

The C-terminal protoxin region of *Bacillus thuringiensis* Cry1Ab toxin has a functional role in binding to GPI-anchored receptors in the insect midgut

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Bacillus thuringiensis Cry toxins are used worldwide for controlling insects. Cry1Ab is produced as a 130-kDa protoxin that is activated by proteolytic removal of an inert 500 amino-acid-long C-terminal region, enabling the activated toxin to bind to insect midgut receptor proteins, leading to its membrane insertion and pore formation. It has been proposed that the C-terminal region is only involved in toxin crystallization, but its role in receptor binding is undefined. Here we show that the C-terminal region of Cry1Ab protoxin provides additional binding sites for alkaline phosphatase (ALP) and aminopeptidase N (APN) insect receptors. ELISA, ligand blot, surface plasmon resonance, and pulldown assays revealed that the Cry1Ab C-terminal region binds to both ALP and APN but not to cadherin. Thus, the C-terminal region provided a higher binding affinity of the protoxin to the gut membrane that correlated with higher toxicity of protoxin than activated toxin. Moreover, Cry1Ab domain II loop 2 or 3 mutations reduced binding of the protoxin to cadherin but not to ALP or APN, supporting the idea that protoxins have additional binding sites. These results imply that two different regions mediate the binding of Cry1Ab protoxin to membrane receptors, one located in domain II–III of the toxin and another in its C-terminal region, suggesting an active role of the C-terminal protoxin fragment in the mode of action of Cry toxins. These results suggest that future manipulations of the C-terminal protoxin region could alter the specificity and increase the toxicity of *B. thuringiensis* proteins.

Insecticidal proteins from the soil bacterium *Bacillus thuringiensis* (Bt)² are used extensively in transgenic plants and sprays to control insect pests (1, 2). These Bt proteins are espe-

cially valuable because they kill some of the world's most harmful pests but are not toxic to people and most other organisms (3–5). Cultivation of crops genetically engineered to produce Bt proteins increased to over 100 million hectares in 2017 (1). Although Bt proteins have provided substantial economic and environmental benefits (1, 2, 6–11), rapid evolution of pest resistance is reducing these advantages (12, 13).

A better understanding of the mode of action of Bt proteins is needed to improve and sustain their efficacy. Many studies have investigated the mode of action of the crystalline (Cry) Bt proteins in the Cry1A family, which kill caterpillar pests and are produced by widely adopted transgenic Bt corn, cotton, and soybeans (1, 2, 14, 15). To exert toxicity, Cry1A proteins bind to insect midgut receptors, such as glycosylphosphatidylinositol-anchored proteins like aminopeptidase N (APN), alkaline phosphatase (ALP) or to a transmembrane cadherin (CAD) to exert toxicity (14, 16). In particular, loops 2 and 3 of domain II of Cry1A toxins are important for binding to midgut receptors (14, 16). The different models of the Bt mode of action described so far include conversion of the full-length Cry1A protoxins by insect midgut proteases to yield activated toxins that bind to insect midgut receptors (14–17). This activation entails removal of ≈40 amino acids from the N terminus and more than 500 amino acids from the C terminus, converting the protoxins (≈130 kDa) into activated toxins (≈65 kDa) (14, 15).

The “classical model” of the Bt mode of action asserts that protoxins must be converted to activated toxins before receptor binding, toxin oligomerization, and pore formation. Thus, this model does not include any role the C-terminal fragment of the protoxin may have in the mode of action of Cry toxins (14, 15). Contrary to this paradigm, bioassays performed against at least 10 resistant strains selected with activated Cry1Ac toxin of four major lepidopteran pests showed that the Cry1Ac protoxin was still able to kill populations resistant to activated toxin, showing 5- to 50-fold higher potency than the activated toxin (18–20). These results with whole insects imply that the intact protoxin or some part of the protoxin other than the activated toxin contributes to toxicity.

In vitro experiments with different fragments of CAD receptor from *Pectinophora gossypiella* and *Manduca sexta* demonstrated that both the protoxin and activated toxin forms of Cry1Ac or Cry1Ab bind to the CAD receptor, specifically to

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This article contains Figs. S1–S3.

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² The abbreviations used are: Bt, *Bacillus thuringiensis*; Cry, crystalline; APN, aminopeptidase N; ALP, alkaline phosphatase; CAD, cadherin; LC₅₀, 50% lethal concentration; BBMV, brush border membrane vesicle; SPR, surface plasmon resonance; PMSF, phenylmethylsulfonyl fluoride; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; CNBr, cyanogen bromide.

C-terminal region of Cry1Ab protoxin binds to APN and ALP

CAD repeats 8–11 (CR8–11) from *P. gossypiella* CAD and to CR7–12 from *M. sexta* (21, 22). Furthermore, both forms promoted different post-binding events in the toxic pathway; two different pathways of oligomerization and pore formation have been described that are based on the interaction of protoxin or the activated toxin with the CAD receptor (18, 21). One oligomer is formed by protease activation of the protoxin after binding to CAD, and a different oligomer is formed by the activated toxin after binding to CAD (21). These oligomers have different sensitivities to temperature and differ in their open probability and conductance (21). In bioassays performed in the cell line CF203 from *Choristoneura fumiferana* showed that both the intact Cry1Ac protoxin without activation and the activated Cry1Ac toxin were toxic to these cells, but the cytological damage to treated cells differed between them (23). The aforementioned *in vitro* experiments performed in the cell line directly tested the effects of intact protoxins because they excluded the proteolytic activation that occurs in insect midguts.

The C-terminal protoxin region of Cry1A toxins has been proposed to be an inert region of the protein that is only involved in crystallization of Cry proteins during the sporulation phase of Bt, and no other role in the mechanism of action of Cry proteins has been attributed to this region (15, 24). However, this C-terminal protoxin region, which is removed during activation, is organized into distinct structural domains (24), where domains V and VII resemble carbohydrate-binding modules and are structurally similar to domains II and III of the activated toxin (24), which mediate binding to midgut receptors (15).

