Multiple Receptors as Targets of Cry Toxins in Mosquitoes[†]

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ABSTRACT: *Bacillus thuringiensis* (*Bt*) produces inclusions that are composed of proteins known as crystal proteins or Cry toxins. Due to their high specificity and their safety to humans and the environment, these Cry toxins are considered to be valuable alternatives to chemical pesticides in insect control programs. It is believed that Cry toxin-induced membrane pore formation is responsible for insect toxicity. The molecular mechanism of pore formation involves recognition and subsequent binding of the toxin to membrane receptors. This binding is accompanied by toxin oligomerization and transfer of domain I helices of the toxin to the lipid−water interface. This toxin insertion creates pores that lyse the cells. Several receptors from lepidopteran, coleopteran, and dipteran insects have been well characterized. This paper provides an overview of the understanding of the interactions between Cry toxin and multiple receptors in mosquitoes, in particular *Aedes aegypti* and reviews the manner by which the receptors were identified and characterized, with a focus on three proteins, cadherin, alkaline phosphatase, and aminopeptidase-N.

KEYWORDS: *Bacillus thuringiensis*, Cry toxins, aminopeptidase, cadherin, alkaline phosphatase, receptor, *Aedes aegypti*

■ CRY TOXIN RECEPTORS

Bacillus thuringiensis (Bt) is a spore-forming pathogenic bacterium distinguished from other members of the *Bacillus* group by its production of crystalline inclusions known as Cry δ-endotoxins. The insecticidal properties of *B. thuringiensis* have been exploited worldwide for the control of insect vectors of human diseases and insect pests in agriculture. Due to the high selectivity and effectiveness of these toxins, their use surged dramatically following the introduction of *cry* genes into plants known as Bt crops.[1,2] Consequently, it is important to elucidate their mode of action to ensure these toxins do not cause deleterious effects on human health and the environment.[1,3] Because *Aedes aegypti* is a principal mosquito vector of several diseases, including dengue and yellow fevers, this paper deals primarily with the mode of action of Cry toxins in this mosquito species.

Upon ingestion by a susceptible mosquito larva, the alkaline midgut environment promotes solubilization of crystalline inclusions releasing the protoxins. Subsequent cleavage by gut proteases results in the formation of active toxins. The activated toxin fragments then bind to specific protein receptors on midgut epithelial cells, leading to membrane insertion and pore formation. These pores allow ions and water to pass though the cells, resulting in swelling, lysis, and death of the insect.[4−8] In the case of lepidopteran insects, sequential binding of Cry1A toxins has been reported. The binding mechanism may initially involve aminopeptidase-N (APN) and alkaline phosphatase (ALP) receptors followed by binding to a cadherin protein. Interaction with the cadherin protein triggers cleavage of helix α1, leading to the formation of oligomeric toxins.[9] These oligomers then bind glycosylphosphatidylinositol (GPI)-anchored proteins in lipid rafts, including APN and ALP, resulting in the insertion of oligomeric toxins into the cell membrane.[10, 11] An alternative model has been proposed that Cry1Ab toxins kill cells by a cascade of signal transduction events.[12] In this model, Cry1Ab first binds the cadherin receptor. This interaction then stimulates a G protein and an adenylyl cyclase, leading to an increase in cyclic AMP and protein kinase A levels, which subsequently leads to cellular alterations resulting in cell death. However, the preponderance of evidence supports the pore-forming model of toxicity.

In any case, specific receptors are necessary for Cry toxin action. Four different protein receptors have been identified in lepidopteran insects: cadherin,[13−15] APN,[16, 17] ALP,[18] and a 270 kDa glycoconjugate.[19] In addition, glycolipids have been implicated.[20] In mosquitoes, besides cadherin, APN, and ALP proteins, an α-amylase has been identified as a novel receptor in *Anopheles albimanus*, one of the malarial disease vectors.[21]

Receptor expression levels have been shown to correlate with Cry toxin activity. For example, in *Manduca sexta*, the three identified protein receptors are expressed in the anterior, middle, and posterior regions of the midgut.[22] These same regions also bind the Cry1A toxins.[23] However, whereas the cadherin receptor protein was observed in all three regions, both the APN and ALP proteins were detected primarily in the posterior midgut. ALP was found at higher levels in the first and second larval instars, whereas APN was the main GPI-anchored Cry1Ab binding protein in the fourth and fifth instars.[10] It appears that in early instars ALP plays a more important role in toxicity than APN proteins. Potentially these differences in expression

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patterns could explain the decreased susceptibility of late *M. sexta* larval instars to Cry1Ab.[10] In addition, it has been reported that the presence of APN activity was not directly correlated with toxin binding.[24] Moreover, no clear relationship could be found between APN activity and the toxicity of Cry proteins,[25, 26] suggesting that the action of Cry toxins is dependent on their presence of protein receptors but not necessarily on their enzymatic activities.[26]

However, interpretation of binding data may be obscured by irreversible associations of Cry toxins with BBMV, as well as its reversible associations.[27−29] In fact, Cry toxin action is relatively complex, likely involving more than one mechanism or one receptor. The idea of multiple receptor bindings may explain why toxin resistance has been linked to the mutations detected in either of the protein receptors, cadherins[30,31] or GPI-anchored protein such as APNs[32] and ALPs.[18, 33] Taken together, these results show that cadherins, ALPs, and APNs are likely the potential receptors for Cry toxins. These receptor proteins will be discussed in more detail with respect to Cry toxin action in *Ae. aegypti* larval mosquitoes.

