Specific Epitopes of Domains II and III of *Bacillus thuringiensis* Cry1Ab Toxin Involved in the Sequential Interaction with Cadherin and Aminopeptidase-N Receptors in *Manduca sexta*

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The *Bacillus thuringiensis* Cry toxins are specific to different insects. In *Manduca sexta* cadherin (Bt-R₁) and aminopeptidase-N (APN) proteins are recognized as Cry1A receptors. Previous work showed that Cry1Ab binds to Bt-R₁, promoting the formation of a pre-pore oligomer that binds to APN leading to membrane insertion. In this work we characterized the binding epitopes involved in the sequential interaction of Cry1Ab with Bt-R₁ and APN. A Cry1Ab immune M13 phage repertoire was constructed using antibody gene transcripts of bone marrow or spleen from a rabbit immunized with Cry1Ab. We identified antibodies that recognize domain II loop 3 (scFvL3-3) or B16–B22 (scFvM22) in domain III. Enzyme-linked immunosorbent assay and toxin overlay binding competition assays in the presence of scFvL3-3, scFvM22, or synthetic peptides showed that domain II loop 3 is an important epitope for interaction with Bt-R₁ receptor, whereas domain III B16 is involved in the interaction with APN. Both scFvL3-3 and scFvM22 lowered the toxicity of Cry1Ab to *M. sexta* larvae indicating that interaction with both receptors is important for *in vivo* toxicity. scFvL3-3 and anti-loop2 scFv (scFv73) promoted the formation of the pre-pore oligomer in contrast to scFvM22. In addition, scFvL3-3 and scFv73 preferentially recognized the monomeric toxin rather than the pre-pore suggesting a conformational change in domain II loops upon oligomerization. These results indicate for the first time that both receptor molecules participate in Cry1Ab toxin action *in vivo*; first the monomeric toxin binds to Bt-R₁ through loops 2 and 3 of domain II promoting the formation of the pre-pore inducing some structural changes, then the pre-pore interacts with APN through β-16 of domain III promoting membrane insertion and cell death.

Crystal proteins (Cry) are widely used as insecticides in agriculture, forestry, and vector transmission due to their high specificity and their safety for the environment. Cry proteins are produced as protoxins of 70–130 kDa that are toxic to larval forms of several insects of different orders as well as to other invertebrates (1). Proteolytic activation of protoxin by midgut proteases produces Cry toxin fragments of 60–65 kDa. Cry toxins then bind to the cell surface where they undergo large-scale irreversible conformational changes to convert them into an oligomeric form capable of inserting into the membrane, causing osmotic lysis of midgut cells and ultimately insect death (1). Receptor binding has been studied extensively as a key step determining insect specificity, toxicity, and resistance of Cry toxins (1, 2). In the case of the lepidopteran insect *Manduca sexta*, at least two Cry1A-binding proteins, a cadherin-like protein (Bt-R₁) and a glycosylphosphatidylinositol (GPI)-anchored aminopeptidase-N (APN), have been described as receptors of Cry1A toxins (3, 4). Previously, we provided evidence showing that binding of monomeric Cry1Ab toxin to Bt-R₁ promotes an additional proteolytic cleavage in the N-terminal end of the toxin (helix α1) facilitating the formation of a pre-pore oligomeric structure that is competent in membrane insertion and that oligomer formation is important for toxicity (5, 6). The pre-pore oligomer has a higher affinity to APN (7, 8). The oligomeric Cry1A structure then binds to the APN receptor leading to its insertion into membrane lipid rafts (7, 8) implying a sequential binding mechanism of Cry1A toxins with Bt-R₁ and APN receptor molecules (8). However, a different mechanism of action of Cry toxins based on the study of the effect of Cry1Ab toxin to cultured *Trichoplusia ni* H5 insect cells expressing *M. sexta* Bt-R₁ (9, 10) was recently proposed. It was proposed that the toxicity of Cry1Ab is mainly due to the interaction of monomeric Cry1Ab toxin with Bt-R₁ by activating a Mg²⁺-dependent adenylyl cyclase/protein kinase A signaling pathway that leads to apoptosis and not to pore formation induced by insertion of oligomeric Cry1Ab into the membrane (9, 10). Therefore, additional experimental evidence is needed.

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2 The abbreviations used are: Cry, crystal protein; BBMV, brush border membrane vesicles; Bt, *Bacillus thuringiensis*; ELISA, enzyme-linked immunosorbent assay; scFv, single-chain Fv antibody fragments; APN, aminopeptidase-N; Bt-R₁, cadherin; GPI, glycosylphosphatidylinositol; Ab, antibody; PBS, phosphate-buffered saline; VL, variable light chain; HL, heavy chain.
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to discriminate between the two models of mode of action of Cry toxins, in particular evidence that determines the role of the APN receptor in toxicity of Cry1A toxins will be valuable.

