A NOVEL *TENEBRIO MOLITOR* CADHERIN IS A FUNCTIONAL RECEPTOR FOR *BACILLUS THURINGIENSIS* CRY3Aa TOXIN *

Jeff Fabrick‡, Cris Oppert§, Marcé D. Lorenzen¶, Kaley Morris¥, Brenda Oppert¶, and Juan Luis Jurat-Fuentes§,

From the ‡USDA ARS U.S. Arid-Land Agricultural Research Center, 21881 North Cardon Lane, Maricopa, AZ 85238, §Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996-4560, ¶USDA ARS Grain Marketing and Production Research Center, 1515 College Ave., Manhattan, KS 66502, and ¥Department of Biochemistry, Kansas State University, Manhattan, KS 66506

Running Title: *T. molitor* cadherin is a Cry3Aa receptor

Address correspondence to: Brenda Oppert, USDA ARS GMPRC, 1515 College Ave., Manhattan, KS 66502 Tel. 785-776-2780; Fax. 785-537-5584; Email: bso@ksu.edu

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Cry toxins produced by the bacterium *Bacillus thuringiensis* (Bt) are effective biological insecticides. Cadherin-like proteins have been reported as functional Cry1A toxin receptors in Lepidoptera. Here we present data that demonstrate a coleopteran cadherin is a functional Cry3Aa toxin receptor. The Cry3Aa receptor cadherin was cloned from *Tenebrio molitor* larval midgut mRNA, and the predicted protein, TmCad1, has domain structure and a putative toxin binding region similar to those in lepidopteran cadherin Bt receptors. A peptide containing the putative toxin binding region from TmCad1 (rTmCad1p) bound specifically to Cry3Aa and promoted the formation of Cry3Aa toxin oligomers, proposed to be mediators of toxicity in lepidopterans. Injection of *TmCad1*-specific dsRNA into *T. molitor* larvae resulted in knockdown of *TmCad1* transcript and conferred resistance to Cry3Aa toxicity. These data demonstrate the functional role of TmCad1 as a Cry3Aa receptor in *T. molitor* and reveal similarities between the mode of action of Cry toxins in Lepidoptera and Coleoptera.

The mode of action of *Bacillus thuringiensis* (Bt)[1] insecticidal Cry toxins has been extensively studied in lepidopteran larvae (1). Our current understanding is that the major factors that contribute to Cry toxicity in insects include solubilization and activation of the crystalline toxin, as well as interactions between toxin and midgut receptors. In lepidopterans, several insect midgut proteins have been proposed as Cry toxin receptors (2). Cry1A receptor functionality has been demonstrated for cadherin proteins from *Bombyx mori* (3,4), *Manduca sexta* (5,6), *Ostrinia nubilalis* (7), and *Heliothis virescens* (8). The specific toxin-binding region in lepidopteran cadherins has been localized proximal to the cell membrane insertion site (9-11). Mutations in toxin binding motifs of lepidopteran cadherin genes are genetically linked to Cry1Ac-resistance in *H. virescens* (12,13), *Helicoverpa armigera* (14,15), and *Pectinophora gossypiella* (9,16).

Interactions between Cry toxins and cadherin receptors and the implications for toxicity have been studied in Lepidoptera more extensively than in any other insect order (2). According to the model proposed by Bravo et al. (17), Cry toxin binding to cadherin is followed by toxin oligomerization. Toxin oligomers reportedly are intermediates required for effective pore formation, and ultimately toxicity (18). A fragment of the BtR1 cadherin from *M. sexta*, corresponding to repeat 12 and containing a critical toxin-binding region, enhanced the activity of Cry1A toxins in Lepidoptera (19) by promoting toxin oligomerization (20). However, other studies in Lepidoptera have found that cadherin fragments can reduce Cry1A toxicity (11,21). An alternative model suggests that Cry toxin binding to cadherin receptors activates intracellular pathways leading to cell death (22). Notably, in

both models cadherin is a critical contact point for Cry toxins that is pivotal for intoxication.

In contrast to the lepidopteran model, relatively little is known about the steps involved in the intoxication process by coleopteran-specific Cry toxins. The solubility of one such toxin, Cry3Aa, was considered a determining factor for toxicity in the coleopteran midgut (23-25). Cry3Aa toxin binding assays and ligand blots with brush border membrane vesicles (BBMV) from *Tenebrio molitor* larval midgut demonstrated binding to a single high affinity (K_d = 17.5 nM) site on an unidentified 144-kDa BBMV protein (26). An ADAM metalloprotease in *Leptinotarsa decemlineata* (27) and a cadherin-like protein in *Diabrotica virgifera virgifera* (28) were proposed as putative Cry toxin receptor proteins. However, neither study demonstrated functional interactions between putative receptor and toxin. More recently, a partial *D. virgifera virgifera* cadherin fragment corresponding to cadherin repeat (CR) domains 8-10 was reported to bind activated Cry3Aa and Cry3Bb toxins and enhance toxin activity in several beetles, suggesting cadherin plays a functional role in Bt intoxication in beetles (29). Another study reported the oligomerization of Cry3Aa toxin after incubation with *L. decemlineata* BBMV, although the proteins involved in oligomerization were not identified (30).

