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Role of Receptors in *Bacillus thuringiensis* Crystal Toxin Activity

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INTRODUCTION

Bacillus thuringiensis is a member of the *Bacillaceae* family and belongs to the *Bacillus cereus* group, which contains *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (146). *B. thuringiensis* isolates have been found worldwide, and 82 different serovars have been reported (102).

B. thuringiensis is pathogenic to insects and can be readily distinguished from other members of the *B. cereus* group by the production of large crystalline inclusions that consist of entomocidal protein protoxins. When activated upon ingestion, these toxins, in addition to other virulence factors, weaken or kill insects and allow *B. thuringiensis* spores to germinate in the insect. The type and number of different protoxins in the crystalline inclusions of *B. thuringiensis* determine a particular strain's toxicity profile. Cry proteins are highly diverse and primarily target insects in the orders Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), and Coleoptera (beetles and weevils) (152); however, some Cry toxins have been reported to kill hymenopterans (wasps and bees) (46) and nematodes (118, 186).

Modes of Action

The transformation of Cry proteins from a relatively inert crystalline protoxin form to a cytotoxic form is a multistep process (152). First, inclusions must be ingested by a susceptible larva. The environment of the midgut promotes crystal solubilization and the consequential release of protoxin. Cleavage sites on the protoxin are recognized and cut by host proteases to produce active toxin that subsequently binds to specific receptors on the midgut epithelium. It is then generally accepted that toxin subunits oligomerize to form pore structures capable of inserting into the membrane. These pores allow ions and water to pass freely into the cells, resulting in swelling, lysis, and the eventual death of the host (96). Recently, an alternative hypothesis has been proposed that suggests Cry toxicity is independent of toxin oligomerization (195, 196). Both of these models will be discussed in more detail in the sections that follow.

Cry Toxins as Biopesticides

The insecticidal properties of *B. thuringiensis* toxins have been exploited commercially, and preparations of spores and crystals have been used to control insects in the orders Lepidoptera, Diptera, and Coleoptera. Such biopesticides have been used for almost 60 years in areas such as forestry management, agriculture, and vector-borne disease control (37, 152). Recently, the use of Cry toxins has increased dramatically following the introduction of *cry* genes into plants (156, 178). These "Bt crops" have thus far proved to be an effective control strategy, and in 2004 Bt maize and Bt cotton were grown on 22.4 million hectares worldwide (79). Such widespread use, however, has led to concerns about the effect Bt crops may have on the environment and on human health (156). These issues—particularly the effect of Bt crops on nontarget organisms (148), food safety (156), and the selection of resistant insect populations (9, 39)—are currently being actively researched.

Toxin Diversity

The remarkable variety of known Cry proteins is the result of a continuing international effort to isolate and characterize new strains of *B. thuringiensis* with the hope of finding toxins with novel properties particularly suited for the control of agronomically or medically important pests. Thousands of strains have been screened and there are currently 143 unique Cry toxins, according to the *B. thuringiensis* Toxin Nomenclature webpage (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

The extraordinary diversity of Cry toxins is believed to be due to a high degree of genetic plasticity. Many *cry* genes are associated with transposable elements that may facilitate gene amplification, leading to the evolution of new toxins (29). In addition, most *cry* genes are found on plasmids, and horizontal transfer by conjugation may result in the creation of new strains with a novel complement of *cry* genes (166, 167).

The large number of known Cry proteins has permitted comparative sequence analyses and has helped to elucidate elements important for both basic toxin function and insect specificity. In 1989, Höfte and Whiteley (70) carried out the first detailed analysis of Cry protein sequence. At that time, 13 holotype Cry proteins were known and assigned to one of four groups based on their insect specificity. Sequence alignment showed a high degree of diversity among the Cry proteins; however, five blocks of conserved amino acids were identified that were found in most sequences. The discovery of new Cry proteins prompted further analysis: first by Bravo in 1997 (17) and then by de Maagd et al. in 2001 (29). In the more recent work, the sequences of proteins from Cry1 to Cry31 were analyzed. Most toxins had some or all of the five conserved blocks identified by Höfte and Whiteley (70), suggesting that these regions may be important for some aspects of toxin stability or function. It was also evident that Cry toxins were generally one of two lengths: either 130 to 140 kDa or approximately 70 kDa. The conserved blocks were present in the N-terminal half of the longer toxins, whereas the C-terminal half constituted a protoxin domain not found in the smaller toxins.

Using domain information derived from the crystal structures of active Cry1Aa, Cry2Aa, and Cry3Aa (described in the next section), de Maagd et al. (29) aligned each of three toxin domains separately and created phylogenetic trees to assess the individual contribution of each domain to insect specificity. The different trees showed that in general, there was a correlation between sequence similarity and insect order specificity but that relatively unrelated clusters could sometimes have similar activities. This suggested that insect specificity may have developed along multiple evolutionary paths. A comparison of the different trees showed that domains I and II had relatively similar tree architectures, suggesting coevolution in many cases. In contrast, the topology of the domain III tree was quite different, and thus it was hypothesized that shuffling in this domain may have contributed to Cry toxin diversity.

TABLE 1. Sequence analysis of representative Cry toxins whose structures have been solved by X-ray crystallography

Toxin	Insect specificity	Total residues	% Sequence identity[a]				Conserved blocks[b]
			1Aa	2Aa	3Aa	4Ba	
Cry1Aa	Lepidoptera	577		23	39	30	1, 2, 3, 4, 5
Cry2Aa	Diptera/Lepidoptera	584			21	20	1, 2
Cry3Aa	Coleoptera	584				36	1, 2, 3, 4, 5
Cry4Ba	Diptera	558					1, 2, 3, 4, 5

[a] Based on structural alignment carried out by Boonserm et al. (11).
[b] As defined by Höfte and Whiteley (70) and de Maagd et al. (29).

Toxin Structure and Function

The three-dimensional structures of Cry toxins have provided considerable insight into the mechanism of toxin function and have helped to explain differences in toxin specificity. To date, seven structures have been solved by X-ray crystallography: Cry1Aa (64), Cry1Ac (32, 108), Cry2Aa (127), Cry3Aa (109), Cry3Ba (45), Cry4Aa (12), and Cry4Ba (11). These toxins show considerable differences in their amino acid sequences and insect specificity but, remarkably, they all have highly similar three domain structures (Table 1; Fig. 1). This is particularly surprising for Cry2Aa, given that it shares only 20 to 23% sequence identity with the other toxins and has only two of the five highly conserved blocks. In this section, the

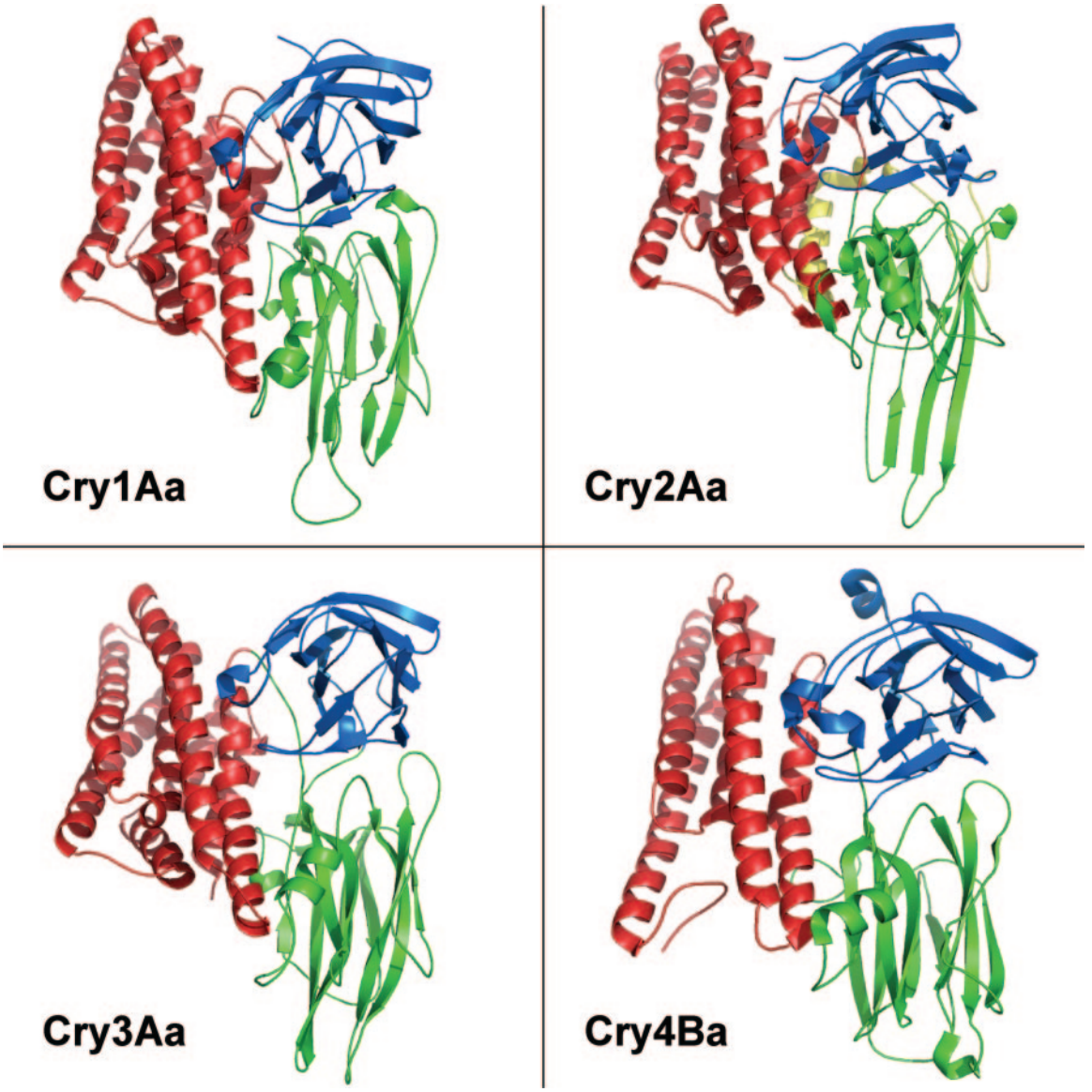


FIG. 1. Crystal structure of Cry1Aa (64) (PDB code, 1CIY), Cry2Aa (127) (PDB code, 1I5P), Cry3Aa (45) (PDB code, 1DLC), and Cry4Ba (11) (PDB code, 1W99). (Adapted from reference 11 with permission from Elsevier.) Domain I, domain II, and domain III are shown in red, green, and blue, respectively. The N-terminal protoxin domain of Cry2Aa is shown in yellow. Images of protein structures in this and subsequent figures were generated using the program PyMol (Warren L. DeLano, DeLano Scientific LLC, San Carlos, CA [http://www.pymol.sourceforge.net]).

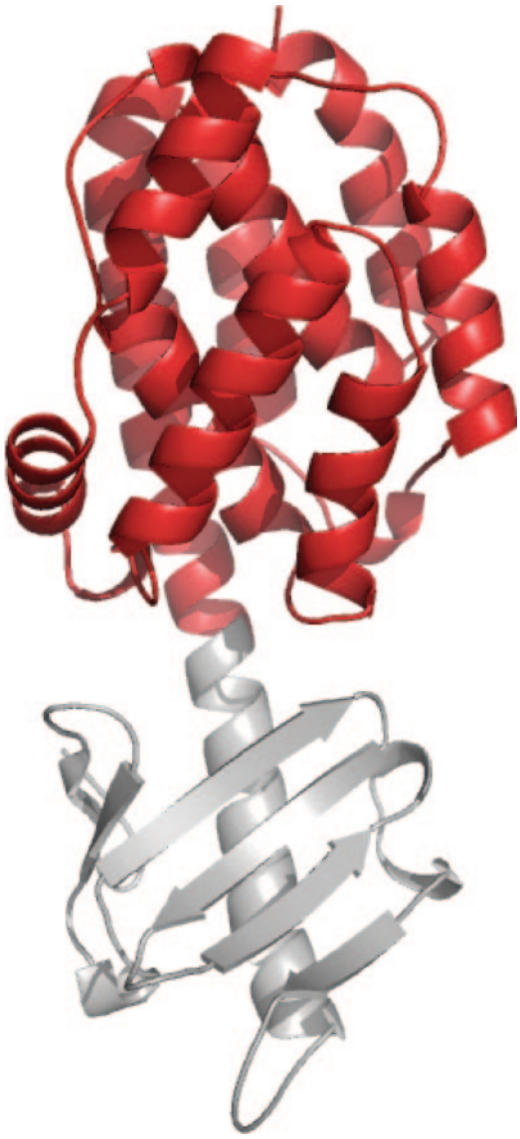


FIG. 2. Crystal structure of colicin N (137) (PDB code, 1A87). The helical bundle with structural similarity to Cry toxin domain I is shown in red.

general structure of each Cry toxin domain will be described and related to its proposed function.

Domain I. Domain I was first described in Cry3Aa by Li et al. (109). It consists of an alpha-helical bundle in which six helices surround a central helix. Each of the outer helices is amphipathic in nature; polar or charged residues are generally solvent exposed and hydrophobic residues, typically aromatic in nature, project towards the central helix. Polar groups are present in the interhelical space, but all are either hydrogen bonded or involved in salt bridges. Most of the helices are longer than 30 Å and would thus be capable of spanning a hydrophobic membrane. These properties, and an overall structural similarity to the pore-forming domain of colicin (137) (Fig. 2), led to the hypothesis that domain I was the major determinant of pore formation in Cry toxins (109). For this theory to be correct, a major conformational change would be necessary to transform domain I from a water-soluble form to a structure capable of membrane insertion. How this transformation occurs is a focus of ongoing research (11, 135, 145).

Domain II. Domain II is formed by three antiparallel β-sheets packed together to form a β-prism with pseudo threefold symmetry (109). Two of the sheets are composed of four strands in a Greek key motif and are solvent exposed. The third sheet packs against domain I and is arranged in a Greek-key-like motif with three strands and a short alpha-helix. Structurally, domain II is the most variable of the toxin domains (11). This is especially true for the apex loops, which differ considerably in length, conformation, and sequence. The lengths of the β-strands are also highly variable, with Cry2Aa and Cry4Ba being the extreme examples. Given this variability, domain II is believed to be an important determinant of toxin specificity. Similarities between the domain II apex and the complementarity-determining region of immunoglobulins suggested a role in receptor binding (109), and extensive mutagenesis studies have provided evidence for this hypothesis (145).

The structure of domain II has been compared to those of other β-prism proteins (28), including vitelline (158) and the plant lectins jacalin (151) and *Maclura pomifera* agglutinin (107). Other proteins with a β-prism fold were identified in the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/Welcome.do) and include *Helianthus tuberosus* lectin (15), artocarpin (141), *Calystegia sepium* lectin (14), and banana lectin (123). Vitelline is found in the vitelline membrane of hen eggs and although its biological function is unknown, it is believed to bind the carbohydrate *N*-acetylglucosamine pentasaccharide at its apex (157). The structure of vitelline is much more symmetrical than that of domain II; each four-β-strand sheet is related by sequence, unlike the β-sheets that comprise domain II of Cry toxins. The protein is also characterized by long, flexible loops at its apex (158), similar to what is observed for some Cry toxins. The plant lectins are part of the jacalin-related superfamily of lectins (139) and are either mannose or galactose specific. Several of these lectins have been cocrystallized with their ligands, and the binding site is invariably at the apex. Banana lectin is unique in that two carbohydrate binding sites have been identified at the apex (123) (Fig. 3). The structural similarity between domain II and lectin domains has led to speculation that domain II may bind to carbohydrates, but this has not yet been demonstrated.

Domain III. Domain III forms a β-sandwich (109). In this arrangement, two antiparallel β-sheets pack together with a "jelly roll" topology. Both sheets are composed of five strands, with the outer sheet facing the solvent and the inner sheet packing against domain II. Two long loops extend from one end of the domain and interact with domain I (64). Domain III shows less structural variability than domain II, and the main differences are found in the lengths, orientations, and sequences of the loops (11). The importance of these differences is particularly evident with Cry1Aa and Cry1Ac, where a loop extension in Cry1Ac creates a unique *N*-acetylgalactosamine (GalNAc) binding pocket implicated in receptor binding (21, 32, 108).

Domain III has been compared to a number of different proteins (28), but its similarity to carbohydrate-binding modules (CBMs) found in microbial glycoside hydrolases, lyases, and esterases is particularly striking. These enzymes generally

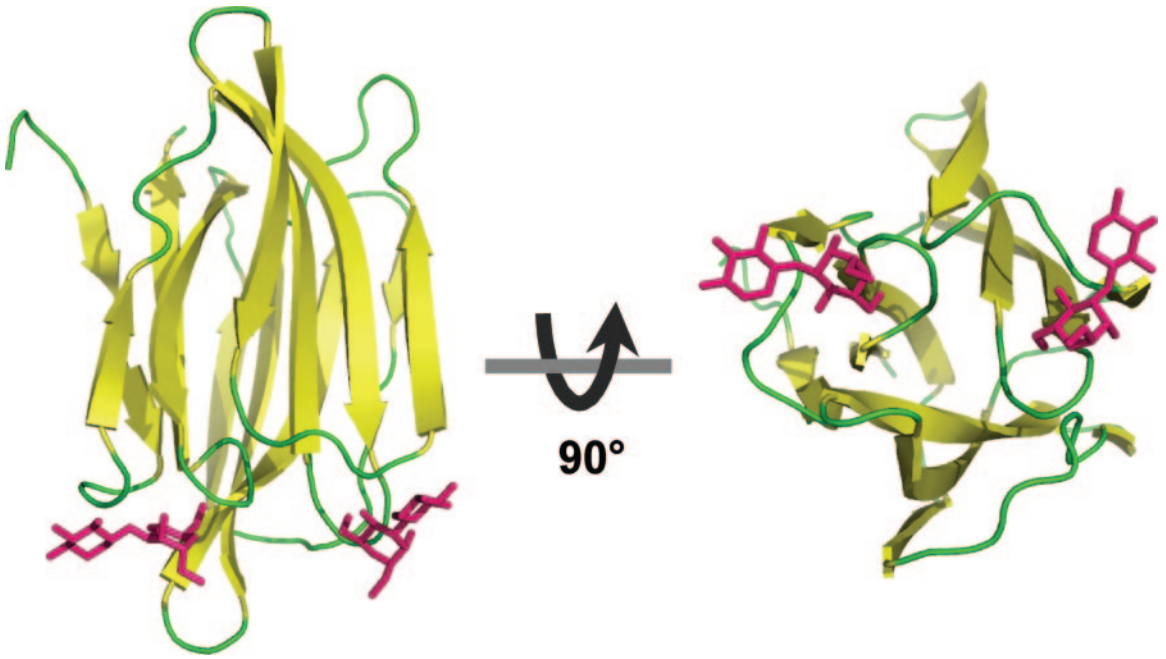


FIG. 3. Crystal structure of banana lectin (yellow/green) bound to laminaribiose (red) at two sites. PDB code, 2BMZ (123).

consist of a catalytic domain linked to one or more CBMs. The function of the CBM is to target the catalytic domain to its polysaccharide substrate (172). This enhances the enzyme's catalytic efficiency by increasing its effective concentration at the substrate surface. The structure of several CBMs in complex with carbohydrate ligands has now been solved, and two binding sites have been identified (140). One site (cleft A) is found at the loops connecting the two β-sheets, and the other (cleft B) is located on the concave surface of one of the β-sheets (Fig. 4). Aromatic residues are important components of each binding site, and in general they are the best-characterized mediators of carbohydrate-protein interactions in CBMs (13). Figure 4 shows an overlay of domain III from Cry1Aa and *Cm*CBM6-2 (the family 6 CBM from *Cellvibrio mixtus* endoglucanase 5a) in complex with two cellotriose molecules (140). As shown, there is significant structural similarity between these domains, suggesting that some Cry toxins may also bind to carbohydrates in these regions. It should be noted, however, that the aromatic residues important for carbohydrate binding in CBMs are generally not conserved in Cry toxins.

