APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2001, p. 872–879 Vol. 67, No. 2
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.2.872–879.2001

Binding Analyses of *Bacillus thuringiensis* Cry δ-Endotoxins Using Brush Border Membrane Vesicles of *Ostrinia nubilalis*

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Received 30 May 2000/Accepted 30 November 2000

Transgenic corn expressing the *Bacillus thuringiensis* Cry1Ab gene is highly insecticidal to *Ostrinia nubilalis* (European corn borer) larvae. We ascertained whether Cry1F, Cry9C, or Cry9E recognizes the Cry1Ab binding site on the *O. nubilalis* brush border by three approaches. An optical biosensor technology based on surface plasmon resonance measured binding of brush border membrane vesicles (BBMV) injected over a surface of immobilized Cry toxin. Preincubation with Cry1Ab reduced BBMV binding to immobilized Cry1Ab, whereas preincubation with Cry1F, Cry9C, or Cry9E did not inhibit BBMV binding. BBMV binding to a Cry1F-coated surface was reduced when vesicles were preincubated in Cry1F or Cry1Ab but not Cry9C or Cry9E. A radioligand approach measured ^{125}I-Cry1Ab toxin binding to BBMV in the presence of homologous (Cry1Ab) and heterologous (Cry1Ac, Cry1F, Cry9C, or Cry9E) toxins. Unlabeled Cry1Ac effectively competed for ^{125}I-Cry1Ab binding in a manner comparable to Cry1Ab itself. Unlabeled Cry9C and Cry9E toxins did not inhibit ^{125}I-Cry1Ab binding to BBMV. Cry1F inhibited ^{125}I-Cry1Ab binding at concentrations greater than 500 nM. Cry1F had low-level affinity for the Cry1Ab binding site. Ligand blot analysis identified Cry1Ab, Cry1Ac, and Cry1F binding proteins in BBMV. The major Cry1Ab signals on ligand blots were at 145 kDa and 154 kDa, but a strong signal was present at 220 kDa and a weak signal was present at 167 kDa. Cry1Ac and Cry1F binding proteins were detected at 220 and 154 kDa. Anti-*Manduca sexta* aminopeptidase serum recognized proteins of 145, 154, and 167 kDa, and anti-cadherin serum recognized the 220 kDa protein. We speculate that isoforms of aminopeptidase and cadherin in the brush border membrane serve as Cry1Ab, Cry1Ac, and Cry1F binding proteins.

Bacillus thuringiensis Cry1Ab toxin is a transgene in commercial corn that controls pest insect larvae. The proposed model for *B. thuringiensis* intoxication involves a three-step process: activation, binding, and pore formation. Activation refers to the specific proteolytic processing of the *B. thuringiensis* protein molecule in the midgut of the susceptible organism. This occurs through a combination of pH and proteolysis. Generally, with ca. 130-kDa protoxins, the C-terminal half and approximately 20 to 30 residues of the N terminus are removed, leaving a ca. 65-kDa activated toxin. Binding refers to the association of the activated toxin with specific proteins located on the apical microvilli of epithelial cells lining the gut. Once bound, the toxin undergoes a conformational change (35) that permits insertion of a helical hairpin into the cell membrane. Ultimately, association with additional toxin molecules through oligomerization leads to the formation of a pore (30). Ion flux through the pore leads to osmotic cell lysis and eventual death of the susceptible organism (reviewed in reference 34).

There is evidence that the evolution of resistance to a particular *B. thuringiensis* toxin may develop through the mutation of one or more midgut proteins that bind the toxin (14). For example, *Plutella xylostella*, which has acquired resistance to Cry1Ac in the field, has a greatly reduced number of binding sites for that toxin (10). In further studies with a different population of *P. xylostella* larvae resistant to Cry1A toxins, Tabashnik and coworkers found no binding of Cry1Ac and that the resistance to Cry1A toxins was reversible (37). The reversal of resistance was correlated with the return of Cry1 binding sites. In laboratory studies with another important crop pest, *Heliothis virescens*, prolonged feeding of Cry1Ac toxin over multiple generations led to high levels of resistance to Cry1A and Cry2A toxins (15). Loss of Cry1Aa, but not Cry1Ac, binding to brush border membrane vesicles (BBMV) from resistant *H. virescens* larvae led to the hypothesis that a Cry1A toxin binding site was altered in the resistant insect population (22). Understanding the patterns of Cry toxin binding to BBMV is relevant to the long-term usage of *B. thuringiensis* Cry proteins for insect control.

Cry1 binding proteins detected on ligand blots of insect BBMV have been identified as members of the aminopeptidase N and cadherin families. Aminopeptidases isolated from *Manduca sexta* BBMV have been identified as Cry1 toxin binding proteins (13, 19, 26, 27). Aminopeptidases have also been identified as Cry1A receptors in BBMV isolated from *Lymantria dispar, H. virescens, P. xylostella*, and *Bombyx mori* (13, 26, 27, 41, 44). A 210-kDa cadherin-like glycoprotein has been

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identified as a Cry1Ab binding protein in BBMV prepared from the midguts of *M. sexta* larvae (39, 40). Although initially detected with Cry1Ab, Cry1Aa and Cry1Ac toxins also bind the cadherin-like protein. A 175-kDa cadherin-like protein was identified as a Cry1Aa binding protein in *B. mori* (31, 32).

Cry1Ab is an especially important insecticidal protein due to its use in commercial transgenic corn. Cry1Ab recognizes a single population of binding sites on the brush border epithelium of *Ostrinia nubilalis*, which Cry1Ac also recognizes (9). In contrast, Cry1Ba toxin recognized an independent toxin receptor. Cry1Fa has high activity against *O. nubilalis* (5) and registration is pending for Cry1Fa for transgenic corn. Cry9Ca is also important due to its high activity against *O. nubilalis*. Cry9Ca recognizes a binding site distinct from the Cry1Ab site (21) and is in commercial development for transgenic corn. The current statuses of the Cry1Fa and Cry9Ca corn registrations are found at the U.S. Environmental Protection Agency website (http://www.epa.gov/oppbppd1/biopesticides/).

The objectives of this study were (i) to measure the capacity of Cry1F, Cry9C, and Cry9E toxins to compete for Cry1Ab binding sites on BBMV from *O. nubilalis*, (ii) to determine the molecular sizes of Cry1Ab, Cry1Ac, and Cry1F binding proteins in *O. nubilalis* BBMV, and (iii) to determine if toxin binding proteins corresponded in molecular sizes to proteins recognized by anti-aminopeptidase N (APN) and anti-cadherin antibodies.

