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Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae

Juan Luis Jurat-Fuentes [a], Michael J. Adang [a,b,*]

[a] *Department of Entomology, University of Georgia, Athens, GA, 30602-2603, USA*
[b] *Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, 30602-2603, USA*

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Abstract

Many pest insect species are effectively controlled by *Bacillus thuringiensis* (Bt) Cry toxins delivered in plants and biopesticides. Since the insect midgut epithelium contains receptors and other molecules that determine Bt toxicity, characterization of these molecules is necessary for sustained usage of Bt toxins. Studies of Bt susceptible and resistant strains of *Heliothis virescens* have provided insights into resistance mechanisms and toxin receptors. For example, the first gene identified as involved in high levels of Cry1Ac resistance in *H. virescens* encodes a cadherin-like protein, a functional Cry1A receptor in Lepidoptera. This manuscript discusses the most updated information on the mode of action of Cry1A toxins obtained from the characterization of resistant mechanisms in *H. virescens* strains. Our studies are focused on biochemical and molecular comparison of a susceptible and three resistant *H. virescens* strains to identify alterations that correlate with toxin resistance. Following this approach we have been able to identify an alkaline phosphatase (HvALP) as a potential receptor and tested the utility of this protein as a marker for resistance to Cry1Ac. Comparison of brush border proteomes from susceptible and resistant larvae has allowed us to identify additional molecules directly involved in the toxicity process.

Keywords: *Bacillus thuringiensis*; Cry1 toxin; *Heliothis virescens*; Resistance; Receptors; Mode of action; Cadherin; Alkaline phosphatase; Proteomics

1. Introduction

The use of Cry toxins from *Bacillus thuringiensis* (Bt) to control insect pests is a revolutionary advancement in crop production. The high specificity, potency, and environmental safety of Bt toxins has led to the wide use of these toxins as insect control proteins in transgenic crops and biopesticides (Betz et al., 2000).

Despite commercial success, the details of Cry toxin mode-of-action remain controversial. The multi-step process of intoxication affects the specificity of each toxin. Therefore, characterization of the steps, especially the identification of functional Cry toxin receptors, is central to both designing improved Cry toxins and the development of strategies to detect and overcome resistance episodes. Even though alterations in toxin solubilization and activation have been detected in resistant insects, alteration of toxin binding to receptors is the most reported resistance mechanism (Ferre and Van Rie, 2002). Because of this, much of the current available information on Cry toxin receptors as well as the intoxication process has been derived from studies of resistant insect strains selected in the laboratory. The goal of this paper is to present updated information on the contributions that this type of research has provided to the current knowledge of Cry1 toxin receptors and the toxin mode of action in *Heliothis virescens* larvae.

Although different proteins have been proposed as Cry1A receptors, most current evidence (Tsuda et al., 2003; Hua et al., 2004), supports the role of cadherin-like proteins as the primary functional receptors, for at least this toxin family. According to the model of Bravo et al. (2004), binding to cadherin results in a conformational change that allows further toxin proteolysis, resulting in formation of

* Corresponding author. Fax: +1 706 542 2279.
E-mail address: adang@uga.edu (M.J. Adang).

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toxin oligomers. These toxin oligomers have increased binding affinity for *N*-aminopeptidases (APNs)[1] and probably other GPI-anchored proteins that localize in specific regions of the membrane called lipid rafts (Zhuang et al., 2002). According to this model, a localized concentration of toxin oligomers on the lipid rafts leads to oligomer insertion, followed by formation of a toxin pore that results in osmotic shock to the midgut cell and ultimately insect death. An alternative model for midgut cell killing has been proposed by Zhang et al. (2005). In this model, binding of the toxin monomer to cadherin activates an intracellular pathway that leads to apoptosis.