The objective of this work was to determine whether the C-terminal region of the protein has a role in Cry toxicity. Our hypothesis was that the C-terminal portion of the protoxin has an active role in Cry toxicity by binding to insect midgut receptors. Here we report the first tests of that hypothesis, performed with different binding assays. Also, we analyzed different single-point Cry1Ab mutants with alterations in domain II that were previously reported to be affected in binding interaction with Cry receptors but were analyzed only as activated toxins (25–31). Overall, our results indicate that the C-terminal fragment of Cry1Ab protoxin is directly involved in the protoxin mode of action by binding to Cry toxin receptors. This region of protoxin contributes to toxicity with additional binding sites to ALP and APN, whereas domain II contributes to CAD binding.

Results

Binding of the C-terminal fragment of Cry1Ab-protoxin to *M. sexta* brush border membrane vesicles and to Cry1Ab receptors

To determine whether the C-terminal portion of Cry1Ab protoxin binds to insect midgut receptors, the C-terminal region of Cry1Ab protoxin was cloned, expressed in *Escherichia coli* cells, and purified by affinity chromatography (Fig. 1). The Cry1Ab protoxin was obtained from solubilized purified crystal inclusions, and the Cry1Ab activated toxin was purified by anion exchange chromatography after protoxin activation with trypsin (Fig. S1). Fig. S2 shows the Western blot analysis of these samples using anti-Cry1Ab toxin or anti-C-terminal antibodies, showing that anti-Cry1Ab toxin antibody cross-reacts

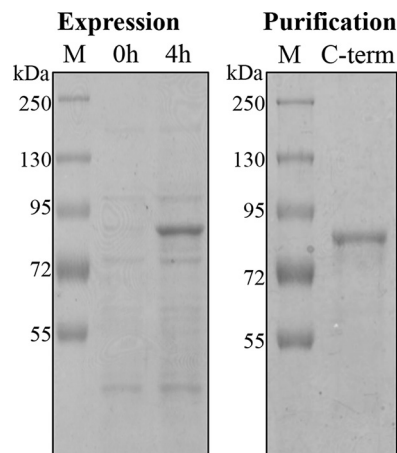


Figure 1. Expression and purification of the recombinant C-terminal region of the Cry1Ab protoxin in *E. coli* cells. The C-terminal (C-term) fragment was purified by affinity chromatography through a nickel affinity column by using the His tag provided in the pET22b cloning vector and eluted with 250 mM imidazole. The samples were analyzed by SDS-PAGE (10% acrylamide) stained with Coomassie Blue. M, molecular size marker; molecular masses are shown in kDa.

with activated toxin and protoxin, but not with the C-terminal fragment and the anti-C-terminal antibody recognized both protoxin and the C-terminal fragment, and did not recognize the activated toxin.

The toxicity of these samples was tested against *M. sexta* neonate larvae. The C-terminal fragment was not toxic to the larvae; no toxicity was observed with 5 $\mu\text{g}/\text{cm}^2$ of this protein. However, the protoxin showed an LC_{50} value of 3.4 ng/cm^2 (95% fiducial limits, 2.5–4.3), in contrast to the activated toxin, which showed an LC_{50} value of 10.3 ng/cm^2 (By 95% fiducial limits, 7.8–13), showing that the protoxin was 3-fold more toxic than the activated toxin.

The binding analysis of purified protoxin, activated toxin, or C-terminal fragment to BBMV from *M. sexta* showed that the C-terminal fragment also interacts with BBMVs with a high apparent binding affinity ($K_d = 25$ nM), similar to the apparent binding affinity of the protoxin ($K_d = 16$ nM) and the activated toxin ($K_d = 18$ nM) (Fig. 2). To determine whether the C-terminal fragment was able to bind to Cry protein receptors such as CAD, APN, or ALP, we performed ELISA binding assays using purified ALP, APN, and CAD (CR7–12 fragment) expressed in *E. coli* cells (Fig. S3). We used the CAD fragment CR7–12 (residues M810–A1485 of *M. sexta* CAD) because this fragment contains all three epitopes of CAD protein involved in Cry1Ab binding (32–34). Our data show that the C-terminal fragment bound to ALP and APN with high affinity (K_d values of 55 ± 9 nM and 24 ± 3 nM, respectively) (Fig. 3A) but showed extremely low absorbance values at 490 nm in the ELISA binding assay in the interaction with CAD (CR7–12). These data were confirmed by kinetic binding studies performed with the immobilized C-terminal fragment in surface plasmon resonance (SPR) assays, which showed that the C-terminal region was able to bind to APN and ALP with high affinity (K_d values of 185 ± 3 nM and 88 ± 5 nM, respectively) but not to CAD (CR7–12) (Fig. 3B). We also performed ligand blot assays of the protoxin, activated toxin, and C-terminal fragment to different concentrations of each receptor molecule (CR7–12, ALP, and APN).

