The *Heliothis virescens* Cadherin Protein Expressed in *Drosophila* S2 Cells Functions as a Receptor for *Bacillus thuringiensis* Cry1A but Not Cry1Fa Toxins[†]

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ABSTRACT: Genetic knockout of the *BtR4* gene encoding the *Heliothis virescens* cadherin-like protein (HevCaLP) is linked to resistance against Cry1Ac toxin from *Bacillus thuringiensis*. However, the functional Cry1Ac receptor role of this protein has not been established. We previously proposed HevCaLP as a shared binding site for *B. thuringiensis* (Bt) Cry1A and Cry1Fa toxins in the midgut epithelium of *H. virescens* larvae. Considering that Cry1Ac and Cry1Fa are coexpressed in second-generation transgenic cotton for enhanced control of Heliothine and *Spodoptera* species, our model suggests the possibility of evolution of cross resistance via alteration of HevCaLP. To test whether HevCaLP is a Cry1Ac and Cry1Fa receptor, HevCaLP was transiently expressed on the surface of *Drosophila melanogaster* Schneider 2 (S2) cells. Expressed HevCaLP bound [^{125}I]Cry1A toxins under native (dot blot) and denaturing (ligand blot) conditions. Affinity pull-down assays demonstrated that Cry1Fa does not bind to HevCaLP expressed in S2 cells or in solubilized brush border membrane proteins. Using a fluorescence-based approach, we tested the ability of expressed HevCaLP to mediate toxicity of Cry1A and Cry1Fa toxins. Cry1A toxins killed S2 cells expressing HevCaLP, whereas Cry1Fa toxin did not. Our results demonstrate that HevCaLP is a functional Cry1A but not Cry1Fa receptor.

The crystalline toxins synthesized by the bacterium *Bacillus thuringiensis* (Bt)[1] are widely used as insect control proteins in transgenic crops and biopesticides. Bt insecticidal toxins are highly specific due to their mode of action. When the toxin is ingested by the insect, the crystalline toxins are solubilized and enzymatically processed to a toxin core. According to the model of Bravo et al. (*1*), toxin monomers bind to cadherin proteins on the midgut epithelium and undergo a conformational change that results in toxin oligomerization and an increase in the affinity of the toxin for aminopeptidase N (APN) binding. Binding of toxin oligomers to APN and possibly alkaline phosphatase results in accumulation of oligomers on specialized membrane regions called lipid rafts, leading to oligomer insertion and pore formation in the cell membrane. The osmotic shock resulting from this membrane permeabilization process is hypothesized to cause cell death by osmotic cell lysis. In an alternative model, binding of toxin monomers to cadherin activates an intracellular apoptotic signaling pathway resulting in cell death (*2*). In either model, cadherin proteins play a key role in the intoxication process.

Cadherin-like proteins have been demonstrated to be functional receptors for Cry1A toxins in *Manduca sexta* (*3*) and *Bombyx mori* (*4*). Traditionally, this family of proteins is involved in cell-to-cell adhesion, and mammalian intestinal cadherins are localized to the basolateral cell-to-cell contact regions (*5*). However, the BtR_1 cadherin from *M. sexta* localizes to microvilli of midgut cells (*6*), a region accessible to Cry1 toxin binding. This cadherin-like protein binds Cry1Aa, Cry1Ab, and Cry1Ac toxins on ligand blots (*7*) and induces toxin susceptibility when expressed in *Drosophila melanogaster* Schneider 2 (S2) or *Trichoplusia ni* Hi5 cells (*2*, *3*). Similarly, the BtR175 cadherin protein from *B. mori* serves as a Cry1Aa functional receptor when expressed in Sf9 and COS7 cells (*4*, *8*). In strains of *Heliothis virescens* (*9*), *Pectinophora gossypiella* (*10*), and *Helicoverpa armigera* (*11*), resistance to Cry1A toxins is linked to alterations in cadherin proteins.

The *H. virescens BtR4* cDNA (*9*) encodes a predicted 1732-amino acid pro-protein (HevCaLP) with a 22-amino acid trans endoplasmic reticulum signal peptide, 11 predicted cadherin repeats, a hydrophobic transmembrane domain, and a cytosolic domain at the C-terminus. There is polymorphism between *H. virescens* cadherins, as the HevCaLP protein (GenBank accession number 15149240) is 98% identical to the *H. virescens* cadherin cloned by Xie et al. (*12*) (GenBank accession number 56159913).

