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Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin

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Abstract Cry toxins form lytic pores in the insect midgut cells. The role of receptor interaction in the process of protoxin activation was analyzed. Incubation of Cry1Ab protoxin with a single chain antibody that mimics the cadherin-like receptor and treatment with *Manduca sexta* midgut juice or trypsin, resulted in toxin preparations with high pore-forming activity in vitro. This activity correlates with the formation of a 250 kDa oligomer that lacks the helix α-1 of domain I. The oligomer, in contrast with the 60 kDa monomer, was capable of membrane insertion as judged by 8-anilino-1-naphthalenesulfonate binding. Cry1Ab protoxin was also activated to a 250 kDa oligomer by incubation with brush border membrane vesicles, presumably by the action of a membrane-associated protease. Finally, a model where receptor binding allows the efficient cleavage of α-1 and formation of a pre-pore oligomeric structure that is efficient in pore formation, is presented. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: δ-Endotoxin; Protoxin activation; Receptor binding; Pre-pore; Membrane insertion

1. Introduction

Bacillus thuringiensis (Bt) is a bacterium that produces insecticidal proteins (Cry) toxic to different insect orders [1]. Cry toxins exert their pathological effect by forming lytic pores in the membrane of insect midgut cells [2].

The Cry proteins are solubilized within the insect gut due to the highly alkaline and reducing conditions of gut lumen. The liberated protoxins are activated by midgut proteases to release the toxin fragment [3]. The three-dimensional structures of some Cry toxins have been resolved [4–6] showing that they are comprised of three domains. Domain I, a seven-α helix bundle, is the pore-forming domain. Domain II and domain III, composed of β-sheets, are involved in receptor binding [7]. Cry toxin binds specifically to its receptors. Two Cry1A toxin receptors have been identified as aminopeptidase-*N* (APN) and cadherin-like (Bt-R_1, Bt-R_{175}) [8–14]. The debate on whether these are functional receptors leading to pore formation is still on-going. In contrast to APN, the expression of the cadherin-like protein from *Bombyx mori*, Bt-R_{175}, on the surface of Sf9 insect cells rendered them sensitive to Cry1Aa toxin [11]. Also, an *Heliothis virescens* population resistant to Cry1Ac toxin is mutated in a cadherin-like gene [15]. In previous work, a scFv antibody (scFv73) that inhibits binding of Cry1A toxins to cadherin-like receptors, but not to APN, and reduced the toxicity of Cry1Ab to *Manduca sexta* larvae was characterized. Interestingly, the CDR3 region of scFv73 shared homology with an eight amino acid epitope of *M. sexta* cadherin-like receptor, Bt-R_1 [16]. Overall, these results suggested that binding to cadherin-like receptor is an important step in the Cry1A toxins mode of action.

Following binding, it is proposed that at least part of the toxin inserts into the membrane resulting in pore formation [1,2]. The intermolecular interaction of Cry1Ab monomers is a necessary step for toxicity [17,18] and a tetrameric oligomer of Cry1Ac was observed in synthetic membranes [19]. However, it has not been established if oligomer formation occurs before (pre-pore) or after toxin insertion. Previous observations in our laboratory showed that trypsin-activated Cry1A protoxins, resulted in toxin preparations with low pore formation activity in vitro, despite the fact that they retained toxicity [20], suggesting an incomplete activation. Also, *M. sexta* midgut juice (MJ) treatment of Cry1Ab protoxin produced proteolytic cleavages inside the toxin. Two additional nicks were identified which remove helices α-1 and α-2 of domain I. The highest in vitro pore formation activity was observed after helix α-1 was removed [20]. Similar results were obtained for Cry1Ac protoxin activated with MJ from *Pieris brassica* [21]. These results suggest that in vivo, further proteolytical cleavages may occur for toxin activation.

In this work we analyzed if receptor interaction, using the scFv73 antibody as model receptor, is a necessary step for an efficient proteolytic activation of Cry1A toxins. We show that Cry1Ab toxin binding to the cadherin-like receptor promotes complete proteolytic activation and a pre-pore formation that is insertion competent leading to the formation of functional ionic pores in vitro.

2. Materials and methods

2.1. MJ isolation and activation of Cry1Ab protoxin

M. sexta larvae were reared on artificial diet. Midgut tissue was dissected from 5th instar larvae. MJ was separated from solid material by centrifugation and filtered through 0.22 μm filters.

