR11-MPED and TM-Cyto, were expressed in *E. coli* and purified as previously described (23). Various amounts of purified peptides were dotted on PVDF filters and probed with ¹²⁵I-labeled Cry4Ba or with ¹²⁵I-labeled Cry4Ba plus unlabeled Cry4Ba (1000-fold) and exposed to X-ray film as above.

Mosquito Larva Bioassay. Soluble Cry4Ba (2) was mixed with purified CR11-MPED or TM-Cyto peptides in 1:100 (toxin:cadherin peptide) mass ratios in distilled water. A total of 10 fourth instar larvae per 2 mL of water with replicates in a six-well Costar culture plate were fed soluble Cry4Ba toxin or a mixture of toxin plus cadherin peptide. Mortalities were scored after 16 h at 27 °C. Bioassays were repeated three times for each treatment.

RESULTS

Cloning and Analysis of *A. gambiae* Cadherin. To identify cadherins in *A. gambiae* larvae that are similar to the lepidopteran cadherins located in the midgut brush border, we searched *A. gambiae* databases using BLAST with the Bt-R₁ cadherin from *M. sexta*. The predicted cadherin sequence (XM_312086) was selected as the most homologous sequence. Using PCR and RACE as diagrammed in Figure 1A, we cloned a cDNA corresponding to this *A. gambiae* cadherin (Figure 1B). The coding sequence encodes a protein, designated AgCad1, of 1735 amino acids with a predicted molecular mass of 195 kDa. Sequence analysis identified a signal peptide at its N-terminus followed by 11 CR, a MPED, a TM domain, and a C-terminal cytoplasmic domain. Ten putative calcium-binding sequences are distributed throughout the extracellular domain. Integrin recognition sequences, RGD (30) and LDV (31–34), are located before CR1 and in CR5, respectively. The cytoplasmic domain consists of 136 amino acids and a calcium-binding recognition sequence (DRD).

***A. gambiae* Cadherin Is Expressed in Midgut Tissue.** The presence of AgCad1 in the midgut of larvae was confirmed by PCR analysis and immunohistochemistry experiments. Gene-specific primers were designed to amplify a 636 bp fragment of AgCad1. PCR amplification using gut cDNA and plasmid pIZT-AgCad1 as templates with gene-specific primers resulted in the expected size products (Figure 2). As a control, PCR primers were designed to a second predicted *A. gambiae* cadherin-like protein, the protein in *A. gambiae* databases most homologous to AgCad1. The PCR product detected using PCAP primers and gut cDNA was smaller in size than the AgCad1 PCR product, and no product was detected with pIZT-AgCad1 as template (Figure 2).

To confirm the presence of AgCad1 protein in midgut tissue, we used α-AgCad1 to probe sectioned gut tissues. The serum is relatively specific for AgCad1 as no cross-reaction was detected to PCAP in Western blot experiments (data not shown). As seen in Figure 3A, immunostaining localized cadherin to the microvilli in the posterior midgut.

