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Selective inhibition of binding of *Bacillus thuringiensis* Cry1Ab toxin to cadherin-like and aminopeptidase proteins in brush border membranes and dissociated epithelial cells from *Bombyx mori*

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Short title: Selective Cry1Ab binding inhibition

Abreviations: Bt, *Bacillus thuringiensis*; APN, aminopeptidase N; BBMV, brush border membrane vesicles; SD, standard deviation; SEM, standard error of the mean.

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SYNOPSIS

Binding analyses with denatured epithelial membrane proteins from *Bacillus thuringiensis* (Bt) evidenced at least two kinds of proteins, aminopeptidases N (APNs) and cadherin-like proteins, as possible receptors for the Cry1A class of Bt toxins. Two alternative models have been proposed, both based on initial toxin binding to a cadherin-like protein, but one involving APN and the other not. We have used two *Bombyx mori* strains (J65 and Kin), which are highly susceptible to Cry1Ab, to study the role of these two types of receptors on Cry1Ab toxin binding and cytotoxicity by means of the inhibitory effect of antibodies. Brush border membrane vesicles (BBMV) of strain J65 incubated with labeled ^{125}I-Cry1Ab revealed a marked reduction in reversible and irreversible binding when anti-BtR175 (a cadherin-like protein) was used for BBMV pretreatment. By contrast, the anti-APN1 antibody specifically affected the irreversible binding, while the reversible binding component was not affected. This is the first time that binding of Cry1Ab to APN1 and to a cadherin-like protein from BBMV in solution has been shown. Dissociated epithelial cells from the Kin strain were used to test the inhibitory effect of the antibodies on the cytotoxicity of Cry1Ab. Preincubation of the cells with the anti-BtR175 antibody conferred protection against Cry1Ab, but not the anti-APN1 antibody. Therefore, our results seem to support the two models of the mode of action of Cry1Ab in Lepidoptera, depending on whether BBMV or intact dissociated cells are used, suggesting that both pathways may co-operate for the toxicity of Cry1A toxins *in vivo*.

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Keywords: Cry1Ab susceptibility, mode of action, toxin receptor binding, APN, reversible binding, irreversible binding.

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INTRODUCTION

Bacillus thuringiensis (Bt) strains produce specific, highly toxic proteins at the moment of sporulation [1]. These proteins accumulate in parasporal aggregations called crystals. The component proteins are called Bt toxins, crystal proteins, Cry proteins, or Cry toxins. Hundreds of such proteins [2] have been identified with activities toward different insects. In spite of the many studies with crystal proteins from the Cry1A subclass (Cry1Aa, Cry1Ab, and Cry1Ac), the detailed mode of action of these insecticidal toxins remains still unclear [3]. Basically, the larva ingests the toxin in a compact crystalline form (protoxin). The toxin is solubilized in the gut and activated by midgut proteases. The active fragment specifically binds to membrane proteins of the brush border of midgut cells eventually causing cell lysis and the death of the insect. There is evidence that toxin binding involves a two-step interaction with the epithelial membrane, with a reversible component and an irreversible one [4-6].

Binding of the toxin to specific midgut receptors is considered a key step in the toxicity of Cry1 type toxins [9]. In fact, a marked reduction in toxin binding to brush border membrane vesicles (BBMV) in solution is considered the most common basis of resistance to these toxins. Binding techniques using denatured proteins, such as ligand blots, have revealed at least two groups of proteins as possible functional Cry1A toxin receptors in different lepidopterans: aminopeptidases (EC 3.4.11.2) (APNs) and a cadherin-like protein. Depending on the insect species, a different set of APN proteins has been proposed to function as Cry1A receptors, such as in the case of *Manduca sexta* [10-12] or of *Bombyx mori* [13-15]. However, only one kind of cadherin-like protein has been described as a putative Cry1A toxin receptor in all insect species [16, 17]. Numerous experiments have shown the possible involvement of both kinds of proteins in the toxicity of Cry1A toxins, such as the work of Gill and Ellar [18], in which the 120-kDa APN of *M. sexta* was expressed transgenically in *Drosophila melanogaster*, or that of Tsuda *et al.* [19] who transfected mammalian cells with the *BtR175* gene of *B. mori*. These studies showed that Cry1A-tolerant insects and mammalian cells, respectively, became susceptible to Cry1A toxins after transgenesis.

Despite the many binding studies using denaturatured APNs and cadherin-like proteins, very few have been done on the involvement of these proteins in Cry1A binding while still associated with their native membranes. It is worth noting the inconsistencies found when

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comparing binding assays using native membranes and ligand blots [9, 20]. Indeed, it is still unclear the role that the above proteins play in toxin-binding and especially on the events that occur following binding. One model proposes that the Cry1Ab toxin first binds to the cadherin-like protein and, after further proteolytic activation and oligomerization, it is transferred to an APN receptor which facilitates the insertion into the membrane with the concomitant formation of pores, finally leading to cell death [7]. An alternative model has been recently proposed in which, after Cry1Ab binding to the cadherin-like receptor, a protein kinase A-dependent pathway is activated leading to the cell death [8]. In this model, binding to APN and pore formation is irrelevant or of marginal relevance.

To shed light into the role of lepidopteran APNs and the cadherin-like protein in Cry1Ab binding we used a model lepidopteran species, *Bombyx mori*. Because of the low toxicity of this toxin to most strains of this insect species (http://www.glfc.scf.rncan.gc.ca/bacillus), we chose two strains (J65 and Kin) which are highly susceptible to Cry1Ab [21]. Highly specific antibodies against APN1, APN3, and the cadherin-like protein (BtR175) were used to try to inhibit binding of Cry1Ab to the above candidate receptor proteins and thus determine their role in the whole binding process.