The high degree of structural similarity among Cry1, 2, 3, and 4 toxins implies that they share a similar mode of action in insects (2,31). To test this hypothesis, we evaluated whether a cadherin in the midgut of a Bt-sensitive coleopteran is a functional receptor for coleopteran-specific Cry3Aa toxin. Our data indicate that Cry3Aa binds specifically to a cadherin-like protein from *T. molitor*. A peptide containing the predicted toxin binding region from *T. molitor* cadherin bound Cry3Aa toxin specifically and promoted Cry3Aa oligomerization in solution. More importantly, we demonstrate that reduced levels of *TmCad1* transcript in actively feeding larvae correlates with a reduction in Cry3Aa toxicity. These results support the hypothesis that *T. molitor* cadherin is a functional Cry3Aa receptor analogous to lepidopteran cadherin receptors and highlights a common interface for Cry toxins in two major insect orders.

EXPERIMENTAL PROCEDURES

Insect Larvae, Bacterial Strains and Toxin Purification - *T. molitor* larvae were from a laboratory colony reared on a diet of 50% rolled oats, 47.5% wheat flour, and 2.5% brewer's yeast at 60% R.H., 28°C, no photoperiod.

Cry3Aa protoxin was purified from sporulated cultures of Bt var. *tenebrionis* following methods described elsewhere (32). Purified Cry3Aa protoxin was quantified using the Coomassie Plus® Protein Assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as standard.

Brush Border Membrane Vesicle (BBMV) Preparation - The isolation of *T. molitor* BBMV was performed as previously described (33). Purified BBMV were quantified as described previously for toxin quantification and were stored at -80°C. N-aminopeptidase (APN) activity was determined by the substrate leucine-ρ-nitroanilide and was used as marker for brush border enzyme enrichment in the BBMV preparations. APN activities were enriched 3-5 fold in the BBMV preparations compared to initial midgut homogenates.

Cloning of TmCad1 *cDNA* - A partial sequence encoding a *T. molitor* cadherin was identified among randomly selected clones from a *T. molitor* larval midgut cDNA library (34). Gene-specific primers were designed based on this sequence, and the complete coding sequence (*TmCad1*) was obtained from larval midgut mRNA by 5'- and 3'-RACE using the GeneRacer™ kit from Invitrogen (Carlsbad, CA) and SuperTaq™ Plus DNA polymerase (Ambion, Austin, TX). Tm1 and Tm2 nucleotide primers (Table 1) were designed from the *TmCad1* sequence in the sense orientation and were used with the GeneRacer 3'-primer to amplify the 3' end. Similarly, PCR primers were designed in the antisense orientation (Tm3, Tm4, Tm5, Tm6, Tm7, Tm8, Tm9, Tm10; Table 1) and were used with the GeneRacer 5'-primer and GeneRacer 5'-nested primer to amplify the missing 5' cDNA fragments. PCR products were gel-purified and inserted into pCR2.1-TOPO or pCR4-TOPO cloning vectors (Invitrogen). DNA sequencing was performed using the GenomeLab™ DTCS Quick Start Kit on a

CEQ8000 DNA sequencer (Beckman-Coulter, Fullerton, CA). Nucleotide primers (Tm11, Tm12, Tm13, Tm14, Tm15) were designed from known *TmCad1* and used to sequence missing internal regions of the subcloned cDNA. The complete *TmCad1* cDNA sequence has been deposited in NCBI (DQ988044).

Sequence Analysis - Multiple sequence alignments were constructed using ClustalW version 1.83 from EMBL-EBI (http://www.ebi.ac.uk/clustalw/). The *TmCad1* sequence and predicted amino acid sequence was compared to those in public databases, and all sequences with significant relatedness were identified.

Percent identity scores were calculated from single pair-wise alignments using AlignX from Vector NTI Advance™ 9.1.0 (Invitrogen) and from ClustalW sequence alignment. Phylogenetic data was obtained from alignments (DIALIGN v. 2.2.1) and used to generate protein distances from the algorithm protdist (Phylip v3.6a3) and bootstrap analysis of one hundred replicates using the Jones, Taylor, and Thorton model of amino-acid substitution (35,36). Phylogenetic trees were made using PHYLIP drawgram (36) with branch lengths made from the Kitsch distance matrix.

Cloning, Expression and Purification of the Predicted TmCad1 Toxin Binding Region - Primers Tm16 and Tm17 were used for PCR with KOD high-fidelity DNA polymerase (EMD Biosciences, San Diego, CA) to amplify a 582-bp region from the *TmCad1* cDNA (nucleotides 3,963 to 4,548). The generated amplicon was gel-purified and inserted into the *Escherichia coli* expression vector pET151-D-TOPO® (Invitrogen) to generate the pET151-rTmCad1p construct. Insertion of the correct sequence into the expression vector was confirmed by sequencing in both directions.

