Differential Protection of Cry1Fa Toxin against *Spodoptera frugiperda* Larval Gut Proteases by Cadherin Orthologs Correlates with Increased Synergism

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The Cry proteins produced by *Bacillus thuringiensis* (Bt) are the most widely used biopesticides effective against a range of crop pests and disease vectors. Like chemical pesticides, development of resistance is the primary threat to the long-term efficacy of Bt toxins. Recently discovered cadherin-based Bt Cry synergists showed the potential to augment resistance management by improving efficacy of Cry toxins. However, the mode of action of Bt Cry synergists is thus far unclear. Here we elucidate the mechanism of cadherin-based Cry toxin synergism utilizing two cadherin peptides, *Spodoptera frugiperda* Cad (SfCad) and *Manduca sexta* Cad (MsCad), which differentially enhance Cry1Fa toxicity to *Spodoptera frugiperda* neonates. We show that differential SfCad- and MsCad-mediated protection of Cry1Fa toxin in the *Spodoptera frugiperda* midgut correlates with differential Cry1Fa toxicity enhancement. Both peptides exhibited high affinity for Cry1Fa toxin and an increased rate of Cry1Fa-induced pore formation in *S. frugiperda*. However, only SfCad bound the *S. frugiperda* brush border membrane vesicle and more effectively prolonged the stability of Cry1Fa toxin in the gut, explaining higher Cry1Fa enhancement by this peptide. This study shows that cadherin fragments may enhance *B. thuringiensis* toxicity by at least two different mechanisms or a combination thereof: (i) protection of Cry toxin from protease degradation in the insect midgut and (ii) enhancement of pore-forming ability of Cry toxin.

Bacillus thuringiensis (Bt) Cry toxins are a family of bacterial pore-forming proteins that are highly toxic to a range of crop pests and disease vectors. Cry proteins are produced as crystals during the sporulation phase. Crystals are ingested, solubilized in the gut lumen to protoxin, and activated by host gut proteases. Activated toxin crosses the peritrophic matrix (PM) and binds cadherin, which is the primary high-affinity receptor for Cry1 toxins on the apical border of midgut microvilli. A current model postulates that toxin interaction with cadherin causes a conformational change in toxin allowing a specific proteolytic cleavage and formation of a prepore toxin oligomer. Evidence suggests that the prepore oligomer has increased affinity for secondary glycosylphosphatidylinositol (GPI)-anchored receptors, such as aminopeptidases (APNs) or alkaline phosphatases (ALPs) localized in lipid rafts. Oligomers insert into the membrane and disrupt membrane integrity by forming lytic pores, which lead directly to insect mortality or indirectly to mortality due to septicemia (5, 7, 21, 45). A recent modification to the pore formation model described above proposes that activated toxin monomers first bind to abundant low-affinity APN receptors before binding to high-affinity cadherin receptors, which results in toxin oligomerization (32). In contrast to the pore formation model, the cell-signaling model (54) proposes that binding of activated toxin monomers to cadherin activates an intracellular signaling pathway, which ultimately results in cell death. However, toxin activation by gut proteases and toxin-receptor interactions are the most essential steps of the *Bacillus thuringiensis* mode of action and are common to all models, and alterations in these steps have been linked with resistance development (14, 30, 31, 51, 52).

A strategy successfully employed to delay development of resistance against chemical pesticides is the use of synergists to enhance toxicity (3). Several synergists of *Bacillus thuringiensis* Cry that show low to moderate synergism of Cry toxicity have been reported. Bt Cry synergists of non-*B. thuringiensis* origin include

zwitermicin (6), endochitinase (42), and avidin (55). Cyt toxin of *B. thuringiensis* subsp. *israelensis*, which shows high-level synergism of mosquitocidal Cry toxins (39, 44, 48), and Bel protein, which enhanced Cry1Ac toxin, are examples of synergists of *B. thuringiensis* origin (12). However, the discovery of *Manduca sexta* cadherin as a Bt Cry synergist is the first time that a receptor has been reported to enhance toxicity (10). The synergistic potential of the *M. sexta* cadherin fragment has encouraged further research in this area (10). Subsequently, the *M. sexta* cadherin peptide CR10-12 (called MsCad in this study) was found to have increased synergy relative to the original CR12 membrane proximal extracellular domain (MPED) peptide (1). Additionally, a synergistic effect has been demonstrated using cadherin fragments from dipterans (mosquito) with mosquitocidal Cry4Ba (19, 35) and from coleopterans (beetle) with beetle-toxic Cry3Aa and Cry3Bb (15, 34), and an additional example of cadherin synergy of lepidopteran-active Cry toxins has been presented (37). Due to the potential of cadherin as a Bt synergist, research is under way to enable commercial use of cadherin fragments either as an additive in Bt formulations or by expression in transgenic Bt plants.

Although several papers on the use of cadherin fragments as Bt synergists have been published (10, 15, 19, 33–37), the mode of action of insect cadherin-based synergists remains to be fully understood. Chen et al. (10) proposed that cadherin fragments in-

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crease the probability of toxin-receptor interaction by anchoring Bt toxins to the membrane. Formation of cadherin-induced toxin oligomers is another mechanism proposed to explain cadherin-based Bt synergism (33, 37). Nevertheless, Cry4Ba toxicity enhancement by an *Anopheles gambiae* cadherin fragment that inhibits toxin binding to brush border membrane vesicle (BBMV) (35) and a *Helicoverpa armigera* cadherin fragment that induces formation of prepore oligomers but reduces Cry1Ac toxicity (23) suggests the existence of alternative or additional synergistic mechanisms.

Cry1Fa, the subject Bt toxin of this paper, is used in genetically modified corn cultivars to control lepidopteran pests. Cry1Fa is highly toxic to *Ostrinia nubilalis*, European corn borer, and has significant but not high-level toxicity to *Spodoptera frugiperda*, fall armyworm (46). Unfortunately, in Puerto Rico, where *S. frugiperda* is a major corn pest, *S. frugiperda* larvae have acquired resistance to Cry1Fa corn and the product was withdrawn from the market (26, 47).