In cadherin proteins from *M. sexta* (*13*) and *B. mori* (*8*), Cry1A toxins bound to the cadherin repeat located most proximal to the cell membrane. A low-affinity Cry1Ac toxin-binding region was found in extracellular repeat 12 on a

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[1] Abbreviations: Bt, *Bacillus thuringiensis*; BBMV, brush border membrane vesicles; HevCaLP, *Heliothis virescens* cadherin-like protein; APN, *N*-aminopeptidase; HvALP, *H. virescens* alkaline phosphatase; BSA, bovine serum albumin; PVDF, polyvinylidiene difluoride Q; HRP, horseradish peroxidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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cadherin protein from *H. virescens* larvae that differs in 22 amino acids from the HevCaLP sequence (*12*). Considering that the Cry1Ac-binding region is conserved in HevCaLP, it can be predicted that Cry1Ac would bind to this region in HevCaLP. Truncated cadherin peptides expressed in *Escherichia coli* containing the Cry1Ac-binding region identified by Xie et al. inhibited only 57% of the Cry1Ab and Cry1Ac toxicity in *H. virescens* or *M. sexta* larvae (*12*). In contrast, comparable amounts of a similar peptide containing the Cry1Ab toxin-binding region from the *M. sexta* BtR_1 cadherin completely inhibited Cry1Ab toxicity toward *M. sexta* larvae (*14*). In agreement with these results, Cry1A toxins bound with the same affinity to both BtR_1 cadherin expressed on insect cells and *M. sexta* BBMV (*3*, *14*), while Cry1Ac bound to the Xie et al. cadherin fragment (*12*) with an ~40-fold lower affinity than it did to *H. virescens* BBMV (*15*). Consequently, either additional regions of the *H. virescens* cadherin are involved in Cry1A toxicity, or complementary receptor molecules exist in this insect that account for part of the binding affinity and susceptibility to Cry1A toxins.

The current Cry1 toxin-binding site model for *H. virescens* larvae suggests that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja toxins share a common binding site, called receptor A, which is the only Cry1Aa binding site (*15*, *16*). Genetic knockout of the HevCaLP cadherin resulted in a lack of Cry1Aa toxin binding (*17*), evidence that this protein is part of the shared A binding site. As a consequence, the binding site model predicts the potential evolution of cross resistance to Cry1A, Cry1Fa, and Cry1Ja toxins by alteration of the shared HevCaLP binding site. In agreement with this hypothesis, Cry1Ac-resistant *H. virescens* larvae that do not express HevCaLP (*17*) exhibit cross resistance to Cry1Fa (*18*). Testing this hypothesis is a key to estimating the risk of resistance evolution against transgenic Bt cotton expressing both Cry1Ac and Cry1Fa toxins for improved pest control.

The goal of this study was to test the hypothesis that HevCaLP is a functional receptor for Cry1A and Cry1F toxins using *Drosophila* Schneider 2 (S2) cells and a fluorescence-based cytotoxicity assay (*3*). To overcome potential limitations due to the existence of multiple Cry1A binding sites in HevCaLP, we used the full-length protein in our expression assays. In these assays, HevCaLP expressed on the surface of S2 cells bound radiolabeled Cry1A toxins under native and denaturing conditions, and this binding resulted in cytotoxicity. However, we did not detect binding of Cry1Fa toxin to HevCaLP, and consequently, this toxin was inactive against S2 cells expressing HevCaLP.

MATERIALS AND METHODS

Expression Vector Construction. A full-length cDNA clone encoding HevCaLP (GenBank accession number AAK85198) in pTOPO was generously provided by L. Gahan (Clemson University, Clemson, SC). The cDNA coding region was excised from pTOPO using *Eco*RI sites, cloned into the EcoRI sites of the pIZT/V5-His vector (Invitrogen), and transformed into *E. coli* strain DH5α. The insert of the resulting clone, pIZT-HvCad, was sequenced in both forward and reverse directions (Molecular Genetics Instrumentation Facility, University of Georgia). The pIZT-MsCad construct (*3*) contains the full-length cDNA clone encoding the BtR_{1a} allele (GenBank accession number AY094541), which differs from BtR1 (GenBank accession number AF319973) in two amino acids.

Preparation and Labeling of Bt Cry1 Toxins. Growth of bacterial strains, purification, and activation of Cry1A toxins were carried out as previously described (*19*). Bt strains HD-37 and HD-73, producing Cry1Aa and Cry1Ac proteins, respectively, were obtained from the *Bacillus* Genetic Stock Center (Columbus, OH), while a Bt strain producing Cry1Fa was obtained from Ecogen Inc. (Langhorne, PA). An *E. coli* strain producing Cry1Ab was provided by L. Masson (Biotechnology Research Institute, Montreal, PQ). Purified activated toxin samples (as determined by 10% SDS−PAGE) were pooled, quantified by the Bradford protein assay (*20*) using BSA as a standard, and stored at −80 °C until they were used for all the experiments. Purified toxins were tested for insecticidal activity before being used (data not shown).

Toxins (1 μg) were radiolabeled with 0.5 mCi of $Na^{125}I$ (Amersham) using Iodo-beads (Pierce) for Cry1Aa and Cry1Ab labeling, or chloramine-T (*21*) for Cry1Ac labeling. On the basis of the input toxin, specific activities were 14−25 μCi/μg of input toxin.

Preparation of BBMV. Midguts were dissected from fifth instar *H. virescens* larvae and stored at −80 °C until they were needed for the preparation of brush border membrane vesicles (BBMV). BBMV were prepared according to the method described in ref *22* and stored in PBS [135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4 (pH 7.5)] at −80 °C. The amount of protein was determined according to the method described in ref *20* using BSA as a standard. Aminopeptidase (APN) activity, using leucine *p*-nitroanilide as a substrate, was used as a marker for brush border enzyme enrichment in the BBMV preparations. APN activities were enriched 6−8-fold in the BBMV preparations compared to those in initial midgut homogenates.

