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Bt-R_{1a} Extracellular Cadherin Repeat 12 Mediates *Bacillus thuringiensis* Cry1Ab Binding and Cytotoxicity*

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The cadherin protein Bt-R_{1a} is a receptor for *Bacillus thuringiensis* Cry1A toxins in *Manduca sexta*. Cry1Ab toxin is reported to bind specific epitopes located in extracellular cadherin repeat (CR) 7 and CR11 on Bt-R_1 (Gomez, B., Miranda-Rios, J., Riudino-Pinera, E., Oltean, D. I., Gill, S. S., Bravo, A., and Soberon, M. (2002) *J. Biol. Chem.* 277, 30137–30143; Dorsch, J. A., Candas, M., Griko, N., Maaty, W., Midboe, E., Vadlamudi, R., and Bulla, L. (2002) *Insect Biochem. Mol. Biol.* 32, 1025–1036). We transiently expressed CR domains of Bt-R_{1a} in *Drosophila melanogaster* Schneider 2 (S2) cells as fusion peptides between a signal peptide and a terminal region that included membrane-proximal, membrane-spanning, and cytoplasmic domains. A domain consisting of CR11 and 12 was the minimal ^{125}I-Cry1Ab binding region detected under denaturing conditions. Only CR12 was essential for Cry1Ab binding and cytotoxicity to S2 cells when tested under native conditions. Under these conditions expressed CR12 bound ^{125}I-Cry1Ab with high affinity (K_{com} = 2.9 nM). Flow cytometry assays showed that expression of CR12 conferred susceptibility to Cry1Ab in S2 cells. Derivatives of Bt-R_{1a} with separate deletions of CR7, 11, and 12 were expressed in S2 cells. Only deletion of CR12 caused loss of Cry1Ab binding and cytotoxicity. These results demonstrate that CR12 is the essential Cry1Ab binding component on Bt-R_{1a} that mediates Cry1Ab-induced cytotoxicity.

Insecticidal Cry1 proteins from *Bacillus thuringiensis* (Bt) are used in biopesticides and transgenic crops (3). Each Cry1 protein is highly, yet selectively, toxic to some lepidopteran species. In a susceptible insect the process of intoxication begins with ingestion of the Cry protein and culminates in insect mortality. Proteinases in the alkaline midgut activate the Cry protein to a toxin that binds with high affinity to receptors in the brush-border epithelium. In the case of the cadherin receptor Bt-R_1, toxin binding initiates a conformational change that probably results in assembling of a pre-pore toxin oligomer (4). Aminopeptidases also bind Cry1 toxins and catalyze toxin-induced pore formation (5–7). An emerging model suggests that after binding cadherin, the toxin binds aminopeptidase and inserts into membrane microdomains called lipid rafts (8). At the midgut level, pore formation in epithelial cells leads to cell lysis and insect mortality.

Cadherin-like proteins are receptors for Cry1A toxins in lepidopteran species. Bt-R_1 from *Manduca sexta* (9) is a classical cadherin protein with predicted adhesive function and Ca^{2+} binding sites (2). This protein contains tandem repeats of homologous extracellular domains, called cadherin repeats (CR),[1] which are numbered from the outermost 1 to the innermost 12, a membrane-proximal extracellular domain (MPED), a membrane-spanning domain, and a cytoplasmic domain. Cry1A toxins bind Bt-R_1 and Bt-R_{1a} with high affinity (9–11) and when expressed on the surface of COS-7 and *Drosophila* S2 cells induce Cry1A cytotoxicity (2, 11). Bt-R_{1a} (AY094541) differs from Bt-R_1 (AF319973) by two amino acid substitutions (H350L and A1189E) (11). The cadherin-like protein BtR175 from *Bombyx mori* serves as a Cry1Aa receptor (12), and when expressed in Sf9 cells it promotes swelling after exposure to Cry1Aa toxin because of formation of ion channels in cell membranes (13). When expressed in mammalian COS-7 cells, BtR175 induced susceptibility to Cry1Aa (14). Elimination or alteration of cadherin-like proteins from the midgut correlates with insect resistance to *B. thuringiensis* Cry proteins in *Heliothis virescens* (15) and *Pectinophora gossypiella* (16).

Regions of domain II of Cry1A toxins bind specific sites on Bt-R_1 (17). Gomez *et al.* (1, 17) identified Cry1Ab toxin binding region 1 (TBR1), consisting of seven amino acid residues located in CR7. Dorsch *et al.* (2) identified a second Cry1Ab binding region (TBR2) within CR11. Recombinant and synthetic peptides containing both amino acid sequences inhibited Cry1Ab toxicity *in vivo* when fed to *M. sexta* larvae (2, 17), demonstrating their involvement in toxicity.