To date, the tertiary structures of six different Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, and Cry4Ba, have been determined by x-ray crystallography (11–16). All these structures display a high degree of similarity with a three-domain organization, suggesting a similar mode of action of the Cry three-domain protein family. The N-terminal domain (domain I) is a bundle of seven \( \alpha \)-helices in which the central helix-\( \alpha \)-5 is hydrophobic and is encircled by six other amphipathic helices, this helical domain is responsible for membrane insertion and pore formation (11–15). Domain II consists of three anti-parallel \( \beta \)-sheets with exposed loop regions, and domain III is a \( \beta \)-sandwich (11–15). Exposed regions in domains II and III are involved in receptor binding (1).

The Cry1Aa, Cry1Ab, and Cry1Ac proteins share more than 95% amino acid sequence identity as protoxins and are selectively toxic to some lepidopteran insect pests. These Cry1A toxins bind to the same receptor molecules in \( M. \ sexta \) (Bt-R\(_1\) and APN) (17, 18). Domain I in the three toxins share more than 98% amino acid sequence identity. However, there are important differences in domains II and III of these toxins. Cry1Ab and Cry1Ac toxins share the same domain II in contrast to Cry1Aa that has a different domain II sharing only 69% identity. In particular, the loop regions involved in receptor interaction are different in Cry1Aa. In contrast, Cry1Aa and Cry1Ab share a very similar domain III, whereas domain III of Cry1Ac shares only 38% identity with both toxins. Because different lepidopteran insects show different sensitivity to these Cry1A toxins, it is likely that the differences in domains II and III influence the receptor binding affinities and the activity of these toxins.

The characterization of the epitopes involved in interaction of Cry toxins with their receptors could give clues on the molecular basis of insect specificity and resistance. In previous work, using a synthetic phage-antibody library, we isolated an scFv antibody (scFv73) that binds to domain II loop 2 (\( \beta 6 \)–\( \beta 7 \) loop) of Cry1A toxins and inhibited binding of Cry1A toxins to Bt-R\(_1\) but did not affect binding to APN (19, 20). Sequence analysis of the CDR3H region of scFv73 led to the identification of an 8-amino acid epitope in Bt-R\(_1\) cadherin repeat 7 (CADR\(_7\), \( ^{\text{869}} \text{HITDTNNK}^{\text{876}} \)) involved in the binding to domain II loop 2 of Cry1A toxins (19, 20). A second binding epitope in Bt-R\(_1\) CADR11 (\( ^{\text{1331}} \text{IPLPASILTVTV}^{\text{1342}} \)) that interacts with domain II loop \( \alpha 8 \) (\( \alpha 8\text{a} \)–\( \alpha 8\text{b} \) loop) and loop 2 of Cry1Ab toxin (21) was recently described. Finally, a third region in CADR12 of Bt-R\(_1\) (amino acids 1363–1464) involved in Cry1Ab interaction and toxicity was identified (22). In the case of the \( Heliotis virescens \) cadherin, this binding region was narrowed to residues 1422–1440 by mutagenesis and shown to bind Cry1Ac domain II loop 3 (\( \beta 10 \)–\( \beta 11 \) loop) (23). Regarding interaction of Cry1A toxins with APN, Cry1Ac toxin binds to APN receptor by means of domain III that specifically recognizes \( N \)-acyetylgalectosamine (GalNAc) moieties in contrast to Cry1Aa and Cry1Ab toxins that show no GalNAc binding capacities (18). Based on the use of monoclonal antibodies that competed binding of Cry1Aa with \( \text{Bombix mori} \) APN, the Cry1Aa–APN interacting epitopes were recently mapped in domain III \( ^{\text{508}} \text{STLRV}^{\text{513}} \) and \( ^{\text{822}} \text{VFTLSAH}^{\text{839}} \) residues, which are exposed and in close proximity in the three-dimensional structure (24, 25).

Binding of Cry1Ab toxin to anti-loop 2 scFv73 antibody or to Bt-R\(_1\), CADR7 or CADR11 peptides facilitates the formation of the pre-pore oligomeric structure \textit{in vitro}, showing that domain II interaction with Bt-R\(_1\) is an important step in the formation of the pre-pore oligomer before toxin inserts into the membrane (6, 21).