■ POTENTIAL RECEPTORS OF CRY TOXINS IN *AEDES AEGYPTI*

Proteomic Identification Approaches. The larval mosquito midgut brush border has distinct structural elements, in which digestive enzymes, ion channel proteins, and various extracellular matrices are located. Identification of the proteome of brush border membrane vesicles (BBMV) is a necessary step in defining potential Cry toxin receptors. Recently, a partial proteome of *Ae. aegypti* midgut BBMV was reported, in which a total of 119 proteins were identified using two complementary proteomic approaches.[34] The most predominant proteins were arginine kinase, fatty acid binding protein, actin, aldehyde dehydrogenase, and protein disulfide isomerase.[34] Metallopeptidases with aminopeptidase activity and alkaline phosphatases, receptor molecules that serve as targets for Cry toxins,[6,10,35−38] were also identified.

In an alternative approach, proteins separated by two-dimensional electrophoresis gel were probed with the Cry4Ba toxin. In this case the toxin bound three ALP isoforms and an aminopeptidase.[39] Other Cry4Ba-binding proteins included the lipid raft proteins, flotillin and prohibitin, the V-ATPase B subunit, and actin. Generally, a cadherin—catenin complex forms a dynamic link with the actin filament network that is involved in the maintenance of cytoskeleton architecture in eukaryotic organisms.[40] Potentially, insertion of the Cry molecule into the membrane may expose regions of the toxin to the cytoplasm, allowing contact with actin, which could lead to disruption of cytoskeletal links and loss of host cell shape and integrity.[39, 41]

Identification of potential receptors through protein—protein interaction is another proteomics approach commonly used.[17, 18, 42−44] Fernandez et al. reported that BBMV proteins purified from Cry11Aa ligand chromatography generated two proteins of 65 and 62 kDa, which bound the Cry11Aa toxin.[36] In addition, the 62 kDa protein was a degraded product of the 65 kDa protein, and both proteins were characterized as GPI-anchored ALP proteins.[36] Recently, a pull-down assay using biotinylated Cry11Aa toxin as a bait purified three protein bands with molecular masses of 140, 95, and 45 kDa from *Ae. aegypti* larval midgut.[45] With the exception of the 45 kDa actin, three of the proteins isolated were identified as APN, two with a mass of

95 kDa. However, the pull-down assay used in this study differed from that utilized by Fernandez et al., in which no APN proteins were observed.[36] Chen et al.[45] incubated biotinylated Cry11Aa toxin directly with BBMV, whereas Fernandez et al.[36] incubated biotinylated Cry11Aa with a pool of proteins released from BBMV using phosphatidylinositol-specific phospholipase C (PI-PLC). Thus, differences in the methods used probably account for the separate identification of APN and ALP proteins.[45] However, it is particularly noteworthy that the cadherin-like mosquito protein was not identified using the proteomic approaches utilized above.[36, 39, 45−47] The high molecular mass of cadherins (≥200 kDa) or its stability could potentially affect its isolation using these approaches in mosquitoes. However, in lepidopteran insects these approaches led to the identification of cadherin as a toxin receptor.[13,14]

Cadherins. Cadherins are single-span transmembrane proteins located primarily within adherens junctions. They also belong to a family of calcium-dependent transmembrane glycoproteins. The presence of cadherins on the cell surface leads to cell sorting, cell adhesion, and morphogenesis. Specific interactions provided by extracellular regions can transfer information intracellularly by interacting with a complex network of cytoskeletal and signaling molecules.[48] Cadherins have long been known as Cry toxin receptors in a variety of insects in the orders Lepidoptera (butterflies and moths),[13,14,49−51] Coleoptera (beetles and weevils),[52] and Diptera (mosquitoes).[47,53] However, the cadherins that bind Cry toxins are distinct from other cadherins that are present within adherens junctions.

The toxin-binding cadherins are localized in the insect midgut to regions in which toxin binding has been observed. In the larval midgut of *Ae. aegypti*, cadherin distribution is observed on the apical side of the distal and proximal caeca and on posterior midgut epithelial cells but not in the apical membranes of anterior midgut.[47] Further studies reveal that Cry4Ba and Cry11Aa toxins are also localized to these sites upon binding.[47,54] Thus, there appears to be a direct correlation between the binding pattern of Cry11Aa and Cry4Ba toxins and the localization of cadherin proteins, leading us to believe that this cadherin serves as one of the main targets of Cry toxin binding within the mosquito gut.