Transient Expression of HevCaLP in Drosophila S2 Cells. Drosophila S2 cells were grown in serum-free insect cell medium (HyQ SFX-Insect, Hyclone) and transfected as previously described (*23*). Plasmid DNA used for transfections was prepared using a Plasmid Maxi Kit (Sigma). Approximately 2×10^6 S2 cells from a confluent culture were suspended in 5 mL of fresh medium and allowed to adhere to 60 mm × 15 mm polystyrene dishes (Falcon) overnight. Plasmid transfection mixtures were prepared by mixing either 5 μg of pIZT or 10 μg of either pIZT-HvCad or pIZT-MsCad plasmid with 1 mL of serum-free insect medium and 20 μL of Cellfectin reagent (Invitrogen). Transfection mixtures were incubated with cells at room temperature for 4 h; subsequently, transfection mixtures were discarded and fresh media added to the plates.

Detection of HevCaLP Expression on the Cell Surface. For localization of expressed HevCaLP on the S2 cell surface, cells were transfected as described above, and 48 h after transfection, cells were collected and seeded on the surface of a microscope slide cover slip. After overnight incubation for adhesion, cells were fixed in PBS containing 2% paraformaldehyde for 30 min. Cover slips were then washed three times for 5 min each with PBS and blocked for 3 h in PBST (PBS and 0.1% Tween 20) with 3% BSA and 2% goat serum. After being blocked, cells were probed with a 1:1000 dilution of anti-HevCaLP serum (*17*) overnight

at 4 °C in blocking buffer. Cells were washed three times for 15 min with blocking buffer at room temperature and then incubated in blocking buffer with a 1:100 dilution of goat anti-rabbit IgG−Texas red fluorophore (Vector Laboratories) for 2 h at room temperature. After being washed three times for 15 min in TBST, cover slips were mounted on microscope slides using Prolong Gold reagent (Molecular Probes). Cells were examined in an Olympus BX60 microscope equipped with appropriate filters for green fluorescent protein (GFP) or Texas red fluorescence.

Immunoblot and Ligand Blot Analyses. At 2.5 days post-transfection, cells were collected and counted in a hemocytometer. For dot blotting, cells were washed three times with PBS, and approximately 5×10^5 cells were blotted per dot on PVDF filters using a Bio-dot apparatus (Bio-Rad). For ligand blots and immunoblots, cells were harvested by centrifugation at 16000*g* for 1 min and then solubilized in 2× Laemmli buffer (Bio-Rad) containing 5% 2β-mercaptoethanol. Samples were heat denatured in a 100 °C bath for 10 min, and then approximately 3×10^5 solubilized cells or BBMV proteins (15 μg) were loaded per lane in SDS−8% PAGE gels. After electrophoresis, separated proteins were transferred to PVDF filters (Millipore) overnight. All filters were blocked with 3% BSA in PBST at room temperature for 1 h.

For immunoblots, filters were probed with a 1:5000 dilution of anti-HevCaLP serum in blocking buffer for 1 h. After the samples had been washed with PBST containing 0.1% BSA, filters were probed with a 1:20000 dilution of an anti-rabbit IgG−horseradish peroxidase conjugate (Sigma) at room temperature for 1 h. HevCaLP protein was detected using an enhanced chemiluminescence substrate (SuperSignal West Pico, Pierce) and photographic film.

For ligand and dot blots, filters were probed with 0.5 nM radiolabeled Cry1A toxin in washing buffer for 1 h at room temperature. After filters had been washed as described above, Cry1A toxin-binding proteins were detected by autoradiography at −80 °C. To compete with nonspecific toxin binding, a 500-fold excess of unlabeled homologous toxin was included in the binding reactions.

Pull-Down Assays. Binding of Cry1Ac and Cry1Fa toxins to HevCaLP transiently expressed in S2 cells or in solubilized BBMV proteins was tested using a pull-down assay. Purified activated toxins (1 mg) were coupled to 0.8 g of cyanogen bromide-activated Sepharose 4B beads (SIGMA) following the manufacturer's instructions. After coupling, beads were blocked with 0.2 M glycine (pH 8.0) for 2 h at room temperature. Beads were pelleted by centrifugation at 10000*g* for 10 min and rinsed seven times with alternate washes using 0.1 M $NaHCO_3$ (pH 8.4), 0.5 M NaCl, 0.1 M sodium acetate (pH 4.0), and 0.5 M NaCl. The final pellet was resuspended in 1.75 mL of solubilization buffer [0.1 M $NaHCO_3$ (pH 8.4), 0.5 M NaCl, 2% CHAPS, 1% Triton X-100, and Complete protease inhibitor tablets (Roche)].