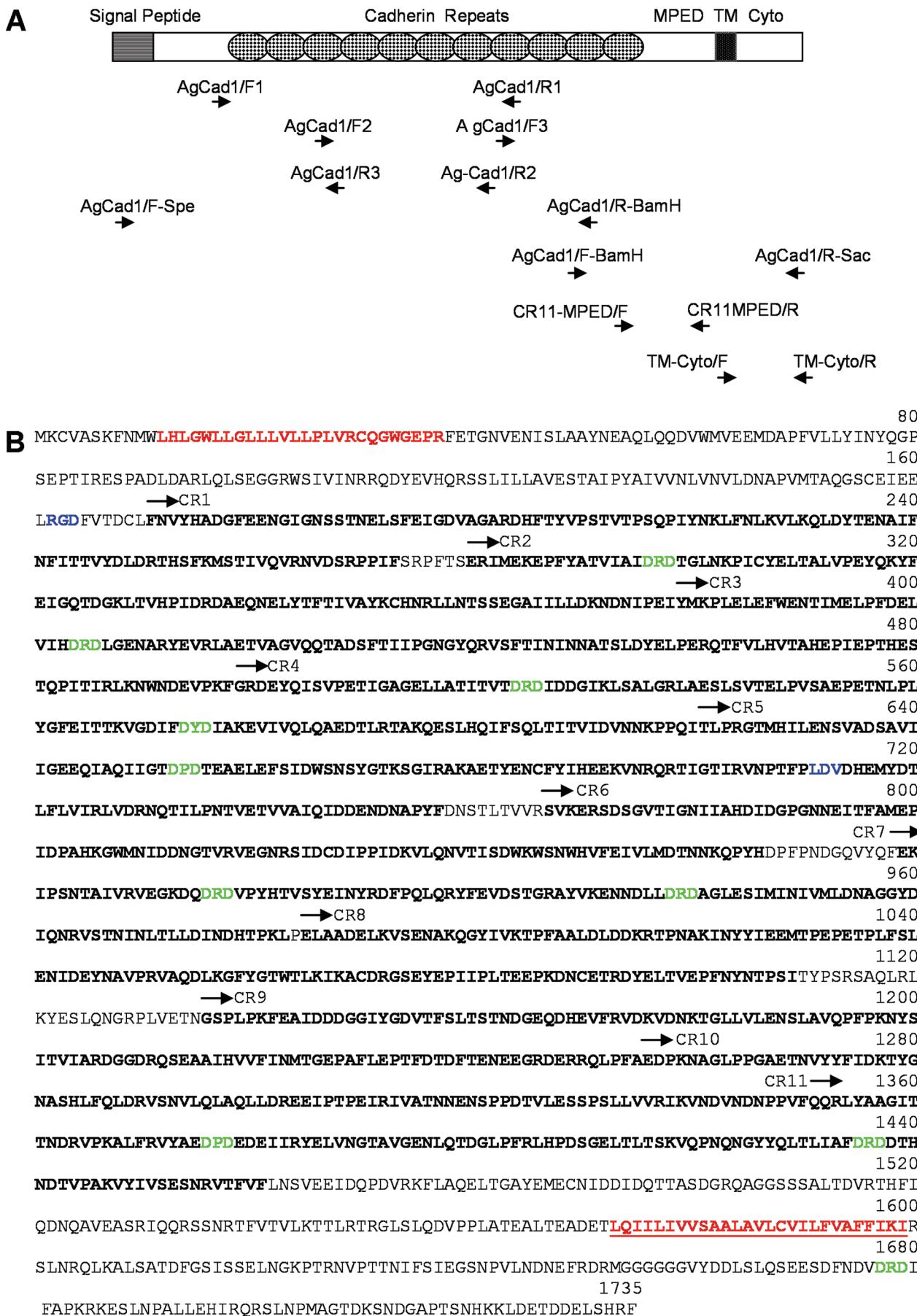


FIGURE 1: (A) Diagram of the *A. gambiae* AgCad1 molecule and primer locations. (B) Protein sequence analyzed using the ISREC ProfileScan server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>). Amino acid sequences representing the CR modules are in bold. Amino acids constituting the putative signal leading peptide and TM (underlined) are in red; putative calcium binding sites are in green; integrin binding sites are in blue.

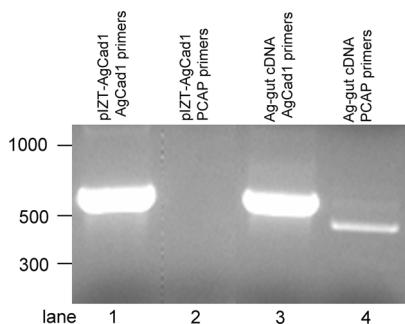


FIGURE 2: Expression of AgCad1 in *A. gambiae* gut cDNA detected by PCR. Lane designations: (1) PCR product of AgCad1 primers with pIZT-AgCad1 template; (2) no product was detected with pIZT-AgCad1 template and PCAP primers; (3) gut cDNA with AgCad1 primers showing the same-sized product as for the pIZTAgCad1 template; (4) gut cDNA with PCAP primers.

