Single Amino Acid Mutations in the Cadherin Receptor from Heliothis virescens Affect Its Toxin Binding Ability to Cry1A Toxins*

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Bacillus thuringiensis Cry protein exerts its toxic effect through a receptor-mediated process. Both aminopeptidases and cadherin proteins were identified as putative Cry1A receptors from Heliothis virescens and Manduca sexta. The importance of cadherin was implied by its correlation with a Cry1Ac resistant H. virescens strain (Gahan, L. J., Gould, F., and Heckel, D. G. (2001) Science 293, 857–860). In this study, the Cry1Ac toxin-binding region in H. virescens cadherin was mapped to a 40-amino-acid fragment, from amino acids 1422 to 1440. This site overlaps with a Cry1Ab toxin-binding site, amino acids 1363–1464 recently reported in M. sexta (Hua, G., Jurat-Fuentes, J. L., and Adang, M. J. (2004) J. Biol. Chem. 279, 28051–28056). Further, feeding of the anti-H. virescens cadherin antiserum or the partial cadherin, which contain the toxin-binding region, in combination with Cry1Ab/Cry1Ac reduced insect mortality by 25.5–55.8% to first instar H. virescens and M. sexta larvae, suggesting a critical function for this cadherin domain in insect toxicity. Mutations in this region, to which the Cry1Ac binds through its loop 3, resulted in the loss of toxin binding. For the first time, we show that the cadherin amino acids Leu1425 and Phe1429 are critical for Cry1Ac toxin interaction, and if substituted with charged amino acids, result in the loss of toxin binding, with a Kd of <10–5 M. Mutation of Gln1450 to an alanine, however, increased the Cry1Ac affinity 10-fold primarily due to an increase on rate. The L1425R mutant can result from a single nucleotide mutation, CTG → CGG, suggesting that these mutants, which have decreased toxin binding, may lead to Cry1A resistance in insects.

Bacillus thuringiensis, a Gram-positive bacterium that produces crystal inclusions during its sporulation stage of growth, is widely used as a biological agent for the control of agricultural pests and of disease vectors (1–3). Additionally, Cry1 proteins, major components of B. thuringiensis crystals, have been expressed in transgenic plants including corn, cotton, and tobacco to combat specific insect pests (2, 4).

Cry proteins, which are composed of three discrete functional domains, exert their toxic effects through a multistage process, which includes solubilization of crystals in susceptible insect midgut followed by proteolytic activation of protoxins to toxins. The activated toxin then binds to midgut epithelial membrane receptors followed by toxin aggregation and insertion into the membrane resulting in the formation of a pore. The toxin-mediated pores, subsequently, disrupt midgut homeostasis, leading to cell lysis and subsequent insect death (1–3). One major step in this process involves toxin binding to membrane receptors. These receptors are either glycosylphosphatidylinositol-anchored aminopeptidase N (5–7) or cadherins (8–11). Binding to the cadherin receptor is required for further toxin activation (12), which is crucial for downstream toxin aggregation and pore formation.

The toxin-binding domains in cadherin receptors are being mapped. In Bombyx mori, a 219-amino-acid residue region (aa 1245–1464) of the cadherin, BtR175, is responsible for Cry1Aa binding (13), whereas three Cry1Ab-binding sites have been mapped in the Manduca sexta cadherin, BtR1. The first site 865NITIHITDTN875, mapped using phage display, is involved in binding loop 2 of Cry1Aa and Cry1Ab toxins (14). A second region, aa 1291–1360, identified to be important for toxin binding (15), was subsequently narrowed to 1331PL-PASILTVTTV1342 and shown to bind loop α-8 (16). A third region, aa 1363–1464, was recently shown to be involved in toxin binding and cytotoxicity (17). In Heliothis virescens, the toxin-binding sites are unknown but are presumably downstream of the transposon insertion site (18) since this insertion leads to insect resistance to the Cry1Ac.

Mutations in the cadherin gene lead to resistance to Cry1A toxins. First demonstrated in a laboratory-selected H. virescens strain (18), such mutations have now been shown in field-isolated Pectinophora gossypiella (19), leading to resistant insects. In both insect species, resistance to Cry1A toxins results from either mutations to premature stop codons or deletions in the cadherin gene. These resistant insects show significant fitness costs (20), resulting in poor larval survival on cotton plants. This high fitness costs suggest that these deletions likely affect cadherin function.