IDENTIFICATION AND VALIDATION OF RECEPTORS

Cry toxin binding to insect midgut epithelial receptors is an important determinant of specificity. The correlation between binding and toxicity was first demonstrated using brush border membrane vesicles (BBMV) prepared from microvilli by use of a technique developed by Wolfersberger (187). Early studies showed that a Cry toxin (Cry1Ba) lethal to *Pieris brassicae* bound specifically to the insect's BBMV but not to BBMV prepared from rat intestine (68). It was later shown that Cry1Ab and Cry1Ba bound specifically and saturably to *P. brassicae* BBMV, whereas only Cry1Ab bound to BBMV prepared from *Manduca sexta* (69). Since both toxins killed *P. brassicae*, but only Cry1Ab killed *M. sexta*, there was a good correlation between binding and toxicity data. With other toxin-insect combinations, the correlation has not always been as

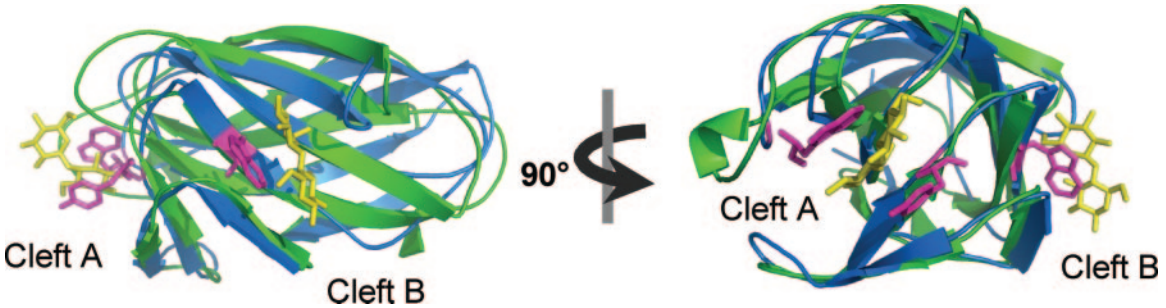
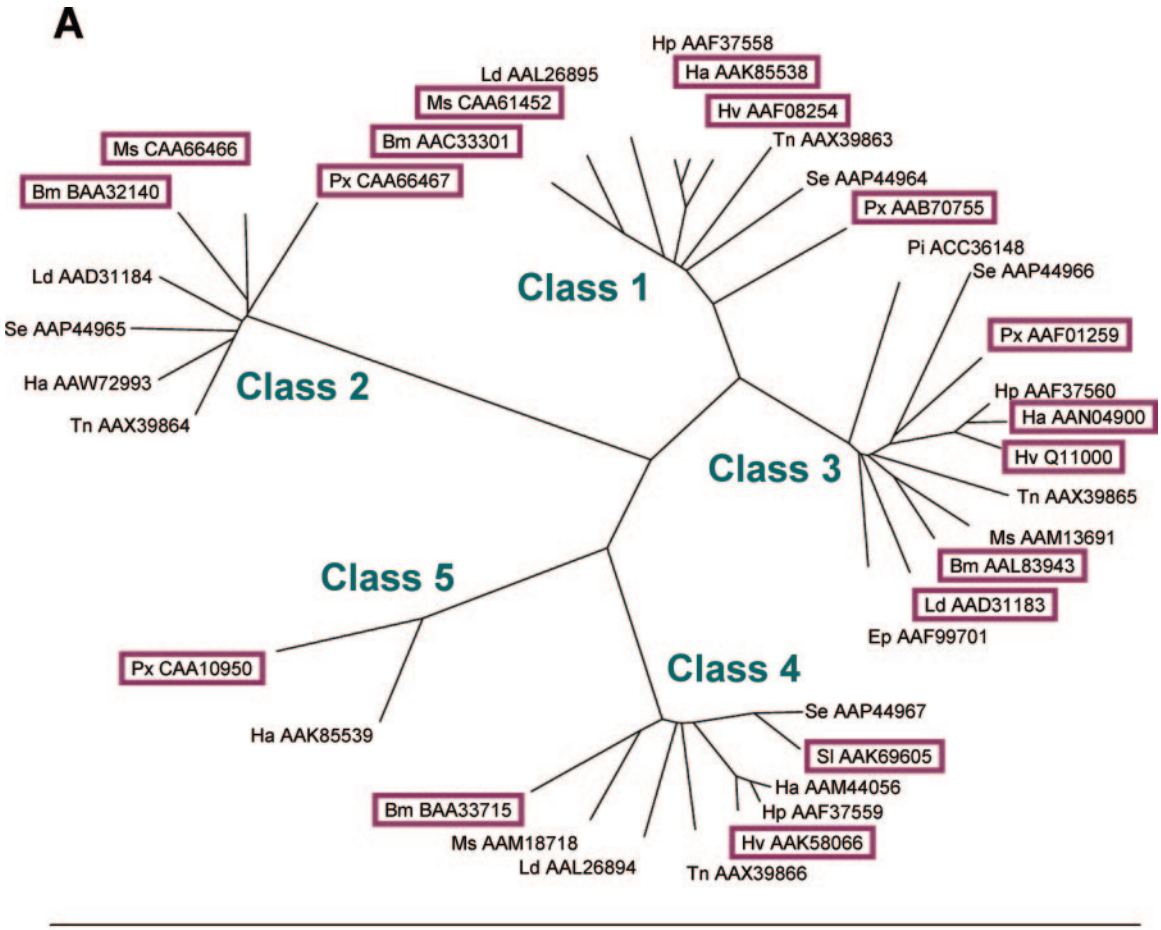


FIG. 4. Crystal structure overlay of the CBM *Cm*CBM6-2 (blue) in complex with two molecules of cellotriose (yellow), and domain III of Cry1Aa (green). Aromatic residues important for carbohydrate binding are shown in magenta. The PDB codes are 1UYY (140) (*Cm*CBM6-2) and 1CIY (64) (Cry1Aa). Clefts involved in CBM carbohydrate binding are indicated (140).



B

Class	1	2	3	4	5
1	61%	26%	38%	30%	30%
2		65%	25%	25%	25%
3			60%	27%	27%
4				67%	35%
5					56%

FIG. 5. Phylogenetic analysis of lepidopteran midgut APN sequences. (A) Phylogenetic tree of representative lepidopteran midgut APN sequences, created using the programs CLUSTALX and DRAWTREE (PHYLIP package). The species name and GenBank accession number are shown for each protein. APNs boxed in purple indicated those reported to interact with Cry toxins. Classes are as proposed by Herrero et al. (67). Species names abbreviations are as follows: Se, *Spodoptera exigua*; Ms, *Manduca sexta*; Ld, *Lymantria dispar*; Hv, *Heliothis virescens*; Ha, *Helicoverpa armigera*; Hp, *Helicoverpa punctigera*; Bm, *Bombyx mori*; Sl, *Spodoptera litura*; Px, *Plutella xylostella*; Pi, *Plodia interpunctella*; Ep, *Epiphyas postvittana*; and Tn, *Trichoplusia ni*. References for binding studies are as indicated in the relevant section of the text. (B) Average amino acid sequence identity within and among the different APN classes.

strong (47, 180). For example, Wolfersberger (188) reported that Cry1Ac was less toxic to *Lymantria dispar* than was Cry1Ab, despite having a relatively stronger binding affinity. This same binding interaction was later studied by Liang et al. (110), who used a two-step interaction scheme to analyze separately the kinetics of reversible and irreversible binding. By this method, it was demonstrated that the rate constant of irreversible binding, rather than the maximum extent of binding, correlated better with toxicity. The general view has been that reversible binding correlates with toxin binding to receptor while irreversible binding equates with the membrane insertion step.

After it was demonstrated that specific high-affinity toxin binding sites were present in the insect midgut, efforts to identify and clone toxin receptors were intensified. Many putative Cry toxin receptors have since been reported, of which the best characterized are the aminopeptidase N (APN) receptors (51, 93, 142, 150) and the cadherin-like receptors (44, 130, 131, 174, 175) identified in lepidopterans. In nematodes, glycolipids are believed to be an important class of Cry toxin receptors (60). Other putative receptors include alkaline phosphatases (ALPs) (38, 85, 86), a 270-kDa glycoconjugate (176), and a 252-kDa protein (73). In the following sections, each receptor class will be discussed with a particular focus on toxin-receptor binding

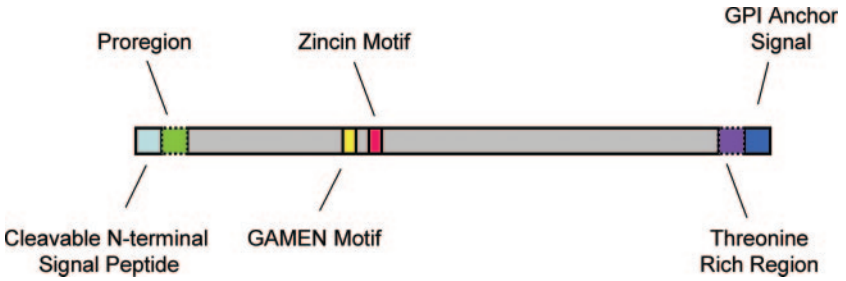


FIG. 6. Schematic representation of a typical lepidopteran APN protein. The proregion and the threonine-rich region, shown with broken lines, have been reported only in some APNs.

interactions and the ability of receptors to confer toxin susceptibility.

APN

The APN family is a class of enzymes that cleaves neutral amino acids from the N terminus of polypeptides. They serve a variety of functions in a wide range of species, but in the lepidopteran larval midgut, they work in cooperation with endopeptidases and carboxypeptidases to digest proteins derived from the insect's diet (185). The proteins belong to the zinc-binding metalloprotease/peptidase superfamily and to a subfamily called the gluczincins (72). Members of this family are characterized by the short zincin motif HEXXH, where X stands for any amino acid, followed by a conserved glutamic acid residue 24 amino acids downstream from the first histidine. The histidines and the last glutamic acid residue serve as zinc ligands, while the first glutamic acid residue is important for enzyme catalysis. A highly conserved GAMEN motif is also believed to form part of the active site (101).

In addition to being studied for their role in digestion, APNs have been extensively studied as putative Cry toxin receptors. Since it was first shown that Cry toxins can bind to APN (93, 150), many different forms have been isolated and characterized. Figure 5 shows the phylogenetic relationship between representative lepidopteran APNs and indicates those that have been reported to interact with Cry toxins. As shown, the APNs have been divided into five different classes (67). The average sequence identity within a class varies from 56% (class 5) to 67% (class 4). Among the different classes, class 2 is the least like the others, with an average sequence identity of only 25 to 26% relative to the other classes, whereas class 1 and class 3 are the most similar, with an average sequence identity of 38%. To date, all known APNs within a particular species have been found to cluster into different classes. In fact, some APNs share higher sequence identity with those in nonlepidopterans than with other APNs in the same species. For example, class 2 APN from *M. sexta* is more similar to APNs in chicken and frog (GenBank accession numbers NP_990192 and AAH85055, respectively) than to class 1 *M. sexta* APN, and yet both *M. sexta* APNs are reported to bind to Cry1Ab (31, 120).

Of the many different APNs that have been studied, several common features have emerged (Fig. 6). The genes encode proteins of approximately 1,000 amino acids that undergo various forms of posttranslational modification to produce mature proteins of between 90 and 170 kDa in size. The proteins have a cleavable N-terminal signal peptide that directs nascent polypeptides to the outer surface of the cytoplasmic membrane. There, they are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (2, 31, 94, 113, 164), in contrast to what is seen for vertebrates, where a hydrophobic N-terminal stalk is used for attachment (155). As will be discussed, glycosylation is important for some Cry toxin-APN interactions, and in many cases the presence of N- or O-linked carbohydrates has been shown biochemically or predicted by sequence analysis (84) (http://www.cbs.dtu.dk/services/). As shown in Fig. 7, the predicted number of O-linked glycosylation sites differs considerably among the different classes of APN, whereas differences in predicted N-linked glycosylation sites are less distinct. Carbohydrate structures including GalNAc are believed to be particularly important for some interactions between Cry1Ac and APN (93).

APN as a Cry-binding protein. Cry1 proteins are toxic to lepidopterans, and several different toxins, including Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1C,a and Cry1Fa, have been shown to bind to APNs (references in the following sections). Based on the experiments carried out so far, APNs and toxins within these families show different patterns of binding. Some APNs bind to multiple Cry toxins and some Cry toxins bind to multiple APNs, and in other cases, unique toxin-APN pairs have been reported. While many toxin-APN binding combinations have yet to be tested, preliminary data are providing some insight into the determinants of receptor binding. In this section, a class-by-class account of APNs and their interactions with Cry toxins will be presented.

(i) Class 1. Class 1 APNs have been identified in nine different lepidopterans. In addition to the features already discussed, class 1 APNs generally have a threonine-rich sequence downstream of the C-terminal GPI signal sequence. This region is believed to have extensive O-linked glycosylation and is thought to form a rigid stalk that elevates the active site of the enzyme above the membrane surface (92, 93). By use of the NetOGlyc 3.1 server (84) (http://www.cbs.dtu.dk/services/NetOGlyc), the number of predicted O-linked glycosylation sites in class 1 APNs has been shown to vary from 6 in *Bombyx mori* to 39in *Helicoverpa armigera* (Fig. 7). In species where native APN has been isolated from the midgut, the correlation between observed molecular mass and the number of predicted O-linked glycosylation sites is strong. For example, APNs from *B. mori*, *M. sexta*, and *Heliothis virescens* are reported to have observed molecular masses of 120 kDa (192),

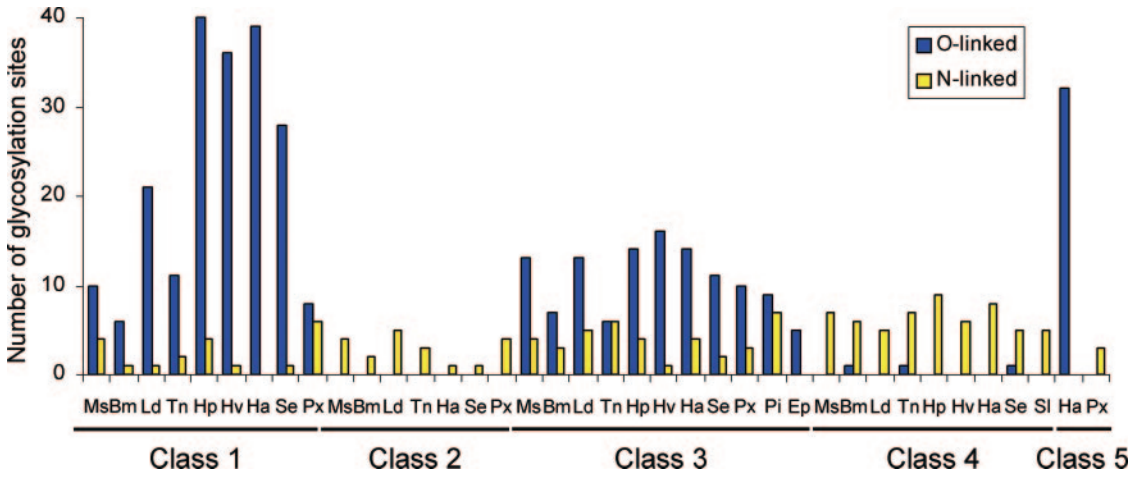


FIG. 7. Comparison of predicted N-linked and O-linked glycosylation sites among representative lepidopteran midgut APNs, sorted by class. Predictions were made using the NetNGlyc 1.0 server and the NetOGlyc 3.1 server (84) (http://www.cbs.dtu.dk/services/). Species name abbreviations and GenBank accession numbers are the same as in Fig. 5.

120 kDa (93), and 170 kDa (116) and totals of 6, 10, and 36 predicted O-linked glycosylation sites, respectively. In *M. sexta*, all 10 O-glycosylation sites are predicted to reside in the C-terminal stalk and are believed to be rich in GalNAc; thus, Knight et al. (92) have proposed that this region is highly likely to be a Cry1Ac binding site.

Five of the nine known class 1 APNs have been tested for their ability to bind to Cry toxins, and the interaction between the 120-kDa *M. sexta* APN and Cry1Ac is perhaps the best studied. APN was initially shown to bind to Cry1Ac by ligand blot analysis (93, 150) and was subsequently purified using a Cry1Ac protoxin affinity column (93). The fact that GalNAc could be used to elute APN from the column and that purified APN could be detected with the GalNAc-specific lectin soybean agglutinin (SBA) suggested that this carbohydrate was involved in toxin binding (93). Using surface plasmon resonance (SPR) analysis, Masson et al. (120) showed that in addition to Cry1Ac, the closely related Cry1Aa and Cry1Ab could also bind to purified native *M. sexta* APN. Cry1Ac was found to bind to APN at two different sites, one of which it shared with Cry1Aa and Cry1Ab. The affinity constants for Cry1Aa, Cry1Ab and Cry1Ac at the common binding site were 28.4 nM, 42.8 nM, and 40.7 to 95.3 nM (depending on the source of toxin), respectively, whereas that at the second Cry1Ac binding site was reported to be 149.4 to 299.3 nM. Unlike Cry1Ac, the interaction between APN and Cry1Aa or Cry1Ab was not inhibited by GalNAc. The more distantly related toxin Cry1Ca was also tested for binding to purified APN, but an interaction could not be detected. A 106-kDa APN has since been reported to bind to Cry1Ca, but the gene encoding this putative receptor has yet to be identified (114).

The interaction between Cry1Ac and exogenously expressed class 1 APN has also been studied. Using ligand blot analysis, Gill and Ellar (50) showed that Cry1Ac could bind to APN expressed in a transgenic line of *Drosophila melanogaster*. In contrast, Luo et al. (115) expressed the same APN in Sf21 cells, and despite its enzymatic activity, glycosylation, and membrane localization, binding to Cry1Ac could not be demonstrated. The authors suggested that posttranslational modification mechanisms in Sf21 cells may be different from those in epithelial cells in the *M. sexta* midgut (115).

A 170-kDa class 1 APN from *H. virescens* has also been identified as a Cry1A-binding protein (116). Like *M. sexta* APN, the first *H. virescens* APN was isolated by Cry1Ac affinity chromatography using GalNAc to elute the protein. By use of SPR analysis, Cry1Aa, Cry1Ab, and Cry1Ac, but not Cry1Ca or Cry1Ea, were shown to interact with the purified APN, and only Cry1Ac binding to APN could be inhibited with GalNAc. Unlike the observation with *M. sexta*, all Cry1A proteins were thought to bind to *H. virescens* APN at two sites, as determined by the observed 2:1 molar ratio of bound toxin to receptor, and by the good fit of experimental data to a two-binding-site model based on kinetics (116). In a later study (8), Cry1Fa was shown to bind to this APN by ligand blot analysis, and thus it appeared that class 1 *H. virescens* APN was not exclusively a Cry1A-binding protein.

Class 1 APN from *B. mori* has also been shown to interact with Cry toxins and is best characterized as a Cry1Aa-binding protein. The 120-kDa APN was released from BBMV with phosphatidylinositol-specific phospholipase C (PI-PLC) and then purified by ion-exchange chromatography (192). This preparation was shown to interact with Cry1Aa by both dot blot and ligand blot analysis. Cry1Aa binding to denatured APN expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* has also been demonstrated, providing evidence that Cry1Aa can interact with APN independently of glycosylation (194). By ligand blot analysis, Cry1Ab has also been reported to bind to *E. coli*-expressed APN (132), whereas Cry1Ac bound only weakly to purified native receptor (193). In contrast, neither Cry1Ab nor Cry1Ac were reported to bind to purified native *B. mori* APN by use of SPR analysis (80), and thus it not clear whether the interaction between APN and these toxins is biologically relevant. Of the three toxins, Cry1Aa is the most toxic towards *B. mori* larvae, and only limited toxicity has been observed with Cry1Ac (49, 99, 104).

Two other species have been reported to produce class 1

APN capable of interacting with Cry toxins: *H. armigera* (142) and *Plutella xylostella* (132). In both cases, binding was studied using exogenously expressed protein. Rajagopal et al. (142) expressed *H. armigera* APN in *Trichoplusia ni* cells by use of a baculovirus expression vector. The expressed protein was found to be membrane associated, catalytically active, and glycosylated and by ligand blot analysis could bind to Cry1Aa, Cry1Ab, and Cry1Ac. To study the interaction of Cry toxins with *P. xylostella* APN, a truncation mutant was expressed in *E. coli* as a GST fusion protein (132). This mutant was based on a previously identified toxin binding region found in a homologous region of *B. mori* APN (193). By ligand blot analysis, both Cry1Aa and Cry1Ab were reported to bind to this truncated mutant (132).

(ii) Class 2. Lepidopteran APNs in class 2 share the least sequence identity with the other classes (Fig. 5). Each member is predicted to be N glycosylated, but in stark contrast to class 1, there are no O-linked glycosylation sites predicted for any of the APNs, and the threonine-rich C-terminal stalk region reported for class 1 APN is completely absent. Interestingly, Cry1Ac has not been reported to bind to any member of class 2, supporting the theory that Cry1Ac binds to APN at the O-glycosylated C-terminal stalk (92). Cry1Aa and Cry1Ab have been reported to bind to class 2 APNs under certain conditions (31, 132), but it remains to be seen whether these interactions are biologically relevant.

Cry1Ab was first shown to bind to class 2 *M. sexta* APN by Denolf et al. (31). This interaction was demonstrated when APN was partially purified using a Cry1Ab affinity column. Using a high-pH carbonate buffer, proteins of many different molecular weights were eluted, in contrast to the Cry1Ac protoxin affinity purification of class 1 APN from *M. sexta* in which a single band was observed following GalNAc elution (93). Nevertheless, by ligand blot analysis Cry1Ab was shown to bind to a 120-kDa band in the purified fraction, and internal amino acid sequence data facilitated cloning of the encoding gene (31). Attempts to express the protein in Sf9 cells were unsuccessful, and thus it was not possible to confirm that the cloned gene actually encoded a Cry1Ab-binding protein.