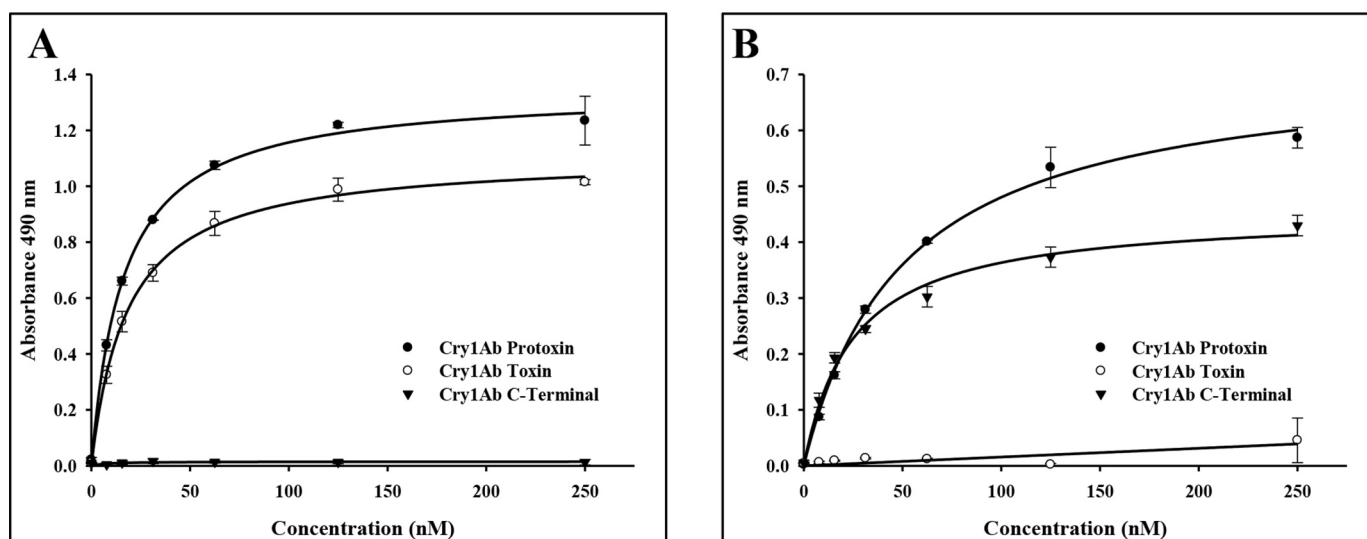


Figure 2. ELISA binding analysis of Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal fragment from Cry1Ab to BBMVs purified from *M. sexta* midgut tissue. A, the binding of these proteins revealed with anti-Cry1Ab antibody that was raised against the activated Cry1Ab toxin. This antibody does not cross-react with the C-terminal fragment (Fig. S1). B, the binding of these proteins revealed with anti-C-terminal antibody that was raised against the purified C-terminal fragment. This antibody does not cross-react with Cry1Ab activated toxin (Fig. S1). A total of 1 μ g of protein of BBMVs was bound to each of the wells of the ELISA plate. Each experiment was performed in duplicate with a total of three repetitions. Standard deviations are shown.

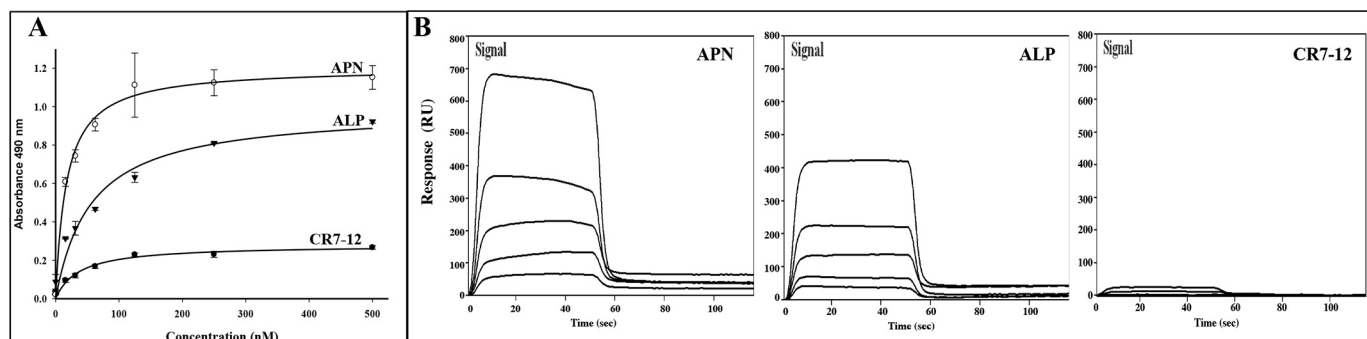


Figure 3. Binding analysis of the C-terminal fragment to CAD, APN, and ALP. A, ELISA binding assays of the C-terminal fragment of Cry1Ab to the purified recombinant CR7-12 fragment, ALP, or APN expressed in *E. coli* cells. Each experiment was performed in duplicate with a total of three repetitions. Standard deviations are shown. B, SPR binding analyses of the C-terminal fragment to Cry1Ab receptors, performed by immobilizing the C-terminal fragment by conventional amine coupling. Sensorgrams of serial doubling dilutions of each purified receptor (CAD (CR7-12), APN, and ALP) are shown. RU, response units.

These data confirmed that the C-terminal fragment did not bind to CAD (CR7-12) but was able to bind to ALP and APN (Fig. 4).

Finally, to demonstrate that the C-terminal fragment of Cry1Ab was able to interact with APN and ALP but not with the complete CAD receptor that is present in the insect BBMVs, pull-down assays with BBMVs proteins from *M. sexta* were performed. Fig. 5 shows that CAD was pulled down by the Cry1Ab protoxin and by the activated toxin but not by the C-terminal fragment, supporting that the C-terminal region of Cry1Ab was unable to bind to the CAD receptor. As expected, we found that APN was pulled down by the Cry1Ab protoxin, activated toxin, and C-terminal fragment (Fig. 5). However, ALP was pulled down strongly by the C-terminal fragment but not by protoxin and only weakly by activated toxin (Fig. 5).

Binding of WT and mutant Cry1Ab protoxins and activated toxins to recombinant CAD, ALP, and APN receptors

We compared the binding interaction of Cry1Ab protoxin and activated toxin with the recombinant purified receptors. Fig. 6A shows the results of ELISA binding assays of WT

Cry1Ab protoxin and activated toxin to CAD (CR12), where the apparent binding affinity was 2.4-fold higher for the activated toxin than the protoxin (Fig. 6A and Table 1). Conversely, for APN and ALP receptors, the apparent binding affinity was higher for protoxin than for activated toxin (11- and 3.7-fold higher for protoxin binding to ALP and APN, respectively, than the activated toxin) (Fig. 6, B and C, and Table 1).

We worked with some Cry1Ab mutant proteins (Cry1Ab-RR368-369AA, Cry1Ab-F371A, and Cry1Ab-G439D) that are located in domain II of Cry1Ab toxin and were characterized previously, showing reduced binding to BBMVs and lower toxicity to *M. sexta* (25-31). It is important to mention that the binding data of these mutants to BBMVs or to purified receptors that were reported before were performed only with activated toxin. Here we compared the binding of the protoxin and the activated toxin from these mutants to purified APN, ALP, and CAD (CR12) receptors. Cry1Ab-RR368-369AA and Cry1Ab-F371A have mutations in domain II loop 2 (16, 25-28). Previous work showed that the activated Cry1Ab-RR368-369AA mutant toxin had reduced binding to APN in SPR