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Abbreviations: BBMV, brush border membrane vesicles; APN, aminopeptidase-*N*; MJ, midgut juice; ANS, 8-anilino-1-naphthalenesulfonate hemimagnesium; Dis-C_3-(5), 3,3′-dipropylthiodicarbocyanine

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Cry1Ab crystals were produced in *Bt* strain 407*cry*$^-$ transformed with pHT315-1Ab [16]. Cry1Ab crystal purification and protoxin solubilization were done as described [16]. For activation, 1–2 mg/ml Cry1Ab protoxin was incubated 1 h with 1/1 ratio of scFv73. Trypsin (1/20 ratio) or MJ (5%) was added and incubated 1 h at 37°C. PMSF (1 mM) was added and samples centrifuged (20 min at 12 000×*g*). For protoxin activation with brush border membrane vesicles (BBMV), 100 nM Cry1Ab protoxin was incubated 15 min with 10 μg BBMV, stopped with PMSF and centrifuged as above. Purification of the activated toxins was done by size exclusion chromatography with Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Sweden) FPLC size exclusion as described [22]. N-terminal sequencing was performed at the Harvard Microchemistry Facility of Harvard University (Cambridge, MA, USA) after 7% SDS–PAGE and transfer onto polyvinylidene difluoride membranes.

2.2. Western blot of Cry1Ab toxin

Activated toxin was separated in SDS–PAGE, transferred onto a nitrocellulose membrane PVDF, blocked with slimed milk (5%) and detected with anti-Cry1Ab polyclonal antibody (1/80 000; 1 h) and visualized with a goat anti-rabbit antibody coupled with horseradish peroxidase (HR) (Sigma, St. Louis, MO, USA) (1/1000; 1 h), followed by SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, USA) as described by the manufacturers.

2.3. Purification and characterization of scFv

Escherichia coli scFv fragments were purified to homogeneity by an Ni-agarose column, as described [16].

2.4. Preparation of BBMV

BBMV from 5th instar *M. sexta* larvae were prepared and dialyzed against 150 mM KCl, 2 mM EGTA, 0.5 mM EDTA, 10 mM HEPES–HCl pH 7.5 as reported [16]. BBMV for activation of Cry1A protoxins were prepared as above without protease inhibitors.

2.5. Fluorescence measurements

Membrane potential was monitored with the fluorescent positively charged dye, 3,3′-dipropylthiodicarbocyanine (Dis-C_3-(5)), in an Aminco Bowman luminescence spectrometer (Urbana, IL, USA) as in [24].

2.6. ANS binding

Binding of 8-anilino-1-naphthalenesulfonate hemimagnesium (ANS, 15 μM, Sigma, St. Louis. MO, USA) was measured as reported [25] at 380/480 nm excitation/emission wavelengths pair.

2.7. Insect bioassay

Bioassays were performed with *M. sexta* neonate larvae as reported [26]. The effective doses were calculated using PROBIT analysis.

3. Results

3.1. Proteolytic activation of Cry1Ab protoxin in the presence of scFv73 antibody

We isolated a single chain antibody (scFv73) that interfered the interaction of Cry1Ab toxin with BBMV from *M. sexta* [16]. The characterization of scFv73 led to the identification of two discrete eight residue regions present in cadherin-like receptors from *M. sexta* and *B. mori*, that were sufficient to compete binding of Cry1Ab and Cry1Aa to cadherin proteins and to negatively affect toxicity of Cry1Ab against *M. sexta* larvae [16]. Therefore scFv73 can be used as a surrogate of cadherin-like receptors.