Using the sequence information derived from class 2 *M. sexta* APN, Denolf et al. (31) were also able to study the corresponding APN in *P. xylostella*. In this case, expression in Sf9 cells was possible, and a 105-kDa glycoprotein with enzymatic activity was produced. Homologous competition binding assays using Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, or Cry9Ca and intact Sf9 cells expressing the APN did not reveal any specific toxin binding, and similar results were obtained with cell-derived membrane preparations. In addition, Cry1Ab binding to APN could not be demonstrated by ligand blot analysis. Since Cry1Ab had previously been shown by ligand blot analysis to bind to a 120-kDa protein in *P. xylostella* BBMV, it was not clear whether the cloned APN was a different protein or whether differences in glycosylation were responsible for the lack of binding. A study by Nakanishi et al. (132) further complicated the matter. This group expressed a putative Cry toxin binding region from class 2 *P. xylostella* APN as a GST fusion protein in *E. coli* and by ligand blotting showed binding to both Cry1Aa and Cry1Ab. In addition, they showed that Cry1Aa and Cry1Ab could bind to the same region in a class 2 APN from *B. mori*. Thus, it seems that further work must be carried out to clarify whether class 2 APNs are genuine Cry1A receptors, and if so, to what extent glycosylation plays a role in toxin binding.

(iii) Class 3. Class 3 is made up of the largest group of known lepidopteran APNs, with members from 11 different species. This class is most closely related to class 1 (Fig. 5), and similarly has a threonine-rich C terminus predicted to be highly glycosylated by the NetOGlyc 3.1 server (84) (http://www.cbs.dtu.dk/services/NetOGlyc/). Class 3 APNs generally have fewer predicted O-linked glycosylation sites than class 1 APNs (Fig. 7) and, of those isolated from BBMV, all have had a molecular mass near 120 kDa. Within this class, binding to Cry1Aa, Cry1Ab, Cry1Ac, Cry1B,a and Cry1Fa has been reported, as will be discussed.

APN from *L. dispar* is perhaps the best-studied member of class 3. It was cloned by Garner et al. (48), and based on sequence identity was believed to be the APN1 previously described by Valaitis et al. (177). In these earlier experiments, APN was released from BBMV by use of PI-PLC and was subsequently purified using a series of chromatographic steps. By ligand blot analysis, Cry1Ac was shown to bind to purified denatured APN. Binding was also tested by SPR analysis and under these conditions Cry1Ac, but not Cry1Aa or Cry1Ab, bound to native APN and Cry1Ac binding could be completely blocked with competing GalNAc (177). The binding of Cry1Ac to APN was reported to occur in a 1:1 ratio, in contrast to the 2:1 ratio reported for the interaction between Cry1Ac and class 1 *M. sexta* APN (120) and class 1 *H. virescens* APN (116). Cloned, exogenously expressed *L. dispar* APN has also been studied (48). Sf9 cells were transformed with a baculovirus vector encoding this APN, and the expressed protein was recognized by an APN-specific antibody; however, ligand blot analysis revealed only weak binding to Cry1Ac. As suggested in other studies, differences in posttranslational modification may account for the differences observed in toxin binding to native and recombinant forms of APN.

The binding of Cry1Ac to class 3 APN purified from *H. virescens* has also been reported (51). In this case, Cry1Ac was shown to bind to purified protein by ligand blot analysis, and the interaction could be blocked with competing GalNAc. It was also reported that Cry1Ac failed to bind to APN prepared by in vitro translation, providing additional evidence that glycosylation, or at least some form of posttranslational modification, was important for binding. Additional characterization was reported by Banks et al. (8), who partially purified a 120-kDa APN believed to be the same as that reported by Gill et al. (51) but whose identity was not confirmed by amino acid sequencing. The study showed that APN could bind to Cry1Ac and Cry1Fa by both affinity chromatography and ligand blotting (8). APN was also shown to react with SBA, a lectin specific for GalNAc, and chemical deglycosylation with periodate eliminated toxin binding.

Another APN from class 3 was identified in *H. armigera* (142). In this study, toxin binding to APN expressed exogenously in *T. ni* cells was studied. The expressed protein was 120 kDa in size, glycosylated, and enzymatically active and was shown by ligand blot analysis to react with Cry1Ac but not with Cry1Ab or Cry1Aa. Although the involvement of GalNAc in binding was not reported, a study by Wang et al. (183) suggests

that glycosylation may not be required for this interaction. This group expressed APN in *E. coli* and showed binding to Cry1Ac with ligand blot analysis.

Class 3 APN from *Epiphyas postvittana* has been studied in both its native and exogenously expressed forms (160). The native protein was purified from detergent-solubilized BBMV proteins by use of a combination of gel filtration and ion-exchange chromatography. APN expressed in Sf9 cells was purified using a similar method. Both Cry1Ac and Cry1Ba could bind to either form of APN by ligand blotting, but in competitive binding assays, neither toxin bound specifically to Sf9 cells expressing APN. To ensure that APN was being expressed on the cell surface, the researchers measured APN activity in cells before and after lysis and found the values to be comparable. Thus, concerns about the relevance of binding demonstrated by ligand blotting were raised (160).

Finally, there is some evidence that class 3 APNs from *B. mori* and *P. xylostella* can bind to Cry1Aa and Cry1Ab, based on reports by Nakanishi et al. (132), where binding to toxin binding regions expressed as GST fusion proteins in *E. coli* was shown by ligand blot analysis.

(iv) Class 4. Like class 2, class 4 lacks the C-terminal threonine-rich tract found in class 1 and class 3. There are currently nine members in this class, and three have been reported to interact with Cry toxins (2, 8, 132).

Class 4 APN from *H. virescens* has been reported to be a Cry1Ac-binding protein. This has been demonstrated using several different methods and was first shown by affinity chromatography, where CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}-solubilized BBMV proteins were passed over a Cry1Ac affinity column and bound proteins eluted with 2 M NaSCN (8). Under these conditions, three major binding proteins were eluted: a 170-kDa protein (class 1 APN), a 120-kDa protein (class 3 APN), and a 110-kDa protein (class 4 APN). Similar results were obtained with Cry1Fa, but no proteins were isolated using Cry1Ea, a toxin previously shown not to bind to *H. virescens* BBMV. That Cry1Ac could bind to class 4 APN following periodate treatment was evidence that glycosylation was not important for binding (8). In addition, SBA did not bind to this APN, suggesting that GalNAc moieties were not present in its glycans. It thus appeared that the interaction between Cry1Ac and class 4 APN differed from the interaction of Cry1Ac with class 1 and class 3 APN, where GalNAc was believed to be an important determinant of binding (93, 116, 177). Cry toxin binding to exogenously expressed class 4 *H. virescens* APN was also reported (7) and here it was shown by fluorescence microscopy that Cry1Ac could bind to S2 cells expressing APN but not to control cells. Attempts were also made to express APN in *E. coli*, but recombinant protein was not produced and thus the importance of glycosylation could not be tested by this method.

Class 4 also includes an APN isolated from *Spodoptera litura*. This species has been reported to be susceptible to Cry1Ca but resistant to Cry1Ac (2). Agrawal et al. (2) examined the binding of Cry1Ca and Cry1Ac to this APN expressed in Sf21 cells and showed that the protein was glycosylated, enzymatically active, and present on the cell surface. Ligand blot analysis showed that Cry1Ca but not Cry1Ac could bind to the denatured form of the protein. Toxin binding to APN was also studied under nondenaturing conditions (2). CHAPS-solubilized APN was incubated with Cry1Ca and immunoprecipitated with anti-Cry1Ca antibodies attached to protein A-Sepharose beads. Using negative control Sf21 cells or omitting Cry1Ca failed to precipitate the APN. A binding assay was also carried out with intact cells, and immunofluorescence showed that Cry1Ca but not Cry1Ac could bind to cells expressing APN. Attempts to express this protein in *E. coli* were unsuccessful, however, and thus the importance of glycosylation in toxin binding was not determined.

There is also some evidence that class 4 *B. mori* APN can bind to Cry1Aa and Cry1Ab, in particular to the proposed toxin binding region described by Nakanishi et al. (132).

(v) Class 5. Class 5 makes up the smallest group of APNs and consists of only two members identified in *P. xylostella* (132) and *H. armigera*. These APNs have an altered form of the highly conserved GAMEN motif, where methionine has been changed to threonine. The effect of mutations in this motif on enzymatic activity have been studied in the related protein insulin-regulated aminopeptidase (101). Here it was shown that mutating methionine to isoleucine, lysine, or glutamic acid decreased activity by 16-fold, decreased activity by 30-fold, or completely abolished activity, respectively. Although the enzymatic activity of the class 5 APNs has not been reported, it seems likely that at least some decrease in activity would be expected.

A comparison of the amino acid sequences of the *P. xylostella* and *H. armigera* APNs shows that there are marked differences in the threonine-rich C-terminal region. *H. armigera* APN has the longest reported open reading frame of any of the lepidopteran APNs and has many threonine residues at the C terminal, 32 of which are predicted to be O glycosylated according to the NetOGlyc 3.1 server (84) (http://www.cbs.dtu.dk/services/NetOGlyc/). In contrast, class 5 APN from *P. xylostella* completely lacks this threonine-rich region and has no predicted O-linked glycosylation sites.

Studies on the binding of class 5 APNs to Cry toxins are limited. The only report is from Nakanishi et al. (132), who showed by ligand blot analysis that a region of *P. xylostella* APN expressed in *E. coli* as a GST fusion protein could bind to Cry1Aa and Cry1Ab. Additional research is needed to determine the significance of these interactions and the general importance of class 5 APNs in mediating Cry toxin susceptibility.

(vi) Other APNs. In addition to the APNs already discussed, three other variants have been reported to bind to Cry toxins but have yet to be cloned and sequenced: a 106-kDa protein from *M. sexta* (114), a 100-kDa protein from the dipteran *Anopheles quadrimaculatus* (1), and a 96-kDa protein from *B. mori* (159). Based on immunoprecipitation experiments, the 106-kDa protein from *M. sexta* appeared to be a Cry1Ca-binding protein, although weaker binding to Cry1Ac was also detected. The N-terminal sequence was nearly identical to that of class 1 *M. sexta* APN (93, 113, 150), whereas an internal sequence—as it was later discovered—was identical to a region of class 2 *M. sexta* APN (31). Whether the preparation of 106-kDa APN used for sequencing contained a single novel fusion of class 1 and class 2 APN or two separate APNs derived from each class was never reported. The 100-kDa protein isolated from *A. quadrimaculatus* was purified from solubilized BBMV and tested for binding to mosquitocidal Cry toxins. By

SPR analysis (1), Cry11Ba, but not Cry2Aa, Cry4Ba, or Cry11Aa, was found to bind to the purified protein. Database searches with the N-terminal sequence of the 100-kDa protein led to its classification as an APN. The 96-kDa protein from *B. mori* was shown to bind to Cry1Ac by ligand blot analysis, and this interaction could be blocked with competing GalNAc (159). The protein was recognized by an antibody with specificity for class 3 *B. mori* APN, but by peptide mass fingerprinting, only 54% of the peptides could be matched. It was thus proposed that the 96-kDa protein was a novel isoform of class 3 APN.

(vii) Summary. The data on Cry toxin binding to APNs are complex, and several factors make this interaction difficult to study. First, several different APNs are believed to be simultaneously expressed in the larval gut. These proteins share sequence identity and can have similarities in properties such as molecular weight, enzymatic activity, and glycosylation. This can make it difficult to purify a particular APN to homogeneity and equally difficult to prove that the protein is pure. Indeed, mass spectrometry analysis of purified class 1 APN from *M. sexta* revealed the presence of contaminating class 3 and class 4 APNs (163). Although exogenous expression of cloned APNs is a possible solution, this can sometimes be difficult (7, 31, 48) and even when successful, tissue- or organism-specific differences in posttranslational modification may eliminate the toxin binding site (48, 115). To further complicate matters, different methods of studying toxin-APN interactions can sometimes give conflicting results (31, 132, 160). This was studied in detail by Daniel et al. (26), who showed that denaturing *M. sexta* APN or Cry1A toxins exposes binding epitopes hidden under nondenaturing conditions. Studies on Cry toxin binding to APN may also be complicated by the presence of cadherins, a second class of Cry toxin receptor particularly sensitive to proteolytic degradation (23, 119, 174). Cadherins have been shown to form approximately 120-kDa degradation products that could possibly be misinterpreted as APN in ligand blot assays (119).

Figure 8 presents a summary of the reported binding between Cry toxins and exogenous or endogenous nondenatured or denatured APN. While it is clear from this figure that several toxin-APN combinations have yet to be explored, some general conclusions can be drawn. Cry1Aa and Cry1Ab are best characterized as class 1 APN-binding proteins. Binding to both endogenous and exogenous forms of APN has been observed for several species and, as shown in Fig. 8, a lack of binding to any class 1 member has been reported in one case only (80). Binding to other APN classes under nondenaturing conditions either has not been reported or has not been observed. Cry1Ac has specificity broader than those of Cry1Aa and Cry1Ab and seems to be primarily a class 1 and class 3 APN-binding protein. Binding to class 1 APNs appears to occur at two sites, one of which it shares with Cry1Aa and Cry1Ab (120). Binding to the other site seems to be GalNAc dependent, and given that both class 1 and class 3 have a threonine-rich region predicted to be highly glycosylated, it is tempting to speculate that Cry1Ac mediates contact to both APNs in this region. As for the remaining toxins, it is difficult to make generalizations about their binding specificity based on the limited data available, and additional studies must be carried out to better characterize these proteins.

APN as a mediator of Cry toxin susceptibility. Since it was demonstrated that Cry toxins can bind to APN, additional studies have been carried out to distinguish between Cry-binding proteins and proteins that confer Cry toxin susceptibility. The following sections describe the various methods that have been used to make this distinction, along with the major findings of these studies.

(i) Permeability. The insecticidal nature of Cry toxins is generally believed to be due to their ability to form pores in the midgut of susceptible organisms (96), and assays have been developed to assess whether putative toxin receptors can enhance pore formation. The $^{86}Rb^{+}$ efflux assay has been used for this purpose, where pore formation is indicated by the release of $^{86}Rb^{+}$ from phospholipid vesicles containing putative receptor. Sangadala et al. (150) used this technique to demonstrate that a mixture of class 1 APN and phosphatase from *M. sexta* could enhance Cry1Ac pore formation. When reconstituted into phospholipid vesicles, these proteins were reported to increase toxin binding by 35% and to enhance toxin induced $^{86}Rb^{+}$ release 1,000-fold relative to protein-free vesicles. Similar results were obtained by Luo et al. (116), who showed that class 1 APN purified from *H. virescens* could enhance Cry1Aa-, Cry1Ab-, or Cry1Ac-induced release of $^{86}Rb^{+}$ but had no effect on Cry1Ca-induced release; Cry1a is a toxin shown not to bind to this class of APN (116). Pore formation has also been studied by measuring toxin channel activity in planar lipid bilayers. Schwartz et al. (153) showed that the inclusion of a purified *M. sexta* receptor complex in phospholipid bilayers caused Cry1Aa, Cry1Ac, and Cry1Ca to form channels at concentrations much lower than that in receptor free membranes. Analysis of this receptor complex by ligand blotting suggested that class 1 APN was the major Cry-binding protein.

(ii) In vitro toxicity. While assays that measure membrane permeability are good indicators of pore formation, they do not necessarily predict whether a receptor will confer toxin susceptibility to an organism. A more direct approach is to test whether Cry toxin-resistant cell lines can be made susceptible by expressing putative toxin receptors. So far, testing APNs by this method has been relatively unsuccessful. Garner et al. (48) expressed class 3 APN from *L. dispar* in Sf9 cells but did not observe cytotoxicity at Cry1Ac concentrations between 0.2 and 50 μg/ml. Because the Sf9 cells expressed APN with a binding affinity for Cry1Ac much lower than that of the native protein, the experiment was somewhat inconclusive. Class 4 APN from *H. virescens* was also tested for its ability to confer toxin susceptibility and was expressed in S2 cells (7). While it was demonstrated that Cry1Ac could bind to APN on the surface of intact cells, cytotoxicity was not observed at a toxin concentration of 30 μg/ml. The limited number of in vitro cytotoxicity-based studies is likely due to difficulties in correctly expressing APN, and obtaining proper glycosylation seems to be the main obstacle (48, 115). If these problems could be resolved, this method of receptor validation may become more useful.

(iii) In vivo toxicity. In vivo methods have also been used to test APN receptors for functionality. Gill and Ellar (50) fed Cry1Ac to transgenic *Drosophila* larvae expressing class 1 APN from *M. sexta* and showed 100% toxicity at a toxin concentration of 50 μg/ml. In comparison, control larvae were resistant to Cry1Ac at concentrations up to 1 mg/ml, the highest con-

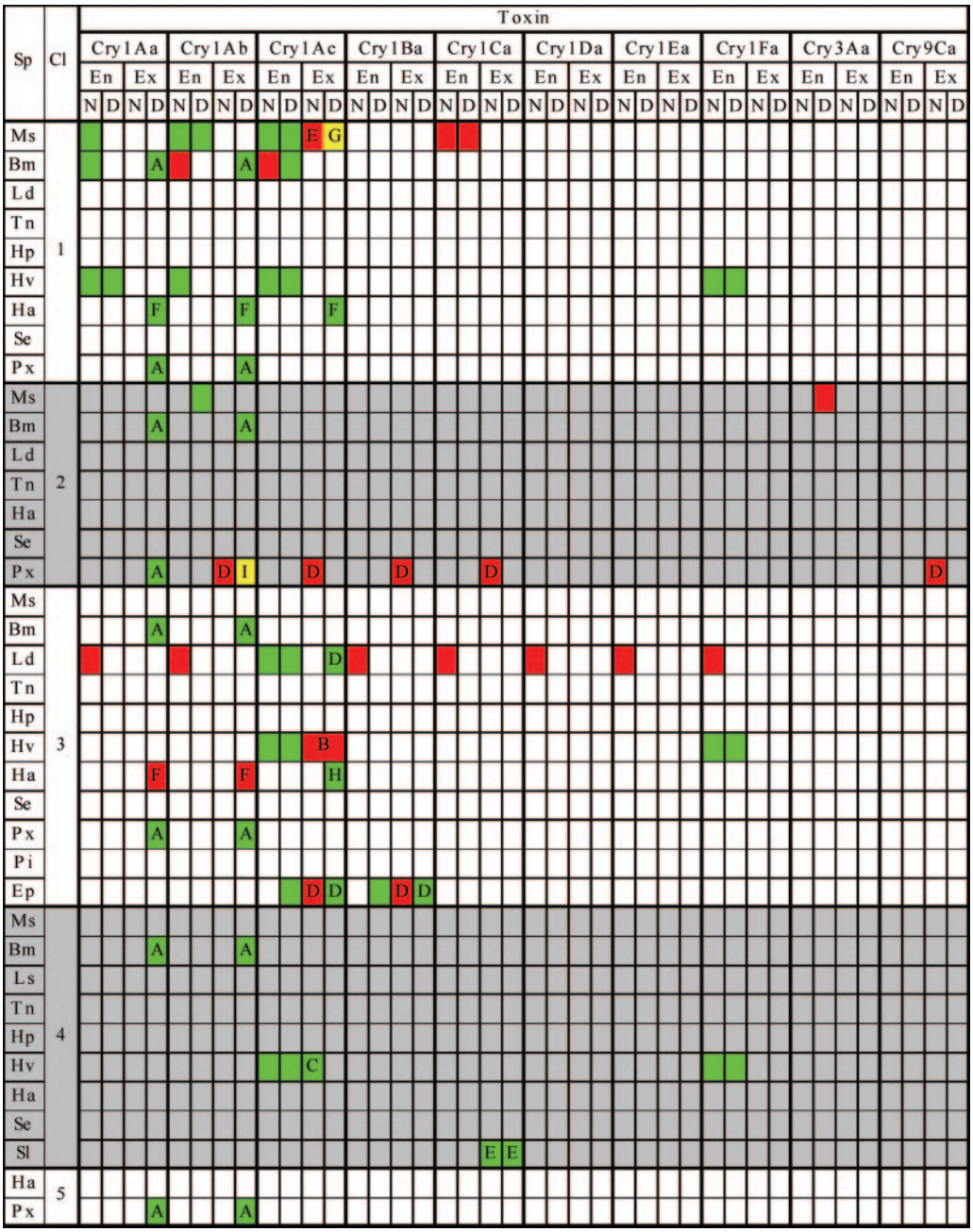


FIG. 8. Summary of reported binding between Cry toxins and endogenous (En) or exogenous (Ex) nondenatured (N) or denatured (D) APNs as discussed and referenced in the preceding sections. Binding, no binding, conflicting reports, and absence of data are indicated by green, red, yellow, or white/gray boxes, respectively. APN was expressed exogenously by *E. coli* (A) in vitro translation (B), S2 cells (C), Sf9 cells (D), Sf21 cells (E), *T. ni* cells (F), *Drosophila* or Sf21 cells (G), *E. coli* or *T. ni* cells (H), or *E. coli* or Sf9 cells (I). In cases where the conditions of binding (denaturing or nondenaturing) were not reported, boxes are merged. Species names are abbreviated as in Fig. 5. Species and class are abbreviated "Sp" and "Cl," respectively.

centration tested. The expression of *M. sexta* APN was confirmed by ligand blotting with Cry1Ac, and although expression levels were low, the receptor binding determinants were apparently intact. These results suggested that APN expressed in the *Drosophila* midgut may be properly glycosylated and that in vivo expression systems may be more suitable for evaluating toxin-receptor interactions than those based on cell lines.

