Domain II Loop 3 of *Bacillus thuringiensis* Cry1Ab Toxin Is Involved in a “Ping Pong” Binding Mechanism with *Manduca sexta* Aminopeptidase-N and Cadherin Receptors*

Received for publication, May 25, 2009, and in revised form, September 22, 2009. Published, JBC Papers in Press, October 6, 2009, DOI 10.1074/jbc.M109.024968

Sabino Pacheco, Isabel Gómez, Ivan Arenas, Gloria Saab-Rincon, Claudia Rodríguez-Almazán, Sarjeet S. Gill, Alejandra Bravo, and Mario Soberón

From the Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca 62250, Morelos, Mexico and the Department of Cell Biology and Neuroscience, University of California, Riverside, California 92506

*Bacillus thuringiensis* Cry toxins are used worldwide as insecticides in agriculture, in forestry, and in the control of disease transmission vectors. In the lepidopteran *Manduca sexta*, cadherin (Bt-R1) and aminopeptidase-N (APN) function as Cry1A toxin receptors. The interaction with Bt-R1 promotes cleavage of the amino-terminal end, including helix α-1 and formation of prepro oligomer that binds to APN, leading to membrane insertion and pore formation. Loops of domain II of Cry1Ab toxin are involved in receptor interaction. Here we show that Cry1Ab mutants located in domain II loop 3 are affected in binding to both receptors and toxicity against *Manduca sexta* larvae. Interaction with both receptors depends on the oligomeric state of the toxin. Monomers of loop 3 mutants were affected in binding to APN and to a cadherin fragment corresponding to cadherin repeat 12 but not with a fragment comprising cadherin repeats 7–12. In contrast, the oligomers of loop 3 mutants were affected in binding to both Bt-R1, but not to APN. Toxicity assays showed that either monomeric or oligomeric structures of Cry1Ab loop 3 mutations were severely affected in insecticidal activity. These data suggest that loop 3 is differentially involved in the binding with both receptor molecules, depending on the oligomeric state of the toxin and also that possibly a “ping pong” binding mechanism with both receptors is involved in toxin action.

*Bacillus thuringiensis* is a bacterium that produces crystalline inclusions formed by insecticidal proteins, called Cry toxins, during the sporulation phase of growth. Cry toxins are toxic to different insect orders as well as to other invertebrates, such as nematodes, mites, and protozoa (1). Cry toxins have been used worldwide in the control of insect pests in agriculture, either as transgenic crops or as spray formulations.

The molecular mechanism proposed to describe the action of Cry1A toxins, which are active against different lepidopteran insect species, involves several steps. After larval ingestion of the crystalline inclusions, these are solubilized in midgut lumen and activated by proteases releasing a toxic 65-kDa fragment that binds, in a sequential manner, with at least two receptors located in midgut microvilli. The first interaction occurs with cadherin protein (Bt-R1) in the case of *Manduca sexta*. This interaction promotes further proteolytic processing of the N-terminal end, including helix α-1 of the toxin, resulting in the formation of a prepro oligomeric structure (2). The oligomer has higher affinity to secondary receptors, which are anchored by glycosylphosphatidylinositol, such as aminopeptidase-N (APN) or alkaline phosphatase in the case of *M. sexta* or *Heliothis virescens*, respectively (3, 4). Glycosylphosphatidylinositol-anchored receptors are located in specific membrane regions called lipid rafts, where the oligomer inserts into the membrane-forming pores, disrupting the osmotic equilibrium and leading to cell death (5, 6). Although this mechanism of action is generally accepted, it may involve additional binding molecules, such as glycolipids, or more than one glycosylphosphatidylinositol-anchored receptor (5, 6). Furthermore, it was shown that Bt-R1, that is normally not located in lipid rafts changes its location to lipid rafts after treatment of *M. sexta* microvilli membranes with Cry1Ab protoxin, suggesting that it remains attached to toxin oligomer after binding to APN (3).

The three-dimensional structures of several Cry toxins with different insect specificity have been solved, including Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, and Cry4Ba (for a review, see Ref. 1). Overall, these proteins show a similar organization in three different domains, suggesting a conserved mode of action as follows. Domain I contains seven α-helices and is involved in oligomer formation, insertion into the membrane, and pore formation (1, 7); domain II consists of three antiparallel β-sheets, and its structure is the most variable of three domains and is implicated in receptor recognition; and domain III is composed of two antiparallel β-sheets and is also involved in receptor binding (1).

Cry1A toxins bind to cadherin proteins of at least six lepidopteran species, *M. sexta*, *Bombyx mori*, *H. virescens*, *Helicoverpa armigera*, *Pectinophora gossypiella*, and *Ostrinia nubilalis* (8–13). Insect cadherins are composed of an ectodomain...