Our goal was to examine the ectodomain units of Bt-R_{1a} for interaction with Cry1Ab and their potential for mediating toxicity in a fluorescent-based *Drosophila* S2 assay (11). We focused on the region from CR7 to CR12 because it includes the reported Cry1A-binding epitopes (2, 17). Truncated Bt-R_{1a} fragments were coexpressed with green fluorescence protein (GFP) in S2 cells and tested for Cry1Ab binding using ligand blots, dot-blots, and binding saturation assays. Cytotoxicity to S2 cells was measured using propidium iodide (PI) and flow cytometry. Results from binding and cell toxicity assays suggest that Cry1Ab has two binding sites located on CR11 and 12 of Bt-R_{1a}. The CR12 domain bound Cry1Ab and conferred susceptibility to Cry1Ab when expressed in S2 cells. Cry1Ab binding to CR11 was dependent on the presence of CR12. Neither toxin binding nor toxicity was detected in cells expressing CR7.

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[1] The abbreviations used are: CR, cadherin repeat; BBMV, brush-border membrane vesicle; BSA, bovine serum albumin; GFP, green fluorescence protein; MPED, membrane-proximal extracellular domain; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinylidene difluoride; S2, Schneider 2; TBR, toxin binding region.

Our results identify CR11 and CR12 as Cry1Ab binding regions and CR12 as the functional Cry1Ab receptor region on Bt-R_{1a}.

MATERIALS AND METHODS

Cloning of Truncated Bt-R_{1a} Fragments—Cadherin *Bt-R_{1a}* (GenBank AY094541) was used for constructing a series of truncated cadherin fragments in the insect cell expression vector pIZT (Invitrogen). Primers were manufactured by Integrated DNA Technologies, Inc. (IDT). The Bt-R_{1a} signal sequence was amplified by PCR with primers 5′-AAAAACTAGTGATTGGCAGCAAAACCATCTGCAGC-3′ and 5′-ACTTCCATGGAGCTAAAACTGCAGGCGCTATAAAC-3′. The resulting PCR product was cloned into pMECA (GenBank AF017063 (18)) using SpeI and NcoI sites to generate pMECA-Cad/S1.9. pMECA-Cad/S1.9 was digested with NcoI and XhoI to eliminate most of the 1.9 kb *Bt-R_{1a}* insert, resulting in the pMECA-Cad construct. A *Bt-R_{1a}* fragment encoding the MPED, and part of the transmembrane domain was amplified by PCR with primers 5′-CTACCCATGGTTCGTCAACACGCTGCAACAGGTCG-3′ and 5′-GAGGCTCGAGTCAGGCGCCGAGTCCGGGCTGGAGT-3′ and cloned into pMECA-Cad in NcoI and XhoI sites to yield plasmid pMECA-Cad-MPED. *Bt-R_{1a}* fragments were amplified by PCR and cloned into pMECA-Cad-MPED using NcoI. CR7 fragment was amplified using primers 5-GAACCCATGGGTGCGCGAGATGTCAGCGGGCGGGC-3′ and 5′-TCTCCCATGGATACACGACGGTATCGAACTTAGTC-3′. A fragment including CR7 to the *BtR_{1a}* stop codon (includes CR7–12) was amplified using primers 5′-GAACCCATGGGTGCGCGAGATGTCAGCGGGCGGGC-3′ and 5′-TCCCTTGAGGTCCATGGCGGTCTCCAGCTCTCCCG-3′. Primers for fragments encoding CR10, CR11, or CR12 to the end stop codon (referred to as CR10–12, CR11–12, and CR-12, respectively) were 5′-ACCTCCATGGTTCGTCTTCCCGACCAACGATGCCG-3′, 5′-ACCTCCATGGGCGTCCTCAGAACATGCTGTCGCTT-3′, and 5′-CACCCCATGGATATCCACAGCGGACTCCATCGGCA-3′ as forward primers, which were used to amplify the fragments with the reverse primer, 5′-GAGGCTCGAGTCAGGCGCCGAGTCCGGGCTGGAGT-3′. Primers 5′-GCCCCCATGGGGAGAACCTAGATTCGCGTCCTCAG-3′ and 5′-CTGTCCATGGCCCTGCGGTGTACAATTCCCTCATA-3′ were used to amplify CR11. To construct the reversed order CR12–11 peptide, CR11 and 12 units were individually PCR amplified with terminal NcoI and XbaI sites. The PCR fragments were digested with XbaI, ligated to form CR12–11, and then CR12–11 was cleaved with NcoI and cloned into pMECA-Cad-MPED.

