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Ligand Specificity and Affinity of BT-R_1, the *Bacillus thuringiensis* Cry1A Toxin Receptor from *Manduca sexta*, Expressed in Mammalian and Insect Cell Cultures

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The *Manduca sexta* receptor for the *Bacillus thuringiensis* Cry1Aa, Cry1Ab, and Cry1Ac toxins, BT-R_1, has been expressed in heterologous cell culture, and its ligand binding characteristics have been determined. When transfected with the BT-R_1 cDNA, insect and mammalian cell cultures produce a binding protein of approximately 195 kDa, in contrast to natural BT-R_1 from *M. sexta*, which has an apparent molecular weight of 210 kDa. Transfection of cultured *Spodoptera frugiperda* cells with the BT-R_1 cDNA imparts Cry1A-specific high-affinity binding activity typical of membranes prepared from larval *M. sexta* midguts. Competition assays with BT-R_1 prepared from larval *M. sexta* midguts and transiently expressed in cell culture reveal virtually identical affinities for the Cry1Aa, Cry1Ab, and Cry1Ac toxins, clearly demonstrating the absolute specificity of the receptor for toxins of the lepidopteran-specific Cry1A family. BT-R_1 therefore remains the only *M. sexta* Cry1A binding protein to be purified, cloned, and functionally expressed in heterologous cell culture, and for the first time, we are able to correlate the Cry1Aa, Cry1Ab, and Cry1Ac toxin sensitivities of *M. sexta* to the identity and ligand binding characteristics of a single midgut receptor molecule.

Upon sporulation, the gram-positive bacterium *Bacillus thuringiensis* produces a variety of insecticidal crystal proteins which exhibit specific toxicities to certain orders of insects, many of them economically important crop pests. The primary site of action of the insecticidal toxins of the various *B. thuringiensis* subspecies resides in the brush border membranes of the midgut epithelia of susceptible insect larvae (2, 5, 30). Until recently, *B. thuringiensis* toxins were grouped according to their specific toxicities toward insects, as described by Höfte and Whiteley (14). The CryI group, for example, is toxic to lepidopteran insect larvae and includes the CryIA family of toxins. Once ingested by sensitive lepidopteran larvae, the CryIA insecticidal crystal proteins, now classified as Cry1Aa through Cry1Ae, are solubilized as 131- to 133-kDa protoxins and midgut proteases in the highly alkaline lepidopteran midgut act to remove the carboxy-terminal portion of the protoxin, producing an active toxin of approximately 60 kDa. The proteolytically activated toxins then bind to a specific membrane receptor(s) in the gut, with both membrane permeability and ion transport ultimately being affected. The immediate gross consequences of toxin ingestion are distortion and enlargement of the midgut epithelial cells, lifting of the cells from the basement membrane, and loss of the epithelia, resulting in death of the insect. The molecular events leading to the ion imbalance and morphological changes seen in insect midgut epithelial cells following toxin ingestion remain speculative, although ion pore formation in the apical membrane by insertion of a portion of the toxin molecule has been considered a likely mechanism (for a review, see reference 18).

Our laboratory previously reported the purification and cDNA cloning of a *B. thuringiensis* toxin receptor, BT-R_1, present in midgut brush border membranes prepared from larvae of the lepidopteran *Manduca sexta* (31, 32). BT-R_1 has a molecular mass of approximately 210 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and an isoelectric point of ~5.5. The BT-R_1 cDNA encodes a polypeptide with a predicted molecular mass of 172 kDa (32). It is a cadherin-like glycoprotein that shows 30 to 60% similarity and 20 to 40% identity to members of the cadherin superfamily of proteins. This receptor was shown in those studies to bind specifically the Cry1Ab toxin of *B. thuringiensis* subsp. *berliner*. The binding of *B. thuringiensis* toxins to midgut brush border membrane vesicles has been reported for numerous insect species, as has the detection of a number of putative toxin receptors. With this report, BT-R_1 remains the only receptor to be successfully expressed as a competent Cry toxin binding protein in a heterologous cell culture system. This accomplishment has permitted the current detailed investigation of the ligand specificity and affinity of the receptor. The three members of the Cry1A protoxin family included in this study, Cry1Aa, Cry1Ab, and Cry1Ac, demonstrate 82 to 90% amino acid identity to one another (14) and exhibit essentially identical toxicities to *M. sexta* larvae as demonstrated by experiments in our laboratory and others (13, 35). The Cry1Ab and Cry1Ac toxins also have been shown previously to compete for the same binding protein(s) in *M. sexta* in heterologous competition binding assays (12, 35). Therefore, it is conceivable that a single receptor protein is responsible for eliciting the pathologic response in *M. sexta* to the highly homologous Cry1A toxins, although this had yet to be demonstrated in the literature. We report here the binding of several *B. thuringiensis* toxins to a number of cell lines that stably and transiently express the *M. sexta* Cry1A receptor BT-R_1. This study provides a better understanding of the ligand specificities and affinities of this receptor and helps correlate toxicity with the presence, identity, and specificity of this receptor within the insect midgut. Ultimately, such information should facilitate the elucidation of the molecular mechanism(s) of action of Cry1A toxin.