Gene silencing has also been used to determine whether APN can confer toxin susceptibility. Rajagopal et al. (143) injected *S. litura* larvae with double-stranded RNA corresponding to a region of the class 4 APN gene and showed a 95% reduction in transcript levels over what was shown for control larvae. In addition, an 80% reduction in APN expression was observed, as determined by immunoblot analysis of BBMV proteins. When treated with Cry1Ca, a 75% reduction in mortality was observed in larvae previously injected with

double-stranded RNA. These results suggest that class 4 APN can confer Cry1Ca susceptibility to *S. litura* and demonstrate that gene silencing may be an effective way to study the biological significance of toxin-receptor interactions.

(iv) Summary. The biological relevance of the Cry toxin-APN interaction has yet to be studied extensively. To date, 17 different APNs have been reported to bind to Cry toxins, and yet only 2 have been shown to mediate toxin susceptibility. Twelve of the 17 APNs have not been studied for functionality by any method. In vivo methods of testing APN functionality have shown considerable promise, and using these methods to study the remaining APNs may lead to a better understanding of the overall importance of this class of Cry toxin receptor.

Cadherin

The cadherin superfamily of proteins is highly diverse and serves a variety of functions, including cell adhesion, migration, cytoskeletal organization, and morphogenesis (4, 65). The expression of cadherins is highly regulated, both spatially and temporally, and is often unique to a particular cell type. The proteins are defined by the presence of repeating calcium-binding domains or cadherin repeats of approximately 110 amino acids in length. Classical cadherins have 5 cadherin repeats (4, 134) but as many as 34 repeats have been reported (34). Some cadherins also have mucin (53), laminin, or epidermal growth factor-like repeats (133). The proteins are glycosylated and are usually anchored to the membrane by a single transmembrane domain, although seven-transmembrane (173) or GPI-anchored variants have also been identified (181).

In 1993, a novel cadherin-like protein was isolated from the midgut epithelium of *M. sexta* by virtue of its binding affinity for Cry1Ab (174). The protein was cloned in 1995 and sequence analysis predicted a signal peptide, 12 cadherin repeats, a membrane proximal extracellular domain, a transmembrane domain, and a small cytoplasmic domain (33, 175). Since then, additional lepidopteran cadherins have been identified, and all have been shown to have a similar domain organization (40, 44, 126, 130, 184). In *M. sexta* cadherin, additional features, such as the cell adhesion sequence HAV (10) and the integrin-binding sequences RGD (149) and LDV (98, 170), have been identified in the ectodomain; however, the functional role of these sequences has not yet been confirmed (33). In contrast, an analysis of the cytoplasmic domain did not reveal sequences known to interact with intracellular proteins such as catenins (33). While classical cadherins are located primarily within adherens junctions involved in cell-cell adhesion (4), lepidopteran cadherin-like proteins have been identified on the apical membrane of midgut columnar epithelial cells (3, 24, 66, 124), the target site of Cry toxins (16, 19, 20, 24). The expression of cadherin has been shown to vary with developmental stage and increases progressively from the first to the fifth instar in *M. sexta* larvae (124). In eggs and adults, however, cadherin expression has not been detected. Although the exact physiological function of midgut cadherins is not known, the tight control of cadherin levels during larval development has been proposed to indicate their importance in maintaining midgut epithelial organization (124).

Lepidopteran cadherin-like proteins have been extensively studied as Cry1A receptors, and there is good evidence to suggest they play a critical role in mediating toxin susceptibility. The following sections describe the best-characterized cadherin-like proteins and their interactions with toxins of the Cry1A family.

BT-R_1 (*Manduca sexta*). The first cadherin-like protein shown to interact with Cry toxins, BT-R_1, was a 210-kDa glycoprotein identified in *M. sexta* BBMV (174). The protein was purified by immunoprecipitation with Cry1Ab followed by two-dimensional gel electrophoresis. Partial sequence information derived from the purified receptor facilitated cloning, and the identified gene was 30 to 60% similar and 20 to 40% identical to other members of the cadherin superfamily (175).

To confirm that BT-R_1 was a genuine Cry1Ab receptor, it was expressed in cultured cells. The protein was first expressed in mammalian COS-7 and HEK-293 cells and by ligand blot analysis was detected as a 195-kDa band by probing with Cry1Ab (175). Cry1Ab binding to intact cells expressing BT-R_1 was also demonstrated, and the measured dissociation constant of 1 nM was similar to that of the native receptor. BT-R_1 was subsequently expressed in insect-derived Sf21 cells and was shown to bind to Cry1Aa and Cry1Ac but not to Cry3Aa and Cry11Aa, which are not toxic to *M. sexta* (91). In addition, competition binding studies showed that Cry1Aa and Cry1Ac could block Cry1Ab binding to membranes prepared from Sf21 cells expressing BT-R_1, suggesting that the toxins bind to a common epitope. Taken together, these results showed that Cry1A toxins could bind to endogenously or exogenously expressed BT-R_1 under both denaturing and nondenaturing conditions.

BT-R_1 was also tested for its ability to confer toxin susceptibility. Initially, HEK-293, COS-7, or Sf21 cells transfected with BT-R_1 failed to show any phenotypic changes when exposed to activated Cry1Ab, even at concentrations as high as 100 μg/ml (91). This unexpected result was explained when a frameshift mutation was discovered in the original cDNA clone (33). A revised analysis of the protein sequence showed that the frameshift mutation occurred upstream of the transmembrane domain, thus explaining why the protein was not embedded in the cytoplasmic membrane in earlier experiments and why the observed molecular weight of exogenously expressed BT-R_1 was less than that of the native protein. Subsequently, the error-free protein localized to the cell surface and rendered COS-7 cells sensitive to Cry1Ab at 0.6 μg/ml (33). S2 cells expressing BT-R_1 were also susceptible to Cry1A toxins, and 12 to 14% of cells were killed by Cry1Aa, Cry1Ab, or Cry1Ac at 20 μg/ml (75). The toxicity of Cry1Ab towards H5 cells expressing BT-R_1 was determined at a range of concentrations, and the 50% lethal concentration was reported to be 65 nM (about 4 μg/ml) (195). These results strongly suggest that BT-R_1 is an important determinant of Cry1A toxin specificity.

BtR175 (*Bombyx mori*). A cadherin-like protein was also identified as a Cry toxin receptor in *B. mori*. In this case, a 175-kDa glycoprotein, BtR175, was identified as a Cry1Aa receptor by immunoprecipitation (130, 131). Partial N-terminal sequencing of the purified receptor led to the cloning of a gene that shared significant homology to the cadherin superfamily of proteins and 69.5% identity to *M. sexta* BT-R_1. The predicted molecular mass of the encoded protein (193.3 kDa) was larger than that of the 175-kDa natural protein, and it was

believed that BtR175 was expressed as a proprotein. This was confirmed when the gene was expressed in Sf9 cells and a 175-kDa band comigrated with BtR175 isolated from BBMV. It was postulated that the sequence ^{288}RPPRWV^{292} may be an endoproteolytic cleavage signal that when cut gives rise to a mature BtR175 with only nine cadherin repeats (130). Interestingly, the proposed cleavage signal is also present in *M. sexta* BT-R_1, where there is no apparent cleavage at this site.

A second group has independently purified and partially sequenced a Cry1Aa receptor with a reported molecular mass of 180 kDa (77). The 103-amino-acid sequence obtained by this group was identical to a region within the sequence of BtR175 previously reported by Nagamatsu et al. (130). A later publication by the same group reported the sequence of three allelic BtR175 variants that differed from BtR175 by one, five, or six amino acids (78). All three receptors bound to Cry1Aa with a similar binding affinity (3.6 to 6.4 nM), although transient expression levels in COS7 cells varied considerably.

To further demonstrate the importance of BtR175 as a toxin receptor, various cell types expressing the gene were tested for cytotoxicity. Nagamatsu et al. (129) showed that exposure to 8 μg/ml Cry1Aa caused BtR175-expressing Sf9 cells, but not control cells, to swell within 15 min, and the number of swollen cells increased for 45 min after the addition of the toxin. These changes were quite similar to those of midgut columnar cells in *B. mori* fed with Cry1Aa and to those of epithelial cells isolated from the midgut and treated with toxin ex vivo (66). In another study, Cry1Aa caused cell swelling and cytotoxicity in mammalian cells expressing BtR175b (an allelic variant of BtR175) (171). Cry1Ab and Cry1Ac had similar albeit weaker effects, in correlation with their lower binding affinities for BtR175 (80, 171). This work demonstrated that BtR175 could confer Cry1A susceptibility outside of an insect system and made it possible to rule out the requirement for other insect specific factors in cytotoxicity.

To determine whether Cry1Aa-induced cell swelling was due to changes in ionic permeability, the membrane currents of Sf9 cells expressing BtR175 with or without the toxin binding region (discussed later) were compared (129). The toxin-induced currents of cells expressing the toxin binding region increased dramatically upon the addition of Cry1Aa, whereas no appreciable difference was observed with the control cells. These results suggested that pore formation leading to aberrations in osmoregulation was responsible for the observed morphological changes in the cells.

HevCaLP (*Heliothis virescens*). In *H. virescens*, genetics preceded biochemistry in identifying a cadherin-like Cry toxin receptor. This was accomplished by Gahan et al. (44), who studied a laboratory strain of *H. virescens*, YHD2, with a high level of recessive resistance to Cry1Ac (resistance ratio, 10,128×). Genetic studies revealed that a single major gene was responsible for 40 to 80% of resistance. With the knowledge that in some insects, resistance is accompanied by a loss in toxin binding, the researchers tested the genes of known Cry toxin-binding proteins to see whether they mapped to the region that conferred resistance. Two genes encoding APNs (class 1 and class 3) were tested for linkage, but they mapped to different regions of the genome. It was known that cadherin-like proteins bound to Cry toxins, but they had yet to be isolated from *H. virescens*. For this reason, the researchers searched for and found a BtR175 homologue in a susceptible strain. The gene was 70% identical to BtR175 and was named HevCaLP. Subsequently, the gene was mapped in resistant insects and found to reside in the resistance locus (44). The allele in resistant strains (r1) differed from the allele in susceptible strains (s1) by the presence of a 2.3-kb insert with hallmarks of a long terminal repeat-type retrotransposon. The insertion introduced a stop codon that truncated the encoded protein prior to the predicted transmembrane domain; thus, an explanation for why the mutation may have conferred resistance was provided.

The importance of HevCaLP as a toxin receptor was further studied by looking at the correlation between expression, binding, and toxin susceptibility in strains believed to have different mechanisms of Cry toxin resistance (89). Initially, it was confirmed that only s1 homozygotes or heterozygotes expressed full-length HevCaLP. It was subsequently shown that HevCaLP expression was necessary for Cry1Aa, but not Cry1Ab or Cry1Ac, binding to BBMV. This was in agreement with earlier studies showing that Cry1Ab and Cry1Ac bind to multiple sites on *H. virescens* BBMV, whereas Cry1Aa binds to a single site (88, 179). Confirmation that Cry1Ab and Cry1Ac could also bind to *H. virescens* cadherin was later provided by Xie et al., who expressed the receptor recombinantly in *E. coli* (190).

More recently, HevCaLP has been expressed in cell lines to determine whether the receptor can confer toxin susceptibility. *Drosophila* S2 cells expressing HevCaLP were sensitive to Cry1Aa, Cry1Ab, and Cry1Ac but, unexpectedly, not to Cry1Fa (87). Human embryonic kidney (HEK) cells expressing *H. virescens* cadherin were also treated with Cry1Ab or Cry1Ac, and although membrane blebbing was observed in some cells, cytotoxicity could not be demonstrated (3). Based on these results, it was proposed that other receptors, such as ALP or aminopeptidase, may be necessary for full toxicity (3, 87).

Cadherin-like proteins in other species. A link between cadherin-like proteins and Cry toxin susceptibility has been demonstrated for several other lepidopteran species. In 2005, two groups independently reported the sequence of a cadherin-like protein in *Ostrinia nubilalis* (25, 40). Flannagan et al. (40) cloned and expressed the putative Cry1Ab receptor in Sf9 cells and showed toxin susceptibility at concentrations as low as 0.1 μg/ml. Morin et al. (126) reported the sequence of a cadherin-like gene in *Pectinophora gossypiella* and identified three mutant alleles linked with resistance to Cry1Ac. In 2005, Xu et al. (191) published the sequence of a cadherin-like gene in *H. armigera* and found that disruption of the gene by a premature stop codon was linked to Cry1Ac resistance. Another group (184) demonstrated Cry1Ac binding to a recombinant *H. armigera* cadherin-like protein and using semiquantitative reverse transcription-PCR showed reduced gene expression in a strain resistant to Cry1Ac. A gene encoding a cadherin-like protein was also cloned from *L. dispar*, and the *E. coli*-expressed protein was reported to bind to Cry1A toxins (89a). In addition, insect cells expressing the gene were rendered toxin susceptible. Finally, the sequences of several other lepidopteran cadherin-like proteins have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html), including those of *P. xylostella*, *Chilo suppressalis*, *Helicoverpa zea*, *Agrotis*

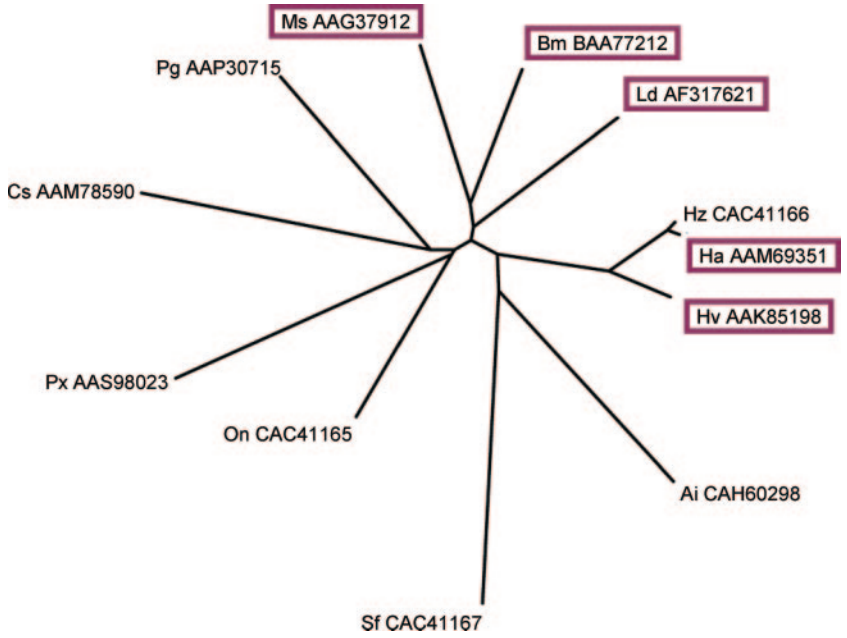


FIG. 9. Phylogenetic tree of lepidopteran cadherin-like proteins deposited in GenBank, created using the programs CLUSTALX and DRAWTREE (PHYLIP package). The species name and GenBank accession number is shown for each protein. Cadherins boxed in purple are those reported to bind to Cry toxins, as discussed in text. Species names abbreviations are as follows: Sf, *Spodoptera frugiperda*; Ms, *Manduca sexta*; Ld, *Lymantria dispar*; Hv, *Heliothis virescens*; Ha, *Helicoverpa armigera*; Hz, *Helicoverpa zea*; Bm, *Bombyx mori*; Px, *Plutella xylostella*; Pg, *Pectinophora gossypiella*; On, *Ostrinia nubilalis*; Cs, *Chilo suppressalis*; and Ai, *Agrotis ipsilon*. Only partial sequence information was available for *P. xylostella*; however, gaps in sequence alignment were excluded from tree construction.

ipsilon, and *Spodoptera frugiperda*. Figure 9 shows the phylogenetic relationship of reported lepidopteran cadherins.

Summary. The preceding sections have presented considerable evidence to support the idea that cadherin-like proteins expressed in the lepidopteran midgut are major determinants of Cry1A specificity. To date, all cloned cadherin genes expressed in cultured cells have been shown to bind to toxin and, when studied, to confer toxin susceptibility. The success of this approach may be due in part to the fact that glycosylation does not seem to be essential for toxin binding, and thus differences in glycosylation between proteins expressed in the midgut and proteins expressed in cultured cells may be irrelevant. Thus, the validation of cadherin-like proteins as genuine toxin receptors is comparatively easier than the validation of APNs, where glycosylation, in some cases, is critical for binding.

Although cadherin-like proteins are clearly important mediators of Cry1A susceptibility, it seems unlikely that they are universal Cry toxin receptors. For instance, the *H. virescens* strain YHD2 expresses a truncated form of HevCaLP and is highly resistant to Cry1A toxins but shows little cross-resistance to Cry2Aa, Cry1Ca, or Cry1Ba (59). Whether APNs, glycolipids, ALPs, or a yet to be discovered class of receptor mediates specificity for these toxins remains to be investigated.

ALP

ALPs have also been identified as Cry toxin receptors. Thus far, the work is very limited by comparison with research on the APN and cadherin-like receptors, and none of the putative receptors have been cloned or shown to have a direct role in toxicity. Nonetheless, preliminary results suggest that ALP may act as a Cry1Ac receptor in *M. sexta* (122, 150) and *H. virescens* (36, 85) and as a Cry11Aa receptor in *Aedes aegypti* (38).

In *H. virescens*, ALP is a 68-kDa GPI-anchored membrane glycoprotein (85). Binding to Cry1Ac was demonstrated by ligand blot analysis of BBMV and appears to be dependent on the presence of an N-linked oligosaccharide containing a terminal GalNAc residue. Interestingly, ALP expression levels were reduced in a resistant strain of *H. virescens*, suggesting a functional role in toxicity. The presence of a GPI anchor and the importance of GalNAc in toxin binding shows clear parallels to APN and its interaction with Cry1Ac (93, 116, 177).

In *M. sexta*, a 65-kDa BBMV protein was identified as a Cry1Ac-binding protein by two-dimensional gel electrophoresis followed by ligand blot analysis (122). It was identified as ALP by database searches of peptide mass fingerprints and by detection with an ALP-specific antibody. The protein was predicted to be GPI anchored but was not present in the pool of proteins released from BBMV by PI-PLC treatment. The role of *M. sexta* ALP in mediating Cry1Ac susceptibility has yet to be established, as has the importance of ALP glycosylation in toxin binding; however, the protein has been shown to colocalize with Cry1A toxins to the microvilli of *M. sexta* midgut epithelial cells (24).

ALP from *A. aegypti* has been reported to have properties similar to those of the lepidopteran ALPs (38). The protein is 65 kDa and is anchored to the membrane by a GPI anchor. It is abundant in BBMV and is estimated to account for 15 to 20% of total protein. Immunofluorescence studies (38) showed that ALP localizes predominantly to the ceca and posterior midgut and has a distribution pattern similar to that of bound

Cry11Aa. Binding to ALP has been demonstrated by ligand blot analysis and by Cry11Aa affinity chromatography (38). Phage displaying ALP-specific peptides blocked Cry11Aa binding to ALP and decreased toxicity, suggesting a functional role for ALP in mediating Cry toxin susceptibility (38).

Based on their similar properties, *A. aegypti* ALP and the same Cry-binding protein reported by Krieger et al. (100) and Buzdin et al. (22) are believed to be the same. In these studies, both Cry11Aa and Cry4Ba were shown to bind to ALP and compete for a common epitope. Interestingly, Cry9Aa also bound to ALP but did not compete with the other toxins and was not toxic to *A. aegypti* (22). Incubation with several carbohydrates, including GalNAc, failed to disrupt the interaction between toxin and ALP, and neither *N*-acetylglucosamine (GlcNAc) nor GalNAc could be used to elute the receptor from Cry11Aa- or Cry4Ba-Sepharose. As such, a role for carbohydrates in binding could not be demonstrated, in contrast to the GalNAc-dependent binding observed between Cry1Ac and *H. virescens* ALP (85).

Glycolipid

Glycolipids are another important class of putative Cry toxin receptor. While an interaction between glycosphingolipids and Cry toxins was first reported in 1986 (30), the importance of this receptor class was demonstrated only recently using the nematode *Caenorhabditis elegans* (60). A number of genetic, genomic, and cell biological tools have been developed for the study of *C. elegans*—tools not available for the study of insects controlled by Cry toxins—that make this organism particularly suitable for elucidating toxin receptors (118). Since nematocidal and insecticidal Cry toxins share sequence similarity (Cry6Aa being the exception), insight gained from *C. elegans* models may lead to advances in the study of Cry toxins active against other more economically important organisms.