Our initial hypothesis was that the CR10-12 peptide of *M. sexta* cadherin (MsCad) (1) would significantly synergize Cry1Fa toxicity to *S. frugiperda*. When data did not support this hypothesis, we hypothesized that the corresponding region of the ortholog cadherin from *S. frugiperda* (called SfCad in this study) would synergize Cry1Fa toxicity to *S. frugiperda*. MsCad and SfCad were then used in a comparative approach to investigate mechanisms of cadherin-based Cry toxin synergism.

MATERIALS AND METHODS

Bacterial strains and toxin purification. The *B. thuringiensis* strain harboring the *cry1Fa* gene was obtained from Ecogen Inc. (Langhorne, PA). The conditions for *B. thuringiensis* cultures, toxin purification, and activation were as described previously by Luo et al. (25). All protein concentrations, unless otherwise specified, were measured by Bio-Rad protein assay using bovine serum albumin (BSA) as the standard (4).

BBMV preparation. Insects for all experiments were purchased from Benzon Research Inc. (Carlisle, PA), and larvae were fed multiple-species artificial diet (Southland Products, Lake Village, AK). Midguts were excised from fourth-instar *S. frugiperda* larvae, and BBMVs were prepared by the MgCl₂ precipitation method (49), with modifications according to Carroll and Ellar (9). The final BBMV pellet was suspended in SET buffer (250 mM sucrose, 5 mM EGTA, 20 mM Tris, pH 7.5) and frozen at -80°C until used.

Preparation of GE. To prepare gut extract (GE), midguts from 10 late-fourth-instar *S. frugiperda* larvae, reared on artificial diet (Southland Products Inc., Lake Village, AR), were dissected, pooled, vortexed, and centrifuged at $13,000 \times g$ for 15 min at 4°C to separate gut extract from the solid materials (31). Supernatant was collected, filtered through a 0.22- μm -pore-size filter, aliquoted, and stored at -80°C .

Cloning, expression, and purification of SfCad and MsCad. The sequence of *S. frugiperda* cadherin, an orthologue of *M. sexta* BtR1 cadherin, was published by Flannagan and Meyer (GenBank accession number [CAC41167](#) [13a]). The amino acid sequence and putative domains of *S. frugiperda* cadherin are shown in Fig. S1 in the supplemental material. The cadherin *M. sexta* BtR1 CR10-12 (MsCad, 35,148 Da) was previously identified to be a Cry synergist (1). A region most similar to MsCad was identified on the *S. frugiperda* cadherin by protein alignment using the ClustalW program (version 1.83; EMBL-EBI). The corresponding region of *S. frugiperda* cadherin (SfCad; amino acids [aa] 1168 to 1479) consists of 311 amino acids that have 51.8% identity with the aligned MsCad (see Fig. S2 in the supplemental material). For expression in *Escherichia coli*, we optimized the SfCad coding region based on *E. coli* codon bias (8) and modified the peptide by adding MK at the N terminus and six histidines at the C terminus, resulting in a 35,068-Da peptide. Both SfCad- and

MsCad-encoding DNAs were synthesized (Genscript, Piscataway, NJ) and then subcloned into an expression vector, pET30a(+) (Novagen, Madison, WI), for high expression in *E. coli*. The expression, solubilization, purification, and quantification methods are described elsewhere (10).

Insect bioassays. The toxicity of Cry1Fa toxin and the synergistic effect of cadherin fragments were evaluated using diet surface bioassays with *S. frugiperda* neonates as described previously by Abdullah et al. (1). Insects were purchased from Benzon Research Inc. (Carlisle, PA). The maximum doses of SfCad and MsCad applied in combination with toxin were tested alone in deionized water as negative controls. Toxin alone and buffer controls were also included with each larval bioassay. Bioassays were scored 7 days after treatment (DAT). Different batches of larvae were used for dose-response and the single-point toxicity enhancement bioassays. Bioassays were repeated three times, and the data were pooled. Probit regression analysis (13) using the EPA probit analysis program (version 1.5) was used to calculate 50% lethal concentrations (LC_{50} s). The enhancement in toxicity is expressed as fold decrease in the LC_{50} of a Cry protein when the protein was used in combination with the cadherin fragments.

Labeling of SfCad and MsCad. SfCad and MsCad were biotinylated using a 50-fold molar excess of sulfo-NHS-LC-biotin according to the manufacturer's (Pierce, Rockford, IL) instructions. The final reaction mixture was dialyzed against 20 mM Na₂CO₃, 150 mM NaCl, pH 9.6, and stored in aliquots at -20°C until needed for binding assays.

For the dot blot experiments with Cry1Fa toxin, SfCad (6 μg) and MsCad (12 μg) were radiolabeled with 0.5 mCi of Na¹²⁵I using the chloramine-T method (16) and stored at 4°C . Specific activities of the labeled peptides were 9.0 $\mu\text{Ci}/\mu\text{g}$ and 7.7 $\mu\text{Ci}/\mu\text{g}$ for SfCad and MsCad, respectively. For the dot blot experiments with BBMVs, SfCad (5 μg) and MsCad (6 μg) were radiolabeled as described above, and the specific activities of the radioligands were 27 $\mu\text{Ci}/\mu\text{g}$ and 7 $\mu\text{Ci}/\mu\text{g}$, respectively.

Labeling Cry1Fa toxin with Alexa Fluor 488. Trypsin-activated Cry1Fa toxin was labeled with Alexa Fluor 488 (Alexa-Cry1Fa toxin) according to the manufacturer's (Invitrogen, Molecular Probes, Inc., Eugene, OR) instructions. The final reaction mixture was dialyzed against 20 mM Na₂CO₃, 200 mM NaCl, pH 9.6, at 4°C , quantified according to the manufacturer's instructions, and stored in aliquots at -20°C until needed for binding assays.

