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Role of GPI-Anchored Membrane Receptors in the Mode of Action of *Bacillus thuringiensis* Cry Toxins

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1. Introduction

Insect pests are the major cause of damage to commercially important agricultural crops. Chemical pesticides have long-term detrimental effects, leading to irreversible damage to the environment and elimination of natural predators. Also, several hundred insect species have developed resistance to one or more chemical insecticides. There is, therefore, a need for environmentally safe pest control to maintain sustainability of the environment. *Bacillus thuringiensis* (Bt) emerged as a valuable biological alternative in pest control, because of its advantages of specific toxicity against target insects, lack of polluting residues and safety to non-target organisms such as humans, other vertebrates and plants, and is completely biodegradable. Bt has been used as a biopesticide in agriculture, forestry and mosquito control and accounts for 95% of the 1% market share of biopesticides in the total pesticide market. However, insect resistance against Bt has been reported in many cases. Insects develop resistance to insecticides through mechanisms that reduce the binding of toxins to gut receptors (de Maagd et al., 2001).

Bacillus thuringiensis Cry toxins have been widely used in the control of insect pests either as spray products or expressed in transgenic crops. These proteins are pore-forming toxins with a complex mechanism of action that involves the sequential interaction with several toxin-receptors. Cry toxins are specific against susceptible larvae and although they are often highly effective, some insect pests are not affected by them or show low susceptibility. In addition, the development of resistance threatens their effectiveness, so strategies to cope with all these problems are necessary. In this chapter we will discuss and compare the different proteins that are involved in the mechanism of action of Cry toxins with special emphasis on GPI-receptors: Aminopeptidases and alkaline phosphatases. We will discuss how the mechanism of toxin-receptor interaction has an important role to design new strategies to improve insecticidal activity of Cry toxins. In addition we will discuss other insect gut proteins that have recently been shown to bind Cry toxins and that may be involved in Cry toxin action.

2. Cry toxins

Bacillus thuringiensis (Bt) is an endospore-forming bacterium characterized by the presence of a protein crystal within the cytoplasm of the sporulating cell. Individual Cry toxin has a defined spectrum of insecticidal activity, usually restricted to a few species in one particular order of Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils), Hymenoptera (wasps and bees), and also to nematodes, respectively (Rajamohan et al., 1998). A few toxins have an activity spectrum that spans two or three insect orders. For example, Cry1Ba is most notably active against the larvae of moths, flies, and beetles. The combination of toxins in a given strain, therefore, defines the activity spectrum of that strain. 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 (Schnepf et al., 1998).

To date, the tertiary structures of seven different Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Ba and Cry8Ea have been determined by X-ray crystallography (Li et al., 1991; Grochulski et al., 1995; Galitsky et al., 2001; Morse et al., 2001; Boonserm et al., 2005; Boonserm et al., 2006; Guo et al., 2009). All these structures display a high degree of similarity with a three-domain organization (Figure 1), suggesting a similar mode of action of the Cry protein family even though they show very low amino acid sequence similarity. Cry toxins are classified by their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 67 groups (Cry1–Cry67). They are globular molecules composed of three structural domains connected by single linkers. Domain I, a seven α-helix bundle, is implicated in membrane insertion, toxin oligomerization and pore formation. Domain II is a beta-prism of three anti-parallel β-sheets packed around a hydrophobic core with exposed loop regions that are involved in receptor recognition, and domain III, is a β-sandwich of two anti-parallel β-sheets. Both domain II and III are implicated in insect specificity by mediating specific interactions with different insect gut proteins (Bravo et al., 2007).

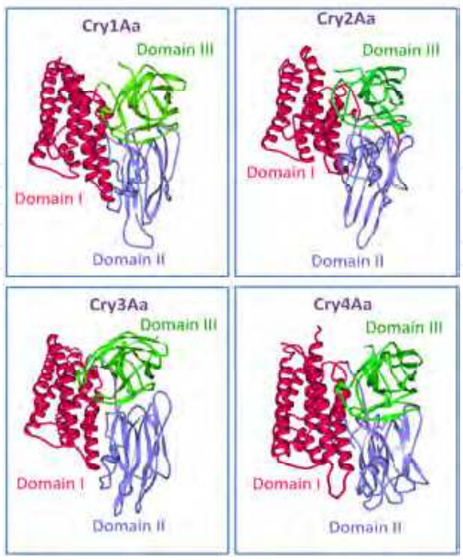

Fig. 1. Crystal structure of Cry1Aa (PDB code, 1CIY); Cry2Aa (PDB code, 1I5P); Cry3A (PDB code, 1DLC) and Cry4Aa (PDB code, 2C9K). Figures were generated using PyMol program.