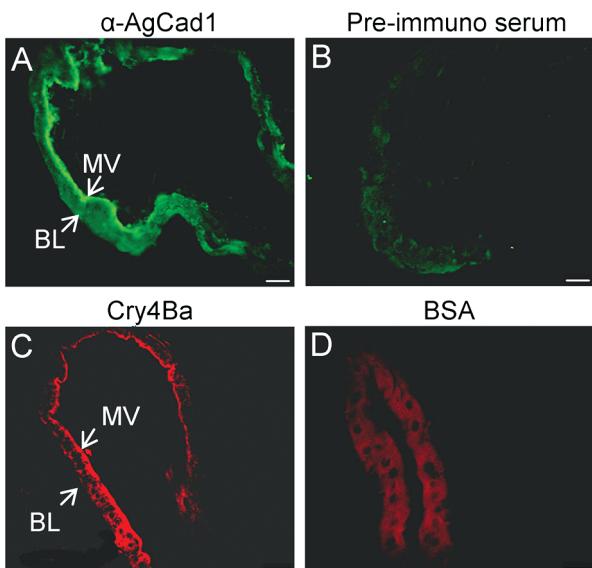


FIGURE 3: Localization of cadherin-like protein in the posterior region of the midgut of larval *A. gambiae*. (A) AgCad1 was immunostained on the microvilli. (B) As a control, midgut sections probed with preimmune sera were not immunostained. (C) Rhodamine-labeled Cry4Ba localized on microvilli of posterior midgut, but labeled BSA (D) did not bind to any part of the midgut (MV, microvilli; BL, basal lamina) (bars, 50 μ m).

The control sections probed with preimmune serum and secondary labeled antibody showed only faint background staining (Figure 3B). We conclude from gene-specific PCR and immunolocalization experiments that AgCad1 is expressed in midgut tissue and the protein is localized on the brush border membrane.

Cry4 toxins bind the apical brush border of midgut cells in the gastric cecae and posterior gut of *A. gambiae* (35). We probed sectioned gut tissue of *A. gambiae* with rhodamine-labeled Cry4Ba, and the results are shown in Figure 3C. Cry4Ba bound to midgut microvilli in the posterior midgut, in a pattern similar to AgCad1 localization. A rhodamine-labeled BSA control showed faint nonspecific binding to gut tissue.

α -AgCad1 Serum and Cry4Ba Detect a 200 kDa Protein in *A. gambiae* BBMV. The molecular size of AgCad1 in brush border membrane was determined to be about 200 kDa by probing blots of BBMV proteins with α -AgCad1 serum (Figure 4A). This size is slightly larger than the 195 kDa predicted size suggesting posttranslational modification, most

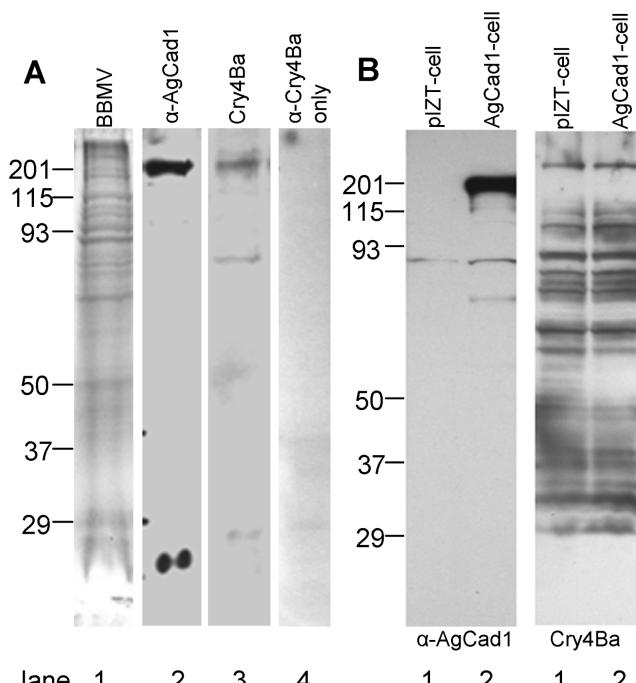


FIGURE 4: Blots of *A. gambiae* brush border membrane proteins and expression of AgCad1 expressed in S2 cells transfected with pIZT-AgCad1. (A) Lane 1: 20 μ g of *A. gambiae* BBMV proteins were stained with CBB. BBMV proteins were separated by SDS-PAGE and then transferred to a PVDF filter. After blocking, filters were probed with α -AgCad1 serum (lane 2) or Cry4Ba/ α -Cry4Ba (lane 3) or α -Cry4Ba alone (lane 4). (B) After blocking, blots of S2 cell proteins were probed with α -AgCad1 or Cry4Ba/ α -Cry4Ba. Lane designations: (1) S2 cells transfected with pIZT vector; (2) cells transfected with pIZT-AgCad1.

likely by glycosylation. The α -AgCad1 serum also detected a 25 kDa peptide that may be a degraded form of AgCad1 or a cross-reactive protein (Figure 4A). When strips from the same blot of *A. gambiae* BBMV proteins were probed with Cry4Ba toxin and α -Cry4Ba serum, a 200 kDa protein was detected (Figure 4A). Proteins of 80 and 28 kDa were also detected by Cry4Ba toxin.