The identification of glycolipids as Cry toxin receptors was made possible by the characterization of chemically mutagenized *C. elegans* strains selected for their resistance to Cry5Ba (61, 118). In total, 200 toxin-resistant lines were isolated, of which 45 were further characterized and found to be mutated at one of five loci (61, 118). Using forward genetics, four genes were identified that could restore toxin susceptibility and were named the *bre* genes for *B. thuringiensis* toxin *re*sistant (61, 62). The first gene to be characterized, *bre*-5, was found to be a member of the β1,3-galactosyltransferase family and encoded a protein most similar to the *Drosophila* protein BRAINIAC (62). Subsequently, *bre-2*, *bre-3*, and *bre-4* were characterized (61). *bre-2* encoded a putative β1-3-glycosyltransferase that shared approximately 25% amino acid identity in its enzymatic domain with other family members. *bre-4* was predicted to encode the previously characterized enzyme UDP-GalNAc:GlcNAc β1-4-*N*-acetylgalactosaminyltransferase (90), and *bre-3* was shown to encode a putative glycosyltransferase homologous to the *Drosophila* protein EGGHEAD. Thus, all resistance genes appeared to encode glycosyltransferases that showed no similarity to putative toxin receptors previously identified in insects.

Several experiments were conducted to determine how the inactivation of glycosyltransferases could lead to the observed toxin-resistant phenotype. Experiments showing that fluorescently labeled Cry5Ba was readily endocytosed by wild-type animals but not by *bre* mutants suggested that the glycosyltransferases synthesized a component necessary for the toxin to interact with intestinal cells (61, 62). Dose-response assays found no difference in the susceptibilities of single and double *C. elegans* mutants to Cry14Aa, providing evidence that the *bre* genes acted in a common pathway (61). Finally, the finding that *bre* mutants failed to produce certain ceramide-based glycolipids that specifically bound to Cry5Ba demonstrated that the *bre* genes function to produce a glycosphingolipid toxin receptor (60).

Because the *Drosophila* proteins EGGHEAD and BRAINIAC were predicted to catalyze consecutive glycosylation reactions involved in glycosphingolipid synthesis (128, 154, 182), it was postulated that glycolipids may also serve as Cry toxin receptors in insects (60). Preliminary results suggested that this might be the case, as glycolipids extracted from the midguts of *M. sexta* were shown to bind to Cry1Aa, Cry1Ab, and Cry1Ac. The importance of this interaction in mediating toxin susceptibility was not reported, however, and remains to be explored.

Other Receptors

Preliminary reports suggest that Cry toxins may bind to two additional types of receptors. The first, a 270-kDa glycoconjugate isolated from *L. dispar* called BTR-270, was shown to bind strongly to Cry1Aa, Cry1Ab, and Cry1Ba, weakly to Cry1Ac, and not at all to Cry1Ca, Cry2Aa, Cry2Ba, and Cry3Aa (176). The receptor has thus far been difficult to characterize; however, it appears to be a highly glycosylated anionic protein that may be a component of the brush border membrane glycocalyx. The second receptor, named P252, was isolated from a Triton X-100-soluble fraction derived from *B. mori* BBMV (73). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, *nsrsid1200748\delrsid1200748* the protein was estimated to have a molecular mass of 252 kDa; however, a 985-kDa homo-oligomer was detected by gel filtration chromatography. The purified receptor bound to Cry1Aa, Cry1Ab, and Cry1Ac under nondenaturing conditions. Lectin binding suggested the presence of N- and O-linked glycans, but GalNAc did not inhibit toxin binding. To characterize the protein further, two internal peptides were sequenced but did not match any proteins in the available databases. Interestingly, however, reanalysis of these peptides for the present review generated significant matches (12/15 and 15/15 identical residues) with a recently described protein called chlorophyllid A-binding protein (ChBP) (121). This protein was identified in the midgut of *B. mori* and was characterized by its ability to bind to a derivative of chlorophyll. The mass of ChBP was estimated to be between 302 and 312 kDa, but unlike for P252, glycosylation could not be detected. Sequence analysis revealed that ChBP was an unusual member of the lipocalin family (41, 42) and contained 15 prototypic lipocalin domains. Whether ChBP and P252 are the same protein remains to be confirmed.

DETERMINANTS OF BINDING

APN

Receptor determinants. While many reports have identified APNs as Cry toxin-binding proteins, little is known about how the molecules interact. As discussed earlier, GalNAc appears to be an important determinant of the Cry1Ac-APN interaction; however, the nature and position of the glycan structure involved in binding have not yet been determined. A recent report on the glycans of class 1 APN from *M. sexta* has provided some insight into the possible determinants of binding. Stephens et al. (163) carried out an extensive characterization of the N-linked oligosaccharides by mass spectrometry. Three of the four glycosylation sites (N295, N623, N752) were linked to highly fucosylated glycans with unusual difucosylated cores. The remaining site (N609) was glycosylated with the paucimannosidic glycan common to insect glycoproteins (168). Interestingly, GalNAc was not identified as a component of any of the glycans, suggesting that N-linked carbohydrates are unlikely to be involved in toxin binding. This finding supports the theory that Cry1Ac interacts with APN at its threonine-rich C-terminal stalk predicted to be O glycosylated with mucin-type (GalNAc-α-O-Ser/Thr) glycans (92, 93).

Attempts have also been made to locate putative toxin binding sites in APNs believed to interact with toxin in a glycan-independent manner. In one study, class 1 APN purified from *B. mori* was proteolytically digested and probed with Cry1Aa in a ligand blot assay (193). The toxin bound to two digestion products, the smaller a 30-kDa fragment believed to consist of amino acids Asp40 to Lys313. Binding was also assessed using fragments of APN expressed as GST fusion proteins in *E. coli*. The smallest fragment that retained toxin binding affinity corresponded to APN amino acids residues Ile135 to Pro198. More recently, Cry1Aa binding to the same APN fragment was demonstrated under nondenaturing conditions by enzyme-linked immunosorbent assay (6).

Cry1Aa and Cry1Ab have also been tested for their abilities to bind to the equivalent toxin-binding fragments in class 1, class 2, class 3, and class 4 APN derived from *B. mori* and *P. xylostella* (132). By ligand blot analysis, both toxins bound to all recombinant APNs, suggesting a conserved, common receptor binding site; however, other results questioned the biological relevance of this finding. It was shown by ligand blot analysis that neither Cry1Aa nor Cry1Ab bound to intact class 2 (90-kDa), class 3 (110-kDa), or class 4 (100-kDa) APN isolated from *B. mori* BBMV (132). This was in agreement with results reported by Jenkins and Dean (80), who showed that Cry1Aa failed to bind to 100-kDa and 110-kDa forms of *B. mori* APN under nondenaturing conditions. It was also shown that Cry1Ab did not bind to class 1 APN purified from *B. mori* BBMV by SPR analysis (80). Since it has been demonstrated that denaturing APN can reveal binding epitopes hidden in the folded protein (26), studies under nondenaturing conditions must be conducted to determine whether the Cry1Aa binding site identified in class 1 *B. mori* APN also binds to Cry1Ab and whether this site is truly conserved among the other APNs.

Toxin determinants. Of the different Cry toxins that have been reported to bind to APN, only Cry1A toxins have been examined further for their determinants of binding. From these studies, three APN binding sites have been proposed. The best characterized site is unique to Cry1Ac and is found on the outer sheet of domain III. At this site, Cry1Ac is believed to bind to APN via a GalNAc-containing glycan. A second binding site shared by both Cry1Ab and Cry1Ac was identified at the apex of domain II and is believed to be formed by surface-exposed loops. The remaining site was identified on Cry1Aa and Cry1Ab and has been proposed to occur near the interface between domain II and domain III.

(i) Domain III. The importance of Cry1Ac domain III as a determinant of APN binding was first shown by Lee et al. (106), who constructed hybrids from Cry1Aa and Cry1Ac and analyzed their binding properties. Under nondenaturing conditions, it was shown that only toxins with domain III derived from Cry1Ac bound strongly to purified *L. dispar* APN. Subsequently, it was shown that GalNAc could completely disrupt this interaction (82). Similar results were obtained by De Maagd et al. (27), who showed that only Cry1Ac/Cry1Ca hybrids constructed with Cry1Ac domain III could bind to purified *M. sexta* APN and that binding could be inhibited with GalNAc.

To understand how Cry1Ac domain III might interact with APN, Burton et al. (21) mutated several residues on the outer sheet of domain III. This region was proposed to form a GalNAc binding pocket based on the structural similarity between domain III and the N-terminal cellulose-binding domain of *Cellulomonas fimi* 1,4-β-glucanase CenC (CBD_{N1}) (83). Residues within and around β-strand 16 were of particular interest based on a preliminary analysis of the Cry1Ac crystal structure that showed a unique conformation in this region (21). In total, 18 single mutants, 1 double mutant, and 1 triple mutant were constructed and tested for binding to *M. sexta* BBMV. Several mutants showed reduced binding, but the greatest decrease was observed with the triple N506D Q509E Y513A mutant. Ligand blot analysis confirmed that this mutation decreased binding to APN. That incubation with GalNAc did not further decrease triple mutant binding to BBMV provided evidence that the interaction between Cry1Ac and *M. sexta* APN at this site was indeed GalNAc dependent. Similar results were obtained in other studies. Jenkins et al. showed that mutants with alanine substitutions at Q509, R511, Y513, or ^{509}QNR^{511} failed to bind to purified *M. sexta* APN (81) and bound to purified *L. dispar* APN with lower affinity (82).

The crystal structure of Cry1Ac, solved in the presence and absence of GalNAc, has helped to further characterize the domain III APN binding site and has provided a structural explanation for why Cry1Ac, but not Cry1Aa, binds to APN in a manner dependent on GalNAc (32, 108). Analysis of the structure revealed a unique 6-residue insertion (^{505}GNNIQ N^{510}) in domain III that was not observed in Cry1Aa and was not predicted by an automatically generated model of Cry1Ac (81). This insertion was shown to project from the outer sheet of domain III and curve back to form a shallow GalNAc-binding cavity. The structure of Cry1Ac with bound GalNAc was very similar to that without bound GalNAc; however, residues in contact with GalNAc were more ordered. In addition, it was observed that the temperature factor of residues in domain I increased upon GalNAc binding. This led to the hypothesis that ligand binding at domain III may increase the mobility of the pore-forming

domain, possibly triggering a conformational change leading to membrane insertion (108). Consistent with this hypothesis, Pardo-López et al. (136) recently reported that GalNAc binding to an oligomeric form of Cry1Ac enhanced toxin membrane insertion.

(ii) Domain II. The loops at the apex of domain II have also been implicated in APN binding. Lee et al. (105) mutated two arginine residues (368 and 369) in loop 2 of Cry1Ab and Cry1Ac that had previously been shown to be important for toxicity and BBMV binding in *M. sexta* and *L. dispar* (144). Consistent with previous results, SPR studies suggested that Cry1Ab bound to *M. sexta* APN at a single site, whereas Cry1Ac bound to both *M. sexta* and *L. dispar* APN at two sites (82, 120). Cry1Ab mutants with alanine or glutamic acid substitutions showed a nearly 10-fold decrease in binding to *M. sexta* APN. The corresponding Cry1Ac mutants also showed reduced binding and kinetic data suggesting that only one of the two proposed binding sites was affected, consistent with the presence of a distinct APN binding site in domain III (see above). Similar results were obtained when the Cry1Ac alanine and glutamic acid mutants were tested for binding to purified *L. dispar* APN. In another study, additional Cry1Ac derivatives with mutations at loop 2, loop 3, and loop α8 were tested for their abilities to bind to purified *L. dispar* APN, and each showed decreased binding to only one of the two proposed APN binding sites (82). A subsequent study showed that loop α8 mutants also decreased binding to purified *M. sexta* APN (103).

In contrast to the results reported above with folded albeit mutated whole toxin, work carried out in the Soberón laboratory found that synthetic peptides derived from the domain II apex loops had no effect on APN binding. Gómez et al. (55, 56) used ligand blot analysis to examine the binding of Cry1Ab to denatured *M. sexta* APN in the presence or absence of synthetic peptides corresponding to sequences from loop 1, loop 2, loop 3, or loop α8. Even in the presence of a 500- to 1,000-fold excess of synthetic peptide, no decrease in binding was observed. Since Cry1Ab detection of APN by ligand blot analysis varies among different laboratories (27, 124, 174), the significance of these results is unclear and requires further investigation.

(iii) Domain II/III interface. A third region that has been proposed to act as an APN binding site was first characterized for Cry1Aa (6). This region was identified at the interface between domain II and III and was shown to interact with the 64-amino-acid toxin-binding fragment of class 1 *B. mori* APN (193). The putative binding site was identified by mapping the epitopes of two monoclonal antibodies that bound to Cry1Aa and blocked its interaction with APN. Based on the results of several binding studies, both antibodies appeared to bind to the outer sheet of domain III at distinct but overlapping sites that included residues ^{508}STLRVN^{513} and ^{582}VFTLSAHV^{589}. Additional studies showed that the APN binding site was close to, but did not include, this region. The interface between domain II and domain III was proposed as a candidate APN binding site based on its proximity to the antibody binding sites and on the presence of conserved residues in this region that are common to Cry toxins with specificity for class 1 *B. mori* APN. Recently, Gómez et al. used synthetic peptides to show that a similar region in Cry1Ab may interact with APN from *M. sexta* (54).

Cadherin

Receptor determinants. To learn more about the involvement of cadherin-like proteins in facilitating Cry toxin susceptibility, several groups have tried to define a toxin binding region. Nagamatsu et al. (129) studied the *B. mori* receptor BtR175. The group expressed truncated variants of BtR175 in Sf9 cells and under nondenaturing conditions identified a region within amino acids 1245 to 1464 necessary for Cry1Aa binding (129). That a variant consisting of amino acids 1108 to 1715 conferred Cry1Aa susceptibility to Sf9 cells but a variant lacking amino acids 1246 to 1390 did not confirmed the functional importance of the toxin binding region. That a synthetic peptide corresponding to BtR175 residues 1296 to 1301 partially blocked the interaction between Cry1Aa and Cry1Ab and BtR175 provided additional support (57).

Toxin binding sites have been studied more extensively in the *M. sexta* cadherin receptor; however, there are some discrepancies over the regions involved in binding. Gómez et al. (55–57) used competition studies to show that synthetic peptides derived from BT-R_1 at 869 to 876 and 1331 to 1342 could interfere with Cry1A binding to denatured cadherin and could mitigate Cry1Ab toxicity to neonates. They also showed that recombinant fragments corresponding to amino acids 831 to 900 and 1291 to 1360 could bind to both nondenatured and denatured forms of Cry1Ab (55). In contrast, Dorsch et al. (33) identified a single toxin binding site. The group expressed truncated BT-R_1 peptides by use of in vitro translation and under denaturing conditions showed that a fragment corresponding to amino acids 1296 to 1465 was sufficient for Cry1Ab binding. Since Cry1Ab did not bind to a fragment containing residues 1 to 1296, there was no evidence for a binding site between residues 831 and 900. Subsequent work by Hua et al. (74) examined Cry1Ab binding to various truncated forms of BT-R_{1a} and under nondenaturing conditions, a fragment consisting of residues 1363 to 1464 was found to be sufficient for binding. This fragment did not include any of the binding sites proposed by Gómez et al. (55–57), but when expressed in S2 cells, it could confer susceptibility to Cry1Ab. It was also found that fragments containing residues 869 to 876 or 1331 to 1342 but lacking residues 1363 to 1464 failed to bind to Cry1Ab under nondenaturing conditions.

Toxin binding sites have also been mapped for cadherins from two other species. Cry1Ac was shown to bind to *H. armigera* cadherin residues 1217 to 1461 expressed as a GST fusion protein in *E. coli* (184), and in *H. virescens* cadherin, a combination of truncation analysis and site-directed mutagenesis was used to identify a Cry1A toxin binding region within amino acids 1422 to 1440 (190).

Since the majority of toxin binding sites appear to be located at or near the most membrane-proximal cadherin repeats (Fig. 10), it is tempting to speculate that binding in this region is important for the toxin's mechanism of action. To test this hypothesis, the toxin susceptibility of cells expressing wild-type cadherin could be compared to cells expressing a recombinant form of cadherin modified to position the toxin binding site away from the membrane.

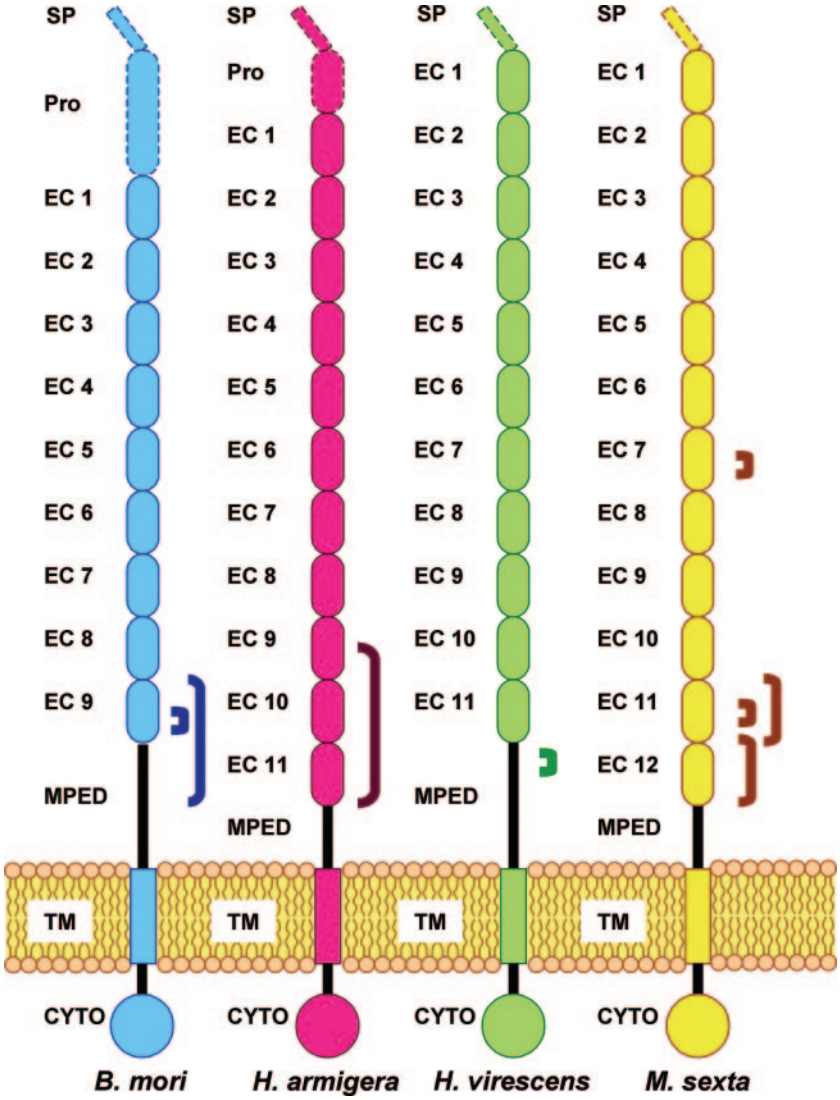


FIG. 10. Domain structure and putative Cry1A toxin binding sites in lepidopteran cadherin-like proteins. The proteins are labeled as follows: PRO, proprotein region; SP, signal peptide; EC, ectodomain; MPED, membrane-proximal extracellular domain; TM, transmembrane domain; CYTO, cytoplasmic domain. Domains are as defined by Nagamatsu et al. (130) (*B. mori*), Wang et al. (184) (*H. armigera*), Gahan et al. (44) (*H. virescens*), and Dorsch et al. (33) (*M. sexta*). Putative toxin binding sites are as reported by Nagamatsu et al. (129) (*B. mori*), Wang et al. (184) (*H. armigera*), Xie et al. (190) (*H. virescens*), and Dorsch et al. (33), Gómez et al. (55–57), and Hua et al. (74) (*M. sexta*). Proteins are illustrated such that homologous regions are horizontally adjacent. Features not expected in the mature form of the protein are outlined with a dashed line.

Toxin determinants. Thus far, studies on the toxin determinants of binding to cadherin are limited. In most cases, putative binding sites have been assessed by testing whether synthetic peptides can disrupt the interaction between toxin and denatured receptor. By this method, Gómez et al. (55, 56) showed that synthetic peptides derived from loop α8 and loop 2 of Cry1Ab and that loop 2 and loop 3 of Cry1Aa were involved in binding to the *M. sexta* cadherin receptor BT-R_1. Additional studies with Cry1Ab suggested that loop α8 and loop 2 bound to two distinct sites on BT-R_1 (55), but as discussed in the previous section, the validity of these toxin binding sites is currently in dispute (33, 74). Similar methods have been used to identify the regions of Cry1Ab and Cry1Ac responsible for binding to denatured CAD3, a recombinant toxin-binding fragment derived from the *H. virescens* cadherin-like protein (190). In the presence of a synthetic peptide corresponding to loop 3, Cry1Ab and Cry1Ac binding to CAD3 was diminished, whereas a loop α8 peptide had no effect on binding. In addition, it was found that a synthetic peptide corresponding to loop 2 decreased Cry1Ab, but not Cry1Ac, binding to CAD3. These apparent differences in CAD3 binding are surprising given that the sequences of Cry1Ab and Cry1Ac in domain II are nearly identical. It would be interesting to see whether additional studies with correctly folded proteins or with domain II mutants support the findings of these preliminary studies.