The cadherin protein shown to interact with Cry11Aa toxin was a 250 kDa glycoprotein identified in *Ae. aegypti* BBMV.[36] This protein, together with other two proteins of 100 and 65 kDa, was detected by ligand blot assay that bound Cry11Aa. The 65 kDa protein was later identified as an ALP protein.[36] The cadherin protein plays a role in Cry11Aa toxin binding to *Ae. aegypti* midgut epithelia because an anti-AaeCad antibody could compete readily with Cry11Aa toxin binding to BBMV. In contrast, an antibody to the sodium—proton exchanger NHE3, which is also expressed in the midgut of *Ae. aegypti* mosquito, did not compete in the assay.[47]

The Cry toxin-binding cadherins have four distinct structural domains: a cytoplasmic domain, a transmembrane domain, a membrane proximal extracellular domain (MPED), and an ectodomain. The ectodomain in mosquitoes consists of 11 cadherin repeats (CR) instead of 12 repeats observed in moths and beetles. In lepidopteran insects the toxin-binding regions are primarily in the nearest cadherin repeats next to the MPED.[55−57] Similarly, in *Ae. aegypti*, the toxin-binding domain was mapped to a C-terminal fragment that contains CR7−CR11, with Cry11Aa having an affinity of ~17 nM for this fragment.[47] Within this C-terminal fragment, CR9−CR11 were found to bind Cry11Aa toxin through domain II loops α8 and 2. Furthermore, a Cry11Aa

Table 1. Toxicity of Purified Cry Toxins to Mosquito Larvae and Their Binding Affinity to Midgut Membrane Proteins

	Cry4Aa [93−95a]	Cry4Ba [61, 93, 94, 96]	Cry4Aa/Cry4Ba [93, 94]	Cry11Aa [45, 47, 96]	Cry11Ba [59, 60, 96]
Ae. aegypti, LC_{50}, ng/mL	1360	300	280	122	7.9
binding to BBMV, K_d, nM	99[b]	520[b]		88[b]	8.2
binding to AeCadherin, K_d, nM				16.7	
binding to AaeAPN1, K_d, nM				8.5	
An. gambiae, LC_{50}, ng/mL	1170	20	380	326	
binding to AgCadherin, CR9−CR11, K_d, nM		13			
binding to AgCadherin, CR11−MPED, K_d, nM		23			
binding to AgALP1, K_d, nM					23.9
binding to AgAPN2, K_d, nM					6.4
An. quadrimaculatus					
binding to APN, K_d, nM					0.56
An. stephensi, LC_{50}, ng/mL	7400	550	300	372	2.2
Cx. pipiens, LC_{50}, ng/mL	400	>20000	63	10	
Cx. quinquefasciatus, LC_{50}, ng/mL	980	>80000	180	1140	3.3
binding to BBMV, K_d, nM				20−30	

[a] References are in parentheses. [b] IC_{50} values are given.

mutant in loop α8, E266A, was unable to bind a peptide fragment that contains CR9−CR11.[47] This binding is in agreement with previous work that showed a loop α8 peptide can compete with Cry11Aa binding to *Ae. aegypti* BBMV.[58] With Cry11Ba, an anticadherin antibody also inhibited toxin binding to *Ae. aegypti* BBMV.[59] A cadherin fragment consisting of CR7−CR11 was able to compete with Cry11Ba binding to BBMV. Hence, as with Cry11Aa, the Cry11Ba toxin-binding region is likely localized to CR9−CR11 of the *Ae. aegypti* cadherin receptor.[60]

The importance of the CRs as a toxin-binding region was further studied by determining the correlation between binding and toxin susceptibility in different mosquito strains.[53,61] A CR11−MPED peptide from the cadherin of *Anopheles gambiae* (AgCad1) larvae acted as a synergist of Cry4Ba's toxicity to the *Anopheles* mosquito.[53] It is believed that this truncated cadherin peptide acts as a receptor, leading to cleavage of helix α1, thereby promoting formation of the oligomeric form of the toxin that binds the GPI-anchored receptors.[62] Subsequently, it was demonstrated that the *Anopheles* CR9−CR11 and CR11−MPED fragments also enhanced Cry4Ba toxicity to *Ae. aegypti* larvae, whereas a cadherin-based fragment isolated from a coleopteran insect, *Diabrotica virgifera virgifera*, did not affect Cry4Ba toxicity.[61,63] Both CR fragments were further tested for their binding affinity with Cry4Ba toxin. Using a one-site saturation model, it was shown that peptides CR9−CR11 and CR11−MPED bound Cry4B with high affinities of 13 and 23 nM, respectively (Table 1). Furthermore, the longer CR9−CR11 fragment was more potent than CR11−MPED in enhancing Cry4Ba activity against *Ae. aegypti*.[61] On the basis of these results, these fragments can be used as synergists to increase Cry toxicity and potentially overcome insect resistance.