For expression, BL21 Star™ (DE3) *E. coli* were transformed with pET151-rTmCad1p and cultures grown as previously described (9). Expressed rTmCad1p peptide containing a histidine tag at the amino terminus was purified using Ni^{2+}-affinity chromatography. Protein was extracted from *E. coli* inclusion bodies, and purification was performed under hybrid denaturing/native conditions as described elsewhere (9). Eluted fractions containing rTmCad1p were pooled and dialyzed against 0.01 M Tris-HCl, pH 8.0, 0.01% Triton X-100®. For most applications (unless noted otherwise), rAcTEV protease (Invitrogen) was used to remove 27 of the 33 additional amino acid residues at the amino terminus of rTmCad1p, including the six histidine tag and a V5 epitope. Purification of rAcTEV protease-treated rTmCad1p was performed according to the manufacturer's recommendations. Purified rTmCad1p was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10-20% Tricine gels (Invitrogen), and protein concentration was determined as for toxin quantification. Concentration and buffer exchange of rTmCad1p was with Centricon® centrifugal filters (Millipore, Bedford, MA).

Binding of Cry3Aa to Purified rTmCad1p - Dot blot overlay binding assays were performed using previously described procedures adapted for Cry3Aa (9,11). For toxin immunodetection, rabbit Cry3Aa antisera was provided by AgDia (Elkhart, IN).

For in-gel binding assays, rTmCad1p was labeled with a fluorescent dye (IR800TmCad1p) using the IRDye® 800CW Protein Labeling Kit (LI-COR Biosciences, Lincoln, NE). The infrared dye forms a stable ester conjugate with the peptide and has an emission maximum of 789 nm. Binding assays were performed according to manufacturer's recommendations (LI-COR). Briefly, approximately 1-3 μg of rTmCad1p with an intact His-tag, Cry3Aa protoxin, or BSA was combined with SDS sample buffer without reducing reagent or heat, and proteins were separated by 10% Bis-Tris SDS-PAGE using MOPS buffer (Invitrogen). After electrophoresis, gels were fixed in 50% isopropanol for 15 min and washed 3X in deionized water for 5 min each. Duplicate gels were either stained with Coomassie blue (Imperial Protein Stain, Pierce) or incubated with 60 ng IRTmCad1p in 10 ml of Odyssey blocking buffer (LI-COR) containing 50 mM $CaCl_2$, with or without 500-fold molar excess unlabeled rTmCad1p, in complete darkness for 1 h at room temperature with gentle shaking. Gels were washed 3X with buffer (0.002 M imidazole-buffered saline with 0.02% Tween 20, KPL, Gaithersburg, MD) and were scanned at 700 and

800 nm on an Odyssey Imager using v. 1.2.15 Odyssey software (LI-COR).

Cry3Aa Oligomerization Assays - Purified Cry3Aa protoxin was labeled with NHS-LC-biotin (Pierce) as described elsewhere (37). Labeled protoxin (1.2 μg) was incubated for 1 h at room temperature with *T. molitor* BBMV (7.8 μg) in 100 μl (final volume) of PBS alone or with either purified rTmCad1p (1.4 μg) or unlabeled Cry3Aa protoxin (24 μg). Unbound Cry3Aa protoxin was removed from BBMV by centrifugation (16,100 x *g* for 10 min) and washing pellets twice in 100 μl of ice-cold binding buffer. BBMV pellets were solubilized in sample buffer (38) and heat-denatured at 95ºC for 5 min prior to separation on 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF overnight at 4°C. Filters were blocked for 1 h at room temperature in PBS pH 7.5, 0.1% Tween-20 (PBS-T), 3% BSA. After blocking, biotinylated Cry3Aa protoxin was detected by streptavidin conjugated to HRP and enhanced chemiluminescence (Western Pico, Pierce).

Knockdown of TmCad1 using RNAi - Initial template DNA was obtained by amplifying gDNA with primers Tm18 and Tm19 (Table 1). After sequence confirmation, a template for *in vitro* transcription was generated from the PCR product using gene-specific primers tailed with the T7 polymerase promoter sequence (Tm20 and Tm21). dsRNA was prepared with the Ambion MEGAscript High Yield Transcription Kit (Applied Biosystems/Ambion, Austin, TX) according to manufacturer protocols. Purified dsRNA was stored at -80°C until injected into *T. molitor* larvae.

Cohorts of *T. molitor* larvae (approximately 1 mo old) were injected with approximately 0.5 μl of filter-sterilized injection buffer (0.1 mM sodium phosphate, pH 7.2, 5 mM potassium chloride, 0.2% (v/v) green food color, n=50) or injection buffer mixed 1:1 with dsRNA (approximately 470 ng/larva; n=38). Fifty additional larvae were used as non-injected control group. Larvae surviving the injection process were reared on diet without toxin under standard rearing conditions until initiation of bioassays.

Quantitative Real Time PCR (qRT-PCR) - Four days post injection, larvae were evaluated for knockdown of *TmCad1*. Two larval guts from each of the control or injection groups were dissected into RNAlater, and total RNA was isolated (RNeasy Mini Kit; Invitrogen, Carlsbad, CA). qRT-PCR was performed on each template using forward (Tm18) and reverse (Tm19) primers (Brilliant II SYBR Green QRT-PCR Master Mix Kit, Mx3000P thermocycler, Stratagene/Agilent, Santa Clara, CA). Relative fold calculations were made with duplicates for each treatment group within the MX3000P software using ribosomal protein S6 (*RPS6*) to normalize gene expression (RPS6f forward primer and RPS6r reverse primer). Data were analyzed by one-way anova with Holm-Sidak pairwise multiple comparisons for statistically significant differences ($p<0.05$).