C-terminal region of Cry1Ab protoxin binds to APN and ALP

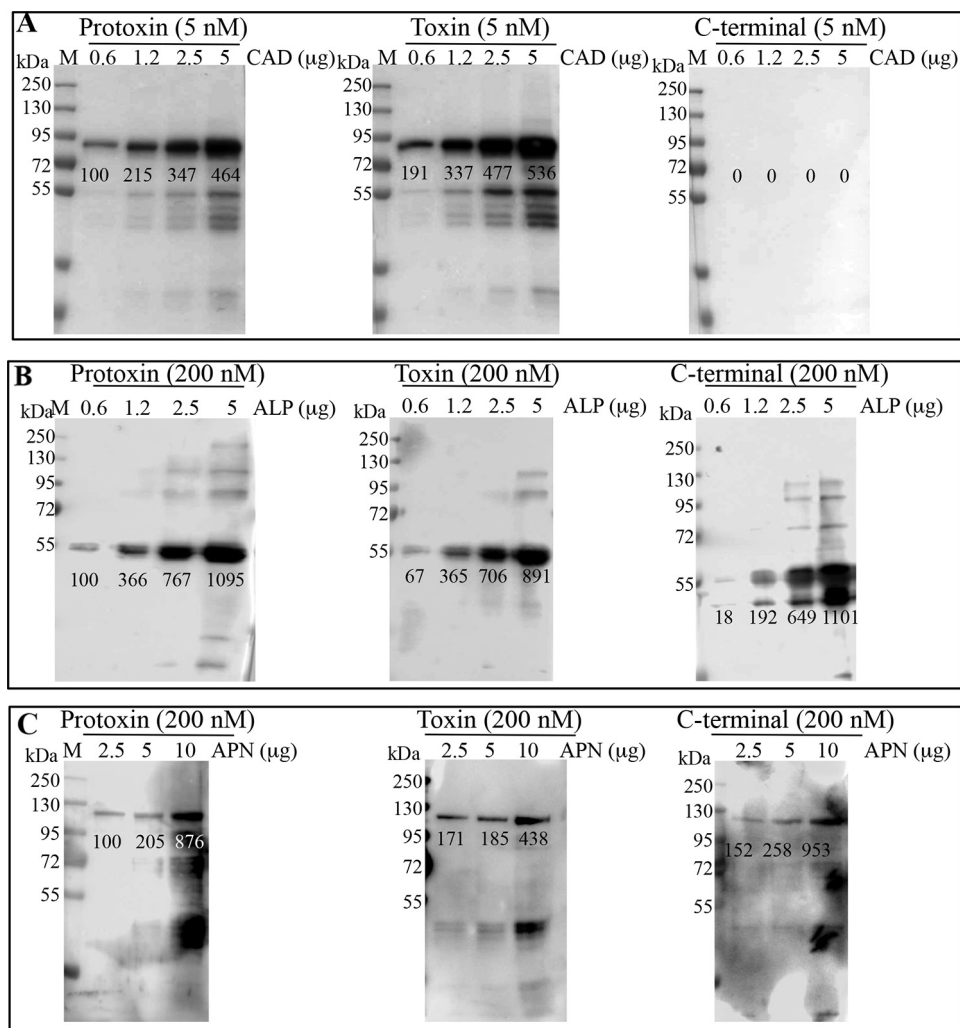


Figure 4. Ligand blot assays of Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal fragment from Cry1Ab to the purified recombinant CAD (CR7-12) fragment, ALP, or APN proteins expressed in *E. coli* cells. Shown are serial dilutions of the recombinant receptor proteins that were loaded in SDS-PAGE and transferred to PVDF membranes. These blots were used for ligand blot binding assays. The numbers under the bands represent the percentage of each band on the blot, calculated after densitometry analysis of the bands using ImageJ software and selecting one band of the protoxin bound to the lower concentration of receptor as 100% reference. Molecular masses are indicated. *A*, ligand blot assays of 5 nM Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal fragment from Cry1Ab to CAD (CR7-12). *B*, ligand blot assays of 200 nM Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal fragment from Cry1Ab to ALP. *C*, ligand blot assays of 200 nM Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal fragment from Cry1Ab to APN. *M*, molecular size marker; molecular masses are shown in kDa.

assays, and the activated form of Cry1Ab-F371A mutant toxin showed reduced binding to *M. sexta* BBMVs (16, 25–28). Previous work showed that the activated form of Cry1Ab-G439D mutant toxin with a mutation in domain II loop 3 showed reduced binding with the CAD (CR12) fragment (29–31). Fig. S1 shows the purified protoxin and the activated toxin molecules from the different mutant proteins. We used the CAD fragment CR12 (residues G1370-A1485 of *M. sexta* CAD) because it is an important Cry1Ab-binding region of CAD (33–35), and the binding phenotype of the Cry1Ab mutants has been reported previously with the CAD binding site CR12 (30). For the three Cry1Ab mutants, the apparent binding affinity of protoxins and activated toxins to CAD was reduced more than 11-fold relative to the WT Cry1Ab, and a final K_d value could not be determined (Table 1 and Fig. 6A). These results imply that both of these domain II exposed loops participate in the binding of protoxin and activated toxin to CAD. In contrast, the apparent binding affinity to ALP and APN was similar for

the protoxins of these three mutants relative to the WT Cry1Ab protoxin (Table 1 and Fig. 6, B and C). The binding analysis of the activated toxin form of these mutants showed that Cry1Ab-F371A did not reduce binding to ALP or APN; Cry1Ab-RR368–369AA reduced binding 1.3-fold to ALP and 1.9-fold to APN, and Cry1Ab-G439D reduced binding 2.7-fold to ALP and 2.9-fold to APN (Table 1, Fig. 6, B and C). These results suggest that loops 2 and 3 of domain II, particularly loop 3, contribute to binding of Cry1Ab activated toxin to ALP and APN. The new data showed that these mutations were not affected in the binding interaction of their protoxin molecules to ALP and APN, supporting that additional regions present in the protoxin are involved in ALP and APN binding.