TOXIN MOLECULAR MECHANISM OF ACTION

Given the number of Cry toxins, putative toxin receptors, and insects currently under investigation, it is perhaps not surprising that the Cry toxin mode of action is controversial.

Even the general view that toxin monomers bind to midgut receptors, oligomerize, and insert into the membrane to form lytic pores has recently been challenged (195, 196). In this section, three contrasting models of Cry1A toxin mode of action will be presented and discussed. The first two models differ considerably in terms of which receptors are important for toxicity and how these receptors facilitate toxin function. The third model combines elements of the first two models and also proposes a functional role for an additional receptor class.

The Bravo Model

The Bravo model is an updated version of the colloid-osmotic lysis model of Knowles and Ellar (96) and proposes that both cadherin and APN receptors are required for full Cry1A toxicity towards *M. sexta* (18). The model is based primarily on experiments involving Cry1Ab interactions with BBMV (18, 58, 197), and suggests that receptor binding is sequential: activated toxin monomer binds initially to BT-R_1 and then to class 1 APN. After binding to BT-R_1, it is proposed that the toxin undergoes a conformational change that facilitates cleavage of helix α-1 by membrane-bound proteases (58). This form of the toxin oligomerizes to form a tetrameric prepore (58) that preferentially binds to APN as a result of an increase in binding affinity (18). APN then directs the prepore to detergent-resistant membranes (DRMs), or lipid rafts, that facilitate membrane insertion to form a lytic pore (18). The generation of as few as 200 copies of this pore, with a reported radius of 0.5 to 1.0 nm, in the columnar cell apical membrane leads to very rapid changes in membrane potential, equilibration of ions across the membrane, influx of water, cell swelling, and eventual lysis (95). In the following sections, experimental evidence for and against this model will be presented.

The theory that solubilized, proteolytically activated Cry toxin monomer binds to BT-R_1 before APN is based primarily on immunoprecipitation experiments with Cry1Ab protoxin and BBMV that show differences in the recoveries of BT-R_1 and APN over time (18). While both BT-R_1 and APN were detected at all time points, the amount of BT-R_1 that coprecipitated with Cry1Ab decreased slightly with time, whereas APN recovery increased by fourfold over the course of the experiment. This, in conjunction with earlier data showing that Cry toxin binds to BT-R_1 with an affinity 100-fold greater than that to APN (150, 174), was taken to indicate that Cry1Ab binding to BT-R_1 occurs before APN binding; however, other explanations of the data are possible. For instance, differences in receptor abundance could explain why maximal levels of BT-R_1 are coprecipitated at all time points whereas APN recovery increases progressively. In *B. mori*, APN was estimated to be 100-fold more abundant than BtR175 on midgut microvilli (66, 131). Similarly, disruption of the gene encoding HevCaLP did not decrease Cry1Ab and Cry1Ac binding to BBMV (89), suggesting that in *H. virescens*, cadherins may also be of low abundance relative to other receptors. If this difference in receptor concentration is also present in *M. sexta*, it would seem likely that high-affinity (1 nM [174]), low-abundance BT-R_1 binding sites are quickly saturated with toxin, whereas low-affinity (100 nM [150]), high-abundance APN binding sites become saturated more slowly. In such a situation, APN may actually function to concentrate Cry1Ab at the surface of the membrane before the toxin is relayed to less abundant cadherin receptors. A similar model has been used to explain how the channel-forming toxin aerolysin might penetrate the glycocalyx to reach receptors on the surface of target epithelial cells: the toxin initially binds to low-affinity carbohydrate structures before being shuttled to high-affinity binding sites on the glycan core of GPI-anchored proteins (117).

After monomeric Cry1Ab binds to BT-R_1, it is believed to undergo a conformational change that facilitates proteolytic cleavage between helices α-1 and α-2. This event exposes a hydrophobic surface that is important for toxin oligomerization (58). While a direct role for native BT-R_1 in facilitating toxin cleavage has not yet been demonstrated, experiments with Cry1Ab and recombinant toxin-binding fragments (55) or the single-chain antibody scFv73 (58) suggest that this may be the case. scFv73 is reported to act as a BT-R_1 mimic. It has been shown to bind to domain II of Cry1Ab and block its interaction with denatured BT-R_1 and to reduce the toxicity of Cry1Ab towards *M. sexta* larvae (57). When scFv73 was incubated with solubilized Cry1Ab protoxin prior to digestion with *M. sexta* midgut juice, the resulting toxin preparation had a high in vitro pore-forming activity relative to toxin prepared in the absence of scFv73. Examination of each preparation by immunoblotting revealed a 250-kDa toxin oligomer found only in the sample preincubated with scFv73. The separation of the oligomer from monomer by gel filtration allowed each species to be tested for pore-forming activity independently, and it was shown that one-fifth the molar concentration of oligomer was almost 2.6-fold more active than monomer. A later study showed that unlike monomeric Cry1Ab, the oligomer formed stable channels with a high probability of being open in a lipid bilayer (147). Analysis of each sample by N-terminal sequencing revealed differences in the sites of proteolytic cleavage: the oligomer between helices α-1 and α-2 (before residue Val-51) and the monomer at the beginning of helix α-1 (before Ile-29). It was thus concluded that binding to BT-R_1 facilitated cleavage of helix α-1, leading to toxin oligomerization and pore formation.

While the results presented above are intriguing, not all data support the hypothesis that Cry1Ab binding to BT-R_1 facilitates cleavage of helix α-1 or that cleavage in this region promotes toxin oligomerization. For example, Miranda et al. (125) treated Cry1Ab with various dilutions of *M. sexta* midgut juice and identified toxin products migrating at 60, 58, and 30 kDa. N-terminal sequencing of the 60-kDa product revealed that in the absence of receptor, cleavage occurred between residues Leu-57 and Gly-58. It was also shown that Cry1Ab protoxin activated either in vitro with *M. sexta* midgut juice or in vivo in *M. sexta* larvae failed to form oligomeric toxin, as determined by immunoblotting. Other studies have questioned whether BT-R_1 has any involvement in toxin oligomerization and even whether the oligomeric species detected by immunoblotting has any role in cytotoxicity. Aronson et al. (5) tested wild-type Cry1Ac and domain I mutants with or without toxicity towards *M. sexta* larvae for their abilities to oligomerize when incubated with BBMV. While a correlation between toxicity and oligomer formation was observed in most cases, the mutant H168R was two- to threefold more toxic than the wild type and yet failed to oligomerize, according to immunoblot analysis. More evidence against the biological relevance of the oligo-

meric Cry1Ab species has come from a study by Zhang et al. (195), who examined Cry1Ab binding to toxin-susceptible cells expressing BT-R_1 (S5) and toxin-resistant control cells (H5). After treatment with Cry1Ab, each cell type was analyzed by immunoblotting. Both monomeric and oligomeric Cry1Ab associated with S5 cells but, surprisingly, the Cry1Ab oligomer also associated with control cells. This result showed not only that the Cry1Ab oligomer could be detected in the absence of BT-R_1 but also that the presence of this species in the cell membrane did not correlate with cytotoxicity, despite its high in vitro pore-forming activity (58, 147). Although these data argue against the biological relevance of the oligomeric species detected by immunoblotting, they do not necessarily rule out the importance of oligomerization in the toxin's mode of action, since the true oligomeric form of the toxin may not be detectable by immunoblotting.

The next step in the Bravo model of toxin mode of action proposes that oligomeric toxin attached to BT-R_1 binds to APN. This is based on experiments showing that relative to the monomer, oligomeric Cry1Ab immunoprecipitates more APN from solubilized BBMV and has a higher affinity for an enriched preparation of APN (18). Once bound to APN, the toxin-receptor complex is then believed to localize to DRM microdomains, where the prepore inserts into the membrane. This hypothesis is based on several experiments. First, an analysis of receptor localization in BBMV prior to toxin exposure revealed that APN and BT-R_1 associate with different membrane fractions: BT-R_1 with the soluble membrane fraction and the majority of APN with the DRM fraction (18). After BBMV was incubated with Cry1Ab protoxin, BT-R_1 was found exclusively in the DRM fraction, along with the majority of Cry1Ab (monomer and oligomer) and APN. It thus appeared that BT-R_1 was recruited to DRMs due to its association with oligomeric Cry1Ab. To confirm the importance of APN in this process, BBMV were pretreated with PI-PLC to reduce APN levels and then incubated with Cry1Ab protoxin (18). As a result of this treatment, a drastic reduction in the amount of Cry1Ab bound to BBMV was observed and Bt-R_1 no longer associated with the DRM fraction.

A functional role for DRMs has been suggested by experiments examining the effect of DRM integrity on pore formation (197). DRM vesicles isolated from *M. sexta* or *H. virescens* BBMV were incubated with methyl-β-cyclodextrin, a reagent that destroys DRMs by extracting cholesterol from the membrane, and compared to control vesicles in an assay measuring pore-forming activity. Methyl-β-cyclodextrin was found to substantially reduce Cry1Ab pore formation in vesicles from both species.

The Zhang Model

In a recent study, the theory that Cry1Ab kills cells exclusively by osmotic lysis has been challenged. Zhang et al. (195) argued that the correlation between pore formation and cytotoxicity has not been adequately demonstrated and proposed an alternative mode of action in which monomeric Cry1Ab binds to BT-R_1 and initiates an $Mg2^+$-dependent signaling cascade that promotes cell death. Additional work suggested that receptor binding activates a signaling pathway involving stimulation of G protein, adenylyl cyclase (AC), increased cyclic AMP (cAMP) levels, and activation of protein kinase A (PKA), leading to destabilization of the cytoskeleton and ion channels and subsequent cell death (196). This section will discuss the experimental evidence upon which the Zhang model is based.

To study the mechanism of Cry1Ab cytotoxicity, Zhang et al. (195, 196) carried out various treatments on toxin-susceptible cells heterologously expressing BT-R_1 (S5) and toxin-resistant control cells (H5). In the presence of Cry1Ab, S5 cells underwent membrane blebbing and ruffling that led to swelling and eventually to cell death. Since calcium had been previously shown to be important for the structural integrity of BT-R_1 (23) and was proposed to mediate BT-R_1-dependent adhesion of *M. sexta* BBMV (63), the effect of calcium on receptor binding and cell death was examined using the divalent cation chelators EDTA and EGTA. Neither chelator affected toxin-receptor binding; however, EDTA (chelator of Ca^{2+} and Mg^{2+}), but not EGTA (Ca^{2+}-specific chelator), completely abolished Cry1Ab-induced cell death (195, 196). It was also shown that the addition of Mg^{2+} to S5 cells pretreated with EDTA restored Cry1Ab-mediated cytotoxicity. Since neither ion was shown to effect Cry1Ab binding to BT-R_1 by immunoblotting, it was proposed that Cry1Ab binding to BT-R_1 was linked to an Mg^{2+}-dependent signaling pathway associated with cell death.

Evidence to suggest that Cry1Ab induces an AC/PKA signaling pathway was based on experiments examining the effect of Cry1Ab and various inhibitors on cAMP production and cytotoxicity in S5 cells (196). This pathway was initially hypothesized to mediate Cry1Ab activity, because many pathway members are Mg^{2+} dependent (162, 165, 198) and the second messenger cAMP had previously been implicated in signaling related to cell death (161, 169). First, it was demonstrated that Cry1Ab treatment stimulated cAMP production in S5 cells, but not in H5 control cells, and that this effect could be reversed by preincubation with EDTA. To test whether G protein activity was involved in Cry1Ab-induced cAMP production, S5 cells were incubated with the G protein α-subunit ($G_{\alpha s}$) antagonist NF449 (71). This compound reduced levels of cytotoxicity by 50%, whereas the inhibitory G protein α-subunit ($G_{\alpha i}$) antagonist NF449 (43) had no effect. The importance of AC in toxin-induced cell death was tested with the inhibitor ddADP, a reagent that blocks substrate utilization. ddADP decreased levels of cytotoxicity, suggesting that the stimulation of AC was also important for Cry1Ab-mediated cell death. The inhibitors H-89 (35) and myristoylated amide 14-22 (PKAI 14-22-amide) (52) were used to test whether PKA was involved in the toxin's mechanism of action. Both inhibitors completely prevented membrane blebbing, cell swelling, and cytotoxicity, and thus PKA appeared to play a critical role in Cry1Ab-induced signaling. Although Cry1Ab stimulation correlated with the production of cAMP, the AC activator forskolin and the cAMP analog pCPT-cAMP were not sufficient to cause cell death. This may indicate that other signaling events originating directly from BT-R_1 or from the interaction of $G_{\alpha s}$ with effectors other than AC work in conjunction with PKA signaling to bring about Cry1Ab-mediated cytotoxicity (196). This has not yet been proven, however, and would be a necessary step towards the validation of this model.

Some caution may be advisable in assessing the Zhang

model, however (196). In a previous study (97), several Cry toxins including Cry1Ab were found to cause an increase in intracellular cAMP in a *Mamestra brassicae* cell line, but the authors concluded that the effects on AC were a secondary effect of the interaction of the toxin with the membrane rather than a direct cause of the cytolytic mechanism. Further work to establish an unambiguous causal connection between cytotoxicity and the rise in cAMP may therefore be necessary to strengthen the Zhang model. Of particular importance would be an examination of how PKA mediates Cry toxicity in vivo, since all work to date has been carried out using cell lines.

The Jurat-Fuentes Model

A third model has recently been proposed to explain the mode of action of Cry1Ac in *H. virescens* (86). The model suggests that cytotoxicity is due to the combined effects of osmotic lysis and cell signaling, and thus elements of both the Bravo model and the Zhang model of toxin mode of action are incorporated. First, activated monomeric Cry1Ac binds to the cadherin-like protein HevCaLP. This results in the activation of an intracellular signaling pathway regulated by phosphatases. Evidence to support the involvement of phosphatases in Cry1Ac-mediated signaling was provided by a proteomic analysis of BBMV that showed that resistant and susceptible strains of *H. virescens* have different levels of intracellular phosphatases (86). Signaling may also be dependent on a direct interaction between Cry1Ac and actin (86, 122), a cytoskeletal protein that interacts with the cytosolic domain of cadherins through tyrosine phosphatases, catenin, and actinin (111). After binding to HevCaLP, monomeric Cry1Ac oligomerizes and then binds to the GPI-anchored proteins APN and—in an extension of the Bravo model—the ALP HvALP (85). Either protein then drives Cry1Ac oligomers into DRMs, where pore formation results in osmotic shock and the activation of signaling pathways leading to cell death. While this model is intriguing, it should be noted that many steps are at present speculative and remain to be confirmed experimentally.

CONCLUDING REMARKS

Since the first Cry toxin receptors were cloned in 1995 (94, 175), much progress has been made in our understanding of toxin specificity and mode of action. By far the best-studied receptors are from lepidopterans, and their interactions with Cry1A toxins are particularly well characterized. Receptors in the APN family have been identified in several lepidopteran species, and 38 members in five different classes have been described. While many studies suggest that APNs can serve as Cry-binding proteins and their ability to mediate pore-formation in vitro is well documented, a direct role in cytotoxicity has been yet to be firmly established. In contrast, a great deal of progress has been made in the functional characterization of cadherin receptors. To date, almost all cloned cadherin genes expressed in cultured cells encode proteins that bind to toxin and, when studied, have been found to confer toxin susceptibility. While it is clear that cadherins play a pivotal role in the Cry1A toxin mode of action, there is currently uncertainty as to whether other putative receptors, known (Table 2) or yet to be discovered, are required for full toxicity.

TABLE 2. Cry toxins for which a putative receptor has been identified[a]

Toxin	Presence of receptor type					
	APN	Cadherin	ALP	Glycolipid	BTR-270	P252
Cry1Aa	+	+		+	+	+
Cry1Ab	+	+		+	+	+
Cry1Ac	+	+	+	+	+	+
Cry1Ba	+				+	
Cry1Ca	+					
Cry1Fa	+					
Cry4Ba			+			
Cry5Ba				+		
Cry9Aa			+			
Cry11Aa			+			
Cry11Ba	+					

[a] See the text for references. +, a putative receptor for the indicated toxin has been identified.

The recent report that Cry toxin activity can be modulated by perturbing intracellular signaling pathways may open up a new avenue of research. In addition to studies showing that inappropriate activation of the AC/PKA pathway can mediate toxin-induced cell death (196), inactivation of the p38 pathway in *C. elegans* apparently increases toxin sensitivity (76). In this way, different signaling pathways may potentiate or mitigate Cry toxicity. At present, the importance of internal signaling as a mechanism of insect resistance is largely unexplored. Research in this area may help to explain why some insects develop resistance without a loss in toxin binding (112, 189).

Important questions about how midgut receptors confer toxicity remain. For Cry1A toxins, binding to cadherin appears to be critical, but the details of this interaction remain elusive. Some studies suggest that cadherins function to convert monomeric toxin into an insertion-competent prepore; however, an oligomeric species has been identified for resistant cells that do not express cadherin. If pore formation is not responsible for cytotoxicity, as has been recently suggested (195, 196), how might loss-of-function mutations that do not affect receptor binding be explained? A better understanding of the interaction between Cry1A toxins and cadherin receptors at a structural level will likely improve our ability to answer such questions.

While progress is being made in our understanding of Cry1A toxicity, comparatively little is known about the remaining Cry toxin families. Based on sequence identity, it seems likely that most Cry toxins share a common three-domain structure; however, binding and genetic studies have shown that cadherins do not mediate cell death in all cases (59, 89). Indeed, nematode-specific toxins apparently utilize a glycolipid receptor (60), and it was recently reported that the *B. thuringiensis* toxin Cyt1Aa synergizes Cry11Aa in mosquitoes by functioning as a membrane-bound receptor (138). As more toxin receptors are discovered and as our understanding of toxin-receptor interactions increases, it will be interesting to see the extent to which Cry toxins utilize a common mode of action.

REFERENCES

1. **Abdullah, M. A., A. P. Valaitis, and D. H. Dean.** 2006. Identification of a *Bacillus thuringiensis* Cry11Ba toxin-binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. BMC Biochem. **7:**16.
2. **Agrawal, N., P. Malhotra, and R. K. Bhatnagar.** 2002. Interaction of gene-