EXPERIMENTAL

Preparation of *B. thuringiensis* toxins

Cry1Aa and Cry1Ab protoxins used for larval toxicity bioassays were prepared as following. Cry1Aa protoxin was prepared from a culture of *B. thuringiensis* serovar *sotto* PAS. Bacteria were cultured on nutrient agar at 25ºC for 2 days. The crystalline inclusions consisted of Cry1Aa2 protoxin [22]. Cry1Ab protoxin was prepared from a culture of recombinant *Escherichia coli* expressing the *cry1Ab* gene [24]. The inclusions that contained Cry1Ab protoxin were partially purified using the method described by Lee *et al.* [4]. Inclusions of Cry1Aa and Cry1Ab were purified using NaBr density gradient centrifugation [24]. Purified inclusions were suspended in sterile distilled water and stored at 4ºC until use. The concentration was estimated using the Lowry assay [25].

Cry1Ab toxin for the *ex-vivo* midgut toxicity assay was expressed from recombinant *E. coli* expressing the *cry1Ab8* gene from *B. thuringiensis* serovar *aizawai* IPL7 strain, which was

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provided by Sumitomo Chemical. *E. coli* was grown at 37ºC in 250 ml of LB medium (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, supplemented with 50 µg/ml ampicillin and 25 mM IPTG) for 16 h and centrifuged. The Cry1Ab protoxin was expressed as an insoluble protein. After incubation in 10 ml of lysis buffer (15 mM Tris-HCl, pH 8.0, 8 mM EDTA, 0.4 mg/ml lysozyme), bacteria were disrupted by sonication, and inclusion bodies were separated from cell debris by repeating the centrifugation and washing with 5% Triton X-100 in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 145 mM NaCl). The inclusion bodies were solubilized and trypsinized, and then the activated toxins were purified using DEAE-cellulose, as described elsewhere [13]. The Bradford method [26] was used to determine Cry1Ab toxin concentration.

Cry1Ab for the binding analysis was prepared according to Sayyed *et al.* [27]. Cry1Ab protoxin was expressed as inclusion bodies in *E. coli* (kindly supplied by Ruud A. de Maagd, Plant Research International, Wageningen, The Netherlands). Protoxin was solubilized and trypsin-activated. It was purified with a MonoQ HR 5/5 anion exchange column (Fast Protein Liquid Chromatography system; Amersham, Uppsala, Sweden). The protein concentration was determined by densitometry after SDS-PAGE.

BSA was used as the standard in all protein quantification methods.

Preparation of brush border membrane vesicles (BBMV)

The Japanese strain No. 65 (J65) of *B. mori* was obtained from the National Institute of Agrobiological Sciences Gene Bank (Tsukuba, Japan). Insects were reared on artificial diet (Silkmate, Nihon-Nosanko, Japan) at 25ºC. The midguts of 5th instar larvae were lyophilized following Hernandez *et al.* [28] and sent to Valencia (Spain), where BBMV were prepared according to the method of Mg^{++} precipitation described by Sayyed *et al.* [29]. The protein concentration was determined using the Bradford method [26] with BSA as the standard. Two batches of BBMV were used for all the binding experiments, one for each replicate.

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Preparation of antibodies and antiserum

Purified antibodies against GST or fusion proteins of GST with BtR175 and APN1, and antiserum specific to APN3, were prepared by immunizing mice following previous reports [15, 30]. For immunoblotting, goat anti-mouse IgG-HRP conjugate (Bio-Rad) was used as the secondary antibody.

Toxicity bioassays

For the larval toxicity assay, suspensions of protoxin inclusions were diluted with sterile distilled water. A four-step serial dilution was made for each protoxin preparation. Four cm^2 of mulberry leaf were smeared with 160 µl of diluted protoxin solution and air dried before use. The air-dried leaf was placed in a plastic Petri dish (90 mm in diameter), which was covered with filter paper (Advantest No.2 filter paper) 70 mm in diameter dampened with a 0.5 ml drop of distilled water to keep the moisture. We used 21-30 newly hatched larvae of strain J65 per piece of leaf. At the end of the second day, the larvae were fed non-treated fresh leaves. Mortality was scored at approximately 48-h intervals for 4 days. The bioassay was performed at 25ºC. Mortality data were analyzed using SPSS v. 7.5 software, and the median lethal concentrations (LC_{50}) of the two protoxins were estimated.

For the *ex vivo* midgut toxicity assay, midguts were dissected from second instar larvae of *B. mori* strain J65, inverted inside out, cut into 2-mm blocks, and incubated at 25ºC in physiological saline (0.9% NaCl) in a 96-well plate. Cry1Ab activated toxin was added, and cell swelling was observed under phase contrast microscopy.

For the cytotoxicity experiments, the Japanese *B. mori* strain Kinshu X Showa (Kin) was used. This is a hybrid race and was purchased from Ueda-Sanshu Co. (Ueda, Japan). Cells were dissociated from dissected midguts as described previously [30]. Cell lysis was assessed by measuring the release of cytosolic lactate dehydrogenase (LDH) [30]. Cells from 4th instar larvae of the Kin strain were preincubated for 1 h with 1 µM of either anti-BtR175, anti-APN1, anti-GTS, or a mixture of anti-BtR175 and anti-APN1 antibodies. Then, 10 nM Cry1Ab was added and incubated for 1.5 h before measuring the release of LDH activity. The experiment was repeated four times. Control experiments of complete cell lysis were performed using 1% of Triton X-100 (w/w).

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Binding experiments

All binding experiments were performed within one month. Association binding experiments were performed according to Van Rie *et al.* [31]. Association and dissociation binding experiments were carried out with ^{125}I-labeled Cry1Ab toxin using the chloramine-T method [31]. The specific activity of ^{125}I-Cry1Ab was 5.1 mCi/mg, as determined using an Enzyme-Linked Immunosorbent Sandwich Assay (ELISA) [31]. Association and dissociation binding experiments with antibody pre-incubation were carried out with another batch of Cry1Ab labeled with a specific activity of 3.7 mCi/mg. All binding experiments were done at least in duplicate.