In contrast, high level resistance observed with many chem-

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1 The abbreviations used are: Cry, crystalline inclusion protein; SPR, surface plasmon resonance; aa, amino acids; RACE, rapid amplification of cDNA ends; NTA, nickel-nitrioltriacetic acid; BtR, B. thuringiensis toxin receptor; CAD, cadherin; HvCad, Heliothis virescens cadherin.

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ical insecticides results from point mutations that do not sub-
stantially affect the target protein function, and single amino
acid changes have been observed in insects resistant to
dieldrin, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, pyre-
throids, and organophosphates (21). In this study, we identified
a critical toxin-binding site in *H. virescens* cadherin and
showed for the first time that point mutations at this site can lead
to substantial decrease in toxin affinity. We also show that
loop 3 of the Cry1Ab and 1Ac toxins bind this site. Selection
and isolation of insects with mutations at these amino acids in
the field would likely lead to the development of highly resist-
ant insect populations, potentially affecting the efficacy of
many transgenic crops expressing these toxins.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Media—**Escherichia coli strains were grown in LB at 37 °C with ampicillin (100 μg/ml). Cry1Ac paras-
poral inclusions were isolated from wild-type *B. thuringiensis* strain
HD73 grown in nutrient broth sporulation medium for 72 h at 30 °C.
Cry1Ab parasporal inclusions were isolated from the acrystalliferous
strain 4Q7cry transformed with pHT315-cry1Ab and grown in HCO
medium (22) for 96 h at 30 °C. Both *H. virescens* (Southland Biopro-
ducts, NJ) and *M. sexta* (Southland Bioprod-
s) and toxin overlay assay. PCR products amplified by different primer
pairs were resequenced to confirm recombination. PCR fragment was amplified from the first stand cDNA with the
sense primer (H11032) and antisense primer, respectively, 5'-TCCTATTGTTT-3'.

**Cloning and Expression of Cadherin Protein—**Two cadherin partial
clones, Hvm-4–62 and Hvm-5–22, 2.9 and 2.1 kb, respectively, were obtained from expressed sequence tag clones (Hvm-5–22 and pFR-CAD-
partial) with opposed or similar hydropathic profile in the predicted
face plasmon resonance (SPR) measurements were performed using a
Biacore X and NTA sensor chips (Biacore). Eluent buffer (0.01 M
HEPES, 0.15 M NaCl, 50 μM EDTA, 0.005% surfactant P20, pH 8.3) was used
throughout the analyses. The ligands, cadherin partial proteins, or
cadherin mutants (24 kDa, homogeneous by SDS-PAGE), at a concen-
tration of 20 ng/ml in eluent buffer, were captured on flow cell 2 at
association and dissociation for 120 and 180 s, respectively. The surface was
used throughout the analyses. The ligands, cadherin partial proteins, or
cadherin mutants (24 kDa, homogeneous by SDS-PAGE), at a concen-
tration of 20 ng/ml in eluent buffer, were captured on flow cell 2 at
densities of less than 100 response units. The analytes (activated
Cry1Ab and Cry1Ac, homogeneous by SDS-PAGE) were injected over both
flour cell 2 at a flow rate of 30 μl/min. The complex was allowed to
associate and dissociate for 120 and 180 s, respectively. The surface was
regenerated with a 3-min injection of regeneration buffer (0.01 M
HEPES, 0.15 M NaCl, 0.55 M EDTA, 0.005% surfactant P20, pH 8.3) at
a flow rate of 600 μl/min. Triplicate injections of each toxin concentration were injected in ran-
daugent over both surfaces, and the response was corrected by double
reference. The data were fitted using global analysis software avail-
able within Biacaluation 3.1 (Biacore). Various concentrations of toxin
were injected over both flow cells, and the response curve on flow cell 1
was subtracted from flow cell 2.

**Hydropathic Pattern Determination—**Hydropathic profiles were
generated using the computer program Hydrophat (28). Peptide se-
quences with opposed or similar hydropathic profile in the predicted
binding regions were selected for analysis. The results obtained were
quantified by linear regression of hydropathic values (Kyte and
Doolittle scale) for each amino acid in the sequence.