Three days after transfection, S2 cells transiently expressing HevCaLP and control cells were collected and resuspended in solubilization buffer at 9.8×10^6 cells/mL. BBMV proteins (6 mg) were centrifuged at 14000*g* for 10 min and then resuspended in 4.5 mL of solubilization buffer. S2 cells and BBMV proteins were incubated in solubilization buffer for 15 min at room temperature and on ice for 1 h. For competition assays, 144 μg of the specific competitor toxin was incubated with the solubilized proteins for 10 min at room temperature before addition of the toxin-coupled beads. Beads with coupled toxin (200 μL) were incubated with solubilized BBMV or S2 cell protein samples (200 μL) in solubilization buffer (final volume of 800 μL) overnight at 4 °C with a constant rotation. As positive controls for binding to HevCaLP, we used Cry1Aa- and Cry1Ac-coupled beads. Beads with no toxin coupled and toxin-coupled beads incubated with solubilization buffer were used as negative controls. Samples were washed (14000*g* for 1 min) four times with 1 mL of ice cold solubilization buffer diluted 1:2 with 0.1 M $NaHCO_3$ (pH 8.4) and 0.5 M NaCl. After a final wash with 1 mL of coupling buffer, S2 cell proteins bound to the toxin beads were released by incubation with 75 μL of 2× Laemmli buffer (Bio-Rad) containing 5% 2β-mercaptoethanol and heat denaturing for 10 min. Samples were then centrifuged at 16000*g* for 3 min and supernatants collected. Samples (45 μL) were loaded on 7.5% SDS−PAGE gels, and after electrophoresis, proteins were transferred to PVDF filters. HevCaLP on blots was detected as described above for immunoblots.

Toxicity Assays. Cytotoxicity assays were carried out as previously reported (*3*) with minor modifications. S2 cells were transfected and incubated at 26 °C for 2.5 days, and then cultures were subdivided and seeded into six-well plates (Falcon) with fresh media and allowed to adhere overnight. The culture medium was replaced with fresh medium containing 20 μg/mL Cry1 toxin (toxin concentration of approximately 330 nM), and cultures were incubated for 4 h at room temperature. Cells were stained with 4 μM (final concentration) propidium iodide (PI) for 10 min and then immediately gated for GPF fluorescence and PI staining using a FACSCalibur (Becton Dickinson) flow cytometer. For GFP fluorescence, cells were excited with a krypton−argon laser, and emission was monitored with a 530/30 nm band-pass filter. GFP-gated cells were then examined for PI fluorescence by monitoring emission with a 585/42 nm band-pass filter.

A previously reported formula (*3*) was used to calculate the percentage of GFP-positive cells in the cell populations killed by Cry1 toxins. The formula accounts for the dead cells (PI-positive) in an untreated population, GFP-positive dead cells that lost GFP due to cell leakage, and the observed transfection efficiency (GPF-positive) for each experiment. The results shown are the means of four independent transfection experiments conducted in triplicate with 10 000 cells being sorted in each replicate.

RESULTS

HevCaLP Expression in S2 Cells. A cDNA encoding full-length HevCaLP protein was transiently expressed in *Drosophila* S2 cells using the pIZT expression vector which contains dual constitutive baculovirus promoters that control HevCaLP and GFP-zeocin expression. Expression of HevCaLP was detected by probing blots of total cell protein with anti-HevCaLP serum. A cross-reactive protein of ~200 kDa was detected only in cells transfected with pIZT-HvCad (Figure 1, lane 2). Smaller amounts of smaller-sized expression products were also detected, suggesting cadherin degradation as previously observed for *M. sexta* BtR_1 cadherin (*24*). In agreement with this observation and as previously

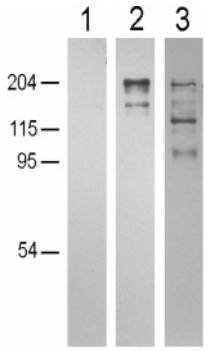

FIGURE 1: Detection of transiently expressed HevCaLP in BBMV and S2 cells transfected with pIZT-HvCad on blots. S2 cells (3×10^5) or BBMV proteins (15 μg) separated by SDS−8% PAGE were transferred to PVDF filters and probed with anti-HevCaLP sera: lane 1, S2 cells transfected with pIZT; lane 2, S2 cells transfected with pIZT-HvCad; and lane 3, *H. virescens* BBMV proteins.

reported (*17*), anti-HevCaLP serum detected at least four proteins of 195, 160, 135, and 100 kDa in brush border membrane vesicles (BBMV) prepared from *H. virescens* larvae (Figure 1, lane 3).

Because expressed HevCaLP had a leader peptide, the processed protein should localize to the exterior of the S2 cell membrane. We tested this possibility by incubating unpermeabilized cells with anti-HevCaLP serum. In these assays, we used GFP expression as a marker for transfection and an anti-rabbit IgG−Texas red conjugate to detect HevCaLP. GFP fluorescence was observed in ~60% of the S2 cells transfected with either pIZT or pIZT-HvCad plasmids. In contrast, only cells transfected with pIZT-HvCad were positive for anti-HevCaLP serum detection (Figure 2). According to the microscopic observations, all the cells transfected with pIZT-HvCad that expressed GFP were positive for HevCaLP expression, although the level of expression varied considerably. Given that cells were not permeabilized before immunodetection, these results are evidence of the expression of HevCaLP on the S2 cell surface.