Discussion

We discovered that ALP and APN from *M. sexta* interact with high affinity with the C-terminal region of Cry1Ab protoxin (Figs. 3–5). The C-terminal fragment was produced in

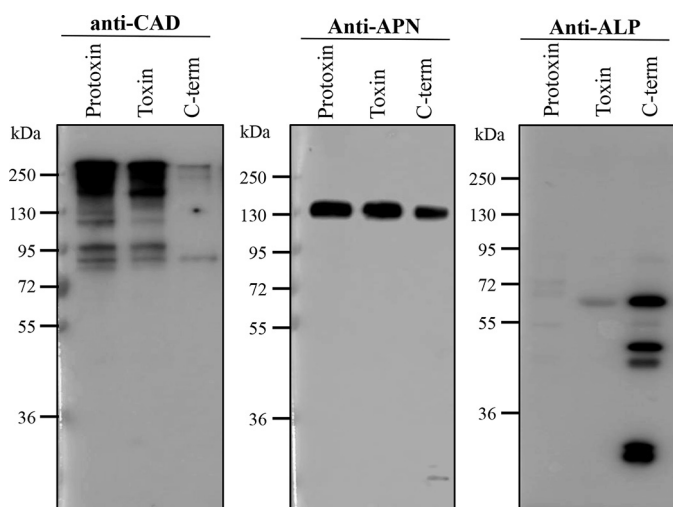


Figure 5. Pull-down assays of BBMV proteins using Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal fragment from Cry1Ab bound to CNBr-agarose. The precipitated BBMV proteins with Cry1Ab protoxin, Cry1Ab activated toxin, and the C-terminal (C-term) fragment from Cry1Ab were revealed with specific antibodies that recognize CAD, APN, or ALP proteins. Molecular masses are indicated.

E. coli because, after treatment of protoxins with trypsin, the C-terminal fragment is cleaved further, making it impossible to purify the C-terminal fragment after protoxin activation with trypsin (36).

The binding affinity of Cry1Ab protoxin to both receptors was higher than activated toxin (Table 1), indicating that C-terminal fragment has additional binding sites for these receptors. These results do not support the notion that the C-terminal region of protoxins is solely involved in crystal formation and has no role in the toxicity of Cry proteins (14, 15, 35, 37). Conversely, they are consistent with the proposition that the C-terminal regions of protoxins contribute to the binding and toxicity of Cry proteins (18, 21). The idea that protoxins participate in toxicity by binding to Cry toxin receptors is also supported by the *in vitro* binding experiments of Cry1Ab and Cry1Ac protoxin, performed with CAD protein from *P. gossypiella* and *M. sexta* larvae (21, 22), with bioassays performed in the cell line CF203 and 10 resistant strains of *Diatraea saccharalis*, *Helicoverpa armigera*, *Helicoverpa zea*, and *Ostrinia nubilalis*, against which protoxin was more potent than activated toxin (18–20).

The new results revealing binding of the C-terminal region of Cry1Ab protoxin to ALP and APN provide evidence of the mechanism by which protoxin could exert toxicity by binding to receptors, leading to toxin oligomerization and pore formation. For protoxins to exert toxicity, it is required that the full protoxin reaches the CAD receptor located in brush border membranes before activation by midgut proteases. Thus, high-affinity binding sites of protoxins to the abundant glycosylphosphatidylinositol-anchored proteins could provide means for the binding of protoxin to brush border membranes before its activation. Fig. 7 shows a model of the mechanism of action of Cry1A protoxin, showing that interaction with APN and ALP helps the protoxin to reach the CAD receptor before activation by midgut proteases. Interaction of protoxin with CAD by means of domain II exposed loops, in the presence of midgut

proteases, would induce the formation of a robust oligomer that displays pore formation activity with a single conductance and high open probability, as demonstrated previously (21).

The new results presented here show that mutations Cry1Ab-RR368-369AA in loop 2 and Cry1Ab-G439D in loop 3 of domain II reduced binding to ALP and APN of the activated toxin but not of the protoxin, indicating differences in binding sites to ALP and APN between Cry1Ab protoxin and activated toxin. The specific sites of protoxin involved in interaction with ALP and APN remain to be elucidated. In particular, it will be useful to test the hypothesis that binding occurs via domains V and VII, which resemble carbohydrate-binding modules and are structurally similar to domains II and III of the activated toxin (24). It also remains to be analyzed whether the C-terminal region binds to the ABC transporter proteins that are important proteins implicated in the toxicity of Cry1A toxins (38–40). Recent data have shown that domain II loops are the regions of Cry1A activated toxins involved in the interaction with the ABCC2 transporter (41).

In contrast to the results with ALP and APN, binding to CAD (CR12) by protoxin and activated toxin was greatly reduced for all three of the Cry1Ab mutants tested (Cry1Ab-RR368–369AA and Cry1Ab-F371A in loop 2 and Cry1Ab-G439D in loop 3). These findings suggest that loops 2 and 3 of domain II are important for binding of both Cry1Ab protoxin and activated toxin to CAD in *M. sexta*. Thus, similar sites of domain II of both molecules participate in CAD interaction.

In other pore-forming toxins, it has been reported that they bind to their receptors as protoxins and that the propeptides, when they are proteolyzed, may display additional functions, such as chaperones that help to keep the protein in question soluble under certain conditions and assist with its secretion or its oligomerization (42). For example, the C-terminal propeptide of aerolysin from *Aeromonas hydrophila* assists in oligomerization and pore formation (43). Also, the *Clostridium septicum* pore-forming α -toxin is secreted as a protoxin, and its propeptide function as a chaperone (44). Here we found that the C-terminal region of Cry1Ab protoxin binds ALP and APN. An additional important implication of this finding is that this region may influence specificity and toxicity. If so, then, similar to achievements observed previously with activated toxins, where it has been shown that domain swapping and site-directed mutagenesis as well as evolution of toxins resulted in improved toxins (45–47), the engineering of the C-terminal region could potentially generate novel Cry proteins that could be more potent against specific target insects or may help to counter insect resistance.