Although some binding epitopes in the toxin have been characterized, little is known about the mechanism by which the Cry1A toxins undergo a sequential interaction with the two receptors molecules. The characterization of the binding epitopes in the pre-pore oligomer and the role of these binding sites in the interaction with both Bt-R\(_1\) and APN is still missing. Furthermore, the characterization of possible structural changes in the toxin epitopes involved in receptor interaction could give clues on the mechanism of differential interaction of monomeric and pre-pore oligomeric structures with Bt-R\(_1\) and APN. Also, the study of the role of the interaction of the pre-pore with APN in Cry1Ab toxicity will be important to determine the role of pre-pore formation in toxicity. In this study, we constructed immune libraries for Cry1Ab toxin and selected specific monoclonal scFv fragments that recognize Cry1Ab domain II loop 3 or domain III \( ^{\text{16}} \text{β}16\text{–}^{\text{22}} \) epitopes and demonstrated that both scFv molecules can inhibit the toxicity to \( M. \ sexta \) larvae. An anti-loop 3 antibody inhibited binding of Cry1Ab to Bt-R\(_1\), whereas an anti-\( ^{\text{16}} \text{β}16\text{–}^{\text{22}} \) antibody inhibited interaction with APN. \textit{In vitro} oligomer formation assays with the selected scFv antibodies showed that only binding of Cry1Ab domain II with Bt-R\(_1\) is involved in oligomer formation in contrast to binding of domain III with APN that did not facilitate the formation of the pre-pore. Overall, these results suggest that interaction of Cry1Ab with both receptor molecules is important for toxicity. Also these data contribute to our understanding of the mechanism involved in the sequential interaction of Cry1Ab toxin with both receptor molecules.

**MATERIALS AND METHODS**

**Purification of Monomeric Cry Toxins**—The acrystalliferous Bt strain 407\( ^{\text{cry}} \) (26) transformed with pH740 plasmid harboring the \( \text{cry1Aa} \) gene (27) or pHT315-\( ^{\text{cry1Ab}} \) (19) were used for Cry1Aa and Cry1Ab production, respectively. Cry1Ac was produced from wild-type Bt strain HD73. Bt strains were grown for 3 days at 29 °C in nutrient broth sporulation medium (28) supplemented with 10 \( \mu \)g/ml erythromycin for Cry1Aa and Cry1Ab. After sporulation, crystals were purified by sucrose gradients as reported (19). The Cry1A protoxins were solubilized and proteolytically activated as reported (19).

**Oligomer Formation Assay**—For activation, 1–2 \( \mu \)g of Cry1Ab protoxin was incubated with scFv molecules in the presence of \( M. \ sexta \) midgut juice as previously reported (6). Purification of the activated toxins was done by size exclusion chromatography with Superdex 200 HR 10/30 (Amersham Biosciences) FPLC size exclusion as described (29). The oligomeric structure was detected by Western blot assays using Cry1Ab polyclonal antibodies as reported (6).
Solubilization of GPI-anchored Proteins—M. sexta brush border membrane vesicles (BBMV) were treated with phospholipase C from Bacillus cereus (Sigma) as previously reported in Ref. 30. Membranes were recovered by ultracentrifugation (90,000 × g for 20 min), and the supernatant was analyzed for aminopeptidase activity. APN enzymatic activity was measured using 1 μg/ml leucine-p-nitroanilide (Sigma) as substrate. BBMV proteins (5 μg) were mixed with APN buffer (0.2 M Tris-HCl, pH 8, 0.25 M NaCl) containing 1 mM leucine-p-nitroanilide. APN enzymatic activity was monitored as change in the absorbance at 405 nm for 10 min at room temperature.

Rabbit Immunization—A New Zealand White rabbit was immunized subcutaneously with a mixture of oligomeric and monomeric Cry1Ab toxin structures obtained after proteolytic activation of Cry1Ab in the presence of scFv73. The rabbit was boosted three times with 1 mg of Cry1Ab toxin structure mixture, mixed with incomplete adjuvant, at 15-day intervals. The bone marrow and spleen were dissected 40 days after the primary immunization.

Phage Display Library Construction—Total RNA was prepared from spleen tissue and bone marrow as described (31). Total RNA and random primer were used for first strand cDNA synthesis using a kit (Roche Applied Sciences), according to the manufacturer’s instructions. From cDNA, heavy and light chain DNA fragments were amplified separately and recombined by three subsequent PCR, essentially as described (31), except that PCR1 and PCR2 were done with Vent DNA polymerase (New England BioLabs, Beverly, MA). Primer sequences for amplification of VL and VH antibody regions have been described before (31). However, primers HH13, HH14, and HH15 sequences were corrected; HH13, 5′-GGCGGATCAGGAGGCGGAGGTTCTGGAGGTGGGAAGTGCMTGACCCAGACCTCCAGCTCCATC-GC-3′; HH14, 5′-GGCGGATCAGGAGGCGGAGGTTCTGGAGGTGGGAAGTGCMTGACCCAGACCTCCAGCTCCATC-GC-3′; HH15, 5′-GGCGGATCAGGAGGCGGAGGTTCTGGAGGTGGGAAGTGCMTGACCCAGACCTCCAGCTCCATC-GC-3′, where M = A/C.

