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Oligomer Formation of Different Cry Toxins Indicates that a Pre-Pore is an Essential Intermediate in the Mode of Action of the Three-Domain Cry Family

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We analyzed *in vitro* formation of Cry1Ab pre-pore oligomers. Our data suggests that a metalloprotease is involved in the cleavage of helix α-1 leading to oligomerization. Optimal oligomer formation was achieved by incubation of Cry1Ab crystals with a monoclonal antibody that mimics a cadherin epitope in a 1:4 ratio at pH 10.5 and in the presence of 5% *M. sexta* midgut juice. Oligomer formation with a high level of pore formation activity was obtained with different Cry1 and Cry3 toxins and with Cry11A toxins. Our data shows that pre-pore formation is a general step required for efficient membrane insertion of three-domain Bt toxins.

B. thuringiensis (Bt) produces parasporal crystalline inclusions that contain proteins (δ-endotoxins) toxic to a broad range of insect species and other invertebrates (1). The molecular mechanism that mediates the insecticidal activity of these toxins is still being elucidated. Basically, it has been described as a multi-step process, which begins upon ingestion of the parasporal inclusions by the susceptible larvae. Specific pH and proteases in the insect gut favor solubilization and proteolytic cleavage of the inclusions. The activated toxins bind to specific receptors on the insect midgut brush border membrane (1). Following binding, at least part of the toxin inserts into the membrane, resulting in pore formation (1). It has been proposed that oligomer formation is a necessary step for pore formation. The oligomeric structure is responsible for the formation of lytic pores (1) followed by cell lysis and insect death. Bt Cry1A toxins bind to two receptors, an aminopeptidase N (APN) protein and a cadherin-like protein (1). We showed that toxin binding depends on the toxin oligomeric sate. The monomeric toxin binds to the cadherin which induces proteolytic processing and oligomerization of the toxin (Fig. 1), while the oligomeric structure binds to the APN which drives the toxin into detergent-resistant membrane microdomains, resulting in pore formation (2, 3). The conformational changes from monomeric to a pre-pore oligomeric form lead to a structure that permits membrane insertion. Our data show that the oligomer, in contrast to the monomer, is able to interact efficiently with phospholipid membranes and to form stable pores (5).

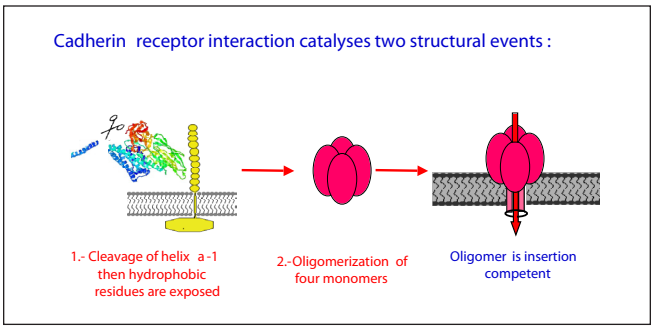

FIG. 1. Cry1A binding to cadherin receptor.

Oligomer formation *in vitro*

We have previously characterized a scFv antibody (scFv73) that inhibits binding of Cry1A toxins to the cadherin receptor, but not to the APN, and that reduces Cry1Ab toxicity to M. sexta larvae (3). Using scFv73 as a surrogate of the cadherin receptor, we demonstrated that binding of Cry1Ab to scFv73 facilitated proteolytic cleavage of helix α-1 of domain I and formation of a 250-kDa tetrameric pre-pore (3). Such oligomer was also formed when the Cry1Ab protoxin was incubated with midgut juice in the presence of M. sexta brush border membrane vesicles (BBMVs) containing native receptor molecules (3). To determine the optimal conditions leading to oligomer formation *in vitro*, we analyzed this process using a Cry1Ab protoxin and scFv73 mixture (1:4 ratio) incubated in the presence of 5% M. sexta midgut juice and revealed by SDS-PAGE electrophoresis and Western blot using an anti-Cry1Ab polyclonal antibody. Figure 2 shows that increasing amounts of 250-kDa oligomers were found when the