cloned and insect cell-expressed aminopeptidase N of *Spodoptera litura* with insecticidal crystal protein Cry1C. Appl. Environ. Microbiol. **68:**4583–4592.
3. **Aimanova, K. G., M. Zhuang, and S. S. Gill.** 2006. Expression of Cry1Ac cadherin receptors in insect midgut and cell lines. J. Invertebr. Pathol. **92:**178–187.
4. **Angst, B. D., C. Marcozzi, and A. I. Magee.** 2001. The cadherin superfamily: diversity in form and function. J. Cell Sci. **114:**629–641.
5. **Aronson, A. I., C. Geng, and L. Wu.** 1999. Aggregation of *Bacillus thuringiensis* Cry1A toxins upon binding to target insect larval midgut vesicles. Appl. Environ. Microbiol. **65:**2503–2507.
6. **Atsumi, S., E. Mizuno, H. Hara, K. Nakanishi, M. Kitami, N. Miura, H. Tabunoki, A. Watanabe, and R. Sato.** 2005. Location of the *Bombyx mori* aminopeptidase N type 1 binding site on *Bacillus thuringiensis* Cry1Aa toxin. Appl. Environ. Microbiol. **71:**3966–3977.
7. **Banks, D. J., G. Hua, and M. J. Adang.** 2003. Cloning of a *Heliothis virescens* 110 kDa aminopeptidase N and expression in *Drosophila* S2 cells. Insect Biochem. Mol. Biol. **33:**499–508.
8. **Banks, D. J., J. L. Jurat-Fuentes, D. H. Dean, and M. J. Adang.** 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ-endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* is not N-acetylgalactosamine mediated. Insect Biochem. Mol. Biol. **31:**909–918.
9. **Bates, S. L., J. Z. Zhao, R. T. Roush, and A. M. Shelton.** 2005. Insect resistance management in GM crops: past, present and future. Nat. Biotechnol. **23:**57–62.
10. **Blaschuk, O. W., R. Sullivan, S. David, and Y. Pouliot.** 1990. Identification of a cadherin cell adhesion recognition sequence. Dev. Biol. **139:**227–229.
11. **Boonserm, P., P. Davis, D. J. Ellar, and J. Li.** 2005. Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J. Mol. Biol. **348:**363–382.
12. **Boonserm, P., M. Mo, C. Angsuthanasombat, and J. Lescar.** 2006. Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. J. Bacteriol. **188:**3391–3401.
13. **Boraston, A. B., D. Nurizzo, V. Notenboom, V. Ducros, D. R. Rose, D. G. Kilburn, and G. J. Davies.** 2002. Differential oligosaccharide recognition by evolutionarily-related β-1,4 and β-1,3 glucan-binding modules. J. Mol. Biol. **319:**1143–1156.
14. **Bourne, Y., V. Roig-Zamboni, A. Barre, W. J. Peumans, C. H. Astoul, E. J. Van Damme, and P. Rougé.** 2004. The crystal structure of the *Calystegia sepium* agglutinin reveals a novel quaternary arrangement of lectin subunits with a b-prism fold. J. Biol. Chem. **279:**527–533.
15. **Bourne, Y., V. Zamboni, A. Barre, W. J. Peumans, E. J. Van Damme, and P. Rougé.** 1999. *Helianthus tuberosus* lectin reveals a widespread scaffold for mannose-binding lectins. Structure **7:**1473–1482.
16. **Braun, L., and B. A. Keddie.** 1997. A new tissue technique for evaluating effects of *Bacillus thuringiensis* toxins on insect midgut epithelium. J. Invertebr. Pathol. **69:**92–104.
17. **Bravo, A.** 1997. Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. J. Bacteriol. **179:** 2793–2801.
18. **Bravo, A., I. Gómez, J. Conde, C. Muñoz-Garay, J. Sánchez, R. Miranda, M. Zhuang, S. S. Gill, and M. Soberón.** 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim. Biophys. Acta **1667:**38–46.
19. **Bravo, A., S. Hendrickx, S. Jansens, and M. Peferoen.** 1992. Immunocytochemical analysis of specific binding of *Bacillus thuringiensis* insecticidal crystal proteins to lepidopteran and coleopteran midgut membranes. J. Invertebr. Pathol. **60:**247–254.
20. **Bravo, A., S. Jansens, and M. Peferoen.** 1992. Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. J. Invertebr. Pathol. **60:**237–246.
21. **Burton, S. L., D. J. Ellar, J. Li, and D. J. Derbyshire.** 1999. *N*-Acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Mol. Biol. **287:**1011–1022.
22. **Buzdin, A. A., L. P. Revina, L. I. Kostina, I. A. Zalunin, and G. G. Chestukhina.** 2002. Interaction of 65- and 62-kD proteins from the apical membranes of the *Aedes aegypti* larvae midgut epithelium with Cry4B and Cry11A endotoxins of *Bacillus thuringiensis*. Biochemistry (Moscow) **67:**540–546.
23. **Candas, M., B. R. Francis, N. B. Griko, E. G. Midboe, and L. A. Bulla, Jr.** 2002. Proteolytic cleavage of the developmentally important cadherin BT-R1 in the midgut epithelium of *Manduca sexta*. Biochemistry **41:**13717–13724.
24. **Chen, J., M. R. Brown, G. Hua, and M. J. Adang.** 2005. Comparison of the localization of *Bacillus thuringiensis* Cry1A delta-endotoxins and their binding proteins in larval midgut of tobacco hornworm, *Manduca sexta*. Cell Tissue Res. **321:**123–129.
25. **Coates, B. S., D. V. Sumerford, R. L. Hellmich, and L. C. Lewis.** 2005. Sequence variation in the cadherin gene of *Ostrinia nubilalis*: a tool for field monitoring. Insect Biochem. Mol. Biol. **35:**129–139.
26. **Daniel, A., S. Sangadala, D. H. Dean, and M. J. Adang.** 2002. Denaturation of either *Manduca sexta* aminopeptidase N or *Bacillus thuringiensis* Cry1A toxins exposes binding epitopes hidden under nondenaturing conditions. Appl. Environ. Microbiol. **68:**2106–2112.
27. **de Maagd, R. A., P. L. Bakker, L. Masson, M. J. Adang, S. Sangadala, W. Stiekema, and D. Bosch.** 1999. Domain III of the *Bacillus thuringiensis* δ-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol. Microbiol. **31:**463–471.
28. **de Maagd, R. A., A. Bravo, C. Berry, N. Crickmore, and H. E. Schnepf.** 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu. Rev. Genet. **37:**409–433.
29. **de Maagd, R. A., A. Bravo, and N. Crickmore.** 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet. **17:**193–199.
30. **Dennis, R. D., H. Wiegandt, D. Haustein, B. H. Knowles, and D. J. Ellar.** 1986. Thin layer chromatography overlay technique in the analysis of the binding of the solubilized protoxin of *Bacillus thuringiensis* var. *kurstaki* to an insect glycosphingolipid of known structure. Biomed. Chromatogr. **1:**31–37.
31. **Denolf, P., K. Hendrickx, J. Van Damme, S. Jansens, M. Peferoen, D. Degheele, and J. Van Rie.** 1997. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. Eur. J. Biochem. **248:**748–761.
32. **Derbyshire, D. J., D. J. Ellar, and J. Li.** 2001. Crystallization of the *Bacillus thuringiensis* toxin Cry1Ac and its complex with the receptor ligand *N*-acetyl-D-galactosamine. Acta Crystallogr. Sect. D **57:**1938–1944.
33. **Dorsch, J. A., M. Candas, N. B. Griko, W. S. Maaty, E. G. Midboe, R. K. Vadlamudi, and L. A. Bulla, Jr.** 2002. Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R(1) in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. Insect Biochem. Mol. Biol. **32:**1025–1036.
34. **Dunne, J., A. M. Hanby, R. Poulsom, T. A. Jones, D. Sheer, W. G. Chin, S. M. Da, Q. Zhao, P. C. Beverley, and M. J. Owen.** 1995. Molecular cloning and tissue expression of FAT, the human homologue of the *Drosophila* fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. Genomics **30:**207–223.
35. **Engh, R. A., A. Girod, V. Kinzel, R. Huber, and D. Bossemeyer.** 1996. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. J. Biol. Chem. **271:**26157–26164.
36. **English, L. H., and T. L. Readdy.** 1989. δ-Endotoxin inhibits a phosphatase in midgut epithelial membranes of *Heliothis virescens*. Insect Biochem. **19:**145–152.
37. **Federici, B. A.** 2005. Insecticidal bacteria: an overwhelming success for invertebrate pathology. J. Invertebr. Pathol. **89:**30–38.
38. **Fernandez, L. E., K. G. Aimanova, S. S. Gill, A. Bravo, and M. Soberón.** 2006. A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochem. J. **394:**77–84.
39. **Ferré, J., and J. Van Rie.** 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **47:**501–533.
40. **Flannagan, R. D., C. G. Yu, J. P. Mathis, T. E. Meyer, X. Shi, H. A. Siqueira, and B. D. Siegfried.** 2005. Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, Ostrinia nubilalis (Hübner) (Lepidoptera: Crambidae). Insect Biochem. Mol. Biol. **35:**33–40.
41. **Flower, D. R.** 1996. The lipocalin protein family: structure and function. Biochem. J. **318:**1–14.
42. **Flower, D. R., A. C. North, and C. E. Sansom.** 2000. The lipocalin protein family: structural and sequence overview. Biochim. Biophys. Acta **1482:**9–24.
43. **Freissmuth, M., S. Boehm, W. Beindl, P. Nickel, A. P. Ijzerman, M. Hohenegger, and C. Nanoff.** 1996. Suramin analogues as subtype-selective G protein inhibitors. Mol. Pharmacol. **49:**602–611.
44. **Gahan, L. J., F. Gould, and D. G. Heckel.** 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science **293:**857–860.
45. **Galitsky, N., V. Cody, A. Wojtczak, D. Ghosh, J. R. Luft, W. Pangborn, and L. English.** 2001. Structure of the insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. Acta Crystallogr. Sect. D **57:**1101–1109.
46. **Garcia-Robles, I., J. Sánchez, A. Gruppe, A. C. Martinez-Ramirez, C. Rausell, M. D. Real, and A. Bravo.** 2001. Mode of action of *Bacillus thuringiensis* PS86Q3 strain in hymenopteran forest pests. Insect Biochem. Mol. Biol. **31:**849–856.
47. **Garczynski, S. F., J. W. Crim, and M. J. Adang.** 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl. Environ. Microbiol. **57:**2816–2820.
48. **Garner, K. J., S. Hiremath, K. Lehtoma, and A. P. Valaitis.** 1999. Cloning and complete sequence characterization of two gypsy moth aminopeptidase-N cDNAs, including the receptor for *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Mol. Biol. **29:**527–535.
49. **Ge, A. Z., N. I. Shivarova, and D. H. Dean.** 1989. Location of the *Bombyx*

mori specificity domain on a *Bacillus thuringiensis* delta-endotoxin protein. Proc. Natl. Acad. Sci. USA **86:**4037–4041.
50. **Gill, M., and D. Ellar.** 2002. Transgenic *Drosophila* reveals a functional *in vivo* receptor for the *Bacillus thuringiensis* toxin Cry1Ac1. Insect Mol. Biol. **11:**619–625.
51. **Gill, S. S., E. A. Cowles, and V. Francis.** 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J. Biol. Chem. **270:** 27277–27282.
52. **Glass, D. B., L. J. Lundquist, B. M. Katz, and D. A. Walsh.** 1989. Protein kinase inhibitor-(6-22)-amide peptide analogs with standard and nonstandard amino acid substitutions for phenylalanine 10. Inhibition of cAMP-dependent protein kinase. J. Biol. Chem. **264:**14579–14584.
53. **Goldberg, M., C. Peshkovsky, A. Shifteh, and Q. Al-Awqati.** 2000. m-Protocadherin, a novel developmentally regulated protocadherin with mucin-like domains. J. Biol. Chem. **275:**24622–24629.
54. **Gómez, I., I. Arenas, I. Benitez, J. Miranda-Rios, B. Becerril, R. Grande, J. C. Almagro, A. Bravo, and M. Soberon.** 2006. Specific epitopes of domains II and III of *Bacillus thuringiensis* Cry1Ab toxin involved in the sequential interaction with cadherin and aminopeptidase-N receptors in *Manduca sexta*. J. Biol. Chem. **281:**34032–34039.
55. **Gómez, I., D. H. Dean, A. Bravo, and M. Soberón.** 2003. Molecular basis for *Bacillus thuringiensis* Cry1Ab toxin specificity: two structural determinants in the *Manduca sexta* Bt-R_1 receptor interact with loops a-8 and 2 in domain II of Cy1Ab toxin. Biochemistry **42:**10482–10489.
56. **Gómez, I., J. Miranda-Rios, E. Rudiño-Piñera, D. I. Oltean, S. S. Gill, A. Bravo, and M. Soberón.** 2002. Hydropathic complementarity determines interaction of epitope ^{869}HITDTNNK^{876} in *Manduca sexta* Bt-R_1 receptor with loop 2 of domain II of *Bacillus thuringiensis* Cry1A toxins. J. Biol. Chem. **277:**30137–30143.
57. **Gómez, I., D. I. Oltean, S. S. Gill, A. Bravo, and M. Soberón.** 2001. Mapping the epitope in cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxin interaction using phage display. J. Biol. Chem. **276:**28906–28912.
58. **Gómez, I., J. Sánchez, R. Miranda, A. Bravo, and M. Soberón.** 2002. Cadherin-like receptor binding facilitates proteolytic cleavage of helix a-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. FEBS Lett. **513:**242–246.
59. **Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. J. Moar.** 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. **88:**1545–1559.
60. **Griffitts, J. S., S. M. Haslam, T. Yang, S. F. Garczynski, B. Mulloy, H. Morris, P. S. Cremer, A. Dell, M. J. Adang, and R. V. Aroian.** 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science **307:** 922–925.
61. **Griffitts, J. S., D. L. Huffman, J. L. Whitacre, B. D. Barrows, L. D. Marroquin, R. Muller, J. R. Brown, T. Hennet, J. D. Esko, and R. V. Aroian.** 2003. Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. J. Biol. Chem. **278:**45594–45602.
62. **Griffitts, J. S., J. L. Whitacre, D. E. Stevens, and R. V. Aroian.** 2001. Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science **293:**860–864.
63. **Griko, N., M. Candas, X. Zhang, M. Junker, and L. A. Bulla, Jr.** 2004. Selective antagonism to the cadherin BT-R_1 interferes with calcium-induced adhesion of epithelial membrane vesicles. Biochemistry **43:**1393–1400.
64. **Grochulski, P., L. Masson, S. Borisova, M. Pusztai-Carey, J. L. Schwartz, R. Brousseau, and M. Cygler.** 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. **254:**447–464.
65. **Gumbiner, B. M.** 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell **84:**345–357.
66. **Hara, H., S. Atsumi, K. Yaoi, K. Nakanishi, S. Higurashi, N. Miura, H. Tabunoki, and R. Sato.** 2003. A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae. FEBS Lett. **538:**29–34.
67. **Herrero, S., T. Gechev, P. L. Bakker, W. J. Moar, and R. A. de Maagd.** 2005. *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four aminopeptidase N genes. BMC Genomics **6:**96.
68. **Hofmann, C., P. Lüthy, R. Hutter, and V. Pliska.** 1988. Binding of the d endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). Eur. J. Biochem. **173:**85–91.
69. **Hofmann, C., H. Vanderbruggen, H. Hofte, J. Van Rie, S. Jansens, and H. Van Mellaert.** 1988. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA **85:**7844–7848.
70. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
71. **Hohenegger, M., M. Waldhoer, W. Beindl, B. Böing, A. Kreimeyer, P. Nickel, C. Nanoff, and M. Freissmuth.** 1998. G_{sa}-selective G protein antagonists. Proc. Natl. Acad. Sci. USA **95:**346–351.
72. **Hooper, N. M.** 1994. Families of zinc metalloproteases. FEBS Lett. **354:**1–6.
73. **Hossain, D. M., Y. Shitomi, K. Moriyama, M. Higuchi, T. Hayakawa, T. Mitsui, R. Sato, and H. Hori.** 2004. Characterization of a novel plasma membrane protein, expressed in the midgut epithelia of *Bombyx mori*, that binds to Cry1A toxins. Appl. Environ. Microbiol. **70:**4604–4612.
74. **Hua, G., J. L. Jurat-Fuentes, and M. J. Adang.** 2004. Bt-R_{1a} extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. J. Biol. Chem. **279:**28051–28056.
75. **Hua, G., J. L. Jurat-Fuentes, and M. J. Adang.** 2004. Fluorescent-based assays establish *Manduca sexta* Bt-R_{1a} cadherin as a receptor for multiple *Bacillus thuringiensis* Cry1A toxins in *Drosophila* S2 cells. Insect Biochem. Mol. Biol. **34:**193–202.
76. **Huffman, D. L., L. Abrami, R. Sasik, J. Corbeil, F. G. Van Der Goot, and R. V. Aroian.** 2004. Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. Proc. Natl. Acad. Sci. USA **101:** 10995–11000.
77. **Ihara, H., T. Uemura, M. Masuhara, S. Ikawa, K. Sugimoto, A. Wadano, and M. Himeno.** 1998. Purification and partial amino acid sequences of the binding protein from *Bombyx mori* for CryIAa δ-endotoxin of *Bacillus thuringiensis*. Comp. Biochem. Physiol. B **120:**197–204.
78. **Ikawa, S., Y. Tsuda, T. Fukada, K. Sugimoto, and M. Himeno.** 2000. cDNA cloning of the Cry1Aa receptor variants from *Bombyx mori* and their expression in mammalian cells. Biosci. Biotechnol. Biochem. **64:**2682–2685.
79. **James, C.** 2004. Preview: global status of commercialized biotech/GM crops: 2004. ISAAA brief no. 32. International Service for the Acquisition of Agri-Biotech Applications, Ithaca, NY.
80. **Jenkins, J. L., and D. H. Dean.** 2001. Binding specificity of *Bacillus thuringiensis* Cry1Aa for purified, native *Bombyx mori* aminopeptidase N and cadherin-like receptors. BMC Biochem. **2:**12.
81. **Jenkins, J. L., M. K. Lee, S. Sangadala, M. J. Adang, and D. H. Dean.** 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Lett. **462:**373–376.
82. **Jenkins, J. L., M. K. Lee, A. P. Valaitis, A. Curtiss, and D. H. Dean.** 2000. Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. J. Biol. Chem. **275:**14423–14431.
83. **Johnson, P. E., M. D. Joshi, P. Tomme, D. G. Kilburn, and L. P. McIntosh.** 1996. Structure of the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC determined by nuclear magnetic resonance spectroscopy. Biochemistry **35:**14381–14394.
84. **Julenius, K., A. Molgaard, R. Gupta, and S. Brunak.** 2005. Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. Glycobiology **15:**153–164.
85. **Jurat-Fuentes, J. L., and M. J. Adang.** 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur. J. Biochem. **271:**3127–3135.
86. **Jurat-Fuentes, J. L., and M. J. Adang.** 2006. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. J. Invertebr. Pathol. **92:**166–171.
87. **Jurat-Fuentes, J. L., and M. J. Adang.** 2006. The *Heliothis virescens* cadherin protein expressed in *Drosophila* S2 cells functions as a receptor for *Bacillus thuringiensis* Cry1A but not Cry1Fa toxins. Biochemistry **45:**9688–9695.
88. **Jurat-Fuentes, J. L., and M. J. Adang.** 2001. Importance of Cry1 δ-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). Appl. Environ. Microbiol. **67:**323–329.
89. **Jurat-Fuentes, J. L., L. J. Gahan, F. L. Gould, D. G. Heckel, and M. J. Adang.** 2004. The HevCaLP protein mediates binding specificity of the Cry1A class of *Bacillus thuringiensis* toxins in *Heliothis virescens*. Biochemistry **43:**14299–14305.
89a. **Jurat-Fuentes, J. L., A. P. Valaitis, and M. J. Adang.** 2005. Abstr. 38th Annu. Meet. Soc. Invertebr. Pathol., abstr. 17.
90. **Kawar, Z. S., I. Van Die, and R. D. Cummings.** 2002. Molecular cloning and enzymatic characterization of a UDP-GalNAc:GlcNAcb-R β1,4-*N*-acetylgalactosaminyltransferase from *Caenorhabditis elegans*. J. Biol. Chem. **277:** 34924–34932.
91. **Keeton, T. P., and L. A. Bulla, Jr.** 1997. Ligand specificity and affinity of BT-R_1, the *Bacillus thuringiensis* Cry1A toxin receptor from *Manduca sexta*, expressed in mammalian and insect cell cultures. Appl. Environ. Microbiol. **63:**3419–3425.
92. **Knight, P. J., J. Carroll, and D. J. Ellar.** 2004. Analysis of glycan structures on the 120 kDa aminopeptidase N of *Manduca sexta* and their interactions with *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Mol. Biol. **34:**101–112.
93. **Knight, P. J., N. Crickmore, and D. J. Ellar.** 1994. The receptor for *Bacillus thuringiensis* CrylA (c) δ-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol. Microbiol. **11:**429–436.
94. **Knight, P. J., B. H. Knowles, and D. J. Ellar.** 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. J. Biol. Chem. **270:**17765–17770.