AgCad1 Expressed in S2 Cells Binds Cry4Ba. Transient expression of AgCad1 in S2 cells provided alternate approaches to test for Cry4Ba binding to AgCad1. S2 cells were transfected with pIZT or pIZT-AgCad1 and probed with either α -AgCad1 serum or Cry4Ba toxin. A 200 kDa AgCad1 was expressed in pIZT-AgCad1 transfected cells (Figure 4B). Although Cry4Ba bound many S2 cell proteins, no Cry4Ba binding was detected to expressed AgCad1 protein (Figure 4B). We considered that AgCad1 expressed by S2 cells was different than AgCad1 expressed on midgut brush border and not detected under denaturing conditions. To facilitate measuring toxin binding under nondenaturing conditions, AgCad1 was partially purified from pIZT-AgCad1-transfected S2 cells using a Ni-affinity column (Figure 5A). The eluted cadherin fraction was dotted in increasing amounts to a membrane filter and then the filter probed with 125 I-Cry4Ba (Figure 5B). As the amount of dotted protein increased, more 125 I-Cry4Ba was bound, and excess unlabeled Cry4Ba (1000-fold) competed for 125 I-Cry4Ba binding (Figure 5B). Since Cry4Ba displayed nonspecific binding to S2 cell proteins on blots, proteins from pIZT vector-transfected cells were applied to a Ni-affinity column and eluted and tested for Cry4Ba binding. This was possible due

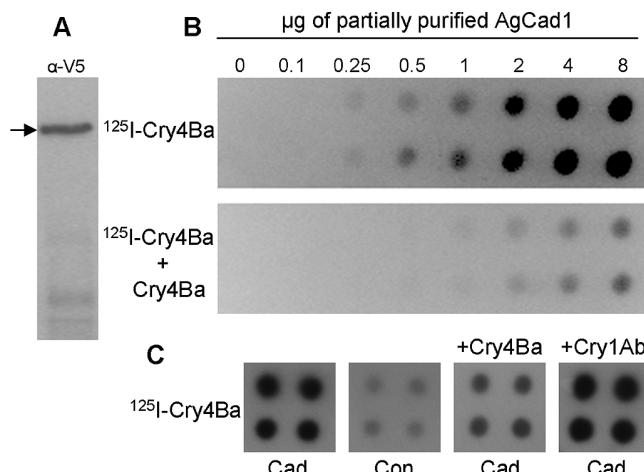


FIGURE 5: Partially purified AgCad1 (A) specifically binds Cry4Ba (B, C). The cadherin expressed on S2 cells was solubilized in CHAPS, and then the soluble proteins were loaded on a nickel-chelating Sepharose column and eluted with imidazole. (A) The partially purified proteins were separated on SDS-PAGE and transferred to a PVDF filter detected with α -V5 sera. The arrow denotes detected cadherin. (B) The proteins were also dotted on a PVDF filter directly and probed with ¹²⁵I-Cry4Ba or with ¹²⁵I-Cry4Ba plus unlabeled Cry4Ba (1000-fold). (C) The proteins purified from pIZT control and pIZT-AgCad1 cells were dotted on a PVDF filter and probed with ¹²⁵I-Cry4Ba or with ¹²⁵I-Cry4Ba plus unlabeled Cry4Ba or Cry1Ab (1000-fold).

to nonspecific binding of S2 cell proteins to the Ni-affinity column (data not shown). While a strong signal was detected for ¹²⁵I-Cry4Ba binding to partially purified AgCad1 protein, a weak signal was detected for dotted S2 cell protein (Figure 5C). The ability of Cry4Ba, but not Cry1Ab (a lepidopteran-active toxin), to compete for ¹²⁵I-Cry4Ba binding (Figure 5C) was further evidence that binding was specific.

