Selective Antagonism to the Cadherin BT-R_1 Interferes with Calcium-Induced Adhesion of Epithelial Membrane Vesicles

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ABSTRACT: BT-R_1 is a member of the cadherin superfamily of proteins and is expressed in the midgut epithelium of *Manduca sexta* during larval development. Previously, we showed that calcium ions influence the structure and stability of BT-R_1 on brush border membrane vesicles (BBMVs) prepared from *M. sexta* midgut epithelium. In the present study, the effects of calcium and Cry1Ab toxin, produced by *Bacillus thuringiensis*, on the adhesive properties of BBMVs were investigated. Addition of calcium to a suspension of BBMVs promoted adhesion and aggregation of the vesicles. Treatment of BBMVs with trypsin or lowering the pH (pH 4.0) of the BBMV suspension abolished calcium-induced vesicle aggregation, whereas treatment with deglycosylating enzymes did not affect the aggregation of vesicles, indicating that adhesion and clustering of BBMVs involves protein−protein interactions. Preincubation of BBMVs with Cry1Ab toxin, which specifically binds to BT-R_1 with high affinity and disrupts the midgut epithelium of *M. sexta*, caused a 50% decrease in calcium-induced vesicle aggregation. The inhibitory effects of the Cry1Ab toxin on BBMV aggregation was blocked completely when the toxin was preincubated with a peptide containing the toxin-binding site of BT-R_1. Cry3A toxin, which is similar in molecular structure to Cry1Ab but does not bind to BT-R_1 and is not toxic to *M. sexta* larvae, did not affect BBMV aggregation. The results of this study demonstrate that the adhesive function of BT-R_1 is compromised by the Cry1Ab toxin, which acts as a selective antagonist, and supports the notion that BT-R_1 is critical in preserving the integrity of larval midgut epithelium in *M. sexta*.

Cell−cell interactions are crucial to development and morphogenetic activities as well as to the maintenance of normal tissues in multicellular organisms (*1*−*5*). The primary mediators of cell−cell interactions include calcium-dependent cell adhesion molecules such as cadherins. Cadherins constitute a large family of transmembrane glycoproteins responsible for cell adhesion and maintenance of the integrity of selective cell−cell interactions (*2*, *6*−*9*). Cadherins also are involved in signal transduction pathways and organization of the cytoskeleton by interacting with other cell adhesion receptors through their ectodomains and with specific cytoplasmic proteins via their cytoplasmic domains (*1*, *2*, *10*−*12*). Cadherin-mediated cell adhesion events are implicated in various biological processes including stimulation and aggregation of subsets of cells and differentiation and physical grouping of cells during tissue organization and epithelial barrier and synapse formation as well as certain pathological disorders such as tumor invasion (*1*, *7*, *13*−*17*). Furthermore, cell−cell interactions occasioned by cadherins are critical for regulating survival of cells in tissues (*18*, *19*).

The functional characteristics of cadherins depend on calcium ions which bind and stabilize the ectodomain structure of cadherins, preserving their adhesive function (*20*−*22*). Removal of Ca^{2+} or changes in the concentration of the ion affects the adhesive activity of cadherins, thereby influencing the initiation and maintenance of intercellular junctions (*6*, *13*, *21*−*24*). Interference with cadherin functions also can lead to pathological conditions including apoptosis or loss of contact inhibition during cell proliferation and cancer (*25*).

Previously, we identified and characterized an atypical cadherin, BT-R_1 (M_r = 210000), that is expressed exclusively in the midgut epithelium during larval development of the tobacco hornworm *Manduca sexta* (*26*−*28*). The molecule has an ectodomain consisting of 12 EC[1] modules that are made of β-sheets structured as cadherin repeats. The ectodomain contains two cell adhesion recognition sequences (HAV) (*29*) and two cell attachment sequences (RGD and LDV) (*30*, *31*), allowing potential interactions between BT-R_1 and other cadherins and integrins (*28*). Like all cadherins, calcium influences the structure of the BT-R_1 ectodomain, and upon removal of calcium, the N-terminus of the ectodomain is clipped by specific proteolysis. Tissue-specific

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[1] Abbreviations: BBMVs, brush border membrane vesicles; DTT, dithiothreitol; EC, ectodomain module; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether) *N*,*N*,*N*,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); TBR, toxin-binding region.

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expression of BT-R_1 during larval development, together with its structural features and associated proteolysis, points to the importance of this adhesion receptor in differentiation and development of midgut epithelium in *M. sexta* (*28*, *32*, *33*).