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MATERIALS AND METHODS

Toxin purification. The recombinant protoxins Cry1Aa, Cry1Ab, and Cry1Ac (*Bacillus* Genetic Stock Center, The Ohio State University) were prepared from *Escherichia coli* JM-103 and trypsinized essentially as described by Lee et al. (21). In addition, the soluble trypsinized 60-kDa toxins were subjected to fast protein liquid chromatography (FPLC) purification over a Mono-Q anion-exchange column (Pharmacia) prior to quantitation, radioiodination, and use in bioassays. Cry3A crystal protein from *B. thuringiensis* subsp. *tenebrionis* was solubilized in 3.3 M NaBr and treated with papain, and the resulting 67-kDa toxin was purified by the method of Li et al. (24). The 65-kDa Cry11A toxin was isolated from *B. thuringiensis* subsp. *israelensis* via differential solubilization as described by Chilcott and Ellar (4) and further purified by anion-exchange FPLC. All toxin protein quantitations were performed by the bicinchoninic acid method (Pierce Chemical Co.) with bovine serum albumin (BSA, fraction V) as a standard.

Radioiodination. All Cry toxins used in this work were iodinated by the chloramine-T method (15) with $Na^{125}I$ purchased from NEN-DuPont. A 10-μg portion of toxin was mixed with 0.5 mCi of $Na^{125}I$ (approximately 5 μl) in 100 μl of 100 mM sodium phosphate buffer (pH 7.0). To this mixture was added 100 μg of chloramine-T, which was allowed to react for 20 s with constant mixing. Label incorporation was halted by the addition of 200 μg of sodium metabisulfite in 50 μl of distilled H_2O, and nonincorporated ^{125}I was removed on a 2-ml Excellulose desalting column (Pierce) equilibrated with phosphate-buffered saline (PBS). Specific activities of labeled toxins were determined by trichloroacetic acid precipitations of the eluates from the desalting columns.

Membrane protein preparations. *M. sexta* eggs were purchased from Carolina Biologicals, and the larvae were reared on Carolina artificial diet at 28°C. Midguts were excised from fifth-instar larvae. Membrane proteins from both isolated insect midguts and cell cultures were prepared essentially as described by Adamo et al. (1), and total protein was determined by the bicinchoninic acid method. Briefly, tissues or cells were homogenized on ice in a tight-fitting glass Dounce homogenizer in 10 volumes of a hypotonic buffer composed of 5 mM Tris · HCl (pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride and 3 mM dithiothreitol (DTT). The mixture was then diluted with an equal volume of ice-cold 5 mM Tris · HCl (pH 7.4) containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3 mM DTT, and a cocktail of protease inhibitors (aprotinin, 10 μg/ml; benzamidine, 1 mM; leupeptin, 1 μg/ml). A low-speed centrifugation (1,000 × *g* for 10 min in a Beckman JA-20 rotor) was used to pellet heavier cellular debris, and a second fraction was then pelleted by ultracentrifugation (100,000 × *g* for 30 min in a Beckman SW60 Ti rotor). This high-speed pellet, which contained essentially all detectable BT-R_1 binding activity, was suspended in 10 mM HEPES (pH 7.4)–10% glycerol–130 mM KCl–3 mM DTT prior to flash-freezing in liquid nitrogen and storage at −80°C.

Expression of BT-R_1 cDNA in mammalian cell culture. The 4,810-bp *Ssp*I-*Sac*I cDNA fragment which contains the open reading frame of BT-R_1 was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and used to transfect mammalian cells by a standard calcium phosphate/DNA precipitation method (3). HEK-293 and COS-7 cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and 10% heat-inactivated calf serum, respectively (both from GIBCO-BRL). For stable expression, the cells were subcultured 24 h after transfection in fresh medium containing 500 μg of active G418 sulfate per ml (Geneticin; GIBCO-BRL). Surviving colonies were isolated in cloning cylinders after approximately 2 weeks of selection, further passaged, and assayed for the ability to bind iodinated Cry1Ab toxin on ligand blots of crude cell lysates. The isolates expressing the highest level of BT-R_1 binding activity per weight were propagated and used to prepare frozen stocks. All mammalian cell culture was performed at 37°C with 5% added CO_2 in humidified Forma Scientific water-jacketed incubators.

Transient expression of BT-R_1 cDNA in insect cell culture. The same 4,810-bp *Ssp*I-*Sac*I cDNA fragment encoding BT-R_1 was used to replace the gp64 coding sequence of the plasmid vector Op-gp64BglATG-STOP described by Kogan and Blissard (19). Transcription of this plasmid is driven by the intact *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (Op*M*NPV) gp64 gene promoter. This ΔOp-gp64/BT-R_1 insect expression construct also retains the gp64 consensus polyadenylation sequences 3′ to the BT-R_1 translation stop codon and minimal BT-R_1 cDNA sequence from either untranslated region. Multiwell culture plates (Corning; six 35-mm wells per plate) were seeded with 1.5×10^6 *Spodoptera frugiperda* Sf21 cells per well in TNM-FH medium (Grace's insect medium with 3.3 g of lactalbumin hydrolysate per liter and 3.3 g of yeastolate per liter [Invitrogen], supplemented with 10% HI-FBS [JRH Biosciences]) for one hour prior to addition of DNA. Sterile DNA was added to 25 mM HEPES (pH 7.1) containing 140 mM NaCl and 125 mM $CaCl_2$. A precipitate was formed when this cocktail was added to the cells, which had been transferred into Grace's insect medium (without supplements)–10% HI-FBS immediately prior to the addition of the DNA. The cells were incubated with the DNA precipitate at 27°C for 4 h, at which time the transfection medium was washed off of the cells and they were returned to TNM-FH medium. Optimization of transfection was carried out by the detection of expressed BT-R_1 via radioiodinated Cry1Ab toxin ligand blots. For large-scale BT-R_1 production in Sf21 cells, 150-cm^2 culture flasks (Corning) were seeded with 8×10^6 cells and transfected with 80 μg of plasmid.