Materials and methods

Purification of Cry1Ab WT and mutant proteins

The Bt 407 strain (48) transformed with pHT315-*cry1Ab* (49), pHT315-*cry1AbRR368–369AA*, pHT315-*cry1AbF371A*, or pHT315-*cry1AbG439D* (16, 30) plasmids was grown for 3 days at 30 °C until complete sporulation in SP medium (0.8% nutrient broth, 1 mM MgSO₄·7H₂O, 13 mM KCl, and 10 mM MnCl₂·4H₂O (pH 7.0) supplemented with 2 ml/liter of sterile solution of 131 mM FeSO₄·7H₂O in 1N H₂SO₄ and 1 ml/liter of

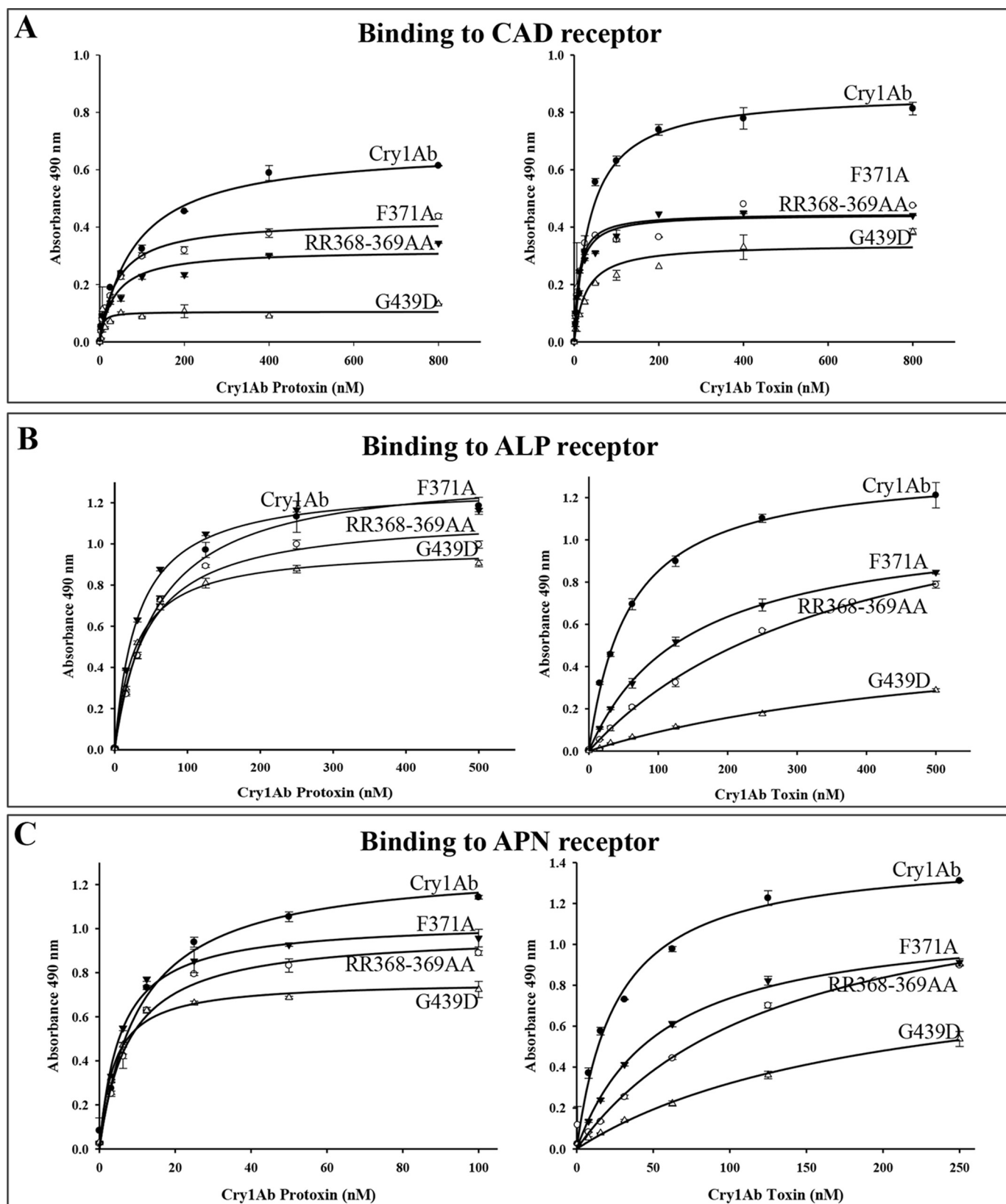


Figure 6. ELISA binding assays of Cry1Ab and Cry1Ab-mutant proteins to the purified recombinant CAD (CR12), ALP, or APN receptors expressed in *E. coli* cells. The three Cry1Ab mutants (Cry1Ab-RR368-369AA, Cry1Ab-F371A, and Cry1Ab-G439D) were affected in toxicity against *M. sexta* larvae. A, binding of protoxin or activated toxin molecules to the purified CAD (CR12) receptor fragment. B, binding of protoxin or activated toxin molecules to purified ALP. C, binding of protoxin or activated toxin molecules to purified APN. Each experiment was performed in duplicate with a total of three repetitions. Standard deviations are shown.

Table 1

Equilibrium dissociation constants (nanomolar) for interactions between wildtype and mutant Cry1Ab protoxins and activated toxins with recombinant CR12, ALP, and APN

NB, no binding.

Type of Cry1Ab	CR12		ALP		APN	
	Protoxin	Toxin	Protoxin	Toxin	Protoxin	Toxin
WT	85 ± 14	35 ± 4	28 ± 2	296 ± 3	79 ± 8	296 ± 5
RR368–9AA	NB	NB	22 ± 3	381 ± 36	55 ± 6	572 ± 53
F371A	NB	NB	16 ± 2	242 ± 48	40 ± 5	214 ± 8
G439D	NB	NB	14 ± 2	806 ± 88	30 ± 4	870 ± 32

sterile 0.5 M CaCl₂) supplemented with erythromycin at 10 µg/ml. Spores/crystals were washed three times in 300 mM NaCl and 10 mM EDTA and then three times with 1 mM phenylmethylsulfonyl fluoride (PMSF) and stored at 4 °C. The crystal inclusions were purified by discontinuous sucrose gradients as described previously (50). Protoxins were solubilized in alkaline buffer (50 mM Na₂CO₃, 0.02% β-mercaptoethanol (pH 10.5)) for 1 h at 37 °C and centrifuged for 20 min at 12,857 × g. For activation, the pH level of the protoxin solution was lowered to 8.5 by adding 1:4 (w/w) of 1 M Tris buffer (pH 8), and 1:50 trypsin (trypsin:toxin) (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin from bovine pancreas, Sigma-Aldrich) was added for 1 h at 37 °C; after this incubation, PMSF (1 mM final concentration) was added. The trypsin-activated toxins were loaded into a HiTrap Q HP column connected to an FPLC system (ÄKTA, GE Healthcare Life Sciences), washed with buffer A (50 mM NaCl and 50 mM Tris buffer (pH 8)), and eluted with a 0–100% gradient of buffer B (1 M NaCl and 50 mM Tris buffer (pH 8.5)). The protein concentration was determined by using the Bradford assay (Bio-Rad) with BSA as the standard. The quality of the samples was analyzed by SDS-PAGE (10% acrylamide) stained with Coomassie Blue and by Western blotting using a specific anti-Cry1Ab antibody (Fig. S1).

