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Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control

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Abstract

Bacillus thuringiensis Cry and Cyt protein families are a diverse group of proteins with activity against insects of different orders - Lepidoptera, Coleoptera, Diptera and also against other invertebrates such as nematodes. Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores. Among this group of proteins, members of the 3-Domain Cry family are used worldwide for insect control, and their mode of action has been characterized in some detail. Phylogenetic analyses established that the diversity of the 3-Domain Cry family evolved by the independent evolution of the three domains and by swapping of domain III among toxins. Like other pore-forming toxins (PFT) that affect mammals, Cry toxins interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding resulting in the formation of a pre-pore oligomeric structure that is insertion competent. In contrast, Cyt toxins directly interact with membrane lipids and insert into the membrane. Recent evidence suggests that Cyt synergize or overcome resistance to mosquitocidal-Cry proteins by functioning as a Cry-membrane bound receptor. In this review we summarize recent findings on the mode of action of Cry and Cyt toxins, and compare them to the mode of action of other bacterial PFT. Also, we discuss their use in the control of agricultural insect pests and insect vectors of human diseases.

Keywords

Cry; Cyt; *Bacillus thuringiensis*; receptor; mode of action; synergism

1. Introduction

Bacillus thuringiensis (Bt) are gram-positive spore-forming bacteria with entomopathogenic properties. Bt produce insecticidal proteins during the sporulation phase as parasporal crystals. These crystals are predominantly comprised of one or more proteins (Cry and Cyt toxins), also called δ-endotoxins. Cry proteins are parasporal inclusion (Crystal) proteins from *Bacillus thuringiensis* that exhibit experimentally verifiable toxic effect to a target organism or have significant sequence similarity to a known Cry protein. Similarly, Cyt proteins are parasporal inclusion proteins from *Bacillus thuringiensis* that exhibits hemolytic (Cytolitic) activity or

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has obvious sequence similarity to a known Cyt protein. These toxins are highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable. Therefore, Bt is a viable alternative for the control of insect pests in agriculture and of important human disease vectors (Bravo *et al.*, 2005).

Bt Cry and Cyt toxins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins undergoing conformational changes in order to insert into, or to translocate across, cell membranes of their host. There are two main groups of PFT: (i) the α-helical toxins, in which α-helix regions form the trans-membrane pore, and (ii) the β-barrel toxins, that insert into the membrane by forming a β-barrel composed of βsheet hairpins from each monomer (Parker and Feil, 2005). The first class of PFT includes toxins such as the colicins, exotoxin A, diphtheria toxin and also the Cry three-domain toxins. On the other hand, aerolysin, α-hemolysin, anthrax protective antigen, cholesterol-dependent toxins as the perfringolysin O and the Cyt toxins belong to the β-barrel toxins (Parker and Feil, 2005). In general, PFT producing-bacteria secrete their toxins and these toxins interact with specific receptors located on the host cell surface. In most cases, PFT are activated by host proteases after receptor binding inducing the formation of an oligomeric structure that is insertion competent. Finally, membrane insertion is triggered, in most cases, by a decrease in pH that induces a molten globule state of the protein (Parker and Feil, 2005).

2. Diversity, structure and evolution of Cry toxins

Cry proteins are specifically toxic to the insect orders Lepidoptera, Coleoptera, Hymenoptera and Diptera, and also to nematodes. In contrast, Cyt toxins are mostly found in Bt strains active against Diptera. The Cry proteins comprise at least 50 subgroups with more than 200 members. Cry proteins are defined as: a parasporal inclusion protein from Bt that exhibits toxic effects to a target organism, or any protein that has obvious sequence similarity to a known Cry protein (Crickmore *et al.*, 1998). Cyt toxins are included in this definition but it was agreed that proteins that are structurally related to Cyt toxins retain the mnemonic Cyt (Crickmore *et al.*, 1998). Primary sequence identity among different gene sequences is the bases of the nomenclature of Cry and Cyt proteins. Additionally, other insecticidal proteins that are not related phylogenetically to the three-domain Cry family have been identified. Among these, are binary-like toxins and Mtx-like toxins related to *B. sphaericus* toxins, and parasporins produced by *B. thuringiensis* (Crickmore *et al.*, 1998).

The members of the three-domain family, the larger group of Cry proteins, are globular molecules containing three structural domains connected by single linkers. One particular feature of the members of this family is the presence of protoxins with two different lengths. One large group of protoxins is approximately twice as long as the majority of the toxins. The C-terminal extension found in the long protoxins is dispensable for toxicity and is believed to play a role in the formation of the crystal inclusion bodies within the bacterium (de Maagd *et al.*, 2001). Cyt toxins comprise two highly related gene families (Cyt1 and Cyt2) (Crickmore *et al.*, 1998). Cyt toxins are also synthesized as protoxins and small portions of the N-terminus and C-terminus are removed to activate the toxin (Li *et al.*, 1996).