**Insect Bioassay—**Bioassays were performed with *H. virescens* or
*M. sexta* neonates using surface-treated food as reported (29). Briefly,
one neonate larva was placed in each well with 9 ng/cm² Cry1Ac toxin
with or without competing cadherin fragments (1000-fold molar excess)
and anti-cadherin antibody, and mortality was recorded after 7 days.
Twelve larvae were assayed for each test, and the assays were repeated
three times.

**RESULTS**

**Isolation of Partial Full-length cDNA Clones Encoding the
H. virescens Cadherin Protein—**Two cDNA clones, Hvm-4–62
and Hvm-5–22, encoding a partial cadherin protein, were obtained
during sequencing of expressed sequence tags from a
*H. virescens* larval midgut library. To obtain a full-length clone,
we then used primers based on the Hvm-5–22 sequence and vector primers to isolate another cDNA clone from this library using a limited growth technique (26). Complete sequencing of this 4.75-kb clone (pFR-CAD-partial) showed that it encoded a partial cadherin protein, which lacked 4 amino acids from the N terminus and 142 amino acids from the C terminus based on FIG. 1. Structure of the *H. virescens* cadherin protein. *H. virescens* cadherin is composed of five domains: (i) signal peptide (SIG); (ii) cadherin repeats (CR); (iii) membrane proximal region (MPR); (iv) transmembrane region (TM); and (v) cytoplasmic domain (CD). The toxin-binding region (TBR) is indicated upstream of the membrane proximal region. The partial clone Hvm-5–22 was used to generate clone CAD0. The wild-type clone pFR-CAD-partial was used to isolate a NdeI/SphI fragment that was used to construct the full-length cadherin clone as indicated under “Materials and Methods.”

FIG. 2. Cry1Ac toxin-binding to partial fragments of *H. virescens* cadherin protein. A, partial cadherin proteins and their abilities to bind Cry1Ac. The numbers refer to amino acid residues in the full-length *H. virescens* cadherin. A strong binding between a protein and Cry1Ab/Cry1Ac is indicated by +, a weaker binding between a protein and Cry1Ab/Cry1Ac is indicated by +/-, whereas no binding between a protein and Cry1Ab/Cry1Ac is indicated by −. Results were obtained from Cry1Ab/Cry1Ac toxin overlay assay using 40 pmol of each purified protein. The Cry1Ac toxin-binding region in *H. virescens* cadherin protein is mapped to aa 1422–1440. B, expression and toxin overlay assay of partial *H. virescens* cadherin proteins. Seven *H. virescens* partial cadherin fragments were generated by PCR or by using restriction sites. cDNA fragments were subcloned and expressed with N-terminal His tag in the pQE system. Purified proteins were silver-stained following SDS-15% PAGE. Each lane contains 40 pmol of purified proteins, which were separated by SDS-PAGE. A toxin overlay assay was performed using 10 nM Cry1Ac after transfer of proteins to Immobilon membranes. Toxin binding abilities was detected with 1:3000 anti-Cry1Ac antibody following the toxin overlay. Partial proteins CAD0, CAD1, CAD2, CAD3, and CAD4 bound Cry1Ac strongly, whereas CAD5 bound to toxins weakly. Protein CAD6 did not bind Cry1Ac.
a reported sequence (18). The C-terminal truncation resulted from the presence of a NotI site, which was used for cDNA synthesis.

Since we could not obtain a full-length cadherin clone, as indicated above, we then used PCR and 3'-RACE to obtain the rest. Since only 4 amino acids were lacking at the N terminus, we used PCR to obtain the 5'-end of the clone using sequences from the pFR-CAD-partial and a published report (18), which became available while this project was underway. However, since there were substantial amino acid differences between pFR-CAD-partial and the published sequence (18), we used RACE to obtain the 3'-end. To construct a full-length cDNA, the 1040-bp 5'-PCR product and the 3860-bp 3'-RACE product were then sequentially cloned into pBluescript vector. The middle NdeI/SphI was then replaced by the NdeI/SphI fragment from the wild-type sequenced cadherin partial clone (pFR-CAD-partial) to generate a full-length cadherin clone.

The deduced amino acid sequence, 1732 amino acids, of *H. virescens* cadherin protein full-length clone has been deposited in GenBank (accession number AY692446). This HvCad protein is 98.4% identical to that identified previously (18) from the same insect species. According to the cloning strategy of the gene in this insect species, showing that there is substantial amino acid variation in the gene in this insect species.