To construct the scFv libraries, scFv PCR products and phagemid vectors pCANTAB 5E and pSyn2 were digested with restriction enzymes SfiI and NotI (New England BioLabs, Beverly, MA). Primer sequences for amplification of VL and VH anti-idiotype peptides were added during the incubation with the synthetic peptides. After panning selection, we randomly picked infected TG1 colonies and amplified their PCR products with the PCR 5′ primer PSYN1 (ATACCTATTGCCTACGGC) and 3′ primer PSYN2 (TTACAAACAGTCTATGGC), or primers JK2 and AW1 (31). To obtain DNA fingerprint of the insert sequences, the PCR products were digested with AluI (New England Biosciences) and resolved on 8% acrylamide gels.

Nucleotide Sequence Determination and Fingerprint Analysis—To determine the diversity of the original libraries and clones after panning selection, we randomly picked infected TG1 colonies and amplified their scFv inserts with the PCR 5′ primer PSYN1 (ATACCTATTGCCTACGGC) and 3′ primer PSYN2 (TTACAAACAGTCTATGGC), or primers JK2 and AW1 (31). To obtain DNA fingerprint of the insert sequences, the PCR products were digested with AluI (New England Biosciences) and resolved on 8% acrylamide gels.

Expression and Purification of Soluble scFv Antibodies—The positive phage clones were subcloned into expression vector PET22b (Novagen). Recombinants were transformed into E. coli BL21(DE3) and induced with 1 mM isopropyl-β-D-galactopyranoside. After incubation at 30°C overnight, the proteins in the periplasm were collected using solution I (30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20% sucrose) and solution II (5...
Preparation of BBMV—M. sexta larvae were reared on an artificial diet. BBMV from third instar M. sexta larvae were prepared as reported (32).

Toxin Binding Assays—Toxin overlay assays to BBMV were performed as described (19–21). To determine the ability of selected scFv to compete with the Cry1Ab toxin, different concentrations of scFv were incubated with biotinylated Cry1Ab toxin in washing buffer before adding the mixture to nitrocellulose membranes. Single gel blots were incubated with different competitors using the PR 150 mini deca-probe (Amersham Biosciences) that was designed to incubate each lane of the blot in different conditions avoiding the need of cutting lanes for different conditions.

For analysis of oligomer binding to GPI-anchored protein extract, microtiter plates were coated with 20 μg of GPI proteins as above. The plates were washed 3 times with PBS and blocked with 200 μl/well of skim milk 2% in PBS for 2 h at 37 °C. 10 nM pure Cry1Ab oligomer was added to the coated wells as above. Cry1Ab oligomer was detected using Cry1Ab polyclonal antibody (1:20,000 dilution) and then a secondary goat anti-rabbit antibody (1:20,000 dilution) forming units of scFv phages were added during incubation with the Cry1Ab oligomer.

Insect Bioassay—Bioassays were performed with M. sexta neonate larvae by the surface contamination method as previously described (19).

RESULTS

Phage Antibody Libraries, Construction, and Characterization—We constructed Cry1Ab-immune rabbit libraries in scFv format that could be used for selection of high-affinity and highly specific antibodies against Cry1Ab toxin. The scFv libraries were constructed as reported previously in three PCR steps using reported primers that allow the amplification of rabbit variable heavy and light chain cDNAs (31). Total RNA was extracted from the spleen and bone marrow of a New Zealand White rabbit previously immunized with a mixture containing both monomeric and oligomeric Cry1Ab toxin structures. After cDNA synthesis by reverse transcription, VH and VL DNA fragment repertoires were amplified separately by PCR using four pairs of primers for heavy chains and three pairs of primers for light chains as reported (31). In the second PCR, a DNA linker coding a (Gly4Ser)3 peptide linker sequence was added using modified 3′-heavy and 5′-light chain primers (31). However, reported primers HH13, HH14, and HH15 were modified because the reported primer sequence contained a misplaced glycine codon (31) (see “Materials and Methods” for primer sequence). Finally, a third PCR was performed to fuse the amplified VH and VL genes DNA fragments by overlapping extension. In the final PCR SfiI and NotI restriction sites were added to the 3′ and 5′ ends, respectively, using primers harboring these restriction sites as reported (31). PCR products of 800 bp from the final PCR were digested with SfiI and NotI restriction enzymes and cloned into the previously digested phagemid vectors pSyn2 or pCANtab that allows the display of the cloned fragment on the surface of M13 phage (19). After E. coli cell transformations, libraries of 2.0 × 106 members were obtained.

To examine the integrity and variability of the libraries, 20 clones of each library were picked at random and analyzed by PCR, 95% of the clones were found to contain scFv DNA fragments of the expected size (data not shown). The diversity of the libraries was determined by digestion of the amplified scFv fragment with the Alul restriction enzyme. The PCR fingerprinting analysis of bone marrow and spleen libraries showed that the libraries contain high variability because all restriction patterns analyzed were different (data not shown).