To analyze the effect of Cry toxin binding to this epitope in the activation process, Cry1Ab protoxin was preincubated with scFv73 at room temperature and treated afterwards with trypsin or with MJ from *M. sexta* larvae. As control, Cry1Ab protoxin was incubated with scFv45 that binds Cry1Ab but has no effect on Cry1Ab binding to Bt-R_1 or in toxicity [16]. MJ or trypsin completely degrade scFv as judged by SDS–PAGE and Western blot (see below). In vitro K^+ permeability assays were performed using a fluorescent positively charged dye (Dis-C_3-(5)) sensitive to changes in membrane potential as reported [24] in isolated *M. sexta* BBMV with the different toxin preparations (Fig. 1A, Table 1). Proteolytic activation of Cry1Ab protoxin incubated with scFv73 and treatment with MJ or trypsin resulted in toxin preparations with high pore formation activities, in contrast with the Cry1Ab protoxin incubated with scFv45 or treated only with MJ or trypsin (Table 1). Also, Cry1Ab protoxin or scFv73 alone or a mixture of both without further treatment with proteases, showed no pore formation activity in vitro (data not shown). Addition of 50 nM of scFv73-MJ-activated Cry1Ab toxin to BBMV loaded with 150 mM KCl and sus-

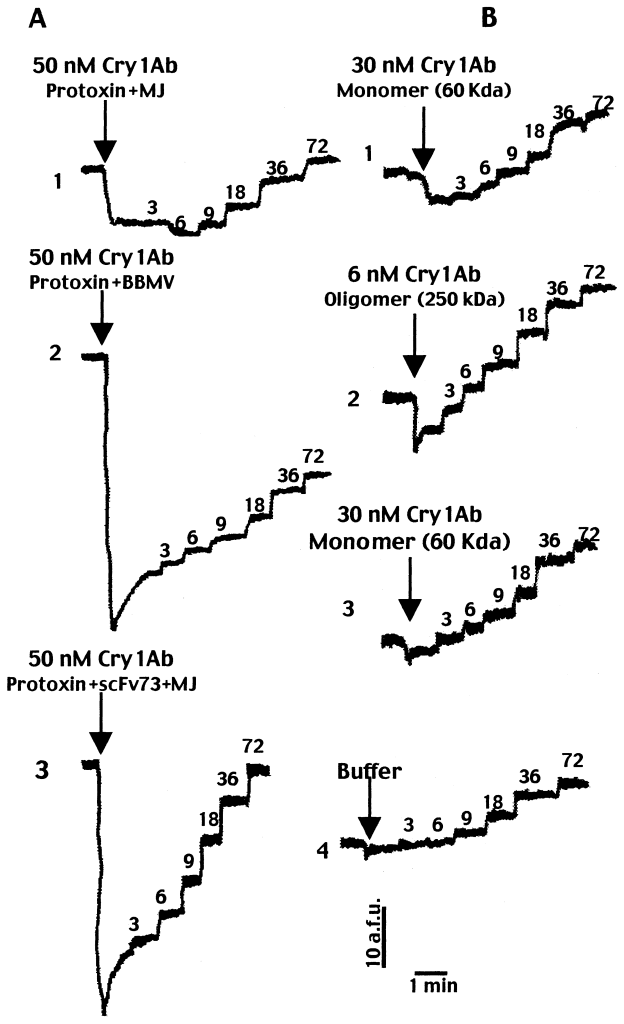

Fig. 1. K^+ permeability across *M. sexta* BBMV induced by Cry1Ab-activated toxins. Changes in distribution of a fluorescent dye (Dis-C_3-(5)) sensitive to change in membrane potential were recorded as 2.5. A: Trace 1, Cry1Ab toxin activated with MJ (50 nM); trace 2, Cry1Ab toxin activated with BBMV (50 nM); trace 3, Cry1Ab toxin activated with scFv73 and MJ (50 nM). B: K^+ permeability of purified Cry1Ab toxins by size exclusion. Trace 1, Cry1Ab 60 kDa monomer (30 nM) (fraction 17 of Fig. 2C); trace 2, Cry1Ab 250 kDa oligomer (6 nM) (fraction 9* of Fig. 2C); trace 3, Cry1Ab 60 kDa monomer (30 nM) (fraction 17* of Fig. 2C); trace 4, control, buffer addition. The arrow on the top of the traces corresponds to the time of toxin addition. An upward deflection indicates a membrane depolarization. Numbers in traces indicate the final K^+ concentrations (mM).