MATERIALS AND METHODS

***B. thuringiensis* strains and toxin purification.** *B. thuringiensis* subsp. *kurstaki* HD-73 was obtained from the *Bacillus* Genetic Stock Culture Collection (Columbus, Ohio). The *cry1Ab* gene was cloned from *B. thuringiensis* subsp. *kurstaki* (strain NRD-12) (29). Cry1Fa, Cry9C, and Cry9E were extracted from formulations of transgenic *Pseudomonas fluorescens* (36).

B. thuringiensis subsp. *kurstaki* HD-73 was grown and Cry1Ac toxin was prepared according to previously published methods (23). Cry1Ab toxin was produced in *Escherichia coli* according to the methods of Masson et al. (29) and toxin was prepared according to the methods of Luo et al. (23). Cry1Fa, Cry9C, and Cry9E toxins were prepared as follows. Protoxins were extracted and activated toxin was produced by incubating a 2 mg/ml *P. fluorescens* crystal suspension in 0.1 M Ca_2CO_3 (pH 11.0) containing 0.1% trypsin (Boehringer Mannheim). Cry1F was activated for 2 h at room temperature and Cry9C preparations were activated for 30 min. A cocktail of proteinase inhibitors (Protease Inhibitor Cocktail Set III; Calbiochem, San Diego, Calif.) was added to trypsin-activated Cry9C toxin. Activated toxins were centrifuged at 150,000 × *g* to remove colloidal lipids and then were purified by fast protein liquid chromatography-Mono Q (Amersham Pharmacia Biotech) ion exchange chromatography as described elsewhere (29). The toxins were fractionated by a 50 to 500 mM linear gradient of NaCl. The toxin peak eluting at 350 mM NaCl was pooled and dialyzed extensively against water with continuous stirring to precipitate the toxins. Precipitated toxins were stored at 4°C until needed, at which time an aliquot was removed and solubilized in HEPES-buffered saline (HBS) (10 mM HEPES [pH 7.4], 150 mM NaCl) and protein concentrations were determined. All protein concentrations were measured by the method of Bradford (2) using bovine serum albumin (BSA) as a standard.

Cry protein preparations for insect bioassays. *cry1Ab, cry1Ac, cry1Fa, cry9C*, and *cry9E* were engineered separately into DOW Agrosciences' inducible plasmid vectors by using standard DNA cloning methods and subsequently were transformed into *P. fluorescens* strains MR818, 843, 872, 1260, and 1264, respectively. Following conventional fermentation and induction, the culture pellets were recovered by centrifugation (10,000 × *g* for 20 min). The cell pellet was washed twice with water and collected by centrifugation as before. The washed pellet was suspended to 10% of its original culture volume in water and lyophilized. The lyophilized materials were quantitatively analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) laser densitometry for toxin content (3). BSA served as the standard. SDS-PAGE was performed according to the method of Laemmli (20). The proteins were stained and destained using Gelcode Bluestain G-250 (Pierce) following the manufacturer's recommendations. Densitometry was performed on a Personal Densitometer SI (Molecular Dynamics). The concentration of the Cry protein was interpolated from the BSA standard curve.

***O. nubilalis* bioassay.** Diet incorporation assays were conducted on *O. nubilalis* larvae to compare Cry1Ab, Cry1Ac, Cry1F, Cry9E, and Cry9C toxicities. The lyophilized toxins were mixed into the bioassay diet and then thoroughly mixed by being vortexed prior to being dispensed into assay plates. Ten doses were used per toxin on a total of 20 first-instar larvae per dose. Mortality was scored after 5 days at 29°C. The 50% lethal concentration (LC_{50}) values and the slopes of concentration-mortality regression lines were obtained using the POLO-PC program (33).

***O. nubilalis* rearing and preparation of BBMV.** *O. nubilalis* eggs were provided by Bruce Lang (DOW Agrosciences, Huxley, Iowa). Eggs were hatched and larvae were grown on an artificial diet preparation (Southland Products, Lake Village, Alaska) at 26°C, 70% relative humidity, with a photoperiod consisting of 12 h of light and 12 h of darkness. Midguts were excised from fifth-instar *O. nubilalis* larvae and frozen on dry ice. About 5 g of midgut tissue (wet weight) was used for each BBMV preparation. BBMV were prepared by the $MgCl_2$ precipitation method (43) with modifications (10). The final BBMV pellet was suspended in 0.3 M mannitol–5 mM EGTA–17 mM Tris-Cl (pH 7.5), and was stored at −70°C.

BBMV were also prepared from whole insects as follows. Larvae were collected and stored at −70°C until needed. Whole frozen insects were added to ice-cold grinding buffer (50 mM sucrose, 2 mM Tris-Cl [pH 7.2], 25 μg of phenylthiourea per ml) in the ratio 1 g of larvae per 10 ml of grinding buffer. Larvae were homogenized for 60 s or until no large insect fragments were visible using a Polytron tissue homogenizer (Braun) at the highest setting. The homogenate was ground further with 15 to 20 strokes of a Dounce homogenizer. $CaCl_2$ was added to 10 mM and the homogenate was stirred at 5°C for 25 min. The mixture was centrifuged at 5,200 rpm (~4,200 × *g*) in a JS-13 rotor (Beckman) for 15 min at 4°C and the pellet was discarded. The supernatant was then reclarified by centrifugation at ~4,200 × *g*. The reclarified supernatant was centrifuged in a JS-13 rotor at 12,500 rpm (~25,000 × *g*) for 25 min and the pellet was resuspended in HBS. BBMV were sonicated to create uniformly sized vesicles of less than 0.5 μm. Sonication was for one min (80 W at 47 kHz). BBMV were cooled on ice for 1 min and the sonication step was repeated.

Leucine aminopeptidase assays on crude homogenate and BBMV were done according to previously published methods (11).

BIAcore instrumentation. The BIAcore 1000 system and CM5 sensor chips were purchased from Pharmacia Biosensor (Piscataway, N.J.). All protein chemical immobilizations were done using the standard BIAcore amino coupling protocol provided with the Pharmacia coupling kit. HBS, the general buffer used with the BIAcore machine, was used as running and diluting buffers for all vesicle experiments. A flow rate of 5 μl/min and a standard BBMV concentration of 0.2 μg of vesicle protein/μl were used for all experiments. Surface regenerations were carried out by injecting two separate 1-min pulses at 5 μl of regeneration solution (1% Zwittergent 3-14) per min. Whole-system cleaning of colloidal lipid vesicles was carried out when needed by injections of 0.5% SDS solution.