Toxin-synergist binding assays. The binding affinities of the cadherin fragments and Cry1Fa toxin were calculated using a microplate-based protein-protein interaction binding assay described previously (34). For both saturation and competition binding assays, microtiter plates were coated with 0.5 μg toxin/well. Plates were incubated with increasing concentrations of biotinylated SfCad or MsCad (0.01 nM to 18 nM) alone or in the presence of a 1,000-fold molar excess of unlabeled SfCad or MsCad to determine total and nonspecific binding. For the competition binding assays, plates were probed with 2 nM biotinylated SfCad in the presence of increasing molar concentrations of nonlabeled SfCad or MsCad to determine homologous and heterologous binding, respectively. Bound biotinylated SfCad or MsCad was detected with horseradish peroxidase (HRP)-conjugated streptavidin and HRP chromogenic substrate as described previously (34). Binding assays were replicated three times. Data were analyzed using SigmaPlot software (version 9; Systat Software, Inc., San Jose, CA), and the curves were fitted on the basis of a best fit of the data to a one-site saturation binding equation or one-site competition equation with the maximum set at 100%.

Dot blot binding assays. For the dot blot experiments, toxin or BBMVs (5 μg) were dotted, in duplicate, onto a polyvinylidene difluoride (PVDF) filter (Millipore, Bedford, MA). For the toxin binding experiments, filters were probed with 0.5 nM ¹²⁵I-labeled SfCad or ¹²⁵I-labeled MsCad alone or in the presence of a 1,000-fold molar excess of nonlabeled SfCad or MsCad. For the BBMV binding experiments, filters were probed with 0.5 nM ¹²⁵I-labeled SfCad or ¹²⁵I-labeled MsCad alone or in the presence of a 1,000-fold molar excess of nonlabeled SfCad, MsCad, or

Cry1Fa. Bound ^{125}I -labeled peptides were detected by autoradiography at -80°C . Binding assays were replicated four times, and representative blots are shown.

AlexaCry1Fa toxin binding assays. To determine the effect of SfCad and MsCad in Cry1Fa toxin binding to *S. frugiperda* BBMV, 200 $\mu\text{g}/\text{ml}$ BBMV was incubated with 2.5 nM AlexaCry1Fa toxin with or without a 10-fold mass ratio of SfCad or MsCad in binding buffer (20 mM Na_2CO_3 , 0.1% Tween 20, 0.15 M NaCl, pH 9.6). The reaction mixtures were incubated at room temperature for 2 h. At the end of incubation, BBMV were pelleted, washed three times with wash buffer (20 mM Na_2CO_3 , 0.1% Tween 20, 0.15 M NaCl, 0.1% BSA, pH 9.6), and solubilized in sodium dodecyl sulfate (SDS) sample buffer (22) by heating the samples for 10 min at 100°C . Proteins were separated using a Criterion 4 to 20% Tris-HCl gradient gel (Bio-Rad, Hercules, CA), and the bound Alexa Fluor 488-conjugated toxins were detected by scanning the gels using a Typhoon imager with 488-nm excitation and 520-nm emission filters (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Assays were replicated three times, and representative data are shown.

Toxin oligomerization and protease K protection assays. The toxin oligomerization and protease K protection experiments were based on the methodology of Aronson et al. (2), with some modifications. To determine toxin oligomer formation, 60 ng of activated Cry1Fa toxin was incubated with or without a 10-fold mass of SfCad or MsCad in NaHCO_3 , 0.25 M NaCl, pH 9.5, at 37°C for 1 h. After 1 h, 20 μg of *S. frugiperda* BBMV was added, followed by further incubation at 30°C for 1 h. For protease K digestion, BBMV plus toxin (preincubated with or without SfCad or MsCad) were incubated as described above; then, protease K (Sigma) was added to a 0.17-mg/ml final concentration, and the suspensions were further incubated at 37°C for 30 min. Protease K-treated Cry1Fa toxin (60 ng) preincubated with or without the peptides and protease K-untreated BBMV plus toxin served as experimental controls. Ten millimolar 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSE; final concentration) was added to the samples that contained BBMV to stop serine protease activity (including protease K), and the mixture was incubated at 37°C for 12 min. Samples were mixed with Tris-SDS as described elsewhere (2) to yield a final SDS concentration of 0.1% and incubated at 65°C for 5 min. Samples were separated on a Criterion 4 to 15% Tris-HCl gel (Bio-Rad, Hercules, CA) and electroblotted onto a PVDF filter. Cry1Fa was detected using rabbit antisera (produced at the Animal Resources Facility, University of Georgia) against Cry1Fa toxin. Goat anti-rabbit IgG horseradish peroxidase, enhanced chemiluminescence substrate, and a FluorChem 8900 digital imaging system (AlphaInnotech, San Leandro, CA) were used to visualize Cry1Fa bands.

In vitro protease protection assays. The effect of SfCad or MsCad on Cry1Fa toxin stability in *S. frugiperda* GE was evaluated by incubating Cry1Fa toxin (100 ng) alone or Cry1Fa toxin that had been preincubated with 1 μg cadherin fragments for 1 h with 1 μl GE in a final volume of 20 μl carbonate buffer (0.1 M Na_2CO_3 , 0.25 M NaCl, pH 9.6) for 5, 10, or 20 min at 30°C . At the end of each time point, toxin proteolysis was terminated by heating the samples for 10 min at 100°C (31). Samples in SDS sample buffer were heated for 10 min at 100°C , separated by gel electrophoresis as described above, and electroblotted onto a PVDF membrane, and residual Cry1Fa toxin was detected using anti-Cry1Fa serum as described above. Experiments were replicated three times, and representative blots are shown.