The association binding assay was performed with different incubation times (0 to 100 min) and 0.2 mg/ml of BBMV with 0.17 nM ^{125}I-labeled Cry1Ab for total binding, and with 3 µM (1,000-fold) unlabeled Cry1Ab for non-specific binding, in 0.1 ml of binding buffer (PBS buffer: 1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4, with 0.1% BSA) at room temperature. Bound toxin was separated from free toxin by centrifugation at 16,100x*g* for 10 min at 4ºC. The pellet was washed twice with 0.5 ml of ice-cold binding buffer. The radioactivity in the pellet was measured in a model 1282 Compugamma CS gamma counter (LKB Pharmacia). Specific binding was calculated by subtracting non-specific binding from total binding.

For the dissociation experiments, an excess (1,000-fold) of unlabeled toxin was added after 100 min of association binding. After different incubation times (0-125 min) the reaction mixture was centrifuged at 16,100x*g* for 10 min at 4ºC. Control experiments followed the same protocol, but PBS buffer was added instead of unlabeled toxin. The non-specific binding was determined by incubating BBMVs with both labeled and excess (1000-fold) unlabeled Cry1Ab for 100 min. The value of the specific binding was obtained from the association binding assay.

Antibody inhibition of each protein was assessed using a 1-h pre-incubation of the BBMVs used in the binding assays with 1 µM specific antibody or a 1:20000 dilution of antiserum in binding buffer at room temperature. Then, the dissociation experiment protocol was followed, as described above, but using 0.34 nM ^{125}I-Cry1Ab. The total specific binding was obtained at 100 min from the association binding experiment with the antibody-pre-incubated BBMV. The integrity of the vesicles was determined by comparing the values with a control experiment using PBS buffer instead of the specific antibody when pre-incubated with the

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BBMV. The stability of the binding association was checked by adding PBS buffer instead of unlabeled toxin. The irreversible binding was measured as the specific binding remaining in a dissociation experiment after 100 min. Reversible binding was calculated as the difference between total specific binding and irreversible binding.

RESULTS

Susceptibility of the strain J65 to Cry1A toxins

The susceptibility of *B. mori* strain J65 to Cry1Aa and Cry1Ab toxins was evaluated using two methods, a larval mortality assay and a cytological assay. The two toxins showed similar toxicities to neonate larvae based on the mortality after 4 days (Table 1). However, although LC_{50} values were similar, the larval mortality responses cannot be considered equal as the slopes were significantly different ($P = 0.04$, t-test), indicating that the response of strain J65 to increasing concentrations of Cry1Aa was faster than the response to Cry1Ab.

Because many strains of B. mori are very susceptible to Cry1Aa but not to Cry1Ab, the unusually high susceptibility of B. mori strain J65 to the Cry1Ab toxin was confirmed by a ex vivo bioassay. Dissected midguts were incubated with two concentrations of toxin (1 and 10 nM) for different periods (1, 2, and 3 h). Microscopic observation of the toxin-exposed midguts revealed the typical effects of Cry proteins [1], including the swelling of midgut cells (Fig. 1). This effect was dependent on the dose of toxin and incubation time, demonstrating that Cry1Ab is toxic to the midgut cells. Control midguts were either not treated with toxin or were exposed for only 1 min; in both cases no effect was observed (Fig. 1).

Kinetics of Cry1Ab binding

Representative kinetics of the binding of Cry1Ab in solution to BBMV from larval midguts of strain J65 insects is shown in Fig. 2. The reversible and irreversible components of the total binding were estimated from an association experiment to measure the total specific binding followed by a dissociation experiment to determine the amount of irreversible binding. BBMV incubated with 0.17 nM ^{125}I-Cry1Ab showed stable specific binding after 100 min (at about 6% of added ligand). The subsequent addition of an excess of unlabeled toxin decreased

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the amount of specific binding, which stabilized after 125 min at about 1.5% of added ligand. This 1.5% represents the irreversibly bound fraction, which was about 25% of the total specific binding that occurred in the association experiment. The difference between the total binding and the irreversible binding (i.e., 4.5% of added ligand) represents the reversible component of the binding and accounted for about 75% of the total specific binding. The non-specific binding, determined by adding an excess of unlabeled toxin to the initial mixture, was about 2.5 ± 0.1% (mean ± SD) of the added ligand. This value remained stable regardless of the length of the experiment. Similarly, control experiments with no addition of unlabeled toxin after the association kinetics experiment showed that the amount of total specific binding was maintained constant for at least 125 min.

Antiserum and antibodies specificity

The specificity of the antiserum raised against APN3 and the antibodies raised against the APN1 and BtR175 proteins of *B. mori* was tested by Western blot analysis with BBMV proteins from the J65 strain (Fig. 3). The observed 115-kDa APN1 band and 110-kDa APN3 band agreed with a previous report [15]. In our hands the anti-APN1 antibody also recognized a 110-kDa band which could represent some cross-reactivity of APN3. However, we can exclude this possibility because, under the same experimental conditions, the anti-APN1 antibody recognized only a 115-kDa band in another strain of *B. mori* which possesses APN3 [21]. It is possible that the 110-kDa protein was a breakdown product of APN1. The 195-kDa band is presumably BtR175, as it was recognized by the anti-BtR175 antibody and its size was similar to the 195-kDa band seen in the middle and posterior midgut regions of *B. mori* on immunoblotting [30]. Nagamatsu *et al.* [32] also purified this 195-kDa band from the BtR175 fraction and visualized it by immunoblotting with anti-BtR175 antibodies. Control experiments performed with antibodies raised against the GST protein did not recognize any band. The results suggest that each antibody specifically interacted with the single class of protein against which it was raised.