---

*This work was supported, in whole or in part, by National Institutes of Health Grant 1R01 AI066014. This work was also supported by Consejo Nacional de Ciencia y Tecnología Grants 83135 and 81639, DGAPA-UNAM Grants IN218608-3 and IN210208, and United States Department of Agriculture Grant 2207-35607-17780.

1 To whom correspondence should be addressed. E-mail: mario@ibt.unam.mx.

2 The abbreviations used are: Bt-R1, cadherin receptor; APN, aminopeptidase-N; CR, cadherin repeat; BBMV, brush border membrane vesicle(s); ELISA, enzyme-like immunosorbent assay; scFv, single-chain Fv; MS, single mutant; MD, double mutant; MT, triple mutant; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
formed by 11 or 12 cadherin repeats (CRs), a transmembrane domain, and an intracellular domain (14). The exposed loops of Cry1A domain II have been directly involved in binding with cadherin in *M. sexta, H. virescens*, and *B. mori* (15–18). Three Cry1Ab binding sites were mapped in CR7, CR11, and CR12 of the *M. sexta* Bt-R, (15, 19). The interaction between Cry1Ab/Cry1Ac toxins and *H. virescens* cadherin was analyzed, showing that loop 3 of domain II binds to CR12 of this receptor (17). Accordingly, loop 3 of Cry1Aa binds the corresponding site of *B. mori* cadherin (Bt-R175) (18). Cry1Ab loop α-8 and loop 2 bind with *M. sexta* Bt-R, CR7 and CR11 epitopes (15).

Regarding interaction of Cry1A toxins with the second receptor, Cry1Ac toxin binds to APN by means of domain III, which recognizes *N*-acetylglucosamine moieties in the receptor (20). Also, recent reports indicate that the region B16–B22 located in domain III of Cry1Aa and Cry1Ab toxins is involved in the interaction with *B. mori* and *M. sexta* APNs, respectively (16, 21). In addition, domain II is involved in Cry1A toxin interaction with APN because mutations in domain II loop regions that affected toxicity were shown to affect binding of Cry1Ac or Cry1Ab to *M. sexta* and *Lymnaea dispar* APNs (22, 23). It is important to mention that these binding studies were performed with monomeric toxins, and these mutations could also affect binding to the cadherin receptor. In another study, scFv antibodies that bind specifically to Cry1Ab domain II loop 2 or loop 3 or to domain III (B16–B22 region) were used to show that these domain II loop regions, in contrast to domain III, undergo a conformational change upon oligomerization, suggesting that this structural change may be involved in the sequential interaction of the Cry1Ab toxin with cadherin and APN receptors (16).

To determine the role of domain II loop 3 residues in the sequential interaction of Cry1Ab toxin with cadherin and APN receptors, we characterized Cry1Ab domain II loop 3 mutants. We analyzed the ability of either monomers or oligomers of these mutants to bind Bt-R, and APN and analyzed their effect on toxicity. Our data show that mutations in domain II loop 3 differentially affect the binding with both receptors, depending on the oligomeric state of the toxin. We propose that possibly a “ping pong” binding mechanism with both receptors is involved in toxin action.

**MATERIALS AND METHODS**

*Site-directed Mutagenesis—*pHT315-Cry1Ab was used as a template for site-directed loop 3 mutagenesis using QuickChange® Multi following the manufacturer’s instructions (Stratagene).

*Purification and Activation of Cry1Ab Toxins—*The acrystallophoric *B. thuringiensis* strain 407 was transformed with pHT315-Cry1Ab or the same plasmid containing loop 3 substitutions. *B. thuringiensis* transformant strains were grown for 3 days at 30 °C in LB medium supplemented with 10 μg/ml erythromycin. After sporulation, crystals were purified by sucrose gradients. For monomer production, Cry1Ab crystals were solubilized in alkaline buffer and activated by trypsin as reported previously (24). In order to obtain oligomeric structure, the crystals were activated with 5% *M. sexta* midgut juice in the presence of scFv73 antibody as described (16). The oligomers were purified by size exclusion chromatography with a Superdex 200HR 10/30 (Amersham Biosciences) fast protein liquid chromatography system. Protein concentration was determined by the Bradford assay, using bovine serum albumin as a standard and the extinction coefficient method, where ε\text{280} = 82,280 M$^{-1}$ cm$^{-1}$ for Cry1Ab toxin.

*Expression and Purification of Cadherin Proteins—*Fragments of cadherin protein (CR7–CR12 or CR12) were cloned into pET22b vector as reported previously (7, 25, 26). These constructions were transformed into *Escherichia coli* ER2566 cells; protein expression was induced by the addition of 1 mM isopropyl-thio-β-galactopyranoside. Proteins expressed as inclusion bodies were solubilized with 8 M urea solution. Bt-R, peptides were purified with nickel affinity columns according to the manufacturer’s instructions (Qiagen) and eluted with 250 mM imidazole in PBS buffer to eliminate urea.