Toxin immobilization on BIAcore CM5 sensor chips. To immobilize toxin to the carboxymethylated dextran (CM5) sensor chip surface, standard amine coupling was used. Carboxyl groups along the CM-dextran chains of the sensor chip surface are activated by exposure (35 μl at 5 μl/min) to a mixture of NHS (0.1 M *N*-hydroxysuccinimide)–EDC [0.1 M *N*-ethyl-*N*-(3-diethylaminopropyl) carbodiimide] (1:1, vol/vol). The resulting succinimidyl ester groups are highly reactive with the free amine group of the N-terminal residue and the solvent-facing lysine or arginine residues of the *B. thuringiensis* protein. Toxin was injected over the surface at 0.1 mg/ml in coupling buffer (20 mM ammonium acetate [pH 4]) with the contact time (i.e., flow rate and injection volume) controlled so as to immobilize the precise quantity desired. In general, approximately 3,000- to 5,000-resonance unit (RU) surfaces were used, representing approximately 3 to 5.5 ng of toxin (1,000 RU equals 1 ng of protein per mm^2). After coupling, unreacted surface ester groups were blocked by exposure to 1 M ethanolamine (pH 8.5). New surfaces were conditioned prior to use by two regenerative detergent pulses as described above.

Protocol for BBMV surface plasmon resonance analyses. *O. nubilalis* BBMV were preincubated with either toxin or an equivalent amount of BSA on ice for 60 min. BBMVs were preincubated with 6 μM toxin in either homologous or heterologous competition experiments. The BBMV mix (35 μl) was injected over an immobilized toxin (or BSA) surface at a rate of 5 μl/min. Using literature-derived dissociation values where *B. thuringiensis* toxins fall in the moderate to high affinity range of 10 nM to 0.1 nM, 6 μM represents a 600- to 6,000-fold excess of competitor toxin.

TABLE 1. Toxicities of nonactivated *B. thuringiensis* Cry proteins to larvae of *O. nubilalis*

Protein	LC_{50}[a] (95% FL)	Slope[b]
Cry1Ab	0.29 (0.12–0.59)	1.46 ± 0.24
Cry1Ac	0.08 (0.06–0.11)	2.01 ± 0.17
Cry1F	0.36 (0.18–1.40)	0.92 ± 0.13
Cry9C	0.21 (0.14–0.32)	2.87 ± 0.34
Cry9E	0.83 (0.50–1.81)	1.69 ± 0.34

[a] LC_{50} values in micrograms of Cry protein per ml of artificial diet. Numbers in parentheses represent the range of results. FL, fiducial limit.
[b] Values represent means ± standard deviations.

Radioligand binding assays. Binding assays were performed as previously described (12) using BBMV isolated from dissected midguts. Purified Cry1Ab (1 μg) was radiolabeled using 0.5 mCi of $Na^{125}I$ (Amersham Pharmacia Biotech) as described previously (12). Specific activity was 64 μCi/μg based on input toxin. To evaluate competitive toxin binding, duplicate samples of BBMV from *O. nubilalis* were incubated with 0.1 nM ^{125}I-Cry1Ab in the presence of different amounts of Cry1Ab, Cry1Ac, Cry1F, Cry9C, or Cry9E toxin. All assays were performed at least two times. Each assay mixture contained 75,000 cpm of ^{125}I-Cry1Ab in 100 μl of Tris-buffered saline (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl) containing 0.1% BSA and 30 μg of BBMV, except for Cry1Ac competition assays, in which case 20 μg of BBMV was present. Assay mixtures were incubated for 60 min at room temperature and samples were centrifuged at 13,000 × *g* for 8 min and the pelleted BBMV was washed twice with ice-cold Tris-buffered saline containing 0.1% BSA. Radioactivity was measured with a Beckman model Gamma 4000 counter. Using the results of these binding experiments, we calculated the dissociation constants (K_d for Cry1Ab and K_{com} for Cry1Ac) and the binding site concentrations (B_{max}) with the LIGAND computer program (Biosoft).

SDS-PAGE, Cry toxin ligand blot, and immunoblot analyses. Toxin preparations were analyzed by SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250. For ligand blot and immunoblot analyses, BBMV were prepared and separated by SDS–7% PAGE on the same day. BBMV samples were loaded in a preparative well adjacent to prestained protein molecular size standards (Bio-Rad, Richmond, Calif.). After separation by electrophoresis, proteins were transferred to a polyvinylidene difluoride Q membrane filter (PVDF) (Millipore) in transfer buffer (38). The PVDF was cut into strips and blocked with 3% BSA in phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 2.7 mM KCl, 136.9 mM NaCl [pH 7.4]) at room temperature with gentle agitation for 1 h. Ligand blotting was done with unlabeled toxin added to a final concentration of 0.01 μg/ml in 0.1% BSA–0.1% Tween 20 in PBS and the PVDF was incubated for 1 h at room temperature. Filter strips were washed three times in 0.1% BSA–0.1% Tween 20 in PBS. The primary antibody was either anti-Cry1Ac or anti-Cry1F toxin rabbit serum diluted (1:30,000 for anti-Cry1Ac and 1:5,000 for anti-Cry1F) in 0.1% BSA–0.1% Tween 20 in PBS. Incubation in anti-Cry toxin serum was for 2 h at room temperature. After being washed three times as described above, filter strips were incubated in 0.1% BSA–0.1% Tween 20 in PBS containing goat anti-rabbit-peroxidase conjugate for 1 h. Detection was performed with an ECL kit (Amersham Pharmacia Biotech). Immunoblotting was done using the same procedure, except primary antibody was prepared against an *E. coli*-expressed portion of *M. sexta* 115-kDa APN (25). The anti-cadherin antibody was provided by D. Dean (Ohio State University).

RESULTS

Insect toxicity. Table 1 shows the results of bioassays conducted with *O. nubilalis*. As previously reported, Cry1Ab, Cry1Ac, Cry1F, and Cry9C are highly toxic to *O. nubilalis* (5, 9, 21). Cry9E also has high activity against *O. nubilalis*.

Toxin and BBMV characterization. Figure 1 shows the purity of the Cry toxins used in surface plasmon resonance, radioligand binding, and ligand blot experiments. Each Cry1A toxin appeared as a single band on an SDS-PAGE gel after staining for unlabeled toxins and autoradiography for ^{125}I-labeled Cry1Ab. While most of the Cry9C toxin was 67 kDa, some 55-kDa protein is visible in Fig. 1 (lane 5). Cry9E-activated toxin is slightly smaller in molecular size than Cry9C. Like Cry9C (21), Cry9E is susceptible to overdigestion by trypsin.