Bioassay of Control and Injected T. molitor *Larvae* - Nine days post-injection, larvae from the control (either uninjected or buffer-injected) and dsRNA-injected treatments were randomly divided into two groups for each treatment. One half of each treatment group was placed on untreated control diet (rearing diet without rolled oats), and the other half was placed on diet containing 14% (w/v) Cry3Aa protoxin (mixed with a ceramic mortar/pestle). Individual larvae were placed into separate wells of a 96-well microtiter plate with 25 mg of either control or Cry3Aa-treated diet. Plates were sealed with gas-permeable membranes (Breathe-Easier, Boston, MA) and placed in a controlled chamber at 60% R.H., 26°C, total darkness. Larval mortality was evaluated after 10 d.

RESULTS

Identification and cloning of T. molitor *cadherin* (TmCad1). Because Cry1A toxins mediate toxicity in lepidopterans through interaction with a midgut cadherin, we designed a strategy to isolate a midgut cadherin from a Bt-sensitive coleopteran. BLAST analysis (39) of a *T. molitor* larval midgut EST database identified a clone with sequence identity to the *M. sexta* cadherin Bt receptor (21). Gene-specific primers were designed based on the EST sequence, and 5'- and 3'-RACE products were generated. A cDNA containing the entire *T. molitor* cadherin

(*TmCad1*) coding sequence was obtained by RT-PCR and confirmed that our results from RACE were consistent with a single, continuous cadherin cDNA. *TmCad1* cDNA consisted of 5,095 bp, with an open reading frame of 4,881 bp encoding a 1,626 amino-acid protein (Fig.1). Both the partial EST and the full-length *TmCad1* cDNA were obtained from *T. molitor* midgut RNA, the target tissue for Cry intoxication. The predicted protein sequence, TmCad1, has a pI of 4.13 and molecular mass of 179,341 Da. Similar to lepidopteran cadherin Bt-receptor proteins, extracellular, transmembrane, and intracellular domains were found in TmCad1 (TMHMM Server v 2.0) and 12 extracellular (EC) repeat domains (Motif Scan of PROSITE database, http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Fig. 2A).

Comparison of the putative toxin-binding region of TmCad1 to the *Tribolium castaneum* genome annotations revealed one ortholog, Tc00222, renamed TcCad1 (XP_971388). Sequence alignments of putative Cry-receptor regions in cadherins from lepidopterans *M. sexta* (MsBtR1), *H. virescens* (HvCad), *P. gossypiella* (PgBtR) and *H. armigera* (HaBtR) with the coleopteran cadherins, TmCad1 and TcCad1, indicated a high degree of similarity in the predicted critical toxin binding region (Fig. 2A). Notably, three lysines (residues 1,359, 1,364, and 1,368) were exclusive to the *T. castaneum* sequence and were not found in MsBtR1, HvCad, PgBtR, HaBtR, or TmCad1.

In a comparison of the full-length lepidopteran and coleopteran cadherin sequences, identity was higher within orders (Table 2). These groups also were retained in phylogenetic analyses (Fig. 2B), suggesting that TmCad1 is an ortholog of TcCad1. Moreover, of all the cadherin-like proteins encoded in the *T. castaneum* genome, TcCad1 shares greater identity with the lepidopteran Bt cadherin receptors. TmCad1 shares >50% identity with TcCad1 and 23-25% identity with lepidopteran cadherin-like proteins. Interestingly, the putative *D. virgifera virgifera* cadherin (DvvCad), purported to be a Bt toxin receptor (28,29) is only 25 and 27% identical to TmCad1 and TcCad1, respectively, similar to values with lepidopteran cadherins (21–24%). Key residues in this putative toxin binding site located in the CR domain immediately adjacent to the membrane proximal region (MPR) were not conserved in DvvCad,(Fig. 2A). Our alignment agrees with Park et al. (29) in that a Cry3 toxin binding domain is not conserved in this CR domain of DvvCad, as it is in *T. molitor* and other lepidopteran cadherins. The lepidopteran cadherins were highly conserved and shared 54-98% identity among members within this order.

Specific binding of Cry3Aa to the predicted toxin-binding region in TmCad1 induces toxin oligomerization. To test the binding of Cry3Aa to TmCad1, we cloned and expressed in *E. coli* the TmCad1 region homologous to the reported Cry1A receptor region in lepidopteran cadherins (10,11). Binding of the purified peptide (rTmCad1p) to Cry3Aa was tested under native (dot blot) and denaturing (in-gel assay) methods. In dot blot assays, rTmCad1p bound Cry3Aa but not BSA, indicating specificity for binding to this peptide (Fig. 3A). Toxin binding was detected with a minimum amount of 0.5 µg of peptide. In an in-gel binding assay, IR-labeled rTmCad1p bound to unlabeled peptide and Cry3Aa protoxin but not to BSA (Fig. 3B right panel), and binding was completely inhibited by the addition of 500-fold molar excess rTmCad1p peptide to the labeled toxin, indicating specificity in the peptide-toxin interaction. These results are evidence that the peptide interacts specifically with itself and with Cry3Aa.

