THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 31, Issue of August 3, pp. 28906–28912, 2001

Printed in U.S.A.

Mapping the Epitope in Cadherin-like Receptors Involved in *Bacillus thuringiensis* Cry1A Toxin Interaction Using Phage Display*

Received for publication, April 5, 2001, and in revised form, May 29, 2001
Published, JBC Papers in Press, May 30, 2001, DOI 10.1074/jbc.M103007200

Isabel Gómez‡, Daniela I. Oltean§, Sarjeet S. Gill§, Alejandra Bravo‡, and Mario Soberón‡¶

From the ‡Instituto de Biotecnología, Departamento de Microbiología Molecular, Universidad Nacional Autónoma de México, Apdo postal 510-3, Cuernavaca, Morelos 62250, México and §Department of Cell Biology and Neuroscience, University of California, Riverside, California 92521

In susceptible lepidopteran insects, aminopeptidase N and cadherin-like proteins are the putative receptors for *Bacillus thuringiensis* (Bt) toxins. Using phage display, we identified a key epitope that is involved in toxin-receptor interaction. Three different scFv molecules that bind Cry1Ab toxin were obtained, and these scFv proteins have different amino acid sequences in the complementary determinant region 3 (CDR3). Binding analysis of these scFv molecules to different members of the Cry1A toxin family and to *Escherichia coli* clones expressing different Cry1A toxin domains showed that the three selected scFv molecules recognized only domain II. Heterologous binding competition of Cry1Ab toxin to midgut membrane vesicles from susceptible *Manduca sexta* larvae using the selected scFv molecules showed that scFv73 competed with Cry1Ab binding to the receptor. The calculated binding affinities (K_d) of scFv73 to Cry1Aa, Cry1Ab, and Cry1Ac toxins are in the range of 20–51 nM. Sequence analysis showed this scFv73 molecule has a CDR3 significantly homologous to a region present in the cadherin-like protein from *M. sexta* (Bt-R_1), *Bombyx mori* (Bt-R_{175}), and *Lymantria dispar*. We demonstrated that peptides of 8 amino acids corresponding to the CDR3 from scFv73 or to the corresponding regions of Bt-R_1 or Bt-R_{175} are also able to compete with the binding of Cry1Ab and Cry1Aa toxins to the Bt-R_1 or Bt-R_{175} receptors. Finally, we showed that synthetic peptides homologous to Bt-R_1 and scFv73 CDR3 and the scFv73 antibody decreased the *in vivo* toxicity of Cry1Ab to *M. sexta* larvae. These results show that we have identified the amino acid region of Bt-R_1 and Bt-R_{175} involved in Cry1A toxin interaction.

Synthetic insecticides cause not only environmental problems, but many have lost their efficacy due to resistance development in the pest insects. *Bacillus thuringiensis* (Bt),[1] a biopesticide, is a viable alternative for the control of insect pests in agriculture and disease vectors of importance in public health. Bt use is also compatible with sustainable and environmentally friendly agricultural practices. Bt produces insecticidal proteins (Cry toxins) during sporulation as parasporal crystals. These crystals are predominantly composed of one or more proteins, also called δ-endotoxins. These toxins are highly specific to their target insect; are safe to humans, vertebrates, and plants; and are completely biodegradable.

The three-dimensional structures of Cry3A and Cry1Aa toxins have been resolved by x-ray diffraction crystallography (1, 2). The two proteins share many similar features and are composed of three domains. Domain I, extending from the N terminus, a seven-helix bundle, is the pore-forming domain. Domain II consists of three anti-parallel β-sheets, and domain III is a β-sandwich of two anti-parallel β-sheets (1, 2). Domains II and III are involved in receptor binding, and domain III additionally protects the toxin from further proteolysis (for reviews, see Refs. 3 and 4).

The mode of action of Cry toxins is a multistage process. Crystal toxins ingested by susceptible larvae dissolve in the alkaline environment of the larval midgut, thereby releasing soluble proteins. The inactive protoxins are then cleaved at specific sites by midgut proteases, yielding 60–70-kDa protease-resistant active fragments. The active toxin then binds to specific membrane receptors on the apical brush border of the midgut epithelium columnar cells (5, 6). Therefore, receptors on the brush border membrane are a key factor in determining the specificity of Cry toxins. This specific binding involves two steps, a reversible followed by an irreversible one (7). After binding, the toxin apparently undergoes a large conformational change leading to its insertion into the cell membrane (1). The Cry toxin molecules then aggregate through toxin-toxin interactions (8), leading to the formation of lytic pores (8–10), which disrupt midgut ion gradients and the transepithelial potential difference. This disruption is accompanied by an inflow of water that leads to cell swelling and eventual lysis, resulting in paralysis of the midgut and subsequent larval death (3, 4).

A number of putative receptor molecules for lepidopteran-specific Cry1A toxins have been identified. In *Manduca sexta*, Cry1Aa, Cry1Ab, and Cry1Ac proteins bind to a 120-kDa aminopeptidase N (APN) (11–13) and to a 210-kDa cadherin-like protein (Bt-R_1) (14, 15). In *Bombyx mori*, Cry1Aa binds to a 175-kDa cadherin-like protein (Bt-R_{175}) (16, 17) and to a 120-kDa APN (18). In *Heliothis virescens*, Cry1Ac binds to two proteins of 120 and 170 kDa, both identified as APN (20, 21). In

* This work was supported in part by Consejo Nacional de Ciencia y Tecnologia (CONACYT) Contract 27637-N, Dirección General de Apoyo al Personal Académico-Universidad Nacional Autónoma de México IN206200 and IN216300, UC MEXUS-CONACYT, United States Department of Agriculture Grant 96-353-0-3820, and the University of California Toxic Substances Research and Training Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 52-73-291618; Fax: 52-73-172388; E-mail: mario@ibt.unam.mx.

[1] The abbreviations used are: Bt, *B. thuringiensis*; APN, aminopeptidase N; BBMV, brush border membrane vesicles; SPR, surface plasmon resonance; CDR, complementary determinant region; PAGE, polyacrylamide gel electrophoresis; scFv, single-chain variable fragment; HBS-P, HEPES-buffered saline with surfactant P-20; LB, Luria broth; NB, nutrient broth.

Plutella xylostella and *Lymantria dispar* APNs were identified as Cry1Ac receptors (11, 22–24). All of these receptor molecule proteins are glycosylated (12, 15, 16, 19). The interaction between toxin and its receptor can be complex. For example, Cry1Ac binds to two sites on the APN purified from *M. sexta*, and only one of these sites is also recognized by Cry1Aa and Cry1Ab (25). Interestingly, binding of Cry1Ac to both receptor sites is inhibited by sugars, which do not inhibit the binding of Cry1Aa and Cry1Ab (25).

There is little information on the receptor domains involved in Cry toxin binding. In *B. mori*, Cry1Aa toxin binds to a conserved APN domain (26). However, the precise regions that are involved in toxin-receptor interactions, including that of the cadherin-like protein, are not known. In an attempt to identify the receptor molecules and map the receptor epitopes involved, we decided to use the phage display technique. Among several approaches used for epitope mapping, phage display has proven to be highly successful (27–31).