All truncated *Bt-R_{1a}* constructs in pMECA-Cad-MPED were excised with SpeI and SacII and cloned into pIZT to yield pIZT-Cad7, pIZT-Cad7–12, pIZT-Cad10–12, pIZT-Cad11, pIZT-Cad11–12, pIZT-Cad12–11, pIZT-Cad12 and pIZT-Cad-MPED. *Bt-R_{1a}* was cloned into pIZT as described previously (11), resulting in the pIZT-Cad construct. Deletions of CR7, CR11, or CR12 in pIZT-Cad-full were constructed using Elongase Enzyme Mix (Invitrogen) to amplify pIZT-CadD7, pIZT-CadD11, and pIZT-CadD12 directly. The primers for D7, D11, and D12 were designed as follows: 5′-CGTCGAATTCATTTACGAGAACGCAACCCACT-3′ and 5′-TCTCGAATTCGCGGAAGTTCTGCTCCAGAGTC-3′; 5′-CACCGAATTCATATCCACAGCGGACTCCATCG-3′ and 5′-CTGAGAATTCGAATCTAGGTTCTCCTTGCGTG-3′; 5′-CTACGAATTCTTCGTCAACACGCTGCAACAGG-3′ and 5′-CTGTGAATTCCCCTGCGGTGTACAATTCCCTC-3′, respectively. PCR fragments were cleaved with EcoRI, purified, and ligated overnight at 15 °C. The coding regions of all Bt-R_{1a} expression plasmid vectors were sequenced in both forward and reverse directions at the Molecular Genetics Instrumentation Facility (University of Georgia).

Transient Expression of Bt-R_{1a} Cadherin and Bt-R_{1a} Fragments in Drosophila S2 Cells—Drosophila S2 cells (Invitrogen) were grown in serum-free insect cell medium (Hyclone) and transfected as described previously (19). DNA for pIZT and pIZT-Cad plasmids was prepared using a Plasmid Maxi Kit (Qiagen). Approximately 1.5×10^6 S2 cells from a confluent culture were resuspended in 5 ml of fresh medium and allowed to adhere to polystyrene dishes (Falcon) overnight. Plasmid transfection mixtures were prepared by mixing either 5 μg of pIZT-truncated cadherins or 20 μg of pIZT-Cad plasmid with 1 ml of serum-free insect medium (Hyclone) and 10 μl of Cellfectin reagent (Invitrogen). 4 ml of medium containing 50 units/ml penicillin-streptomycin mixture (Invitrogen) were added to the cultures, and cells were incubated at 25 °C for 2.5 days.

Purification and Labeling of Cry1Ab Toxin—Procedures for production and purification of Cry1Ab-containing inclusion bodies in an *Escherichia coli* strain carrying the *B. thuringiensis* NRD-12 *cry1Ab* toxin gene are described elsewhere (20). Activated toxin was prepared and purified according to Ref. 21. Purified toxin was quantified by the Bradford protein assay (22) using BSA as standard and stored at −80 °C until used.

Purified Cry1Ab toxin (10 μg) was radiolabeled with 0.5 mCi of $Na^{125}I$ (Amersham Biosciences) using IODO-BEADS (Pierce) following the manufacturer's instructions. Specific activity, based on input toxin, was 4.1 μCi/μg.

Brush-border Membrane Vesicle (BBMV) Preparation—Midguts were dissected from 2nd-day 5th instar *M. sexta* larvae and stored at −80 °C until used to prepare BBMV. BBMV were prepared according to Ref. 23 and stored at −80 °C in 300 mM mannitol, 5 mM EGTA, 17 mM Tris, pH 7.5, until needed. The protein amount was determined according to Ref. 22 using BSA as standard.

Immunoblot and Ligand Blot Analysis—Drosophila S2 cells were seeded and transfected as described above. For dot-blotting, transfected cells were collected and counted with a hemocytometer. Duplicate aliquots of 5×10^5 cells were resuspended in PBS (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5) containing 0.1% BSA, and dot-blotted on PVDF filters (Millipore Corp., Bedford, MA) using a Bio-dot apparatus (Bio-Rad). Filters were blocked in blocking buffer (PBS, 3% BSA, and 0.1% Tween 20) for 1 h and incubated with 0.25 nM ^{125}I-labeled Cry1Ab for 1 h in washing buffer (PBS, pH 7.5, 0.1% BSA, and 0.1% Tween 20). Nonspecific binding was detected by including 500-fold excess Cry1Ab with radiolabeled toxin in the incubation mixtures. After washing, filters were exposed to x-ray film at −80 °C overnight.

For immunoblots and ligand blotting, 1×10^7 transfected S2 cells were harvested by centrifugation at $400 \times g$ for 2 min followed by three washes in PBS. Cell pellets were suspended in SDS-PAGE sample buffer (24) and separated on a SDS-polyacrylamide 4–20% gel (Bio-Rad) as described previously (11). After electrophoresis, proteins were transferred to PVDF filters. Filters were blocked with 5% skim milk in PBST (PBS + 0.1% Tween 20) for 1 h at room temperature before probing with anti-Bt-R_1 serum in PBST containing 0.1% skim milk for 2 h or with 0.25 nM ^{125}I-Cry1Ab for 1 h.

Bound antibodies were visualized by probing with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and chemiluminescent substrate (ECL; Amersham Biosciences). S2 cell proteins binding ^{125}I-Cry1Ab on ligand blots were detected by autoradiography at −80 °C.