As with *M. sexta* BrR (9) and *B. mori* BrR175 (11) cadherins, this *H. virescens* protein contains a 23-aa N-terminal signal peptide, 12 cadherin repeats, a membrane proximal region, a transmembrane domain, and a cytoplasmic portion on the C terminus (Fig. 1). The *H. virescens* cadherin has 63.9 and 65.4% identity to *M. sexta* and *B. mori* cadherins, respectively. These cadherins are distantly related to classical cadherins, albeit they all contain typical cadherin repeats that imply their involvement in cell-cell adhesion. Neither integrin recognition sequence RGD/LDV nor cadherin/cadherin-binding sequence HAV that is present in *M. sexta* cadherin (15) exists in the *H. virescens* cadherin.

![Fig. 3. Competition of loop region peptides of Cry1Ab and Cry1Ac to the partial cadherin fragment CAD3. A, toxin overlay detection of the CAD3 protein by Cry1Ab toxin in the presence of loop a-8 peptide, 250 and 500 molar excess (lanes 2 and 3); in the presence of loop2 peptide (lanes 4 and 5), loop 3 peptide (lanes 6 and 7), and loop 2 and 3 peptides together, 300 and 600 molar excess (lanes 8 and 9). B, toxin overlay detection of the CAD3 protein by Cry1Ac toxin as in A, except that it also includes loop 1 peptide. Toxin binding in the absence of any peptide is shown in lanes 1.](http://www.jbc.org/)

| Crystal | Treatment | Mortality |
|---------|-----------|-----------|
|         |           |           |           |
|         |           | M. sexta | *H. virescens* |
| Cry1Ab  | Toxin     | 97.4 ± 4.4 | 97.2 ± 4.8 |
|         | Toxin + CAD0 | 48.7 ± 2.2 | 43.4 ± 2.9 |
|         | Toxin + anti-CAD | 70.0 ± 1.3 | 68.4 ± 1.4 |
|         | Toxin + pre-serum | 97.6 ± 4.1 | 97.8 ± 3.9 |
| Cry1Ac  | Toxin     | 98.4 ± 2.8 | 98.3 ± 2.9 |
|         | Toxin + CAD0 | 47.2 ± 4.8 | 45.2 ± 4.2 |
|         | Toxin + anti-CAD | 74.2 ± 1.3 | 71.5 ± 6.3 |
|         | Toxin + pre-serum | 97.4 ± 4.4 | 97.1 ± 5.1 |

**TABLE I**

Toxicity of Cry1A crystals to 1st instar larvae can be alleviated by anti-*H. virescens* cadherin-like protein antiserum and partial cadherin-like proteins that are involved in toxin binding.

Cry1Ac in toxin overlay assays (Fig. 2B). Further, in bioassays with neonate *H. virescens* and *M. sexta*, the CAD0 protein and an anti-CAD0 antibody (1000-fold molar excess) both attenuated Cry1Ab and Cry1Ac toxicity (Table I). In contrast, feeding of Cry1A toxins with preimmune serum had no effect on mortality, showing that this cadherin domain plays a role in toxicity.
To further delineate the toxin-binding region, a series of clones was then generated either by PCR or by suitable restriction enzyme digestion (Fig. 2A). The corresponding partial proteins were expressed in E. coli M15, purified using Ni-NTA agarose, and then analyzed with Cry1Ac toxin overlay assays. As shown in Fig. 2B, proteins CAD0, CAD1, CAD2, CAD3, and CAD4 bound Cry1Ac strongly, whereas protein CAD5 bound Cry1Ac slightly weakly. Protein CAD6 did not bind to Cry1Ac. These data show that amino acids 1422–1440 are apparently important for Cry1Ac binding, but amino acid residues beyond this region may be required for stronger binding. This region, aa 1422–1440, also falls with the Cry1Ab toxin domain, aa 1364–1464, recently mapped for the Manduca sexta BtR1 receptor (17). Further, since the small protein CAD5, which does not include these amino acid residues, aa 1422–1440, still bound Cry1Ac, it is clear that additional regions can also bind Cry1Ac, albeit with likely lower affinity.