Binding of Cry1A Toxins to Expressed HevCaLP. We previously reported that genetic knockout of the *BtR4* gene results in a lack of HevCaLP and a reduced level of binding of Cry1Aa toxin to *H. virescens* BBMV (*17*). Expression of HevCaLP on the surface of S2 cells allowed us to test binding of Cry1A toxin to HevCaLP devoid of the Cry1A binding aminopeptidases and alkaline phosphatase present in larval BBMV. Blots of proteins from S2 cells transfected with pIZT or pIZT-HvCad were probed with ^{125}I-labeled Cry1Aa, Cry1Ab, or Cry1Ac toxins (Figure 3A). S2 cells transfected with pIZT-MsCad were used as a positive control for toxin binding (*3*), and competition with a 500-fold excess of unlabeled homologous toxin to establish specificity of binding. All tested [^{125}I]Cry1A toxins bound a 210 kDa protein in S2 cells transfected with pIZT-MsCad, with the binding signal of Cry1Ab being the most intense. In blots of proteins from S2 cells expressing HevCaLP, each [^{125}I]Cry1A toxin bound specifically to a 200 kDa band (Figure 3A). [^{125}I]Cry1A toxins bound to additional proteins from S2 cells, yet binding was not challenged by excess unlabeled toxin, suggesting nonspecificity.

Because the denaturing conditions of ligand blotting can expose toxin binding sites not present under nondenaturing

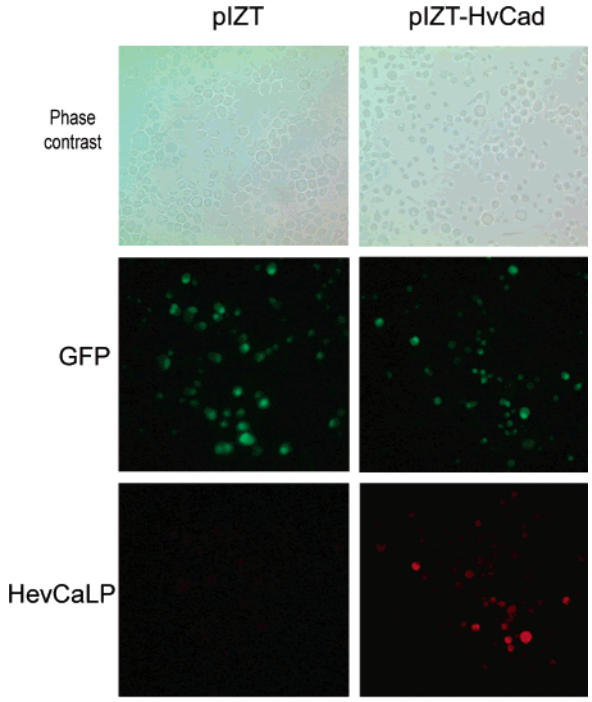

FIGURE 2: Immunocytochemical detection of HevCaLP expression on the surface of S2 cells. Cells were transfected with pIZT (left) or pIZT-HvCad (right). After transfection, cells were fixed and probed with antisera against HevCaLP. Proteins detected by the antisera were visualized using anti-rabbit sera conjugated to a Texas red fluorophore. Cells were examined for differential interference contrast (top), GFP expression (center), or HevCaLP expression on the cell surface (bottom).

conditions (*25*), we bound [^{125}I]Cry1A toxin to cells expressing HevCaLP under native conditions using dot blotting. Cells expressing BtR_{1a} were used as positive controls for binding. As shown in Figure 3B, all [^{125}I]Cry1A toxins bound to S2 cells expressing BtR_{1a}. As predicted by the ligand blot results, all the [^{125}I]Cry1A toxins bound to cells expressing HevCaLP, although with less intensity than the binding observed for cells expressing BtR_{1a}. In dot blotting experiments, [^{125}I]Cry1Ac was the toxin displaying the strongest binding to cells expressing HevCaLP.

Binding of Cry1Fa to HevCaLP. Because radiolabeling inactivates Cry1Fa toxin (*19*), we used pull-down assays with toxin coupled to Sepharose beads to test the binding of toxin to HevCaLP expressed in S2 cells or in *H. virescens* BBMV (Figure 4). No proteins were detected when using Sepharose beads incubated with solubilized cells (Figure 4, No toxin) or toxin-coupled beads incubated with solubilization buffer alone (Figure 4, No cells). In agreement with the results from ligand and dot blotting, both Cry1Aa and Cry1Ac toxin-coupled beads pulled down HevCaLP from solubilized S2 cells transfected with pIZT-HvCad or BBMV, but not from control cells transfected with pIZT (Figure 4, lanes 1Aa and 1Ac). As observed in the ligand blots, the HevCaLP signal was slightly more intense for Cry1Ac-coupled beads than for the Cry1Aa samples. Even though Cry1Fa-coupled beads pulled down several BBMV proteins (data not shown), Cry1F did not pull down HevCaLP from cells transfected with pIZT-HvCad or BBMV (Figure 4, lane 1Fa).