Specific Binding of ^{125}I-Cry1Ab to Cadherin Fragments Expressed on S2 Cells—Saturation of specific ^{125}I-Cry1Ab binding to S2 cells transfected with pIZT or pIZT-cadherin fragments was measured according to Ref. 11. Increasing amounts of ^{125}I-Cry1Ab were incubated with a constant number of S2 cells (1.6×10^5 S2 cells) for 1 h at room temperature in 100 μl of binding buffer (PBS plus 0.1% BSA). Binding reactions were stopped by centrifugation, and cell pellets were washed once with 1 ml of ice-cold binding buffer. Radioactivity of the final pellets was counted in a Beckman model 4000 Gamma detector. Nonspecific binding was measured as the amount of ^{125}I-Cry1Ab toxin bound in the presence of unlabeled Cry1Ab toxin at a concentration 100-fold higher than the highest ^{125}I-Cry1Ab concentration used in the assays. Specific binding was calculated as the difference between total and nonspecific binding. The dissociation constant (K_{com}) and concentration of binding sites (B_{max}) for ^{125}I-Cry1Ab were calculated using the KELL software (BIOSOFT, Cambridge, UK). Data shown are the means of two independent experiments done at least in duplicate.

Cell Toxicity Assays—Toxin treatments were conducted on two dishes containing 3×10^6 transfected S2 cells, two samples of 10,000 cells from each dish being analyzed by flow cytometry. Fresh medium with 20 μg/ml Cry1Ab toxin (~330 nM toxin concentration) was added, and dishes were incubated with gentle shaking for 2.5 h. Cells were then stained with 4 μM (final concentration) of PI for 10 min and then gated for size determination by comparing forward and side scattered light dual parameters using a BD Biosciences FACSCalibur instrument. For GFP fluorescence gating, cells were excited with 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. We used the formula described previously (11) to calculate the percentage of GFP-positive cells in the pIZT-Cad cell populations killed by Cry1Ab toxin. This formula accounts for the dead cells (PI-positive) in an untreated population, GFP-positive dead cells that lost GFP because of cell leakage, and the observed transfection efficiency (GPF-positive) for an experiment. The results are the mean of three independent experiments done at least in triplicate.

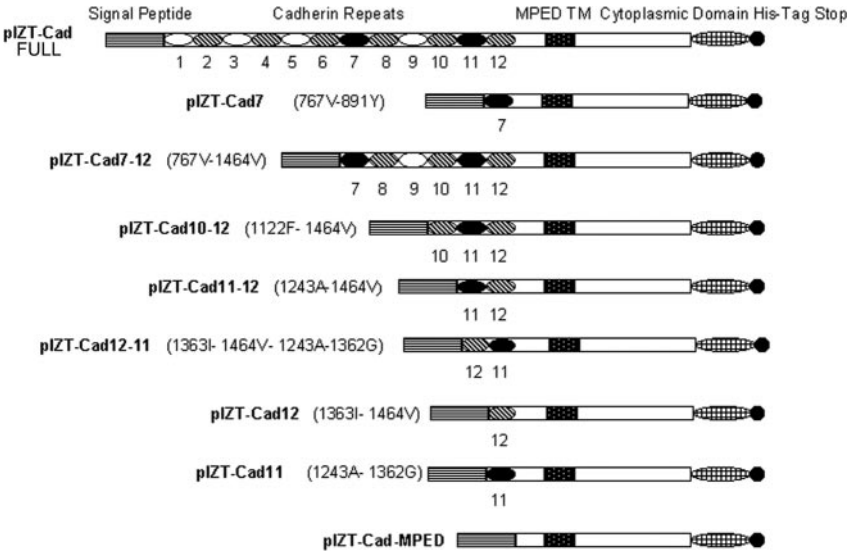

FIG. 1. **Diagram of truncated Bt-R$_{1a}$ cadherin ectodomains expressed on the surface of *Drosophila* S2 cells using the vector pIZT-V5-His.** Plasmids are designated for the cadherin repeat units encoded. *Numbers* in *parentheses* indicate the amino acid residues of the truncated cadherin start and end positions. CR7 and 11 (in *black*) contain TBR1 and 2, respectively.

RESULTS

Truncated Bt-R$_{1a}$ peptides were expressed on the surface of S2 cells to identify regions that bound Cry1Ab and induced cytotoxicity. Previous studies identified Cry1Ab binding regions TBR1 (1) and TBR2 (2), amino acid residues 865–875 and 1291–1360, respectively, which are located in CR7 and CR11. Truncated versions of *Bt-R$_{1a}$* were constructed in pIZT so that the repeated CR units of Bt-R1a were preceded by the signal peptide and followed by the C-terminal region (including the membrane-proximal extracellular, membrane-spanning, and cytoplasmic domains) (Fig. 1). For example, pIZT-Cad7, referred to subsequently as Cad7, was designed to express the CR7 unit attached to the MPED on the S2 cell surface. pIZT-Cad7–12, abbreviated Cad7–12, would be expressed as CR7–12 attached to the MPED. Full-length and truncated Bt-R$_{1a}$ peptides were detected on immunoblots of S2 cell protein by sera against Bt-R$_1$ (Fig. 2). Immunorecognition of full-length Bt-R$_{1a}$, Cad7–12, and the smallest peptide, Cad-MPED, was stronger than for the other truncated peptides.