2. *Heliothis virescens* as a model

The tobacco budworm (*H. virescens*) is an important model for investigating Cry toxin receptors and other molecules involved in toxin action Importantly, the first transgenic Bt crop commercialized in the USA, Bt cotton, was developed to express Cry1Ac, the most active toxin against this insect, for field budworm control. Information on both Cry toxin receptors and resistance mechanisms evolved by this pest are vital to maintain the utility of Bt plant technology.

Van Rie et al. originally proposed a model of three populations of Cry1A toxin binding sites in midguts from *H. virescens* larvae (Van Rie et al., 1989). Subsequently, the model was extended to include Cry1Fa and Cry1Ja binding to these three sites (Jurat-Fuentes and Adang, 2001). Proteins belonging to the A population of binding sites are recognized by Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja toxins. Both Cry1Ab and Cry1Ac recognize the B population of binding sites, while Cry1Ac is the only toxin recognizing proteins in population C. This model of multiple binding sites for Cry1Ac directly correlates with this toxin being the most active against *H. virescens*.

Several proteins have been proposed as components of site A, including the *H. virescens* cadherin-like protein HevCaLP (Jurat-Fuentes et al., 2004), and the 170- and 130-kDa *N*-aminopeptidases (APNs) (Luo et al., 1997; Oltean et al., 1999). The components of the B group of binding sites have not been extensively studied, and only a 130-kDa protein has been associated to this site (Jurat-Fuentes and Adang, 2001). Several proteins of less than 100-kDa in size are predicted to form part of the C binding site.

3. Resistant *H. virescens* strains

Our studies of Bt resistant *H. virescens* have focused on discovering biochemical alterations that correlate with resistance in three strains of *H. virescens*. The YHD2 strain, selected with Cry1Ac crystals, developed 10,000-fold resistance to this toxin and cross-resistance to Cry1A and Cry1Fa but not Cry2A toxins (Gould et al., 1995). In brush border membrane vesicles (BBMVs) prepared from larvae of this strain only Cry1Aa binding was altered (Lee et al., 1995). When analyzed at the genetic level, most of the Cry1Ac-resistance in YHD2 was linked to knockout of the gene encoding the HevCaLP cadherin protein (Gahan et al., 2001). As Cry1Ac selection continued on YHD2, a more resistant strain (called YHD2-B) was generated that is more than 73,000-fold resistant to Cry1Ac and still cross-resistant to Cry1A and Cry1Fa toxins (Jurat-Fuentes et al., 2002). The HevCaLP knock-out genotype seems to be fixed in this strain and the increased level of resistance suggested the presence of additional resistance mechanisms (Jurat-Fuentes et al., 2004).

The CXC and KCBhyb strains were independently generated by selecting the progeny larvae of backcrosses between moths from susceptible and Cry1Ac-resistant strains on diet containing Cry2Aa toxin. The parental resistant strain for the CXC strain was the CP73-3 strain, while KCB was the resistant parent used to generate the KCBhyb strain. Individuals from both the CP73-3 and the KCB strains were about 200-fold resistant to Cry1Ac and also cross-resistant to Cry2Aa (Gould et al., 1992; Forcada et al., 1999). Subsequent selection with Cry2Aa resulted in increased resistance to Cry2Aa, a toxin that has a distinct mode of action when compared to Cry1A toxins (English et al., 1994). This cross-resistance event is especially relevant when considering that Cry2Ab toxin is 'stacked' with Cry1Ac in second generation Bt cotton. When compared to BBMV from susceptible larvae, binding of Cry1A toxins to vesicles prepared from the CXC strain was unaltered, while only Cry1Aa binding was reduced in BBMV from the KCBhyb strain (Jurat-Fuentes et al., 2003).