In the Lepidoptera model, interactions between Cry1A toxin and cadherin proteins result in toxin oligomerization (1). There is mounting evidence that toxin oligomers are crucial for toxicity (18,40). Similarly, because the *D. virgifera virgifera* CR8-10 cadherin peptide enhanced the activity of Cry3 toxins in several beetles, it was suggested that cadherins may promote the formation of pre-pore toxin oligomers, which thereby enhances toxicity (29). To evaluate whether TmCad1 can promote Cry3Aa oligomerization, we incubated biotinylated Cry3Aa protoxin with rTmCad1p and *T. molitor* BBMV proteins and detected the formation of toxin oligomers (Fig. 3C). Biotinylated Cry3Aa protoxin bound to *T. molitor* BBMV (Fig. 3C, lane 2), and the binding was specific as determined by competition with unlabeled protoxin (Fig. 3C, lane 3). Incubation of biotinylated Cry3Aa protoxin with *T. molitor* BBMV proteins and rTmCad1p resulted in the

formation of increased molecular mass Cry3Aa oligomers (Fig. 3C, lane 4). The molecular mass of the oligomers corresponds to that of dimers (~130 kDa), trimers (~195 kDa), and tetramers (~260 kDa) of the 65 kDa protoxin. Oligomers were also detected when Cry3Aa protoxin was incubated with BBMV proteins, although a much longer exposure of blots to film was necessary for detection (Fig. 3C, lane 5), denoting lower levels of oligomerization than in the presence of rTmCad1 peptide. These results support the role of TmCad1 in promoting Cry3Aa toxin oligomerization.

RNAi knockdown of TmCad1 *results in decreased susceptibility to Cry3Aa toxin*. To test the role of TmCad1 as functional Cry3Aa receptor *in vivo*, *T. molitor* larvae were injected with either buffer or *TmCad1*-specific dsRNA. The amount of *TmCad1* transcript in each treatment group was analyzed and compared to that of the noninjected control larvae using qRT-PCR (Fig. 4A). Four days post injection, the amount of *TmCad1* transcript was significantly reduced in larvae injected with *TmCad1* dsRNA (~80% less transcript than the basal level found in noninjected larvae). This knockdown was stable in larvae two weeks post injection (data not shown).

Injected larvae from both treatment groups were evaluated nine days post injection for their ability to survive Cry3Aa toxin-treated diets (Fig. 4B). After 10 days, there was no mortality in the dsRNA-injected groups fed either control or Cry3Aa-treated diet. This sharply contrasted with the mortality observed in noninjected/control larvae on Bt-treated diet. About 27 and 33% of noninjected and buffer-injected larvae, respectively, died on Bt-treated diet. In comparison, only 4 and 12% mortalities were observed in the same treatments when larvae were fed control diets. Therefore, lower levels of *TmCad1* expression in larvae directly correlated with survival on Cry3Aa-treated diet, demonstrating the functional role of this cadherin as putative Cry3Aa receptor.

DISCUSSION

Considering that coleopterans are some of the most damaging pests to field crops, forests, and stored products, research to develop effective biopesticides against beetle pest species is highly relevant and urgent. Most coleopteran-specific Cry toxins have limited host range and efficacy that prevent their widespread use in agriculture. Recently, transgenic corn expressing the coleopteran Cry toxin Cry3Bb, has been developed to control coleopteran rootworms (*Diabrotica* spp.). Although treated refugia are part of the mandated resistance management for Cry3Bb corn, the threat of resistance to this transgenic technology is significant (41). Recent reports (42,43) of field-evolved resistance to Bt crops in lepidopteran pests highlights the need to counter this threat, particularly where toxins are not completely effective at controlling target insects. Understanding the mode of action of coleopteran-specific Bt toxins will aid in the development of novel Bt biopesticides with increased efficacy, as well as in the development of resistance detection and management strategies.

Cadherins are Cry toxin receptors in lepidopteran larvae (3,5-8), and also have been reported as putative receptors for mosquitocidal Cry toxins (44). In this report, we present evidence to support the hypothesis that a cadherin protein is a functional Cry toxin receptor in coleopteran larvae, the first demonstration of Cry3Aa receptor functionality for a coleopteran cadherin. In a previous report of a cadherin-like gene from *D. virgifera virgifera* (*DvvCad*), biochemical evidence was not provided to support the hypothesis that DvvCad acts as a functional receptor for Cry toxins (28). Another study demonstrated that a partial DvvCad fragment bound Cry3Aa and Cry3Bb and slightly enhanced Cry3 toxicity when together were fed to insect larvae (29). Interestingly, this region differs from the Cry3Aa toxin binding region reported here for TmCad1. The Cry3Aa toxin binding region in TmCad1 corresponds to the reported membrane proximal region in DvvCad (28,29). The DvvCad potentiating fragment (CR8-10) corresponds to CR9-11 in TmCad1, suggesting that beetle cadherins may possess different or multiple Cry3 toxin binding domains. Alternatively, there may be another cadherin homolog in *D. virgifera virgifera*. Our data indicates that there is little sequence conservation in DvvCad corresponding to toxin binding region 3 for *M. sexta* (10) and TmCad1. The MPR region was not tested for

DvvCad, and CR9-11 was not tested for TmCad1. It is possible that these unique sites may be important for differences in susceptibility to different Cry3 toxins.