Inhibition of Cry1Ab binding using specific antibodies

In order to determine which membrane proteins were involved in the binding of Cry1Ab, BBMV from the J65 strain were preincubated for 1 h with antibodies against candidate receptors before starting the binding assays. Significant changes in the binding kinetics after incubation

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with the antibodies were interpreted as the participation of the specific protein recognized by the antibody in the binding process.

In relation to the control with just buffer, no significant effect (one-way ANOVA with the Bonferroni post-test, $P > 0.05$) on total specific binding was observed in samples preincubated with either anti-APN3 antiserum or anti-GST antibody (Fig. 4). However, significant reduction in total specific binding (one-way ANOVA with the Bonferroni post-test, $P < 0.05$) was observed when anti-BtR175 and anti-APN1 antibodies were used. Anti-BtR175 produced a greater reduction (84%) than anti-APN1 (55%). Control experiments were performed to determine the possible effect of the antibody incubation on the non-specific binding; no variation in this component of binding was observed.

Analysis of the effect of antibody preincubation on the reversible and irreversible components of binding revealed a marked reduction of both components when using anti-BtR175 (Fig. 5), with values not significantly different (t-test, $P > 0.05$) from the values obtained for non-specific binding. By contrast, anti-APN1 specifically affected the amount of irreversible binding (to the same level as the non-specific binding) (Fig. 5A), while the reversible binding component was not affected (t-test, $P > 0.05$) (Fig. 5B).

Inhibition of Cry1Ab cytotoxicity to dissociated midgut cells

The effect of anti-APN1 and anti-BtR175 antibodies on the action of Cry1Ab on intact cells (as opposed to BBMV) was measured using the Kin strain, another *B. mori* Cry1Ab susceptible strain [21]. The release of cytosolic LDH (as an indicator of cell lysis) upon incubation of dissociated cells with Cry1Ab demonstrated the cytotoxicity of this protein *ex vivo* (Fig. 6). Preincubation of the cells with anti-BtR175 antibody significantly reduced the cytotoxic effect. However, neither anti-APN1 nor anti-GTS (control) antibodies significantly decreased the cytotoxicity of Cry1Ab in the assay conditions. In addition, anti-APN1 did not show any additive effect when combined with the anti-BtR175 antibody.

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DISCUSSION

Bombyx mori has been shown to be tolerant to Cry1Ab and Cry1Ac proteins and susceptible to Cry1Aa. However, its is not surprising that different strains can show variation in susceptibility, as it was found in other lepidopteran species [33]. In fact, the relative toxicities of Cry1Aa, Cry1Ab, or Cry1Ac vary markedly in different reports [4, 23, 34-37]. Strains J65and Kin are atypical strains in that they are highly susceptible to Cry1Ab (this work and 21), to a similar extent than to Cry1Aa. Our experiments analyzing several susceptible and resistant *B. mori* strains indicated that the resistance of several strains does not depend on a loss of toxin-activating activity or an excess of toxin-degrading activity of the midgut fluid (data not shown), but rather on the direct susceptibility of midgut epithelial cells to the toxin (Fig. 2).

The association binding kinetics of toxins with BBMV in solution provide information on the full binding process, but dissociation kinetics experiments are needed to determine the relevance of each binding component, reversible and irreversible. In fact, Liang *et al.* [6] showed that the rates of irreversible binding of Cry1A toxins, rather than the binding affinities, were directly related to toxicity in *Lymantria dispar* BBMV. Other studies have shown that loss of toxicity and decreased irreversible binding without change in reversible binding parameters occur simultaneously by mutating toxins in domain I or II [5, 39]. The association of Cry1Ab toxin with membranes from *B. mori* has been previously shown using BBMV in solution using a Cry1Ab tolerant strain [37]. However, with the tolerant strain the amount of irreversible binding observed was much lower than in our study (8% vs. 30%, referred to the total specific binding). Ihara *et al.* [37] proposed a direct relationship between Cry toxin toxicity and irreversible binding in *B. mori*. Therefore, the higher Cry1Ab irreversible binding contribution to the total binding in strain J65 compared with that in the tolerant strain of Ihara *et al.* [37] must be responsible for the high Cry1Ab susceptibility of J65.

Our study using specific antibodies to block toxin binding allowed the identification of the molecules involved in the binding process. Denaturating techniques, such as ligand-blot analysis, in several lepidopteran species have determined that Cry1Ab binds to several types of APNs [7, 10] and also to cadherin-like proteins, such as Bt-R_1 in *M. sexta* [40] and OnBt-R_1 in

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Ostrinia nubilalis [41]. In *B. mori*, Nakanishi *el al.* (2002) reported binding of Cry1Ab to APN1 but not to BtR175, and no other study has provided data on the binding of Cry1Ab to BtR175 of *B. mori*. However, our data suggest that Cry1Ab toxin binds to the putative BtR175 receptor in strain J65. This is somewhat expected given that the strain we have used is susceptible to this toxin and that cadherin-like proteins are thought to play an important role in the toxic action of Cry1A toxins in lepidopterans [16, 19, 30, 42, 43]. An observation which supports our finding is that Nagamatsu *et al.* [32] showed the inhibition of Cry1Aa binding to BBMV of *B. mori* after incubating the BBMV with anti-BtR175 antibody, and Cry1Aa and Cry1Ab toxins have been shown to share binding sites in *B. mori* [37].