To date, the tertiary structures of six different three-domain Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba have been determined by X-ray crystallography (Fig. 1) (Li *et al.*, 1991;Grochulski *et al.*, 1995;Morse *et al.*, 2001;Galitsky *et al.*, 2001; Boomserm *et al.*, 2005; Boomserm *et al.*, 2006). All these structures display a high degree of similarity with a three-domain organization, suggesting a similar mode of action of the Cry three-domain protein family. The N-terminal domain (domain I) is a bundle of seven α-helices in which the central helix-α5 is hydrophobic and is encircled by six other amphipathic helices; and this helical domain is responsible for membrane insertion and pore-formation. Domain II consists

of three anti-parallel β-sheets with exposed loop regions, and domain III is a β-sandwich (Li *et al.*, 1991;Grochulski *et al.*, 1995;Morse *et al.*, 2001;Galitsky *et al.*, 2001; Boomserm *et al.*, 2005; Boomserm *et al.*, 2006). Exposed regions in domain II and domain III are involved in receptor binding (Bravo *et al.*, 2005). Domain I shares structural similarities with other PFT like colicin Ia and N and diphtheria toxin, supporting the role of this domain in pore-formation. In the case of domain II, structural similarities with several carbohydrate-binding proteins like vitelline, lectin jacalin, and lectin Mpa have been reported (de Maagd *et al.*, 2003). Domain III, shares structural similarity with other carbohydrate-binding proteins such as the cellulose binding domain of 1,4-β-glucanase C, galactose oxidase, sialidase, β-glucoronidase, the carbohydrate-binding domain of xylanase U and β-galactosidase (de Maagd *et al.*, 2003). These similarities suggest that carbohydrate moieties could have an important role in the mode of action of three-domain Cry toxins. Interestingly, in the nematode *C. elegans*, mutations in *bre* genes involved in the synthesis of certain glycolipids lead to Cry5 resistance showing that glycolipids are important receptor molecules of Cry5 (Griffits *et al.*, 2005).

Cyt proteins, on the other hand, have a single α-β domain comprising of two outer layers of α-helix hairpins wrapped around a β-sheet (Li *et al.*, 1996, Fig 1). Cyt toxin is structurally related to volvatoxin A2, a PFT cardiotoxin produced by a straw mushroom *Volvariella volvacea* (Lin *et al.*, 2004).

An analysis of the phylogenetic relationships of the isolated domains of members of the three-domain Cry family revealed interesting features regarding the creation of diversity in this protein family (Bravo, 1997; de Maagd *et al.*, 2001). Domains I and II have coevolved. The analysis of domain III sequences, revealed a different topology due to the fact that several examples of domain III swapping among toxins occurred (Bravo, 1997; de Maagd *et al.*, 2001). Some toxins with dual specificity (coleopteran, lepidopteran) are clear examples of domain III swapping among coleopteran and lepidopteran specific toxins. This suggests that domain III swapping could create novel specificities. In this regard, *in vitro* domain III swapping of certain Cry1 toxins resulted in changes in insect specificity (Bosch *et al.*, 1994; de Maagd *et al.*, 2000). The independent evolution of the three structural domains and domain III swapping among different toxins generated proteins with similar mode of action but with very different specificities (Bravo, 1997; de Maagd *et al.*, 2001).

3. Mode of action of three-domain Cry toxins in lepidopteran insects

The mode of action of Cry toxins has been characterized principally in lepidopteran insects. As mentioned previously, it is widely accepted that the primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells (Aronson and Shai, 2001; de Maagd *et al.*, 2001, Bravo *et al.*, 2005). Nevertheless, it has been recently suggested that toxicity could be related to G-protein mediated apoptosis following receptor binding (Zhang *et al.*, 2006). Cry proteins pass from crystal inclusion protoxins into membrane-inserted oligomers that cause ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding 60–70 kDa protease resistant proteins (Bravo *et al.*, 2005). Toxin activation involves the proteolytic removal of an N-terminal peptide (25–30 amino acids for Cry1 toxins, 58 residues for Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. Figure 2 shows a schematic representation of the Cry protoxin structure and their protease cleavege sites. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (de Maagd *et al.*, 2001; Bravo *et al.*, 2005) before inserting into the membrane. Toxin insertion leads to the formation of lytic pores in microvilli of apical membranes (Aronson and Shai, 2001; Bravo *et al.*, 2005). Subsequently cell lysis and disruption of the midgut epithelium releases the cell contents

providing spores a germinating medium leading to a severe septicemia and insect death (de Maagd *et al.*, 2001; Bravo *et al.*, 2005).

One interesting feature of Cry toxin activation is the processing of the N-terminal end of the toxins. The 3-dimensional structure of Cry2Aa protoxin showed that two α-helices of the N-terminal region occlude a region of the toxin involved in the interaction with the receptor (Morse *et al.*, 2001). Also, it was found that a Cry1Ac mutant that retained the N-terminus end after trypsin treatment binds nonspecifically to *M. sexta* membranes and was unable to form pores on *M. sexta* brush border membrane vesicles (BBMV) (Bravo *et al.*, 2002). Therefore, processing of the N-terminal end of Cry protoxins may unmask a domain II hydrophobic patch involved in toxin-receptor or toxin-membrane interaction.

3.1 Receptor binding in lepidopteran larvae

For Cry1A toxins, at least four different binding-proteins have been described in different lepidopteran insects; a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Vadlamudi *et al.*, 1995; Knight *et al.*, 1994; Jurat-Fuentes *et al.*, 2004; Valaitis *et al.*, 2001). Figure 3 shows a representation of the four types of putative Cry1A-receptor molecules characterized so far. The role of toxin-receptor interaction has been particularly well described in *Manduca sexta*. In this insect, at least two Cry1A-binding proteins, a CADR protein (Bt-R_1) and a GPI-anchored APN, have been described as receptors (Vadlamudi *et al.*, 1995; Knight *et al.*, 1994). Cadherins are transmembrane proteins with a cytoplasmic domain and an extracellular ectodomain with several cadherin repeats, 12 in the case of Bt-R_1 (Vadlamudi *et al.*, 1995). The ectodomain contains calcium-binding sites, integrin interaction sequences and cadherin binding sequences. Surface plasmon resonance experiments showed that the binding affinity of monomeric Cry1A toxins to the *M. sexta* Bt-R_1 is in the range of 1 nM (Vadlamudi *et al.*, 1995), while that of APN is in the range of 100 nM (Jenkins and Dean, 2000).