Bead Extraction of AgCad1 Expressed on Dm S2 Cells. Bead extraction experiments provided a second approach for testing Cry4Ba binding to cadherin expressed in S2 cells. Cry4Ba was coupled indirectly to protein A beads via an α -Cry4Ba antibody (Experimental Procedures), and the bead complex was incubated with S2 cells expressing AgCad1. Extracted proteins were separated by SDS-PAGE and blotted to a membrane filter, and AgCad1 was detected with α -V5 mouse antibody. Using α -V5 mouse antibody to detect the C-terminal V5 epitope tag on expressed AgCad1 circumvented detection of rabbit antibodies attached to the Cry4Ba-bead complex. AgCad1 was detected in S2 cells, primarily as a mixture of 200, 55, and 29 kDa bands (Figure 6, “AgCad1 cell lysate” lane). The Cry4Ba-bead complex extracted the three AgCad1 peptides, and as more Cry4Ba was added to the bead complex, more AgCad1 was extracted. Some AgCad1 was extracted by the bead-protein A and the bead-protein A- α -Cry4Ba (Figure 6, lanes “Beads” and “0 μ g”). The extracted 50 kDa protein correlated with the presence of antibody on the beads, but not Cry4Ba toxin. Since the 55 and 29 kDa peptides bound Cry4Ba and were detected by α -V5 antibody, those fragments probably correspond to C-terminal fragments of AgCad1. The Cry4Ba extraction experiments are further evidence that Cry4Ba binds AgCad1 and suggest that Cry4Ba binds at the C-terminal region of AgCad1. A binding site near the C-terminus is consistent with the current model for Cry1A toxin binding to lepidopteran cadherins, where the Cry1A toxins

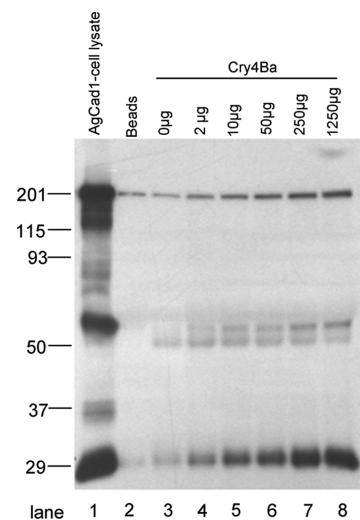


FIGURE 6: Cry4Ba-bead extraction of AgCad1 expressed in S2 cells. Cadherin expressed in S2 cells was solubilized by CHAPS and incubated with a complex of protein A beads- α -Cry4Ba bound with different amounts of Cry4Ba toxin. After being washed to remove loosely adhering proteins, the binding complex was boiled in SDS sample buffer, separated by SDS-PAGE, and then transferred to a PVDF filter. AgCad1 was detected by α -V5 epitope serum. Lane designations: (1) total cell lysate of S2 cells transfected with pIZT-AgCad; (2) beads alone; (3) beads with protein A- α -Cry4Ba; (4–8) complete bead complex with increasing amounts of Cry4Ba.

bind the cadherin repeat nearest the C-terminus of the protein (21, 22, 36).

The CR11-MPED Region of AgCad1 Enhances Cry4Ba Toxicity to A. gambiae Larvae and Binds Cry4Ba Toxin. In Chen et al. (23) we reported the unexpected observation that the CR12-MPED region of Cry1A-binding cadherin from *M. sexta* enhanced *in vivo* Cry1A toxicity to lepidopteran larvae (23). We (23) also reported that Cry1Ab binding to CR12-MPED was necessary for toxin enhancement properties. Based on the data in Chen et al. (23) and evidence that Cry4Ba bound a C-terminal fragment of AgCad1 (Figure 6, lanes 2–8), we expressed the comparable CR11-MPED region from AgCad1 in *E. coli* and tested the purified 24 kDa peptide with Cry4Ba in bioassays of mosquito larvae. As shown in Figure 7, The CR11-MPED peptide increased the toxicity of Cry4Ba against *A. gambiae* fourth instar larvae. Cry4Ba alone at 0.25 μ g/mL killed 20% of the larvae, whereas Cry4Ba:CR11-MPED (1:100 mass ratio) increased mortality to 92.5%. The peptide alone was not toxic to *A. gambiae* larvae. A similarly sized peptide comprised of the TM-Cyto region reduced Cry4Ba toxicity to only 5% larval mortality.