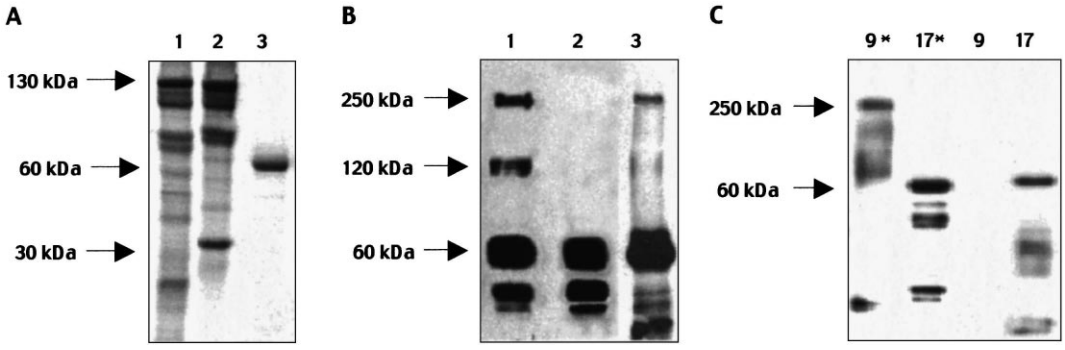

Fig. 2. SDS–PAGE and Western blots of activated Cry1Ab toxin. A: SDS–PAGE (10% acrylamide): lane 1, Cry1Ab protoxin; lane 2, Cry1Ab protoxin with scFv73; and lane 3, Cry1Ab protoxin with scFv73 and treated 1 h with MJ. B: Western blotting (SDS–PAGE 7% acrylamide) detected with an anti-Cry1Ab polyclonal antibody. Lane 1, Cry1Ab protoxin activated with scFv73 and MJ; lane 2, Cry1Ab protoxin activated only with MJ; lane 3, Cry1Ab protoxin activated with *M. sexta* BBMV. C: Western blotting (SDS–PAGE 12% acrylamide) of samples from size exclusion chromatography of Cry1Ab protoxins activated with scFv73 and MJ (*) or with MJ. Proteins were revealed with anti-Cry1Ab polyclonal antibody. Numbers in C are the retention times of the different fractions.

pended in 150 mM MeGluCl produced a fast hyperpolarization (Fig. 1A). The response of the Dis-C_3-(5) dye to KCl additions also increased, when compared to the control in which the same amount of buffer was added. After each KCl addition a new membrane potential is established, and a depolarization is produced. Preincubation of Cry1Ab protoxin with scFv73 was necessary since simultaneous addition of scFv73 and MJ proteases resulted in no pore formation (Table 1). Activation was further improved when scFv73 was preincubated with Cry1Ab protoxin at 37°C, suggesting a better interaction at this temperature (Table 1).

Bioassays were performed with toxin preparations obtained by MJ treatment in the presence or absence of scFv73. Table 2 shows that toxin preparations obtained in the presence of scFv73 showed similar LC_{50} than Cry1Ab protoxin and 10 times higher toxicity than toxin treated with MJ in absence of scFv73.

3.2. *In vitro pore formation activity correlates with oligomer formation*

Fig. 2A shows a SDS–PAGE of different samples of Cry1Ab protoxin. A major band of 130 kDa was observed in the Cry1Ab protoxin samples in the absence or presence of scFv73 (Fig. 2A, lanes 1 and 2), an additional 30 kDa band was also observed in the sample incubated with scFv73 that corresponds to the molecular mass of the scFv molecule. In contrast, in the Cry1Ab protoxin sample incubated with scFv73 and treated with MJ only a 60 kDa band was observed suggesting that scFv73 was degraded by the protease treatment (Fig. 2A, lane 3). Western blot of Cry1Ab-activated proteins, using an anti-Cry1Ab polyclonal antibody, was performed. Fig. 2B shows that a major 60 kDa protein and lower molecular mass proteins were obtained as reported [20]. However, additional higher molecular mass bands (120 and 250 kDa) that reacted with poly-Cry1Ab, were observed in toxin preparations obtained in the presence of scFv73. The molecular sizes of these proteins correspond to dimers and tetramers of Cry1Ab. The samples containing the oligomers were treated at different temperatures before loading the gels. The same amount of the 250 kDa oligomer was observed at 50°C, 80°C or boiling, implying that oligomers were highly stable (data not shown). To analyze if scFv73 was part of the oligomer, a Western blot using anti-mic tag antibody was performed showing that the scFv73 was not part of the oligomer (data not shown). To further characterize the putative oligomers, Cry1Ab toxin preparations obtained by MJ treat-