Cloning of the C-terminal fragment

The C-terminal fragment was amplified with specific primers: ForC-ter, 5'-TAT CTG GGA TCC TCG AAT TGA ATT TGT TCC GGC AG-3'; RevC-ter, 5'-GAG CTC GAA TTC AAT TCC TCC ATA AGA AGT AAT TCC-3'. The PCR product was digested with BamHI and EcoRI and cloned into pET-22b (Novagen, Madison, WI). Plasmids were DNA-sequenced in the facilities of the Instituto de Biotecnología, Universidad Nacional Autónoma de México.

Toxicity assays against *M. sexta* larvae

Bioassays were performed with neonate larvae using five concentrations of Cry1Ab protoxin, activated toxin, or C-terminal fragment solutions that were poured onto the surface of the diet. We used 24-well polystyrene plates and one plate per dose in triplicate. Mortality was analyzed after 7 days, and the 50% lethal concentration (LC₅₀) was calculated with Probit LeOra software. Negative controls without protein addition were included.

Preparation of BBMVs from *M. sexta* larvae

The *M. sexta* colony was maintained on an artificial diet under laboratory conditions at 28 ± 2 °C and 65% ± 5% relative

humidity under a 12:12 (light-dark) photoperiod. The midgut tissue was dissected from third-instar larvae. The BBMVs were prepared as described previously (51) and stored at –70 °C. The BBMV protein concentrations were determined with the Lowry DC protein assay (Bio-Rad) using BSA as the standard. The enrichment of APN (5-fold) and ALP (4-fold) activities in the BBMVs in relation to the homogenate was analyzed as reported previously (16).

Expression and purification of recombinant Cry receptors and the C-terminal fragment

CAD (CR12 and CR7–12), ALP, and APN from *M. sexta* larvae were cloned in pET-22b and expressed in *E. coli* cells (52–54). The CAD fragments CR12 (G1370-A1485) and CR7–12 (M810-A1485) were expressed in *E. coli* ER2566. APN, ALP, and the C-terminal fragment were expressed in *E. coli* BL21 (DE3) (Invitrogen). Expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, and inclusion bodies were solubilized with 8 M urea as reported previously (52–54). The recombinant proteins were purified through a nickel affinity column that bound the recombinant proteins through the His tag peptide added in the pET-22b cloning vector. Proteins were eluted with 250 mM imidazole in PBS as described previously (29, 52–54). The quality of the samples was analyzed by SDS-PAGE (10% acrylamide) stained with Coomassie Blue and by Western blotting using a specific anti-C terminus antibody (Figs. S1 and S2).

ELISA binding assays

ELISA plates were coated with 0.5 µg of *M. sexta* CAD (CR12 or CR7–12), APN, or ALP in 100 µl of PBS/well overnight at 4 °C or with 1 µg of BBMVs. Plates were washed three times with PBS, blocked with 200 µl/well of PBS-M (PBS and 2% skim milk) for 2 h at 37 °C and washed three times with PBS. Different concentrations of protoxin or activated toxins of WT or mutants or the C-terminal fragment from Cry1Ab were added to a total 100-µl volume of PBST (PBS and 0.1% Tween 20) for 1 h at 37 °C. The unbound proteins were removed by three washes with PBST and three washes with PBS. The bound proteins were detected using 100 µl of PBST buffer containing anti-Cry1Ab (1:20,000) polyclonal antibody for 1 h at 37 °C or anti-C-terminus (1:30,000) polyclonal antibody. After three washes with PBST and three washes with PBS, we added 100 µl of PBST containing anti-rabbit HRP-conjugated antibody (1:20,000) (Santa Cruz Biotechnology) for 1 h at 37 °C. Finally, three washes with PBST were done, and proteins were incubated with 100 µl/well of substrate mixture (2 mM O-phenylenediamine (Sigma) and 0.05% H₂O₂ in 0.1 M phosphate buffer (pH 5.0)). The reaction was stopped with 60 µl of 5 M HCl and measured at A_{490 nm} using an ELISA plate reader. Each experiment was performed in duplicate with three repetitions. Data were analyzed with the SigmaPlot program (version 12.0) and adjusted with Scatchard plot analysis.

The anti-C-terminus polyclonal antibody was raised in a New Zealand White rabbits after subcutaneous immunization with purified C-terminal fragment. The rabbit was boosted three times with 1 mg of the C-terminal fragment mixed with incomplete Freund's adjuvant at 15-day intervals. Blood serum

C-terminal region of Cry1Ab protoxin binds to APN and ALP

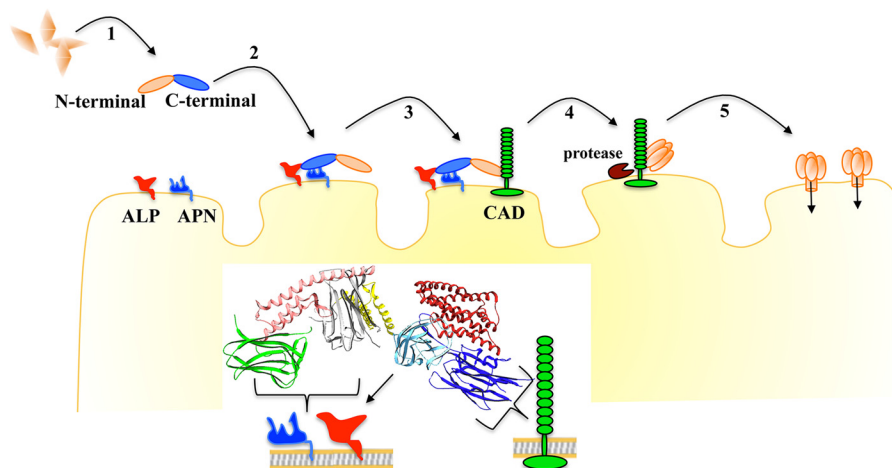


Figure 7. Proposed mechanism of action of Cry protoxins from Bt. 1, the parasporal crystals are solubilized into protoxin. 2, the C-terminal region of protoxin binds with highly abundant APN and ALP receptors. 3, the protoxin binds to the CAD receptor by the loops of domain II. 4, proteases activate the protoxin, inducing oligomer formation. 5, the oligomer inserts into the membrane, forming a pore that kills the cell. The interaction with APN and ALP with the C-terminal region helps the protoxin to reach the CAD receptor before proteolytic activation, and the interaction of protoxin with CAD in the presence of midgut proteases induces the formation of a robust oligomer that displays high pore formation activity with single conductance and a high open probability, as demonstrated previously (18).