In vivo protease protection assays. *In vivo* protease protection assays were performed on fourth-instar *S. frugiperda* larvae starved for 1 h prior to force feeding. The larvae were force fed 2 μl treatment solution containing 0.2 μg Cry1Fa toxin alone or Cry1Fa toxin that had been preincubated for 1 h with 1 μg SfCad or MsCad in 20 mM Na_2CO_3 , pH 10, using a Gilmont syringe (Cole-Parmer, Vernon Hills, IL) fitted with a 26-gauge blunt-end needle. The larvae were then placed on artificial diet until dissected. Midguts from 8 larvae per treatment per time point were dissected, and the midgut tissue was separated from gut content, including the PM. The tissue and the gut content were pooled separately in 80 μl SET buffer,

10 μl 100 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μl 50 $\times$ protease inhibitor cocktail (Complete mini EDTA-free tablet; Roche, Indianapolis, IN) and homogenized on ice using a 2-ml Potter-Elvehjem glass tissue grinder for 30 to 60 s.

A microplate-based bicinchoninic acid protein assay kit compatible with reducing agents (Pierce Biotechnology, Rockford, IL) was used to quantify the total protein content of the samples as per the manufacturer's instructions. On the basis of the total protein concentration, 600 μg protein was solubilized in SDS sample buffer (22) by boiling. Samples were centrifuged, the supernatant was separated using a Criterion 12.5% gel (Bio-Rad, Hercules, CA) electroblotted onto a PVDF membrane, and the residual Cry1Fa toxin was detected using antisera raised against Cry1Fa toxin as described above. The Cry1Fa bands were quantified by densitometry using a FluorChem 8900 imager (AlphaInnotech, San Leandro, CA). Imager software was used to subtract background density values from regions containing a toxin band. Experiments were replicated three times, and representative blots are shown.

Membrane permeability assays. Light-scattering assays were performed with a stop-flow spectrofluorimeter (model RSM 1000; On-Line Instrument Systems, Bogart, GA) essentially as described previously, with slight modifications (25). BBMV from *S. frugiperda* were prepared 1 day before the assay as described above, except that the final BBMV pellet was suspended in HEPES buffer (25 mM sucrose, 2.4 mM MgCl_2 , 9 mM HEPES/KOH, pH 7.5) and stored on ice. All treatment mixtures were prepared fresh in HEPES/KCl buffer (25 mM sucrose, 2.4 mM MgCl_2 , 0.27 M KCl, 9 mM HEPES/KOH, pH 7.5). Assays were initiated by simultaneously injecting 0.25 ml (75 μg) of BBMV and 0.25 ml of a treatment mixture into the cuvette in the spectrofluorimeter sample compartment. The treatment mixtures consisted of various concentrations of Cry1Fa alone or Cry1Fa mixed with either SfCad or MsCad in KCl buffer. The different concentrations of SfCad and MsCad used with Cry1Fa were tested alone as negative controls. Treatments were replicated five times per experiment, and data from two experiments were pooled to calculate the means and standard errors.

The shrinkage response signal, measured by determining the increase in the scattered light signal, was determined by comparing the light scattered from BBMV coinjected with buffer having the same osmotic strength (HEPES buffer) and the light scattered from BBMV coinjected with KCl buffer. The mean scattered light signal was converted to percentage of volume recovered using the formula $[(KCl_t - \text{Treat}_t)/(KCl_t - \text{NS}_t)] \times 100$, where KCl_t is the mean signal (at time t) obtained for BBMV mixed with the KCl buffer, Treat_t is the mean signal (at time t) obtained for BBMV mixed with various treatments, and NS_t is the mean signal (at time t) obtained for BBMV mixed with the HEPES buffer. SigmaPlot, version 9, was used to plot data and fit curves (Systat Software, Inc., San Jose, CA).

Statistical analysis. Mortality data from the bioassays were normalized using arcsine-square root (x) transformation and subjected to analysis of variance (ANOVA) with the significance level set at 0.05. When significant, means were separated using a Fisher protected least-significant-difference test (LSD) to compare the treatment means with the toxin-alone treatment. BBMV binding data were analyzed using ANOVA, and when significant, means were compared with the peptide-alone treatment using LSD. All calculations were performed using PROC GLM and PROC Univariate of the Statistical Analysis System (SAS 2002-2003, version 9.1; SAS Institute, Cary, NC). Data are presented in the original scale.

RESULTS

We tested the ability of MsCad (Fig. 1A) to enhance Cry1Fa toxicity to *S. frugiperda* using various concentrations of Cry1Fa applied to diet surface while maintaining a constant 1:5 Cry1Fa/MsCad mass ratio. The LC_{50} of Cry1Fa alone was 491 ng/cm^2 (95% fiducial limit, 400 to 637 ng/cm^2), while that of Cry1Fa-MsCad was 275 ng/cm^2 (95% fiducial limit, 229 to 331 ng/cm^2) (a 1.8-fold increase in Cry1Fa toxicity against *S. frugiperda* larvae). In

FIG 1 Comparison of Cry1Fa and Cry1Ab toxicity enhancement by SfCad and MsCad. (A) Schematic diagram of *M. sexta* and *S. frugiperda* cadherins and SfCad and MsCad. Cadherin domains: SP, signal peptide; CR, cadherin repeat; MPED, membrane proximal extracellular domain; TM, transmembrane domain; CYTO, cytoplasmic domain. (B and C) *S. frugiperda* neonates were treated with increasing concentrations of Cry1Fa toxin alone or in combination with a 5-fold mass excess of SfCad or MsCad (B) or 100 μ g/cm² Cry1Fa toxin alone or in combination with a 5- to 40-fold mass excess of either SfCad or MsCad (C). Peptide-alone treatments were included as controls. Experiments were replicated three times, and the means $\pm$ SEMs are reported. Vertical lines indicate SEMs. In panel C, letters above the histograms indicate significant differences between toxin control and toxin plus SfCad or MsCad treatments, as determined by ANOVA ($P = 0.05$) and Fisher protected LSD.

view of the possibility that a fragment of *S. frugiperda* cadherin may enhance Cry1Fa toxicity to *S. frugiperda* better than MsCad, we identified the most similar region in *S. frugiperda* cadherin. The MsCad region (311 aa) aligns to *S. frugiperda* cadherin residues 1168 to 1479 (311 aa) with 51.6% sequence similarity (see Fig. S2 in the supplemental material). The SfCad region was cloned into *E. coli* for protein production, and SfCad was purified from inclusion bodies (see Fig. S3 in the supplemental material). The synergistic effect of SfCad was quantified as described for MsCad. SfCad lowered the Cry1Fa LC₅₀ to 94 ng/cm² (95% fiducial limit, 83 to 105 ng/cm²), a 5.2-fold significant increase in toxicity compared to Cry1Fa toxin-alone treatment (Fig. 1B). The values in LC₅₀s are considered significantly different if the 95% fiducial limits do not overlap (43). These results showed that SfCad was a significantly better Cry1Fa toxicity enhancer than MsCad against *S. frugiperda*.