Identification of Anti-loop 3 and Anti-domain III Phage Antibodies—To study the role of domain II and III regions in the binding interaction with receptor molecules, we selected scFv-M13 phages by panning that recognize Cry1Ab-domain II loop 3 or domain III because these regions have been shown to be involved in interaction with Bt-R1 or APN (23, 25). Previously we characterized an scFv antibody (scFv73) that binds domain II loop 2 and inhibits Cry1Ab toxin binding with Bt-R1 but not with APN (19, 20).

Novel panning procedures were performed to select the desired specific scFv antibodies. For the identification of anti-loop 3 scFv antibodies, the panning procedure consisted in two panning rounds against whole Cry1Ab toxin and a third panning round against a biotinylated synthetic peptide with an amino acid sequence corresponding to Cry1Ab loop 3 (biotin-loop3, Table 1). Simultaneously, a third panning round was performed against biotinylated Cry1Ab toxin. After the third round of panning, scFv antibodies were preferentially retained by Cry1Ab as shown by the higher number of exit colony forming units obtained for biotinylated Cry1Ab (107 colony forming units/ml) in comparison to biotin-loop3 peptide (104 colony forming units/ml). Fingerprinting analysis of 20 clones selected against the biotin-loop 3 peptide revealed five different restriction patterns (Fig. 1). The five different scFv-M13 phages were tested in ELISA for binding to Cry1Aa or Cry1Ab toxins. Fig. 2 A shows that all five clones bound to Cry1Ab toxin but not to Cry1Aa, which has a different loop 3 amino acid sequence. The scFv phage

**TABLE 1**

| Name       | Sequence                      | Description                                     |
|------------|-------------------------------|------------------------------------------------|
| Biotin-loop 3 | Biotin-SGSSG-FRSGFSNSSVS     | Cry1Ab residues 436-379 fused to biotin-SGSSG peptide |
| Loop2      | LYRREPNNQDINNOQ               | Cry1Ab residues 366-379                         |
| Loop3      | FRSGFSNSSGVS1IR               | Cry1Ab residues 436-449                         |
| DIII-1     | QGISTLRVNVITA                 | Cry1Ab residues 506-517                         |
| DIII-2     | VPTLSAHVFN                    | Cry1Ab residues 583-592                         |
scFv3-L3 was selected and Fig. 2B shows that binding of scFv3-L3 to Cry1Ab was competed by a loop 3 synthetic peptide but not by loop 2, β16 or β22 synthetic peptides (Fig. 2B).

To select scFv antibodies that recognize Cry1Ab domain III, we performed a panning procedure that counter-selected scFv-M13 phages that recognize Cry1Ac toxin that has a different domain III and very similar domains I and II. After two rounds of panning against Cry1Ab, a third round against Cry1Ac was performed. The non-binding Cry1Ac phages were recovered, amplified, and subjected to a final panning round against Cry1Ab in the presence of soluble Cry1Ac to ensure binding to Cry1Ab of phages that do not recognize Cry1Ac. Fifty colonies from the fourth round were amplified by PCR and characterized by fingerprinting. Five different restriction patterns were identified (Fig. 1). Analysis of binding to Cry1A proteins revealed that four of these scFv phages bind to Cry1Ab but not to Cry1Ac, suggesting that these scFv antibodies bind domain III of Cry1Ab (Fig. 2C). Previously it was demonstrated that Cry1Aa domain III STLRVN513 and VFTLSAHV589 residues, corresponding to β16 and β22, were involved in binding with B. mori APN (25). These amino acid regions are in close proximity in the Cry1Aa structure (25). To determine whether any of the selected anti-domain III scFv-M13 phages recognize these binding epitopes, binding of these five scFv-M13 phages to Cry1Ab was performed in the presence of synthetic peptides corresponding to the β16 or β22 regions of Cry1Ab. Fig. 2B shows that binding of phage scFvM22 to Cry1Ab was inhibited by the two domain III synthetic peptides corresponding to Cry1Ab β16 or β22 and not by the synthetic peptides of domain II loops, indicating that scFvM22 binds to this domain III amino acid epitope.

**Effect of Anti-loop 3 and Anti-β-16–β-22 scFv Molecules on Cry1Ab-Receptor Interaction**—To determine whether the selected scFv protein fragments inhibit the Cry1Ab-receptor interaction, we performed toxin overlay assays using *M. sexta* BBMV. Fig. 3A shows that the anti-loop 3 scFvL3-3 phage particles inhibited binding of biotinylated Cry1Ab to Bt-R1 (210 kDa) in contrast to the anti-β16–β22 scFvM22 phage particles that did not affect this interaction. Interestingly, APN (120 kDa) binding was competed by scFvM22 phage in contrast to scFvL3-3 phage that did not compete this interaction (Fig. 3A). To determine whether binding competition of scFv molecules was not due to steric hindrance due to the size of the scFv molecule, binding of Cry1Ab toxin with blotted BBMV proteins was performed in the presence of synthetic peptides corresponding to Cry1Ab binding epitopes. Fig. 3B shows that loop 3 synthetic peptide competed the binding of Cry1Ab to Bt-R1 but not to APN. In contrast, synthetic peptide DIII-1 (corresponding to the β16) competed the binding of Cry1Ab to APN but had no effect on Bt-R1 binding (Fig. 3B). Synthetic peptide DIII-2 (corresponding to β22) did not compete the binding of Cry1Ab to Bt-R1 nor to APN. These results show that domain II loop 3 is involved in Bt-R1 binding, whereas domain III β16 from Cry1Ab is important for the interaction with *M. sexta*-APN.