2.1 Structure-function relationship

Domain I was immediately recognized as being equipped for pore formation, since shares structural similarities with other PFT like colicin Ia and N and diphtheria toxin, supporting the role of this domain in pore-formation. Isolated domain I fragments have been demonstrated to partition into model membranes to form pores (de Maagd et al., 2003); conversely, the domains II–III segment expressed without domain I was shown to bind to midgut membrane (Flores et al., 1997). Domain II was suspected to determine the specificity, because it represents the most divergent part of the toxin sequence, and exchanging domain II, or domains II and III, between closely related toxins has resulted in active hybrids showing altered specificity. Site-directed mutagenesis in its three hypervariable apical loops have identified residues involved in specific binding to membranes of several lepidopteran and coleopteran insects (Rajamohan et al., 1998; Schnepf et al., 1998). The specific binding consisted of reversible and irreversible steps, but only the irreversible binding is correlated with toxicity. More recent domain-exchange studies have found that toxicity of the hybrids to the insect host followed the movement of domain III, which would point to domain III as being responsible for the prerequisite step in toxicity, namely receptor binding. Site-directed mutagenesis in domain III located a small number of residues affecting membrane binding affinity and toxicity (de Maagd et al., 2001). However, direct observation of a toxin with a bound specificity determinant from the insect receptor is still needed to identify the receptor-binding site.

2.2 Molecular mode of action

The mode of action of Cry toxins has been characterized principally in lepidopteran insects and involves several steps and interactions with different receptors that depend on the oligomeric state of the toxin in a ping-pong binding mechanism. Cry1A toxins are produced as crystal inclusion bodies, which need to be ingested by the susceptible larvae to be toxic. These crystals are dissolved in the alkaline and reducing environment of the larval midgut, releasing soluble protoxins of 130 kDa. The inactive protoxins are then cleaved by midgut proteases yielding 60 kDa monomeric toxins (Soberon et al., 2009).

In figure 1, the activated monomeric toxins bind to highly abundant low affinity receptors, glycosylphosphatidylinositol (GPI)-anchored proteins, such as aminopeptidase N (APN) and alkaline phosphatase (ALP), localizing the toxin in the brush border microvilli. Specifically, loop 3 of domain II and β-16 of domain III are involved in this first interaction (Pacheco et al., 2009; Arenas et al., 2010). After this, the toxin binds to low abundant cadherin receptor, in a high affinity and complex interaction involving participation of loop 2, loop 3, and loop α-8 of domain II the toxin. Binding with cadherin facilitates additional protease cleavage of the N-terminal end of the toxin eliminating helix α-1 of domain I (Gomez et al., 2002). This cleavage induces assembly of an oligomeric form of the toxin. The conformational changes in toxin oligomers results in 100-fold increased binding affinity to APN and ALP receptors, through loop 2 (Bravo et al., 2004; Arenas et al., 2010). After the oligomers bind to these receptors they insert into membrane microdomains, creating pores in the apical membrane of midgut cells causing osmotic shock, bursting off the midgut cells and finally ending with the death of the insect (Soberon et al., 2009).

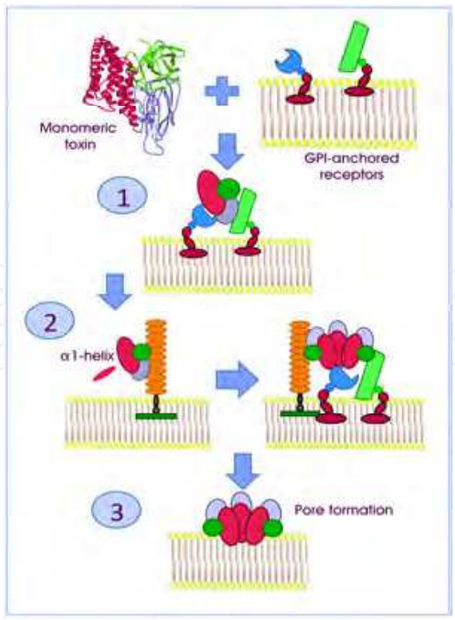

Fig. 2. Model of the mode of action of Cry toxins. 1) First interaction between monomeric toxin with low affinity-high abundant GPI-anchored receptors. 2) Second interaction occurs between monomeric toxin with cadherin receptor to induce oligomerization of the toxin. 3) The interaction of oligomeric toxin with GPI-anchored receptors result in pore formation.

2.3 Specifity=Binding receptor?

Although a given toxin has quite a narrow host range *in vivo*, *in vitro* many are non-specific and activated forms are even capable of forming pores in simple lipid bilayers. However, excessively high concentrations are required for lipid bilayer pore formation and the resulting pores have properties different than those formed in the presence of insect midgut membrane components. Many proteins have been identified in the midgut membrane that are capable of binding to Bt toxins but the ability to bind does not always correlate with susceptibility. For example, the pink bollworm (*Pectinophora gossypiella*) is susceptible to Cry1Ab and Cry1Ac toxins, both of which bind to a common site. A strain of pink bollworm (AZP-R) resistant to these two toxins could no longer bind Cry1Ab but, surprisingly, Cry1Ac binding was unaffected (Gonzalez-Cabrera et al., 2003). One explanation for such a result is that not all toxin binding is productive; that is, not all toxin binding results in pore-formation. Nevertheless, for several insect midgut proteins have been shown to have functional relevance to the toxic mechanism. The first is aminopeptidase N, whose expression in a *Drosophila* cell line resulted in an acquired susceptibility to toxin (Gill & Ellar 2002) and whose silencing by RNAi in a susceptible insect resulted in a reduced sensitivity to toxin (Rajagopal et al., 2002). Another membrane protein, cadherin, demonstrated a high toxin affinity, and mutations in cadherin genes result in a loss of sensitivity (Gahan et al., 2001; Morin et al., 2003).