The interaction of Cry1A toxins with the Bt-R_1 receptor is rather complex involving at least three binding epitopes in the two molecules. Using a synthetic phage-antibody library, Gómez *et al.*, 2001, 2002a characterized an scFv antibody (scFv73) that binds to domain II loop 2 (β6-β7 loop) of Cry1A toxins. This antibody inhibited binding of Cry1A toxins to Bt-R_1 but not to APN. Sequence analysis of the CDR3H region of scFv73 led to the identification of an eight amino acid epitope in Bt-R_1 CADR repeat 7 (^{869}HITDTNNK^{876}) involved in toxin binding to domain II loop 2 of Cry1A toxins (Gómez *et al.*, 2001, 2002a). Additionally, another binding epitope in Bt-R_1 CADR repeat 11 (^{1331}IPLPASILTVTV^{1342}) that interacts with domain II loop α8 (α8a-α8b loop) and loop 2 of Cry1Ab toxin was identified (Gómez *et al.*, 2003). Finally, a third region in CADR repeat 12 of Bt-R_1 (residues 1363–1464) involved in Cry1Ab interaction and toxicity was reported (Hua *et al.*, 2004). In the case of the *Heliothis virescens* CADR, site-directed mutagenesis narrowed this binding region to residues 1422 to 1440 and shown to bind Cry1Ac domain II loop 3 (β10-β11 loop) (Xie *et al.*, 2005).

The most frequent mechanism of resistance to Cry toxins involves a change in receptor binding (Ferré and Van Rie, 2002). In the case of the laboratory selected *H. virescens* Cry1Ac-resistant line YHD2, it was shown that a single mutation was responsible for 40 to 80% of Cry1Ac resistance levels and shown to be linked to a retrotransposon insertion in the CADR gene (Gahan *et al.*, 2001). Also, characterization of CADR alleles in field-derived and laboratory selected strains of *Pectinophora gossypiella* (pink bollworm) and *Helicoverpa armigera* revealed different mutated CADR alleles that were associated with Cry1Ac resistance (Morin *et al.*, 2003; Xu *et al.*, 2005).

Regarding APN, Cry1Ac toxin binds to APN receptor by means of domain III that specifically recognizes N-acetylgalactosamine (GalNAc) moieties in contrast to Cry1Aa and Cry1Ab toxins that show no GalNAc binding capacities (Masson *et al.*, 1995). Based on the use of monoclonal antibodies that competed binding of Cry1Aa with *Bombyx mori* APN, the Cry1Aa-APN interacting epitopes were recently mapped in domain III β16 (^{508}STLRVN^{513}) and β22 (^{582}VFTLSAHV^{589}) residues (Atsumi *et al.*, 2005). Surface plasmon resonance binding studies of Cry1Ab mutants with pure *M. sexta* APN showed that domain II loop 2 and loop 3 are also involved in APN recognition (Jenkins and Dean, 2000). In the case of the lepidopteran insect *L. dispar*, a sequential binding mechanism was proposed in the interaction of Cry1Ac with APN (Jenkins *et al.*, 2000). Cry1Ac domain III first interacts with APN GalNAc sugar moieties facilitating the subsequent interaction of domain II loop regions with another region in this receptor (Jenkins *et al.*, 2000). In addition, it was described that a Cry1C-resistant *Spodoptera exigua* colony did not express the APN1 (Herrero *et al.*, 2005).

In the case of ALP receptor, it was demonstrated that in the *H. virescens* resistant line YHD2 part of the Cry1Ac resistant phenotype is due to mutations that lower the production of the GPI-anchored ALP receptor (Jurat-Fuentes *et al.*, 2004). In *M. sexta*, proteomic analysis of BBMV Cry1Ac-binding proteins also revealed ALP as a putative receptor molecule (McNall and Adang, 2003).

3.2 Pre-pore formation

As mentioned previously several PFT form a soluble oligomeric structure before membrane insertion. In the case of Cry1Ab toxin, binding of this toxin to *M. sexta* Bt-R_1 promotes an additional proteolytic cleavage in the N-terminal end of the toxin (helix α-1) facilitating the formation of a pre-pore oligomeric structure that is important for insertion into the membrane and for toxicity (Gómez *et al.*, 2002b; Rausell *et al.*, 2004a). Incubation of Cry1Ab protoxin with the single chain antibody scFv73 that mimics the CADR receptor or with the toxin-binding peptides of Bt-R_1 (CADR repeats 7 and 11), and treatment with *M. sexta* midgut juice, resulted in toxin preparations with the formation of a 250 kDa oligomer that lacked the helix α-1 of domain I (Gómez *et al.*, 2002b, 2003). It was reported that oligomeric structures of Cry1Ab and Cry1Ac increase 100–200 fold their binding affinity to the APN receptor, showing apparent dissociation constants of 0.75–1 nM (Gómez *et al.*, 2003, Pardo *et al.*, 2006). The oligomer, in contrast to the 60 kDa monomer, was membrane insertion competent as judged by measuring toxin membrane insertion using the intrinsic fluorescence of tryptophan residues and also by the analysis of membrane permeability using black lipid bilayers (Rausell *et al.*, 2004a). The pore activity of the Cry1Ab oligomeric structure analyzed in synthetic planar lipid bilayers revealed different kinetic characteristics from the monomeric Cry1Ab toxin. First, pore formation by pure oligomer preparations was observed at much lower toxin concentrations than the monomeric toxin and secondly, the kinetics were different since oligomeric Cry1Ab showed stable channels that had a high open probability in contrast to the monomeric toxin that showed an unstable opening pattern (Rausell *et al.*, 2004a). The formation of Cry oligomeric structures has been demonstrated for Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da, Cry1Ea, Cry1Fa and Cry3 toxins (Gómez *et al.*, 2002b; Rausell *et al.*, 2004a, 2004c; Muñoz-Garay *et al.*, 2006). In all cases, the Cry toxin samples containing oligomeric structures correlated with high pore activity, in contrast to monomeric samples that showed marginal pore-formation activity, supporting the hypothesis that oligomer formation is a necessary step in the mechanism of action of Cry toxins.