*Secondary Structure Analysis of CR12 and CR7–CR12 by CD Spectroscopy—*CD spectra were recorded with a JASCO model J-715 spectropolarimeter equipped with a Peltier temperature control supplied by Jasco. Spectra were collected from 200 to 240 nm. Buffer conditions were 10 mM potassium phosphate, pH 7.6, and 25 °C. Eight replicate spectra were collected on each sample to improve signal/noise ratio. The final purified protein (CR12 or CR7–CR12) concentration was 0.3 mg/ml, and spectra were collected in a 0.01-cm path length cell. The secondary structure prediction was performed using the CDSSSTR algorithm, which requires data from 200 to 240 nm (27–29).

*Expression and Purification of scFv Antibodies—*The soluble scFv73 and scFv3L3 antibodies were expressed in *E. coli* BL21 (DE3) cells and purified (16, 24).

*Preparation of BBMV—*Insect midguts of fourth instar *M. sexta* larvae were dissected and used to prepare brush border membrane vesicles by differential precipitation using MgCl₂ (30) and stored at −70 °C until use.

*Toxin Overlay Assay—*One hundred micrograms of BBMV protein were loaded in a single long lane, separated by 9% SDS-PAGE, and electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences). Single gel blots were incubated with different Cry1Ab proteins 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. Ten parallel troughs milled in one side of the upper plate become individual incubation chambers when the unit is assembled. After blocking with PBS-M (PBS, 5% skim milk), the membrane was incubated with 5 μM wild type or mutant Cry1Ab toxins. Unbound toxins were removed by washing the membrane with PBS-T (PBS, 0.1% Tween 20). The membrane was then incubated with rabbit anti-Cry1Ab antibody followed by secondary anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Biosciences). Blots were visualized using luminol (ECL™, Amersham Biosciences).

*Binding Assay of Cry1Ab Toxins with BBMV—*For binding assays of wild type and mutant Cry1Ab toxins to BBMV, 10 μg of BBMV protein were incubated in binding buffer (PBS, 0.1% bovine serum albumin, 0.1% Tween 20) with 5 nM Cry1Ab or loop 3 mutants. The unbound toxin was removed by centrifugation (10 min at 14,000 rpm). BBMV were washed three times with binding buffer and suspended in 10 μl of PBS, 3 μl of...
Cry1Ab Loop 3 Binding with M. sexta Aminopeptidase-N and Cadherin

Laemmli sample buffer 4\times (0.125 \text{ m Tris/HCl}, 4\% SDS, 20\% glycerol, 10\% β-mercaptoethanol, 0.01\% bromphenol blue). The samples were separated by 10\% SDS-PAGE and electro-transferred to polyvinylidene difluoride membrane. The membrane was revealed using rabbit anti-Cry1Ab (1:5000) and secondary anti-rabbit (1:5000) conjugated with horseradish peroxidase antibodies.

For competition binding assays, Cry1Ab toxin was labeled with biotin using biotinyl-N-hydroxysuccinimide ester according to the manufacturer’s instructions (Amersham Biosciences). Ten micrograms of BBMV protein were incubated in binding buffer (PBS, 0.1\% bovine serum albumin, 0.1\% Tween 20) with 5 nM biotinylated Cry1Ab toxin in the presence or absence of a severalfold molar excess of unlabeled wild type or Cry1Ab-activated mutants. The unbound toxin was removed by centrifugation (10 min at 14,000 rpm). BBMV were washed three times with binding buffer and suspended in 10 \mu l of PBS, 3 \mu l of Laemmli sample buffer 4\times.

The samples were separated by 10\% SDS-PAGE and electro-transferred to polyvinylidene difluoride membrane. The membrane was incubated with streptavidin-peroxidase conjugate (Amersham Biosciences) for 1 h, and blots were visualized using luminol (Amersham Biosciences).

APN Purification—Aminopeptidase-N was purified from BBMV of fifth instar M. sexta larvae as reported (31). BBMV were incubated in solubilization buffer (1\% CHAPS, 5 mM EDTA, 0.1 \text{ m NaCl}, 1 \text{ mM phenylmethylsulfonyl fluoride}, 20 \text{ mM Tris/HCl, pH 8.5}) for 2 h at 4 °C. After centrifugation, supernatant was recovered and dialyzed in buffer A (2 \text{ mM MgCl}_2, 2 \text{ mM KCl}, 20 \text{ mM Tris/HCl, pH 8.5}). The sample was loaded into an ion exchange column (Mono-Q) connected to a fast protein liquid chromatography system, APN was eluted using a 0–1M NaCl gradient, and finally the eluted APN was visualized by SDS-PAGE and Western blot. The activity of purified APN was determined using 1-leucine-\(p\)-nitroanilide as substrate as reported previously (32).