3. Cry toxins receptors

As mentioned previously, Cry toxins are highly selective and kill only a limited number of insect species. This selectivity is mainly due to the interaction of Cry toxins with larval

proteins located in the midgut epithelium cells. The crucial role of this receptor binding for toxicity is emphasized by the observation that insects selected for resistance to a Cry toxin often have no or reduced binding capacity for that toxin(Ferre & Van Rie 2002). A major research effort has taken place in the identification of insect proteins that bind Cry toxins and mediate toxicity. Among these, two major types of receptors have been identified: transmembrane proteins, such as cadherins, and proteins anchored to the membrane such as the GPI-anchored proteins that have been proposed to be involved in the action of Cry toxins (Gomez et al., 2007).

3.1 What kind of molecules are receptors for Cry toxins?

After it was demonstrated that specific high-affinity toxin binding sites are present in the midgut of susceptible insects, efforts to identify and clone this molecules has been intensified. Several putative Cry toxin receptors have since been reported, of which the best characterized are the aminopeptidase N (APN) receptors and the cadherin-like receptors identified in lepidopterans. In nematodes, glycolipids are believed to be an important class of Cry toxin receptors. Other putative receptors include alkaline phosphatases (ALPs), a 270-kDa glycoconjugate, and a 252-kDa protein (Pigott & Ellar 2007).

Protein	Insect order	Species
Cadherin	Lepidoptera	*Manduca sexta, Heliothis virescens, Ostrinia nubilalis, Helicoverpa armigera, Bombix mori, Pectinophora gossypiella*
	Diptera	*Anopheles gambiae, Aedes aegypti*
	Coleoptera	*Tenebrio molitor* *Diabrotica virgifera virgifera*
BTR-270	Lepidoptera	*Lymantria dispar*
Chlorophyllide-binding protein (252 protein)	Lepidoptera	*Bombix mori*
ADAM 10 metalloprotease	Coleoptera	*Leptinotarsa decemlineata*

Table 1. Receptors of Cry toxins (non-GPI anchored to the membrane) described in different insects.

3.1.1 Cadherins

Cadherin proteins represent a large family of glycoproteins that are responsible for inter-cellular contacts. These proteins are composed of an ectodomain formed by 11 to 12 cadherin repeats (CR), a transmembrane domain and an intracellular domain (Bel & Escriche 2006). In the case of CADR of the lepidopteran *Manduca sexta*, it was shown that this protein is located in the microvilli of midgut cells (Chen et al., 2005). Cry1A toxins binds

to cadherin proteins of at least six lepidopteran species, *Manduca sexta*, *Bombyx mori*, *Heliothis virescens*, *Helicoverpa armigera*, *Pectinophora gossypiella* and *Ostrinia nubilalis* (Pigott & Ellar 2007).

The role of cadherin-like proteins as Cry1A toxin receptor was supported by the selection of a *Heliothis virescens* Cry1Ac-resistant line YHD2 that has a retrotransposon insertion mutation in the cadherin-like gene (Gahan et al., 2001). In addition, the characterization of CADR alleles in field-derived and in laboratory-selected Cry toxin-resistant strains of the cotton pest *Pectinophora gossypiella* (pink bollworm) revealed three mutated CADR alleles that were associated with Cry toxin resistance (Morin et al., 2003).

The interaction of Cry1A toxins with the CADR receptor is a complex process. Three regions in CADR proteins have been shown to interact with three domain II loop regions. Cry1Ab loop 2 interacts with CADR residues ^{865}NITIHITDTNN^{875} located in repeat 7, whereas loops α-8 and 2 interact with CADR residues ^{1331}IPLPASILTVTV^{1342} located in repeat 11. A third Cry1A binding region was located in CADR in the repeat 12. In the case of *H. virescens* cadherin, this binding epitope was narrowed to ^{1423}GVLTLNFQ^{1431} and demonstrated that it interacts with Cry1Ac domain II loop 3 (Gomez et al., 2007).

A cadherin protein from *T. molitor* was identified as a Cry3Aa binding protein, and it was shown to facilitate Cry3Aa oligomer formation. Moreover, silencing of the cadherin gene by feeding dsRNA showed that the silenced beetles were resistant to Cry3Aa indicating an active role of cadherin on Cry3Aa toxicity (Fabrick et al., 2009). A cadherin protein was also identified as a Cry3Aa receptor in *Diabrotica virgifera virgifera*. A fragment of this cadherin protein containing the membrane proximal cadherin repeats 8-10 bound Cry3Aa and Cry3Bb toxins with high affinity (Kd of 12 and 1.4 nM, respectively) and enhanced Cry3Aa and Cry3Bb toxicity to different coleopteran insects (Park et al., 2009).