The extent of MsCad- and SfCad-mediated enhancement of Cry1Fa toxicity was further tested using 100 ng/cm² Cry1Fa toxin alone or with 1:5 to 1:40 mass ratios of MsCad or SfCad. As shown in Fig. 1C, the level of MsCad-mediated enhancement of Cry1Fa toxicity was not significantly different from that of the toxin-alone treatment. In contrast to the MsCad results, SfCad significantly enhanced Cry1Fa at a 1:5 Cry1Fa/SfCad ratio (increased mortality from 31% to 64%), and mortality was further increased (about 91% mortality) when the Cry1Fa toxin/cadherin ratio was increased from 1:5 to 1:10 (Fig. 1C). As a control, BSA did not enhance Cry1Fa toxicity (data not shown). The highest concentration of the cadherin peptides was not toxic to larvae.

SfCad and MsCad bind Cry1Fa saturably and with high affinity. Since cadherin fragment binding to toxin usually correlates with synergism of insecticidal activity (10, 33), we tested MsCad and SfCad for binding to Cry1Fa toxin. While both cadherin fragments specifically bound Cry1Fa toxin in binding enzyme-linked immunosorbent assays (ELISAs), SfCad had more nonspecific binding to Cry1Fa toxin (Fig. 2B). Binding affinities were calculated from ELISA data using a one-site saturation binding equation. MsCad and SfCad bound Cry1Fa with similar affinities: binding affinity (K_D) values were 5.8 ± 0.7 and 2.2 ± 0.5 nM, respectively (Fig. 2A and B). Homologous and heterologous competition of biotin-SfCad binding to Cry1Fa was tested by the addition of increasing amounts of unlabeled cadherin fragment to the binding reactions. As seen in Fig. 2C, MsCad and SfCad competed biotin-SfCad binding to Cry1Fa toxin, evidence that MsCad and SfCad share a population of sites on Cry1Fa toxin. However, the shared SfCad and MsCad population of binding sites appears to be low-affinity sites, as 50% competition was not attained until about 1 μ M unlabeled peptide was in the binding reaction. Whether or not SfCad and MsCad share a high-affinity binding site on Cry1Fa cannot be concluded from the data.

Binding to toxin was also tested by incubating ¹²⁵I-labeled MsCad or SfCad with PVDF filters dotted with Cry1Fa toxin, visualizing bound toxin by autoradiography, and quantifying bound toxin by counting radioactivity. Specificity of binding was assessed by adding 1,000-fold excess unlabeled peptide to the binding reaction. As seen in Fig. 2 (insets), ¹²⁵I-labeled SfCad and ¹²⁵I-labeled MsCad bound Cry1Fa toxin with some specificity, and according to the radioactivity counts, Cry1Fa bound more ¹²⁵I-labeled SfCad (14 ± 0.1 nmol) than ¹²⁵I-labeled MsCad (7 ± 1.0 nmol).

¹²⁵I-labeled SfCad but not ¹²⁵I-labeled MsCad specifically binds to *S. frugiperda* BBMVs. We tested the ability of ¹²⁵I-labeled cadherin fragments to bind to BBMVs prepared from larvae. BBMVs were spotted to PVDF membranes and probed with labeled cadherin peptides alone or in the presence of 1,000-fold nonlabeled cadherin peptide (Fig. 3). ¹²⁵I-labeled SfCad but not ¹²⁵I-labeled MsCad bound to BBMVs, and binding was competed by unlabeled SfCad (Fig. 3A). Binding was not competed by Cry1Fa toxin, suggesting that SfCad interacts with BBMVs using different binding sites than the Cry1Fa toxin. Spots were excised and counted for radioactivity, and data are presented in Fig. 3B as nmol peptide bound per μ g of BBMV protein. This analysis of bound peptides clearly showed the specific affinity of ¹²⁵I-labeled SfCad for BBMVs prepared from *S. frugiperda* larvae. The ability of SfCad to bind both toxin and BBMVs suggests that SfCad may function as a surrogate receptor for the Cry1Fa toxin. This may occur by binding of SfCad first to toxin and then binding of the

FIG 2 Saturation and characteristics of MsCad and SfCad competitive binding to Cry1Fa toxins. (A and B) Ninety-six-well microtiter plates coated with Cry1Fa toxin were probed with increasing molar concentrations of biotinylated MsCad (A) or SfCad (B) to determine total binding. Bound biotinylated peptide was detected with a streptavidin-HRP conjugate and substrate. Nonspecific binding was determined by probing the plates with increasing molar concentrations of biotinylated MsCad or SfCad plus a 1,000-fold molar excess of nonlabeled MsCad or SfCad, respectively. Specific binding was calculated by subtracting nonspecific binding from total binding. (Insets) Binding of equal concentrations of MsCad and SfCad to Cry1Fa (A and B) toxin spotted in duplicate on a PVDF membrane and probed with 0.5 nM 125 I-labeled MsCad (A) or 125 I-labeled SfCad (B) alone or in the presence of a 1,000-fold excess of unlabeled SfCad (B) or MsCad (A), respectively. (C) Microtiter plates coated with Cry1Fa toxin were probed with 2 nM biotinylated (biot) SfCad in the presence of increasing molar concentrations of nonlabeled SfCad or MsCad to determine homologous and heterologous binding. Experiments were replicated three times, and the means $\pm$ SEMs are reported. Vertical lines indicate SEMs.

complex to BBMVs, or alternatively, it is possible that SfCad binds first to BBMVs and then to Cry1Fa.