In this study, we focused on the interaction of Cry1A toxins with brush border membrane vesicles from susceptible insects. We report here the identification of one scFv antibody whose CDR3 region shares extensive homology with an 8-amino acid region present in the cadherin-like receptors Bt-R_1 and Bt-R_{175} from two lepidopteran insects. This 8-amino acid region competes with the binding of Cry1Ab and Cry1Aa to Bt-R_1 and Bt-R_{175}, suggesting that we identified the Cry1A toxin-binding epitopes in the cadherin-like receptor protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media—Escherichia coli strains were grown in Luria broth (LB) at 37 °C either with ampicillin (100 μg/ml) or erythromycin (250 μg/ml), while Bt strains were grown in nutrient broth sporulation medium (NB) at 30 °C with or without erythromycin (7.5 μg/ml). The acrystalliferous strain 407*cry*$^-$ (32) transformed with pHT409 (33) harboring the *cry1Aa* gene or pHT315–1Ab harboring the *cry1Ab* gene was used for Cry1Aa and Cry1Ab production. Cry1Ac was produced from the wild type Bt strain HD73.

Purification of Cry1A Toxins and Cry1Ab Protein Fragments—Bt strains containing the *cry1Ab*, *cry1Aa*, and *cry1Ac* genes were grown for 3 days in NB. The spores and crystals were harvested and washed with buffer containing 0.01% Triton X-100, 50 mM NaCl, 50 mM Tris-HCl, pH 8.5. Crystals were isolated by sucrose gradients as previously described (34). These crystals were solubilized and activated by trypsin (1:50, w/w) for 2 h, and the proteins were purified by anion exchange chromatography (Q-Sepharose) as described (34, 35). The purified toxins were concentrated in dialysis bags (Spectra/Por, cut-off 12–14 kDa; Fisher) covered with polyethylene glycol 8000, dialyzed against 1000 volumes of buffer A (150 mM *N*-methylglucamine chloride, 10 mM HEPES, pH 8), and stored at 4 °C until used. Toxins were apparently homogeneous as determined by SDS-PAGE and silver staining.

Phage Display Library, Selection, and Sequencing of Clones—The Nissim synthetic phage-antibody library used in this work was kindly provided by the Cambridge Center for Protein Engineering (Cambridge, UK). This library, with a diversity of 1×10^8 clones, contains a diverse repertoire of *in vitro* rearranged VH genes containing a random VH-CDR3 of 4–12 amino acid residues in length (36). Cry1Ab-binding phages were isolated by panning using immunotubes (Nunc), which were coated with (100 μg/ml) Cry1Ab toxin overnight at room temperature. After each round of selection, individual clones were analyzed for their ability to bind Cry1Ab by enzyme-linked immunosorbent assay. The helper phage VCS-M13 (Stratagene) was used to rescue phages from individual colonies of infected *E. coli* TG-1. Expression of soluble fragments from single infected *E. coli* HB2151 colonies (36–38) was induced by isopropyl thiogalactoside. Bacterial supernatants containing phage or scFv fragments were screened for toxin binding by enzyme-linked immunosorbent assay. DNA fingerprinting was performed by amplifying the scFv insert using primers LMB3 (5′-CAGGAAACAGCTATGAC) and fd-SEQ1 (5′-GAATTTTCTGTATGAGG) followed by digestion with the frequently cutting enzyme *Bst*NI as described (37). CDR3 sequence was determined using the primer CDRFOR (5′-CAGGGTACCTTGGCCCCA) (38).

Purification and Characterization of scFvs—For purification of scFv molecules, scFv genes were subcloned into the pSyn vector (38) and used to transform *E. coli* TG1. scFv fragments were purified to homogeneity as follows. Selected clones were cultured at 37 °C in 2× TY (supplemented with 100 μg/ml ampicillin and 0.1% glucose) until they reached an OD of 0.7 at 600 nm. Production of soluble scFv was induced by the addition of 0.5 mM isopropyl thiogalactoside to the culture and grown for 4 h at 25 °C. The scFv was collected from the periplasm. Soluble periplasmic extracts were obtained by osmotic shock at 4 °C using lysis buffer containing 200 mg/ml sucrose, 1 mM EDTA, 300 mM Tris-HCl, pH 8. The supernatant was applied to a nickel-agarose column, which was washed with PBS, and the scFv was eluted with 2 ml of 250 mM imidazole, 0.2% azide in PBS.

Western Blotting of Cry1Ab Domain I and Domain II-III Polypeptides—DomI and DomII-III-H6 of the Cry1Ab toxin were individually expressed in BL21 *E. coli* cells as described (39).[2] Briefly, an overnight culture of pDomII-III-H6 (or pDomI) transformed cells was grown at 37 °C in LB medium (200 μg/ml ampicillin). This culture was used to inoculate 100 ml of LB medium (1:100 dilution). The cells were grown to an OD of 0.5–0.6 and induced with 1 mM isopropyl thiogalactoside. After 3 h of growth, the cells were centrifuged and suspended in 3 ml of buffer A (50 mM $NaSO_4$, 300 mM NaCl, pH 8). Cells were then sonicated on ice (two 1-min bursts) and centrifuged (10 min at 12,000 × *g*). Soluble proteins (10 μg) were separated by 10% SDS-PAGE, and Western blot analysis was performed as described (35), using bacterial supernatants containing phage or scFv fragments. For scFv fragments, a c-Myc antibody (Sigma) (1:1000 dilution) was used, followed by incubation with a secondary goat anti-mouse antibody conjugated with peroxidase (Sigma) (1:1000 dilution). For clone M13–19, an anti-M13 antibody conjugated to peroxidase (Sigma) (1:1000 dilution) was utilized as described (34, 35). Blots were visualized using luminol (ECL; Amersham Pharmacia Biotech).

Preparation of Brush Border Membrane Vesicles (BBMVs)—M. sexta eggs were kindly supplied by Dr. Jorge Ibarra (CINVESTAV, Irapuato), and *B. mori* eggs were obtained from Carolina Biological Supply Co. *M. sexta* and *B. mori* larvae were reared on an artificial diet and fresh mulberry leaves, respectively. BBMVs from fifth instar *M. sexta* or *B. mori* larvae were prepared as reported (41) except that neomycin sulfate (2.4 μg/ml) was included in the buffer (300 mM mannitol, 2 mM dithiothreitol, 5 mM EGTA, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 150 μg ml^{-1} pepstatin A, 100 μg ml^{-1} leupeptin, 1 μg ml^{-1} soybean trypsin inhibitor, 10 mM HEPES-HCl, pH 7.4).