Recently, an *Ae. aegypti* colony having a low level of resistance to *Bt. israelensis* was identified from a field collection. Preliminary identification of resistance genes identified a N-cadherin as well as two other proteins including an APN as potential toxin targets.[64] The N-cadherin is expressed in the larval midgut but is not known to bind any mosquitocidal toxins to date.

Mutations of mosquito cadherin genes will likely lead to lower larval sensitivity to single Cry toxins. However, unlike lepidopteran active strains, mosquitocidal active strains, such as *Bt israelensis*, produce multiple toxins with different modes of action. Consequently, the development of high-level mosquito resistance to these mosquitocidal strains has been lacking. In large part this lack of resistance development is due to the presence of Cyt1A, which acts to delay resistance development[65] by acting as a surrogate receptor for the mosquitocidal Cry toxins.[66] In contrast, mosquito resistance to *B. sphaericus* has been rapid in the field.[67−69]

ALPs. There is increasing evidence that ALPs are Cry toxin receptors in various insect species.[10,18,36,70−72] In the *Ae. aegypti* mosquito, preliminary reports suggested mosquitocidal Cry toxins bound proteins of 65 and 62 kDa.[73,74] Using a ligand blotting technique, the binding of biotinylated Cry toxins to these proteins was shown to be reversible, and both Cry4Ba and Cry11Aa toxins competed for binding to these two proteins.[73] The 65 kDa protein lacked leucine aminopeptidase activity, and the 62 kDa protein was a degradation product of the 65 kDa protein. Interestingly, Cry toxins inactive against *Ae. aegypti* larvae, such as the lepidopteran active Cry9Aa toxin, either fail to bind to the 65 and 62 kDa proteins or bind but did not compete for Cry11Aa toxin binding.[74] On the basis of these results, the 65 and 62 kDa proteins are likely to be Cry4Ba and Cry11Aa toxin receptors in gut epithelial cells of *Ae. aegypti* larvae.

Further identification of ALP as Cry toxin receptors in *Ae. aegypti* was made possible by ligand blot analysis between Cry11Aa toxin and a pool of proteins released from BBMV by PI-PLC treatment.[36] Three proteins of 200, 100, and 65 kDa were identified to bind the Cry11Aa toxin. The 65 kDa protein was purified by affinity chromatography with Cry11Aa toxin, and this protein was later characterized as a GPI-anchored ALP enzyme.[70] The specific activity of this ALP was enriched up to 6-fold after PI-PLC treatment of BBMV and Cry11Aa affinity chromatography, suggesting an abundance of ALP proteins in *Ae. aegypti* BBMV.[36]

Immunofluorescence studies have shown that ALPs are located predominantly in gastric caeca and posterior midgut epithelial cells. The distribution pattern is similar to that of the cadherin protein and bound Cry11Aa toxin.[47] Further studies have shown that phages displaying ALP-specific peptides decreased toxicity against *Ae. aegypti* larvae. Domain II loop α8 of Cry11Aa toxin was involved in the interaction with ALP, because