Table 1
K^+ permeability of Cry1Ab-activated toxins

Sample	$m-m^a_{int}$ (K^+ permeability)	% K^+ permeability
Protox. Cry1Ab+scFv73+trypsin	0.202	41
Protox. Cry1Ab+scFv73+MJ	0.392	78
Protox. Cry1Ab+scFv45+trypsin	0.049	10
Protox. Cry1Ab+scFv45+MJ	0.027	5
Protox. Cry1Ab+MJ	0.045	9
Protox. Cry1Ab+scFv73+MJ at 37°C	0.498	100
Protox. Cry1Ab+scFv73+MJ (mixed)[b]	0.087	18
Protox. Cry1Ab+BBMV	0.143	29
Protox. Cry1Ab+MJ (monomer)[c]	0.049 (0.079)[e]	10 (16)
Protox. Cry1Ab+scFv73+MJ (monomer)[c]	0.047 (0.076)	10 (16)
Protox. Cry1Ab+scFv73+MJ (oligomer)[d]	0.122 (1.02)	25 (207)

Values are means of four different determinations. Standard deviations were less than 10%. 50 nM toxin for each assay except where indicated.
[a]The differences of the slope of the curve of each activated toxin (m_{tox}) minus the slope of the control trace in which the same amount of buffer was added [m_{int}] are presented.
[b]Cry1Ab protoxin and scFv73 were not preincubated and were mixed along with the MJ.
[c]30 nM toxin. Proteins after size excusion chromatography of activated Cry1Ab toxin as indicated.
[d]60 nM toxin. Proteins after size excusion chromatography of activated Cry1Ab toxin as indicated.
[e]Numbers in parentheses are values obtained considering 50 nM protein concentration as the rest of the treatments.

ment in the presence or absence of scFv73 were subject to size exclusion chromatography and poly-Cry1Ab detection. Fig. 2C shows that a major fraction containing the monomer (60 kDa) was obtained in both toxin preparations (retention time 17 min). In this fraction, lower molecular mass proteins that co-eluted with the 60 kDa monomer were observed. These smaller proteins are the product of proteolysis within domain II that co-eluted with the monomer since they remain attached after nicking [20]. However, in the toxin preparation obtained in the presence of scFv73 the 250 kDa oligomer (retention time 9 min), was observed (Fig. 2C). The 120 kDa protein was not observed after size exclusion. The same samples of the chromatography were blotted and revealed using a monoclonal 4D6 that recognizes an epitope in domain I of Cry1Ab [23]. 4D6 only recognized the Cry1Ab monomer (data not shown).

Finally, pore formation activity of the 250 kDa oligomer and of 60 kDa monomers obtained after size exclusion chromatography was determined. Fig. 1B and Table 1 show that only the oligomer had pore formation activity in vitro.

3.3. Oligomer formation involves cleavage after helix α-1

The amino-terminal sequences of the 60 kDa monomer and the 250 kDa oligomer were determined. The amino terminal sequence of the monomer (^{29}IETGYTP) corresponds to the beginning of helix α-1, in contrast to the oligomer (^{51}VPGAG) that was located between helices α-1 and α-2, showing that the helix α-1 was removed.

3.4. ANS binding to Cry1Ab monomer and oligomer

ANS binds to solvent-accessible clusters of non-polar residues [27], which are relatively rare in the native state of soluble proteins. We analyzed ANS binding to the 250 kDa oligomer, and to the 60 kDa monomer. Fig. 3 shows that only the toxin preparation containing the oligomer bound ANS.

3.5. Activation of Cry1Ab toxin with membrane-associated proteases

Cry1Ab protoxin was activated by incubation with BBMV preparations isolated without protease inhibitors for different times and analyzed by SDS–PAGE and Western blot with poly-Cry1Ab. Incubation of Cry1Ab protoxin with BBMV prepared in the absence of proteinase inhibitors for 15 min was enough to digest the protoxin to a 60 kDa polypeptide (Fig. 2B) suggesting the existence of proteases associated with the BBMV. The formation of the 250 kDa oligomer (Fig. 2B), which showed in vitro pore formation activity (Fig. 1A, Table 1) was also observed.