Our results of binding inhibition using antibodies showed that both APN1 and BtR175 are involved in Cry1Ab binding to *B. mori* BBMVs in solution. The use of anti-BtR175 antibody practically completely abolished Cry1Ab binding, affecting both its reversible and irreversible components. However, the anti-APN1 antibody only inhibited the irreversible component of Cry1Ab binding (Figs. 4 and 5). These results can be explained by a model in which binding is the result of sequential steps involving these two proteins, such as the model proposed by Bravo *et al.* [7]. In this model the initial binding of Cry1Ab toxin to the cadherin-like protein would be reversible, in order to drive the toxin to the APN to cause pore formation. This last step would require stable irreversible binding to permit the lytic activity. Our results are in agreement with this model in that blocking the first step (the cadherin-like receptor) inhibited the whole binding process, while blocking the second step (the APN1 receptor) only inhibited the irreversible binding component, allowing the reversible binding to the cadherin-like receptor to take place. In addition, our experiments identified APN1 of *B. mori* as the specific APN involved in this model, because the treatment with anti-APN1 antibody totally blocked the irreversible binding. This is also in agreement with the reported functional interaction of a Cry1A (Cry1Ac) with APN1 in *Helicoverpa armigera* [44] and with Bravo *et al.* and Gómez *et al.* [7, 45] because the 120-kDa APN of *M. sexta* in the proposed model is homologous to *B. mori* APN1 [46]. No other APNs or other proteins seem to be significantly involved in binding in *B. mori*, given that we have been able to inhibit essentially all binding by using highly specific antibodies.

Our results with the antibody inhibition of the cytotoxic action of Cry1Ab are in complete agreement with previous studies in *B. mori* in which the involvement of APN1 in the cytotoxicity of Cry1Aa or Cry1Ac seemed not to be relevant [30] in this type of *ex vivo* assays and that only

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binding to the BtR175 receptor seemed relevant. In our hands, either the anti-APN antibody cannot prevent efficiently the binding of the toxin in this type of assay or other mechanisms of toxicity are acting in live cells as opposed to BBMV. The latter possibility would be in agreement with the model proposed by Zhang *et al.* [8], in which binding to the cadherin-like protein would be sufficient to provoke the death of detached cells. It is possible that both mechanisms (pore formation and the signaling pathway) can act simultaneously *in vivo* (in the intact larva) and that depending on the experimental system used the results can be the consequence of one or another. In addition, the relative contribution of both types of toxicity pathways can be different depending on the lepidopteran species considered.

Our work provides the first evidence that Cry1Ab toxin binds to APN1 and to BtR175 cadherin-like protein in BBMV in solution. All previous evidences showing the binding of Cry1Ab to these receptors have been obtained either under denatured conditions or with purified proteins out of their membrane environment. Furthermore, our results seem to support the two models of the mode of action of Cry1Ab in Lepidoptera. On the one hand, our results are in agreement with the sequential involvement of BtR175 and APN1 in the binding to BBMV [7]. On the other hand, the assays with dissociated cells are in agreement with the involvement of the cadherin-like receptor in triggering a signaling pathway [8]. It is very likely that both pathways contribute to the toxicity of Cry1A toxins *in vivo*. If confirmed, it would be desirable that further studies can determine the relative contribution of both mechanisms to the *in vivo* toxic effect of Cry1A toxins.

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TABLES

Table 1. Susceptibility of neonate larvae of the J65 strain of *B. mori* to Cry1Aa and Cry1Ab protoxins. The parameter LC_{50}, measured as ng protein/cm^2 mulberry leaf, is the concentration that caused 50% mortality after 4 days and is given with the 95% confidence limits (CL_{95}). The assays were replicated three times.

Protoxin	LC_{50}	CL_{95}	Slope ± S.E.M.
Cry1Aa	35	23–46	7.2 ± 1.7
Cry1Ab	34	5–266	2.0 ± 0.3

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FIGURE LEGENDS

Figure 1. *Ex vivo* susceptibility of midgut epithelial cells from the *B. mori* strain J65 to Cry1Ab. Each midgut was incubated with 0, 1, or 10 nM Cry1Ab toxin and observed under phase contrast microscopy 1, 60, 120, and 180 min later. The arrows indicate midgut epithelial cells that swelled and the bar represents 200 μm.

Figure 2. Association and dissociation kinetics of the binding of ^{125}I-Cry1Ab to larval BBMV from *B. mori* strain J65. The arrow indicates the addition of a 1000-fold excess of unlabeled toxin. This moment represents the end of the association experiment and the start of the dissociation experiment. The separation of the experiments is indicated by the vertical dotted line. Values represent the total specific binding as percentage of the added ligand. Duplicate experiments were performed and the error bars represent SEM.

Figure 3. SDS-PAGE and Western blot analysis of BBMV proteins from *B. mori* strain J65. BBMV proteins were separated by 5% (A) or 7% (B) SDS-PAGE and then transferred to nitrocellulose membranes. Western blot analysis was performed with anti-BtR175 (lane 2), anti-GST (lane 3), or anti-APN1 antibodies (lane 5), or with anti-APN3 antiserum (lane 6). Lanes 1 and 4, were stained with Coomassie blue.

Figure 4. Association binding experiments of ^{125}I-Cry1Ab and BBMV from *B. mori* J65. BBMV were preincubated for 1 h with different antibodies (anti-GST, anti-APN1, or anti-BtR-175), antiserum (anti-APN3), or buffer (control) before adding the labeled toxin. Values represent the total specific binding (as percentage of the added ligand) after 100-min of incubation. At least two experiments were performed, and the error bars indicate the SEM.

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Figure 5. Dissociation binding experiments of ^{125}I-Cry1Ab and BBMV from *B. mori* J65 strain. BBMV were preincubated for 1 h with anti-APN1 or anti-BtR-175 antibodies or buffer (control) before adding the labeled toxin. After a 100-min incubation with the labeled toxin, a 1000-fold excess of unlabeled Cry1Ab was added and the incubation was continued for 100 min. The remaining specific binding accounted for the irreversible component of binding (A). The reversible component of binding (B) was calculated as the total specific binding minus the irreversible binding. Values represent the total specific binding as percentage of the added ligand. At least two experiments were performed, and the error bars indicate the SEM (the apparent lack of error bar in the control in (A) is due to the similar values obtained for the replicates).