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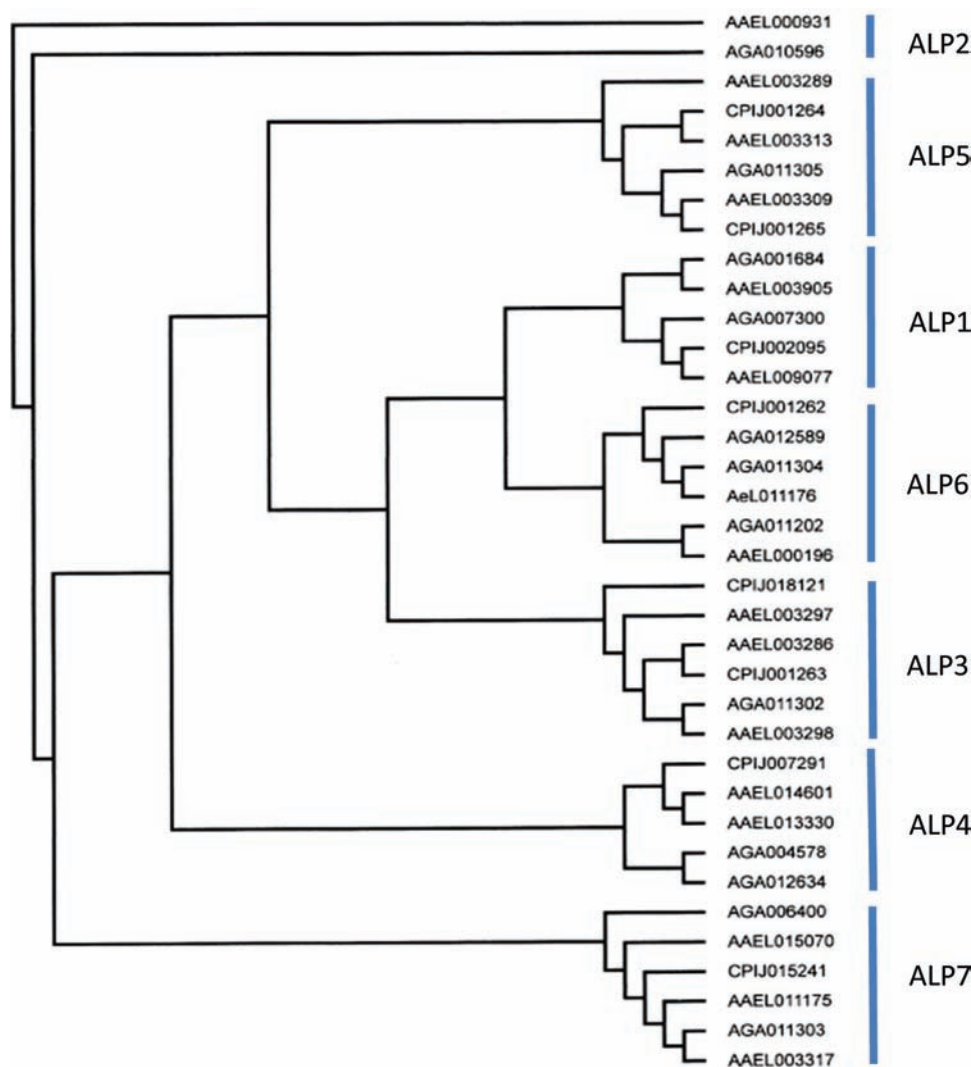
Figure 1. Phylogenetic tree obtained from ClustalX alignment followed by bootstrap analysis using the maximum likelihood method of mosquito alkaline phosphatases. Protein sequences were obtained from Vectorbase for the three mosquito species, *Anopheles gambiae* (Agam Gene build 3.5; sequences have the prefix AGA); *Aedes aegypti* (AaegL Gene build 1.2; sequences have the prefix AAEL); and *Culex pipiens* (CpipJ Gene build 1.2; sequences have the prefix CPIJ). These prefixes are used as in noted in Vectorbase.

the binding of Cry11Aa and the displayed peptide phages was specifically attenuated by a peptide with a sequence corresponding to loop α8.[36] The putative ALP receptors have been subsequently cloned and characterized.[70] Of three cloned ALPs, the ALP1 isoform (AAEL009077) was shown to bind Cry11Aa and the displayed peptide phage that specifically binds the midgut ALP−Cry11Aa receptor. Furthermore, two Cry11Aa regions (R59−G102 and N257−I296) that bind ALP1 were mapped by examining Cry11Aa binding to nine overlapping peptides of ALP1. By using a peptide spot array of the Cry11Aa domain III together with site-directed mutagenesis, it was shown that the ALP1 R59−G102 region binds Cry11Aa through domain II loop α8, whereas the ALP1 N257−I296 region interacts with Cry11Aa through domain III [561]RVQSQNSGNN[570] located in β18−β19. Conclusions drawn from these studies were that the Cry11Aa domains II and III are involved in binding two distinct binding sites in the ALP1 receptor.[70]

Experiments carried out with Cry4Ba toxin and toxin-overlay assays were used to identify the toxin-binding BBMV protein

complexes in *Ae. aegypti*.[54] It was reported that the domain II−III fragment reproducibly reacted with the same *Ae. aegypti* BBMV proteins as did the Cry4Ba toxin. One of these proteins was a 60 kDa protein, a size that approximates that of an ALP. However, the isolated domain III fragment did not bind these BBMV proteins, suggesting domain II of Cry4Ba toxin is essential for interaction with *Ae. aegypti* midgut proteins.[54]

More recently, BBMV competitive assays revealed that the Cry11Ba binding to *Ae. aegypti* BBMV could compete with *Aedes* ALPs. It was also demonstrated that AaeALP1 more readily competes off the binding of Cry11Ba toxin to BBMV than do AaeALP2 (AAEL000931) and AaeALP3 (AAEL003286), suggesting that ALP1 could be more important in the interaction with Cry11Ba than the other ALP isoforms.[60] Thus, the ALP protein is an essential receptor molecule that mediates Cry11Aa toxicity and also is involved in the binding interaction of Cry4Ba and Cry11Ba with *Ae. aegypti* BBMV.