Effect of SfCad and MsCad on AlexaCry1Fa toxin binding to BBMVs. We analyzed whether SfCad increases Cry1Fa toxin binding to *S. frugiperda* BBMVs. As shown in Fig. 4A (lanes 2 and 3), although AlexaCry1Fa toxin bound BBMVs, there was limited competition by unlabeled Cry1Fa toxin. Preincubation of toxin with SfCad did not increase AlexaCry1Fa binding to BBMVs compared to untreated toxin (Fig. 4A).

SfCad and MsCad increase Cry1Fa oligomerization but not toxin membrane insertion. We assessed the ability of SfCad and MsCad to induce Cry1Fa oligomer complexes without and with *S. frugiperda* BBMVs. As shown in Fig. 4B (lanes 1 to 3), preincuba-

FIG 3 Binding of 125 I-labeled SfCad and MsCad to *S. frugiperda* BBMVs. (A) *S. frugiperda* BBMVs (5 μ g) spotted on a PVDF membrane were probed with either 125 I-labeled SfCad or 125 I-labeled MsCad (0.5 nM) alone or in the presence of a 1,000-fold excess of unlabeled SfCad, MsCad, or Cry1Fa. (B) Quantitative representation of the amount of 125 I-labeled peptides bound to the BBMVs spots in panel A. Uppercase or lowercase letters to the right of the bars indicate significant differences determined by ANOVA ($P = 0.05$) and Fisher protected LSD.

tion of Cry1Fa toxin with a 10-fold mass ratio of either MsCad or SfCad increased formation of toxin aggregates, which presumably represent prepore toxin oligomer complexes (≥ 180 kDa). There was no difference in the amount of prepore oligomer induced by the two peptides. Interestingly, the peptide-induced oligomers observed in the absence of BBMVs were not detected when BBMVs were added to the mixture (Fig. 4B, lanes 4 to 6). To possibly clarify this issue, we used a protease K protection assay (2, 29) to reveal membrane-inserted toxin monomers and oligomers. As shown in Fig. 4B (lanes 7 to 9), protease K treatment resulted in the degradation of Cry1Fa toxin, except for an ~ 37 -kDa fragment, and oligomers were not detected. Total degradation of Cry1Fa toxin by protease K in the absence of BBMVs (Fig. 4B, lanes 10 to 12) suggested that the ~ 37 -kDa fragment observed in the presence of BBMVs is the membrane-inserted part of Cry1Fa toxin. Preincubation with cadherin fragments did not affect membrane insertion, suggesting that cadherin-based synergism of Cry toxin is not mediated through an increase in membrane insertion. The insertion of a smaller ~ 37 -kDa fraction of Cry1Fa toxin in the membrane is in agreement with a recent analysis of Cry1Ab membrane insertion where domain I is inserted in the membrane and domains II and III remain exposed to the solvent (53).

SfCad and MsCad prolong stability of Cry1Fa toxin in *S. frugiperda* digestive fluid. Insect midgut proteases are essential for the activation of Bt protoxins (17), have a role in toxin specificity, and are sometimes involved in resistance (27, 30). Therefore, the effect of the cadherin fragments on Cry1Fa toxin stability in *S. frugiperda* gut extract was tested *in vitro*. Both SfCad and MsCad showed stabilization of Cry1Fa at the 5-min time point compared to toxin-alone treatment (Fig. 5A and C). At the 10-min time point, however, more residual toxin was observed in the SfCad-pretreated lane than the MsCad-pretreated or toxin-alone lane, suggestive of prolonged toxin stabilization by SfCad in the *S. frugiperda* gut extract (Fig. 5A and C).

SfCad and MsCad prolong stability of Cry1Fa toxin in *S. frugiperda* midgut. To further demonstrate the protective effect of cadherin fragments against gut proteases, *S. frugiperda* larvae were force fed Cry1Fa toxin alone or toxin preincubated with MsCad or SfCad, and residual Cry1Fa toxin was evaluated at different time

FIG 4 Comparison of SfCad- and MsCad-induced Cry1Fa toxin binding, oligomerization, and membrane insertion. (A) The binding of Cry1Fa toxin to *S. frugiperda* BBMVs was tested by incubating ^{Alexa}Cry1Fa toxin alone or with a 1,000-fold molar excess of unlabeled Cry1Fa toxin with BBMVs (A, lanes 2 and 3). The effect of SfCad and MsCad on ^{Alexa}Cry1Fa binding to BBMVs was tested by incubating SfCad or MsCad preincubated ^{Alexa}Cry1Fa toxin with BBMVs (A, lanes 4 and 5). (B) The ability of the cadherin fragments to facilitate Cry1Fa prepore oligomeric complex is tested in lanes 1 to 3. The ability of the cadherin fragments to facilitate oligomeric structure in BBMVs is tested in lanes 4 to 6. The ability of the cadherin fragments to facilitate Cry1Fa toxin membrane insertion in BBMVs in a protease K (Prot. K) protection assay is tested in lanes 7 to 9. Lanes 10 to 12, complete degradation of Cry1Fa toxin by protease K in the absence of BBMVs. Reactants are indicated above the blot. Residual toxin was detected using Cry1Fa antibody.

points after force feeding. As seen in Fig. 5B and D, peptide-preincubated Cry1Fa was relatively more stable in the gut than the toxin alone at the 2- and 4-h postfeeding times tested here. While both peptides protected Cry1Fa in the midgut, SfCad provided better Cry1Fa protection than MsCad, as evidenced by the higher residual Cry1Fa in the SfCad-treated animals. The relatively larger amount of Cry1Fa in the peptide-Cry1Fa-treated larvae may explain enhancement of Cry1Fa toxicity by the peptides, while the larger amount of Cry1Fa in SfCad-treated larvae may explain why SfCad is a better Cry1Fa enhancer than MsCad. These results provided additional support for the idea that one of the modes of action of the synergistic peptides involved stabilization of Cry toxin in the presence of cadherin peptides in the gut. The prolonged stability of toxin could increase the probability of the toxin inflicting more damage to the gut membrane via increased binding, insertion, and pore formation.