4. Role of HevCaLP in resistance to Cry1 toxins

The HevCaLP component of receptor A seems to be especially critical to toxin action as the lack of HevCaLP in midgut epithelium correlated with high levels of resistance to Cry1Ac. Loss of HevCaLP also caused reduced Cry1Aa binding to BBMV from larvae of the YHD2 and KCBhyb strains, while Cry1Ab and Cry1Ac binding remained unchanged (Lee et al., 1995; Jurat-Fuentes et al., 2004). These results supported the role of HevCaLP as part of receptor A, the only Cry1Aa binding site in the model. Considering the binding site model, these observations also suggested that site B (binds Cry1Ab and Cry1Ac) and site C (binds C) are less important for toxicity. Interestingly, CXC larvae have HevCaLP, so resistance in this strain is most likely due to alternative resistance mechanisms. This observation suggests that assays to detect *BtR4* knockout and reduced HevCaLP levels would detect one type of Bt resistance in field collected insects.

The functional role of HevCaLP as a Cry1A toxin receptor was investigated using the *Drosophila* S2 cell expression

[1] *Abbreviations used:* APN, *N*-aminopeptidase, Bt, *Bacillus thuringiensis*, BBMV, brush border membrane vesicles, GPI, glycosyl-phosphatidylinositol, HevCaLP, *Heliothis virescens* cadherin-like protein, HvALP, *Heliothis virescens* alkaline phosphatase, PMF, peptide mass fingerprinting SBA, soybean agglutinin.

approach that previously established BtR1a as a functional Cry1A receptor (Hua et al., 2004). As predicted from the toxin-binding site model, HevCaLP expressed on the S2 cells bound Cry1A and Cry1Fa toxins (Jurat-Fuentes and Adang, manuscript in preparation). In agreement with in vivo toxicity data (Jurat-Fuentes and Adang, 2001), Cry1Ac was the most active toxin against cells transiently expressing HevCaLP, followed by Cry1Aa and Cry1Ab (Jurat-Fuentes and Adang, in preparation). Unexpectedly, Cry1Fa was inactive against these cells, suggesting that binding of this toxin to HevCaLP is not sufficient to confer toxicity. This phenomenon was previously observed for a 110-APN from *H. virescens* that bound Cry1Fa to S2 cells, but did not promote cytotoxicity (Banks et al., 2003).

Although cytotoxicity was detected in the cell assays, Cry1Ac killed only 20% of the cells at high toxin concentrations. These results suggest the participation of additional receptors from the brush border epithelium in toxicity, as predicted by the three-site model for Cry1Ac binding to BBMV from *H. virescens* and a recently proposed model (Bravo et al., 2004). Co-expression of HevCaLP with additional putative receptor proteins on cultured cells should provide more insight into the role of multiple receptors on the Cry toxin mode of action.

There is no evidence for interactions between Cry2A and cadherin proteins in midgut epithelia. This conclusion agrees with previous reports demonstrating lack of binding competition between Cry1A and Cry2Aa toxins in *H. virescens* (Jurat-Fuentes and Adang, 2001). In Cry2Aa-resistant larvae from the KCBhyb strain, disruption of the *Btr4* gene was detected (Jurat-Fuentes et al., 2003). However *Btr4* disruption in YHD2-B larvae not conferring cross-resistance to Cry2Aa is further evidence that HevCaLP is not a functional receptor for this toxin. This hypothesis is also supported by lack of Cry2Aa cytotoxicity against S2 cells transiently expressing HevCaLP (Jurat-Fuentes and Adang, in preparation).

5. HvALP as a Cry1Ac receptor involved in resistance

Continuous selection of larvae from the YHD2 strain with Cry1Ac resulted in a strain called YHD2-B that has 73-fold higher resistance than YHD2 and reduced Cry1Ab and Cry1Ac binding (Jurat-Fuentes et al., 2002). These changes imply that Cry1Ac binding sites other than HevCaLP are involved in toxicity to *H. virescens* larvae. One approach to revealing additional receptor alterations involved in resistance is the comparison of midgut epithelium proteins prepared from susceptible and resistant larvae.