As in lepidopteran insects, cadherin proteins have been identified in *Ae. aegypti* and *An. gambiae* showing binding to Cry11Aa and Cry4Ba respectively (Hua et al., 2008; Chen et al., 2009). In *Ae. aegypti*, cadherin also serves a receptor of Cry11Ba toxin that was isolated from the Bt var *jegathesan* strain but showed lower affinity to Cry4Ba protein (Gill et al., 2011). An *An. gambiae* cadherin fragment containing the Cry4Ba binding site enhanced the toxicity of Cry4Ba in both *An. gambiae* and *Ae. aegypti* larvae suggesting its active role as a receptor of Cry4Ba in these mosquitoes species. In the case of *Ae. aegypti* cadherin, it was shown that an anti-cadherin antibody competed binding of Cry11Aa to *Ae. aegypti* BBMV. In both *Ae. aegypti* and *An. gambiae*, cadherin is located in the microvilli of the caeca and in the microvilli of the posterior gut cells, that are the same sites where Cry11Aa and Cry4Ba bind (Hua et al., 2008; Chen et al., 2009; Park et al., 2009)

3.2 Other molecules

In lepidopteran insects, another proteins and molecules different from cadherin have been identified as a 270 kDa glycoconjugate and a 250 kDa protein named P252 (Pigott & Ellar 2007).

The 270 kDa glycoconjugate was identified as Cry1Ac binding protein in *L. dispar* (Valaitis et al., 2001). Recently *B. mori* P252 that binds Cry1Ac was identified as a choraphyllide-binding protein (Pandian et al., 2008). In addition, glycolipids were proposed to act as Cry toxin receptors in lepidopteran insects as was demonstrated for the nematode *Caenorhabditis*

elegans (Griffitts et al., 2005). In the case of *L. decemlineata*, an ADAM 3 metalloprotease was identified as Cry3Aa receptor. Binding of Cry3Aa to ADAM-3 through domain II loop 1 enhanced Cry3Aa pore-formation activity suggesting that this binding interaction is important for Cry3Aa toxicity (Ochoa-Campuzano et al., 2007). The only GPI-anchored protein identified in coleopteran insects as a putative Cry receptor was an ALP from *A. grandis* that bound Cry1B toxin (Martins et al., 2010).

3.2.1 GPI-anchored receptors: APN, ALP and glucosidase

Glycosylphosphatidylinositol-anchored proteins (GPI proteins) are eukaryotic exoplasmic membrane proteins that play very diverse biological functions including hydrolytic enzyme activity, transmembrane signaling, intracellular sorting and cell adhesion interaction. Besides these biological functions, GPI proteins such as alkaline phosphatases (AP-GPI), 5-nucleotidase, dipeptidase, aminopeptidase P, have been used primarily as markers of plasma membranes during their purification procedure. Later, most of GPI proteins were found to be resistant to Triton X-100 solubilization at low temperature in kidney brush border membranes, whereas transmembrane enzymes were solubilized (Nosjean et al., 1997). Similar observations were done on brush border membranes from insects where APN and ALP are both GPI-anchored proteins; these proteins are proposed to be selectively included in lipid rafts that are conceived as spatially differentiated liquid-ordered microdomains in cell membranes. The APN and ALP in *M. sexta* and *H. virescens*, in contrast to the CADR receptors, were shown to be located in lipid rafts. The interaction of pore-forming toxins with lipid rafts could result in additional cellular events, including toxin internalization, signal transduction and cellular response (Zhuang et al., 2002; Bravo et al., 2004). In table 2 are presented the GPI-ancored proteins identified as Cry toxins receptors.

The first Cry1A toxin-binding protein that was described was an APN protein in the lepidopteran *M. sexta*. This protein was glycosilated and anchored to the membrane by a GPI anchor. Since then, other GPI-anchored APNs have been recognized as Cry toxin receptors in different lepidopteran species such as *H. virescens*, *Spodoptera litura*, *H. armigera*, *B. mori*, *Lymantria dispar*, *Plutella xylostella*, and in the dipteran *An. Quadrimaculatus*, and *A. aegypti*. Phylogenetic analyses suggest that in lepidopteran insects there are at least five different APN families and at least three of them have been shown to bind Cry1 toxins in different insect species (Gomez et al., 2007; Pigott & Ellar 2007).