SfCad and MsCad enhance pore formation by Cry1Fa. The effect of cadherin fragments on the pore-forming ability of Cry1Fa toxin was analyzed. Pore-forming ability was indirectly quantified by measuring membrane permeability using the vesicle light-

FIG 5 Cadherin-mediated protection of Cry1Fa toxin in *S. frugiperda*. (A) *In vitro* analysis of Cry1Fa toxin degradation in *S. frugiperda* gut extract in the presence or absence of cadherin peptides. Cry1Fa toxin alone or pretreated with SfCad or MsCad was incubated with 5% (vol/vol) gut extract at 30°C for 5, 10, or 20 min. At the end of each time point, toxin proteolysis was terminated by heating the samples for 10 min at 100°C. (B) *In vivo* analysis of residual Cry1Fa in the tissue and gut content of *S. frugiperda* in the presence or absence of SfCad and MsCad. Midguts were extracted at various time points after treatment, and the tissue was separated from the gut content along with the PM. (A and B) Samples were homogenized, separated by SDS-PAGE, and electroblotted to a PVDF membrane, and the residual Cry1Fa was detected using antisera against Cry1Fa. (C and D) The band intensities of the residual Cry1Fa toxin in panels A and B, respectively, were used to calculate fold differences in band intensities compared to toxin-alone treatment. G. Cont., gut content.

scattering assay previously described by Carroll and Ellar (9). The principle behind the vesicle light-scattering assay is that in the presence of external KCl, the amount of scattered light rapidly increases due to vesicle shrinkage, while membrane permeabilization causes a decrease in the scattered light due to vesicle swelling (Fig. 6A). In agreement with Luo et al. (25), when *S. frugiperda* BBMVs were made permeable by increasing the doses of Cry1Fa, the scattered light signal decreased (Fig. 6A). The percentage of volume recovered for increasing concentrations of Cry1Fa is shown in Fig. 6B. A linear regression equation was used to calculate osmotic swelling rate at various concentrations of Cry1Fa used (percent volume recovered per minute). A dose-response standard curve was obtained on the basis of the best fit ($R^2 = 0.99$) of the osmotic swelling rates (y) of increasing concentrations of Cry1Fa (x) to a logarithmic regression equation: $y = -10.35 + 5.90(\ln x)$ (Fig. 6C). Since cadherin fragments alone slightly lowered the swelling rate of vesicles relative to the HEPES/KCl treatment (Fig. 6C), the swelling rate for cadherin peptide-alone treat-

FIG 6 SfCad and MsCad increase Cry1Fa-induced permeabilization of *S. frugiperda* BBMV. (A) Direct traces of scattered light (V, volts) from HEPES buffer-treated, KCl buffer-treated, toxin-treated, and cadherin-treated BBMV. Scattered light traces of only the highest concentration of cadherin-treated BBMV and two representative concentrations of toxin-treated BBMV were shown here. For clarity, the traces obtained during the initial 0.012 min after the stopped-flow shots were omitted. This removed the initial traces that showed the initial very rapid increase in scattered light intensity as the vesicles shrank in the presence of KCl. (B) The effect of treatment was determined by converting the scattered light signal into the percentage of volume recovered. For clarity, results only for Cry1Fa treatments at various concentrations are shown. (C) Osmotic swelling rates (slopes of the curves from panel B) plotted against the concentration of Cry1Fa to show a dose-response standard curve. (D) Osmotic swelling rates for Cry1Fa alone and Cry1Fa with cadherin fragments. The ratios of the calculated equivalent Cry1Fa concentration over the actual Cry1Fa concentration are shown above each bar. The ratios indicate how much more toxin would have been needed to obtain the same level of membrane permeability.

ments (see Table S1 in the supplemental material) was added to the osmotic swelling rates (γ) of the treatments containing Cry1Fa with cadherin peptides; this addition was based on the assumption that the dampening effect on vesicle swelling also occurs when cadherin peptides are mixed with Cry1Fa. The sum values of Cry1Fa-cadherin peptide rates plus cadherin-alone rates were plotted in the equation to calculate equivalent Cry1Fa concentrations. The ratios of the calculated equivalent Cry1Fa concentration over the actual Cry1Fa concentration in the treatment represent the synergistic factor. As shown in Fig. 6D, a 2- to 3-fold increase in osmotic swelling rate with two graded concentrations of Cry1Fa and peptides suggested that the peptides enhanced toxicity by increasing the pore-forming ability of the toxin (Fig. 6D). Nevertheless, the similar increase in osmotic swelling rates by both peptides suggested that pore formation was not a factor that contributed to the differential Cry1Fa toxicity enhancement by the peptides.

DISCUSSION

In this paper, we demonstrate the greater ability of SfCad relative to MsCad to synergize Cry1Fa toxicity to *S. frugiperda* larvae and investigated the mechanisms underlying the observed synergy.

High-affinity Cry binding to cadherin peptide is a prerequisite for the peptide's synergistic effect (10, 37). SfCad has a predicted toxin binding region (TBR) located in CR12 that is 62% identical and similar in physicochemical properties to the MsCad high-affinity Cry1Ab TBR (11, 18) (see Fig. S1 and S2 in the supplemental material). Both cadherin fragments bound Cry1Fa with high affinity (2 nM for SfCad and 8 nM for MsCad) and shared a common binding site on Cry1Fa toxin. High-affinity Cry1Fa recogni-

tion of SfCad is consistent with cadherin having a Cry1 receptor role in *S. frugiperda*, like it does in *Manduca* and other lepidopteran species (reviewed in Pigott and Ellar, 2007 [41]). However, high-affinity binding to cadherin peptide did not lead to high MsCad synergism of Cry1Fa toxicity, suggesting that other synergistic mechanisms may influence the synergistic ability of cadherin peptides.