**Oligomer Formation of Cry1Ab in the Presence of Anti-loop 3 or Anti-domain III scFv Antibodies**—Previous work demonstrated that binding of Cry1Ab protoxin to Bt-R1 peptides CADR7 or CADR11 or to scFv73 antibody, which mimics these cadherin epitopes (6, 19, 21), facilitated the proteolytic cleavage of domain I helix α1 resulting in the formation of a pre-pore oligomeric structure that is capable of membrane insertion (6, 21). To determine whether the scFvL3-3 or scFvM22 antibodies...
facilitate the formation of the pre-pore structure, Cry1Ab protoxin was proteolytically activated in the presence of each one of these pure scFvL3-3 or scFvM22 molecules. Fig. 4 shows that when Cry1Ab protoxin was activated in the presence of scFvL3-3, a 250-kDa oligomer was produced. In contrast, when Cry1Ab protoxin was activated in the presence of the scFvM22 molecule only monomeric 60-kDa Cry1Ab toxin was produced (Fig. 4).

Binding of scFv to Cry1Ab Monomeric and Oligomeric Structures—The pre-pore oligomeric Cry1Ab structure gains 200-fold affinity to APN receptor suggesting some structural changes in the receptor-binding epitopes of the Cry1Ab toxin upon oligomerization (8). To determine whether domain II loops 2 and 3 of Cry1Ab pre-pore oligomer recognizes both Cry1Ab structures. These results suggest that domain II loops 2 and 3 of Cry1Ab might suffer a conformational change upon oligomerization or are buried in the oligomeric structure, in contrast to $\beta_16-\beta_22$ that has a similar conformation in both monomeric and oligomeric structures.

Domain III $\beta_16-\beta_22$ epitope involved in Cry1Ab pre-pore oligomer with M. sexta GPI-anchored Proteins.—The results shown so far demonstrated that the Cry1Ab monomer interacts with APN by domain III $\beta_16$ and that this binding epitope has a similar structure in both monomeric and oligomeric structures. To determine whether domain III $\beta_16$ was involved in the interaction of the Cry1Ab pre-pore to APN, binding of pure oligomeric Cry1Ab pre-pore to proteins obtained after phosphatidylinositol-phospholipase C treatment of BBMV was performed in the presence of scFvM22 or scFvL3-3 phages as competitors. APN is GPI-anchored and phosphatidylinositol-phospholipase C cleaves out GPI anchored proteins (4, 30). Fig. 5B shows that scFvM22-phage competed the binding of Cry1Ab oligomer to GPI-anchored proteins in contrast to scFvL3-3 phage that had no effect in this interaction.

Effect of scFv Phage on Toxicity of Cry1Ab Toxin in M. sexta Larvae—To test the effects of selected scFv phages on the toxicity of Cry1Ab toxin to M. sexta larvae, bioassays were performed using the different scFv phages in combination with Cry1Ab toxin. First instar M. sexta larvae were fed Cry1Ab toxin either alone or with the Cry1Ab toxin preincubated with $10^9$ phage particles of the different monoclonal scFv antibodies. As control we included an scFv phage particle (scFvS1) that was selected by biopanning against Cry1Ab toxin and that showed similar binding to Cry1Aa, Cry1Ab, or Cry1Ac in ELISA (data not shown). None of the phages were toxic to M. sexta larvae.}

**FIGURE 3.** Binding of Cry1Ab toxin to M. sexta BBMV proteins by toxin overlay assay. A, toxin overlay assay in the presence of scFv phage particles as competitors ($10^8$ or $10^9$ plaque-forming units as competitors); B, toxin overlay assay in the presence of synthetic peptides as competitors. Numbers indicate the molar excess of each synthetic peptide; lanes C, control without competitor. Representative results of at least four repetitions.

**FIGURE 4.** Pre-pore oligomer formation induced by binding of Cry1Ab with selected scFv molecules. Western blot of Cry1Ab toxin after incubation with scFv phages ($1/4$ ratio) and activated with M. sexta midgut juice. Reaction was stopped with $1$ mM phenylmethylsulfonyl fluoride and centrifuged 20 min at 12,000 × g. Supernatants were loaded into SDS-PAGE and Cry1Ab structures were detected with polyclonal anti-Cry1Ab antibody. Lane 1, proteolytic activation in the presence of anti-loop 2 scFv73 antibody; lane 2, activation in the presence of anti-loop 3 scFv3L-3 antibody; lane 3, activation in the presence of anti-domain III scFvM22 antibody.