Interaction of HvCad with Loop Regions of Cry1A Toxins—Gomez et al. (16, 31) previously demonstrated that competition assays with the loop regions peptides facilitates identification of which loop region is involved in toxin binding. We therefore incubated the loop peptides of Cry1Ab and Cry1Ac during toxin overlay assays. Fig. 3 shows that loop 3 is involved in Cry1Ab and 1Ac binding to CAD3, showing that this cadherin domain binds to a different Cry1A toxin loop than those reported in other studies (14, 16, 31). The lower level of displacement of Cry1Ac binding than that of Cry1Ab by the loop peptides could be due to the higher affinity of the Cry1Ac toxin to the cadherin receptor (32). Loop 3 of both toxins and loop 1 of Cry1Ac do not play a role in toxin binding to the CAD3 protein (Fig. 3A). However, loop 2 could be involved in some Cry1Ab binding to the cadherin receptor (32). Loop α of both toxins and loop 1 of Cry1Ac do not play a role in toxin binding to the CAD3 protein (Fig. 3A).

With this knowledge, and previous evidence (16) that indicates that the Cry1A proteins can interact with their receptors through amino acid sequences displaying inverted hydropathic profiles, we then analyzed whether the loop 3 regions of Cry1Ac and Cry1Ab, FSNSSVSII, share a similar hydropathic pattern with the toxin-binding domain mapped above. Fig. 4 shows that loop 3 had an inverse hydropathic pattern to specific amino acids, GVLTLNFQ, of the putative toxin-binding region mapped above, amino acids 1422–1440.

Role of Specific Amino Acids in the H. virescens Cadherin Toxin-binding Domain—With this information, we used alanine scanning to further refine the toxin-binding residues. The CAD3 protein was used because it had high toxin binding (Fig.

### Table II

| Mutant | Nucleotide switch | Amino acid switch |
|--------|------------------|------------------|
| M1425  | CTG → GAG        | Leu → Glu        |
| M1425a | CTG → CGG        | Leu → Arg        |
| M1428  | AAC → GCC        | Asn → Ala        |
| M1428a | AAC → GAC        | Asn → Asp        |
| M1428b | AAC → AAG        | Asn → Lys        |
| M1429  | TTC → GAC        | Phe → Asp        |
| M1429a | TTC → GGC        | Phe → Arg        |
| M1430  | CAG → GCC        | Gln → Ala        |
| M1430a | CAG → GAG        | Gln → Glu        |
| M1430b | CAG → CGG        | Gln → Arg        |
2B), overlapped with CAD0, which inhibited Cry1Ac toxicity in vivo (Table I), and it similarly could inhibit Cry1Ac toxicity in insect bioassays (data not shown). Three or four amino acids in the putative toxin-binding region were mutated at a time to generate six mutants, numbers 7–12 (Fig. 5A), that covered amino acids 1423–1440. The mutant proteins were purified using His tag affinity chromatography and analyzed in Cry1Ac toxin overlay assays. None of these mutant proteins could bind Cry1Ac (Fig. 5B, lanes 7–12), indicating that this domain was critical for toxin binding as predicted by the hydropathic analysis.

To further define the limits of the toxin-binding domain, we constructed additional mutants on both sides of this putative toxin-binding region, aa 1422–1440. In total, we obtained 17 mutants, mutants 1–15 and 17 and 18 (Fig. 5A). No mutants could be obtained for amino acids 1407AVQ1409 (putative mutant 16). All mutant proteins were purified, and their toxin binding abilities were analyzed in Cry1Ac toxin overlay assays. As shown in Fig. 5B, in addition to mutant 14 and mutant 17, the toxin binding capacities of mutants 6–12 were completely lost. These data suggest that a critical toxin-binding site is from amino acids 1407 to 1443. Amino acids in these regions may be critical for toxin binding. This large domain also suggests that loop 3 of Cry1Ab and Cry1Ac toxins recognizes its cadherin receptor, probably through a three-dimensional, rather than a linear, epitope.