Since Cry1Aa and Cry1Fa share receptor site A in BBMV (*15*, *16*), we tested the possibility that Cry1Fa would inhibit binding of Cry1Aa toxin to HevCaLP on S2 cells. As shown

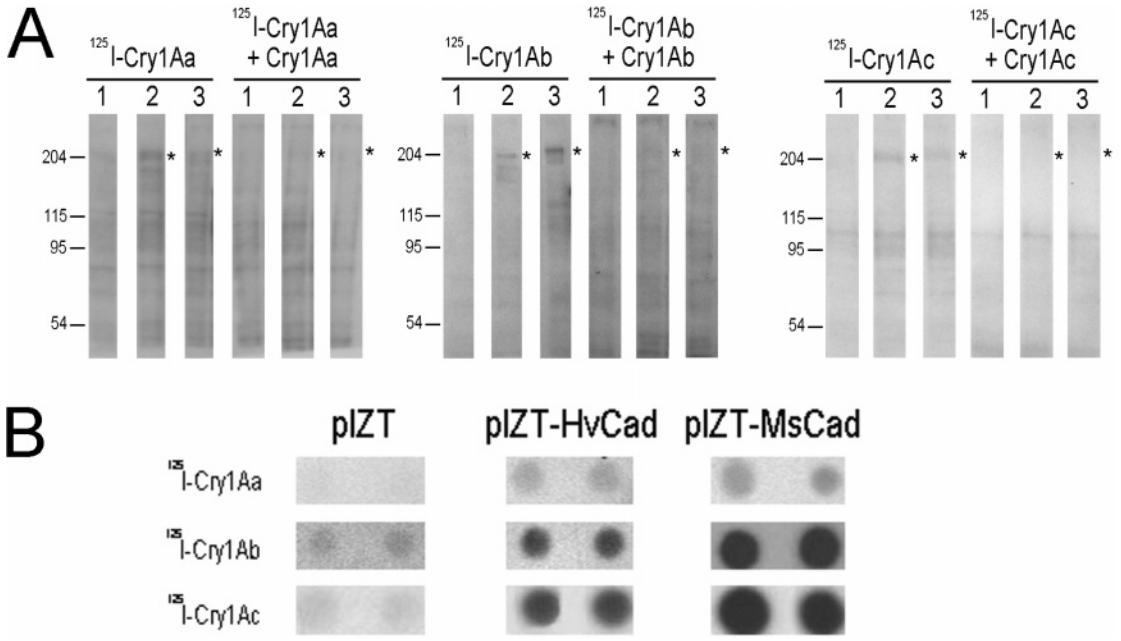

FIGURE 3: Detection of binding of [^{125}I]Cry1A to S2 cells expressing HevCaLP or BtR_{1a} on ligand blots (A) or dot blots (B). For ligand blots, S2 cell proteins were separated by SDS−8% PAGE and then transferred to PVDF filters. Filters were probed with [^{125}I]Cry1A toxins in the absence or presence of a 500-fold excess of unlabeled homologous competitor. Bound toxin was detected by autoradiography at −80 °C. The time of film exposure was determined by the appearance of the ~200 kDa band. Asterisks denote the position of HevCaLP as detected by probing the same filters with sera against HevCaLP: lane 1, S2 cells transfected with pIZT; lane 2, S2 cells transfected with pIZT-HvCad; and lane 3, S2 cells transfected with pIZT-MsCad. For dot blots, cells were dotted on PVDF filters and probed with [^{125}I]Cry1A toxins. Bound toxin was detected as it was for ligand blots.