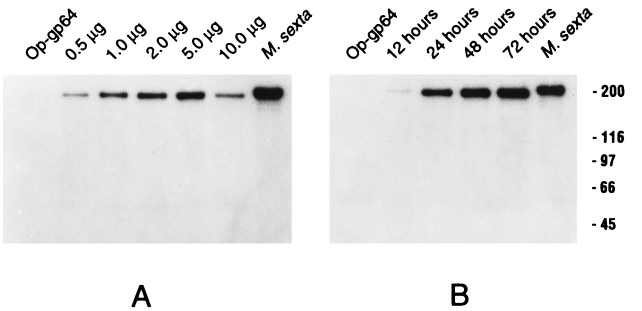

FIG. 1. Optimization of BT-R_1 expression in Sf21 insect cell cultures. We seeded 35-mm culture wells with 1.5×10^6 Sf21 cells and transfected them with the ΔOp-gp64/BT-R_1 construct. In each case, 50 μg of cellular proteins was solubilized by boiling in SDS sample buffer. The proteins were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with ^{125}I-Cry1Ab toxin. (A) Results of a plasmid dose-response experiment where all cells were harvested at 48 h posttransfection. (B) Results of a time course experiment where each well was transfected with 5 μg of DNA and the cells were harvested at the indicated times. In both experiments, the following controls are indicated: Sf21 cells transfected with the parent plasmid Op-gp64, and 50 μg of membrane proteins prepared from *M. sexta* larvae midguts. Positions of molecular mass markers (in kilodaltons) are indicated on the right.

Ligand blotting. For ligand-blotting studies, proteins were denatured by boiling in SDS loading buffer (20) and separated by electrophoresis through discontinuous SDS–7.5% polyacrylamide gels. Separated proteins were blotted to polyvinylidene difluoride (PVDF) membranes (Millipore) by a semidry blotting technique (Milliblot; Millipore) and blocked for at least 2 h with Tris · HCl-buffered saline (pH 8.0) containing 5% nonfat dry milk powder, 5% glycerol, 0.5% Tween 20, and 0.025% sodium azide. The PVDF membranes were then incubated with ^{125}I-Cry1A toxins (approximately 1 nM) for 2 h at 26°C, washed three times (30 min each) with blocking buffer, and exposed overnight to Kodak X-Omat AR autoradiography film at −80°C. Competition blots were handled identically except for the addition of unlabeled Cry toxin competitor to the blocking buffer containing iodinated toxin prior to use on the ligand blot.

Competition binding assays. Nonsolubilized membrane proteins (50 μg) were incubated with ^{125}I-Cry1A toxin (1.0 nM) in the presence of increasing concentrations (0 to 1 μM) of unlabeled Cry toxin in a final assay volume of 100 μl of PBS (pH 7.4)–0.2% BSA. Nonspecific binding was measured as bound radioactivity in the presence of 1 μM unlabeled toxin (at least a 1,000-fold excess of unlabeled ligand). All values are reported as percentages relative to the binding detected in the absence of competitor. All competition reactions were allowed to proceed for 30 min at room temperature with gentle mixing in microcentrifuge tubes which had previously been blocked with PBS–0.2% BSA. The tubes were then placed in an Eppendorf 5415C microcentrifuge and centrifuged at maximum speed for 5 min at 26°C to pellet the bound ^{125}I-Cry1A toxin. Each pellet was washed at least four times by vortexing in 500 μl of PBS–0.2% BSA followed by centrifugation. The amount of ^{125}I in the final pellet was determined with a Beckman Gamma 5500 gamma counter.

Quantitation of competition ligand blots. After exposure to X-Omat AR film, competition ligand blots were placed on imaging screens of a Bio-Rad GS525 molecular imager system for approximately 2 h. BT-R_1 bands were sampled at high resolution with Molecular Analyst software, and total counts in the sampling areas were recorded. Identical sampling in the transfection control lanes was subtracted as background. All bar graph values in Fig. 5 are reported as percentages relative to the binding detected in the absence of competitor.

RESULTS

Optimization of BT-R_1 expression in Sf21 cell cultures. BT-R_1 was expressed in *S. frugiperda* Sf21 cell culture by using a plasmid construct containing promoter and polyadenylation sequences derived from the Op*M*NPV gp64 gene (19). The level of BT-R_1 expression in Sf21 cells varied greatly, depending upon transfection conditions. Expression was optimized by ligand blot analyses of crude cell lysates following transfection with the ΔOp-gp64/BT-R_1 construct (Fig. 1). For expression studies with 35-mm culture wells, optimal expression levels were achieved when 5 μg of DNA was used to transfect cells (Fig. 1A), which then were harvested for study 48 h posttransfection (Fig. 1B). Figure 1 also shows the molecular mass difference between the natural glycoprotein (210 kDa) de-