Since our analysis using hydropathic complementarity also predicted that single amino acid changes could affect Cry1A toxin binding to the cadherin receptor, we then tested 4 amino acids individually. Fig. 4 shows inverse hydropathic patterns to the loop 3 region of Cry1Ac toxin, in particular the residues Leu1425, Asn1428, Phe1429, and Gln1430. To disrupt this complementarity, we introduced primarily charged residues at these sites (Table II) by mutagenesis of CAD3. The single amino acid mutated partial cadherin fragments generated were expressed, using the pQE30 plasmid vector, as His6-tagged fusion proteins, and the Cry1Ac binding activities of these mutants were analyzed using toxin overlay assays. The proteins, M1425, M1425+, M1429+, and M1429, containing single amino acid changes from a hydrophilic amino acid to a hydrophobic amino acid, completely lost their toxin binding abilities in overlay assays (Fig. 6). In contrast, substitution of a hydrophobic amino acid with alanine or a positively charged amino acid, as in M1425+, M1428+, M1429+, M1428, M1428+, M1430+, M1430, and M1430+, were immobilized on NTA chip and analyzed using Cry1Ab (data not showed) and Cry1Ac (Fig. 7 and Table III). The mutants, M1425, M1425+, M1429+, and M1429+, all had Keq values of less than 10^{-5} M. The M1428, M1428+, and M1428+ mutants all showed decreased affinity predominantly due to decreased on rates, k_{on}. In contrast, three mutants, M1430+, M1430, and M1430, had increased k_{off}. Both M1430+ and M1430 also had increased k_{off} rates, whereas M1430 had k_{off} rates similar to that of the wild-type CAD3. The overall effect of these differences is that M1430 had about a 10-fold increase in affinity, as compared with wild-type CAD3, with a K_{D} of 9.9 \times 10^{-9} M, whereas the K_{D} values of M1430+ and M1430 are 28 and 131 \times 10^{-9} M, respectively. The affinity data obtained are consistent with the ability of the toxin binding observed in overlay assays (Fig. 6).

**Involvement of the Partial Cadherin Protein in Cry1A Toxicity**—Since the 25-kDa partial cadherin protein CAD3 (aa 1381–1556) contains the toxin-binding site, we then evaluated whether this protein and the mutants could inhibit Cry1Ac toxicity. Thus, first instar larvae were fed with Cry1Ac toxins either alone or together with a 1000-fold molar excess of CAD3, M1425+, or M1428+ proteins. The toxicity of Cry1Ac against *H. virescens* was reduced by about 32% when the toxin was mixed with the CAD3 protein (Table IV). However, Cry1Ac toxicity was reduced by only 13–15% by either of the mutant proteins. The inability to suppress toxicity more significantly is likely due to protease-mediated degradation of the CAD3 protein and its mutants in the insect midgut. Nevertheless, we are able to conclude that single amino acid changes in the *H. virescens* cadherin toxin-binding sites can affect the Cry1A toxicity.

**DISCUSSION**

Cadherins, aminopeptidases, and other proteins/lipids are identified as Cry1A toxin-binding molecules from different insect species (5–9, 11, 13, 18, 33–36). These molecules bind to Cry1A toxins differentially and are important in the receptor-mediated toxicity of *B. thuringiensis* Cry toxins. Of these, the cadherin protein has been shown to be essential since mutation of its gene by a retrotransposon insertion results in insect resistance to the Cry1A toxins (18). However, more recent evidence suggests that aminopeptidases also appear to have a functional role. For example, the silencing of an aminopeptidase by RNA interference resulted in decreased insect sensitivity to Cry1A toxins (37), and in vivo expression of an aminopeptidase in Cry1A-insensitive *Drosophila melanogaster* resulted in these transformed insects becoming toxin-sensitive (38).

Since the cadherin protein is critical for initial toxin binding in *H. virescens*, we mapped the Cry1Ab/1Ac toxin-binding regions in this protein. This binding domain from aa 1422–1440

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**Table I**

| CAD3 single amino acid mutant | M1428 | M1428+ | M1429 | M1429+ | M1430 | M1430+ | M1430+ | M1429- | M1429+ |
|-----------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Binding affinity (k_{D})    |       |       |       |       |       |       |       |       |       |
| k_{off} (10^{-9} M)         |       |       |       |       |       |       |       |       |       |
| k_{on} (10^{-9} M)          |       |       |       |       |       |       |       |       |       |

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**Fig. 6.** Toxin overlay assay of single amino acid mutants of the CAD3 protein. The mutated CAD3 protein was probed with Cry1Ac toxin as described in Fig. 5. Four mutants showed binding in these assays. Mutant numbers are as described in Table II.
Fig. 7. Surface plasmon resonance analyses of Cry1Ac binding to partial *H. virescens* cadherin protein CAD3 and its mutants. A, a sensogram of varying concentrations (6.25–100 nM) of Cry1Ac binding to immobilized CAD3 protein on an NTA chip. RU, relative response units. B–D, sensograms of Cry1Ac toxin binding (at the concentrations indicated) to CAD3 mutants M1428, M1428/H11002, and M1430, respectively. E, comparative sensogram of the Cry1Ac toxin at 25 nM binding to immobilized CAD3 protein and its various mutants.
corresponds to the region within cadherin repeat 12, the last repeat. Thus, the Cry1A resistance observed in the *H. virescens* mutant (18) results from a loss of the toxin-binding site in the cadherin molecule because the retrotransposon insert was upstream of this toxin-binding site.