The diversity of ALPs present in mosquitoes is much greater than the three ALPs identified above. Depending on the level of
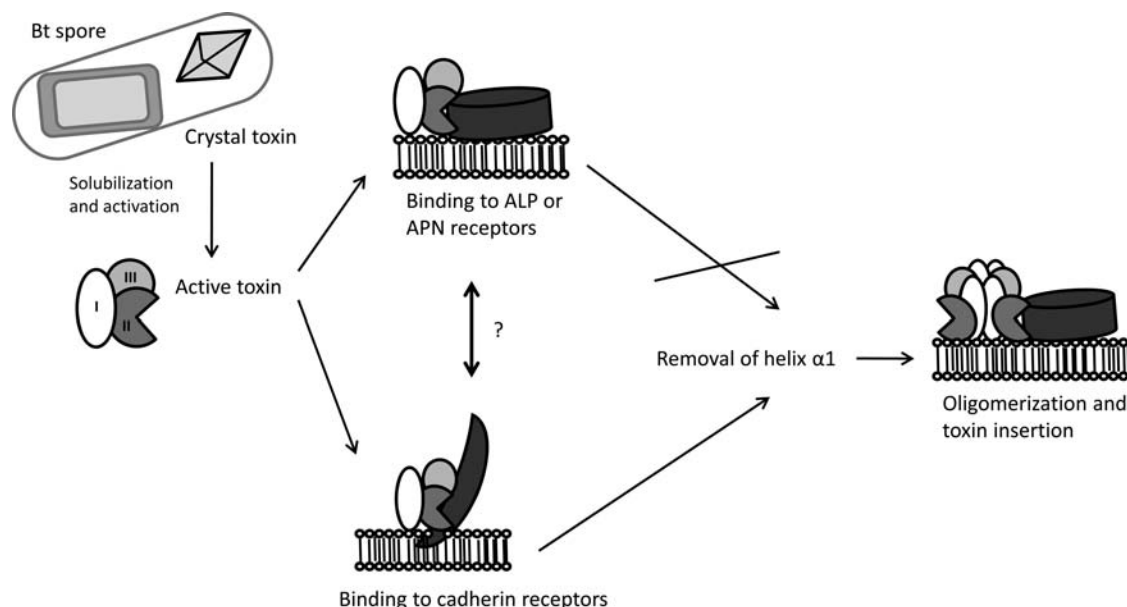
Figure 2. Models of Cry toxin mechanism in mosquitoes. Upon ingestion by a mosquito larva, the alkaline midgut environment promotes solubilization of crystalline inclusions releasing the protoxins, which are cleaved by gut proteases, resulting in the formation of active toxins. Toxin binding occurs first with the cadherin protein triggering cleavage of helix α1, leading to the formation of oligomeric toxins, which then bind anchored APN or ALP proteins in lipid rafts, resulting in the insertion of oligomeric toxins into the cell membrane. Alternatively, because mosquitocidal toxins have high affinity to APN and ALP receptors, it is possible that toxin binding initially involves APN and ALP receptors followed by binding to a cadherin protein.

diversity chosen, there are likely seven ALP families in mosquito species (Figure 1). Moreover, there appears to be significant gene diversity within these seven major ALP family classes. When compared with ALP sequences from other insects including *Drosophila melanogaster*, there is even more significant diversity among the ALP families (data not shown).

APNs. APNs are membrane proteins that function to cleave amino acids at the N-terminus of polypeptides. They commonly serve, along with other enzymes, in the digestion of proteins derived from the insect's diet.[75] These proteins have long been identified as Cry toxin receptors in various insect species.[16,76−84] Recently, a deletion mutation of the APN gene was associated with Cry1Ac resistance in *Helicoverpa armigera*,[84] confirming that APN proteins may play an important role in the mechanism of Cry toxicity.

APNs require a signal peptide to direct nascent polypeptides to the outer surface of the cytoplasmic membrane, where they are attached by a GPI anchor.[5,85,86] The APNs also undergo post-translational modifications through N- and O-glycosylation, including that by *N*-acetylgalactosamine (GalNAc), which is considered to be important for interactions between Cry1A toxins and APNs.[42,87−89] However, some APNs are believed to bind toxins in a glycan-independent aspect as discussed below. Collectively, these modifications give mature proteins of between 90 and 170 kDa in size, which affect the protein structure, stability, molecular recognition, and signaling activities.

Less is known of APNs as Cry toxin receptors in mosquitoes. In *Anopheles quadrimaculatus* and *An. gambiae*, APNs were identified as putative receptors for the Cry11Ba toxin.[90,91] Both of these APNs showed high affinity for the Cry11Ba toxin. For instance, an APN from *An. quadrimaculatus* binds Cry11Ba with a K_d of 0.56 nM, whereas a 106 kDa APN from *An. gambiae* binds the same toxin with an apparent affinity of 6.4 nM (Table 1). This is in contrast to the binding affinity of Cry1A toxins to lepidopteran APNs that are in the range of 100 nM, suggesting that APN

binding in mosquitoes may have a different role in the initial binding steps of mosquitocidal Cry toxins. It is believed that high-affinity toxin binding occurs first to the more abundant GPI-anchored proteins and then to cadherin (Figure 2). Nevertheless, no experimental evidence to date supports this possibility in mosquitoes. A partial AgAPN2 fragment expressed in *Escherichia coli* was able to bind Cry11Ba toxin in a dot-blot experiment and a microtiter plate binding assay,[91] suggesting this APN protein binds toxins in a glycan-independent manner. The 60 kDa APN (AgAPN2) from *An. gambiae* has only about a 46% homology to an APN from the sequenced genome (Agam P3.5 Gene Build, Vectorbase), whereas the *An. quadrimaculatus* APN has homology to a number of APNs from *An. gambiae*.