ELISA Binding Assay of Cry1Ab Toxins—ELISA plates were coated with 1 \mu g of either cadherin protein fragment (CR7–CR12 or CR12), APN from M. sexta, or scFv antibodies (scFv73 or scFv3L3) in 100 ml of PBS/well overnight at 4 °C. The plates were washed three times with PBS, blocked with 200 \mu l/well PBS-M for 2 h at 37 °C, and washed three times with PBS. Different non-saturated concentrations of monomeric or oligomeric Cry1Ab were used in the different binding assays as indicated. The unbound toxins were removed by three washes with PBS-T and three washes with PBS. The bound toxins were detected using rabbit anti-Cry1Ab and anti-rabbit conjugated with horseradish peroxidase antibodies. Finally, ortho-phenylenediamine (Sigma) plus H_2O_2 was used as substrate for detection. The reaction was stopped adding 50 \mu l of 1 \text{ m H}_2\text{SO}_4 and measured at 490 nm using an ELISA microplate reader. Data were analyzed using GraphPad Prism software (version 5.0b), and data curves were transformed by the Scatchard equation for obtaining relative binding affinities (K_a).

Insect Bioassay—Bioassays were performed with M. sexta neonate larvae by the surface contamination method (2). Different doses of toxin-activated proteins (from 0.1 to 2000 ng/cm^2) were applied onto the diet surface contained in 24-well polystyrene plates (Cell Wells, Corning Glass). A total of 24 larvae/plaque were fed with different doses of trypsin-activated toxins. The plates were incubated at 28 °C with 65 ± 5% relative humidity and a light/dark photoperiod of 16/8 h. Mortality was recorded after 7 days, and the 50% lethal concentration (LC_{50}) was analyzed with Probit software. For single dose assays, 2 ng/cm^2 of pure oligomer or 25 ng/cm^2 of monomer samples were used with 24 larvae per concentration, and mortality was recorded after 5 days.

RESULTS

Construction of Cry1Ab Loop 3 Mutants—Previously, we reported that loop 3 of Cry1Ab toxin showed a hydrophobic complementary pattern with the binding epitope of H. virescens cadherin, suggesting a putative interaction through a complementary hydrophobic profile (17). Here we introduce amino acid substitutions in loop 3 of Cry1Ab toxin designed to disrupt the hydrophobic profile of this region. Three mutants were constructed, a single point mutant S446V (named MS), a double mutant S441R/N442V (MD), and the combination of these substitutions in a triple mutant S441R/N442V/S446V (named MT). After mutagenesis, plasmids were sequenced and transformed into an acrystalliferous B. thuringiensis strain, and cells were sporulated to produce crystal proteins. The crystalline inclusions produced by the loop 3 mutants, MS, MD, and MT, and the Cry1Ab toxin were solubilized and activated with trypsin. Fig. 1A shows that trypsin activation of the loop 3 mutants produced a 65-kDa protein similar to the Cry1Ab toxin. To determine whether these mutations affect oligomer formation we activated mutant proteotoxins with M. sexta midgut juice in the presence of scFv73 antibody. Antibody scFv73 was previously shown to mimic a cadherin binding region and facilitates formation of the oligomer structure when the Cry1Ab protoxin was proteolytically activated in the presence of this molecule (2, 24). Fig. 1B shows the Cry1Ab oligomers revealed by Western blot using a Cry1Ab antibody that recognizes both the monomeric and the oligomeric structures. The MS and MD mutants produced 40% lower yields of oligomer than the Cry1Ab protein, as revealed by scanning the optical density of the 250-kDa oligomer band (Fig. 1B). However, the MT mutant produced significantly lower levels of oligomer than Cry1Ab or MS and MD proteins (Fig. 1B).

To determine if loop 3 mutations affect the structure of other regions of domain II, we analyzed the binding of two anti-domain II antibodies (scFv73 and scFv3L3) that recognize either domain II loop 2 or loop 3, respectively, and compete with binding of Cry1Ab with Bt-R1 in toxin overlay assays (16, 24, 33). Anti-loop 2 scFv73 antibody bound to the trypsin-activated mutants and to Cry1Ab toxin, indicating the same toxin conformation in this binding epitope (Fig. 1C). In contrast, the anti-loop 3 scFv3L3 antibody only recognized the Cry1Ab toxin, indicating that loop 3 mutations specifically affected the binding capacities of the loop 3 amino acid region (Fig. 1D).