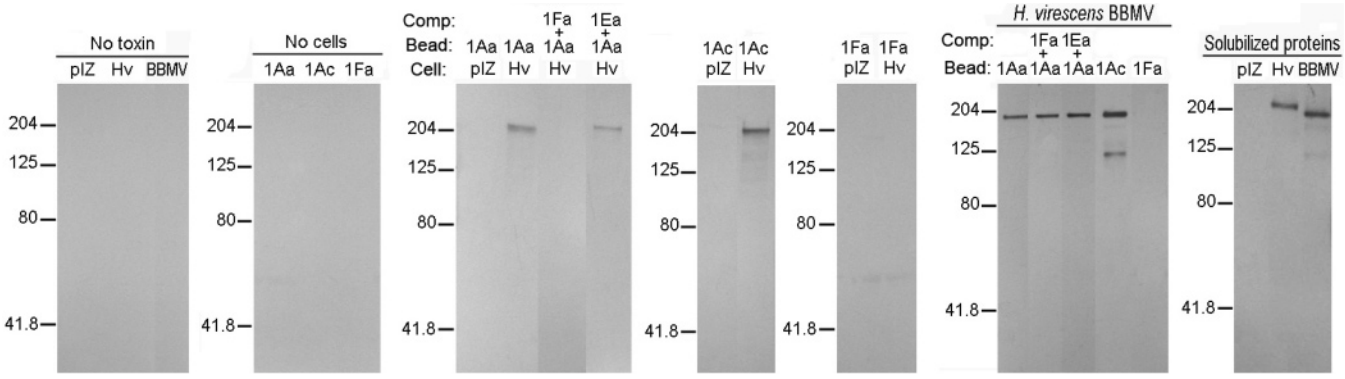

FIGURE 4: Pulled-down HevCaLP expressed on S2 cells or from *H. virescens* BBMV by Cry1 toxins (Cry1Aa, Cry1Ac, and Cry1Fa) coupled to Sepharose beads. Solubilized S2 cells transfected with pIZT (pIZ lanes), pIZT-HvCad (Hv lanes), or BBMV proteins were incubated with Sepharose beads alone (No toxin) or with beads coupled with Cry1Aa, Cry1Ac, or Cry1Fa as designated above each lane. To test the competition of Cry1Fa for binding of Cry1Aa toxin to HevCaLP, Cry1Fa was incubated with the solubilized BBMV or cell proteins before adding the Cry1Aa-coupled beads. As a negative control for Cry1Aa toxin binding competition, we used Cry1Ea. Cell or BBMV proteins bound to the toxin beads were solubilized, separated by SDS−7.5% PAGE, and blotted on PVDF filters. HevCaLP on the filters was detected with sera against the protein and enhanced chemiluminescence. Controls of Cry1Aa-, Cry1Ac-, and Cry1Fa-coupled beads incubated with solubilization buffer alone (No cells) and detection of HevCaLP in solubilized S2 cells or BBMV proteins (Solubilized proteins) are also shown. Results are representative of five independent experiments conducted with four independently transfected cell cultures and two independently purified toxin batches. BBMV results are representative of four independent experiments.

in Figure 4, a Cry1F preincubation step resulted in greatly reduced amounts of HevCaLP being pulled down from S2 cells by Cry1Aa-coupled beads. Conversely, preincubation of solubilized BBMV proteins with Cry1Fa did not affect binding of Cry1Aa to HevCaLP. When solubilized BBMV or cell proteins were preincubated with Cry1Ea, a toxin that does not compete for binding of Cry1Aa to *H. virescens* BBMV (*15*), the amounts of HevCaLP pulled down by Cry1Aa-coupled beads were comparable to controls (Figure 4, lanes 1Aa and 1Ea).

Cytotoxicity Assays. Cry1 toxin binding is necessary but not sufficient to cause insect mortality (*19*, *26*). To test whether binding of Cry1 toxin to HevCaLP mediates cytotoxicity, we used a flow cytometry approach and PI to detect cells permeabilized by toxin treatment (*3*). Using GFP fluorescence as a transfection marker and the previously reported formula (*3*), we calculated the number of transfected cells responding to toxin treatment. Each Cry1A toxin was cytotoxic to S2 cells expressing the BtR_{1a} protein, with Cry1Ac being the most active toxin (Figure 5). In the case of cells expressing HevCaLP, similar levels of toxicity were observed after treatment with Cry1Aa or Cry1Ab toxins, while Cry1Ac was the most active toxin, with almost 15% dead cells after toxin treatment. Differential toxin suscepti-

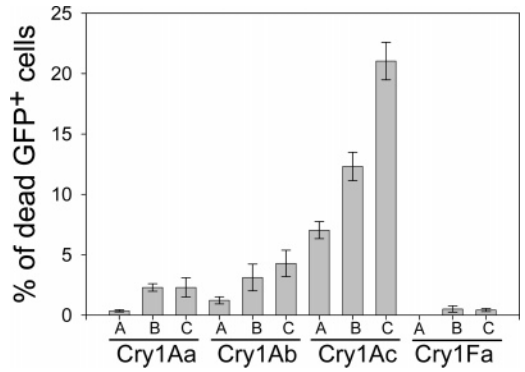

FIGURE 5: Percentage of dead GFP-positive cells after treatment with the indicated Cry1 toxins. S2 cells were transfected with pIZT (column A), pIZT-HvCad (column B), or pIZT-MsCad (column C), and 3 days after transfection, cells were treated with activated toxins as described in Materials and Methods. Cells were collected and incubated with propidium iodide before 10 000 cells per treatment were gated using a FACSCalibur (Becton Dickinson) flow cytometer. Each cell was gated for live (PI−) or dead (PI+) and GFP fluorescence (GFP+ or GFP−). The mean percentages of dead GFP+ cells are shown in the graph as calculated using a formula to account for background mortality and GFP− cells in the cultures (*3*). Bars denote the standard error of the mean percentage calculated from cytotoxicity assays carried out in triplicate with cells from four independent transfection events.

bility directly correlated with toxicities observed in bioassays with *H. virescens* larvae (*15*). Cry1Ac also exhibited some cytotoxicity toward control cells, although at a level lower than that to cells expressing HevCaLP. In agreement with the binding assays, Cry1Fa did not kill cells expressing HevCaLP, even when using 80 μg/mL toxin (data not shown), evidence that expression of HevCaLP is not sufficient to confer Cry1Fa susceptibility.