Since CR11-MPED enhanced Cry4Ba toxicity, and toxin binding was required for CR12-MPED enhancement of Cry1A toxicity (23), Cry4Ba was tested for the ability to bind CR11-MPED from AgCad1. Increasing amounts of the truncated AgCad1 peptides (CR11-MPED or TM-cyto) were dotted onto a membrane filter, and the filter was probed with ¹²⁵I-labeled Cry4Ba. Binding was visualized through autoradiography to X-ray film. Labeled Cry4Ba bound to CR11-MPED stronger than to TM-Cyto on dot blots, though both bound at low levels (Figure 7). Excess unlabeled Cry4Ba toxin (1000-fold) partially reduced the binding to CR11-MPED but had no effect on binding to the TM-Cyto peptide.

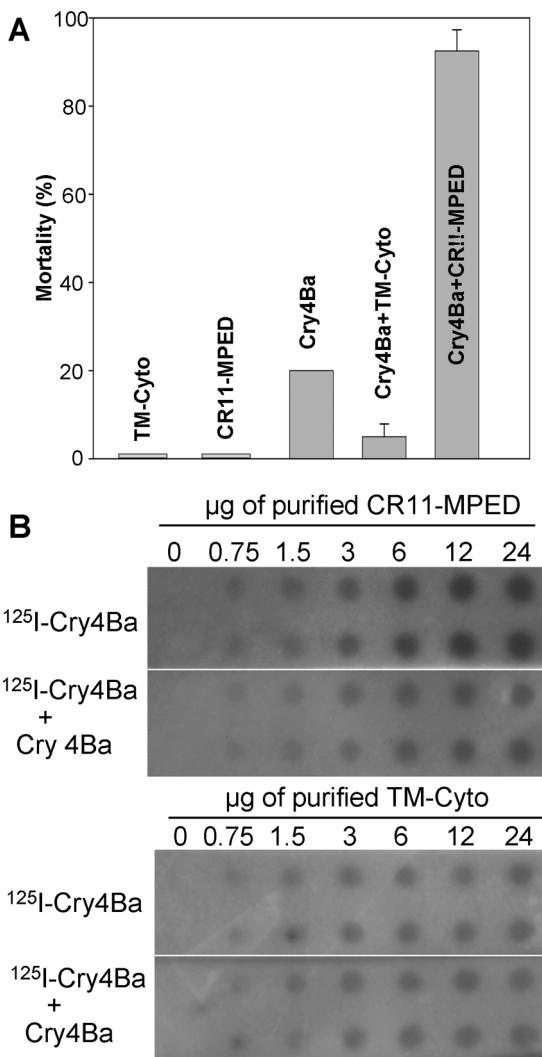


FIGURE 7: CR11-MPED peptide enhances Cry4Ba toxicity and displays limited Cry4Ba binding on dot blots. (A) Bioassay of Cry4Ba on *A. gambiae* with or without truncated cadherin fragments. Fourth instar larvae were put in bioassay wells with 2 mL of distilled water; each well contained 10 larvae. The concentration of Cry4Ba toxin was 0.25 μ g/mL with 100-fold of truncated peptides in mass ratio. Control groups contained the same amounts of peptides as in test groups. Each column represented the mean \pm SE from four replicates which were composed of 10 \times 4 *A. gambiae* larvae. (B) CR11-MPED or TM-Cyto peptides were spotted in duplicate on a membrane filter and probed with ¹²⁵I-Cry4Ba alone or in the presence of a 1000-fold excess of unlabeled Cry4Ba.

DISCUSSION

In this study we present an analysis of a cadherin-like protein, AgCad1, which is expressed in the midgut of *A. gambiae* larvae. We present supporting data to show that AgCad1 is a binding protein and possibly a functional receptor for Cry4Ba toxin. AgCad1 bound Cry4Ba toxin in BBMV prepared from larvae and when expressed in S2 cells. A truncated fragment of AgCad1, CR11-MPED, enhanced Cry4Ba toxicity to the mosquito larvae.

AgCad1 is homologous to midgut cadherins that function as Cry1A toxin receptors (e.g., Bt-R₁) in lepidopteran larvae. Bt-R₁ is located on the apical membrane of midgut columnar epithelial cells (28, 37–39), unlike classical cadherins, which are located mainly within adherens junctions involved in cell–cell adhesion (40). Like Bt-R₁, the mosquito cadherin was also localized on the apical membrane in the gut region

of the larva. Previous research shows that the apical region of the posterior gut in *A. gambiae* binds Cry4A protein (35). In agreement with those authors, we also localized Cry4Ba binding to the brush border of the posterior gut (Figure 3C). This pattern of binding correlates with the presence of receptors.