Qualitative Binding Assays with Isolated BBMV—Binding and competition analyses of Cry1Aa-c toxins to *M. sexta* and *B. mori* BBMV were performed as previously described (35). Amino acid sequences of synthetic peptides used for competition experiments were the following: CDR3–73 (RITQTTNRAA), BtR1-CRY (HITDTNNKAA), BtR175-CRY1 (QIIDTNNKAA), BtR175-CRY2 (LDETTNVLAA), and PepL1 (TDAHRGEYYW). Toxins were biotinylated using biotinyl-*N*-hydroxysuccinimide ester (Amersham Pharmacia Biotech), and binding analyses were performed in 100 μl of binding buffer (PBS, 0.1% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.6). Ten micrograms of BBMV protein were incubated with 10 nM biotinylated toxin, and the unbound toxin was removed by centrifugation for 10 min at 14,000 × *g*. The pellet containing BBMV and the bound biotinylated toxin was suspended in 100 μl of binding buffer and washed twice. Finally, the BBMVs were suspended in 10 μl of PBS, pH 7.6, and an equal volume of 2× sample loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue) was added. The samples were separated by SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes. The biotinylated protein was visualized by incubating with streptavidin-peroxidase conjugate (1:4000 dilution) for 1 h, followed by luminol (ECL; Amersham Pharmacia Biotech). For competition experiments, biotinylated Cry1A toxins were incubated with different concentrations of scFvs or peptides in PBS for 1 h at room temperature before incubating toxin with BBMV.

Toxin Overlay Assays—Protein blot analysis of BBMV preparations was performed as described previously (35, 41). Ten micrograms of BBMV protein were separated by 9% SDS-PAGE and electrotransferred to nitrocellulose membranes. After blocking, the membranes were incubated for 2 h with 10 nM biotinylated Cry1A toxins. Unbound toxin was removed by washing the membrane three times with washing buffer for 10 min, and the bound toxin was identified by incubation with streptavidin-peroxidase conjugate (1:5000) for 1 h and visualized using luminol (ECL; Amersham Pharmacia Biotech). For competition experiments, biotinylated Cry1A toxins were incubated with different concentrations of scFvs or peptides in washing buffer (0.1% Tween 20, 0.2%

[2] A. Bravo, R. Meza, and A. Lorence, unpublished results.

BSA in PBS) for 1 h at room temperature before incubating toxin with nitrocellulose membranes.

Biosensor (SPR) Analysis of scFv73 Affinities to Cry1A—All surface plasmon resonance (SPR) measurements were performed using a Biacore X and CM5 sensor chips (Biacore). HBS-P buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 005% surfactant P20) was used throughout the analyses. The ligand, scFv73 (30 kDa, apparently homogeneous based on SDS-PAGE) at a concentration of 25 μg/ml in 20 mM ammonium acetate, pH 5, buffer, was immobilized on flow cell 2 using a standard amine-coupling kit (Biacore) at densities of less than 150 response units. The surfaces of both flow cells were activated for 5 min at a flow rate of 10 μl/min. Following ligand immobilization on flow cell 2, both flow cells were blocked with a 5-min injection of 1 M ethanolamine at a flow rate of 10 μl/min. The analytes (65 kDa, apparently homogenous based on SDS-PAGE) were injected over both flow cells at a flow rate of 30 μl/min. The complex was allowed to associate and dissociate for 120 and 180 s, respectively. The surfaces were regenerated with a 1-min injection of 1 mM HCl. Triplicate injections of each toxin concentration were injected in random order over both surfaces, and the responses were corrected by double referencing (42). The data were fitted using global analysis software available within Biaevaluation 3.1 (Biacore). Competition experiments were performed by injection of a 10- and 200-fold molar excess of scFv in combination with Cry1Ab and Cry1Aa toxins. Carbohydrate inhibition studies with GalNAc were carried out using 600 nM Cry1Ac and 20 μM GalNAc. As an additional control, we immobilized a non-Cry1A-binding scFv4E that was obtained by panning against a different antigen onto flow cell 1 at similar levels as scFv73. Various concentrations of toxin were injected over both flow cells, and the response curve on flow cell 1 was subtracted from flow cell 2. Using this control flow cell configuration, identical Cry1Ab binding curves were obtained compared with using the ethanolamine-blocked control surface.

Insect Bioassay—Bioassays were performed with *M. sexta* neonate larvae using surface-treated food with 9 ng/cm^2 as reported (5), and mortality was recorded after 7 days.

RESULTS

Characterization of scFv Antibodies That Bind Cry1Ab Toxin—A library of 10^8 human single chain antibody fragments (scFv) with variability in the CDR3 region (5–12 amino acids) (36) was used to select a population of phages that bound Cry1Ab toxin. After eight rounds of panning, 98% of the M13 phages bound Cry1Ab (data not shown). To characterize the phages isolated, we amplified the variable regions by PCR and digested the products with *Bst*NI restriction enzyme. Analysis of 50 phage clones showed three different restriction patterns (data not shown). One of these patterns was found in 48 of the clones analyzed (representative clone scFv45), while the other two patterns were each represented by one clone. DNA sequence analysis of the CDR3 region was determined for 10 clones of the most abundant restriction pattern (including scFv45) and also for the two clones representing the other two unique restriction patterns (scFv19 and scFv73). Three different amino acid sequences were present in the CDR3 regions of the clones analyzed (scFv19, RTSPRLTPKHR; scFv73, ITQTTNR; scFv45, NPRIPP).

We used Western blot analysis to determine which Cry1Ab toxin domain bound the three scFv antibodies. This analysis was performed using the scFv antibodies against membrane blots containing protein extracts from *E. coli* expressing either Cry1Ab domain I or domains II-III. Fig. 1 shows that the three scFv clones recognized the 44-kDa domain II-III polypeptide but not the 30-kDa domain I polypeptide. To analyze whether domain II or III was recognized by these scFv proteins, we determined if the three M13-scFv phages bound Cry1Ac toxin, since this toxin shares 98% identity with Cry1Ab toxin in domain II but only 38% identity in domain III. Enzyme-linked immunosorbent assay binding analysis showed that the three scFv antibodies also recognized Cry1Ac toxin (data not shown), suggesting that the three scFv fragments bound to domain II of Cry1Ab toxin.

Competition of Cry1Ab Toxin Binding to M. sexta BBMV with

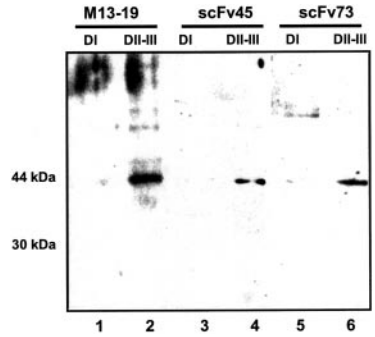

FIG. 1. **Binding of scFv molecules to Cry1Ab toxin domains.** Protein extracts of *E. coli* strains expressing domain I (*lanes 1*, *3*, and *5*) or domains II-III (*lanes 2*, *4*, and *6*) were detected with M13–19 (*lanes 1* and *2*), scFv45 (*lanes 3* and *4*), or scFv73 (*lanes 5* and *6*) as described under "Materials and Methods." Molecular masses of domain I and domain II-III polypeptides are 30 and 44 kDa, respectively.