Truncated Bt-R$_{1a}$ peptides expressed from Cad7–12, Cad10–12, and Cad11–12 bound ^{125}I-Cry1Ab on blots (Fig. 2). However, the individual CR units (7, 11, or 12) contained in Cad7, Cad11, and Cad12 and the MEPD (*i.e.* Cad-MPED) did not bind Cry1Ab. In agreement with (2), the Cry1Ab binding region detected on ligand blots is contained in CR11–12. To test whether the order of the CR units is important for toxin binding, we produced Cad12–11 in S2 cells. Expressed Cad12–11 did not bind Cry1Ab on blots, suggesting that position of CR11 and 12 is crucial for Cry1Ab binding on blots.

Because proteins on blots are denatured, and denaturation can expose epitopes that do not occur in natural conditions (25, 26), we tested Cry1Ab binding to Bt-R$_{1a}$ peptides under native conditions. S2 cells expressing full-length and truncated cadherin peptides were dotted on filters and probed with ^{125}I-Cry1Ab. As seen in Fig. 3, peptides containing CR12 attached to the MPED (full-length cadherin, Cad7–12, Cad10–12, Cad11–12, and Cad12) bound ^{125}I-Cry1Ab. As is apparent in Fig. 3 and when radioactivity of the individual dots was counted (data not shown), Cad11–12 containing CR11 and 12 bound more Cry1Ab toxin than the other peptides. In contrast to the ligand blot results, CR11 was not required for Cry1Ab binding to nondenatured Bt-R$_{1a}$. CR12 was the only repeated unit essential for Cry1Ab binding. The reversed order peptide, Cad12–11, did not bind Cry1Ab on dot-blots, suggesting either that the CR11 and 12 order is involved in binding, or the membrane-proximal region participates with CR12 in binding

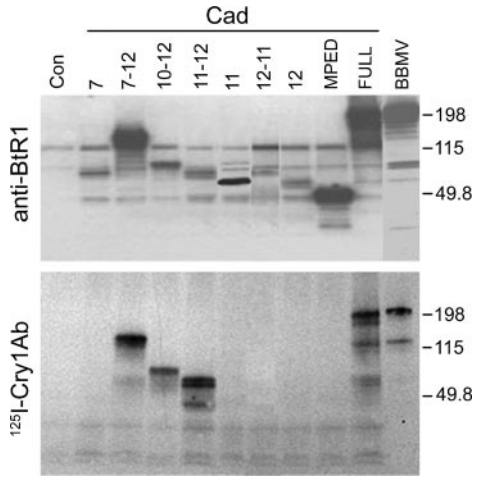

FIG. 2. **Expression of full-length and truncated Bt-R$_{1a}$ in S2 cells and Cry1Ab toxin binding to expressed protein on ligand blots.** Cell proteins were separated by SDS-PAGE and then transferred to PVDF filters. After blocking, filters were probed with anti-Bt-R$_1$ serum or ^{125}I-Cry1Ab. *Con lane*, S2 cells transfected with pIZT vector; other *lanes* correspond to Bt-R$_{1a}$ constructs shown in Fig. 1; BBMV, from *M. sexta*.

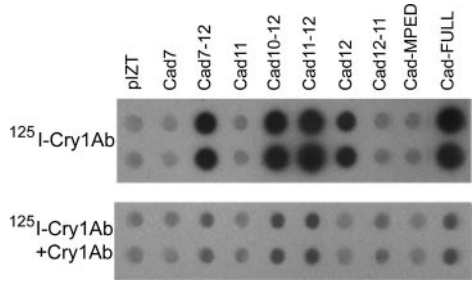

FIG. 3. **Cry1Ab binding to truncated Bt-R$_{1a}$ expressed on S2 cells under nondenaturing conditions.** S2 cells (5×10^5) were dot-blotted on PVDF filters. After blocking, the filters were probed with ^{125}I-Cry1Ab or ^{125}I-Cry1Ab plus 500-fold excess unlabeled Cry1Ab.

or spatially positions CR12 proximal to the membrane surface.

Bt-R$_{1a}$ expressed on S2 cells demonstrated saturable and high affinity (K_{com} = 3 nM) binding to Cry1Ab (11). To calculate the parameters of Cry1Ab binding to expressed Bt-R$_{1a}$ fragments, we performed Cry1Ab binding saturation assays by incubating a constant number of S2 cells with increasing concentrations of ^{125}I-Cry1Ab. Results from saturation binding