3.3 Membrane insertion

After exposure of BBMV to Cry1A toxins, these toxins were found associated with lipid rafts microdomains and it was reported that the integrity of these microdomains was essential for *in vitro* Cry1Ab pore-forming activity (Zhuang *et al.*, 2002). The Bt-R_1 receptor is located in

soluble membrane in contrast with APN and ALP receptors, which are attached to the membrane by GPI anchors, and are preferentially partitioned into lipid rafts (Zhuang *et al.*, 2002). Lipid rafts are detergent-insoluble lipid microdomains enriched in cholesterol and sphingolipids, and GPI-anchored proteins (Simons and Toomre, 2000). Like their mammalian counterparts, *H. virescens* and *M. sexta* lipid rafts are enriched in cholesterol, sphingolipids, and GPI-anchored proteins (Zhuang *et al.*, 2002). Lipid rafts have been implicated in membrane and protein sorting, and in signal transduction (Simons and Toomre, 2000). Also, they have been described as portals for different viruses, bacteria and toxins. Different bacterial PFT interact with receptors located in lipid rafts and this is a crucial step in the oligomerization and insertion of PFT into the membrane (Cabiaux *et al.*, 1997). In the case of Cry1A toxins, the APN receptor has been implicated in Cry1A toxin insertion, since cleavage of APN by phosphatidylinositol phospholipase C treatment, that cleaves out the GPI anchored proteins, substantially decreased the levels of Cry1Ab oligomer in insoluble membranes and reduced drastically the pore-formation activity of the toxin (Bravo *et al.*, 2004). In addition, APN incorporation into lipid bilayers enhanced Cry1Aa pore-formation activity (Schwartz *et al.*, 1997). Using tryptophan fluorescence analysis, structural changes observed after binding of the oligomeric Cry1Ac toxin to the APN receptor were studied by analyzing the binding of GalNAc to the Cry1Ac toxin, since GalNac is a binding determinant in the Cry1Ac-APN interaction (Pardo *et al.*, 2006). The *in vitro* interaction of GalNAc with oligomeric Cry1Ac induced a conformational change in the toxin and enhanced its insertion into lipid membranes indicating that the interaction of the pre-pore oligomer of Cry1A toxins with APN is important for facilitating membrane insertion (Pardo *et al.*, 2006).

It has been proposed that proteins must partially unfold to facilitate membrane insertion and channel formation. In the case of PFT active against mammalian cells, unfolding is triggered by acidic pH (Parker and Feil, 2005). Acidic pH could be encountered upon cell internalization of the toxins in acidic membrane compartments, and also in the membrane surface that has an acidic pH that could be up to 2 pH units lower than the bulk pH (Parker and Feil, 2005). Interestingly, lepidopteran and dipteran insects have a basic pH (up to pH 11) in their midgut lumen (Dow J.A.T. 1986). Unfolding analyses of pure Cry1Ab structures at different pHs demonstrated that the molten globe state of the pre-pore complex was induced by alkaline pH (Rausell *et al.*, 2004b). These analyses also showed that the pre-pore and membrane inserted oligomer, have a more flexible conformation than the monomeric toxin (Rausell *et al.*, 2004b). Although not proven, it may be possible that the conformational change observed after interaction of the pre-pore oligomer with APN could be related to molten globule state since, as mentioned previously, this interaction facilitates membrane insertion (Pardo *et al.*, 2006). Additionally, in the membrane-inserted pore, only domain I was protected from heat denaturation, suggesting that it may be inserted into the membrane in contrast to domains II and III (Rausell *et al.*, 2004b). Finally, the alkaline pH induced a looser conformation of the membrane-inserted domain I that is important for an active channel formation (Rausell *et al.*, 2004b).

Based on the data described above, we described a model involving the sequential interaction of Cry1A toxins with Bt-R_1 and APN receptor molecules. First, the interaction of monomeric Cry1A toxins with Bt-R_1 facilitates the formation of a pre-pore oligomeric structure that gains binding-affinity to APN, the pre-pore toxin binds APN, a conformational change occurs and a molten globule state of the toxin is induced, the pre-pore is inserted into lipid rafts inducing pore formation and cell swelling (Bravo *et al.*, 2004; Fig 4A).

4. Mode of action of Cry and Cyt toxins in mosquitoes

Bt subsp. *israelensis* (Bti) is highly toxic to different *Aedes*, *Culex* and *Anopheles* mosquito species that are vectors of human diseases (Margalith and Ben-Dov, 2000). This bacterium

produces crystal inclusions composed of Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba toxins (Berry *et al.,* 2002). As mentioned previously, the mosquitocidal active Cry proteins Cry11Aa, Cry4A and Cry4B share similar structures with the lepidopteran active toxin Cry1Aa suggesting a similar mode of action of these Cry proteins in mosquitoes.