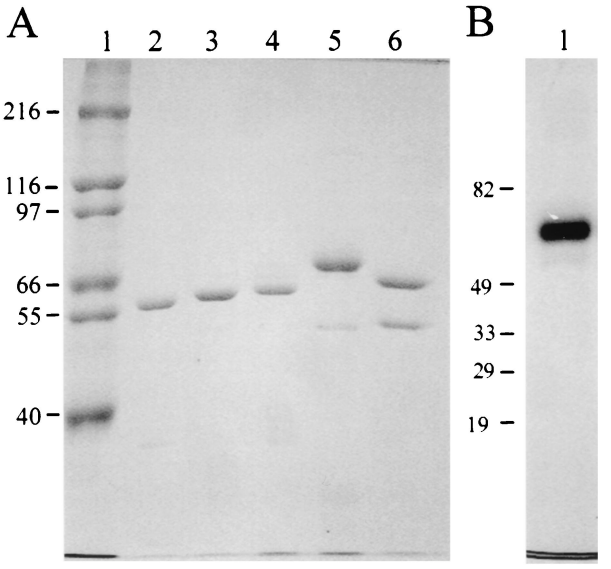

FIG. 1. SDS-PAGE and autoradiography of purified Cry toxins. (A) Coomassie blue-stained SDS-PAGE gel. Lane 1, molecular size markers; lane 2, Cry1Ab; lane 3, Cry1Ac; lane 4, Cry1F; lane 5, Cry9C; lane 6, Cry9E. (B) Autoradiography of ^{125}I-labeled toxin. Lane 1, Cry 1Ab. The numbers on the left of each panel are molecular masses (in kilodaltons).

BBMV were prepared from dissected midguts and whole *O. nubilalis* larvae. During the purification process, samples were taken and assayed for leucine aminopeptidase activity (43). Specific activity was calculated to be 20 U (optical density at 405 nm/min)/mg of BBMV protein for preparations from dissected midguts and 17 U/mg of BBMV protein for preparations from whole larvae. Aminopeptidase specific activity for dissected midgut homogenate was 1.1 U/mg of protein, resulting in an 18-fold enrichment in the final BBMV preparation.

Surface plasmon resonance: control experiments—Cry1Ab surface. Control experiments on the Cry1Ab surface were performed to ascertain any anomalous interactions with the preincubated Cry toxin (Cry-Cry interactions), or alternatively, the BBMV and the surface. When 6 μM Cry1Ab was injected over an immobilized surface (5,000 RU) of Cry1Ab, no evidence of interaction was observed (data not shown). From these experiments it was shown that Cry1Ab does not stick to either the immobilized Cry1Ab or the dextran surface of the chip. Similarly, *O. nubilalis* BBMV stick poorly to either the immobilized BSA or the dextran surface of the chip.

Surface plasmon resonance: competition experiments—Cry1Ab surface. Competition of Cry1F, Cry9C, and Cry9E for Cry1Ab binding sites on BBMV was measured by surface plasmon resonance analysis. The basic approach was to inject purified BBMV that had been preincubated with a toxin over an immobilized toxin surface of the same type (homologous competition) or a different type (heterologous competition). A typical representation of Cry1Ab homologous inhibition is shown in Fig. 2A. Taking a time point 60 s after the start of

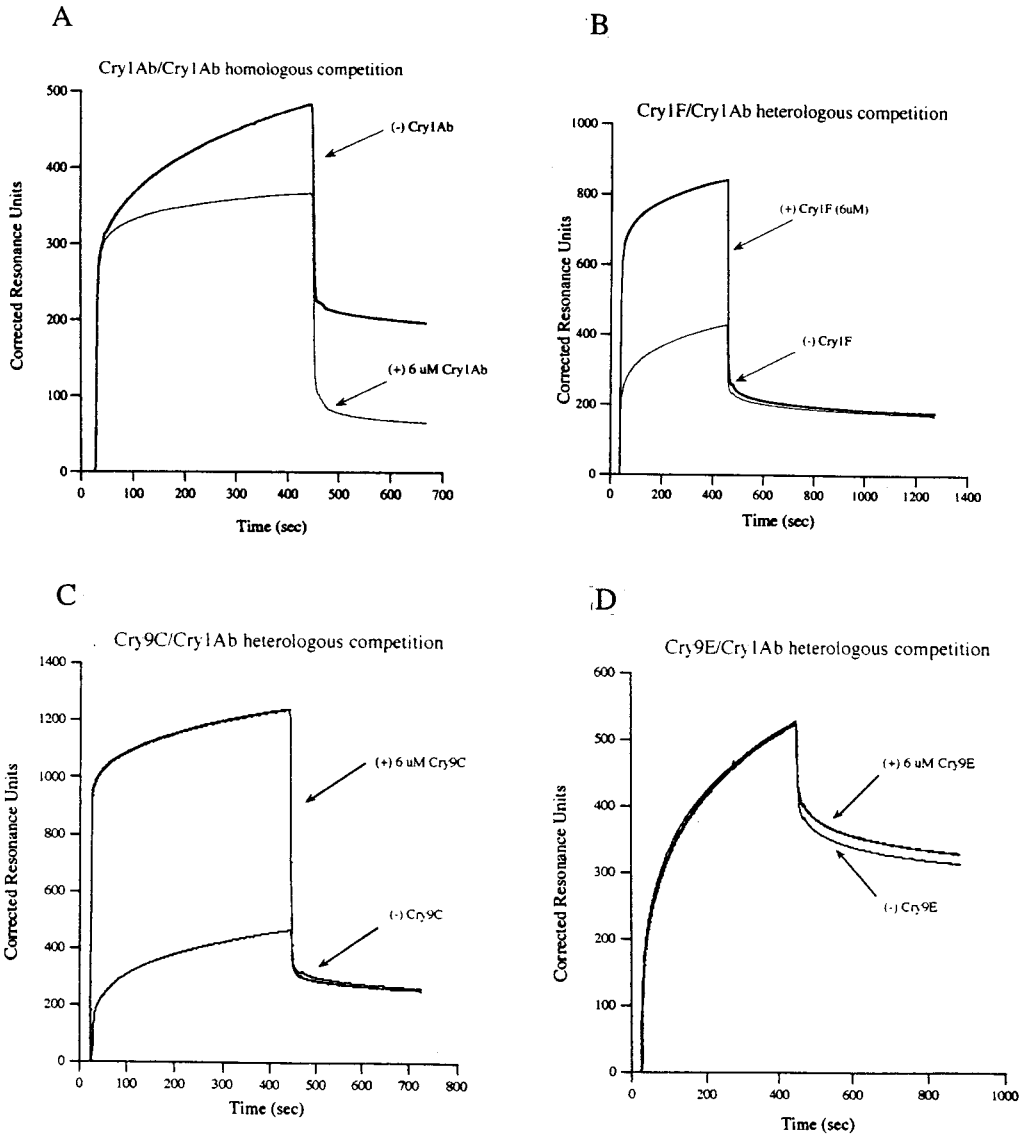

FIG. 2. Homologous and heterologous competition of Cry toxins for Cry1Ab sites on BBMV purified from *O. nubilalis* larvae. Vesicles were preincubated for 60 min on ice with BSA, Cry1Ab (A), Cry1F (B), Cry9C (C), or Cry9E (D). BBMV were then injected over a surface of immobilized Cry1Ab (4,000 to 5,000 RU). All response curves were adjusted for the mass action of the injected buffer components.

wash-off we see approximately 64% inhibition of binding, or alternatively, 36% nonspecific binding.