BT-R_1 also is a target receptor for Cry1A toxins of the entomopathogenic soil bacterium *Bacillus thuringiensis* (*27*, *28*). The toxicity of the Cry toxins, as well as the susceptibility of an insect larva to the toxins, depends on high-affinity interaction with BT-R_1 in the midgut epithelium. The toxin binds to BT-R_1 and exerts some or all of its toxicity through this interaction in the larva (*27*). Toxin binding to BT-R_1 causes swelling of the epithelial cells and detachment of the cells from the tissue, leading to destruction of the integrity of the tissue (*28*), suggesting that Cry toxins interfere with cell−cell interactions in the larval midgut epithelium. Previously (*33*), we showed that calcium ions affect the structure and stability of BT-R_1. In the present study, we performed turbidity experiments to determine the effects of calcium and Cry1Ab toxin on the adhesive properties of brush border membrane vesicles (BBMVs) prepared from *M. sexta* larval midguts. BBMVs are microsome-like vesicles, largely oriented with the right side out (*34*, *35*), and their surface composition, distribution, and organization of lipids, proteins, and carbohydrates resemble those of larval midgut cells, including stem, columnar epithelial, and goblet cells. Significantly, BT-R_1 is an integral component of the midgut epithelial cells and is present on the BBMVs. The results demonstrate that calcium mediates adhesion of BBMVs and that Cry1Ab toxin selectively interferes with calcium-dependent adhesion of the vesicles by interacting with BT-R_1. Furthermore, prevention of the binding of Cry1Ab toxin to BT-R_1 by a soluble peptide derived from the toxin-binding region of BT-R_1 completely abolishes the interference by toxin of calcium-induced BBMV aggregation. These findings suggest that there is a mechanistic link between the influence of calcium on BT-R_1 and its molecular interactions and that BT-R_1 is critical to calcium-dependent cell adhesion events in the midgut epithelium of *M. sexta* larva.

EXPERIMENTAL PROCEDURES

Preparation of M. sexta BBMVs. Hornworm eggs and the diet for larvae were obtained from the Carolina Biological Supply Co. (Burlington, NC). BBMVs were prepared according to the Mg^{2+}/EGTA precipitation method (*36*, *37*). Briefly, midgut tissue isolated from 5th instar larvae were homogenized in an isoosmotic buffer containing 300 mM mannitol, 5 mM EGTA, and 17 mM Tris·HCl, pH 7.5. BBMVs were enriched by two successive precipitations with 12 mM $MgCl_2$ and differential centrifugation at 3000*g* and 10000*g*. BBMVs obtained using this protocol have been shown to be tightly sealed and predominantly (∼94%) oriented right side out (*34*, *35*). All preparations were stored at −70 °C in buffer A (10 mM Hepes, pH 8.0, and 10% glycerol).

Construction of Truncated BT-R_1 Fragments, Cloning, and Expression. The cloning, expression, and preparation of TBR peptide was accomplished as previously described (*28*). Briefly, a DNA fragment encoding the toxin-binding region of BT-R_1 was generated by digesting the BT-R_1 cDNA with the restriction enzymes *Hinc*II and *Sac*I. The fragment was subcloned using standard cloning methods into the plasmid vector pET-30b (Novagen, Madison, WI) for polyhistidine tagging and expression in *E. coli* BL21(DE3) cells. Recombinant bacterial colonies were selected and grown overnight in 5 mL of LB medium at room temperature. A 100 mL sample of LB medium was then inoculated with 2 mL of the overnight culture, and the culture was grown to $OD_{600\ nm} = 0.65$. Protein expression in the culture was induced by the addition of a 1 M solution (100 μL) of isopropyl β-thiogalactoside (IPTG). The induced culture was incubated for 4 h at room temperature, and the *E. coli* cells were homogenized by sonication. His-tagged proteins were purified on a nickel affinity column packed and activated as described by Novagen. Purified protein was concentrated and desalted on Centricon10 spin columns (Centricon) and assayed by the bicinchoninic acid method (Pierce Chemical Co.) with bovine serum albumin (BSA; fraction V) as a standard.