As in lepidopteran insects, in mosquitoes the crystals ingested by susceptible larvae dissolve in the alkaline gut environment, releasing soluble proteins. In the case of the 70 kDa Cry11Aa protoxin, proteolytic activation using gut extract removes 28 residues from the N-terminal and cleaves intramolecularly resulting in 34 and 32 kDa fragments (Dai and Gill, 1993), but these two fragments remain associated and retain toxicity (Fig. 2) (Yamagiwa et al., 2004). This is also the case for the 130 kDa Cry4Ba protoxin where N-terminal, C-terminal and intra-molecular cleavage results in an active toxin comprised of two protein fragments of 18 kDa and 46 kDa (Fig. 2) (Angsuthanasombat *et al.,* 1993; Komano *et al.,* 1998). As mentioned previously, Cyt toxins are also synthesized as protoxins and small portions of the N-terminus and C-terminus are removed to activate the toxin (Armstrong *et al.,* 1985, Gill *et al.,* 1987; Li *et al.,* 1996). In the case of Cyt2Aa, 32 amino acid residues and 15 amino acid residues from the N-terminal and the C-terminal ends are removed by proteinase K treatment producing a monomeric protein with hemolytic activity (Koni and Ellar, 1994).

4.1 Receptor binding in mosquito midgut membranes

It is proposed that Cry toxins bind to specific protein receptors in the microvilli of the mosquito midgut cells. In contrast, Cyt toxins do not bind to protein receptors but directly interact with membrane lipids inserting into the membrane and forming pores (Thomas and Ellar, 1983; Gill *et al.,* 1987; Li *et al.,* 1996; Promdonkoy and Ellar 2003) or destroying the membrane by a detergent like interaction (Butko, 2003).

In the mosquitocidal Cry4A, Cry4B and Cry11Aa toxins, domain II loop regions have been implicated in toxin-receptor interaction and specificity. Cry4Ba shows no toxicity to *Culex* species in contrast to Cry4Aa toxin that is toxic to *Culex* larvae. Mutagenesis of loop 3 region of Cry4Ba to mimic that of Cry4Aa introduced toxicity to *Culex* species (Abdullah *et al.,* 2003). In addition, mutagenesis of loop 1 and loop 2 of Cry4Ba abolished toxicity to *Aedes* and *Anopheles* larvae (Abdullah *et al.,* 2003). Recently, it was demonstrated that redesigning the Cry1Aa domain II loop amino acid regions to resemble that of Cry4Ba resulted in a Cry1Aa mutant with moderate insecticidal activity against *C. pipiens* larvae (Liu and Dean, 2006). Also, in the case of Cry11Aa toxin, qualitative binding competition assays with synthetic peptides corresponding to predicted Cry11Aa domain II exposed regions revealed that loop α-8, β-4 region and loop 3 were important for binding to *Ae. aegypti* BBMV. Mutagenesis of putative loop α-8 residues confirmed that this region is important for Cry11Aa interaction with *Ae. aegypti* BBMV and toxicity (Fernández *et al.,* 2005). Overall these results show that domain II loop regions are very important for Cry toxin-receptor interaction in mosquitoes.

In the case of Cry11Aa and Cry4Ba mosquitocidal toxins, binding-proteins of 65 and 62 kDa were identified in BBMV from *Ae. aegypti* larvae by toxin overlay assays (Buzdin *et al.,* 2002). Recent work, identified three *Ae. aegypti* midgut proteins of 200 kDa, 100 kDa and 65 kDa that bound Cry11Aa toxin suggesting the presence of multiple receptor molecules for Cry toxins in the mosquito membranes. Of these proteins, the 100 and 65 kDa were shown to be anchored to the membrane by GPI (Fernández *et al.,* 2006). However, GPI-anchored proteins purified after Cry11Aa affinity chromatography revealed only the 65 kDa. This protein was identified as a GPI-anchored ALP enzyme (Fernández *et al.,* 2006). In addition, *Ae. aegypti* GPI-ALP protein was shown to be involved in the toxicity of Cry11Aa. Two peptide displaying phages, P1.BBMV and P8.BBMV, that specifically bound the 65 kDa ALP, competed with the binding of the toxin to BBMV, and interfered with the toxicity of Cry11Aa toxin (Fernández

et al., 2006). Overall these results show that the GPI-ALP is an important receptor molecule that mediates Cry11Aa toxicity to *Ae. aegypti* larvae.

In *Anopheles quadrimaculatus*, a GPI-anchored APN was identified as a putative Cry11Ba receptor (Abdullah *et al.,* 2006). This APN protein bound Cry11Ba with high affinity but did not interact with Cry4Ba or Cry11Aa toxins (Abdullah *et al.,* 2006).

As in lepidopteran insects, several Cry receptors in mosquitoes are attached to the membrane by GPI anchors, including ALP and APN (Fernández *et al.,* 2006; Abdullah *et al.,* 2006). Also, in the case of the Bin toxin produced by *B. sphaericus*, a GPI-anchored α-glucosidase is the functional receptor in *Culex pipiens* (Darboux *et al.,* 2001). As mentioned previously, these and other GPI-anchored proteins are proposed to be selectively included in lipid rafts. Therefore it seems that targeting GPI-anchored proteins may be a general strategy of PFT, including Cry toxins, to interact with their target cells.

Further work is needed to determine the role of Cry-receptor molecules in mosquitoes and if a pre-pore oligomeric structure is also a membrane-insertion intermediate in these toxins. However, the conserved structure of Cry toxins, the role of domain II in receptor interaction and the presence of multiple receptor molecules, some anchored to the membrane by GPI, suggests that the mode of action of Cry toxins in mosquitoes could be similar to that characterized in lepidopteran insects. Moreover, the role in intracellular signaling, as observed with the Cry1Ab toxin (Zhang *et al.,* 2006), has to be evaluated for mosquitocidal active Cry toxins.