**FIGURE 5.** Domain III $\beta_16-\beta_22$ epitope involved in Cry1Ab pre-pore interaction with APN. A, binding of anti-loop 2 scFv73, anti-loop 3 scFv3L-3, or anti-domain III scFvM22 to pure Cry1Ab monomeric or oligomeric structures. B, binding of pure oligomer to GPI-anchored proteins from M. sexta BBMV without competitor (C) or in the presence of $10^3$ scFvM22 or scFvL3-3 phage particles. Bars are S.D. of three repetitions. Statistical significance: A, p < 0.01; B, p < 0.05.
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TABLE 2
Toxicity of Cry1Ab toxin to M. sexta larvae in the presence of scFv-phages competitors

| Treatment | Mortalitya (%) |
|-----------|----------------|
| Cry1Ab    | 95 ± 0.8       |
| Cry1Ab + scFv3L-3 | 17 ± 1.6 |
| Cry1Ab + scFvM22  | 30 ± 5.5       |
| Cry1Ab + scFvS1   | 96 ± 1          |

*Expressed as mean ± S.D. (n = 3) using 24 larvae. For each treatment 10 ng/cm² of Cry1Ab toxin plus 10⁸ colony forming units of scFv-displaying phage were used.

(data not shown). Table 2 shows that both scFvL3-3-M13 and scFvM22-M13 phages inhibited 70–80% toxicity of the Cry1Ab toxin. In contrast, scFvS1 phage particles had no effect on Cry1Ab toxicity.

DISCUSSION

We have previously proposed a model involving the sequential interaction of Cry1A toxins with cadherin-like protein (Bt-R₁) and GPI-anchored APN receptor molecules. First, the interaction of monomeric Cry1A toxins with Bt-R₁ facilitates the formation of a pre-pore oligomeric structure that gains binding affinity to APN, after APN binding the Cry1A pre-pore inserts into lipid rafts inducing osmotic unbalance and cell swelling (8). However, the molecular mechanism that determines the sequential interaction of monomeric and pre-pore oligomeric structures with both receptors is not understood. In this work we identify the binding epitopes of monomeric and oligomeric Cry1Ab structures to their corresponding receptor (cadherin Bt-R₁ for the monomeric structure and APN for the oligomeric structure) and determined for the first time the important role of both receptors in the Cry1Ab toxicity in vivo.

In the case of Bt-R₁, domain II loops a8, 2, and 3 have been shown to have an important role in Bt-R₁ recognition (19–21, 23). In agreement with this, anti-loop 3 scFvL3-3 antibody, or the previously reported anti-loop 2 scFvV73, inhibited the interaction with Bt-R₁ (19, 20). Sequence analysis of the five anti-loop 3 antibodies revealed that all of them shared a similar CDR1 of the variable light chain. Binding competition analysis of anti-loop 3 antibodies to Cry1Ab toxin using synthetic peptides corresponding to the amino acid sequences of the six predicted CDR regions in scFvL3-3 revealed that only peptide CDR1-L competed the binding indicating that anti-loop 3 binds Cry1Ab loop 3 using the CDR1-L region. The CDR1-L amino acid sequence (162QASQSIVS169) showed a similar hydrophatic profile to the Bt-R₁ sequence (1412NAQTGVLTI1419) located in CADR12. This amino acid region corresponds to the loop 3-binding epitope of H. virescens cadherin 1421QTGVLTL-NFQ1431 (23), suggesting that both M. sexta and H. virescens cadherins recognize loop 3 similar binding epitopes.

Data from several laboratories, including this report, have recognized that Cry1A domain III has an important role in APN recognition (25, 33). Cry1Ac toxin binds M. sexta APN receptors through domain III binding to GalNac residues (18, 33), whereas it was proposed that Cry1Aa binds B. mori APN through domain III β16 (508STLRVNS1513) and β22 (682VFTL- SAHV588) residues (25). In this work we show that Cry1Ab binds M. sexta APN principally by the domain III β16 epitope because scFvM22 antibody that binds β16 and β22 amino acid regions competed the binding of Cry1Ab with APN. Nevertheless, Cry1Ab binding competitions with synthetic peptides DIII-1 (corresponding to β16 residues) or DIII-2 (corresponding to β22) revealed that only the β16 region is involved in interaction with APN in contrast to the β22 region. Mutagenesis of β16 residues will be helpful to further narrow this APN binding epitope. Interestingly, the β16 –β22 epitope has a similar structure in both the monomeric and oligomeric structures because scFvM22 recognized both toxin structures with similar efficiencies and scFvM22 inhibited the interaction of the pre-pore oligomer with GPI-anchored protein extracts. These results indicate that this domain III epitope is involved in the interaction of the oligomer with APN.