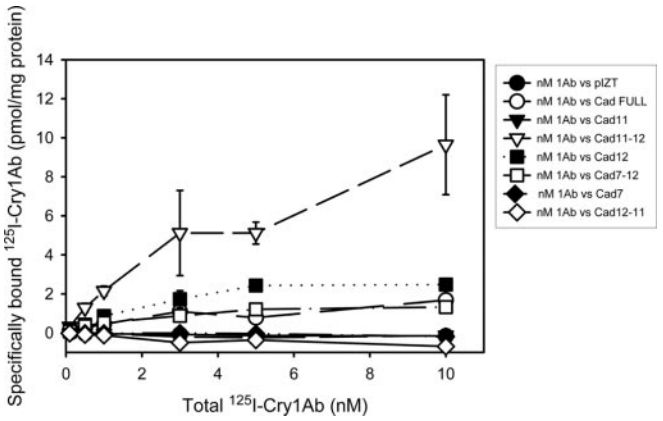

FIG. 4. **Saturation of ^{125}I-Cry1Ab binding to S2 cells expressing truncated cadherin.** Cells transfected with the indicated plasmid were incubated with increasing ligand (^{125}I-Cry1Ab) concentrations for 1 h. Data shown are specific binding values determined by subtraction from total binding of nonspecific binding in the presence of 1,000 nM unlabeled toxin. *Bars* depict the S.D. calculated from at least three replicates.

assays agreed with dot-blot binding assays. ^{125}I-Cry1Ab bound saturably to Cad7–12, 11–12, 12, and full-length cadherin (Fig. 4). Although quantitative binding analyses to Cad11–12 showed a single high affinity binding site, the increased binding detected at 10 nM ^{125}I-Cry1Ab may suggest the presence of lower affinity interactions at higher toxin concentrations. Although all Bt-R_{1a} fragments that bound Cry1Ab displayed similar binding affinities (Table I), the concentration of binding sites was higher for Cad11–12 and Cad12 than for Cad7–12 or full-length cadherin. Furthermore, Cad11–12 displayed a 2-fold higher concentration of binding sites than Cad12, a result consistent with the dot-blot binding experiment. A plausible explanation for these results is that toxin binding to TBR2 in CR11 and an epitope in CR12 increases the number of toxin molecules bound per expressed cadherin fragment.

We recently described a S2 cell cytotoxicity assay based on the fluorescent detection of PI in cells permeabilized by Cry toxin (11). In this system Bt-R_{1a} is a functional receptor for Cry 1A toxins. To investigate the role of CR11 and 12 in Cry1Ab toxicity, we used flow cytometry to measure quantitatively the percentage of cytotoxic response induced by Cry1Ab in S2 cells expressing different truncated fragments. Coexpression with GFP provided a method to monitor transfection efficiency, and we used PI to detect cell death. Cytotoxicity was quantified using a formula reported previously (11), which relates both transfection and cytotoxicity to background cell death in control (mock transfected) cells. As presented in Fig. 5, Cry1Ab was similarly cytotoxic to cells expressing Cad7–12, Cad10–12, Cad11–12, Cad12, and full-length Bt-R_{1a} cadherin. On the other hand, Cry1Ab was not cytotoxic to cells expressing Cad7, Cad11, or Cad-MPED. These results are evidence that CR12 contains the minimal functional receptor epitope in Bt-R_{1a} necessary for Cry1Ab cytotoxicity.

To confirm CR12 as the essential Cry1Ab toxin functional receptor domain, we separately deleted CR7, CR11, or CR12 in full-length cadherin (Fig. 6). Each cadherin clone with a deleted CR unit was expressed in S2 cells (Fig. 6*A*). In ligand blots, labeled Cry1Ab bound to CadD7 and Cad-full, but not to CadD11 or CadD12 (Fig. 6*B*). CR11 and CR12 units are both required for Cry1Ab binding when cadherin is denatured. Under the native conditions of dot-blotting, cells expressing CadD7, CadD11, or Cad-full bound Cry1Ab toxin (Fig. 6*C*). In cytotoxicity assays, Cry1Ab toxin was cytotoxic to 6.7 ± 0.57% of cells expressing CadD7, 7.5 ± 0.58% of cells expressing CadD11, and 8.5 ± 0.59% of cells expressing full cadherin.

TABLE I
Dissociation constants (K_{com}) and concentration of receptors (B_{max}) calculated from ^{125}I-Cry1Ab toxin binding saturation assays with S2 cells expressing Bt-R_{1a} fragments

Bt-R_{1a} fragment	K_{com} [a]	B_{max} [b]
	nM	*fmol/mg protein*
Cad-full	3.5 ± 1.2	625 ± 15
Cad7–12	2.0 ± 0.1	505 ± 22
Cad11–12	3.5 ± 1.0	3,319 ± 627
Cad12	2.9 ± 0.8	1,407 ± 45

[a] Values shown are nM ± S.E.
[b] Values shown are fmol/mg of protein ± S.E.

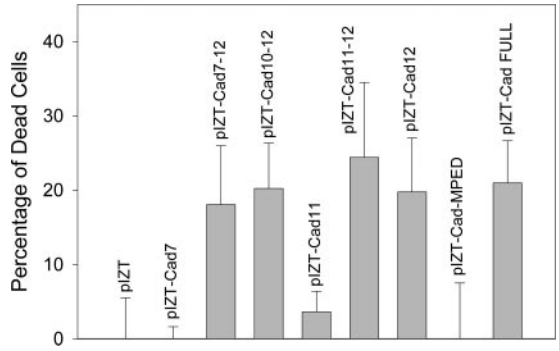

FIG. 5. **Percentage of GFP$^+$/PI$^+$ (dead) S2 cells transfected with truncated cadherins in pIZT after treatment with Cry1Ab toxin for 2.5 h.** Transfected cells were treated and gated by fluorescence-activated cell sorter as described in Ref. 11. The formula used in Ref. 11 was then used to calculate percentages of GFP expressed in cells that die (PI-positive) after toxin treatment. Values shown are the mean of three independent experiments with different transfected cell cultures conducted at least in triplicate. *Bars* denote S.D.