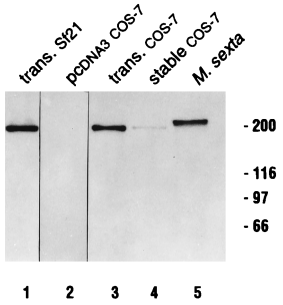

FIG. 2. Ligand blot of mammalian and insect cells expressing the BT-R_1 cDNA. Total cellular proteins were analyzed by solubilization in SDS sample buffer, separation by SDS-PAGE, blotting, and incubation with ^{125}I-Cry1Ab toxin. Lanes: 1, protein extracts from Sf21 cells transfected with the ΔOp-gp64/BT-R_1 plasmid; 2 through 4, COS-7 cells transfected with the parent pcDNA3 plasmid and cells both transiently and stably expressing the pcDNA3/BT-R_1 construct, respectively; 5, 50 μg of membranes prepared from *M. sexta* midguts. Positions of molecular mass markers (in kilodaltons) are indicated on the right.

tected in *M. sexta* larval midguts and the approximately 195-kDa receptor expressed in the cell cultures. The molecular mass of this expressed protein was incorrectly reported as 210 kDa in HEK-293 human cell cultures in the initial cloning paper by Vadlamudi et al. (32). Attempts have been made to express the BT-R_1 cDNA in several commonly used cell lines, but all those tested to date produce a receptor with a slightly lower molecular mass than that of the natural BT-R_1 in *M. sexta* midguts.

BT-R_1 expression was high in the optimized transient Sf21 and COS-7 cultures (Fig. 2, lanes 1 and 3), and stable expression in COS-7 cells resulted in a very low level of protein production (lane 4). Treatment of membranes prepared from *M. sexta* midguts and the transiently transfected insect cells with *N*-glycosidase A demonstrated that the expressed BT-R_1 is glycosylated in cell cultures, as is natural BT-R_1 in *M. sexta* midguts. However, because of the difference in the molecular mass of the two receptors, glycosylation of each molecule probably varies (data not shown).

Ligand specificity and affinity of BT-R_1. Investigation of the affinity of BT-R_1 expressed in Sf21 cells for Cry1A toxins indicated that even though it is not posttranslationally processed identically to the protein in *M. sexta*, the binding affinity and specificity of expressed BT-R_1 with respect to recombinant Cry1Ab were virtually identical to those of the 210-kDa *M. sexta* protein. To test the ligand specificity of the expressed BT-R_1 and the native protein to other Cry toxins, competitive binding assays were performed with membranes prepared from both *M. sexta* larval midguts and Sf21 cells transiently expressing BT-R_1. Binding assays were performed in the presence of increasing concentrations of unlabeled Cry1Ab, Cry3A, and Cry11A toxins (Fig. 3). Figure 3A and B depict the binding characteristics of *M. sexta* midgut membranes and transiently transfected Sf21 cell membranes, respectively. Unlabeled Cry1Ab inhibited the binding of ^{125}I-labeled Cry1Ab to membranes prepared from both *M. sexta* midguts (Fig. 3A) and BT-R_1-transfected cells (Fig. 3B), whereas neither the coleopteran-specific Cry3A nor the dipteran-specific Cry11A showed any detectable interference with Cry1Ab binding. A ligand competition-blotting experiment performed with the same Sf21 materials shows a similar decrease in binding with increasing concentrations of unlabeled competitor, as can be seen in the boxed insert in Fig. 3B. For this competition ligand blot, identical amounts (50 μg) of membrane protein prepared from transiently transfected Sf21 cells were separated by SDS-PAGE (7.5% polyacrylamide) and electroblotted to a PVDF membrane, which was then cut into strips prior to incubation in blocking buffer. Following a 12-h incubation in blocking buffer (see Materials and Methods), the PVDF strips were incubated in blocking buffer containing a premix of labeled toxin and unlabeled competitor. In this experiment, the presence of a 10-fold excess of unlabeled toxin (10 nM) reduced the amount of labeled toxin that bound to the receptor by approximately 90%. These blots clearly show that the presence of a 500-fold excess of unlabeled toxin (500 nM) was sufficient to essentially block all detectable binding.

The affinity of BT-R_1 to Cry1Aa and Cry1Ac also was determined in similar experiments by heterologous competition binding assays with the recombinant Cry1A toxins. The results (Fig. 4) revealed the relative ability of these two toxins to