Feeding *H. virescens* larvae with either anti-cadherin antiserum or partial cadherin proteins containing the toxin-binding region in combination with Cry1A proteins alleviated insect mortality 25.5–55.6%. These data suggest that the toxin-binding region identified in this study is not only involved in Cry1A toxin binding but also plays an important role in Cry1A toxicity. The fact that only part of the mortality was alleviated suggests that other cadherin domains or proteins might also be involved in Cry1A toxicity. Thus, toxin binding seems to be a subtle and complex process in which several proteins and binding domains, as well as protein conformational changes, are involved. A recent model (46) shows that cadherin is critical for

**Table III**

| Ligand    | $k_{on}$ ($\times 10^4$ M$^{-1}$ s$^{-1}$) | $k_{off}$ ($\times 10^{-3}$ s$^{-1}$) | $K_D$ (M) |
|-----------|------------------------------------------|------------------------------------|------------|
| CAD3      | 4.95                                     | 4.77                               | 9.65 $\times 10^{-8}$ |
| M1428     | 0.154                                    | 4.74                               | 3.08 $\times 10^{-6}$ |
| M1428$^+$ | 0.190                                    | 2.89                               | 1.52 $\times 10^{-6}$ |
| M1428$^-$ | 0.045                                    | 13.1                               | 2.91 $\times 10^{-5}$ |
| M1430     | 45.2                                     | 4.48                               | 9.91 $\times 10^{-9}$ |
| M1430$^+$ | 35.5                                     | 10.1                               | 2.84 $\times 10^{-8}$ |
| M1430$^-$ | 12.9                                     | 16.9                               | 1.31 $\times 10^{-7}$ |

**Table IV**

| Crystal   | Treatment | Mortality of *H. virescens* % |
|-----------|-----------|-------------------------------|
| Cry1Ac    | Toxin     | 99.0 ± 1.7                    |
|           | Toxin + CAD3 | 67.0 ± 5.7                  |
|           | Toxin + M1425$^-$ | 86.0 ± 1.7                |
|           | Toxin + M1428 | 84.0 ± 1.7                  |
initial toxin binding followed by further proteolytic cleavage and oligomerization (12), binding to aminopeptidases in lipid rafts (25), and insertion in the cell membrane forming pores (39).

In *B. mori*, Cry1Aa binds to a region covering aa 1108–1464 of the BtR172 cadherin receptor (13), whereas the three Cry1Ab toxin-binding regions in *M. sexta* BtR1 have been mapped to aa 865–875 (site 1 (14, 31), 1331–1342 (site 2 (15, 16), and 1363–1464 (site 3 (17)). Consequently, the toxin-binding region and amino acids identified here overlap with site 3 observed in both *B. mori* (13) and *M. sexta* (17). Although our constructs, CAD1–3, contain site 2, the Cry1Ac toxin showed negligible binding to this site as observed by the absence of toxin binding in our CAD3 mutants (*e.g.* mutant M1425'), which had an intact site 2.

Each of the three toxin-binding sites identified includes a number of amino acids. Although it is not clear which specific amino acids of sites 1 and 2 are critical, we show here that a number of specific amino acids are required for toxin binding in site 3. This is not surprising since each of the sites interacts with specific loops of the Cry1A toxins: site 1 binds loop 2 (14, 31), site 2 binds loop α8 (16), and site 3 binds loop 3. Toxin binding to multiple sites on the cadherin receptor is probably essential for changing the toxin conformation, which facilitates cleavage of the α1 helix that promotes subsequent oligomerization (12).