In general, no significant competition was observed when BBMV were preincubated in Cry1F, Cry9C, or Cry9E toxin (Fig. 2B through D). The inference is that the three toxins recognize and bind to receptors separate from the Cry1Ab receptor on the BBMV surface. This is apparent in all heterologous sensorgrams, where the decreasing slopes of the competitor and noncompetitor curves were the same (essentially superimposable), showing that the mass accumulation on the surface occurs at the same rate. By comparison, the slopes of the homologous competition curves are quite different. As clearly demonstrated in Fig. 2A, when competition occurs the slope of the curve containing a competitor is reduced compared to that of BBMV without a competitor.

Surface plasmon resonance: control experiments—Cry1F surface. A typical result for a Cry1F surface experiment is shown in Fig. 3A, in which 6 μM Cry1F was injected over an immobilized surface (3,000 RU) of Cry1F. Taking a time point 60 s after the start of wash-off we see approximately 72% inhibition, or alternatively, 28% nonspecific binding. The competition numbers are relatively similar to those previously reported for Cry1Ab.

Surface plasmon resonance: competition experiments—Cry1F surface. In the case of Cry1Ab preincubated vesicles, competition was observed against immobilized Cry1F surface (Fig. 3B). Interestingly, no competition was observed in the reverse configuration, i.e., when Cry1Ab was immobilized. In general, no significant competition was observed in two types of competitive experiments (Cry9C- and Cry9E-preincubated

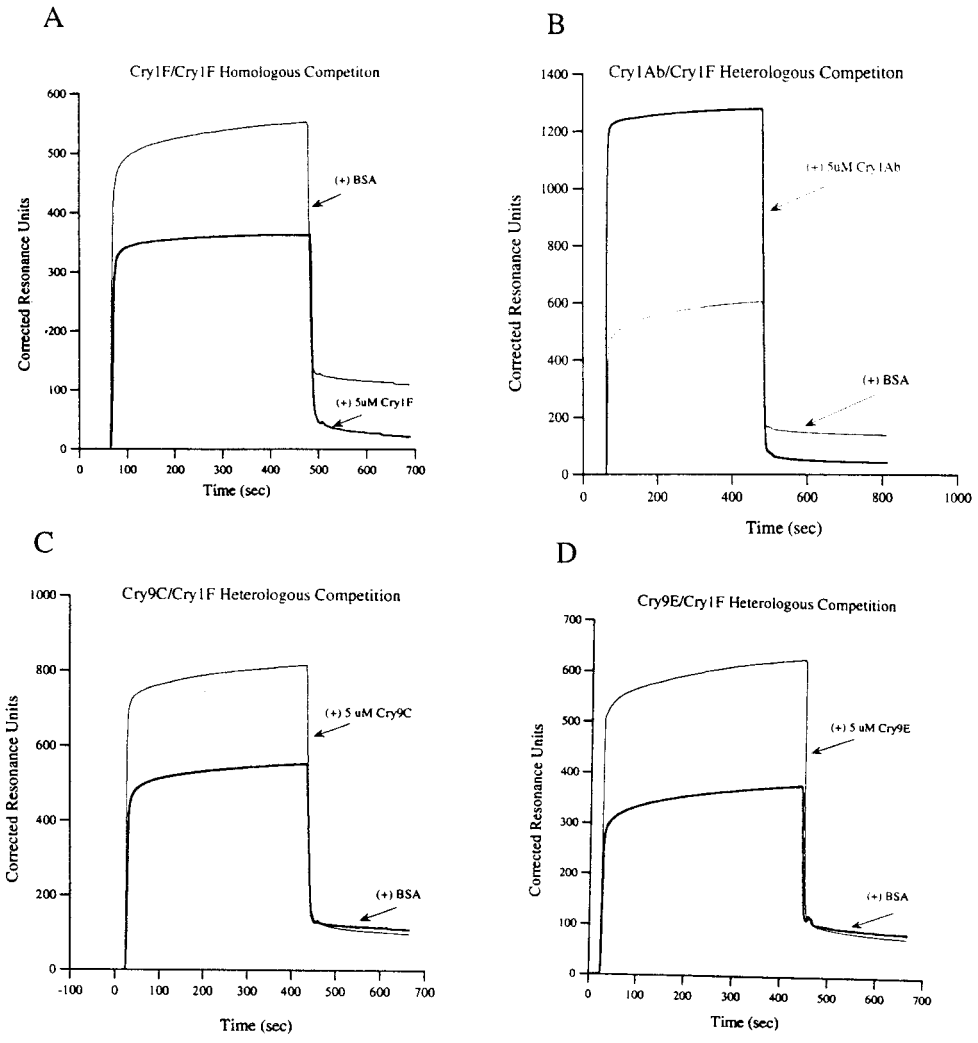

FIG. 3. Homologous and heterologous competition of Cry toxins for Cry1F sites on BBMV purified from *O. nubilalis* larvae. Vesicles were preincubated for 60 min on ice with BSA, Cry1F (A), Cry1Ab (B), Cry9C (C), or Cry9E (D). BBMV were then injected over a surface of immobilized Cry1F (2,000 to 4,000 RU).

BBMV), indicating that the Cry9 toxins bind to a separate receptor (or receptors) from Cry1F on the *O. nubilalis* BBMV surface (Fig. 3B and C). The 50-kDa forms of Cry9E and Cry9C can compete for the same Cry9 receptor on the *O. nubilalis* BBMV. The same holds true for the 65-kDa forms of the toxins (data not shown).

Radioligand competition binding. A qualitative binding experiment was done to identify a concentration of BBMV from *O. nubilalis* suitable for competition binding experiments. ^{125}I-labeled Cry1Ab was incubated with varyious concentrations of BBMV. Maximal specific binding of Cry1Ab was observed at concentrations of greater than 200 μg of vesicle protein per ml (data not shown). This value for maximal Cry1Ab binding is comparable to the value determined previously (1). Competition binding experiments were performed with 300 μg of vesicle protein per ml for all competing toxins, except for Cry1Ac competition assays (200 μg of vesicle protein per ml). Maximal ^{125}I-Cry1Ab binding ranged from 6 to 16% for individual competition binding experiments.