Selected scFv Antibodies—The three anti-Cry1Ab scFv genes were subcloned into a plasmid to incorporate a hexahistidine tag and then expressed and purified from *E. coli*. Only scFv73 and scFv45 were produced in *E. coli* in high quantities, and scFv19 was therefore not analyzed further. To determine if the selected scFv antibodies could compete with the binding of Cry1Ab toxin to its receptor, we performed two different binding assays. In the first protocol, a qualitative binding assay, biotinylated Cry1Ab toxin was incubated in solution with BBMV. The bound toxin was visualized following SDS-PAGE and electrotransfer of the proteins to nitrocellulose membranes. Fig. 2*A* shows that both scFv antibodies compete with the binding of Cry1Ab toxin to *M. sexta* BBMV, although scFv73 competes more efficiently than scFv45 (Fig. 2*A*, *lanes 4* and *6*). The second protocol, toxin overlay assays, allows the identification of BBMV proteins that interact with Cry1Ab. BBMV proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose. Then biotinylated Cry1Ab toxin was incubated with the membranes, and the proteins that bound the toxin were detected with streptavidin coupled to peroxidase. Fig. 2*B* shows that both the 120-kDa aminopeptidase (APN) (13) and the 210-kDa cadherin-like (Bt-R$_1$) (15) proteins bound biotinylated Cry1Ab. The scFv45 did not compete with Cry1Ab binding to either the 120- or 210-kDa proteins. In contrast, scFv73 competed with the binding of Cry1Ab to the 210-kDa protein but not to the 120-kDa protein.

The scFv73 CDR3 Shares Significant Homology with M. sexta and B. mori Cadherin-like Proteins—Since scFv73 competed with the binding of Cry1Ab toxin to the Bt-R$_1$ receptor from *M. sexta*, we compared the amino acid sequence of the CDR3 region to that of Bt-R$_1$ and APN. The CDR3 amino acid sequence of scFv73 (RITQTTNR) shares 71% similarity with an 8-amino acid region present in Bt-R$_1$ (GenBank™ accession number AAG37912, ^{869}HITDTNNK^{876}) from *M. sexta* and 66% similarity to the corresponding region of Bt-R$_{175}$ protein from *B. mori* (GenBank™ accession number BAA77212, ^{873}IIDTNNK^{880}). In addition, a second 6-amino acid region in Bt-R$_{175}$ (^{1296}LDETTN^{1301}) shares 71% similarity with scFv73 CDR3. No significant homology with APN was found. The CDR3 regions of scFv45 and scFv19 had no homology with either Bt-R$_1$ or APN.

Binding Affinities of scFv73 to Cry1A—The binding affinity of Cry1Ab toxin to purified Bt-R$_1$ has been reported to be 0.7 nM using ^{125}I-labeled toxin (15). To determine the binding affinity of the scFv73 CDR3 region to Cry1A toxins we performed real time binding kinetics by SPR. SPR analyses showed that Cry1Aa, Cry1Ab, and Cry1Ac toxins bound immobilized scFv73. Toxin binding curves were globally fitted to various

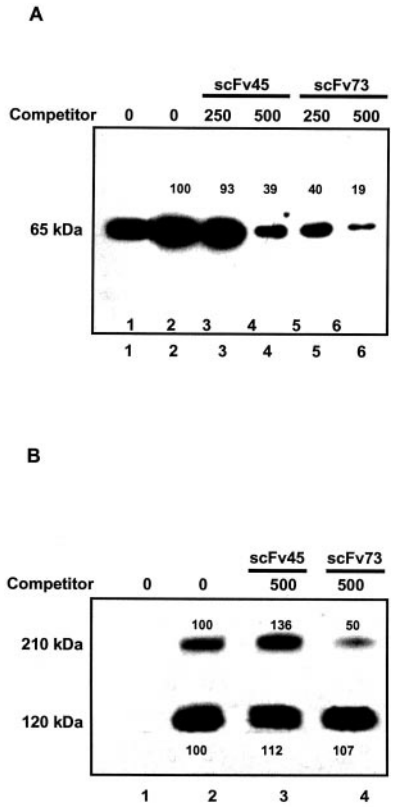

FIG. 2. **The scFv73 antibody competes binding of Cry1Ab to *M. sexta* BBMV and to Bt-R_1.** *A*, qualitative binding of Cry1Ab to *M. sexta* BBMV. *Lane 1*, biotinylated Cry1Ab toxin as marker; *lane 2*, binding of Cry1Ab to BBMV; *lanes 3* and *4*, binding of Cry1Ab with a 250- and 500-fold molar excess of scFv45, respectively; *lanes 5* and *6*, binding of Cry1Ab with a 250- and 500-fold molar excess of scFv73, respectively. *B*, toxin overlay assays of Cry1Ab to *M. sexta* BBMV. *Lane 1*, *M. sexta* BBMV; *lane 2*, binding of Cry1Ab; *lane 3*, competition of Cry1Ab with a 500-fold molar excess of scFv45; *lane 4*, binding of Cry1Ab with a 500-fold molar excess of scFv73. Cry1Ab (10 nM) was used in *lanes 2–6*. Molecular weights of Cry1Ab-binding proteins are indicated on the *left*. *Numbers within* the *images* represent the percentage of signal relative to Cry1Ab binding without competitors as determined by scanning optical density of bands in blots.

binding models and the best fit (χ^2 value of 1) was found using a Langmuir binding model that indicated a 1:1 toxin/receptor stoichiometry. The binding responses were reproducible during three separate scFv73 immobilizations, which indicated that amine coupling did not affect the Cry1Ab binding site on scFv73. The overall affinity (K_d of 39.7 nM) for Cry1Ab binding to scFv73 was obtained from the apparent rate constants (k_{on} and k_{off} values) generated by the binding model (Fig. 3*A*, Table I). The Cry1Aa and Cry1Ac affinities were 51.1 and 20.5 nM, respectively (Table I). scFv73 coinjected with Cry1Ab or Cry1Aa toxin completely inhibited (100%) toxin binding to the immobilized scFv73 (Fig. 3*B*). In contrast, scFv45, only inhibited 5% of the Cry1Ab and Cry1Aa binding to scFv73. These results indicate that the scFv73 and scFv45 bind to different sites on Cry1Aa and Cry1Ab. Bovine serum albumin, a protein of similar size as the Cry1A toxins did not bind scFv73 at any of the concentrations tested.

Cry1Ac binding to immobilized scFv73 was identical in the absence or presence of GalNAc (data not shown). GalNAc efficiently competes the binding of Cry1Ac to APN by binding to a pocket located in domain III (25, 44, 45). This suggests the Cry1Ac epitope that binds scFv73 is different than that known for initially binding to APN, and as our results suggest, is located on domain II.