SPR binding studies of Cry1Ab mutants with pure M. sexta APN showed that domain II loops 2 and 3 are also involved in APN recognition (34). These data seem to be in disagreement with the finding that an anti-loop 2 scFvV73 or anti-loop 3 scFvL3-3 phages did not inhibit the interaction of monomeric Cry1Ab with APN and the oligomeric pre-pore with soluble GPI-anchored proteins. In the case of the lepidopteran insect Lymantria dispar, a sequential binding mechanism was proposed in the interaction of Cry1Ac with APN (35). Cry1Ac domain III first interacts with APN GalNAC sugar moieties facilitating the subsequent interaction of domain II loop regions with another region in this receptor (35). This binding mechanism could explain the binding competition of anti-domain III scFvM22 phage particles to APN in contrast to anti-domain II loop antibodies scFvV73 and scFvL3-3, because competing the first binding event will inhibit the binding of domain II loop regions.

The scFv molecules characterized in this work were useful in determining the role of Cry1Ab binding to both receptor molecules in the formation of the pre-pore oligomeric structure. Anti-domain II loop scFv molecules, scFvV73 or scFvL3-3, facilitated the formation of the pre-pore oligomeric structure. Because these scFv molecules inhibited Bt-R₁ binding and not APN recognition, these data are in agreement with a model where binding of monomeric Cry1Ab toxin to Bt-R₁ through domain II loop regions induces a conformational change resulting in the formation of the pre-pore structure. In contrast, anti-domain III antibody scFvM22 did not facilitate the formation of the oligomeric structure, suggesting that the interaction of monomeric Cry1Ab with APN has no consequence in pre-pore formation.

Previous work demonstrated that Cry1Ab pre-pore oligomer bound APN with a 200-fold increased apparent binding affinity in comparison to monomeric Cry1Ab, suggesting that structural changes of certain binding epitopes in the toxin occur upon oligomerization (8). Interestingly, the anti-loop 2 scFvV73 and anti-loop 3 scFvL3-3 antibodies bound preferentially the Cry1Ab monomeric structure in comparison to the oligomeric structure, suggesting a possible conformational change upon oligomerization in the domain II loop 2 and 3 regions. Because domain II loops 2 and 3 seems to suffer a conformational change upon oligomerization that affects recognition by specific monoclonal antibodies, it could be possible that changes in domain II loops 2 and 3 may be involved in the sequential inter-
action with Bt-R1 and APN. This structural change could lead to loose binding of oligomer to Bt-R1, and enhance binding of oligomer to APN. The nature of the structural change of the domain II loop region in the oligomer remains to be characterized, we cannot rule out that domain II loop regions may be buried into the pre-pore oligomer affecting the interaction of monoclonal antibodies (scFv73 and scFvL3-3) that mimic the Bt-R1 binding sites. Determination of binding affinities of pure pre-pore oligomeric structures of several point mutants in domain II loop regions of Cry1Ab to APN and Bt-R1 will be helpful in understanding the role of these regions on Cry1Ab pre-pore binding to APN. Overall these results show that the constructed immune scFv libraries against Cry1Ab toxin are a valuable resource for identifying regions in the toxin involved in different steps of the toxin mode of action.

Finally, it was recently proposed that the toxicity of Cry1Ab is mainly due to the interaction of monomeric toxin with Bt-R1, by apparently activating a Mg$^{2+}$-dependent adenyl cyclase/protein kinase A signaling pathway in midgut cells that leads to apoptosis and not to lytic pore formation induced by oligomer membrane insertion (9, 10). This was proposed based on the study of the effect of Cry1Ab toxin to cultured Trichoplusia ni H5 insect cells expressing M. sexta Bt-R1 (9, 10). Although not recognized by the authors, the data presented by Zhang et al. (9) showed that toxicity of Cry1Ab toxin to H5 cells expressing Ms-Bt-R1 correlated with increased oligomer formation. In addition, these authors found that activation of adenyl cyclase using a direct activator (forskolin) of membrane adenyl cyclase does not affect cell viability even though it was expected that the increased levels of cAMP would activate protein kinase A under treatment with forskolin. The authors concluded that other effectors could be involved in Cry1Ab toxicity (10). In any case, it should be pointed out that the situation in vivo on intact larvae midgut cells could be very different from cultured insect cells. Our data show that scFvM22, which specifically blocks binding of Cry1Ab to APN without affecting the interaction of Cry1Ab with Bt-R1, severely attenuated the toxicity of Cry1Ab toxin in vivo, indicating that the interaction of Cry1Ab toxin with Bt-R1 is not enough to kill the larvae and that interaction with both receptor molecules is necessary for complete toxicity in M. sexta larvae. These data are in agreement with the model of the sequential participation of Bt-R1 and APN in toxin membrane insertion, pore formation, and toxicity of Cry1A toxins.

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