Conversely, Cry1Ab toxin treatment did not affect control cells (0.17 ± 0.37%) or cells expressing CadD12 (0.05 ± 0.78%). These data are in agreement with the truncated cadherin data, supporting CR12 as the Cry1Ab functional receptor domain in *M. sexta* BtR_{1a} cadherin.

DISCUSSION

Cadherin-like proteins are functional *B. thuringiensis* Cry1A toxin receptors in lepidopteran larvae. Elimination or alteration of binding to receptors is an important mechanism of insect resistance to *B. thuringiensis* toxins. In tobacco hornworm (*M. sexta*) larvae, the Bt-R_1 cadherin-like protein has been resolved as a functional Cry1A toxin receptor (2). In Ref. 11 we described an assay for Cry toxin binding and cytotoxicity based on expression of Bt-R_{1a} on the surface of S2 cells.

The ectodomain CR units of Bt-R_{1a} were expressed on the surface of S2 cells and tested for their Cry1Ab binding abilities under denaturing and native conditions. Binding to sites in CR11 and CR12 was dependent on whether the target CR peptide was denatured (ligand blots) or native (dot-blots and saturation binding assays). TBR2 in CR11 was discovered by Dorsch *et al.* (2) as the consequence of the loss of Cry1Ab binding to truncated Bt-R_1 peptide on blots. Those authors also reported inhibition of Cry1Ab larval toxicity by a truncated Bt-R_1 peptide containing TBR2. More recently, loop 2 and α-8 in Cry1Ab domain II were identified as the Cry1Ab regions that interact with TBR2 (27). In agreement with Dorsch *et al.* (2), we observed that CR11 contains a region necessary for detection by Cry1Ab on blots. For example, although the Cad11–12 peptide bound Cry1Ab on ligand blots, elimination of CR11 caused loss of binding (Fig. 2; Cad11, Cad12). Unexpectedly, CR11 alone and the reversed construct Cad12–11 did not bind Cry1Ab on ligand or dot-blots and did not promote Cry1Ab cytotoxicity. These results may seem in conflict with (2); how-

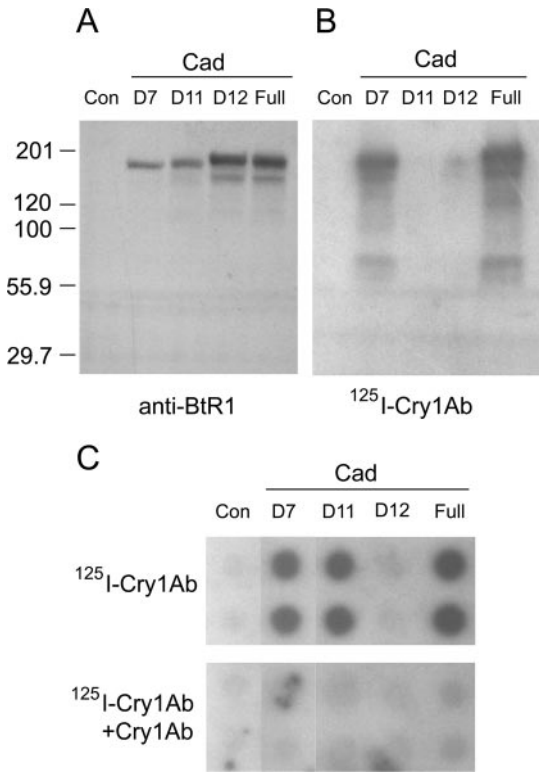

FIG. 6. **Detection of Bt-R$_{1a}$ expression in S2 cells (full-length or with deleted individual cadherin repeat units) by immunoblot (*A*), Cry1Ab ligand blot (*B*), or dot-blot (*C*).** Cell proteins were separated by SDS-PAGE then transferred to PVDF filters. After blocking, filters were probed with anti-Bt-R$_1$ serum (*A*) or ^{125}I-Cry1Ab (*B*). In dot-blots (*C*), cells were dotted on PVDF filters and after blocking, filters were probed with ^{125}I-Cry1Ab in the absence (*upper panel*) or presence of unlabeled Cry1Ab toxin (*lower panel*).

ever, the TBR2 region proposed by those authors contained part of CR12. Therefore CR11 and an epitope in CR12 would be required for detection of Cry1Ab binding on blots. In contrast to the ligand blot results, TBR2 binds toxin under native conditions as the short peptide ^{1331}IPLASILTVTV^{1342} located on CR11 decreased toxin binding and Cry1Ab toxicity to *M. sexta* larvae (27). Our quantitative binding data, discussed below, are additional evidence that Cry1Ab binds TBR2 under native conditions when CR12 is present.