4. Discussion

The accepted mode of action of Cry toxins involves solubilization of Cry proteins, proteolytic activation, receptor binding, membrane insertion, toxin oligomerization and finally pore formation leading to cell swelling and lysis [7]. It is accepted that the pore is formed by the aggregation of several monomers [17–19]. However, it is still unclear if oligomer formation occurs before or after membrane insertion. Neither is clear if receptor binding is important in the process of oligomer formation. In this work we provide evidences for an active role of receptor binding in the process of proteolytic activation. Moreover, our data support the formation of a pre-pore structure that is capable of inserting into membranes. Thus, the proposed mode of action of Cry toxins now resembles the one proposed for several other pore-forming toxins in which proteolytic cleavage after receptor binding promotes pre-pore oligomer formation as a prerequisite for membrane insertion [25,28–31]. We show here that cadherin binding promotes efficient proteolytic activation of Cry1Ab toxin that leads to dimer and tetrameric oligomer formation. However, the dimer was consistently observed in lower amounts than the tetramer (Fig. 2B) and could not be detected after separation by size exclusion suggesting that the dimer could be an unstable intermediate in the formation of the tetramer. The tetrameric oligomer correlates with higher in vitro pore formation activity and enhanced Cry1Ab toxicity (Fig. 1B). The similar activation with scFv73 and MJ was obtained for Cry1Aa protoxin (data not shown).

Cry toxins do not form spontaneous oligomers in solution in vitro [22], suggesting that in vivo the oligomer formation may occur either after receptor binding or by lateral movement of monomers in the membrane. Our data support that the Cry1Ab oligomer is formed after receptor binding and before membrane insertion, forming a pre-pore structure that is insertion competent. The oligomer preferentially bound ANS that specifically reacts with solvent-accessible clusters of non-polar residues suggesting that oligomerization is accompanied by exposure of a hydrophobic surface. Binding of Cry1Ab toxin to the cadherin epitope analyzed in this work [16] seems to be a key step in the process of protease cleavage and pre-pore formation. However, the efficiency of Cry1Ab activation in vitro in the presence of scFv73 was low since a large proportion of the toxin remained monomeric (Fig. 2). Affinity of scFv73 to Cry1A toxins was in the range of 20–50 nM [16] in contrast with 1 nM of purified Bt-R_1 [10], suggesting that additional epitopes in the receptor may contribute to the binding interaction, or other receptors might be involved

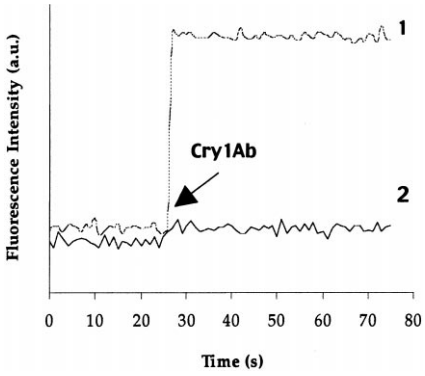

Fig. 3. ANS binding to Cry1Ab protoxin activated in the presence of scFv73 and MJ (1) or with MJ (2). Samples correspond to proteins shown in Fig. 2B, lanes 1 and 2 respectively. After 25 s 1.5 μg protein of the Cry1Ab samples was added.

Table 2
Toxicity of Cry1Ab-activated toxins to *M. sexta*

Treatment	LC_{50} (ng/cm^2)[a]
Protoxin Cry1Ab	1 (0.5–1.7)
Protoxin Cry1Ab+scFv73+MJ	1.6 (0.78–3.01)
Protoxin Cry1Ab+MJ	20.7 (14.3–67.4)

[a]LC_{50} values and (95% confidence limits) calculated by probit analysis.

(e.g. APN). Another possibility, to explain the low efficiency is that since scFv73 is degraded by the protease treatment, most of the Cry1Ab proteins loose scFv73 binding before a proper cleavage. The activation with BBMV was less efficient than with scFv73. It is likely that cadherin concentration in BBMV was low compared with the excess of scFv73 used (1/1 ratio). Also, the fact that BBMV were purified in the absence of proteinase inhibitors could reduce even more the concentration of active receptor. This remains to be analyzed.

Our data indicate that cadherin receptor binding is involved in proteolytic activation of Cry1Ab protoxin, promoting the formation of an oligomeric structure which exposes hydrophobic residues and is active for pore formation activity.

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