Another report indicated that a membrane ADAM metalloprotease from *L. decemlineata* is a receptor for Cry3Aa toxin (27). That study reported the presence of an ADAM-recognition motif in domain II of Cry3Aa, and a peptide containing this sequence motif prevented Cry3Aa toxin pore formation in BBMVs from *L. decemlineata*. In another study (45), inhibition of *L. decemlineata* ADAM metalloproteinase activity resulted in increased pore formation. However, the significance of Cry3Aa-ADAM interaction in toxin oligomerization or receptor interaction has not been delineated.

Lepidopteran cadherins function as receptors for Cry1A toxins by promoting toxin oligomerization (46) and/or by activation of intracellular death pathways (22). The TmCad1 peptide containing the toxin binding region, amino acid residues 1,322-1,516, was demonstrated to bind both rTmCad1p and Cry3Aa protoxin. In addition, TmCad1p promoted the formation of Cry3Aa toxin oligomers, similar to those found when the toxin was incubated with BBMV. These toxin oligomers may represent important pre-pore oligomeric structures, similar to those demonstrated to insert efficiently into membranes in Lepidoptera and promote pore formation (46) and toxicity (18). Cry3Aa oligomeric structures also have been reported after incubation of Cry3Aa protoxin with *L. decemlineata* BBMV (30).

As expected from the predicted functional Cry3Aa receptor role of TmCad1, reduction of TmCad1 expression in *T. molitor* by RNAi resulted in increased survival on Cry3Aa-toxin treated diet. Interestingly, reduced expression of TmCad1 did not result in observable fitness costs that adversely affected larval health. Furthermore, larvae injected with dsRNA suffered no mortality on control diets. Fitness costs also were not observed in some laboratory-selected Cry-resistant lepidopteran strains with cadherin mutations (47). While the specific function of Cry toxin-binding cadherins in the insect gut is unknown, it is possible that its primary function may occur in a developmental stage other than that used for knockdown. Alternatively, analogous proteins may rescue the specific function carried out by Cry-binding cadherins in these insects, as has been suggested in cadherin-mutant insects that do not express the toxin binding cadherin and are toxin insensitive.

Our study reports binding of Cry3Aa to a specific cadherin receptor protein from *T. molitor* and is the first demonstration that this protein functions as a receptor for the toxin within Coleoptera. The finding that lepidopteran and coleopteran insects share significant similarity in Cry toxin receptor regions further supports the hypothesis that cadherins are a common target in insects for Cry toxins (Fig. 5). Further research is necessary to establish the existence of additional conserved steps in the mode of action of Cry toxins in Coleoptera and Lepidoptera. A thorough comparative analysis of potential toxin binding regions within beetle cadherins also is needed. To date, low efficacy and the lack of knowledge regarding the mode of action of Cry toxins in coleopterans have limited the commercialization of coleopteran-specific Cry toxins. Characterization of the molecules directly involved in the mode of action of Cry toxins in Coleoptera will provide the tools necessary to increase the efficacy of Cry-based biopesticides against economically important beetles.

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FOOTNOTES

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[1]The abbreviations used are: Alkaline phosphatase, ALP; aminopeptidase, APN; *Bacillus thuringiensis*, Bt; brush border membrane vesicles, BBMV; cadherin repeat, CR; ectodomain, EC; expressed sequence tag, EST; green fluorescent protein, GFP; horseradish peroxidase, HRP; infrared, IR; membrane proximal region, MPR; open reading frame, ORF; phosphate buffered saline, PBS; polyvinylidene difluoride, PVDF; quantitative real time polymerase chain reaction, qRT-PCR; recombinant *T. molitor* cadherin-1 peptide, rTmCad1p; RNA interference, RNAi; sodium dodecylsulfate polyacrylamide gel electrophoresis, SDS-PAGE; *Tribolium castaneum* cadherin, TcCad

FIGURE LEGENDS

Figure 1. cDNA sequence and deduced ORF of TmCad1 with position of rTmCad1p highlighted in gray (residues 1,322-1516). The various domains of TmCad1 are underlined, including the membrane signal peptide with double underline, the twelve cadherin-repeat regions with solid arrows, the transmembrane domain with dashed arrows, and the cytoplasmic domain with dotted arrows.