95. **Knowles, B. H., and J. A. Dow.** 1993. The crystal δ-endotoxins of *Bacillus thuringiensis*: models for their mechanism of action in the insect gut. Bioessays **15:**469–475.
96. **Knowles, B. H., and D. J. Ellar.** 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim. Biophys. Acta **924:**507–518.
97. **Knowles, B. H., and R. W. Farndale.** 1988. Activation of insect cell adenylate cyclase by *Bacillus thuringiensis* δ-endotoxins and melittin. Toxicity is independent of cyclic AMP. Biochem. J. **253:**235–241.
98. **Komoriya, A., L. J. Green, M. Mervic, S. S. Yamada, K. M. Yamada, and M. J. Humphries.** 1991. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. J. Biol. Chem. **266:**15075–15079.
99. **Kondo, S., N. Tamura, A. Kunitata, M. Hattori, A. Akashi, and I. Ohmori.** 1987. Cloning and nucleotide sequencing of two insecticidal δ-endotoxin genes from *Bacillus thuringiensis* var. *kurstaki* HD-1 DNA. Agric. Biol. Chem. **51:**455–463.
100. **Krieger, I. V., L. P. Revina, L. I. Kostina, A. A. Buzdin, I. A. Zalunin, G. G. Chestukhina, and V. M. Stepanov.** 1999. Membrane proteins of *Aedes aegypti* larvae bind toxins Cry4B and Cry11A of *Bacillus thuringiensis* ssp. *israelensis*. Biochemistry (Moscow) **64:**1163–1168.
101. **Laustsen, P. G., S. Vang, and T. Kristensen.** 2001. Mutational analysis of the active site of human insulin-regulated aminopeptidase. Eur. J. Biochem. **268:**98–104.
102. **Lecadet, M. M., E. Frachon, V. C. Dumanoir, H. Ripouteau, S. Hamon, P. Laurent, and I. Thiéry.** 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. J. Appl. Microbiol. **86:**660–672.
103. **Lee, M. K., J. L. Jenkins, T. H. You, A. Curtiss, J. J. Son, M. J. Adang, and D. H. Dean.** 2001. Mutations at the arginine residues in α8 loop of *Bacillus thuringiensis* δ-endotoxin Cry1Ac affect toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N. FEBS Lett. **497:**108–112.
104. **Lee, M. K., R. E. Milne, A. Z. Ge, and D. H. Dean.** 1992. Location of a *Bombyx mori* receptor binding region on a *Bacillus thuringiensis* delta-endotoxin. J. Biol. Chem. **267:**3115–3121.
105. **Lee, M. K., F. Rajamohan, J. L. Jenkins, A. S. Curtiss, and D. H. Dean.** 2000. Role of two arginine residues in domain II, loop 2 of Cry1Ab and Cry1Ac *Bacillus thuringiensis* δ-endotoxin in toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N. Mol. Microbiol. **38:**289–298.
106. **Lee, M. K., B. A. Young, and D. H. Dean.** 1995. Domain III exchanges of *Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. Biochem. Biophys. Res. Commun. **216:**306–312.
107. **Lee, X., A. Thompson, Z. Zhang, H. Ton-that, J. Biesterfeldt, C. Ogata, L. Xu, R. A. Johnston, and N. M. Young.** 1998. Structure of the complex of *Maclura pomifera* agglutinin and the T-antigen disaccharide, Galb1,3GalNAc. J. Biol. Chem. **273:**6312–6318.
108. **Li, J., D. J. Derbyshire, B. Promdonkoy, and D. J. Ellar.** 2001. Structural implications for the transformation of the *Bacillus thuringiensis* δ-endotoxins from water-soluble to membrane-inserted forms. Biochem. Soc. Trans. **29:**571–577.
109. **Li, J. D., J. Carroll, and D. J. Ellar.** 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature **353:**815–821.
110. **Liang, Y., S. S. Patel, and D. H. Dean.** 1995. Irreversible binding kinetics of *Bacillus thuringiensis* CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J. Biol. Chem. **270:**24719–24724.
111. **Lilien, J., and J. Balsamo.** 2005. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of β-catenin. Curr. Opin. Cell Biol. **17:**459–465.
112. **Liu, Y. B., B. E. Tabashnik, L. Masson, B. Escriche, and J. Ferré.** 2000. Binding and toxicity of *Bacillus thuringiensis* protein Cry1C to susceptible and resistant diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **93:**1–6.
113. **Lu, Y. J., and M. J. Adang.** 1996. Conversion of *Bacillus thuringiensis* Cry1Ac-binding aminopeptidase to a soluble form by endogenous phosphatidylinositol phospholipase C. Insect Biochem. Mol. Biol. **26:**33–40.
114. **Luo, K., Y. J. Lu, and M. J. Adang.** 1996. A 106 kDa form of aminopeptidase is a receptor for *Bacillus thuringiensis* CryIC δ-endotoxin in the brush border membrane of *Manduca sexta*. Insect Biochem. Mol. Biol. **26:**783–791.
115. **Luo, K., J. R. McLachlin, M. R. Brown, and M. J. Adang.** 1999. Expression of a glycosylphosphatidylinositol-linked *Manduca sexta* aminopeptidase N in insect cells. Protein Expr. Purif. **17:**113–122.
116. **Luo, K., S. Sangadala, L. Masson, A. Mazza, R. Brousseau, and M. J. Adang.** 1997. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. **27:**735–743.
117. **MacKenzie, C. R., T. Hirama, and J. T. Buckley.** 1999. Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance. J. Biol. Chem. **274:**22604–22609.
118. **Marroquin, L. D., D. Elyassnia, J. S. Griffitts, J. S. Feitelson, and R. V. Aroian.** 2000. *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. Genetics **155:**1693–1699.
119. **Martínez-Ramírez, A. C., S. González-Nebauer, B. Escriche, and M. D. Real.** 1994. Ligand blot identification of a *Manduca sexta* midgut binding protein specific to three *Bacillus thuringiensis* CryIA-type ICPs. Biochem. Biophys. Res. Commun. **201:**782–787.
120. **Masson, L., Y. J. Lu, A. Mazza, R. Brousseau, and M. J. Adang.** 1995. The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. **270:**20309–20315.
121. **Mauchamp, B., C. Royer, A. Garel, A. Jalabert, M. Da Rocha, A. M. Grenier, V. Labas, J. Vinh, K. Mita, K. Kadono, and G. Chavancy.** 2006. Polycalin (chlorophyllid A binding protein): a novel, very large fluorescent lipocalin from the midgut of the domestic silkworm *Bombyx mori* L. Insect Biochem. Mol. Biol. **36:**623–633.
122. **McNall, R. J., and M. J. Adang.** 2003. Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. Insect Biochem. Mol. Biol. **33:**999–1010.
123. **Meagher, J. L., H. C. Winter, P. Ezell, I. J. Goldstein, and J. A. Stuckey.** 2005. Crystal structure of banana lectin reveals a novel second sugar binding site. Glycobiology **15:**1033–1042.
124. **Midboe, E. G., M. Candas, and L. A. Bulla, Jr.** 2003. Expression of a midgut-specific cadherin BT-R_1 during the development of *Manduca sexta* larva. Comp. Biochem. Physiol. B **135:**125–137.
125. **Miranda, R., F. Z. Zamudio, and A. Bravo.** 2001. Processing of Cry1Ab δ-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. **31:**1155–1163.
126. **Morin, S., R. W. Biggs, M. S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L. J. Gahan, D. G. Heckel, Y. Carriere, T. J. Dennehy, J. K. Brown, and B. E. Tabashnik.** 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. USA **100:**5004–5009.
127. **Morse, R. J., T. Yamamoto, and R. M. Stroud.** 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure (Cambridge) **9:**409–417.
128. **Müller, R., F. Altmann, D. Zhou, and T. Hennet.** 2002. The *Drosophila melanogaster* brainiac protein is a glycolipid-specific β1,3*N*-acetylglucosaminyltransferase. J. Biol. Chem. **277:**32417–32420.
129. **Nagamatsu, Y., T. Koike, K. Sasaki, A. Yoshimoto, and Y. Furukawa.** 1999. The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. FEBS Lett. **460:**385–390.
130. **Nagamatsu, Y., S. Toda, T. Koike, Y. Miyoshi, S. Shigematsu, and M. Kogure.** 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. Biosci. Biotechnol. Biochem. **62:**727–734.
131. **Nagamatsu, Y., S. Toda, F. Yamaguchi, M. Ogo, M. Kogure, M. Nakamura, Y. Shibata, and T. Katsumoto.** 1998. Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA (a) toxin. Biosci. Biotechnol. Biochem. **62:**718–726.
132. **Nakanishi, K., K. Yaoi, Y. Nagino, H. Hara, M. Kitami, S. Atsumi, N. Miura, and R. Sato.** 2002. Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella*-–their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry1A toxin. FEBS. Lett. **519:**215–220.
133. **Nakayama, M., D. Nakajima, T. Nagase, N. Nomura, N. Seki, and O. Ohara.** 1998. Identification of high-molecular-weight proteins with multiple EGF-like motifs by motif-trap screening. Genomics **51:**27–34.
134. **Nollet, F., P. Kools, and F. van Roy.** 2000. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. J. Mol. Biol. **299:**551–572.
135. **Pardo-López, L., I. Gómez, C. Muñoz-Garay, N. Jiménez-Juarez, M. Soberón, and A. Bravo.** 2006. Structural and functional analysis of the pre-pore and membrane-inserted pore of Cry1Ab toxin. J. Invertebr. Pathol. **92:**172–177.
136. **Pardo-López, L., I. Gómez, C. Rausell, J. Sánchez, M. Soberón, and A. Bravo.** 2006. Structural changes of the Cry1Ac oligomeric pre-pore from *Bacillus thuringiensis* induced by *N*-acetylgalactosamine facilitates toxin membrane insertion. Biochemistry **45:**10329–10336.
137. **Parker, M. W., F. Pattus, A. D. Tucker, and D. Tsernoglou.** 1989. Structure of the membrane-pore-forming fragment of colicin A. Nature **337:**93–96.
138. **Pérez, C., L. E. Fernandez, J. Sun, J. L. Folch, S. S. Gill, M. Soberón, and A. Bravo.** 2005. *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proc. Natl. Acad. Sci. USA **102:**18303–18308.
139. **Peumans, W. J., E. J. Van Damme, A. Barre, and P. Rouge.** 2001. Classification of plant lectins in families of structurally and evolutionary related proteins. Adv. Exp. Med. Biol. **491:**27–54.
140. **Pires, V. M., J. L. Henshaw, J. A. Prates, D. N. Bolam, L. M. Ferreira, C. M. Fontes, B. Henrissat, A. Planas, H. J. Gilbert, and M. Czjzek.** 2004. The

crystal structure of the family 6 carbohydrate binding module from *Cellvibrio mixtus* endoglucanase 5a in complex with oligosaccharides reveals two distinct binding sites with different ligand specificities. J. Biol. Chem. **279:**21560–21568.

141. **Pratap, J. V., A. A. Jeyaprakash, P. G. Rani, K. Sekar, A. Surolia, and M. Vijayan.** 2002. Crystal structures of artocarpin, a *Moraceae* lectin with mannose specificity, and its complex with methyl-α-D-mannose: implications to the generation of carbohydrate specificity. J. Mol. Biol. **317:**237–247.
142. **Rajagopal, R., N. Agrawal, A. Selvapandiyan, S. Sivakumar, S. Ahmad, and R. K. Bhatnagar.** 2003. Recombinantly expressed isozymic aminopeptidases from *Helicoverpa armigera* midgut display differential interaction with closely related Cry proteins. Biochem. J. **370:**971–978.
143. **Rajagopal, R., S. Sivakumar, N. Agrawal, P. Malhotra, and R. K. Bhatnagar.** 2002. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J. Biol. Chem. **277:**46849–46851.
144. **Rajamohan, F., J. A. Cotrill, F. Gould, and D. H. Dean.** 1996. Role of domain II, loop 2 residues of *Bacillus thuringiensis* CryIAb δ-endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J. Biol. Chem. **271:**2390–2396.
145. **Rajamohan, F., M. K. Lee, and D. H. Dean.** 1998. *Bacillus thuringiensis* insecticidal proteins: molecular mode of action. Prog. Nucleic Acid Res. Mol. Biol. **60:**1–27.
146. **Rasko, D. A., M. R. Altherr, C. S. Han, and J. Ravel.** 2005. Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiol. Rev. **29:**303–329.
147. **Rausell, C., C. Muñoz-Garay, R. Miranda-CassoLuengo, I. Gómez, E. Rudiño-Piñera, M. Soberón, and A. Bravo.** 2004. Tryptophan spectroscopy studies and black lipid bilayer analysis indicate that the oligomeric structure of Cry1Ab toxin from *Bacillus thuringiensis* is the membrane-insertion intermediate. Biochemistry **43:**166–174.
148. **Romeis, J., M. Meissle, and F. Bigler.** 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nat. Biotechnol. **24:**63–71.
149. **Ruoslahti, E.** 1996. RGD and other recognition sequences for integrins. Annu. Rev. Cell Dev. Biol. **12:**697–715.
150. **Sangadala, S., F. S. Walters, L. H. English, and M. J. Adang.** 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and $^{86}Rb^{+}$-K^{+} efflux *in vitro*. J. Biol. Chem. **269:**10088–10092.
151. **Sankaranarayanan, R., K. Sekar, R. Banerjee, V. Sharma, A. Surolia, and M. Vijayan.** 1996. A novel mode of carbohydrate recognition in jacalin, a *Moraceae* plant lectin with a β-prism fold. Nat. Struct. Biol. **3:**596–603.
152. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:**775–806.
153. **Schwartz, J. L., Y. J. Lu, P. Sohnlein, R. Brousseau, R. Laprade, L. Masson, and M. J. Adang.** 1997. Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. FEBS Lett. **412:**270–276.
154. **Schwientek, T., B. Keck, S. B. Levery, M. A. Jensen, J. W. Pedersen, H. H. Wandall, M. Stroud, S. M. Cohen, M. Amado, and H. Clausen.** 2002. The *Drosophila* gene brainiac encodes a glycosyltransferase putatively involved in glycosphingolipid synthesis. J. Biol. Chem. **277:**32421–32429.
155. **Semenza, G.** 1986. Anchoring and biosynthesis of stalked brush border membrane proteins: glycosidases and peptidases of enterocytes and renal tubuli. Annu. Rev. Cell Biol. **2:**255–313.
156. **Shelton, A. M., J. Z. Zhao, and R. T. Roush.** 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu. Rev. Entomol. **47:**845–881.
157. **Shimizu, T., and K. Morikawa.** 1996. The β-prism: a new folding motif. Trends Biochem. Sci. **21:**3–6.
158. **Shimizu, T., D. G. Vassylyev, S. Kido, Y. Doi, and K. Morikawa.** 1994. Crystal structure of vitelline membrane outer layer protein I (VMO-I): a folding motif with homologous Greek key structures related by an internal three-fold symmetry. EMBO J. **13:**1003–1010.
159. **Shitomi, Y., T. Hayakawa, D. M. Hossain, M. Higuchi, K. Miyamoto, K. Nakanishi, R. Sato, and H. Hori.** 2006. A novel 96-kDa aminopeptidase localized on epithelial cell membranes of *Bombyx mori* midgut, which binds to Cry1Ac toxin of *Bacillus thuringiensis*. J. Biochem. (Tokyo) **139:**223–233.
160. **Simpson, R. M., and R. D. Newcomb.** 2000. Binding of *Bacillus thuringiensis* δ-endotoxins Cry1Ac and Cry1Ba to a 120-kDa aminopeptidase-N of *Epiphyas postvittana* purified from both brush border membrane vesicles and baculovirus-infected Sf9 cells. Insect Biochem. Mol. Biol. **30:**1069–1078.
161. **Skalhegg, B. S., and K. Tasken.** 2000. Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA. Front. Biosci. **5:**678–693.
162. **Sprang, S. R.** 1997. G protein mechanisms: insights from structural analysis. Annu. Rev. Biochem. **66:**639–678.
163. **Stephens, E., J. Sugars, S. L. Maslen, D. H. Williams, L. C. Packman, and D. J. Ellar.** 2004. The N-linked oligosaccharides of aminopeptidase N from *Manduca sexta*: site localization and identification of novel N-glycan structures. Eur. J. Biochem. **271:**4241–4258.
164. **Takesue, S., K. Yokota, S. Miyajima, R. Taguchi, H. Ikezawa, and Y. Takesue.** 1992. Partial release of aminopeptidase N from larval midgut cell membranes of the silkworm, Bombyx mori, by phosphatidylinositol-specific phospholipase C. Comp. Biochem. Physiol. B **102:**7–11.
165. **Tesmer, J. J., R. K. Sunahara, R. A. Johnson, G. Gosselin, A. G. Gilman, and S. R. Sprang.** 1999. Two-metal-ion catalysis in adenylyl cyclase. Science **285:**756–760.
166. **Thomas, D. J., J. A. Morgan, J. M. Whipps, and J. R. Saunders.** 2001. Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in laboratory culture, river water, and dipteran larvae. Appl. Environ. Microbiol. **67:**330–338.
167. **Thomas, D. J., J. A. Morgan, J. M. Whipps, and J. R. Saunders.** 2000. Plasmid transfer between the *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* in laboratory culture and soil and in lepidopteran and coleopteran larvae. Appl. Environ. Microbiol. **66:**118–124.
168. **Tomiya, N., S. Narang, Y. C. Lee, and M. J. Betenbaugh.** 2004. Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines. Glycoconj. J. **21:**343–360.
169. **Tortora, G., and F. Ciardiello.** 2002. Protein kinase A as target for novel integrated strategies of cancer therapy. Ann. N. Y. Acad. Sci. **968:**139–147.
170. **Tselepis, V. H., L. J. Green, and M. J. Humphries.** 1997. An RGD to LDV motif conversion within the disintegrin kistrin generates an integrin antagonist that retains potency but exhibits altered receptor specificity. Evidence for a functional equivalence of acidic integrin-binding motifs. J. Biol. Chem. **272:**21341–21348.
171. **Tsuda, Y., F. Nakatani, K. Hashimoto, S. Ikawa, C. Matsuura, T. Fukada, K. Sugimoto, and M. Himeno.** 2003. Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm). Biochem. J. **369:**697–703.
172. **Tunnicliffe, R. B., D. N. Bolam, G. Pell, H. J. Gilbert, and M. P. Williamson.** 2005. Structure of a mannan-specific family 35 carbohydrate-binding module: evidence for significant conformational changes upon ligand binding. J. Mol. Biol. **347:**287–296.
173. **Usui, T., Y. Shima, Y. Shimada, S. Hirano, R. W. Burgess, T. L. Schwarz, M. Takeichi, and T. Uemura.** 1999. Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell **98:**585–595.
174. **Vadlamudi, R. K., T. H. Ji, and L. A. Bulla, Jr.** 1993. A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*. J. Biol. Chem. **268:**12334–12340.
175. **Vadlamudi, R. K., E. Weber, I. Ji, T. H. Ji, and L. A. Bulla, Jr.** 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. **270:**5490–5494.
176. **Valaitis, A. P., J. L. Jenkins, M. K. Lee, D. H. Dean, and K. J. Garner.** 2001. Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. Arch. Insect Biochem. Physiol. **46:**186–200.
177. **Valaitis, A. P., A. Mazza, R. Brousseau, and L. Masson.** 1997. Interaction analyses of *Bacillus thuringiensis* Cry1A toxins with two aminopeptidases from gypsy moth midgut brush border membranes. Insect Biochem. Mol. Biol. **27:**529–539.
178. **Van Rie, J.** 2000. *Bacillus thuringiensis* and its use in transgenic insect control technologies. Int. J. Med. Microbiol. **290:**463–469.
179. **Van Rie, J., S. Jansens, H. Höfte, D. Degheele, and H. Van Mellaert.** 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. **186:**239–247.
180. **Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett, and H. Van Mellaert.** 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science **247:**72–74.
181. **Vestal, D. J., and B. Ranscht.** 1992. Glycosyl phosphatidylinositol-anchored T-cadherin mediates calcium-dependent, homophilic cell adhesion. J. Cell Biol. **119:**451–461.
182. **Wandall, H. H., J. W. Pedersen, C. Park, S. B. Levery, S. Pizette, S. M. Cohen, T. Schwientek, and H. Clausen.** 2003. *Drosophila egghead* encodes a b1,4-mannosyltransferase predicted to form the immediate precursor glycosphingolipid substrate for brainiac. J. Biol. Chem. **278:**1411–1414.
183. **Wang, G. R., G. M. Liang, K. M. Wu, and Y. Y. Guo.** 2005. Gene cloning and sequencing of aminopeptidase N3, a putative receptor for *Bacillus thuringiensis* insecticidal Cry1Ac toxin in *Helicoverpa armigera* (Lepidoptera: Noctuidae). Eur. J. Entomol. **102:**13–19.
184. **Wang, G. R., K. M. Wu, G. M. Liang, and Y. Y. Guo.** 2005. Gene cloning and expression of cadherin in midgut of *Helicoverpa armigera* and its Cry1A binding region. Sci. China Ser. C **48:**346–356.
185. **Wang, P., X. Zhang, and J. Zhang.** 2005. Molecular characterization of four midgut aminopeptidase N isozymes from the cabbage looper, Trichoplusia ni. Insect Biochem. Mol. Biol. **35:**611–620.
186. **Wei, J. Z., K. Hale, L. Carta, E. Platzer, C. Wong, S. C. Fang, and R. V. Aroian.** 2003. *Bacillus thuringiensis* crystal proteins that target nematodes. Proc. Natl. Acad. Sci. USA **100:**2760–2765.

187. **Wolfersberger, M. G.** 1984. Enzymology of plamsa membranes of insect intestinal cells. Am. Zool. **24:**187–197.
188. **Wolfersberger, M. G.** 1990. The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experientia **46:** 475–477.
189. **Wright, D. J., M. Iqbal, F. Granero, and J. Ferré.** 1997. A change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only in part responsible for field resistance to *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*. Appl. Environ. Microbiol. **63:**1814–1819.
190. **Xie, R., M. Zhuang, L. S. Ross, I. Gómez, D. I. Oltean, A. Bravo, M. Soberón, and S. S. Gill.** 2005. Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J. Biol. Chem. **280:**8416–8425.
191. **Xu, X., L. Yu, and Y. Wu.** 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. **71:**948–954.
192. **Yaoi, K., T. Kadotani, H. Kuwana, A. Shinkawa, T. Takahashi, H. Iwahana, and R. Sato.** 1997. Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. Eur. J. Biochem. **246:** 652–657.
193. **Yaoi, K., K. Nakanishi, T. Kadotani, M. Imamura, N. Koizumi, H. Iwahana, and R. Sato.** 1999. *Bacillus thuringiensis* Cry1Aa toxin-binding region of *Bombyx mori* aminopeptidase N. FEBS Lett. **463:**221–224.
194. **Yaoi, K., K. Nakanishi, T. Kadotani, M. Imamura, N. Koizumi, H. Iwahana, and R. Sato.** 1999. cDNA cloning and expression of *Bacillus thuringiensis* Cry1Aa toxin binding 120 kDa aminopeptidase N from *Bombyx mori*. Biochim. Biophys. Acta **1444:**131–137.
195. **Zhang, X., M. Candas, N. B. Griko, L. Rose-Young, and L. A. Bulla, Jr.** 2005. Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R_1 expressed in insect cells. Cell Death Differ. **12:**1407–1416.
196. **Zhang, X., M. Candas, N. B. Griko, R. Taussig, and L. A. Bulla, Jr.** 2006. A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. Proc. Natl. Acad. Sci. USA **103:**9897–9902.
197. **Zhuang, M., D. I. Oltean, I. Gómez, A. K. Pullikuth, M. Soberón, A. Bravo, and S. S. Gill.** 2002. *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. J. Biol. Chem. **277:**13863–13872.
198. **Zimmermann, G., D. Zhou, and R. Taussig.** 1998. Mutations uncover a role for two magnesium ions in the catalytic mechanism of adenylyl cyclase. J. Biol. Chem. **273:**19650–19655.