The APN has been implicated in toxin insertion, since cleavage of APN by phosphatidyl-inositol specific phospholipase C treatment which cleaves out the GPI anchored proteins substantially decreased the levels of Cry1Ab incorporation into insoluble lipid raft membranes (Bravo et al., 2004) and drastically reduced the pore formation activity of the toxin assayed in BBMV from *Trichoplusia ni* (Lorence et al. 1997). In addition, the incorporation of APN into the lipid bilayer enhanced Cry1Aa pore formation activity (Schwartz et al., 1997). The sugar GalNAc in the APN receptor is an important epitope in the interaction with Cry1Ac toxin (Burton et al., 1999; de Maagd et al., 1999). In the case of the lepidopteran *Lymantria dispar*, it was proposed that the monomeric Cry1Ac toxin interacts with APN following a sequential binding model. In this model, APN is first recognized by domain III of Cry1Ac through the GalNAc moiety, followed by a protein-protein contact of the domain II loop region of Cry1Ac. The first contact is fast and reversible, and mutations close to a domain III cavity affect this initial binding, while mutations in domain II affect the

rate constants of the second interaction step which is slower and tighter (Jenkins et al., 2000). Li et al. reported that the binding of GalNAc to monomeric Cry1Ac correlates with an increase of temperature factors in the pore-forming domain I. However, there was no indication of a clear conformational change in the monomeric-Cry1Ac toxin (Li et al., 2001). In contrast, the fluorescence spectroscopy studies of Cry1Ac in its oligomeric state showed that GalNAc induces a conformational change in domain III of the oligomeric structure of Cry1Ac in the vicinity of the sugar pocket. The interaction of Cry1A-oligomer with GalNAc enhanced membrane insertion of the soluble pre-pore oligomeric structure, supporting the model that interaction of the Cry1A pre-pore with GPI-anchored receptors facilitates membrane insertion and pore-formation. The APN-oligomer interaction may be especially critical when low toxin protein concentrations reach the midgut epithelium, conditions that may occur in vivo in the larvae gut where the Cry toxins are exposed to high concentration of proteases (Pardo-Lopez et al., 2006).

A resistant *S. exigua* population that is resistant to Cry1Ca toxin was shown to lack the RNA transcript of APN-1, suggesting that this APN is involved in Cry1C toxicity to this insect species (Herrero et al., 2005). Finally, in the case of *S. litura*, silencing an APN with dsRNA resulted in a lower susceptibility to Cry1Ca toxin, also indicating a role of APN in Cry1C toxicity in this insect species (Herrero et al., 2005).

Two APN isoforms (AaeAPN1 and AaeAPN2) were identified in *Ae. aegypti* by Cry11Aa pull down experiments. Protein fragments from both APN isoforms were produced in *E. coli* and shown to inhibit binding of Cry11Aa to BBMV, suggesting their active role in Cry11Aa binding to insect membranes (Chen et al., 2009). In the case of *An. gambiae* and *An. quadrimaculatus* larvae, two APN's were also identified as Cry11Ba binding proteins. Interestingly, Cry11Ba binds both *An. quadrimaculatus* and *An. gambiae* APN molecules with a very high binding affinity of 0.56 nM and 6.4 nM respectively (Abdullah et al., 2006; Zhang et al., 2008). These results suggest that APN may have a more important role in the toxicity of Cry11Ba in these two Anopheline species. In fact, it was recently shown that certain *A. gambiae* APN protein fragments enhanced Cry11Ba toxicity as has been shown for cadherin protein fragments (Zhang et al., 2008).

In regard to the APN binding epitopes involved in Cry toxin interaction, a region of 63 residues (I135-P198) involved in Cry1Aa binding was identified in *B. mori* APN1 (Nakanishi et al., 2002). The domain III of Cry toxins is involved in the APN–Cry interaction, as shown by the interchange of domain III between Cry1Ac and Cry1Ab toxins (de Maagd et al., 1999). Domain III residues of Cry1Aa ^{508}STRVN^{513} and ^{582}VFTLSAHV^{589} were shown to be involved in binding I135-P198 APN fragment (Atsumi et al., 2005).

ALPs are found in all animals and as is expected are mainly localized in microvilli of columnar cells and of insect midgut epithelium cells (Eguchi 1995). ALPs can be divided into two groups: soluble (s-ALP) and membrane-bound (m-ALP) (Eguchi et al., 1990; Itoh et al., 1991). In insects, both ALPs are found in larval midgut epithelium cells; however, they are expressed in different cell types. The s-ALP is found exclusively in the cavity of goblet cells and in the apical region of the midgut; whereas, m-ALP is localized in the brush border membrane of columnar cells and particularly restricted to the middle and posterior midgut. Moreover, s- and m-ALPs show distinct differences in enzymatic activity (such as optimal pH) and also the structure of sugar side chain, suggesting that they perform different functions *in vivo* (Eguchi et al., 1990).