Figure 2. A. Schematic showing conserved domain structure of insect cadherin Cry receptors and alignment of critical toxin binding region. Cadherins include those from *M. sexta* [MsBtR1, AAG37912, (36)], *H. virescens* [HvCad, AAK85198, (13)], *P. gossypiella* [PgBtR, AAP30715, (16)], *H. armigera* [HaBtR, ABF69362, (43)], *T. molitor* (TmCad1), and the putative *T. castaneum* ortholog (TcCad1; XP_971388). The extracellular (EC), transmembrane (T), and intracellular (IC) domains are illustrated. A Cry toxin-binding region (TBR) is found in the cadherin repeat region (numbered) closest to the membrane-proximal-region (MPR). The critical toxin-binding sequence of lepidopteran cadherins is aligned with the corresponding region from TmCad1, TcCad1, and the *D. virgifera virgifera* cadherin (DvvCad, AAV88529). The Cry1A critical toxin binding regions in *M. sexta* (42) and *H. virescens* (11) cadherins are highlighted in yellow; lysine residues in the corresponding region of TcCad1 that are potentially disruptive to toxin binding are highlighted in red. B. Phylogenetic tree analysis of insect cadherins using PHYLIP v3.6a3. Protein sequences used in analysis are from *M. sexta* (MsBtR1), *B. mori* (BmCad, BAA99404), *H. virescens* (HvCad), *H. armigera* (HaBtR), *P. gossypiella* (PgBtR), *L. dispar* (LdCad, AAL26896), *O. furnacalis* (OfCad, ABL10442), *O. nubilalis* (OnCadA1, AAT37678), *P. xylostella* (PxCad, ABU41413), *C. suppressalis* (CsCad, AAM78590), *T. castaneum* (TcCad1), *T. molitor* (TmCad1), *D. virgifera virgifera* (DvvCad) and the additional *T. castaneum* cadherins; (Tc01129; XP_971786), Starry night (TcStan; XP_968232), Fat protein (TcFat; XP_971084), and *D. melanogaster* Fat protein (DmFat, NP_477497).

Figure 3. Purified rTmCad1p binds Cry3Aa and promotes oligomerization. A) Dot blot overlay binding assay of 0.1-10 μg of rTmCad1p incubated with Cry3Aa toxin and detected with Cry3Aa antisera. B) Coomassie stained protein gel scanned at 700 nm (left) and in-gel toxin binding with IR800TmCad1p and competition assay with unlabeled rTmCad1p scanned at 800 nm (right):

lane 1, rTmCad1p; lane 2, Cry3Aa; lane 3, BSA. C) Detection of Cry3Aa oligomerization using Western blotting. BBMV proteins (7.8 μg, lane 1) were incubated with 1.2 μg of biotinylated Cry3Aa protoxin alone (lane 2), in the presence of a 24-fold excess of unlabeled Cry3Aa protoxin (lane 3), or with 1.4 μg of rTmCad1p (lane 4). After incubation, unbound Cry3Aa toxin was washed by centrifugation and final pellets used for electrophoresis and transferring to PVDF filters. Bound Cry3Aa toxin was detected with streptavidin-conjugated to HRP and enhanced chemiluminescence (Western Pico, Pierce). Lane 5 represents the sample in lane 2 after a twenty-fold longer exposure to film than the other lanes. Arrows indicate the position of putative Cry3Aa dimers, trimers and tetramers.

Figure 4. A. Reduction in *TmCad1* expression in larvae. RNA was obtained from *T. molitor* larvae, including controls either noninjected or buffer-injected, or larvae injected with dsRNA, and the relative amount of *TmCad1* transcript in each treatment group was compared, normalized to the expression of RPS6. Data with the same letter are not statistically different ($p<0.05$). B. Bioassay of larvae from treatment groups (either controls or dsRNA-injected) with control or Cry3Aa-treated diets, as indicated on the left. The number of larvae in each bioassay group is indicated to the right of each bar (* two larvae from the buffer-injected control group on control diet escaped at day 8 and were removed from the final calculation).

Figure 5. Similarities between the Cry toxin mode of action in Lepidoptera and Coleoptera. Cadherins are key receptors for Cry toxins in both orders. In Lepidoptera, Cry1A toxins are protease activated (a) and bind to cadherin receptors on the apical surface of the midgut membrane (b). Cell-death results from either the activation of an intracellular signaling pathway or through the formation of toxin oligomers (c) that bind to N-aminopeptidase (APN) and/or alkaline phosphatase (ALP) (d) to insert in the membrane and form pores that result in cytotoxicity due to osmotic imbalance (e). In Coleoptera, Cry3 protoxin interacts with a gut-specific ADAM metalloproteinase. Toxin or protoxin binding to cadherin results in toxin oligomerization and eventually cell death. Further research is necessary to characterize post-binding steps in the coleopteran model.

Tables

Table 1. Nucleotide primers used to obtain the full-length *TmCad1* cDNA by 5'- and 3'-RACE, internal sequence in *TmCad1* cDNA fragments in cloning vectors, generate the partial rTmCad1p toxin-binding region fragment for protein expression, dsRNA synthesis, and qRT-PCR amplification of *TmCad1* and *RPS6*.