Binding Assays of Cry1Ab Loop 3 Mutants with M. sexta BBMV Proteins—To determine the effect of loop 3 mutations in binding to both cadherin and aminopeptidase receptors, toxin overlay assays were performed. In these assays, BBMV proteins were separated by SDS-PAGE and blotted to polyvinylidene
Cry1Ab Loop 3 Binding with M. sexta Aminopeptidase-N and Cadherin

FIGURE 1. Cry1Ab domain II loop 3 mutants are structurally stable. A, SDS-PAGE electrophoresis pattern of trypsin-activated Cry1Ab (lane 2), MS (lane 3), MD (lane 4), and MT (lane 5) toxins. Lane 1, molecular weight marker. B, Western blot of toxin-activated samples in the presence of scFv73 antibody, Cry1Ab (lane 1), MS (lane 2), MD (lane 3), and MT (lane 4). The 250-kDa oligomer and 60-kDa monomer bands are shown. Numbers below the 250 kDa band represent the percentage of oligomer concentration after scanning this band. C and D, ELISA binding analysis of 2.5 nM trypsin-activated Cry1Ab loop 3 mutants to anti-loop 2 scFv73 antibody (C) or anti-loop 3 scFv3L3 (D).

FIGURE 2. Cry1Ab loop 3 mutants are affected in binding to M. sexta BBMV proteins. A, toxin overlay binding assays of Cry1Ab (lane 1), MS (lane 2), MD (lane 3), and MT (lane 4) toxins to blotted M. sexta BBMV proteins. The 210-kDa protein corresponds to Bt-R1, and the 120-kDa protein corresponds to APN. The numbers represent the percentage of binding after scanning bands. B, binding assays of 10 nM Cry1Ab (lane 1), MS (lane 2), MD (lane 3), and MT (lane 4) toxins with M. sexta BBMV. The 65-kDa protein corresponds to bound toxins recovered after centrifugation of BBMV samples and revealed with anti-Cry1Ab antibody as described under “Materials and Methods.” The numbers represent the percentage of binding after scanning bands. C, binding competitions of biotinylated Cry1Ab toxin to BBMV using different excess of unlabeled Cry1Ab (○), MS (●), MD (▲), and MT (×) proteins.

APN in native conformation, we performed ELISA binding saturation assays of the activated Cry1Ab, MS, MD, and MT toxins to two cadherin fragment proteins produced in E. coli and to APN protein purified from M. sexta midgut tissue. Previously, it was shown that the apparent binding dissociation constant of Cry1Ab monomeric toxin with Bt-R1 was in the range of 1 nM (8). Also, it was shown that binding with APN was in the range of 100 nM (20). We previously cloned and produced in E. coli two cadherin fragments corresponding to CR7–CR12 (residues Met810–Ala1485) and to CR12 (residues Gly1370–Ala1485) of M. sexta Bt-R1 (7, 25, 26). It was reported that Cry1Ab bound to a cadherin peptide (CR12-MPED) similar to CR12 with a binding affinity of 9.5 nM (34). Fig. 3 shows the analysis of folding of cadherin fragments CR7–CR12 and CR12 by circular dichroism spectra, indicating that both polypeptides have a similar content of α-helices and β-strand structures, with the β-fold structure the most abundant secondary structure. Fig. 4 shows that the Cry1Ab loop 3 mutants were not affected in binding to CR7–CR12 cadherin fragment but were affected in binding to CR12 and APN. Calculation of apparent binding affinities by Scatchard plots (not shown) revealed that Cry1Ab and the three loop 3 mutants bound CR7–CR12 with very high binding affinity (Kd = 0.2 nM). In the case of CR12, the
MT mutant showed a 250-fold reduction on binding affinity, whereas MT and MD showed a 70- and 80-fold reduction in binding in comparison with Cry1Ab (Kd = 9.5 nM). Finally, the MS, MD, and MT mutants were severely affected upon binding with APN, showing a 28-, 14-, and 23-fold reduction in binding affinity to APN, respectively, in comparison with Cry1Ab (Kd = 85 nM). The apparent binding affinities obtained by the saturation ELISA binding assays are in the range of those previously reported for cadherin fragment CR12-MPED and APN (20, 34). However, binding to CR7–CR12 showed a very high apparent dissociation of 0.2 nM, 5-fold higher than the reported binding affinity of 0.1 nM Cry1Ab and loop 3 mutant oligomers with the cadherin fragments revealed that the loop 3 mutations affected significantly the binding of the oligomer with both CR7–CR12 and CR12 cadherin fragments (Fig. 5, B and C). As controls, we performed ELISA binding assays using monomers that confirmed that loop 3 mutations affected monomer binding with CR12 but not with CR7–CR12 (Fig. 5, D and E).

Interaction of Cry1Ab Loop 3 Mutant Oligomers with M. sexta APN—To determine the effect of the loop 3 mutations on the binding of Cry1Ab oligomer with APN, we performed ELISA binding assays with APN purified from M. sexta BBMV, using either oligomeric or monomeric structures of each mutant and of Cry1Ab. As above, non-saturated concentrations were used in the ELISA binding assays. Fig. 6A shows that 0.1 nM Cry1Ab, MD, MS, and MT oligomers bound similarly to APN protein. In contrast, 25 nM monomeric MD, MS, and MT proteins were affected in binding to APN in contrast to the wild type Cry1Ab monomer (Fig. 6B).