was obtained. Specificity and sensitivity were determined in a ligand blot assays of the C-terminal fragment spotted on nitrocellulose strips and analyzed with different concentrations of the polyclonal anti-C-terminus antibody (from 1:10,000 to 1:50,000 dilutions) and the secondary goat anti-rabbit HRP antibody (1:30,000 dilution). All procedures involving animals were conducted according to the ethical guidelines of the Instituto de Biotecnología, Universidad Nacional Autónoma de México.

Biosensor (SPR) analysis of C-terminal binding to Cry1Ab-receptors

The SENSi-Q instrument was used to perform SPR measurements. Running buffer (HBS buffer (pH 7.4) containing 0.1 M HEPES, 1.5 M NaCl, and 0.005% (v/v) Tween 20) was freshly prepared, filtered (pore size of 0.22 μm), and degassed. The C-terminal fragment of Cry1Ab was immobilized onto a COOH functionalized sensor chip (ICX Nomadics) by conventional amine coupling at densities of less than 1500 response units (RU). Then 1 M ethanolamine at a flow rate of 10 $\mu\text{l}/\text{min}$ for 5 min was injected to block flow cells. The analytes (CAD (CR7–12), APN, or ALP) were injected at a flow rate of 25 $\mu\text{l}/\text{min}$. Serial doubling dilutions of Cry1Ab receptors were analyzed, and the surface was regenerated with a 1-min injection of 20 mM NaOH. Injections were performed three times for each receptor protein concentration. The data were analyzed using SensiQ Software Qdat version B.02. This software employs nonlinear regression and the Levenberg–Marquardt algorithm to fit experimental data to a binding interaction model that defines the interaction.

Ligand blot assays

Serial dilutions of the recombinant CAD (CR7–12), ALP (5, 2.5, 1.25, and 0.625 μg), and APN (10, 5, and 2.5 μg) proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was blocked with PBS supplemented with 5% skimmed milk, and blots were incubated for

1 h at room temperature with Cry1Ab protoxin, activated toxin, or the C-terminal fragment. We used 5 nM protein in washing buffer (0.1% Tween 20 in PBS) for the ligand blot analysis with CR7–12 and 200 nM protein for the ligand blot analysis with ALP and APN. Unbound proteins were removed by washing three times in washing buffer for 15 min. Bound toxin and protoxin were identified with anti-Cry1Ab polyclonal antibody (1:20,000 dilution) and bound C-terminal fragment with anti-C-terminus polyclonal antibody (1:20,000 dilution) for 1 h at room temperature. After washing, the membrane was incubated with the secondary goat anti-rabbit HRP antibody (1:20,000 dilution) for 1 h at room temperature. After washing the excess of unbound secondary antibody, proteins were visualized with Super Signal chemiluminescence substrate (Pierce).

Pull-down assays

BBMVs from *M. sexta* were solubilized for 2 h at 4 °C in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1 mM PMSF containing 1% CHAPS (v/v). Undissolved material was removed by centrifugation at 100,000 $\times g$ for 1 h at 4 °C. We used 100 μg of purified Cry1Ab activated toxin, protoxin, or C-terminus that was incubated with 200 μl of CNBr-agarose (GE Healthcare) in 0.1 M sodium phosphate buffer (pH 7.5) at 4 °C overnight. The noncoupled proteins were removed by centrifugation. The unreacted CNBr groups were blocked with 0.1 M Tris-HCl (pH 8) at room temperature for 2 h. The resin was washed five times with 500 μl of PBS. The CNBr beads coupled to protoxin, activated toxin, or the C-terminal fragment were incubated with 200 μg of solubilized BBMV proteins in 500 μl for 1 h at 4 °C; the unbound BBMV proteins were removed by centrifugation at 18,400 $\times g$ for 10 min at 4 °C. The coupled CNBr-agarose beads containing the bound proteins from BBMV were washed five times with 500 μl of PBS supplemented with 1 M NaCl, followed by five washes with 500 μl of PBS to remove unbound proteins. The proteins that remained bound to the CNBr protoxin-, activated toxin-, or C-terminal-agarose beads were dissociated after 5 min at 100 °C in 50 μl of loading buffer (100 mM

TrisCl, 200 mM DTT, 4% SDS (w/v), 0.2% bromphenol blue (w/v), and 20% glycerol (v/v) (pH 6.8)). As a negative control, the activated CNBr-agarose was incubated without Cry1Ab protein, blocked as described above, and incubated with solubilized BBMV. The pulled down proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes that were used in Western blotting assays using anti-CAD, anti-ALP, and anti-APN polyclonal antibodies as described below.

Western blotting assays

The anti-Cry1Ab, anti-CAD, anti-ALP, and anti-APN polyclonal antibodies were raised previously in our laboratory. The anti-C-terminal antibody was raised for this work as reported above. For Western blot assays, the PVDF membranes were blocked with 5% skimmed milk in PBS buffer (pH 7.4) plus 0.1% Tween 20 for 1 h at room temperature. The membranes were rinsed once with the same buffer. The different proteins were detected after 1-h incubation with the corresponding polyclonal antibody (anti-CAD 1:20,000, anti-ALP and anti-APN 1:10,000, anti-Cry1Ab 1:20,000, and anti-C-terminus 1:20,000 dilutions) and for 1 h with goat anti-rabbit HRP secondary antibody (Santa Cruz Biotechnology) (1:20,000 dilution) and finally visualized by incubation with Super Signal chemiluminescence substrate (Pierce) according to the manufacturer's instructions.

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