Identification of the Binding Region of Bt-R_1 and Bt-R_{175} to Cry1Ab and Cry1Aa Toxins by Competition Experiments with Synthetic Peptides—We performed binding competition experiments to determine if the Bt-R_1 region that shares sequence homology with scFv73 CDR3 plays a role in receptor binding to Cry1Ab. Synthetic peptides corresponding either to scFv73 CDR3 (CDR3–73) or to the corresponding region in Bt-R_1 (BtR1-CRY) were used as competitors. Two alanine residues were added to each peptide at the C terminus to facilitate synthesis. Fig. 4*A* shows that the two synthetic peptides, CDR3–73 and BtR1-CRY, decreased Cry1Ab toxin binding to *M. sexta* BBMV (*lanes 4* and *6*). This competition was specific, since an unrelated ten amino acid peptide (PepL1, *lane 2*) did not compete the binding of Cry1Ab. In contrast, Cry1Ac binding to *M. sexta* BBMVs was not competed by BtR1-CRY (*lane 8*). Cry1Aa binding to *M. sexta* BBMVs was also inhibited by BtR1-CRY peptide (data not shown). Toxin overlay assays also confirmed that both synthetic peptides competed Cry1Ab binding to the 210-kDa protein and that the BtR1-CRY synthetic peptide competed more efficiently (Fig. 4*B*, *lanes 6–8*) than CDR3–73 (Fig. 4*B*, *lanes 2–5*). However, these peptides did not substantially inhibit Cry1Ab binding to the 120-kDa protein (Fig. 4*B*). Fig. 4*C* shows that the BtR1-CRY peptide also competed binding of Cry1Aa toxin to the 210-kDa protein. In contrast to that observed with Cry1Ab binding, CDR3–73 showed greater competition than with BtR1-CRY. In our experimental conditions, we could not detect binding of Cry1Ac to the 210-kDa protein (data not shown).

As mentioned previously, scFv73 CDR3 shares significant amino acid homology with two regions in the *B. mori* Cry1Aa receptor Bt-R_{175}. To determine if the regions identified in Bt-R_{175} could also compete with the binding of Cry1Ab and Cry1Aa toxins to *B. mori* BBMV, competition binding experiments of Cry1Aa and Cry1Ab to *B. mori* BBMV were performed. Fig. 5*A* (*lanes 2–7*) shows that binding of Cry1Aa toxin to BBMV in solution was not competed efficiently with either of the two synthetic peptides: one that corresponds to the similar region mapped for Bt-R_1 (BtR175-CRY1) and the second that corresponds to the amino acid sequence of the second site in Bt-R_{175} (BtR175-CRY2). Toxin overlay assays showed that BtR175-CRY2 peptide competed with Cry1Ab binding to the 175-kDa protein (Fig. 5*B*, *lanes 7* and *8*) more efficiently than competition of Cry1Aa binding (Fig. 5*C*, *lanes 7* and *8*). In contrast, BtR175-CRY1 competed poorly with both Cry1Ab and Cry1Aa binding to the 175-kDa protein (Fig. 5, *B* and *C*, *lanes 5* and *6*).

Involvement of the Epitope Mapped in Bt-R_1 in Cry1Ab Toxicity—To determine if the epitope mapped in Bt-R_1 involved in Cry1Ab toxin interaction interferes with Cry1Ab toxicity to *M. sexta* larvae, bioassays were performed using the different scFv antibodies and synthetic peptides in combination with Cry1Ab toxin. First instar larvae were fed Cry1Ab toxin either alone or the Cry1Ab toxin previously incubated with a 300-fold molar excess of the different proteins or peptides. Table II shows that the toxicity of Cry1Ab toxin was reduced by 50% when the toxin was incubated with scFv73 or the synthetic peptides CDR3–73 or BtR1-CRY. In contrast, treatment with scFv45, which has a different epitope, had little effect on Cry1Ab toxicity. None of the peptides were toxic to *M. sexta* larvae (data not shown).

DISCUSSION

In susceptible insects, Cry toxin specificity correlates with receptor recognition (8, 9). The identification of epitopes involved in Cry toxin-receptor interactions could provide insights into the mechanism of insect specificity and the mode of action of these toxins. Furthermore, receptor epitope mapping offers tools for improving the specificity and toxicity of Bt toxins. To facilitate the identification of these receptor epitopes, we uti-

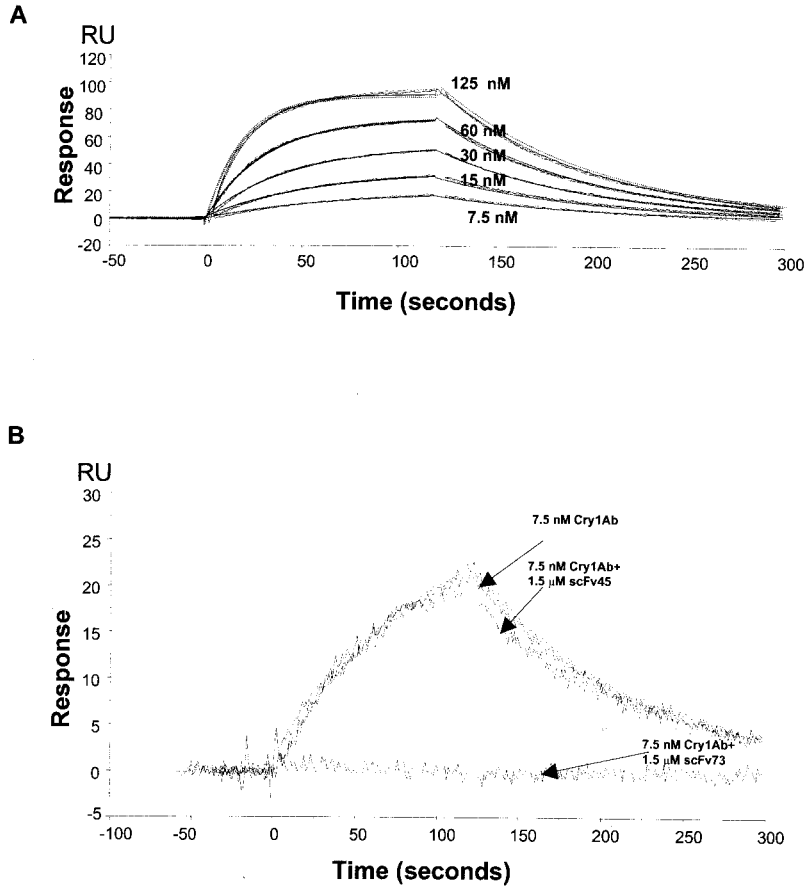

FIG. 3. **Surface plasmon resonance analyses of Cry1Ab binding to scFv.** *A*, sensogram of various Cry1Ab toxin concentrations binding to immobilized scFv73. *Dotted lines* represent curves generated by the one-site binding model. *B*, sensogram of Cry1Ab (7.5 nM) binding to immobilized scFv73 in the presence and absence of a 200-fold molar excess of soluble scFv73 or scFv45.

TABLE I
Binding kinetics of Cry1Aa-c to scFv73

k_{on} is the association rate constant; k_{off} is the dissociation rate constant; K_d is the apparent affinity (k_{off}/k_{on}).