4.2 Synergism of Cyt and Cry toxins

A major threat to the use of Bt is the appearance of insect resistance, which has been documented in the field with lepidopteran insects (Ferré and Van Rie, 2002). However, no resistance has been observed in the field in mosquito species controlled with Bti (Becker, 2000). The lack of resistance to Bti is due to the presence of the Cyt1Aa protein in the crystal (Georghiou and Wirth, 1997). *Culex quinquefasciatus* populations resistant to Cry4Aa, Cry4Ba or/and Cry11Aa have been selected under laboratory conditions but resistance to the Cry proteins could not be selected in the presence of Cyt1Aa protein (Wirth *et al.,* 1997). Also, Cyt1Aa suppresses the resistance of the *Cx. quinquefasciatus* Cry-resistant populations (Wirth *et al.,* 1997). In addition, synergism between Cyt1Aa and the Cry proteins of Bti has been observed (Chilcott and Ellar, 1988; Angsuthanasombat *et al.,* 1992; Georghiou and Wirth, 1997); the activity of the Bti crystals is much higher than that of the isolated proteins. Recently, it was demonstrated that Cyt1Aa protein synergizes Cry11Aa toxicity by functioning as a receptor molecule (Pérez *et al.,* 2005). Binding of Cry11Aa to *Ae. aegypti* BBMV was enhanced by membrane-bound Cyt1Aa and the high affinity interaction between Cyt1Aa and Cry11Aa in solution and in membrane bound state was determined. The epitopes of Cyt1Aa that bind Cry11A were identified. The role of loop β6-αE and part of β7 of Cyt1Aa in binding Cry11Aa was confirmed by heterologous competition assays using synthetic peptides corresponding to these regions and by site-directed mutagenesis (Pérez *et al.,* 2005). Previously, it was suggested that Cyt proteins insert into the membrane by means of the βsheets structures leaving the β6-αE loop exposed (Li *et al.,* 1996). Similarly, the loop α8, β4 and loop 2 regions of Cry11Aa were identified as the specific epitopes involved in the binding interaction with Cyt1Aa (Pérez *et al.,* 2005). As mentioned previously, the domain II loop α-8 is involved in the interaction of Cry11Aa with ALP receptor (Fernández *et al.,* 2006). Mutagenesis of specific amino acid residues in both toxins demonstrated that binding between these two toxins correlated with synergism (Pérez *et al.,* 2005). These data indicate that Cry11Aa binds to membrane-inserted Cyt1Aa resulting in the insertion of Cry11Aa into the mosquito membranes. This interaction of Cry11Aa is similar to its binding with its natural receptor. Consequently, this is the first example of an insect pathogenic bacterium that not only produces a toxin but also its functional

receptor, thereby promoting toxin binding to target membranes and toxicity to the mosquito (Pérez *et al.*, 2005). Figure 4B shows the proposed mode of action of Cry and Cyt in *Ae. aegypti*.

5. Applications of Cry toxins

Three major applications of Bt toxins have been achieved: (i) in the control of defoliator pests in forestry, (ii) in the control of mosquitoes that are vectors of human diseases, and (iii) in the development of transgenic insect resistant plants.

One of the most successful applications of Bt has been the control of lepidopteran defoliators, which are pests of coniferous forests mainly in Canada and United States. In both countries, the control of forests defoliators relies mostly on the use of Bt strain, HD-1, producing Cry1Aa, Cy1Ab, Cry1Ac and Cry2Aa toxins (van Frankenhuyzen, 2000; Bauce *et al.*, 2004). Successful application of Bt is highly dependent on proper timing, weather conditions and high dosage of spray applications. These factors combine to determine the probability of larvae ingesting a lethal dose (van Frankenhuyzen, 2000; Bauce *et al.*, 2004). The use of Bt in the control of defoliators has resulted in a significant reduction in the use of chemical insecticides for pest control in the forests.

As mentioned previously, Bti is highly active against disease vector mosquitoes like *Ae. aegypti* (vector of dengue fever), *Simulium damnosum* (vector of onchocerciasis) and certain *Anopheles* species (vectors of malaria). Its high insecticidal activity, the lack of resistance to Bti, the lack of toxicity to non-target organisms and the appearance of insect resistant populations to chemical insecticides resulted in a rapid implementation of Bti as an alternative control method of mosquito and black fly populations (Becker, 2000). In 1983, a control program for the eradication of onchocerciasis was launched in eleven countries of Western Africa using Bti since *S. damnosum* populations had developed resistance to larvicidal organophosphates (Guillet *et al.*, 1990). Presently, more than 80 % of this region is protected by Bti applications and 20 % with the chemical larvicide, temephos. Furthermore, control of onchocerciasis has protected over 15 million children without the appearance of black fly resistance to Bti (Guillet *et al.*, 1990). This success of vector control using Bti will certainly increase its use around the world. However, the low activity of Bti to certain vector mosquitoes, mainly Anophelines, will require the isolation of other Bt strains with novel *cry* genes more effective against these important disease vectors.

The development of transgenic crops that produce Bt Cry proteins has been a major break through in the substitution of chemical insecticides by environmental friendly alternatives. In transgenic plants the Cry toxin is produced continuously, protecting the toxin from degradation and making it reachable to chewing and boring insects. Cry protein production in plants has been improved by engineering *cry* genes with a plant biased codon usage, by removal of putative splicing signal sequences and deletion of the carboxy-terminal region of the protoxin (Schuler *et al.*, 1998). The use of insect resistant crops has diminished considerably the use of chemical pesticides in areas where these transgenic crops are planted (Qaim and Zilberman, 2003). Interestingly, the use of Bt-cotton in countries like China, Mexico and India showed that the use of this Bt-crop had a significant positive effect on the final yield and a reduction in the use of chemical pesticides, since in these countries the yield loss is mainly due to technical and economical constrains which are overcome in part by the use of insect resistant crops (Qaim and Zilberman, 2003; Toenniessen *et al.*, 2003).