GPI-anchored Cry toxin-binding receptors [a]				
ORDER	**SPECIES**	**PROTEIN**	**BINDING TOXIN**	**IDENTIFICATION METHOD**
Lepidoptera	***Manduca sexta***	Class 1 APN	Cry1Ac[11], Cry1Aa, Cry1Ab[14]	Affinity chromatography [11], Chromatography purification, ligand blot, SPR [14]
		Class 2 APN	Cry1Ab5 [5]	Affinity chromatography, ligand blot [5]
		ALP	Cry1Ac [15]	PLPC treatment, 2D-SDS-PAGE-Mass spectrometry [15]
	Bombix mori	Class 1 APN	Cry1Aa [23], Cry1Aa, Cry1Ab [16]	Ion exchange chromatography purification [23], Heterologous expression, toxin overlay assay [16]
		Class 3 APN, Class 4 APN	Cry1Aa, Cry1Ab [16]	Heterologous expression, toxin overlay assay [16]
	Helicoverpa armigera	Class 1 APN	Cry1Aa, Cry1Ab, Cry1Ac [18]	Heterologous expression, toxin overlay assay [18]
		Class 3 APN	Cry1Ac [18]	Heterologous expression, ligand blot [18]
		ALP	Cry1Ac [21]	Anion exchange chromatography [21]
	Heliothis virescens	Class 1 APN	Cry1Aa, Cry1Ab, Cry1Ac [13], Cry1Fa [3]	Affinity chromatography, SPR [13] Affinity chromatography, ligand blot [3]
		Class 3 APN	Cry1Ac [9]	Affinity chromatography, ligand blot [9]
		Class 4 APN	Cry1Fa [3]	Affinity chromatography, SPR [3]
		ALP	Cry1Ac [10]	PLPC treatment, western blot [10]
	Lymantria dispar	Class 1 APN	Cry1Ac [22]	Immunolocalization [22]
		Class 3 APN	Cry1Ac [8]	Chromatographic purification [8]
	Plutella xylostella	Class 1 APN, Class 2 APN, Class 3 APN, Class 5 APN	Cry1Aa, Cry1Ab [16]	Heterologous expression, toxin overlay assay [16]
	Spodoptera litura	Class 3 APN, Class 5 APN	Cry1C [2]	*In silico* identification, heterologous expression, toxin overlay assay [2]
	Epiphyas postvittana	Class 3 APN	Cry1Aa, Cry1Ba [19]	Ion exchange chromatography purification [19]
Diptera	***Aedes aegypti***	AaeAPN1, AaeAPN2	Cry11Aa [4]	Pull-down, heterologous expression, ligand blot [4]
		APN	Cry4Ba [4]	2D SDS-PAGE, ligand blot [4]
		APN2778, APN2783, APN5808	Cry4Ba [20]	*In silico* identification, RNA interference [20]
		ALP	Cry4Ba [4], Cry11Aa [7]	2D SDS-PAGE, ligand blot [4], PLPC treatment, ligand blot [7]
	Anopheles albimanus	α-amilase	Cry4Ba, Cry11Aa [8]	PLPC treatment, ligand blot [8]
	Anopheles quadrimaculatus	APN	Cry11Aa [1]	Anion-exchange chromatography, enzymatic activity, SPR [1]
	Anopheles gambiae	APN	Cry11Ba [24]	*In silico* identification, heterologous expression, PLPC treatment, anion- exchange chromatography [24]
Coleoptera	***Anthonomus grandis***	ALP	Cry8Ka5 [17]	2D-SDS-PAGE, ligand blot, mass spectrometry [17]

(a) Compiled from: (1) Abdulah et. al., 2006; (2) Agrawal et. al., 2002; (3) Banks et. al., 2001; (4) Bayyareddy et. al., 2009; (5) Chen et. al., 2009; (6) Denolf et. al., 1997; (7) Fernández et. al., 2006; (8) Fernández-Luna et. al., 2010; (9) Garner et. al., 1999; (10) Gill et. al., 1995; (11) Jurat-Fuentes & Adang 2004; (12) Knight et. al., 1994; (13) Luo et. al., 1997; (14) Masson et. al., 1995; (15) McNall & Adang 2003; (16) Nakanishi et. al., 2002; (17) Nakasu et. al., 2010; (18) Rajagopal et. al., 2003; (19) Simpson & Newcomb 2000; (20) Saengwiman et. al., 2011; (21) Santosh & Singh P, 2011; (22) Valaitis et. al., 1997; (23) Yaoi et. al., 1997; (24) Zhang et. al., 2008.

Table 2. Description of GPI-anchored receptors founded in insects that bind to Cry toxins.

A GPI-anchored ALP that binds Cry toxins has been described in the lepidopterans *M. sexta*, *H. virescens* and *H. armigera*; in the dipteran *Ae. aegypti*, and in the coleopteran *Anthonomus grandis* (McNall et al., 2003; Jurat-Fuentes et al., 2004; Martins et al., 2010; Ning et al., 2010). In the case of *M. sexta*, ALP protein that bind Cry1A toxins was shown to be located in the microvilli of epithelial cells that is the site of action of Cry1A toxins (Chen et al., 2005). Also, an *H. virescens* laboratory selected population, YHD2, contained a retrotransposon insertion in the cadherin gene (Gahan et al., 2001). However, the mutation in the cadherin gene only accounted for 40-80% of the resistance phenotype. Additional mutations were responsible for the rest of the resistant phenotype in YHD2. These additional mutations were shown to affect GPI-ALP production, indicating that ALP is likely a functional receptor of Cry1Ac toxin in *H. virescens* (Jurat-Fuentes & Adang 2004).