Toxicity Effects of Cry1Ab Loop 3 Mutations—We determined the effect of the Cry1Ab loop 3 mutations on toxicity against M. sexta larvae. Table 1 shows that the activated MS and MD mutants had a 9-fold reduction in mortality, whereas MT had a 70-fold reduction in mortality when the LC50 lethal values were compared with activated Cry1Ab protein. To determine the effect on mortality in the context of both monomeric and oligomeric structures, we performed a single dose toxicity assay of pure monomer or oligomeric structures. Table 1 shows that a single dose of Cry1Ab monomer (25 ng/cm2) or Cry1Ab oligomer (2 ng/cm2) resulted in 95% larval mortality. In contrast, the same doses of monomeric and oligomeric structures of the three loop 3 mutants were severely affected in toxicity to M. sexta larvae (Table 1).

DISCUSSION

Interaction of pathogens with their target cells involves specific recognition of surface molecules to modulate cell recognition, membrane insertion, or cell internalization. In the case of bacterial pore-forming toxins, the interaction of single receptors seems to be a general strategy, although in the case of several toxins, such as diphtheria, anthrax protective antigen, or aerolysin, more than one surface molecule is involved in the binding and mode of action of these toxins (35–37). With viruses, sequential interaction with several surface molecules is important for infection where structural changes of the viral proteins are involved in target cell interactions (38, 39).

Regarding insecticidal Cry1A toxins, membrane insertion is the result of the sequential interaction with at least two receptor molecules in the lepidopteran M. sexta, Bt-R1, and APN (3). Binding of Cry1Ab toxin with Bt-R1 facilitates removal of helix α-1, triggering toxin oligomerization (2). The oligomer gains binding affinity to the second receptor, APN, and this interaction enhances insertion of the oligomer into the membrane (3, 40). We previously hypothesized that sequential interaction of the Cry1Ab toxin with the two receptors involves structural changes of binding epitopes upon oligomerization (16). By use

3 I. Arenas and I. Gómez, unpublished data.
of scFv antibodies that bind with two different domain II loop regions, loop 2 and loop 3, or with the domain III/B16–B22 region, we showed that antibodies to domain II loop recognized preferentially the monomeric structure rather than the oligomer, in contrast to the anti-domain III scFv molecule that recognized equally both structures. This finding suggests a subtle structural change in domain II loop 2 and 3 binding regions upon oligomerization of the toxin (16). Here we show that the loop 3 mutations have a differential effect on binding to both Bt-R1 and APN, depending on the oligomeric state of the toxin (monomer versus oligomer structures). Thus, structural changes that occur upon oligomerization also affect Cry1Ab toxin binding capacities.

In this study, we mutagenized domain II loop 3 to determine its role in the in vivo binding to both Bt-R1, and APN receptors. Published data regarding the role of this binding region in the interaction with both receptor molecules are incomplete because binding of loop 3 mutant oligomers with the two receptor molecules was not previously analyzed. Mutagenesis studies of Cry1Ab and Cry1Ac loop 3 previously performed indicated that this amino acid region was important for binding with M. sexta BBMV and toxicity (40, 41). In addition, alanine substitutions of loop 3 residues in Cry1Ab and Cry1Ac toxins showed a correlative effect on APN binding, suggesting that their effects on toxicity were due to defects in this binding (22, 23). However, it was also shown that loop 3 is the cognate bind-
ing region of CR12 in H. virescens cadherin and in Bt-R1 receptors (17) (Fig. 4), suggesting that this toxin region may be involved in the interaction with both Bt-R1 and APN receptors.

Toxin overlay binding analysis of loop 3 mutants confirmed the hypothesis that this amino acid region may be involved in the interaction with both Bt-R1 and APN receptors because binding with both of these proteins was greatly reduced, data that correlated with the observed reduced binding with M. sexta BBMV. However, binding analysis of monomeric or oligomeric structures to non-denatured Bt-R1 fragment or APN revealed unexpected results that indicate that the mode of action of Cry1Ab toxin may involve multiple binding interactions with both receptor molecules during the intoxication process. ELISA binding assays showed that loop 3 mutations had a significant effect on the binding of monomeric toxin with APN. In the case of aerolysin, another pore-forming toxin, the high affinity interaction with Bt-R1 is present in much lower concentrations (42). We therefore speculate that monomeric Cry1Ab binds first with the high abundance low affinity APN site before the high affinity interaction with Bt-R1.