Figure 4 shows plots of data from competition experiments performed with ^{125}I-Cry1Ab and unlabeled competitor toxins. ^{125}I-Cry1Ab bound to BBMV from *O. nubilalis* with high affinity (K_d = 1.2 nM ± 1.0 nM). The determined B_{max} for Cry1Ab binding sites was 0.23 ± 0.13 pmol per mg of BBMV. As expected from previously published results (9), the presence of Cry1Ac prevented Cry1Ab from binding to BBMV. The determined K_{com} for Cry1Ac binding was 7.5 nM ± 2.4 nM and B_{max} was 0.98 ± 0.33 pmol per mg of BBMV. Cry1F reduced the amount of ^{125}I-Cry1Ab bound only at the highest concentration of Cry1F tested. ^{125}I-Cry1Ab binding was 49% of the maximal in the presence of 1,000 nM Cry1F (Fig. 4). Cry9C and Cry9E toxins did not compete for Cry1Ab binding sites.

Ligand blotting. Ligand blotting was done to identify the molecular sizes of Cry1Ab, Cry1Ac, and Cry1F binding proteins in BBMV isolated from *O. nubilalis* midgut tissue. Cry1Ab recognized proteins of 145, 154, and 220 kDa (Fig. 5, lane 2). Cry1Ac and Cry1F binding proteins were detected at

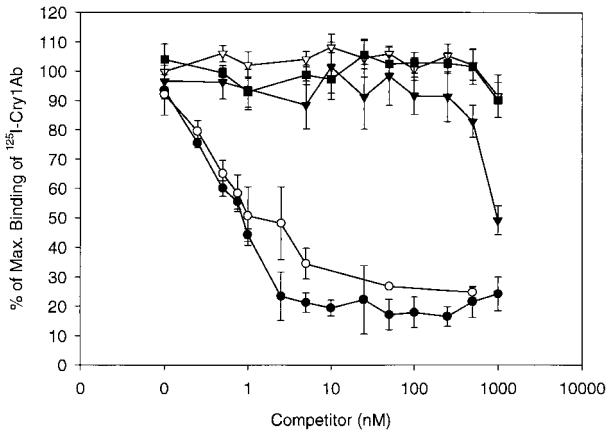

FIG. 4. Competition between ^{125}I-labeled Cry1Ab and unlabeled Cry1Ab (●), Cry1Ac (○), Cry1F (▼), Cry9C (▽), and Cry9E (■) toxins. *O. nubilalis* BBMV were incubated with ^{125}I-labeled Cry1Ab at a concentration of 0.1 nM plus different concentrations of unlabeled toxins. Binding was expressed as a percentage of the maximum amount of radiolabeled toxin bound during incubation in the absence of competitors. Each data point is a mean based on the results of two independent experiments using duplicate samples. Standard deviation between samples is shown by error bars.

154 kDa and 220 kDa. A weak signal for Cry1Ab and Cry1F is also visible at 167 kDa. Additionally, each toxin recognized a protein or aggregate that barely migrated into the analytical gel. The similarity between Cry1Ac and Cry1F binding patterns on ligand blots was striking, while Cry1Ab recognition differed by detecting an additional protein of 145 kDa.

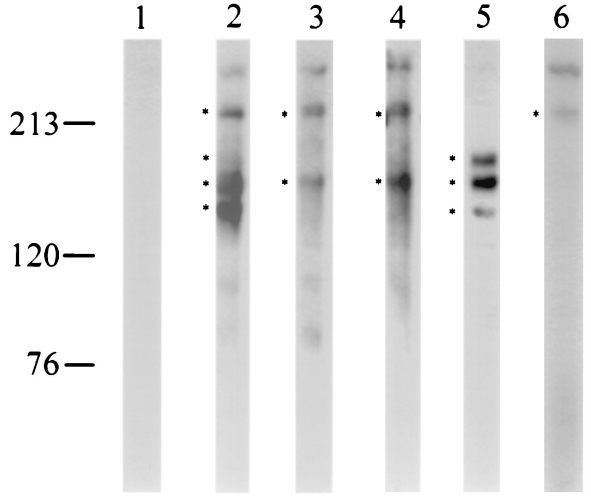

FIG. 5. Toxin ligand blotting and immunoblotting analyses of midgut proteins of *O. nubilalis*. Lane 1, incubation without toxin and with anti-Cry1Ac serum; lane 2, incubation with 0.01 μg of Cry1Ab per ml and anti-Cry1Ac serum; lane 3, incubation with 0.01 μg of Cry1Ac per ml and anti-Cry1Ac serum; lane 4, incubation with 0.01 μg of Cry1F per ml and anti-Cry1F antiserum; lane 5, incubation with anti-APN serum; lane 6, incubation with anti-cadherin serum. Secondary antibody was anti-rabbit-peroxidase and detection was by enhanced chemiluminescence. Bands indicated by asterisks are discussed in the text. An unmarked band is visible near the top of lanes 2 through 6. The numbers on the left are molecular masses (in kilodaltons).

Because aminopeptidases are Cry1 toxin binding proteins in other insect species, we probed a strip of blotted *O. nubilalis* BBMV protein with antibody prepared against *E. coli*-expressed *M. sexta* APN (Fig. 5, lane 5). This strip was taken from the same filter as that used for Cry1 toxin ligand blots. Anti-APN detected proteins of 145, 154, and 167 kDa in *O. nubilalis* BBMV (Fig. 5, lane 5). Of these three putative aminopeptidases, Cry1Ab, Cry1Ac, and Cry1F toxins recognized the 154-kDa protein. The 145-kDa protein, and to some extent, the 167-kDa protein, are recognized only by Cry1Ab. Anti-cadherin serum recognized the 220-kDa protein (Fig. 5, lane 6).

DISCUSSION

Our primary objective was to determine if Cry1F and Cry9 toxins recognize the Cry1Ab binding site (or sites) on BBMV from *O. nubilalis*. The results from surface plasmon resonance measurements and radioligand binding experiments are in agreement. There appear to be several Cry toxin binding sites and/or receptors in the midgut epithelia of *O. nubilalis*. As expected from prior results (9), Cry1Ac effectively competes for the Cry1Ab binding site. Cry9C and Cry9E appear to compete for a binding site or sites different from those of Cry1Ab and Cry1Ac. Cry9C is known to recognize a site different from the Cry1Ab site (21).