The predicted AgCad1 protein has the features expected of a member of the cadherin superfamily. AgCad1 has 11 cadherin repeats compared to 12 cadherin repeats in Bt-R₁, and both cadherin proteins contain an MPED followed by a predicted membrane spanning region. Similar to lepidopteran cadherins, the cytoplasmic domain of AgCad1 does not have sequences predicted to interact with intracellular proteins such as catenins (21). AgCad1 has 29% identity with Bt-R₁ in pairwise alignment. A parologue of AgCad1 in *A. gambiae* (PCAP; XM_321513.2) shows 18% identity and an orthologue in *D. melanogaster* cad88C/15646 shows 17% identity. The function of cad88C is not reported in the literature. Bel and Escriche (41) noted that in zebrafish and mammals an orthologue of lepidopteran midgut cadherins, cadherin 23, is involved in maintenance of hair bundles (stereocilia) of the inner ear, related to signal mechanotransduction.

AgCad1 was detected as a 200 kDa protein in BBMV prepared from *A. gambiae* larvae. Although the same-sized protein bound Cry4Ba on ligand blots (Figure 4A), Cry4Ba did not bind to S2 cell-expressed AgCad1 on ligand blots (Figure 4B). This was the case when S2 cell protein was either run directly on blots or enriched by partial purification. In contrast, Cry4Ba bound partially purified AgCad1 in dot blot experiments, and binding was competed by unlabeled toxin (Figure 5B). The dot-blot results and the observation that Cry4Ba extracted nondenatured AgCad1 from S2 cells suggest that the tertiary structure of AgCad1 may contribute to Cry4Ba binding. There is precedence for this explanation as Cry1Ab binds a motif on Bt-R₁ comprised of the N- and C-terminal ends of Bt-R₁ brought together by secondary structure (42).

The CR11-MPED region of AgCad1 bound Cry4Ba toxin on dot blots; however, the binding signal was considerably weaker than seen with the full-length AgCad1, and competition by unlabeled Cry4Ba was less obvious (Figures 5B and 7B). In contrast, the comparable peptide, CR12-MPED from Bt-R₁, gave a strong signal on dot blots and bound toxin at a high-affinity site ($K_d = 9$ nM) and low-affinity sites ($K_d = 1 \mu$ M) (23). Although we were unable to quantify the dot blot binding data shown in Figure 7 and calculate the affinity value, qualitatively the data suggest that the affinity of Cry4Ba for the CR11-MPED peptide is much lower than the affinity of Cry1Ab for CR12-MPED from Bt-R₁. Xie et al. (22) determined the Cry1Ac binding motif in *Heliothis virescens* cadherin as GVLTLNFQ, which is located in the last repeat of the cadherin. A similar region, GVLTLNIQ, present in *M. sexta* Bt-R₁, affects binding and Cry1A toxicity on lepidopteran larvae (23). CR12-MPED-mediated Cry1A toxin enhancement was significantly reduced when the high-affinity Cry1A binding epitope (GVLTLNIQ) within the cadherin peptide was deleted. It is interesting to note that a similar conserved region, GELTLTSKVQ, is located within the last repeat of the *A. gambiae* cadherin molecule.

When we reported synergism of Cry1Ab toxicity to *M. sexta* by CR12-MPED from Bt-R₁, the results were unexpected (23), because others had reported inhibition of toxicity

in similar experiments (21, 22, 43). Enhancement of Cry4Ba toxicity to *A. gambiae* larvae by CR11-MPED indicates that the toxicity enhancement properties of cadherin fragments extends at least to Cry toxins active against dipteran larvae. In Chen et al. (23), we demonstrated that the last repeat of cadherin not only bound Cry1Ab toxin but also bound to the brush border membrane of midgut (23). We suggested that Cry1Ab binding to the CR12-MPED may promote the switch of toxin from monomer to oligomer according to the Bravo model (44).

Overall, the data demonstrate that midgut cadherin, AgCad1, is a Cry4Ba binding protein and putative receptor. Further investigations of the interaction of Cry4Ba, and other mosquitocidal Cry toxins, with midgut molecules are needed to establish the role of midgut cadherin in the intoxication process.

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