Therefore, loop 3 is important for Cry1Ac interaction with the cadherin receptor prior to toxin insertion. Indeed, Cry1Ab loop 3 mutants have reduced initial binding to *H. virescens* midgut brush border membranes and show decreased toxicity (40). The greatest loss of toxicity was observed with the G439A and F440A mutants in loop 3 of Cry1Ac. These amino acids were predicted by hydropathic complementarity (Fig. 4) to interact within the toxin-binding region aa 1422–1440 (Fig. 2). It is, therefore, not surprising that mutation of the *H. virescens* cadherin in this region results in significantly reduced toxin binding to both Cry1Ab and Cry1Ac since loop 3 of both toxins is identical. Consequently, it can be predicted that the G439A and F440A loop 3 mutants (40) will show decreased binding to site 3.

Loop 3 has been demonstrated to be involved in initial binding to a high affinity binding site (40). Data from Hua et al. (17) with *M. sexta* support the role of site 3 being a high affinity binding site, in which not affinity was obtained with a partial cadherin (containing only site 3) expressed in *Drosophila* S2 cells. Our data also show that site 3 has higher affinity than site 2 since we observe a substantial loss of toxin binding and lower affinity with mutant proteins, which have an intact site 2 but not site 3. The lower affinity observed in our study as compared with that in Hua et al. (17) potentially arises from using *E. coli*-expressed proteins and using SPR instead of 125I-labeled proteins. Cadherin proteins, BtR1 and BtR175, have been reported to have affinities of 0.7 and 2.6 nM to Cry1A toxins determined by 125I-labeled toxin (8) and SPR (30), respectively.

In this study, we also show that single amino acid changes in site 3 can have a profound effect on toxin binding. Of the 4 amino acids we mutated in site 3, Leu1425, Asn1428, Phe1429, and Gln1430, changes in Leu1425 and Phe1429 have the greatest effect on toxicity. Introduction of charged residues at either of these sites results in the loss of toxin binding (Kd values of < 10–5 M). Substitution at Asn1428 also decreased toxin binding and affinity; however, substitution of Gln1430 with alanine or glutamic acid increased toxin affinity, primarily to an increased rate, kcat.

The laboratory-selected Cry1Ac-resistant *H. virescens*, which has a retrotransposon that causes truncation of the cadherin transcript (18), is resistant to the toxin because a truncated cadherin protein, if expressed, would not be an integral membrane protein and would not have the toxin-binding sites. In *P. gossypiella*, three resistant alleles have been identified in the field, two of which, *r1* and *r3*, contain deletions, whereas the third, *r2*, has a premature stop codon (19). The cadherin in *P. gossypiella* with the allele *r2* would not be an integral membrane protein and would not have sites 2 and 3, and would, therefore, be resistant to Cry1A toxins. However, the cadherins in insects with alleles *r1* and *r3* retain all three toxin-binding sites and are predicted to be integral membrane proteins. Thus, the mechanism of resistance in these insects is not as easily defined. Nevertheless, all three alleles have a substantial fitness cost (20) (also expected with *H. virescens* resistant strain), probably resulting from the loss of cadherin function. This high fitness cost has likely contributed to the lack of failures in the control of *H. virescens* and *P. gossypiella* on cotton, although the frequency of such resistant alleles in the field is high (41, 42).

In contrast, resistance to chemical insecticides, including pyrethroids, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, dieldrin, and organophosphates, has arisen primarily from point mutations in target sites for these insecticides and is fairly widespread in the world (21). For example, the resistance to dieldrin allele, *rdr*, has an A302S mutation in the γ-aminobutyric acid-gated chloride channel (43); the *krd* alleles that confer 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane and pyrethroid resistance contain leucine-to-phenylalanine substitutions in the IIS6 transmembrane segment of the sodium channel (44); and a G137D mutation in a carboxyesterase results in resistance to the organophosphate, diazinon (45).

Therefore, a substantial change in toxin binding to the cadherin receptor that results from single amino acid changes has a major practical significance with respect to development of resistance to Cry1A toxins. Because there is substantial polymorphism in the *H. virescens* cadherin (the HvCad reported here, AY692445, is only 98.4% identical to that reported by Gahan et al. (18)), it is likely that such mutations are present in the field. It is critical, therefore, to evaluate the frequency of such mutations in the toxin-binding sites, particularly in site 3, in field populations. For example, the L1425R mutant can result from a single nucleotide mutation, CTG → CGG. The presence of such mutants, which have reduced toxin binding, could potentially lead to resistance to Cry1A toxins in field populations of insects.

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Single Amino Acid Mutations in the Cadherin Receptor from *Heliothis virescens* Affect Its Toxin Binding Ability to Cry1A Toxins

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