Toxin	k_{on}	k_{off}	K_d
	$\times 10^5\ M^{-1}\ s^{-1}$	$\times 10^{-3}\ s^{-1}$	$\times 10^{-8}\ M$
Cry1Aa	1.16	5.91	5.11
Cry1Ab	3.2	12.7	3.97
Cry1Ac	3.69	7.57	2.05

lized phage display technology, which has accelerated the identification of protein epitopes involved in protein-protein interactions (28, 29, 30, 31).

In *M. sexta*, two proteins bind Cry1A toxins, a 120-kDa APN (13, 43) and a 210-kDa cadherin-like protein (Bt-R_1) (15). Expression of these putative receptor proteins in heterologous cell lines did not render the cells sensitive to Cry1A toxins (11, 15, 46). An exception is the expression of the cadherin-like protein from *B. mori* (Bt-R_{175}) in SF9 cells, which become responsive to Cry1Aa toxin (17).

Bt-R_1 receptor shares 20–40% identity to members of the cadherin superfamily of proteins. Like other cadherins, Bt-R_1 contains an extracellular domain with 11 repeats, a transmembrane domain and a small cytoplasmic domain (15). Using phage display, we identified a scFv antibody that competed with Cry1Aa and Cry1Ab binding to BBMV and decreased Cry1Ab toxicity to *M. sexta.* The CDR3 variable region of this antibody, ITQTTNR, has 71% homology to a region in Bt-R_1 (^{869}HITDTNNK^{876}), located before the eighth repeat in the extracellular domain. Synthetic peptides corresponding to these epitopes inhibit binding of Cry1A toxins to BBMV. The data suggest that Bt-R_1 plays an important role in the binding of Cry1Aa and Cry1Ab toxins to brush border membranes in *M. sexta*. This epitope is also conserved in cadherin-like proteins isolated from the susceptible insects *L. dispar*[3] (GenBank™ accession number AF317621) and *B. mori.* In *B. mori*, a similar region (^{873}IIDTNNK^{880}), with less homology (61%) to CDR3 of scFv73, was found.

In *B. mori*, the region of Bt-R_{175} responsible for Cry1Aa binding was mapped by deletion analysis to residues 1245–1391, which includes the first 112 amino acid residues of the membrane proximal region (17). Interestingly, a six-amino acid sequence within this region (^{1296}LDETTN^{1301}) has significant homology (71%) with scFv73 CDR3. Competition experiments with peptides that correspond to the two regions in Bt-R_{175} with homology to scFv73 CDR3 showed that the second epitope was responsible for Cry1Aa and Cry1Ab binding. Our results suggest that the binding epitopes for Cry1A toxins in Bt-R_1 and Bt-R_{175} are different. The epitopes mapped in this work are present in several Cry1A-susceptible insects supporting the notion that cadherin-like proteins are important receptors for these toxins.

Competition of Cry1Aa and Cry1Ab binding to BBMV proteins by synthetic peptides was more efficient in *M. sexta* than in *B. mori*. In *M. sexta*, >250-fold molar excess of the peptide was required, whereas in *B. mori* a >750-fold molar excess was needed. This difference may be because the Bt-R_{175}-Cry1Aa toxin interaction depends on a native receptor conformation, since Cry1Aa binding to Bt-R_{175} could not be detected under denaturing conditions (16, 17). In contrast, *M. sexta* Bt-R_1 binds Cry1A toxins in both native and denaturing conditions (15). Although we were able to detect binding of Cry1Aa and Cry1Ab toxins to Bt-R_{175} in toxin overlay assays (denaturing conditions), this binding was much weaker than observed with

[3] A. Valaitis, personal communication.

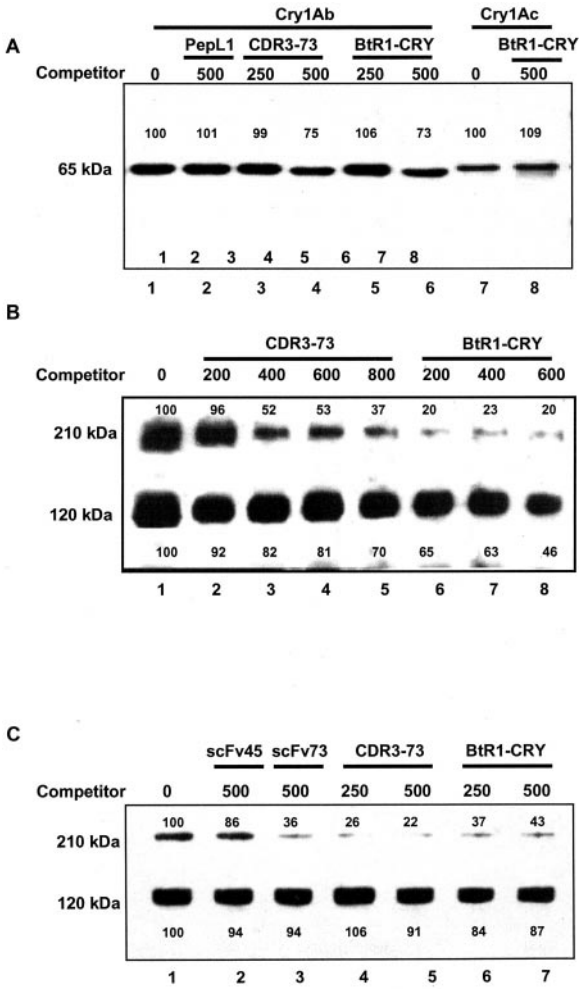

FIG. 4. **Synthetic peptides homologous to scFv73 CDR3 and to Bt-R_1 compete with Cry1Ab binding to Bt-R_1.** *A*, qualitative binding of Cry1Ab to *M. sexta* BBMV. *Lane 1*, binding of Cry1Ab to BBMVs; *lane 2*, binding of Cry1Ab with a 500-fold molar excess of peptide PepL1; *lanes 3* and *4*, binding of Cry1Ab with a 250- and 500-fold molar excess of peptide CDR3–73, respectively; *lanes 5* and *6*, binding of Cry1Ab with a 250- and 500-fold molar excess of peptide BtR1-CRY, respectively; *lane 7*, binding of Cry1Ac to BBMV; *lane 8*, binding of Cry1Ac with a 500-fold molar excess of peptide BtR1-CRY. *B*, toxin overlay assays of Cry1Ab to *M. sexta* BBMV. *Lane 1*, binding of Cry1Ab; *lanes 2–5*, competition of Cry1Ab with a 200-, 400-, 600- and 800-fold molar excess of peptide CDR3–73, respectively; *lanes 6–8*, competition of Cry1Ab with a 200-, 400-, and 600-fold molar excess of peptide BtR1-CRY, respectively. *C*, toxin overlay assays of Cry1Aa to *M. sexta* BBMV. *Lane 1*, binding of Cry1Aa; *lane 2*, competition with a 500-fold molar excess of scFv45; *lane 3*, competition with a 500-fold molar excess of scFv73; *lanes 4* and *5*, competition of Cry1Aa with a 250- and 500-fold molar excess of peptide CDR3–73, respectively; *lanes 6* and *7*, competition of Cry1Aa with a 250- and 500-fold molar excess of peptide BtR1-CRY, respectively. Molecular weights of Cry1A proteins are indicated on the *left*. *Numbers within* the *images* represent the percentage of signal relative to Cry1A binding without competitors as determined by scanning optical density of bands in blots.