BBMV Aggregation Assay. Calcium-induced aggregation of BBMVs was determined by solution turbidity assays that monitor the change in absorbance at 400 nm (*38*, *39*, *40*). To remove residual calcium from the BBMVs, 1 mL of vesicle suspension (0.5 mg/mL protein) was added to EDTA (dissolved in water) to reach a final concentration of 3 mM. The suspension was incubated for 1 h at room temperature. Then the BBMVs were washed with buffer A, centrifuged at 8000*g* for 10 min, and resuspended in buffer A for turbidity experiments. Turbidity measurements were performed using a Beckman UV/vis spectrophotometer. The effect of CrylAb toxin on calcium-induced BBMV aggregation was determined using various concentrations (10, 50, 100, and 150 nM) of the toxin. The influence of TBR on CrylAb action was determined as follows. A 500 μL sample of the BBMV suspension (0.25 mg/mL protein) was preincubated with 100 nM Cry1Ab toxin with or without TBR (10 μM) for 20 min at room temperature. Absorbance was recorded every 15 s following addition of calcium for 20 min. Appropriate blanks consisting of vesicles without protein and protein without vesicles were prepared, and their absorbance at 400 nm was subtracted from the sample values. The disaggregation of the BBMVs upon addition of 10 mM EDTA to the suspension also was monitored as it was done for aggregation.

Proteolytic removal of BBMV surface proteins was accomplished as follows. BBMV samples were incubated with trypsin for 16 h at room temperature with an enzyme-to-protein ratio of 1:5. The reaction was stopped with addition of PMSF (1 mM). The trypsin-treated samples were washed in buffer A, and centrifuged at 8000*g* for 10 min in a microcentrifuge. The pellet was resuspended in buffer A for the vesicle aggregation assay. The extent of protein cleavage was monitored by SDS−PAGE followed by immunoligand blotting. The Cry1Ab toxin was used to detect toxin-binding proteins in both the control and trypsin-treated BBMV suspensions.

All aggregation experiments were performed using 0.5 mL of BBMV (0.25 mg/mL protein) suspended in buffer A at pH 8.0 except for the experiment using buffer at lower pH. The effect of lower pH on calcium-induced aggregation was tested using BBMVs suspended in a buffer composed of 10 mM glycine, pH 4.0, and 10% glycerol.

Deglycosylation of membrane proteins was performed using a PNGase F kit from New England Biolabs (NEB), Beverly, MA. Membrane vesicles (200 μg of protein) were incubated for 16 h at 37 °C in 50 mM sodium phosphate buffer, pH 7.5, containing 3 mM PMSF in the absence or presence of 7000 NEB-defined units of PNGase F. The samples were washed in 20 mM Hepes, pH 8.0, and centrifuged at 8000*g* for 10 min in a microfuge. The pellet was resuspended in buffer A for the aggregation assay.

The effect of Cry3Aa toxin on BBMV aggregation was determined using 100 nM toxin. Preincubation of the BBMVs with Cry3Aa toxin and turbidity measurements were the same as described for the Cry1Ab toxin.

Microscopy. The aggregation and dissociation of BBMVs were examined by phase-contrast microscopy. Micrographs were obtained immediately after the aggregation experiments. A 10 μL sample of the BBMV suspension was placed on a glass slide and examined at 200× magnification using a NIKON TE600.

Ligand Blot Experiments. Gel electrophoresis was carried out according to the procedure of Laemmli (*41*). Trypsin-treated and untreated BBMV proteins separated by SDS−PAGE were blotted to PVDF membranes (Millipore) using a semidry blotting apparatus (Owl Scientific). The blotted membranes were blocked for 2 h at room temperature with Tris·HCl-buffered saline (TBS; pH 8.0) containing 5% nonfat dry milk powder, 5% glycerol, and 0.1% Tween 20. Binding was detected by anti-Cry1Ab polyclonal antibody (1:60000, 1 h) and visualized with a goat antirabbit antibody coupled with horseradish peroxidase (Sigma) (1:3000, 1 h) using appropriate detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS

Calcium-Induced BBMV Aggregation. To study the function of adhesion receptors and to test whether toxins that target adhesion receptors can disrupt cell adhesion, we developed an in vitro assay that detects adhesion events by monitoring the aggregation of BBMVs generated from midgut epithelial tissue of *M. sexta*. The aggregation and disaggregation of BBMVs were monitored by changes in turbidity of BBMV suspensions and by light microscopy (Figure 1). Adhesion receptors require calcium for structural stability and function (*21*−*24*). To understand the influence of calcium on the adhesion molecules present in the midgut epithelial tissue, BBMVs were prepared in the absence of calcium and aggregation was monitored following addition of calcium. Calcium-induced BBMVs aggregate in a dose-dependent fashion, with a half-maximal aggregation at approximately 1 mM calcium and a near-saturating aggregation at 5 mM calcium (Figure 1A). On the basis of a calcium titration curve, it was determined that 5 mM $CaCl_2$ was sufficient to induce aggregation in this system. These levels are within the physiological range of calcium ion concentrations in the larval gut of *M. sexta* (*42*, *39*). No noticeable BBMV aggregation was detected upon addition of 10 mM magnesium or 150 mM sodium in the suspension, indicating specificity for calcium over these other cations (not shown). At 5 mM calcium, the BBMVs aggregated rapidly and reached a maximum within 10−20 min (Figure 1B). The aggregated vesicles became completely disaggregated within 10 min upon addition of EDTA (10 mM), and the absorbance of the suspension returned to its precalcium level (Figure 1C). Disaggregation of the BBMVs, upon addition of EDTA, demonstrates that calcium indeed promotes adhesion of vesicles and leads to aggregation, and that the increase in the optical density in the suspension represents vesicle aggregation but not fusion of the vesicles, which usually is typified by an irreversible increase in the turbidity of the suspension upon addition of calcium (*43*, *44*). Light micrographs of BBMVs with no calcium added showed crowded fields of uniformly dispersed vesicles (Figure 1D), whereas BBMVs in the presence of 5 mM calcium showed large clumps of vesicles with only a few individual vesicles within 20 min (Figure 1E). Calcium-aggregated vesicles became dispersed (Figure 1F), and the suspension appeared indistinguishable from the pre-calcium light micrograph (Figure 1D), which also contained only individual vesicles. Apparently, BBMV aggregation is completely reversible and does not involve vesicle fusion. Extended exposure (e.g., 24 h) of BBMVs to EDTA promotes proteolysis of the BT-R_1 ectodomain at the N-terminus, leading to the removal of the first five EC modules (*33*). To test whether proteolysis affects adhesion events in this system, BBMVs were disaggregated by chelating the calcium with EDTA (10 mM) for 24 h. After 24 h, these vesicles could be fully reaggregated by adding calcium at a concentration that was 5 mM in excess of the EDTA (data not shown). Apparently, EC modules 1−5 of the BT-R_1 ectodomain are not required for calcium-mediated BBMV aggregation.

Effect of Trypsin Treatment, pH, and Deglycosylating Enzymes on BBMV Aggregation. To determine whether aggregation is a result of protein−protein interactions at the surface of the BBMVs, vesicle aggregation experiments were performed using trypsin-treated BBMVs (Figure 2). Trypsin treatment of BBMVs completely abolished calcium-induced aggregation (Figure 2A). Immunoligand blots of BBMV proteins from trypsin-treated (inset, lane 2) and untreated (inset, lane 1) vesicles using Cry1Ab toxin showed virtual elimination of toxin-binding proteins in the trypsin-treated sample (inset, lane 2). Cry1Ab toxin bound to BT-R_1 (inset, lane 1, arrow) as well as to several other proteins in the untreated BBMVs. Trypsin treatment did not alter the physical integrity of the vesicles, as observed by light microscopy (not shown); however, it most likely clipped or removed proteins at the surface of the BBMVs. Protein bands separated by SDS−PAGE varied in trypsin-treated and untreated BBMV samples (data not shown). The aggregation of BBMVs was prevented when the vesicles were suspended in a buffer at pH 4.0 (Figure 2B), indicating that charge distribution and structural conformation of the proteins at the surface as well as electrolytic properties of the suspension are important for interactions between the vesicles (*45*, *46*). Treatment of BBMVs with deglycosylating enzymes had virtually no effect on vesicle aggregation (Figure 2C), demonstrating that glycosylated residues probably are not involved in BBMV aggregation. Overall, these results indicate that BBMV clusters are established through protein−protein interactions.

Effect of Cry1Ab Toxin and TBR Peptide on BBMV Aggregation. Because cadherins are important in calcium-mediated cell−cell adhesion and because the Cry1Ab toxin binds with high affinity and specificity to BT-R_1, the effect