Figure 6. Effect of anti-BtR175 and anti-APN1 antibodies on the cytotoxicity of Cry1Ab on *B. mori* dissociated midgut epithelial cells as measured by the release of cytosolic LDH. Cells from 4th instar larvae of the Kin strain were preincubated for 1 h with 1 µM of either anti-BtR175, anti-APN1, anti-GTS, or a mixture of anti-BtR175 and anti-APN1 antibodies. Then, 10 nM Cry1Ab was added and incubated for 1.5 h before measuring the release of LDH activity. The sample labeled with a "–" sign denotes a control without toxin and the control without antibodies is labeled as "Cry1Ab".Values represent the mean (with SEM) of four replicates. Asterisks indicate a significant difference ($p<0.01$) with the control without antibodies (labeled as Cry1Ab), as determined by Student's *t*-test. Complete lysis with 1% of Triton X-100 gave an O.D. value of 0.38.

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Figure 1

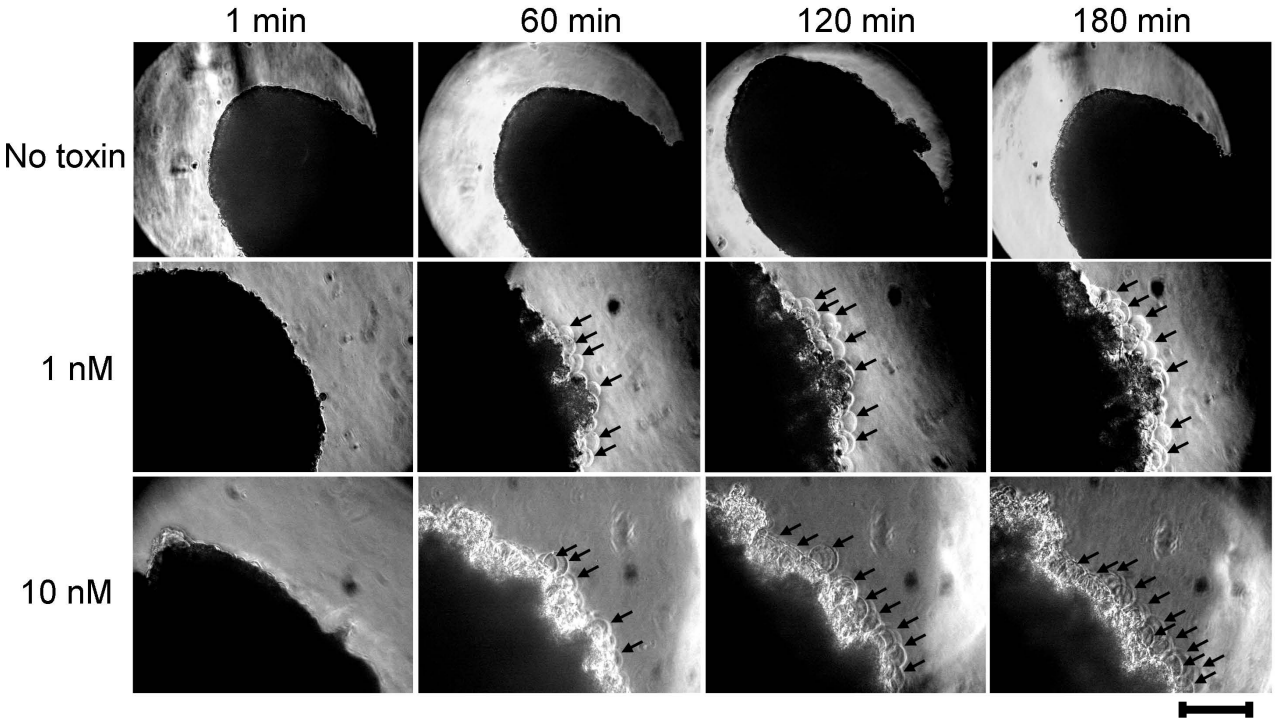

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Figure 2

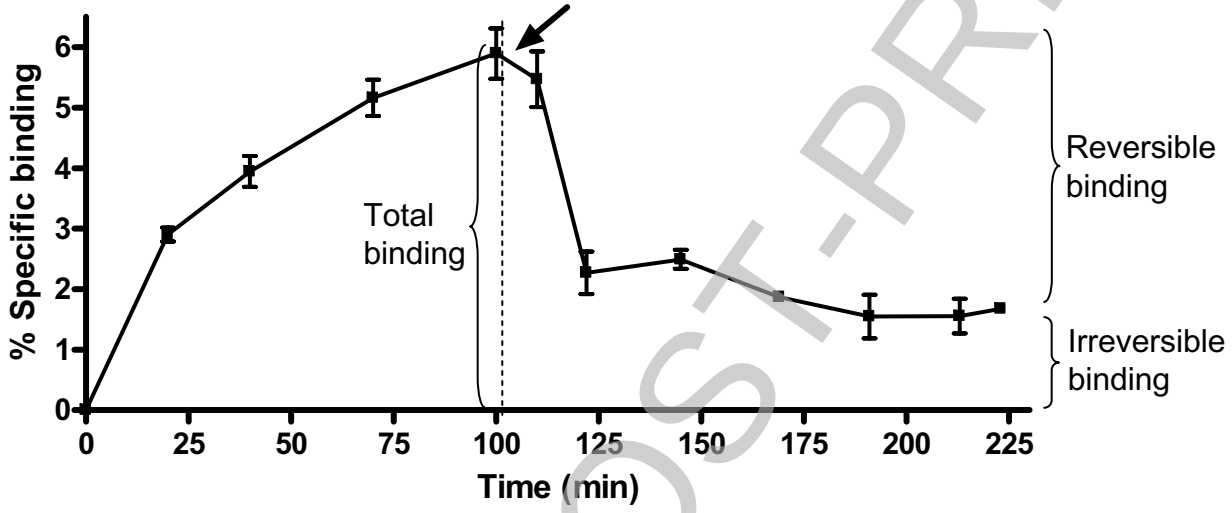

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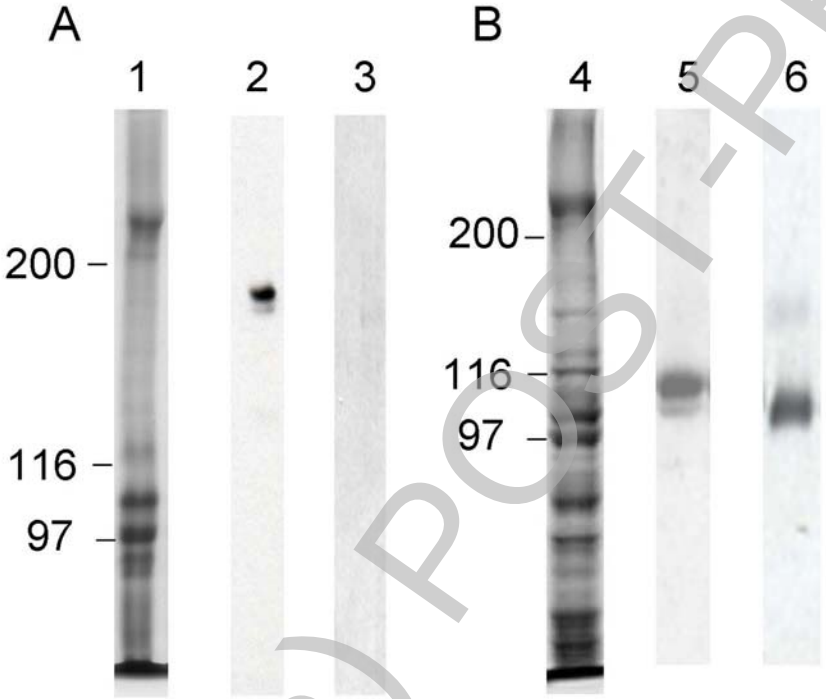
Figure 3



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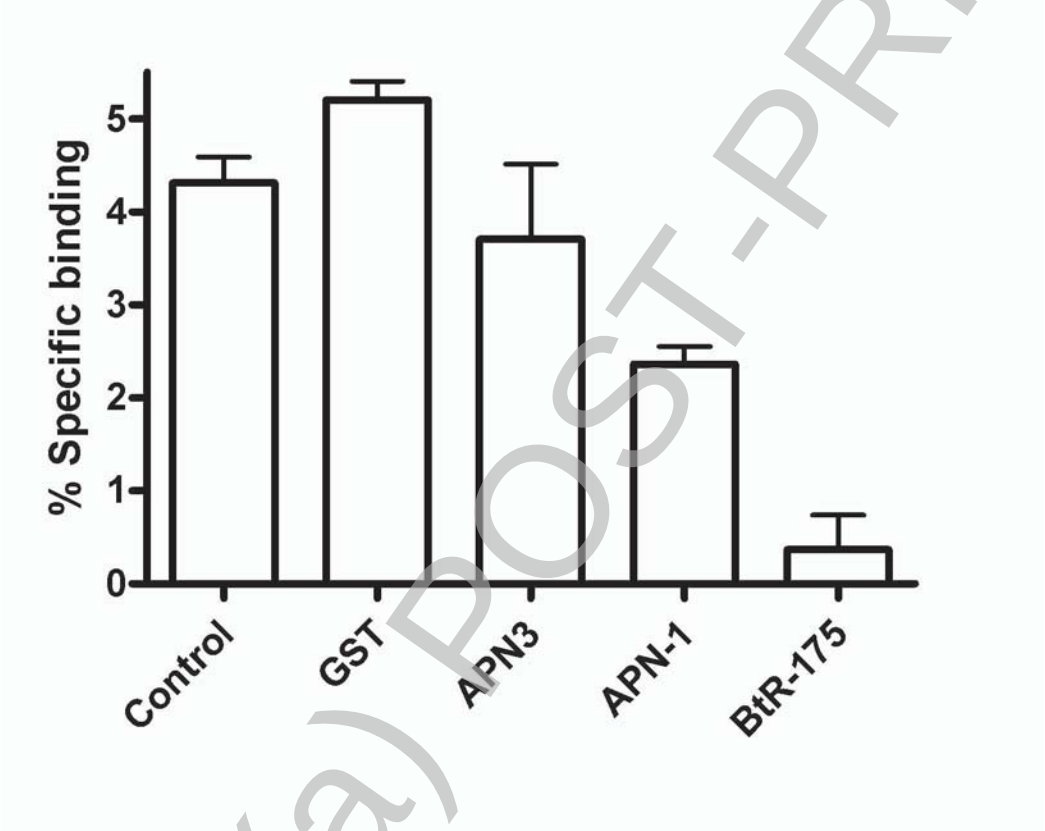