Comparison of the brush border protein profiles of YHD2-B larvae to proteins from the YDK susceptible strain detected lower levels of soybean agglutinin (SBA) binding to at least two BBMV glycoproteins (Jurat-Fuentes et al., 2002). The rationale for selecting SBA as a probe was that since both SBA and Cry1Ac recognize GalNAc epitopes (Burton et al., 1999), we predicted that reduced glycosylation of brush border proteins might correlate with increased Cry1Ac resistance. This was the case, except that additional studies revealed that the reduced SBA binding was due to reduced protein levels and not to altered glycosylation. One of these proteins was identified as a membrane-bound form of alkaline phosphatase (HvALP) that was GPI-anchored to the cell membrane (Jurat-Fuentes and Adang, 2004). N-glycosidase treatment of BBMV proteins reduced Cry1Ac binding to HvALP, suggesting N-linked carbohydrate groups are recognized by toxin. More specifically, Cry1Ac inhibited SBA binding to HvALP, suggesting that both proteins competed for terminal GalNAc residues on sugars that are N-linked to HvALP. Alkaline phosphatase has previously been identified as a Cry1Ac binding protein in lepidopteran midgut, and it has been proposed as a toxin receptor (English and Readdy, 1989; Sangadala et al., 1994; McNall and Adang, 2003; Jurat-Fuentes and Adang, 2004). Reduced amounts of a brush border alkaline phosphatase in resistant insects support a functional role for this enzyme in toxin action.

We predicted that if HvALP was reduced in resistant larvae, this reduction might be detected as reduced phosphatase activity in larval brush border membrane vesicle (BBMV) preparations. Total phosphatase activity was reduced 3-fold in BBMV from YHD2-B larvae when compared to YDK vesicles. Furthermore, larvae from the F1 generation of crosses between YDK and YHD2-B adults, which are susceptible to Cry1Ac toxin, had phosphatase levels and HvALP amounts similar to their YDK parents (Jurat-Fuentes and Adang, 2004), clear evidence of a correlation between HvALP levels and resistance to Cry1Ac. When testing total phosphatase activity in BBMV from susceptible strains compared to vesicles from resistant strains, specific phosphatase activity was at least 50% reduced in all three resistant strains (YHD2-B, CXC, and KCBhyb). The activity assay results agreed with the protein blot data and suggest that phosphatase activity measurements may be an efficient approach to detect resistant larvae.

6. Analysis of receptor alterations in resistant insects: proteomic analyses

Proteomic analyses utilizing two-dimensional (2D) gel electrophoresis and protein identification by mass spectrometry have elucidated novel toxin binding proteins and protein alterations that correlate with resistance in Bt resistant insects (Candas et al., 2003; McNall and Adang, 2003). Actin and alkaline phosphatase were identified as novel Cry1Ac receptors in the BBMV proteome from *M. sexta* (McNall and Adang, 2003). Homologues of these proteins have been identified as Cry1Ac toxin binding sites in the *H. virescens* BBMV proteome (Krisnamoorthy et al., manuscript in preparation).

Cry1Ac binding to actin is problematical since members of the actin family are intracellular components of cytoskeleton. However, it is possible that conformational

changes in Cry1Ac upon binding to cadherin facilitate interactions between part of the toxin and intracellular actin. The activation of intracellular apoptotic pathways after Cry1A toxin binding to cadherin has been hypothesized to be involved in Cry1 toxicity (Zhang et al., 2005). Actin interacts through tyrosine phosphatases, catenin, and actinin with the cytosolic domain of cadherin proteins to activate intracellular pathways in response to extracellular signals (Lilien and Balsamo, 2005).

A targeted 2D-gel electrophoretic analysis that compared BBMV prepared from larvae of susceptible (YDK) and resistant (YHD2-B, CXC, and KCBhyb) *H. virescens* strains detected reduced levels of HvALP in all resistant strains tested (Jurat-Fuentes and Adang, in preparation). As mentioned above, HvALP is a Cry1Ac binding protein and putative receptor. Since CXC BBMV bound Cry1Ac with the same affinity as BBMV from the susceptible YDK larvae (Jurat-Fuentes et al., 2003), reduced HvALP activity correlates with resistance, but not reduced Cry1Ac binding. However, the existence of multiple GPI-anchored proteins that bind Cry1A toxins, including APNs and HvALP suggests that HvALP may be just one of alternative GPI-anchored toxin receptors necessary for intoxication. Although this hypothesis needs to be tested, it highlights the potential use of HvALP as a Cry1Ac resistance marker to detect resistant larvae in the field. As shown in Table 1, testing for reduced phosphatase specific activity or reduced HvALP levels differentiated resistant strain larvae from susceptible larvae.