The Cad12 peptide bound ^{125}I-Cry1Ab under native conditions (dot-blot and quantitative binding assays), but binding was not detected on ligand blots. Dorsch *et al.* (2) also did not detect a binding region in CR12 on blots. However, those authors did not analyze the Cry1Ab binding properties of ectodomain CR12 under native conditions, emphasizing the need to perform binding studies under conditions that retain functional proteins. In our saturation binding assays we detected increased Cry1Ab binding to Cad11–12 compared with Cad12. Additionally, the Cad11–12 saturation binding curve (Fig. 4) shows increased binding between 5 and 10 nM ligand concentrations. These results can be explained by either the existence of binding sites in both ectodomains or by conformational changes associated with concurrent expression of both ectodomains that increase the amount of bound toxin. Because Cad11 did not bind Cry1Ab, we presume that toxin binding to CR11 is dependent on prior binding of toxin to CR12. Thus, after binding to CR12, a conformational change in either the toxin or Bt-R$_{1a}$ may promote binding to CR11. Gomez *et al.* (27) proposed a sequential interaction of loops of domain II from Cry1Ab with TBR2 that could lead to proteolytic activation of Cry1Ab to a pre-pore toxin-oligomer structure.

Gomez *et al.* (17) identified a Cry1Ab toxin binding epitope, called TBR1, on Bt-R$_1$ through its amino acid sequence identity with an scFv antibody (scFV73) that blocked toxin binding to Bt-R$_1$ cadherin on blots. TBR1 corresponds to residues 865–876 located in CR7 of Bt-R$_1$. Additionally, a truncated peptide that included TBR1 bound Cry1Ab toxin on blots (27). TBR1 is bound by loop 2 of Cry1Ab domain 2 (1, 27). In agreement with Dorsch *et al.* (2), we did not observe Cry1Ab binding associated with CR7 on blots. Neither did we detect Cry1Ab binding to the CR7 unit as presented by the Cad7 peptide on dot-blots or in quantitative binding assays. Additionally, deletion of CR7 in full-length cadherin did not eliminate Cry1Ab binding in ligand or dot-blots or cause loss of cytotoxicity in cell assays. The different techniques to test for Cry1Ab binding used by Gomez and colleagues may account for the discrepancies. Furthermore, because S2 cells expressing Cad7 did not bind or were not susceptible to Cry1Ab toxin, potential toxin binding to these sites would not be related to cytotoxicity.

Full-length Bt-R$_{1a}$ had a lower concentration of receptors than both Cad11–12 and Cad12, which resulted in lower amounts of Cry1Ab toxin bound to S2 cells expressing Cad-full compared with Cad11–12 and Cad12. However, because S2 cells expressing Cad11–12, Cad12, or the full-length Cad displayed the same level of susceptibility to Cry1Ab toxin, the increased binding to Cad11–12 and Cad12, although specific, was not related directly to cytotoxicity. The substantial amount of Bt-R$_{1a}$ and truncated Bt-R$_{1a}$ peptides detected in transfected cells (Fig. 2) suggests that receptor was not limiting. In Ref. 11 we discuss factors that may limit the toxicity of Cry1A toxins to S2 cells. For example, the low pH 6.4 of the cell culture medium relative to the alkaline lepidopteran midgut may not be optimal for toxin insertion into the membrane. Midgut trypsin-like proteinases involved in toxin processing and pore formation (28) are lacking from the S2 system. At the epithelial membrane level, toxin is known to sequester into lipids rafts whose components include Cry1Ab-binding aminopeptidase (8, 29). Although not perfected as a model for midgut epithelial cells, the S2 assay system served to identify CR12 as the critical domain of Bt-R$_{1a}$.

Our results clearly identify ectodomain CR12 as a critical Cry1Ab receptor epitope in Bt-R$_1$. CR12 was necessary and sufficient for Cry1Ab binding in both dot-blots and saturation binding assays. Furthermore, this ectodomain was the minimum region necessary to confer cell susceptibility to Cry1Ab to the same level as full-length Bt-R$_{1a}$. Nagamatsu *et al.* (13) observed that a mutant of the *B. mori* cadherin, BtR175, lacking CR9, failed to mediate Cry1Aa toxicity in Sf9 cells. Our results and those of Nagamatsu *et al.* (13) indicate that the CR unit adjacent to the membrane-proximal region is critical to Cry1 toxin binding and pore formation. For BtR175, the critical unit is CR9, and for Bt-R$_{1a}$ the critical unit is CR12.

Experiments using the S2 cell expression and FACS approach aimed at defining the specific amino acids from CR12 involved in Cry1Ab binding are currently being performed in our laboratory. Results obtained from this work will shed light on *B. thuringiensis* toxin pathogenesis as well as enable the design of optimized toxins with improved efficacy. Knowledge of the functional receptor region would also facilitate detection of changes resulting in toxin binding alteration, the most reported mechanism of resistance to *B. thuringiensis* toxins.

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