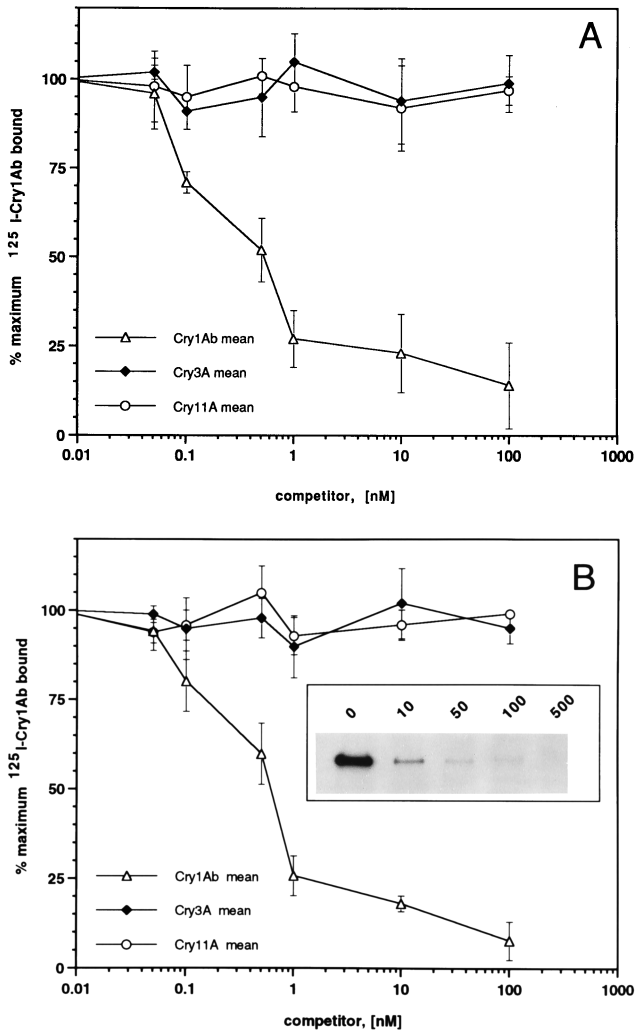

FIG. 3. Competition binding assays demonstrating BT-R_1 affinity and specificity for lepidopteran-specific Cry1Ab toxin. The membranes were suspended in PBS–0.2% BSA contained in microcentrifuge tubes. ^{125}I-Cry1Ab was added to approximately 1.0 nM simultaneously with the indicated concentrations of unlabeled Cry1Ab, Cry3A, and Cry11A. (A) Results obtained with 50 μg of *M. sexta* midgut membranes; (B) data obtained with the same amount of membranes prepared from transiently transfected Sf21 cells. Values shown throughout are means of experiments performed in triplicate, with standard deviations indicated. The boxed insert in panel B demonstrates a competition blot experiment performed with proteins from transfected Sf21 cells analyzed by SDS-PAGE and blotting to PVDF membranes followed by incubation with 1.0 nM ^{125}I-Cry1Ab and the indicated concentrations (nanomolar) of unlabeled Cry1Ab competitor.

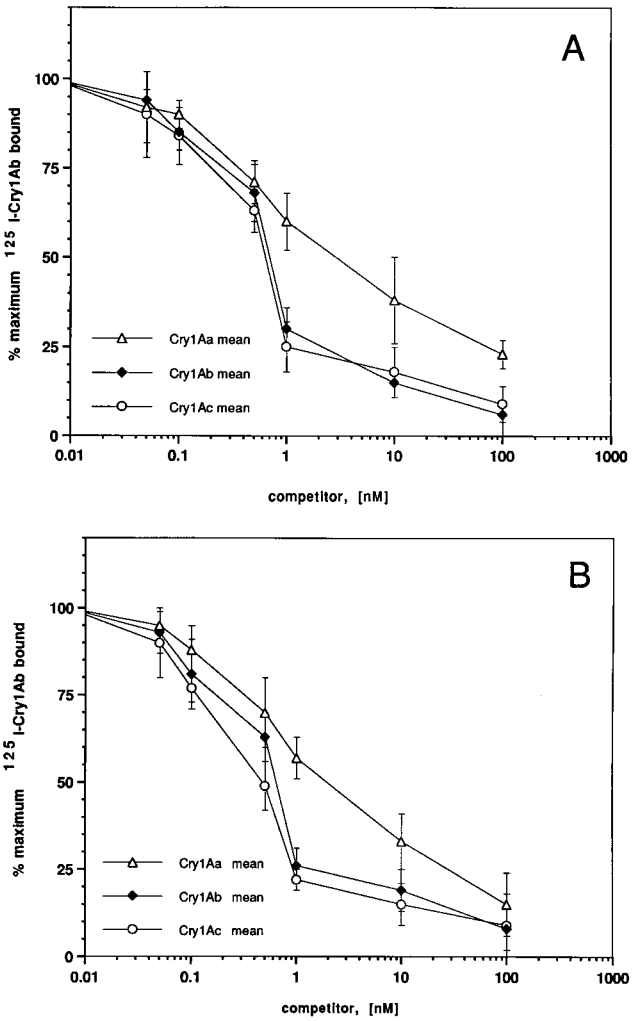

FIG. 4. Heterologous competition binding assays demonstrating the specificity of BT-R_1 for the Cry1A toxins. The membranes were suspended in PBS–0.2% BSA in microcentrifuge tubes, and ^{125}I-Cry1Ab was added to approximately 1.0 nM simultaneously with the indicated concentrations of unlabeled Cry1Aa, Cry1Ab, and Cry1Ac. (A) Results obtained with *M. sexta* midgut membranes; (B) data obtained from membranes prepared from transiently transfected Sf21 cells.

inhibit the binding of iodinated recombinant Cry1Ab toxin to both *M. sexta* membrane preparations and BT-R_1-transfected Sf21 cell membranes compared to homologous Cry1Ab competitions. Figure 4A displays the competition curves obtained with iodinated Cry1Ab on membranes prepared from *M. sexta* midguts. A duplicate experiment with membranes prepared from BT-R_1-transfected Sf21 cells (Fig. 4B) revealed essentially identical results. Cry1Ab and Cry1Ac competed for ^{125}I-Cry1Ab binding statistically to the same extent, whereas Cry1Aa competed to a slightly lesser degree in both cases. The presence of 1 nM unlabeled Cry1Ab and Cry1Ac toxins consistently reduced the binding of iodinated Cry1Ab by approximately 75%, whereas the presence of 100 nM competitor (approximately a 100-fold excess) reduced the binding of these toxins essentially to background levels. Scatchard analyses of Cry1Ab homologous competition binding data have demonstrated this high-affinity binding to BT-R_1 (31).