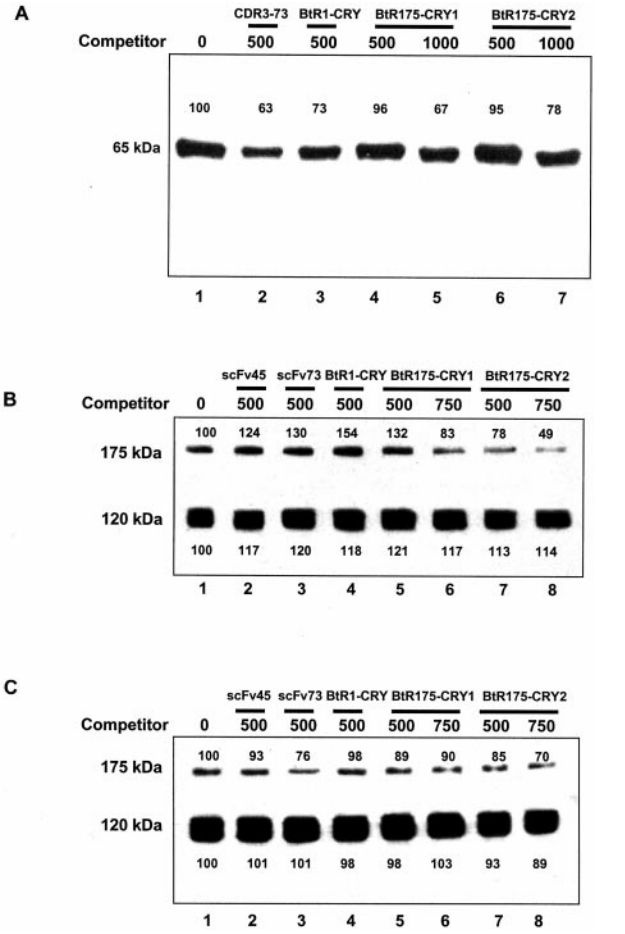

FIG. 5. **Synthetic peptides homologous to *B. mori* Bt-R_{175} compete binding of Cry1Ab and Cry1Aa to the receptor.** *A*, binding of Cry1Aa to *B. mori* BBMV; *lane 1*, binding of Cry1Aa; *lane 2*, binding of Cry1Aa with a 500-fold molar excess of peptide CDR3–73; *lane 3*, binding of Cry1Aa with a 500-fold molar excess of peptide BtR1-CRY; *lanes 4* and *5*, binding of Cry1Aa with a 500- and 1000-fold molar excess of peptide BtR175-CRY1; *lanes 6* and *7*, binding of Cry1Aa with a 500- and 1000-fold molar excess of peptide BtR175-CRY2. *B*, toxin overlay assays of Cry1Ab to *B. mori* BBMV. *Lane 1*, binding of Cry1Ab; *lane 2*, competition of Cry1Ab with a 500-fold molar excess of scFv45; *lane 3*, competition of Cry1Ab with a 500-fold molar excess of scFv73; *lane 4*, competition of Cry1Ab with a 500-fold molar excess of peptide BtR1-CRY; *lanes 5* and *6*, competition of Cry1Ab with a 500- and 750-fold molar excess of peptide BtR175-CRY1, respectively; *lanes 7* and *8*, competition of Cry1Ab with a 500- and 750-fold molar excess of peptide BtR175-CRY2, respectively. *C*, toxin overlay assays of Cry1Aa to *B. mori* BBMV. *Lane 1*, binding of Cry1Aa; *lane 2*, competition of Cry1Aa with a 500-fold molar excess of scFv45; *lane 3*, competition of Cry1Aa with 500-fold molar excess of scFv73; *lane 4*, competition of Cry1Aa with a 500-fold molar excess of peptide BtR1-CRY; *lanes 5* and *6*, competition of Cry1Aa with a 500- and 750-fold molar excess of peptide BtR175-CRY1, respectively; *lanes 7* and *8*, competition of Cry1Aa with a 500- and 750-fold molar excess of peptide BtR175-CRY2, respectively. Molecular weights of Cry1A proteins are indicated on the *left*. *Numbers within* the *images* represent the percentage of signal relative to Cry1A binding without competitors as determined by scanning optical density of bands in blots.

M. sexta Bt-R_1 (Figs. 4*B* and 5*B*). This could also explain the low competition observed with synthetic peptides in Cry1Aa and Cry1Ab binding to *B. mori* BBMV (Fig. 5*A*). Cry1Aa and Cry1Ab toxins share the same binding site in *B. mori* BBMV, with Cry1Aa toxin having at least a 10-fold higher toxicity due to a higher binding affinity (47). These affinity differences may explain why the peptide BtR175-CRY2 competes more efficiently with Cry1Ab binding to Bt-R_{175} than the binding of Cry1Aa (Fig. 5, *B* and *C*), since less peptide is needed to compete the binding of a protein with lower affinity.

The epitope of Cry1Ab toxin involved in the interaction of

TABLE II
Toxicity of Cry1Ab protein to M. sexta larvae in the presence or absence of competitors

Treatment	Mortality[a]
Cry1Ab[b]	97 ± 2.3
Cry1Ab + scFv45	89 ± 4.6
Cry1Ab + scFv73	55 ± 1.1
Cry1Ab + CDR3–73	48 ± 5.9
Cry1Ab + BtR1CRY	49 ± 4.2
Control	0

[a] Percentage of 48 larvae per treatment ± S.D. of three experiments.
[b] 9 ng/cm^2 of toxin; competitors used were 300-fold molar excess.

this toxin with the Bt-R_1 receptor has not been mapped. However, mutations in loop 2 and loop 3 of Cry1Ab domain II affect initial binding to *M. sexta* BBMV (7, 42, 44). Mapping the epitopes in Cry1Aa and Cry1Ab toxins that interact with scFv73 antibody could help in determining the epitopes of these toxins involved in the interaction with cadherin-like receptors.

This is the first report that has mapped the receptor epitopes involved in Cry toxin binding and toxicity. Defining the toxin and the receptor epitopes involved in the specific interaction of these proteins could have a significant impact in the design of more efficient Bt toxins and also help explain the high specificity of these toxins.

Acknowledgments—We thank Baltazar Becerril for scFv4E and discussions; Didier Lereclus for Bt strain 407*cry*$^-$ and pHT409; Juan Carlos Almagro and Juan Miranda for fruitful discussions; Martín Peralta for performing initial experiments; and Laura Lina, Jorge Sanchez, and Oswaldo Lopez for technical assistance.