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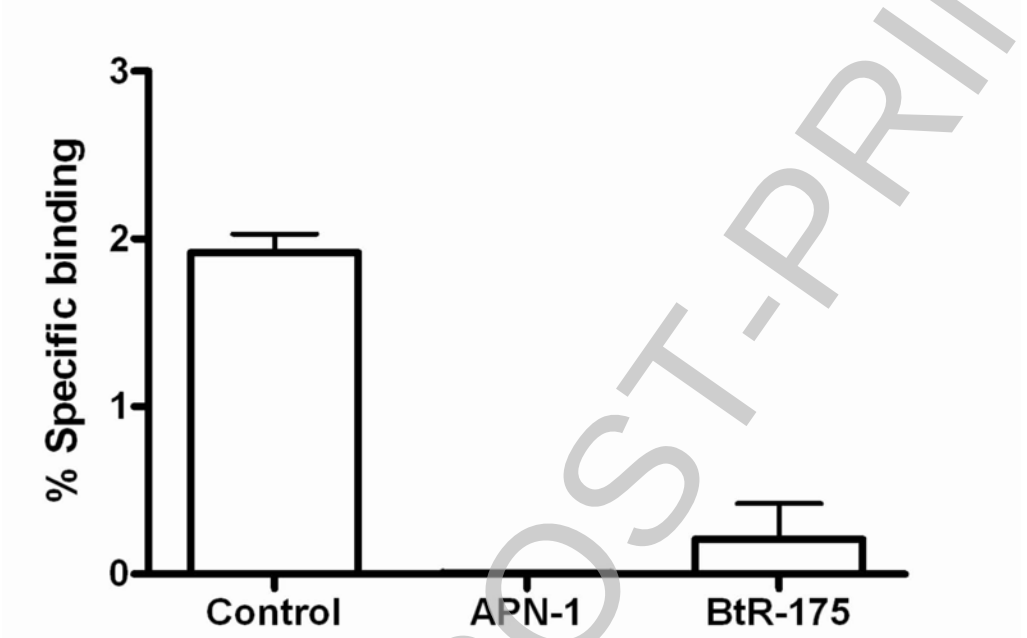
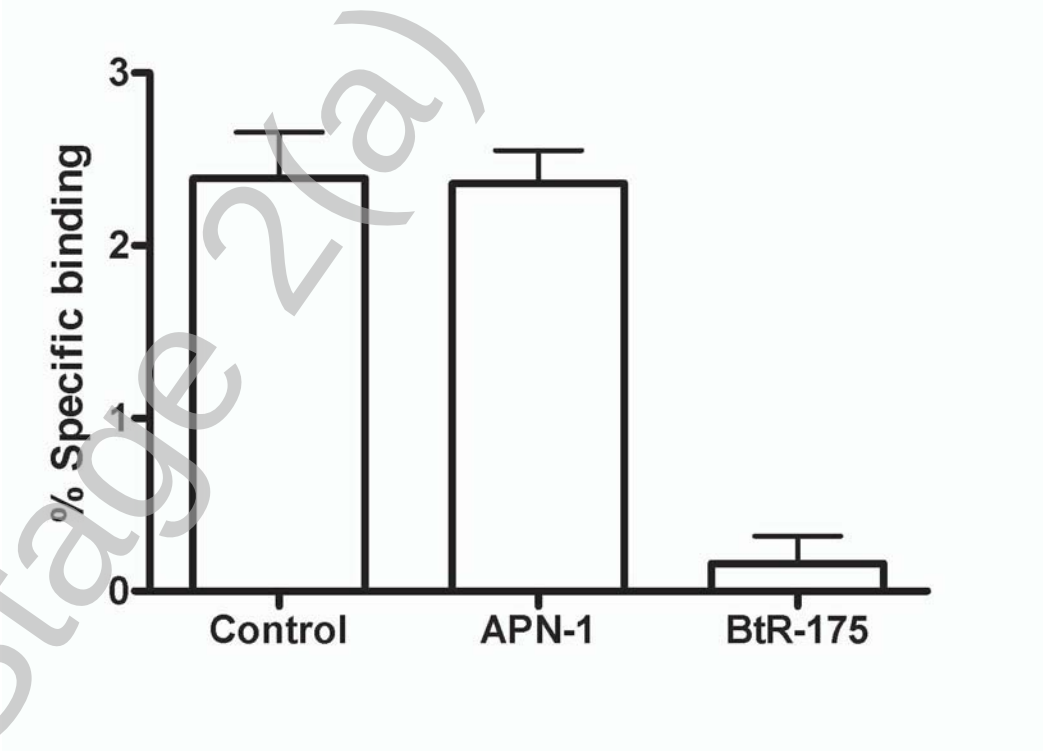
Figure 5

A



B



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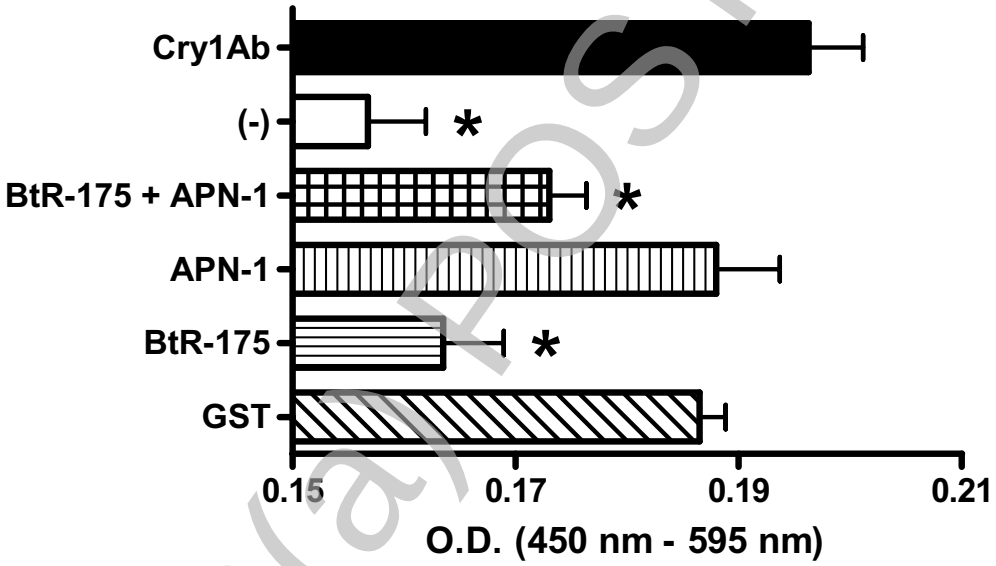

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