Identification of toxin binding proteins via competition ligand blotting. To confirm the identity of the receptor involved in our competition binding experiments, we carried out homologous competition ligand binding studies with the same membrane protein preparations isolated from both transiently transfected Sf21 cells and larval *M. sexta* midguts. Representative examples of results of competition binding assays performed with proteins separated by SDS-PAGE and blotted to PVDF membranes are shown in Fig. 5. In the case of Sf21-expressed BT-R_1 (Fig. 5, lane 2 of each blot), ^{125}I-labeled Cry1Aa, Cry1Ab, and Cry1Ac all bound the 195-kDa expressed receptor and the presence of approximately 500-fold unlabeled competitor reduced the ^{125}I-labeled toxin binding essentially to background levels (500 nM blots in Fig. 5). Membranes from *M. sexta*, on the other hand, demonstrated very different binding characteristics on ligand blots with regard to Cry1Ac (lane 3 of each blot). Whereas Cry1Aa and Cry1Ab bound only to the 210-kDa BT-R_1 protein, ^{125}I-Cry1Ac recognized multiple proteins with various molecular masses. However, even in the presence of up to 500 nM unlabeled Cry1Ac competitor, the only binding which was eliminated, or even appreciably reduced, was that of the 210-kDa BT-R_1. As can be seen in the bar graphs for Cry1Ac, 500 nM unlabeled competitor reduced the binding to the receptor expressed in cell culture by approximately 90%; these results correspond well to those obtained from the competition binding assays in Fig. 3 and 4. Computer analysis of ^{125}I counts bound to the 210-kDa BT-R_1 band on the *M. sexta* blots provided evidence that Cry1Ac binding to this protein was essentially eliminated in the presence of a 500-fold excess of competitor (the bar graph of BT-R_1-bound ^{125}I in *M. sexta* is reduced only by approximately 60% due to interference from neighboring or underlying Cry1Ac binding, as can be visually confirmed in the ligand blot photographs). On the other hand, it is apparent that the majority of the ^{125}I-Cry1Ac binding to blots of *M. sexta* midgut proteins is to the numerous bands which do not exhibit any appreciable reduction in binding, even in the presence of a 500-fold excess of unlabeled competitor. Taken together with the competition binding assays shown in Fig. 3 and 4, these data clearly demonstrate that the BT-R_1 protein expressed in cell culture shows the ligand affinity and specificity characteristic of the natural protein derived from larval *M. sexta* midgut tissues.

Functionality of the expressed BT-R_1 protein. Having established that the expressed BT-R_1 receptor is capable of specifically binding the Cry1A toxins, we attempted to determine whether expression of BT-R_1 itself in a heterologous cell culture system would impart cell sensitivity to Cry1Ab toxin. Transfected HEK-293, COS-7, and Sf21 insect cells expressing BT-R_1 were incubated with activated Cry1Ab toxin, and cell integrity was monitored by inverted phase-contrast microscopy. When exposed to Cry1Ab toxin (up to 100 μg/ml) for 2 h, none of the transfected cells exhibited any observable phenotypic changes. Also, we failed to detect the formation of ^{51}Cr-permeable pores upon loading HEK-293 cells with ^{51}Cr prior to toxin exposure (33). Additional studies with ^{125}I-labeled toxins have failed to detect significant amounts of toxin binding to the intact cells in culture. We therefore deduce that although the expressed protein exhibits binding characteristics typical of *M. sexta* midgut membrane preparations, it is not being processed to the plasma membrane of the culture cells used in this study.

DISCUSSION

It is generally accepted that the crystal toxins of *B. thuringiensis* either directly or indirectly disrupt the ion balance of midgut epithelial cells after their interaction with specific receptors on the luminal surface of the midgut epithelium. Most animal cells utilize various combinations of Na^+, K^+, and Cl^-