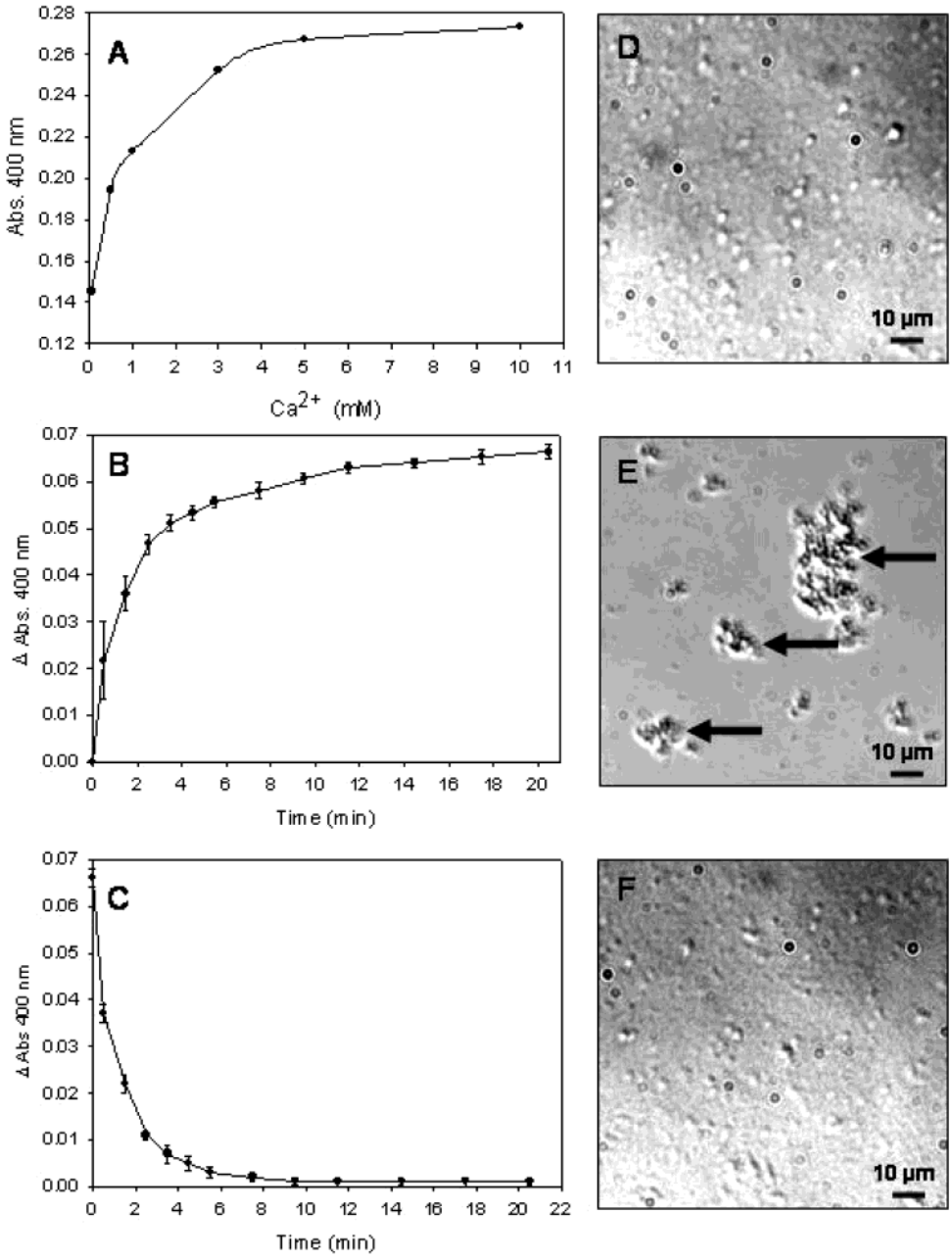

FIGURE 1: Calcium-induced BBMV aggregation. (A) The extent of BBMV aggregation was monitored spectrophotometrically upon addition of different amounts of calcium. (B) BBMV aggregation upon addition of 5 mM calcium. (C) Disaggregation of BBMV aggregates upon addition of 10 mM EDTA. (D) Light micrograph of the BBMV suspension in the absence of calcium. (E) Micrograph of the BBMV suspension 20 min after addition of 5 mM calcium. Arrows indicate clusters of BBMV aggregates. (F) Micrograph of disaggregated BBMV aggregates after addition of 10 mM EDTA. Graph values for the experiments described in this figure and in all other figures represent values normalized by subtracting the appropriate blanks (BBMVs without any treatment) from the test readings.

of the toxin on calcium-induced BBMV adhesion and aggregation was investigated (Figure 3). There was a significant decrease (50%) in calcium-induced aggregation of BBMV preincubated with Cry1Ab toxin (100 nM) (Figure 3A), demonstrating that Cry1Ab toxin interferes with calcium-induced vesicle adhesion. The toxin had no affect on vesicle aggregation in the absence of calcium (Figure 3A). Also, it did not disrupt calcium-induced aggregates (not shown). With preaggregated vesicles, the toxin most likely cannot access those BT-R_1 molecules located between adhered vesicles. Maximum inhibition of BBMV aggregation by the toxin (up to 150 nM) did not exceed 50%, indicating that other adhesion molecules that do not bind toxin most likely are present on vesicles (Figure 3B). Alternatively, toxin binding may weaken but not completely disrupt the adhesion interactions occasioned by BT-R_1 molecules so that maximum toxin binding reduces but does not completely abolish vesicle aggregation.