Cry1F has multiple binding sites on *O. nubilalis* BBMV. It is likely that one site is recognized with high affinity and a second site is recognized with low affinity. In surface plasmon resonance experiments Cry1F did not inhibit BBMV binding to a Cry1Ab surface (Fig. 2). However, Cry1Ab-preincubated BBMV showed reduced binding to a Cry1F surface (Fig. 3). Also, high doses of Cry1F reduced ^{125}I-Cry1Ab binding to vesicles (Fig. 4). These results are explained if Cry1F has low affinity for the Cry1Ab binding site. The lack of high-affinity Cry1F competition was unexpected. In *P. xylostella*, Cry1Fa and Cry1Ab share a high-affinity binding site (16) and Cry1A-resistant *P. xylostella* larvae are cross resistant to Cry1F (37). Our Cry1F vesicle binding data suggest that the *P. xylostella* model, whereby Cry1Ab and Cry1F both bind with high affinity to a common site, does not apply to *O. nubilalis* BBMV. Because functional Cry1 toxin binding is typified by affinity binding constants in the nanomolar range (42), it is possible that Cry1F recognition of the Cry1Ab site is not related to Cry1F toxicity.

Ligand and immunoblot analyses yielded insights into Cry1Ab, Cry1Ac, and Cry1F recognition of BBMV proteins. Each toxin recognized a 154-kDa protein (probably an APN) and a 220-kDa protein (probably a cadherin-like protein). Cry1Ab also recognized a 145- and 167-kDa APN. Previous studies of Cry1Ab and Cry1Ac binding proteins in *M. sexta* provide comparisons with our results. In *M. sexta*, Cry1Ab and Cry1Ac share binding sites on BT-R_1, the 210-kDa cadherin-like protein (18). The 220-kDa Cry1 binding protein in *O. nubilalis* is probably homologous to the 210-kDa protein called BT-R_1 in *M. sexta* due to detection by the anti-cadherin (BT-R_1) serum. Cry1Ab also binds to APN in *M. sexta*. Luo et al. (24) affinity selected a 106-kDa APN by using immobilized Cry1Ab, and Masson et al. (28) showed that Cry1Ab recognizes a binding site on 115-kDa APN purified from *M. sexta* BBMV. Some of the confusion about multiple Cry1 binding

proteins is explained by analyses of mutated Cry1Ab and Cry1Ac toxins and ligand blotting. Basically, domain II of Cry1Ab recognizes the 210-kDa protein (7) while interaction with APN is specified by domain III (8). A triple mutant in Cry1Ac at amino acid residues Asn506, Gln509, and Tyr513 showed reduced binding to *M. sexta* APN on ligand blots (4). Jenkins et al. (17) recently reported that a triple Cry1Ac mutant, 509–511 (GluAsnArg-AlaAlaAla), had eliminated APN binding and reduced BBMV binding but retained binding to a band of >200 kDa on ligand blots. Our results are in agreement with *M. sexta* studies where Cry1Ab and Cry1Ac recognize multiple molecules in the brush border membrane, one molecule being an isoform of APN and the other molecule being a cadherin-like protein. Further, Cry1F binding to the 154- and 220-kDa proteins on ligand blots suggests that Cry1F also has multiple binding determinants, possibly specified independently by domains II and III.

O. nubilalis BBMV have proteins of 145, 154, and 167 kDa that are detected by anti-APN serum. Our APN antiserum was prepared with a 30-kDa peptide from Ms-APN-1 expressed in *E. coli* (25). APN comprises a family of at least two genes in Lepidoptera (6). Chang et al. (6) observed that members of the two APN families are more closely related to gene family members within other lepidopteran species (about 60%) than to the other gene family within the same species (about 26%). Since our anti-Ms-APN-1 serum reacted poorly with Ms-APN-2 (106-kDa APN) (data not shown), *O. nubilalis* APN detected on immunoblots may be more closely related to Ms-APN-1 relative to Ms-APN-2. We do not know if the three aminopeptidases detected in *O. nubilalis* are separate gene products or the same aminopeptidase glycosylated differently.

Our vesicle binding analyses of Cry1F, Cry9C, and Cry9E binding are evidence that the Cry1F and Cry9 toxins are compatible with Cry1Ab for *O. nubilalis* pest management. It would be interesting to extend our ligand blot analyses of *O. nubilalis* BBMV proteins through the use of mutated toxins and purified and/or expressed binding proteins. We can then clarify the relationship between toxin-receptor interaction and in vivo toxin potency.

ACKNOWLEDGMENTS

This research was funded by DOW Agrosciences.

We thank Ben McGraw (Department of Entomology, DOW Agrosciences, Indianapolis, Ind.) for conducting insect bioassays.