Regarding the interaction of Cry1Ab monomer with Bt-R1 receptor, previous work showed that domain II, loop α-8, loop 2, and loop 3 are involved in the binding of Cry1Ab toxin to this receptor (2, 16, 32). Loop 2 was the cognate binding epitope of the CR7 region (16–22) (33); loop α8 and loop 2 interact with the CR11 region (133–141) (15), whereas loop 3 binds to the CR12 region of the cadherin receptors in H. virescens and B. mori (17, 18). Monomeric structures of the three loop 3 mutants characterized here were only affected in the binding with the CR12 fragment of Bt-R1 and not with a Bt-R1 fragment corresponding to CR7–CR12 (Fig. 4), confirming that Cry1Ab loop 3 binds CR12 and that binding with the CR7–CR12 fragment also involves loops α-8 and 2.

Loop 3 mutations had no effect on the binding interaction of the Cry1Ab oligomeric structure with APN but had a significant effect on oligomer binding with Bt-R1 (Figs. 5 and 6). These results suggest that when oligomer is formed after interaction with Bt-R1, it remains bound with Bt-R1 through loop 3 and that oligomer interaction with APN involves other regions as previously reported, corresponding to the domain III β16–β22 region (16, 21). Furthermore, the formation of such a protein complex (Bt-R1-Cry1Ab-APN complex) was previously suggested because Bt-R1 was mobilized into lipid rafts after toxin interaction (5). What could be the role of such a protein complex (Bt-R1-oligomer-APN) in Cry1Ab toxicity? Loop 3 mutations severely affected the toxicity of Cry1Ab oligomer, suggesting that this protein complex may be important for toxicity. However, engineered Cry1Ab- and Cry1Ac-modified toxins (Cry1AbMod and Cry1AcMod) lacking helix α-1 formed oligomers in the absence of cadherin interaction and killed M. sexta larvae that were silenced for the cadherin gene, showing that cadherin interaction is not important for toxicity mediated by Cry1Ab oligomer (25). Therefore, the effect of loop 3 mutations on oligomer toxicity should be due to postbinding APN events.

Previously, it was shown that the APN binding through N-acetylglactosamine moieties enhances membrane insertion of Cry1Ac toxin (43). It is possible that domain II loop 3 mutations affect structural changes that are triggered by APN and necessary for membrane insertion of the Cry1Ab oligomer. In this regard, it has been shown that mutations in domain II loop 2 Phe871 retain binding with M. sexta BBMV but are affected in membrane insertion (44, 45).

The results shown here indicate that Cry1Ab toxin specificity is determined by a complex binding mechanism with two different receptor molecules that depends on the oligomeric state of the toxin. A “ping pong” binding mechanism may occur where domain II loop 3 may be involved in the first binding event with the high abundance low affinity APN receptor. This interaction is followed by a high affinity binding to Bt-R1 receptor that involves participation of other regions of domain II in addition to loop 3, such as loops α-8 and 2. Interaction with Bt-R1 triggers cleavage of helix α-1 and oligomer formation. The oligomeric structure gains binding affinity with APN through other regions of the toxin, such as the domain III β16–β22 region, but remains bound with Bt-R1 through loop 3. Finally, residues in domain II loop 3 may be also involved in post-APN binding events important for toxicity, such as oligomer membrane insertion (Fig. 7).

Acknowledgment—We thank Lizbeth Cabrera for technical assistance.

REFERENCES
1. Bravo, A., Gill, S. S., and Soberón, M. (2007) Toxicon 49, 423–435
2. Gómez, I., Sánchez, J., Miranda, R., Bravo, A., and Soberón, M. (2002) FEBS Lett. 513, 242–246
3. Bravo, A., Gómez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., Zhuang, M., Gill, S. S., and Soberón, M. (2004) Biochim. Biophys. Acta 1667, 38–46
4. Jurat-Fuentes, J. L., and Adang, M. J. (2004) Eur. J. Biochem. 271, 3127–3135
5. Zhuang, M., Oltean, D. I., Gómez, I., Pullikuth, A. K., Soberón, M., Bravo, A., and Gill, S. S. (2002) J. Biol. Chem. 277, 13863–13872
6. Griffiths, J. S., Haslam, S. M., Yang, T., Garczynski, S. F., Mullloy, B., Morris, H., Cremer, P. S., Dell, A., Adang, M. J., and Aroian, R. V. (2005) Science 307, 922–925
7. Jiménez-Juárez, N., Muñoz-Garay, C., Gómez, I., Saab-Rincon, G., Damian-Almazo, J. Y., Gill, S. S., Soberón, M., and Bravo, A. (2007) J. Biol. Chem. 282, 21222–21229
8. Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H., and Bulla, L. A., Jr. (1995) J. Biol.
9. Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S., and Kogure, M. (1998) *Biosci. Biotechnol. Biochem.* **62**, 727–734