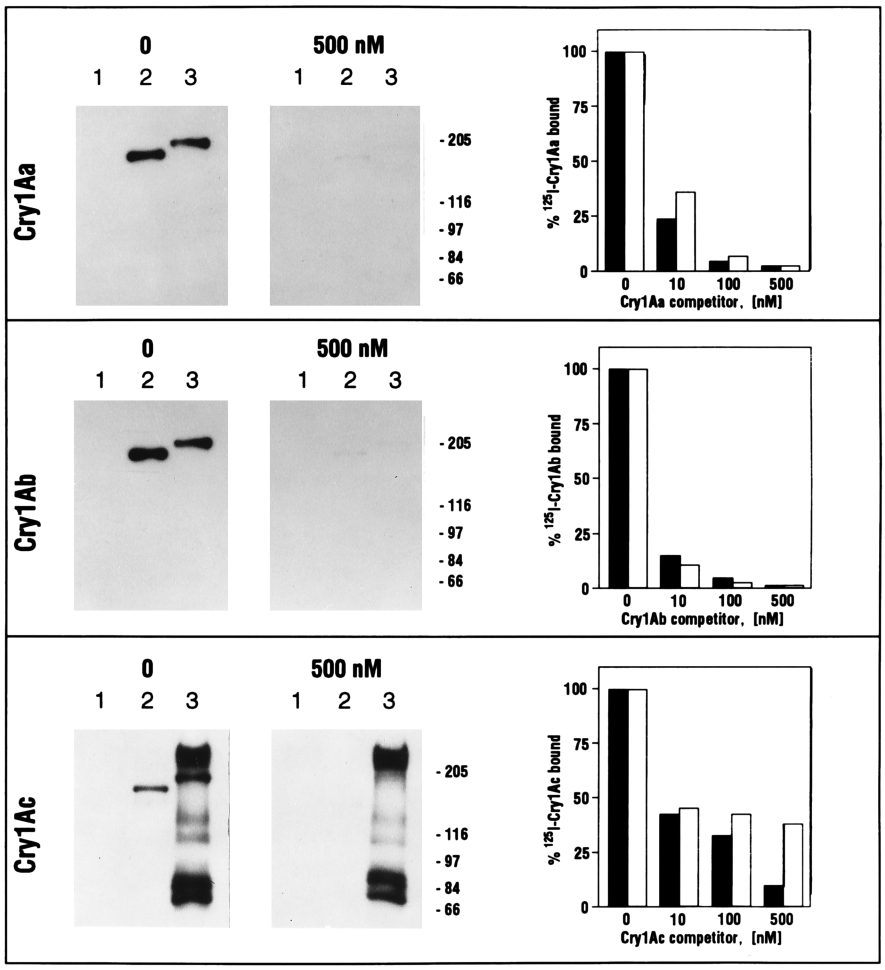

FIG. 5. Homologous competition ligand blots demonstrating the binding characteristics of ^{125}I-labeled Cry1Aa, Cry1Ab, and Cry1Ac toxins to BT-R_1 proteins in both Sf21 cells and *M. sexta* midgut membranes. The toxins are indicated at the left of each panel, and the amount of unlabeled competitor toxin is indicated above each blot. The concentration of labeled toxin in all experiments was approximately 1.0 nM. In all cases, lane 1 contains proteins prepared from Sf21 cells transfected with the parent Op-gp64 plasmid, lane 2 contains membranes from Sf21 cells transiently expressing BT-R_1, and lane 3 contains membranes prepared from *M. sexta* larva midguts. Positions of molecular mass markers (in kilodaltons) are indicated to the right of the blots. Bar charts were obtained by quantitation of the ^{125}I signal corresponding to the BT-R_1 bands, with lane 1 subtracted as background. Solid bars represent binding to the 195-kDa expressed protein (data from lane 2), and open bars represent quantitations of binding to the 210-kDa natural BT-R_1 protein in *M. sexta* (data from lane 3).

channels along with the Na^+/K^+- and H^+/K^+-ATPases to maintain a constant resting potential across the plasma membrane. However, in the case of the lepidopteran midgut, the apical plasma membrane of columnar epithelial cells has been demonstrated to regulate K^+ efflux and influx only indirectly through a reliance upon neighboring goblet cell H^+/K^+-ATPases (18). This unique situation has been proposed to make the epithelial lining of the lepidopteran midgut susceptible to the toxins of *B. thuringiensis*, which are generally believed to produce pores in the membrane. Any major influx of K^+ across the apical membrane of the columnar cell will result in depolarization of the membrane with subsequent efflux of H^+ down the large pH gradient normally maintained in the highly alkaline midgut. Alternatively, it has been postulated (18) that the formation of even relatively few nonselective ion pores would result in an osmotic stress that the lepidopteran midgut epithelia is ill equipped to counter.

The initial binding studies of BT-R_1 prior to the cDNA cloning indicated that this protein exhibited the degree of specificity and affinity required to render it a candidate receptor for Cry1Ab toxin in *M. sexta* midguts (25, 31). In these particular reports, the 210-kDa BT-R_1 receptor was the only binding protein detected on ligand blots with ^{125}I-Cry1Ab as a probe on *M. sexta* midgut membrane preparations. Following the cloning of the BT-R_1 cDNA, it became apparent that the only significant sequence homology found in the protein databases with this novel protein consisted of some identity to members of the cadherin superfamily of cell adhesion proteins (32). Based upon the deduced amino acid sequence of the cDNA, BT-R_1 is postulated to contain one transmembrane domain near the C terminus and several cadherin-like repeat sequences in the putative extracellular domain. To date, the function of this cadherin-like protein in the midgut is unknown, although various studies recently have been published implicating a role for cadherins in apoptotic events which result in programmed cell death (10, 11, 29). It is conceivable that BT-R_1 somehow elicits Cry toxin pathology through an apoptotic signaling event, inducing the swelling and destruction of midgut epithelium which is seen following toxin ingestion. Regardless of the mechanism of action, BT-R_1 remains to date the only *M. sexta* Cry1Aa or Cry1Ab binding protein

detected in our laboratory and the only cloned Cry1Aa or Cry1Ab receptor in the current literature.

Cry1Ac binding in *M. sexta* also has been extensively studied, and multiple forms of aminopeptidase N (APN) have been shown to bind ^{125}I-labeled Cry1Ac in midgut brush border membrane vesicles from this insect (7, 8, 16, 17, 23, 28, 34). These digestive proteases are very abundant throughout the gut and function to remove amino-terminal residues from proteins bound in the lumen of the larval digestive tract. Enriched APN receptor complexes, however, demonstrate a relatively low affinity for Cry1Ac, with a reported K_d of approximately 100 to 200 nM (26, 28). The relatively low affinity of APN for Cry1Ac, along with the fact that the various APN complexes fail to bind either Cry1Aa or Cry1Ab on ligand blots, has prompted us to pursue the characterization of the Cry1A receptor BT-R_1 in efforts to determine the identity of the receptor(s) responsible for Cry1A toxin sensitivity in *M. sexta* larvae.