The role of this receptor class was demonstrated only recently in *Ae. aegypti*.[45] In this study, *Ae. aegypti* APNs, named AaeAPN1 (AAEL012778) and AaeAPN2 (AAEL008155), were isolated and identified as Cry11Aa-binding proteins in a biotinylated Cry11Aa toxin pull-down assay. As bait in the purifying process, Cry11Aa toxin bound four protein bands with molecular masses of 140, 95, 45, and 32 kDa.[45] The 32 kDa protein is a fragment of Cry11Aa toxin. Three pulled-down proteins were identified as APNs; the 140 kDa protein was AaeAPN1, whereas the 95 kDa consisted of two proteins identified as AaeAPN2 and AaeAPN3 (AAEL012774). AaeAPN1 was cloned and a partial fragment expressed in *E. coli*.[45] This fragment was able to bind Cry11Aa, suggesting the interaction of AaeAPN1−Cry11Aa is also glycan-independent.[45] Further studies revealed that the full-length AaeAPN2 and two of its fragments, AaeAPN2b and AaeAPN2e, bound Cry11Aa toxin and they also competed with Cry11Aa binding to *Ae. aegypti* BBMV. The data suggest amino acids 569−641 form part of the Cry11Aa toxin-binding region in AaeAPN2 (J. Chen and S. S. Gill, unpublished work). Similarly in AaeAPN1, one of the Cry11Aa-binding regions is localized to amino acids 525−778.[45] However, it should be noted that these

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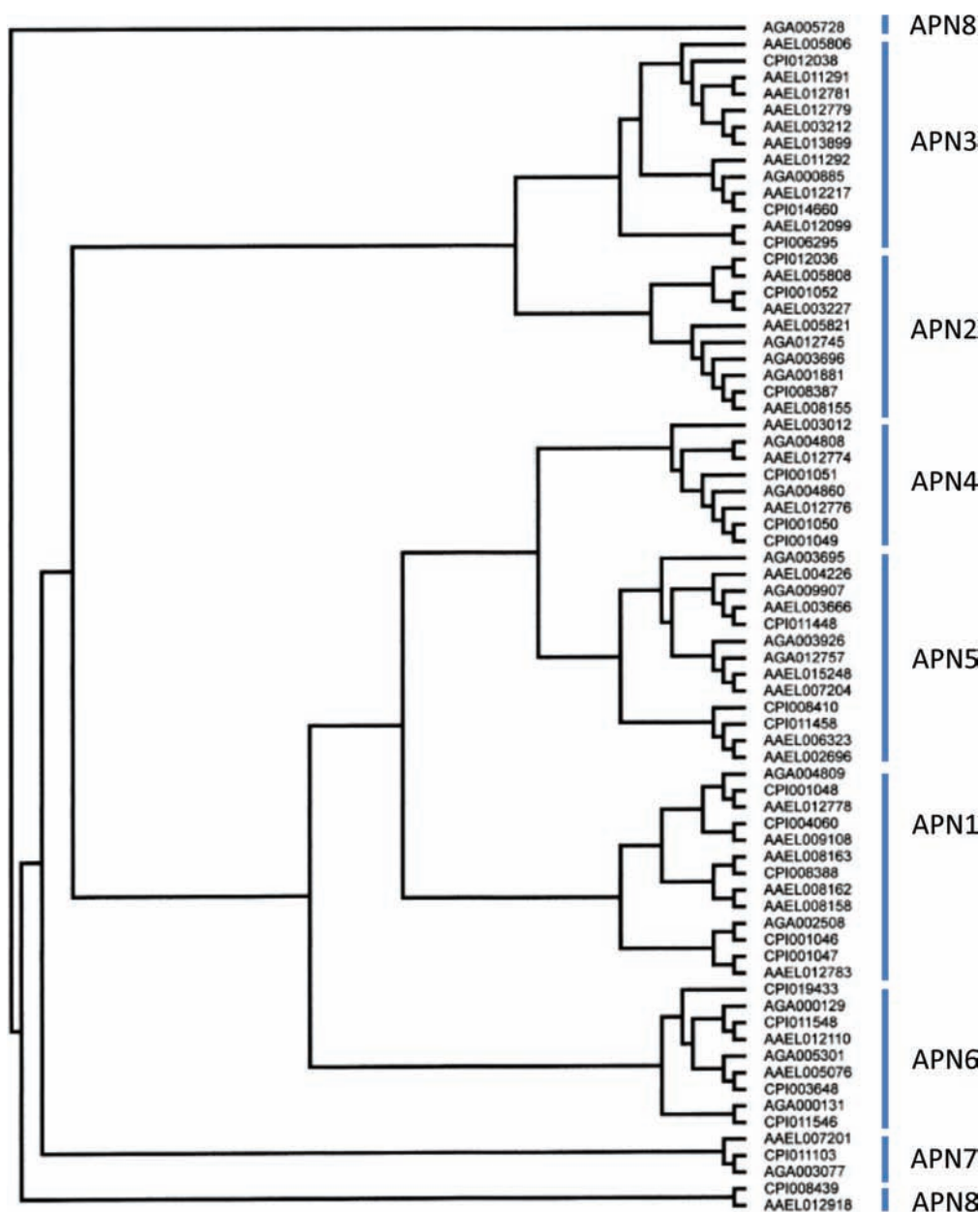
Figure 3. Phylogenetic tree obtained from ClustalX alignment followed by bootstrap analysis using the maximum likelihood method of mosquito aminopeptidases. Protein sequences were obtained from Vectorbase for the three mosquito species *Anopheles gambiae* (Agam Gene build 3.5; sequences have the prefix AGA), *Aedes aegypti* (AaegL Gene build 1.2; sequences have the prefix AAEL), and *Culex pipiens* (CpipJ Gene build 1.2; sequences have the prefix CPIJ).