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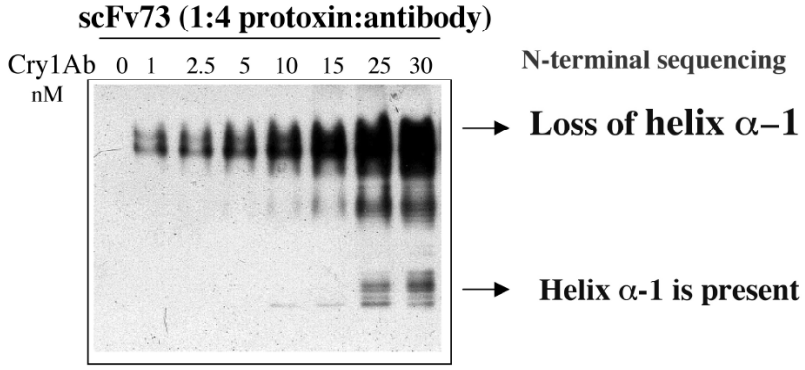

FIG. 2. Activation of Cry1Ab protoxin with M. sexta midgut juice in the presence of scFv73 antibody that mimics that of a cadherin receptor.

amount of Cry1Ab protein was increased. Additionally, we determined the effect of pH, of different Cry1Ab: scFv73 ratios and of different concentrations of midgut juice on oligomer formation. The optimal conditions for *in vitro* production of oligomer were found to be pH 10.5, 5% midgut juice and a Cry1Ab protoxin/scFv73 ratio of 1:4 at 37°C.

A metalloprotease is involved in oligomer formation

To characterize the protease involved in the cleavage of helix α-1 and the formation of the oligomer, *in vitro* oligomer formation was performed in the presence of protease inhibitors: cysteine proteases (E64), serine proteases (PMSF), aspartic proteases (pepstatin), elastase-like serine proteases (elastinal) and metalloproteases (EDTA). The only protease inhibitor that inhibited oligomer formation was EDTA, suggesting that a metalloprotease is involved in helix α-1 cleavage and in oligomer formation.

Different members of the 3D Cry family form pre-pore oligomers

To determine if the pre-pore structure is a general step taking place in the mode of action of three-domain Bt toxins, we analyzed the oligomer formation of different Cry toxins. In the case of Cry1 toxins, Cry1Aa, Cry1Ca, Cry1Da, Cry1Ea, Cry1Fa and Cry1Ga protoxins were proteolytically activated by midgut juice in the presence of *M. sexta* BBMVs. Except for Cry1Ga toxin, all these toxins are toxic to *M. sexta* larvae. Analysis of oligomer formation revealed that Cry1Ga did not form oligomers, in contrast to the other Cry1 toxins that produced oligomers in the presence of *M. sexta* BBMVs. Pore formation assays revealed that high level of pore formation activity correlated with oligomer formation.

Previous work demonstrated that activated Cry3 toxins (Cry3A, Cry3B and Cry3C) produced oligomers *in vitro* in the presence of coleopteran BBMVs (5). The oligomeric form of Cry3 toxins showed high pore formation activity in contrast to monomeric toxins (5). Furthermore, we investigated the possibility for the mosquitocidal Cry11Aa toxin to form oligomers in the presence of *Aedes aegypti* BBMVs or in the presence of Cyt1Aa, which was recently shown to be a functional Cry11Aa receptor (4). Cry11Aa oligomers were produced when the protoxin was activated with either trypsin or *M. sexta* midgut juice and in the presence of either *A. aegypti* BBMVs or Cyt1Aa toxin.

These results suggest that pre-pore oligomer formation is a general step of three-domain Bt toxins and that it is necessary for efficient membrane insertion.

Acknowledgments

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