Comparison of susceptible and resistant *H. virescens* BBMV proteomes has identified a group of protein spots that are up-regulated in the YHD2-B strain but down-regulated in the CXC and KCBhyb strains when compared to the susceptible YDK proteomes (Jurat-Fuentes and Adang, in preparation). Database searches with the peptide mass fingerprints (PMFs) of these protein spots matched to intracellular phosphatases. This information agrees with previous proteomic data suggesting a role for intracellular proteins in Cry1A mode of action and with the model of Zhang et al. (2005). Interestingly, this family of phosphatases is also involved in responses to extracellular stimuli through the activation of intracellular pathways via lipid rafts (Magee et al., 2002). Though speculative, altered regulation of phosphatases may have an effect via either of the proposed mechanisms of cell death by Cry toxins. Further research is needed to characterize this protein and its physiological role in lepidopteran larvae.

7. Conclusions and future prospects

Studies of Bt resistant strains of *H. virescens* and other insect species provide a means to discover critical features of Cry toxin action. For example both HevCaLP and HvALP were identified as putative Cry1 receptors by detection of their alteration in resistant larvae (Gahan et al., 2001; Jurat-Fuentes and Adang, 2004). Proteins homologous to HevCaLP have been proposed as a mechanism of resistance to Cry1Ac in *Helicoverpa armigera* (Xu et al., 2005), and *Pectinophora gossypiella* (Morin et al., 2003). Based on this information, testing for cadherin gene alterations was proposed as a potential method to detect resistance in the field (Morin et al., 2003). While this approach may be useful, testing for cadherin disruption did not differentiate resistant CXC larvae from susceptible larvae (Jurat-Fuentes et al., 2004). However, all resistant strains tested, including CXC, had lower alkaline phosphatase activity when compared to susceptible larvae. Possibly, testing for reduction of phosphatase activity may be a more sensitive method to differentiate susceptible from resistant larvae. The characterization of the genetic alteration responsible for the lower levels of alkaline phosphatase in resistant larvae is crucial to develop more sensitive and specific DNA-based testing methods.

Proteomic approaches have been successfully used to identify novel Cry toxin binding proteins and proteins that are differentially expressed in resistant larvae. Proteomic-based analyses are limited by the low percentage of lepidopteran sequences available in the databases. This reduced number of sequences translates in low probability matches in the PMF searches. The existence of highly conserved amino acid stretches helps to obtain matches in databases to proteins that most probably share a similar function. Increasing numbers of lepidopteran protein sequences in the databases should help to obtain higher probability matches to more accurately identify proteins directly involved in the toxicity process.

From all the information gathered by comparing midgut proteins from susceptible and resistant *H. virescens* larvae, a revised mode of action for Cry toxins can be proposed (Fig. 1). According to this model, binding to cadherin (HevCaLP) and GPI-anchored proteins (APN and HvALP) has a significant role for toxicity. Both binding events would result in the activation of intracellular signaling

Table 1
Summary of toxin binding, HevCaLP, gut protease, phosphatase activity, and HvALP phenotypes observed for susceptible (YDK) and resistant (YHD2-B, CXC, and KCBhyb) strains of *H. virescens*

Strain	Cry1Ac RR[a]	Cry2Aa RR	^{125}I-Cry1A binding[b]	HevCaLP phenotype	Gut protease alterations	Phosphatase activity	HvALP levels
YDK	NA	NA	1Aa, 1Ab, 1Ac	+	–	Wild type	Wild type
YHD2-B	73,703	10	—	–	+	Reduced	Reduced
CXC	289	>250	1Aa, 1Ab, 1Ac	+	+	Reduced	Reduced
KCBhyb	187	>250	1Ab, 1Ac	–	–	Reduced	Reduced

[a] Resistance ratio (LC_{50} resistant strain/LC_{50} YDK). N/A, not applicable.
[b] As detected in BBMV binding assays.