A model for the synergistic effect of cadherin fragments was suggested by the ability of some cadherin fragments to bind Cry toxin and BBMV (10, 37). This model would be analogous to Cyt toxin of *B. thuringiensis* subsp. *israelensis*, where Cyt binds midgut membrane and functions as a surrogate Cry11Aa receptor (38). ¹²⁵I-labeled SfCad bound *S. frugiperda* BBMV and was competed by unlabeled SfCad, unlike labeled MsCad, which did not bind BBMV. The ability of SfCad to bind BBMV was consistent with SfCad enhancing Cry1Fa toxicity and the surrogate receptor model. However, the lack of a peptide-mediated increase in Cry1Fa toxin binding to *S. frugiperda* BBMV suggests that the ability to increase binding to BBMV is not an important determinant of the synergistic ability of a cadherin fragment. A cadherin fragment from *Anopheles gambiae* that synergizes Cry4Ba toxicity inhibited Cry4Ba binding to BBMV (35), which further causes the role of cadherin-BBMV bindability in toxin synergism by cadherin fragments to be questioned. An alternative explanation is that it may not be necessary that SfCad increase the amount of toxin binding to have a positive effect on pore formation and Cry1Fa toxicity. Further research, such as knocking out SfCad binding to BBMV by mutational analyses, could test the relevance of the cadherin-BBMV binding property to the synergistic effect.

Another possibility is that SfCad and not MsCad induces prepore oligomer formation. Studies show that the *M. sexta* cadherin synergist-containing CR12 region, a high-affinity binder (10, 33), induces Cry1Ab oligomerization. A *Helicoverpa armigera* cadherin fragment was also shown to facilitate oligomerization of Cry1Ac toxin in the presence of midgut proteinases (37). The observed increased Cry1Fa oligomer formation in the presence of SfCad and MsCad agrees with the reported correlation between increased formation of toxin oligomers and enhanced activity of toxin binding cadherin fragments (33, 37). However, unlike the Cry1Ab oligomers that are stable in *M. sexta* BBMVs (20), the Cry1Fa oligomers observed here were transient and disappeared in the presence of *S. frugiperda* BBMVs. It is possible that the Cry1Fa oligomers observed without BBMVs were present in BBMVs but were unstable under our BBMV solubilization conditions. It is also possible that the formation of prepore toxin oligomer may have a detrimental effect on toxicity, as was reported in *Helicoverpa armigera*, where Cry1Ac toxin oligomer formation induced by *H. armigera* cadherin fragments reduced Cry1Ac toxicity (23). Consequently, the induction of prepore toxin oligomers by cadherin fragments or other synergists cannot be used as a consistent predictor of a synergistic effect on *B. thuringiensis* toxicity.

Gut proteases activate Cry protoxin to its active toxin form and can conversely deactivate toxin by proteolytic degradation in naturally tolerant insects and in insects that have evolved resistance to Bt Cry proteins (27, 30). A better protective, i.e., stabilization, effect by SfCad would account for why SfCad is a better Cry1Fa toxicity enhancer than MsCad. Data from our *in vitro* gut extract assay showed that both SfCad and MsCad protected Cry1Fa from proteolytic degradation and SfCad protected Cry1Fa for a longer duration than MsCad. In agreement with the *in vitro* experiment, results from the *in vivo* experiment further confirmed the protective role played by the peptides. The larger amount of Cry1Fa detected in the membrane and the gut content of the peptide-Cry1Fa-treated larvae correlated with the enhancement of toxicity by the peptides, while higher residual Cry1Fa in SfCad-treated larvae explained why SfCad is a better Cry1Fa enhancer than MsCad. A similar observation, where MsCad stabilizes Cry1Ab toxicity in the *S. exigua* midgut (unpublished data), suggests that cadherin-mediated stabilization of toxin may well be a general mode of action for cadherin-based Bt Cry synergists. Although the exact mechanism of protection is unknown, high-affinity interaction between the peptides and the toxins points to the possibility that the peptides physically protect critical proteolytic sites on the toxin, limiting digestion in the insect gut.

According to the pore formation model, Cry toxin-induced pore formation is an important step in the mode of Bt Cry toxin action (9, 24, 40, 50). Although both monomeric and prepore oligomeric complexes of Cry toxin form pores, prepore oligomeric complexes have higher pore-forming ability than monomeric Cry toxins (28). In agreement with previous reports (25, 28), our light-scattering assay showed that Cry1Fa toxin increased the permeability of *S. frugiperda* BBMVs in a dose-dependent function with toxin concentration. The observed 2- to 3-fold increase in the rate of pore formation by the cadherin-treated Cry1Fa toxin correlated with higher Cry1Fa enhancement by SfCad but not MsCad. Since the cadherin fragments did not increase the amount Cry1Fa toxin binding or membrane insertion, the increase in the rate of pore formation by the cadherin-treated

toxin suggests that the cadherin fragments promote more efficient pore formation by the membrane-inserted toxins. Similar to the oligomeric form of Cry toxin that forms better pores (28), high-affinity toxin-cadherin interaction may modify toxins to form more efficient or stable pores in the membrane. Although both SfCad and MsCad can increase the rate of pore formation *in vitro*, cadherin-mediated protection of Cry1Fa toxin in the midgut will affect the amount of toxin available for membrane insertion and pore formation. Therefore, the greater SfCad-mediated protection of Cry1Fa toxin in the midgut along with the general ability of cadherin fragments to increase the rate of pore formation makes SfCad a better Cry1Fa toxicity enhancer in *S. frugiperda*.

In conclusion, our results showed that like most cadherin-based Bt synergists, *S. frugiperda* cadherin was also a Cry1Fa toxin binding protein and may be a receptor. With regard to the modes of action of cadherin-based Bt synergists, cadherin-mediated protection of Cry toxin in the midgut and enhancement of pore-forming ability of Cry toxin by cadherin fragments likely act in concert to enhance Cry toxicity. The knowledge gained through this study may lead to better design of Bt and its synergists to prolong the utility of *B. thuringiensis* as a valuable resource in crop pest management.

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