Primer	Orientation	Position	Primer DNA Sequence
Tm1	Sense	4538-4563	5'-TGAAAGCGTGGTTGATCGGTGTTTCG-3'
Tm2	Sense	4648-4676	5'-TCCAGTACCAAATTCGGGTCGCAAGAG-3'
Tm3	Antisense	4152-4179	5'-GGCATCAGCTTTGTGATTTTCCGGCTCT-3'
Tm4	Antisense	4018-4042	5'-TGTCCAGGTCGAGGTTAGATGGAGT-3'
Tm5	Antisense	4055-4079	5'-TCTCCGGATTGCGTATTCATGGTAA-3'
Tm6	Antisense	3864-3893	5'-TCAAACACTGGAGATTCGTCGTTCTGGTCT-3'
Tm7	Antisense	3788-3811	5'-GCTTGTCAGCGTTAGATGACTGAA-3'
Tm8	Antisense	3734-3753	5'-GAGCGGTTGTTTAAGGGTGA-3'
Tm9	Antisense	2906-2929	5'-TGTCACCTTCATCGTCATCTTTCC-3'
Tm10	Antisense	1388-1412	5'-TCATCGTTGCATATCATTTAGGTTGA-3'
Tm11	Sense	1830-1853	5'-CGACGCAGATTTGGAGTTCTCGAT-3'
Tm12	Antisense	2267-2290	5'-CAACCCAGTCGGGAGTGTTCTCAT-3'
Tm13	Sense	377-404	5'-TCAAGAACTTGGACGACGAACATCCGAC-3'
Tm14	Antisense	883-909	5'-GGCATCCACCGTAGCGAAGTTGTTCTC-3'
Tm15	Antisense	1023-1044	5'-AATGTCTTCAAGGATCAGCAGT-3'
Tm16	Sense	Adapter	5'-CACCGAGCACGAGGACACTGACAT-3'
Tm17	Antisense	4526-4548	5'-CTACCACGCTTTCAAAATTGCTTCCA-3'
Tm18	Sense	1982-2001	5'- AGGAAACACAACCTGGCAAC-3'
Tm19	Antisense	2454-2473	5'- CCATGACAGGAACATTGTCG-3'
Tm20	Sense	1982-2001	5'- taatacgactcactatagggAGGAAACACAACCTGGCAAC-3'
Tm21	Antisense	2215-2234	5'- taatacgactcactatagggTCCATGTCAGCGTCAGTAGC-3'
RPS6f	Sense	401-420	5'- GGCCCAAGCGAGCATCTAAC-3'
RPS6r	Antisense	567-584	5'- GAGCGCCAACCTGTGACG-3'

Table 2. Percent identity scores from full-length sequence alignments of cadherins from *M. sexta* (MsBtR1, AAG37912), *B. mori* (BmCad, BAA99404), *L. dispar* (LdCad, AAL26896), *H. armigera* (HaBtR, ABF69362), *H. virescens* (HvCad, AAK85198), *O. nubilalis* (OnCadA1, AAT37678), *P. xylostella* (PxCad, ABU41413), *O. funarcalis* (OfCad, ABL10442), *P. gossypiella* (PgBtR, AAP30715), *C. suppressalis* (CsCad, AAM78590), *T. castaneum* (TcCad1, XP_971388), *T. molitor* (TmCad1; DQ988044), and *D. virgifera virgifera* (DvvCad, AAV88529). Scores in bold were calculated from single pair-wise alignments using AlignX from Vector NTI Advance 9.1.0. Italicized scores were calculated from the multiple alignment of protein sequences using ClustalW version 1.83 from EMBL-EBI (http://www.ebi.ac.uk/clustalw/). Gray-highlighted cells indicate coleopteran sequences.

	MsBtR1	BmCad	LdCad	HaBtR	HvCad	OnCadA1	PxCad	OfCad	PgBtR	CsCad	TcCad1	TmCad1	DvvCad
MsBtR1	100	*70*	*64*	*61*	*63*	*60*	*57*	*60*	*58*	*56*	*23*	*23*	*22*
BmCad	**70**	100	*67*	*63*	*64*	*63*	*57*	*63*	*60*	*57*	*24*	*24*	*21*
LdCad	**64**	**67**	100	*63*	*63*	*62*	*56*	*62*	*59*	*55*	*24*	*25*	*24*
HaBtR	**61**	**63**	**64**	100	*84*	*59*	*55*	*59*	*55*	*55*	*24*	*25*	*23*
HvCad	**62**	**64**	**63**	**85**	100	*60*	*56*	*59*	*56*	*55*	*23*	*24*	*22*
OnCadA1	**59**	**62**	**61**	**58**	**59**	100	*57*	*98*	*57*	*58*	*24*	*24*	*23*
PxCad	**57**	**57**	**56**	**54**	**55**	**57**	100	*57*	*54*	*51*	*24*	*23*	*26*
OfCad	**60**	**63**	**61**	**59**	**59**	**98**	**57**	100	*57*	*58*	*24*	*25*	*23*
PgBtR	**58**	**60**	**58**	**55**	**55**	**57**	**54**	**57**	100	*54*	*24*	*23*	*22*
CsCad	**55**	**57**	**55**	**55**	**55**	**57**	**51**	**57**	**54**	100	*24*	*24*	*22*
TcCad1	**22**	**24**	**23**	**22**	**22**	**23**	**22**	**22**	**22**	**23**	100	*52*	*28*
TmCad1	**21**	**22**	**23**	**23**	**23**	**23**	**22**	**23**	**23**	**22**	**51**	100	*26*
DvvCad	**21**	**22**	**24**	**22**	**23**	**22**	**22**	**23**	**22**	**22**	**27**	**25**	100