10. Gahan, L. J., Gould, F., and Heckel, D. G. (2001) *Science* **293**, 857–860

11. Xu, X., Yu, L., and Wu, Y. (2005) *Appl. Environ. Microbiol.* **71**, 948–954

12. Morin, S., Biggs, R. W., Sisterson, M. S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L. J., Heckel, D. G., Carrière, Y., Dennehy, T. J., Brown, J. K., and Tabashnik, B. E. (2003) *Proc. Nat. Acad. Sci. U.S.A.* **100**, 5004–5009

13. Flannagan, R. D., Yu, C. G., Mathis, J. P., Meyer, T. E., Shi, X., Siqueira, H. A., and Siegried, B. D. (2005) *Insect. Biochem. Mol. Biol.* **35**, 33–40

14. Bel, Y., and Escriche, B. (2006) *Gene* **381**, 71–80

15. Go´mez, I., Dean, D. H., Bravo, A., and Sobero´n, M. (2003) *Biochemistry* **42**, 10482–10489

16. Go´mez, I., Arenas, I., Benitez, I., Miranda-Ríos, J., Becerril, B., Grande, R., Almagro, J. C., Bravo, A., and Sobero´n, M. (2006) *J. Biol. Chem.* **281**, 34032–34039

17. Xie, R., Zhuang, M., Ross, L. S., Gomez, I., Oltean, D. I., Bravo, A., Soberon, M., and Gill, S. S. (2005) *J. Biol. Chem.* **280**, 8416–8425

18. Masson, L., Lu, Y. J., Mazza, A., Broussseau, R., and Adang, M. J. (1995) *J. Biol. Chem.* **270**, 20309–20315

19. Atsumi, S., Mizuno, E., Hara, H., Nakanishi, K., Kitami, M., Miura, N., Tabunoki, H., Watanabe, A., and Sato, R. (2005) *FEBS J.* **275**, 4913–4926

20. Rajamohan, F., Hussain, S. R., Cotrill, J. A., Gould, F., and Dean, D. H. (1996) *J. Biol. Chem.* **271**, 25220–25226

21. Smedley, D. P., and Ellar, D. J. (1996) *Microbiology* **142**, 1617–1624

22. Chen, J., Brown, M. R., Hua, G., and Adang, M. J. (2005) *Cell Tissue Res.* **321**, 123–129

23. Pardo-López, L., López, L., Gómez, I., Tabashnik, B. E., and Bravo, A. (2007) *Science* **318**, 1640–1642

24. Pacheco, S., Gómez, I., Gill, S. S., Bravo, A., and Soberón, M. (2009) *Peptides* **30**, 583–588

25. Whitmore, L., and Wallace, B. A. (2004) *Nucleic Acids Res.* **32**, W668–W673

26. Whitmore, L., and Wallace, B. A. (2008) *Biopolymers* **89**, 392–400

27. Wolfersberger, M., Lüthy, P., Maurer, A., Parenti, P., Sacchi, F. V., Giordana, B., and Hanozet, G. M. (1987) *Comp. Biochem. Physiol.* **86A**, 301–308

28. Sreerama, N., and Woody, R. W. (2000) *Anal. Biochem.* **287**, 252–260

29. Whitmore, L., and Wallace, B. A. (2004) *Nucleic Acids Res.* **32**, W668–W673

30. Wolfersberger, M., Lüthy, P., Maurer, A., Parenti, P., Sacchi, F. V., Giordana, B., and Hanozet, G. M. (1987) *Comp. Biochem. Physiol.* **86A**, 301–308

31. Sangadala, S., Azadi, P., Carlson, R., and Adang, M. J. (2001) *Insect. Biochem. Mol. Biol.* **32**, 97–107

32. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

33. Gahan, L. J., Gould, F., and Heckel, D. G. (2001) *Science* **293**, 857–860

34. Bel, Y., and Escriche, B. (2006) *Gene* **381**, 71–80

35. Gahan, L. J., Gould, F., and Heckel, D. G. (2001) *Science* **293**, 857–860

36. Flannagan, R. D., Yu, C. G., Mathis, J. P., Meyer, T. E., Shi, X., Siqueira, H. A., and Siegried, B. D. (2005) *Insect. Biochem. Mol. Biol.* **35**, 33–40

37. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

38. Flannagan, R. D., Yu, C. G., Mathis, J. P., Meyer, T. E., Shi, X., Siqueira, H. A., and Siegried, B. D. (2005) *Insect. Biochem. Mol. Biol.* **35**, 33–40

39. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

40. Flannagan, R. D., Yu, C. G., Mathis, J. P., Meyer, T. E., Shi, X., Siqueira, H. A., and Siegried, B. D. (2005) *Insect. Biochem. Mol. Biol.* **35**, 33–40

41. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

42. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

43. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

44. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307

45. Lawrence, A., Darszon, A., and Bravo, A. (1997) *FEBS Lett.* **414**, 303–307