The approximately equal toxicity of Cry1Aa, Cry1Ab, and Cry1Ac to *M. sexta* larvae has been previously demonstrated (13, 35). In our laboratory, we also routinely derive 50% lethal concentrations from these three toxins of approximately 5 to 10 ng/cm^2 by using FPLC-purified toxins on neonatal and first-instar larvae of *M. sexta*. Heterologous competition binding assays (Fig. 4) indicate that Cry1Ab and Cry1Ac compete with ^{125}I-Cry1Ab for the same high-affinity binding site in *M. sexta* midgut preparations, data that also been previously reported by other groups (12, 35). There often appears to be a discrepancy, however, when competition assay data are compared to ligand blot data published in the literature, a point recently highlighted in studies of *Lymantria dispar* larvae by Lee and Dean (22). Cry1Aa, Cry1Ab, and Cry1Ac all bound to *L. dispar* brush border membrane vesicles with high affinity, as demonstrated by homologous competition assays. Heterologous competition experiments indicated that all three toxins were competing for the same binding site. Ligand blots, however, demonstrated a striking difference in binding patterns among the toxins. Cry1Aa and Cry1Ab bound only to a 210-kDa protein, whereas Cry1Ac bound only to a 120-kDa band. In the case of *L. dispar*, the lack of a single common receptor on ligand blots remains puzzling, and the apparent discrepancy between the ligand blots and competition assays has yet to be resolved. It may be important, however, that these studies did not include competition binding experiments on the ligand blots; therefore, no conclusions regarding the specificity of binding detected in the competition assays could be made. While we cannot explain the lack of a 210-kDa Cry1Ac binding protein in reports from other groups (7, 8, 16, 17, 28, 34), we can state that although we did detect bands of hybridization around 100 to 120 kDa in *M. sexta*, these bands had relatively low intensity and none exhibited a reduction in ^{125}I-Cry1Ac binding when excess unlabeled ligand was added to the hybridization buffer. Figure 5 shows examples of such competition ligand blots in *M. sexta* and clearly demonstrates the importance of these experiments in attempts to correlate competition binding data with the presence and identity of receptors demonstrating both high affinity and specificity. Given the multitude of Cry toxin binding proteins that have been identified to date, such a correlation will become increasingly important in the future to distinguish between toxin binding proteins and physiologically relevant toxin receptors.

In a recent study of *M. sexta*, Du and Nickerson (6) brought to light Cry1Ac binding characteristics which indicate that this particular Cry1A toxin recognizes and binds to the biotin moiety of certain biotinylated proteins in *M. sexta*. This study also demonstrated that Cry1Ac binds to biotinylated BSA, pyruvate carboxylase, and ovalbumin but not to biotin itself or the non-biotinylated counterparts of these proteins. Therefore, we believed that it was important to demonstrate that BT-R_1 exhibited the following characteristics: (i) that Cry1Aa, Cry1Ab, and Cry1Ac do have a single common receptor, BT-R_1, as proved by the competition ligand blots in Fig. 5 and by similar ligand blotting of the solubilized, gel filtration purified BT-R_1 (9); (ii) that heterologous competition binding assays with ^{125}I-Cry1Ab indicate that the three Cry1A toxins studied compete with one another for binding of this high-affinity Cry1Ab site; and (iii) that the binding data are consistent with the equal toxicities of the Cry1Aa, Cry1Ab, and Cry1Ac toxins to *M. sexta* larvae. The data reported in this work clearly satisfy these requirements and demonstrate that the 210-kDa BT-R_1 protein is the single common receptor for the Cry1A toxins in *M. sexta* larvae. Taken together with the high degree of sequence homology among these toxins, these results further suggest that BT-R_1 is involved in the pathologic response to the Cry1A toxins.

A possible reason for the differences in relative intensity between the 100- to 120-kDa Cry1Ac binding proteins detected in this study and those reported elsewhere may lie in part in the midgut membrane preparation methods used. Work reported elsewhere by this laboratory demonstrates the instability of the 210-kDa BT-R_1 under certain experimental conditions (9), a finding which may also be important in discussions of toxin binding protein detection. We believe that it is unlikely that the use of different insect populations is responsible for any differences reported between our studies and those of others. Regardless, as outlined above, the 210-kDa BT-R_1 remains the only Cry1A binding protein which has demonstrated the characteristics we consider necessary to be considered a viable candidate receptor.

The results of the present study indicate that expression of BT-R_1 in cell culture confers specific, high-affinity binding of the lepidopteran-specific Cry1A toxins to membranes prepared from these cultures, although binding to intact cells has not been detected. This latter result implies that although numerous existing cell lines are capable of expressing a binding-competent receptor, they cannot properly modify the receptor and do not target it to the cell surface. In *M. sexta* larvae, BT-R_1 expression is highly tissue specific, with significant expression being found only in midgut tissues (27). This observation, along with the molecular mass differences between natural *M. sexta* and cell culture-expressed receptor molecules reported here, suggests that BT-R_1 in *M. sexta* larvae undergoes unique midgut-specific posttranslational processing and trafficking events which the experimental cell lines cannot accommodate. On the other hand, the expressed BT-R_1 protein retains the high affinity and specificity for the Cry1A lepidopteran-specific toxins in a manner essentially identical to that of the *M. sexta* protein, indicating that glycosylation of the protein is not an overriding factor for ligand identification and binding. Therefore, cell culture-expressed BT-R_1 will continue to be extremely useful for studies of receptor-ligand interactions and has allowed us to demonstrate the high affinity and specificity of BT-R_1 for the Cry1Aa, Cry1Ab, and Cry1Ac toxins of *B. thuringiensis*. Additional work on the biochemical nature of BT-R_1 will be necessary to elucidate the molecular mechanism(s) that mediates Cry toxin pathogenesis in the tobacco hornworm, *M. sexta.*

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