Blocking the interaction of Cry toxins with GPI-anchored receptors has been useful in some cases to show the role of these proteins in Cry insecticidal activity. In the case of *M. sexta*, a scFv-phage that bound Cry1Ab toxin through β16-β22 of domain III blocked binding of Cry1Ab with APN but not with Bt-R1 and inhibited the toxicity of Cry1Ab in bioassays (Gomez et al., 2006). Nevertheless, in *B. mori* detached midgut cells, an anti-APN antibody did not affect toxicity of Cry1Aa in contrast to an anticadherin antibody that inhibited toxicity, suggesting either that this APN may not be involved in toxicity or that other additional GPI-anchored proteins or lipids could substitute APN function (Hara et al., 2003; Ibiza-Palacios et al., 2008). In *Ae. aegypti*, a peptide-phage that bound the 65 kDa ALP competed binding of the Cry11Aa to BBMVof mosquito and inhibited Cry11Aa toxicity in bioassays, suggesting that GPI-anchored ALP is a functional receptor of Cry11Aa (Fernandez et al., 2006).

Recently, ligand blot assays indicated that a 70 kDa GPI-anchored protein present in midgut brush border membrane vesicles of *A. albimanus* interacts with Cry4Ba and Cry11Aa toxins. This protein was identified as an a-amylase by mass spectrometry and enzymatic activity assays (Fernandez-Luna et al., 2010).

The fact that similar Cry binding proteins are involved in the mechanism of action of Cry toxins in both lepidopteran and dipteran insects suggests the Cry toxins have a conserved mode of action. However, the precise role of the Cry toxins receptors identified in mosquitoes in the mode of action of Cry toxins still remains to be determined. As in lepidopteran insects cadherin binding might facilitate oligomer formation while binding of Cry oligomer to GPI-anchored ALP or APN receptors might be necessary to facilitate membrane insertion. Nevertheless, the high binding affinity of Anopheline APN's to Cry11Ba is substantially different from what has been reported in lepidopteran insects and further studies on the differential role of APN and cadherin in monomer/oligomer binding in mosquitoes are necessary to determine the precise role of toxin binding to these receptor molecules.

3.3 Other insect molecules that bind Cry toxins identified using proteomic approaches

Proteomic approaches based on two-dimensional (2D) gel electrophoresis and mass spectrometry have been used to discover novel Bt toxin binding proteins and elucidate changes in midgut proteins associated with Bt resistance. Using this approach ALP was identified as a Cry1Ac binding protein in brush border of *Manduca sexta* and *Heliothis virescens* (McNall & Adang 2003; Krishnamoorthy et al., 2007). This identification was validated in *H. virescens* when ALP was demonstrated as a functional receptor molecule and

loss of the enzyme correlated with Bt resistance to Cry1Ac (Jurat-Fuentes & Adang 2004). Candas et al. used differential-in-gel electrophoretic (DIGE) analysis to compare Bt susceptible and resistant larvae of *Plodia interpunctella*. These authors detected increased levels of midgut enzymes associated with oxidative metabolism and altered migration of an F_1F_o-ATPase in resistant larvae when compared to susceptible proteins on 2D gels. Candas et al. also detected reduced levels of alkaline chymotrypsin in the resistant *P. interpunctella* larvae associated with reduced capacity for protoxin activation (Candas et al., 2003).

Additional Cry1Ac binding proteins in lepidopteran brush border preparations detected by 2DE ligand blots approach includes actin, aminopeptidase, vacuolar-ATPase subunit A and a desmocollin-like protein (McNall & Adang 2003; Krishnamoorthy et al., 2007). A proteomics-based approach using differential-in-gel electrophoretic (DIGE) analysis quantified altered levels of specific proteins in Bt susceptible and resistant larvae of *Plodia interpunctella* (Candas et al., 2003). Those authors detected changes in the levels of APN, V-ATPase and an F_1F_0-ATPase in resistant larvae.

The analysis of Cry4Ba binding proteins by mass spectrometry in *Ae. aegypti* BBMV, revealed two lipid rafts associated proteins, flotillin and prohibitin, as well as cytoplasmic actin, besides ALP and APN, thus suggesting that additional proteins as well as intracellular proteins may have an active role in the mode of action of Cry toxins in mosquitoes (Bayyareddy et al., 2009).