6. Final remarks

The mode of action of Cry toxins is a multi-step process that involves the interaction with several receptor molecules leading to membrane insertion and cells lysis. The characterization

of the mode of action of Cry toxins in other susceptible organisms will be important to fully understand the mode of action of this family of proteins. Also, the identification of receptor molecules and binding epitopes will help in the development of strategies to cope with the potential problem of insect resistance. In addition, screening of novel Cry proteins with novel insect and receptor specificities will be fundamental for the development of novel products for the control of different insect pests.

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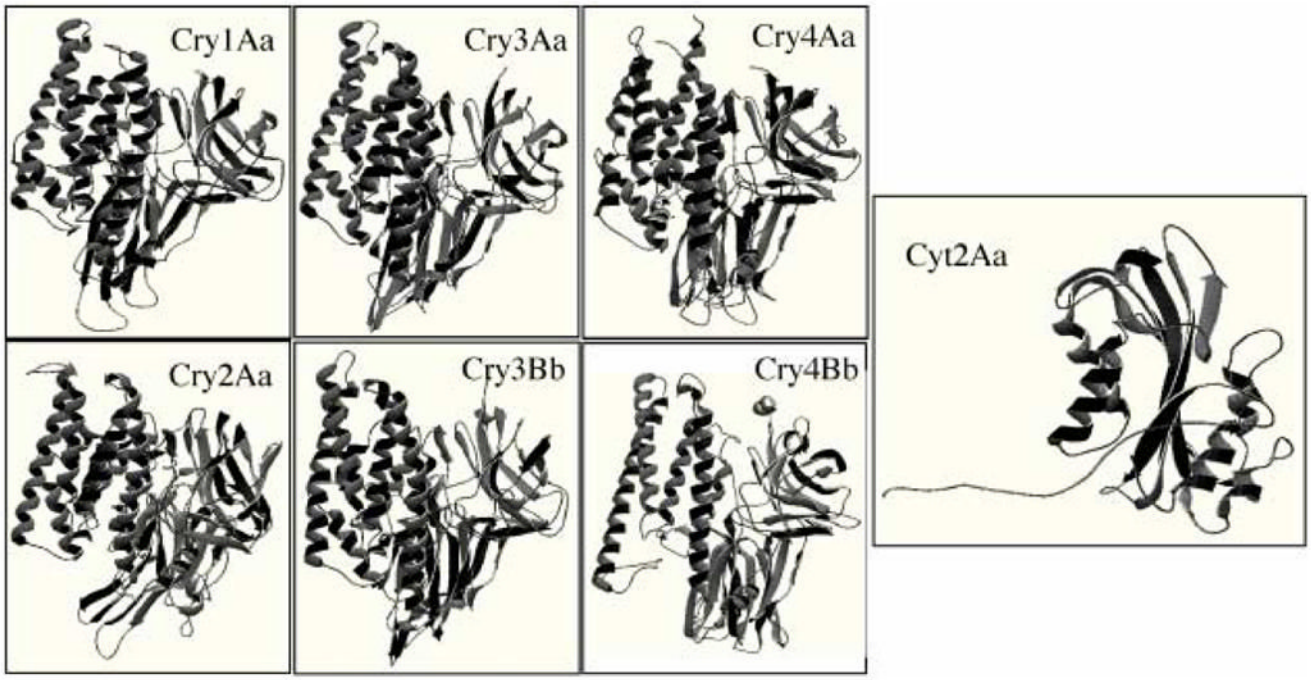

Figure 1.
Three dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis* Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Bb and Cyt2A.

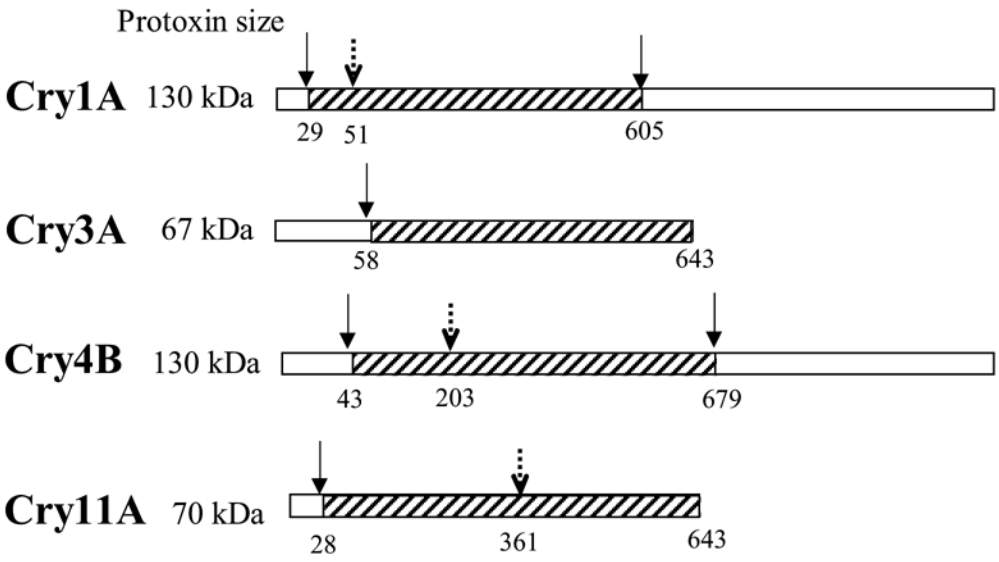

Figure 2.