DISCUSSION

Previously (*17*), we hypothesized HevCaLP to be a component of binding site A, the shared binding site for Cry1A, Cry1Ja, and Cry1Fa toxins present in *H. virescens* BBMV (*15*, *16*). Given that both Cry1Ac and Cry1Fa are expressed in second-generation Bt cotton, we were especially interested in testing whether HevCaLP is a functional receptor for both Cry1Ac and Cry1Fa toxins. Our results with HevCaLP expressed on the surface of S2 cells are evidence of the role of this cadherin as a Cry1A but not a Cry1Fa receptor.

The level of toxicity observed in our cell assays is comparable to previous reports with BtR_{1a} cadherin expressed on S2 cells (*3*), yet it is lower than we predicted for a functional receptor. Higher cytotoxicity levels in cells expressing BtR_1 were reported in ref *2* using Hi5 cells. Although we attempted to reproduce these results by expressing HevCaLP in Hi5 cells, the high level of susceptibility of this cell line to Cry1Ac (*27*) made cytotoxicity analyses problematical. Higher toxicity levels were observed when increasing Cry1Ac concentrations were used, although background toxicity also increased proportionally (data not shown). Low cytotoxicity levels suggest that either the assay conditions are not optimal for Cry1A toxicity or additional receptor molecules are required to produce higher cytotoxicities. However, both Cry1Ac and Cry1Fa killed up to 95% of Hi5 cells in cytotoxicity assays under the same conditions that were used for S2 cells, suggesting that both toxins are fully active under the conditions used for the assay. It is still possible, however, that the conditions used are not optimal for the intoxication steps effected by HevCaLP.

The alternative explanation that more than one receptor is required to obtain high levels of toxicity has been previously proposed (*1*, *3*). Midgut alkaline phosphatases (*28*) and aminopeptidases (*29*, *30*) have been reported as alternative Cry1Ac receptors in *H. virescens*. However, the 110 kDa aminopeptidase from *H. virescens* that binds Cry1Ac and Cry1Fa toxins did not promote cytotoxicity when expressed on S2 cells (*23*). Reduced levels of an alkaline phosphatase (HvALP) correlate with resistance to Cry1Ac toxin, even though the specific functional role of this protein as a toxin receptor has not been established. Coexpression of HevCaLP, HvALP, and aminopeptidases on insect cell cultures should help characterize the specific role of each of these proteins in the intoxication process.

As described in other Lepidoptera (*31*, *32*), Cry1Fa binds to a Cry1A binding site in BBMV from *H. virescens* larvae (*15*). Previously, we reported binding of Cry1Fa to a 210 kDa protein detected in blots of BBMV proteins from *H. virescens* larvae (*15*) and among the Cry1Ac binding proteins purified from *H. virescens* BBMV using an affinity chromatography approach similar to our pull-down assays (*33*). On the basis of the molecular size and Cry1Ac binding features, we predicted this 210 kDa protein to be HevCaLP. However, our pull-down assay results do not support this prediction. Either the 210 kDa protein recognized by Cry1Fa on blots is not HevCaLP, or Cry1Fa's affinity for HevCaLP is too weak to precipitate HevCaLP in the pull-down assay. Regardless, Cry1F toxin was inactive against S2 cells transiently expressing cadherin, evidence that HevCaLP is not a functional receptor for Cry1Fa.

Interestingly, we detected inhibition of interactions between Cry1Aa toxin and HevCaLP by Cry1Fa in our pull-down assays with solubilized S2 cell proteins but not with BBMV. Inhibition of Cry1Aa−HevCaLP interactions may be explained if Cry1Fa binds HevCaLP only at a low-affinity site. In this model, Cry1Fa reduces the level of Cry1Aa-bead pull down of HevCaLP in S2 cells, but not in solubilized BBMV where high-affinity sites for Cry1Fa are present. This explanation is consistent with previous reports of Cry1Fa competing with binding of Cry1Aa toxin to BBMV (*15*).

According to our results, binding of Cry1A toxin to HevCaLP results in cell mortality. Therefore, HevCaLP alteration would potentially result in resistance to Cry1A but not Cry1Fa toxins. Consequently, Cry1Fa cross resistance observed in Cry1Ac-resistant *H. virescens* strains (*18*) that do not express HevCaLP (*17*) is probably due to the existence of additional resistance mechanisms in these larvae. In agreement with this hypothesis, resistance in larvae from the CP73-3 strain, which are cross-resistant to Cry1Fa (J. L. Jurat-Fuentes, unpublished observation), is not linked to alterations in *BtR4* (*35*). Similarly, even though a shared Cry1A−Cry1Fa receptor exists in *Plutella xylostella* larvae (*32*), Cry1Ac resistance and Cry1Fa cross resistance in specific strains of this insect (*36*) are not linked to alterations in cadherin genes (*37*). Further research aimed at identifying the shared Cry1Ac−Cry1Fa toxin functional receptors is needed to design measures to delay the evolution of

Cry1Ac−Cry1Fa cross resistance. Considering that our results identify HevCaLP as a key Cry1A receptor, this cadherin should be one of the molecules to be considered for resistance monitoring and Cry1A toxin improvement.

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