Protein	Insect	Reference
ABC transporter	*Heliothis virescens* (Lepidoptera)	Gahan *et. al.,* 2010
	Helicoverpa armigera (Lepidoptera)	Chen *et. al.,* 2010
V-ATPase	*Heliothis virescens* (Lepidoptera) (A subunit)	Krishnamoorthy *et. al.,* 2007
	Anthonomus grandis (Coleoptera) (A subunit)	Nakasu, 2010
	Helicoverpa armigera (Lepidoptera) (B subunit)	Chen *et. al.,* 2010
	Aedes aegypti (Diptera) (E subunit)	Bayyareddy *et. al.,* 2009
Heat Shock Proteins	*Helicoverpa armigera* (Lepidoptera)	Chen *et. al.,* 2010
	Anthonomus grandis (Coleoptera)	Nakasu, 2010
Actin	*Manduca sexta* (Lepidoptera)	McNall *et al.*, 2003
	Heliothis virescens (Lepidoptera)	Krishnamoorthy, *et al.*, 2007
	Helicoverpa armigera (Lepidoptera)	Chen *et. al.,* 2010
	Aedes aegypti (Diptera)	Bayyareddy *et al.*, 2009
ATP sintase	*Aedes aegypti* (Diptera)	Bayyareddy, *et al.*, 2009
Flotilin	*Aedes aegypti* (Diptera)	Bayyareddy *et al.*, 2009
Prohibitin	*Aedes aegypti* (Diptera)	Bayyareddy *et al.*, 2009

Table 3. Receptors of Cry toxins described in different insects using proteomic analysis.

3.4 How these receptors are involved in resistance to Cry toxins

Resistance to Cry toxins can be developed by mutations in the insect pests that affect any of the steps of the mode of action of Cry toxins. The most common mechanism of toxin resistance in insect pests until now is the reduction in toxin binding to midgut cells, that in different insect species include mutations in Cry toxin receptors (Gahan et al., 2001; Ferre & Van Rie 2002; Morin, Biggs et al. 2003; Zhang, Hua et al. 2008). In fact, the most frequently phenotype of insect resistance, denoted as "Mode 1 of Resistance", is characterized by the reduction of one Cry1A toxin binding, cross resistance of Cry1Aa, Cry1Ab and Cry1Ac and lack of resistance to Cry1C. In several lepidopteran insects, the mode 1 of resistance is linked to mutations in the cadherin gene (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). In field conditions three lepidopteran insect pests have evolved resistance to formulated Bt products, *Plodia interpunctella*, *Plutella xylostella* and *T. ni* [McGaughey, 1985; Tabashnik, et al, 1994; (Janmaat & Myers, 2003). In recent years, at least four cases of resistance to Bt crops have been documented, *H. zea* to Bt-cotton expressing Cry1Ac in United States, *S. frugiperda* to Bt-corn expressing Cry1F in Puerto Rico, *Busseola fusca* to Bt-corn expressing Cry1Ab in South Africa and *P. gossypiella* to Bt-cotton expressing Cry1Ac in India (Gill et al., 2011).

Recently, a resistant allele of a *H. virescens* resistant population was identified as a mutation in a gene coding for an ABC transporter molecule. This mutation affected binding of Cry1A toxin to brush border membrane vesicles indicating that this ABC transporter molecule is a novel Cry1A toxin receptor probably involved in the later stages of oligomer membrane insertion (Gahan et al., 2010). Also it was reported the correlation between reduced ALP protein, activity, and mALP expression levels in strains of three species in the Noctuidae Family with diverse resistance phenotypes against Cry toxins (*Heliothis virescens*, *Helicoverpa armigera* and *Spodoptera frugiperda*) (Jurat-Fuentes et al., 2011). Finally, in a recent work it was showed the role of aminopeptidase the resistance to the Bt toxin Cry1Ac in the cabbage looper, *Trichoplusia ni*, evolved in greenhouses, is associated with differential alteration of two midgut aminopeptidases N, APN1 and APN6, conferred by a trans-regulatory mechanism (Tiewsiri & Wang, 2011).

4. Conclusion

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 susceptible organisms will be important to fully understand the mode of action of this family of proteins. In the case of GPI-anchored receptors, APN and ALP have been identified in different insect species as Cry toxin binding molecules and in several insects they have been shown to be important for toxin action. In the case of *M. sexta*, both APN and ALP have been shown to have a similar role since Cry1Ab toxin show similar binding affinities to both molecules depending on the oligomer state of the toxin. Thus it seems that APN and ALP have redundant roles in the action of Cry toxins. Nevertheless, it was shown that ALP could have a predominant role in toxicity since it was preferentially express in young instar larvae that are more sensitive to the toxin in contrast to APN that was expressed later in larval development. Nevertheless, it could still be possible that in certain insect species both APN and ALP act as a complex receptor or that APN or ALP could be differentially important in toxicity depending in the insect species.

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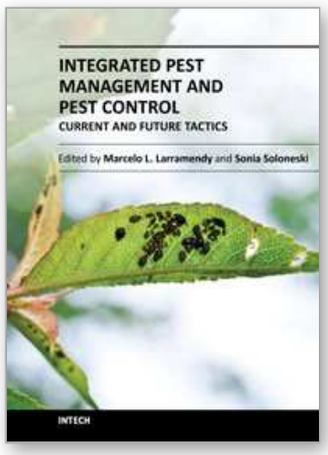

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