Relative length of Cry protoxins and position of protease digestion. White boxes represent the protoxin and striped boxes represent the activated toxin. Solid arrows show the amino- and carboxy- terminal cleavage sites of the activated toxins. Doted arrows show the intramolecular cleavages. Cleavage of Cry1A at residue 51 resulted in loss of helix α-1 and pre-pore formation. Cleavage of Cry4B resulted in two fragments of 18 and 46 kDa, while Cry11A resulted in two fragments of 34 and 32 kDa.

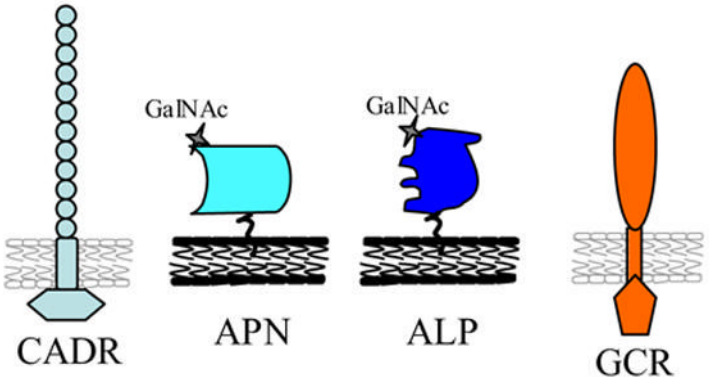

Figure 3.
Receptor molecules of Cry1A proteins. CADR, cadherin receptor; APN, aminopeptidase-N, ALP, alkaline phosphatase, GCR, 270 kDa glyco-conjugate receptor.

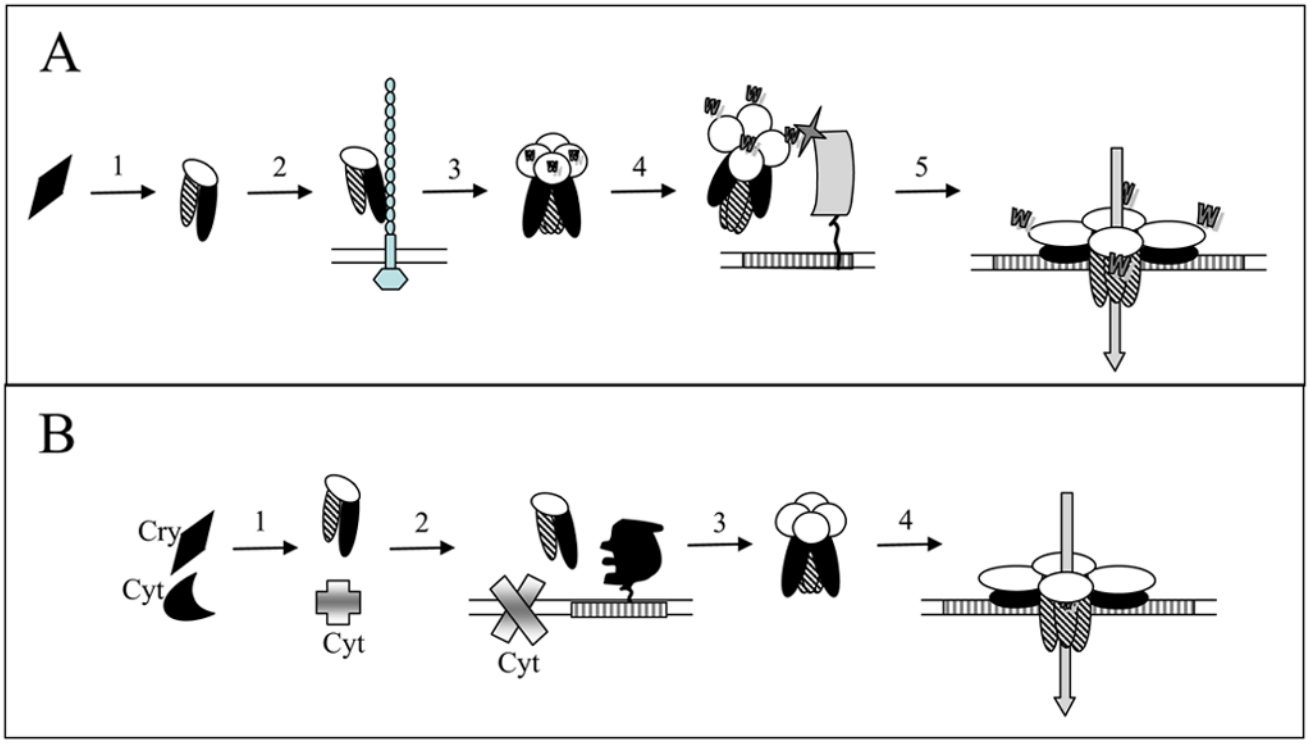

Figure 4.

Model of the mode of action of Cry and Cyt toxins. Panel A, sequential interaction of Cry toxins with different receptor molecules in lepidopteran larvae. (1) Solubilization and activation of the toxin; (2). Binding of monomeric Cry toxin to the first receptor (CADR or GCR), conformational change is induced in the toxin and α-helix 1 is cleaved; (3) Oligomer formation; (4) Binding of oligomeric toxin to second receptor (GPI-APN or GPI-ALP), a conformational change occurs and a molten globule state of the toxin is induced; (5) insertion of the oligomeric toxin into lipid rafts and pore formation. Panel B, role of Cyt and Cry toxins in the intoxication of dipteran larvae. (1) Cry and Cyt toxins are solubilized and activated; (2) Cyt toxin inserts into the membrane and Cry toxin binds to receptors located in the membrane (ALP or Cyt toxin); (3) oligomerization of the Cry toxin is induced; (4) oligomer is inserted into the membrane resulting in pore formation.