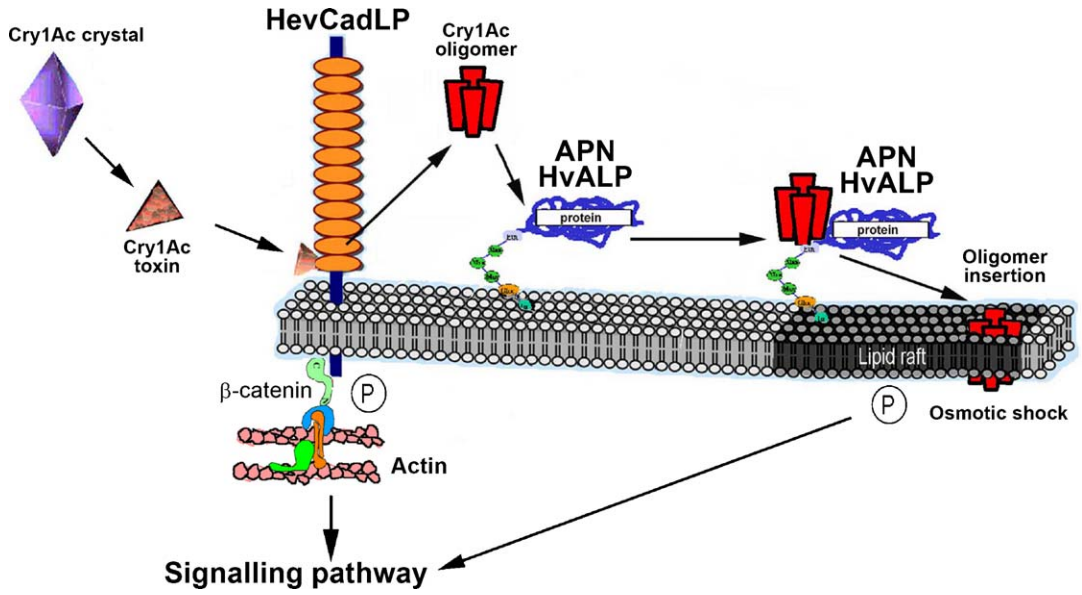

Fig. 1. Proposed model for the mode of action of Cry1Ac toxin in *Heliothis virescens* larvae. Solubilized Cry1Ac crystals (purple) are activated to crystal monomers (orange) that bind to HevCaLP. This binding results in activation of intracellular signaling pathways regulated by phosphatases (P). Part of the Cry1Ac toxin may interact with actin and intracellular phosphatases. After binding to HevCaLP, toxin monomers are processed and form oligomers that bind to GPI anchored proteins (HvALP, APN). These proteins are concentrated in lipid rafts, and toxin binding may induce lipid raft aggregation. Concentration of toxin on lipid rafts probably has a dual effect by inducing toxin insertion forming pores and activating intracellular signaling pathways. These signaling cascades are generally regulated by the same type of phosphatases that regulate signaling through cadherins. Both the intracellular signals, which may activate apoptotic responses, and osmotic shock induced by toxin pore formation contribute to cell killing.

cascades, through toxin binding to cadherin or aggregation of GPI-anchored proteins on lipid rafts, which can result in apoptosis. In this model, osmotic shock due to toxin pore formation and apoptosis are not mutually exclusive, and it is possible that both events take place and have a direct effect in toxicity. Additional research on the specific intracellular pathways activated by Cry intoxication as well as the role of HvALP in toxicity and resistance is needed to better characterize the functional Cry toxin receptors and the intoxication process.

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