REFERENCES

1. Li, J., Carroll, J., and Ellar, D. J. (1991) *Nature* **353,** 815–821
2. Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J. L., Brousseau, R., and Cygler, M. (1995) *J. Mol. Biol.* **254,** 447–464
3. Pietrantonio, P. V., and Gill, S. S. (1996) in *Biology of the Insect Midgut* (Lehane, M. J., and Billingsley, P. F., eds) pp. 345–372, Chapman and Hall, London
4. Schnepf, H. E., Crickmore, N., Van Rie, J., Dereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., and Dean, D. H. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 775–806
5. Bravo, A., Jansens, S., and Peferoen, M. (1992) *J. Invertebr. Pathol.* **60,** 237–246
6. Hofmann, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S., and Van Mellaert, H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7844–7848
7. Dean, D. H., Rajamohan, F., Lee, M. K., Wu, S.-J., Chen, X. J., Alcántara, E., and Hussain, S. R. (1996) *Gene* (*Amst.*) **179,** 111–117
8. Soberón, M., Perez, R. V., Nuñez-Valdéz, M. E., Lorence, A., Gómez, I., Sánchez, J., and Bravo, A. (2000) *FEMS Microbiol. Lett.* **191,** 221–225
9. Lorence, A., Darszon, A., Díaz, C., Liévano, A., Quintero, R., and Bravo A. (1995) *FEBS Lett.* **360,** 217–222
10. Schwartz, J. L., Garneau, L., Masson, L., Brousseau, R., and Rousseaeu, E. (1993) *J. Membr. Biol.* **132,** 53–62
11. Denolf, P., Hendrickx, K., VanDamme, J., Jansens, S., Peferoen, M., Egheele, D., and VanRie, J. (1997) *Eur. J. Biochem.* **248,** 748–761
12. Garczynski, S. F., and Adang, M. J. (1995) *Insect Biochem. Mol. Biol.* **25,** 409–415
13. Knight, P. J. K., Crickmore, N., and Ellar, D. J. (1994) *Mol. Microbiol.* **11,** 429–436
14. Belfiore, C. J., Vadlamudi, R. K., Osman, Y. A., and Bulla, L. A., Jr. (1994) *Biochem. Biophys. Res. Commun.* **200,** 359–364
15. Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H., and Bulla, L. A., Jr. (1995) *J. Biol. Chem.* **270,** 5490–5494
16. Nagamatsu, Y., Toda, S., Yagamuchi, F., Ogo, M., Kogure, M., Nakamura, M., Shibata, Y., and Katsumoto, T. (1998) *Biosci. Biotechnol. Biochem.* **62,** 718–726
17. Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A., and Furukawa, Y. (1999) *FEBS Lett.* **460,** 385–390
18. Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., Iwahana, H., and Sato, R. (1997) *Eur. J. Biochem.* **246,** 652–657
19. Knowles, B. H., Knight, P. J. K., and Ellar., D. J. (1991*) Proc. R. Soc. Lond. B* **245,** 31–35
20. Gill, S. S., Cowles, E. A., and Francis, V. (1995) *J. Biol. Chem.* **270,** 27277–27282
21. Oltean, D. I., Pullikuth, A. K., Lee, H-K., and Gill, S. (1999*) Appl. Environ. Microbiol.* **65,** 4760–4766
22. Luo, K., Tabashnik, B. E., and Adang, M. J. (1997) *Appl. Environ. Microbiol.* **63,** 1024–1027
23. Lee, M. K., You, T. H., Young, B. A., Cotrill, J. A., Valaitis, A. P., and Dean, D. H. (1996) *Appl. Environ. Microbiol.* **62,** 2845–2849
24. Valaitis, A. P., Lee, M. K., Rajamohan, F., and Dean, D. H. (1995) *Insect Biochem. Mol. Biol.* **25,** 1143–1151
25. Masson, L., Lu, Y.-J., Mazza, A., Brousseau, R., and Adang, M. J. (1995*) J. Biol. Chem.* **270,** 20309–20315
26. Nakanishi, K., Yaoi, K., Shimada, N., Kadotani, T., and Sato, R. (1999) *Biochim. Biophys. Acta* **1432,** 57–63
27. Cortese, R., Felici, F., Galfre, G., Luzzago, A., Monaci, P., and Nicosia, A. (1994) *Trends Biotechnol.* **12,** 262–267
28. DeLeo, F., Yu, L., Burritt, J. B., Loetterle, L. R., Bond, C. B., and Jesaitis, A. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7110–7114
29. Demangel, C., Maroun, R. C., Rouyre, S., Bon, C., Mazié, J. -C., and Choumet, V. (2000) *Eur. J. Biochem.* **267,** 2345–2353
30. Luzzago, A., Felici, F., Tramontano, A., Pessi, A., and Cortese, R. (1993) *Gene* (*Amst.*) **128,** 51–57
31. Szardenings, M., Törnoth, S., Mutulis, F., Muceniece, R., Keinänen, K., Kuusinen, A., and Wikberg, J. E. S. (1997) *J. Biol. Chem.* **272,** 27943–27948
32. Lereclus, D., Arantès, O., Chaufaux, J., and Lecadet, M.-M. (1989) *FEMS Microbiol. Lett.* **60,** 211–218
33. Arantès, O., and Lereclus, D. (1991) *Gene* (*Amst.*) **108,** 115–119
34. Thomas, W. E., and Ellar, D. J. (1983) *J. Cell Sci.* **60,** 181–197
35. Aranda, E., Sanchez, J., Perferoen, M., Güereca, L., and Bravo, A. (1996) *J. Invertebr. Pathol.* **68,** 203–212
36. Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Lidgley, C., Lane, D., and and Winter, G. (1994) *EMBO J.* **13,** 692–698
37. Hoogenboom, H. R., and Winter, G. (1992) *J. Mol. Biol.* **227,** 381–388
38. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., MacCafferty, J., Griffiths, A. D., and Winter, G. (1991) *J. Mol. Biol.* **222,** 581–597
39. Flores, H., Soberón, X., Sánchez, J., and Bravo, A. (1997) *FEBS Lett.* **414,** 313–318
40. Low, N. M., Holliger, P., and Winter G. (1996) *J. Mol. Biol.* **260,** 359–368
41. Wolfersberger, M., Lüthy, P., Maurer, A., Parenti, P., Sacchi, F. V., Giordana, B., and Hanozet, G. M. (1987) *Comp. Biochem. Physiol.* **86A,** 301–308
42. Myszka, D. G. (1999) *J. Mol. Recognit.* **12,** 279–284
43. Burton, S. L., Ellar, D. J., Li, J., and Derbyshire, D. J. (1999) *J. Mol. Biol.* **287,** 1011–1022
44. Jenkins, J. L., Lee, M. K., Valaitis, A. P., Curtiss, A., and Dean, D. H. (2000) *J. Biol. Chem.* **275,** 14423–14431
45. Garczynski, S. F., Crim, J. W., and Adang, M. J. (1991) *Appl. Environ. Microbiol.* **57,** 2816–2820
46. Keeton, T. P., and Bulla, L. A. (1997*) Appl. Environ. Microbiol.* **63,** 3419–3425
47. Ihara, H., Kuroda, E., Wadano, A., and Himeno, M. (1993) *Biosci. Biotechnol. Biochem.* **57,** 200–204