REFERENCES

1. **Bai, C., D. Degheele, S. Jansens, and B. Lambert.** 1993. Activity of insecticidal crystal proteins and strains of *Bacillus thuringiensis* against *Spodoptera exempta*. J. Invertebr. Pathol. **62:**211–215.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Brussock, S. M., and T. C. Currier.** 1990. Use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis to quantify *Bacillus thuringiensis* δ-endotoxins, p. 78–87. *In* L. A. Hickle and W. L. Fitch (ed.), Analytical chemistry of *Bacillus thuringiensis*. American Chemical Society, Washington, D.C.
4. **Burton, S. L., D. J. Ellar, J. Li, and D. J. Derbyshire.** 1999. N-Acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Mol. Biol. **287:**1011–1022.
5. **Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. B. Johnson, and C. Gawron-Burke.** 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J. Bacteriol. **173:**3966–3976.
6. **Chang, W. X. Z., L. J. Gahan, B. E. Tabashnik, and D. G. Heckel.** 1999. A new aminopeptidase from diamondback moth provides evidence for a gene duplication event in Lepidoptera. Insect Mol. Biol. **8:**171–177.
7. **de Maagd, R. A., M. S. G. Kwa, H. ven der Klei, T. Yamamoto, B. Schipper, J. Vlak, W. J. Stiekema, and D. Bosch.** 1996. Domain III substitution in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl. Environ. Microbiol. **62:**1537–1543.
8. **de Maagd, R. A., P. L. Bakker, L. Masson, M. J. Adang, S. Sangadala, W. Stiekema, and D. Bosch.** 1999. Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol. Microbiol. **31:**463–471.
9. **Denolf, P., S. Jansens, M. Peferoen, D. Degheele, and J. Van Rie.** 1993. Two different *Bacillus thuringiensis* delta-endotoxin receptors in the midgut brush border membrane of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyrallidae). Appl. Environ. Microbiol. **59:**1828–1837.
10. **Ferre, J., M. D. Real, J. Van Rie, S. Jansens, and M. Peferoen.** 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. USA **88:**5119–5123.
11. **Garczynski, S. F., and M. J. Adang.** 1995. *Bacillus thuringiensis* CryIA(c) δ-endotoxin binding aminopeptidase in the *Manduca sexta* midgut has a glycosyl-phosphatidylinositol anchor. Insect Biochem. Mol. Biol. **25:**409–415.
12. **Garczynski, S. F., J. W. Crim, and M. J. Adang.** 1991. Identification of putative brush border membrane binding proteins specific to *Bacillus thuringiensis* delta-endotoxin by protein blot analysis. Appl. Environ. Microbiol. **57:**2816–2820.
13. **Gill, S., E. A. Cowles, and V. Francis.** 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J. Biol. Chem. **270:**27277–27282.
14. **Gould, F.** 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. **43:**701–726.
15. **Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva, and W. J. Moar.** 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **89:**7986–7990.
16. **Granero, F., V. Ballester, and J. Ferre.** 1996. *Bacillus thuringiensis* crystal proteins Cry1Ab and Cry1Fa share a high affinity binding site in *Plutella xylostella* (L.). Biochem. Biophys. Res. Commun. **224:**779–783.
17. **Jenkins, J. L., M. K. Lee, S. Sangadala, M. J. Adang, and D. H. Dean.** 2000. Binding of *Bacillus thuringiensis* CryIAc toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Lett. **462:**373–376.
18. **Keeton, T. P., and L. A. J. Bulla.** 1997. Ligand specificity and affinity of BT-R_1, the *Bacillus thuringiensis* Cry1A toxin receptor from *Manduca sexta*, expressed in mammalian and insect cell cultures. Appl. Environ. Microbiol. **63:**3419–3425.
19. **Knight, P. J. K., N. Crickmore, and D. J. Ellar.** 1994. The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane is aminopeptidase N. Mol. Microbiol. **11:**429–436.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
21. **Lambert, B., L. Buysse, C. Decock, S. Jansens, C. Piens, B. Saey, J. Seurinck, K. Van Audenhove, J. Van Rie, A. Van Vliet, and M. Peferoen.** 1996. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. Appl. Environ. Microbiol. **62:**80–86.
22. **Lee, M. K., F. Rajamohan, F. Gould, and D. H. Dean.** 1995. Resistance to *Bacillus thuringiensis* CryIA δ-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. **61:**3836–3842.
23. **Luo, K., D. Banks, and M. J. Adang.** 1999. Toxicity, binding, and permeability analyses of four *Bacillus thuringiensis* Cry1 δ-endotoxins by use of brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. Appl. Environ. Microbiol. **65:**457–464.
24. **Luo, K., Y. Lu, and M. J. Adang.** 1996. A 106-kDa form of aminopeptidase is a receptor for *Bacillus thuringiensis* CryIC δ-endotoxin in the brush border membrane of *Manduca sexta*. Insect Biochem. Mol. Biol. **26:**33–40.
25. **Luo, K., J. McLachlin, M. R. Brown, and M. J. Adang.** 1999. Expression of *Manduca sexta* aminopeptidase in insect cells. Protein Expr. Purif. **17:**113–122.
26. **Luo, K., S. Sangadala, L. Masson, A. Mazza, R. Brousseau, and M. J. Adang.** 1997. The *Heliothis virescens* 170-kDa aminopeptidase functions as 'Receptor A' by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. **27:**735–743.
27. **Luo, K., B. E. Tabashnik, and M. J. Adang.** 1997. Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant diamondback moths (*Plutella xylostella*). Appl. Environ. Microbiol. **63:**1024–1027.
28. **Masson, L., Y. Lu, A. Mazza, R. Brosseau, and M. J. Adang.** 1995. The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. **270:**20309–20315.
29. **Masson, L., G. Prefontaine, L. Peloquin, P. C. K. Lau, and R. Brousseau.** 1989. Comparative analysis of the individual protoxin components in p1

crystals of *Bacillus thuringiensis* subsp. *kurstaki* isolate NRD-12. Biochem. J. **269:**507–512.
30. **Masson, L., B. E. Tabashnik, Y. B. Liu, and J. L. Schwartz.** 1999. Helix 4 of the *Bacillus thuringiensis* Cry1Aa toxin lines the lumen of the ion channel. J. Biol. Chem. **274:**31996–32000.
31. **Nagamatsu, Y., S. Toda, T. Koike, Y. Miyoshi, S. Shigematsu, and M. Kogure.** 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. Biosci. Biotechnol. Biochem. **62:**727–734.
32. **Nagamatsu, Y., S. Toda, F. Yamaguchi, M. Ogo, M. Kogure, M. Nakamura, Y. Shibata, and T. Katsumoto.** 1998. Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. Biosci. Biotechnol. Biochem. **62:**718–726.
33. **Russell, R. M., J. L. Robertson, and N. E. Savin.** 1977. POLO: a new computer program for probit analysis. Bull. Entomol. Soc. Am. **23:**209–213.
34. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal. Microbiol. Mol. Biol. Rev. **62:**775–806.
35. **Schwartz, J.-L., M. Juteau, P. Grochulski, M. Cygler, G. Prefontaine, R. Brousseau, and L. Masson.** 1997. Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. FEBS Lett. **410:**397–402.
36. **Soares, G. G., and T. C. Quick.** 1992. MVP, a novel bioinsecticide for the control of diamondback moth, p. 129–137. *In* N. S. Talekar (ed.), Proceedings of the second international workshop on the management of diamondback moth and other crucifer pests. Asian Vegetable Research and Development Center, Taipei, Taiwan, Republic of China.
37. **Tabashnik, B. E., N. Finson, F. R. Groeters, W. J. Moar, M. W. Johnson, K. Luo, and M. J. Adang.** 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc. Natl. Acad. Sci. USA **91:**4120–4124.
38. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
39. **Vadlamudi, R. K., T. H. Ji, and L. A. Bulla, Jr.** 1993. A specific binding protein from Manduca sexta for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*. J. Biol. Chem. **268:**12334–12340.
40. **Vadlamudi, R. K., E. Weber, I. Ji, T. H. Ji, and L. A. Bulla, Jr.** 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. **270:**5490–5494.
41. **Valaitis, A., M. K. Lee, F. Rajamohan, and D. H. Dean.** 1995. Brush border membrane aminopeptidase-N in the midgut of the gypsy moth serves as the receptor for the CryIA(c) δ-endotoxin of *Bacillus thuringiensis*. Insect Biochem. Mol. Biol. **25:**1143–1151.
42. **Van Rie, J., S. Jansens, H. Hofte, D. Degheele, and H. Van Mellaert.** 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *B. thuringiensis* delta-endotoxins. Appl. Environ. Microbiol. **56:**1378–1385.
43. **Wolfersberger, M. G., P. Luthy, A. Maurer, P. Parenti, V. F. Sacchi, B. Giordana, and G. M. Hanozet.** 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol. **86:**301–308.
44. **Yaoi, K., T. Kadotani, H. Kuwana, A. Shinkawa, T. Takahashi, H. Iwahana, and R. Sato.** 1997. Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. Eur. J. Biochem. **246:** 652–657.