regions in the two APNs are located toward the C-terminal part of the respective proteins. These binding regions differ from the observed Cry1Aa binding site in a *Bombyx mori* APN, which is localized to the N-terminal region.[83,92] Interestingly, Sf21 cells expressing either AaeAPN1 or AaeAPN2 showed no increased sensitivity to Cry11Aa toxicity. It is noteworthy that the molecular mass of AaeAPN1 in Sf21 cells was lower than that of these proteins in BBMV. Therefore, it is possible that posttranslational modifications in Sf21 cells might differ from that observed in the epithelial cells of *Ae. aegypti* midgut and, also, improper glycosylations could affect toxin binding.[45]

Depending on the level of divergence considered, APNs can be divided into eight classes.[91] It is evident, however, that mosquito APNs show significant differences from lepidopteran APNs. There is a great diversity in APNs among the three mosquito

species for which genomes have been sequenced. Indeed, phylogenetic analysis of *An. gambiae*, *Ae. aegypti*, and *Culex pipiens* APNs shows that eight major families can be readily classified among these three mosquito species (Figure 3).

■ TOXIN RECEPTOR EXPRESSION

Although high-level receptor expression is observed in tissues and cell types that bind Cry toxins, the expression of cadherins, ALPs, and APNs is not limited to these tissues. In fact, all three receptor types are also expressed in the adult female midgut and in the Malpighian tubules (data not shown). Clearly these proteins have functions that are critical for these tissues.

The tissue distribution patterns of the three receptor types have been examined in greater detail with immunofluorescence

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using specific antibodies. For example, *Aedes* cadherin has been localized to the apical side of the distal and proximal caeca and on the posterior midgut epithelial cells, but its expression is not observed in the anterior midgut.[47] This pattern of expression suggests some regionality in cadherin expression in the larval midgut. Cellular distribution in the adult midgut or in Malpighian tubules has not been determined. As noted earlier, AaeALP1 expression is similar to that of cadherin, and hence it is expressed in gastric caeca and posterior midgut epithelial cells.[47]

In the larval *Aedes* midgut AaeAPN1 showed a distinct expression pattern, with expression observed in the apical side of posterior midgut epithelial cells but not in the anterior midgut and gastric caeca cells.[45] This expression pattern is similar to that observed in *M. sexta* gut, in which APN was preferentially expressed in the posterior gut epithelial cells. In contrast, AaeAPN2 was expressed not in posterior midgut cells but in the anterior midgut and gastric caeca cells (J. Chen, K. G. Aminova, and S. S. Gill, unpublished work).

Microarray experiments performed using the gene set obtained from the recently sequenced *Ae. aegypti* genome were used to assess the effect of toxin exposure on the expression patterns of cadherins, ALPs, and APNs. Low- or high-level toxin exposure had no effect on cadherin expression (data not shown). Similarly, the expression of AaeALP1 (AAEL009077), which bound Cry11Aa and also binds Cry11Ba, did not change following Cry11Aa exposure. In contrast, AaeALP2 (AAEL000931) and AaeALP4 (AAEL013330), which bind these toxins poorly, showed significant decrease in expression levels following Cry11Aa exposure. A similar pattern was observed with APNs. It thus appears that toxin exposure had little effect on the receptor expression patterns.

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