Inhibition of BBMV aggregation by Cry1Ab toxin was neutralized completely when the toxin was preincubated with the toxin-binding region (TBR) of BT-R_1 (Figure 4A), indicating that TBR prevents binding of the toxin to the surface of BBMVs and that the inhibitory effect of the toxin on calcium-induced vesicle aggregation is dependent on the specific interaction of toxin with BT-R_1. The TBR peptide itself did not promote vesicle aggregation in the absence of calcium (Figure 4A). On the other hand, another toxin, Cry3Aa, which has a molecular structure similar to that of

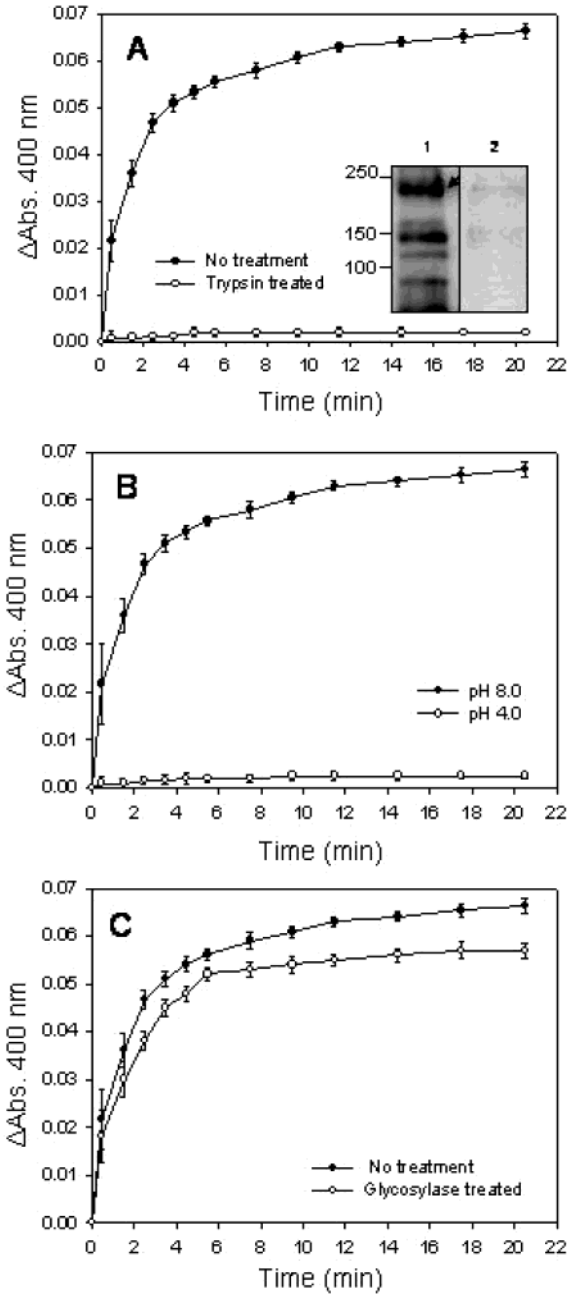

FIGURE 2: Effect of trypsin, low pH, and deglycosylation on calcium-induced BBMV aggregation. The effects of trypsin, pH, and deglycosylation on calcium-induced aggregation of BBMVs were monitored by turbidity measurements. (A) Aggregation of trypsin-treated BBMVs (125 μg of BBMV proteins) was determined upon addition of 5 mM calcium. Binding of Cry1Ab toxin to untreated (inset, lane 1) and trypsin-treated (inset, lane 2) BBMV proteins was determined by immunoligand blotting with Cry1Ab. The arrow in the inset indicates the M_r = 210000 BT-R_1 protein band. (B) Aggregation of BBMVs suspended in buffer at pH 4.0 was monitored upon addition of calcium. (C) Aggregation of BBMVs treated with glycolytic enzymes upon addition of calcium was determined.

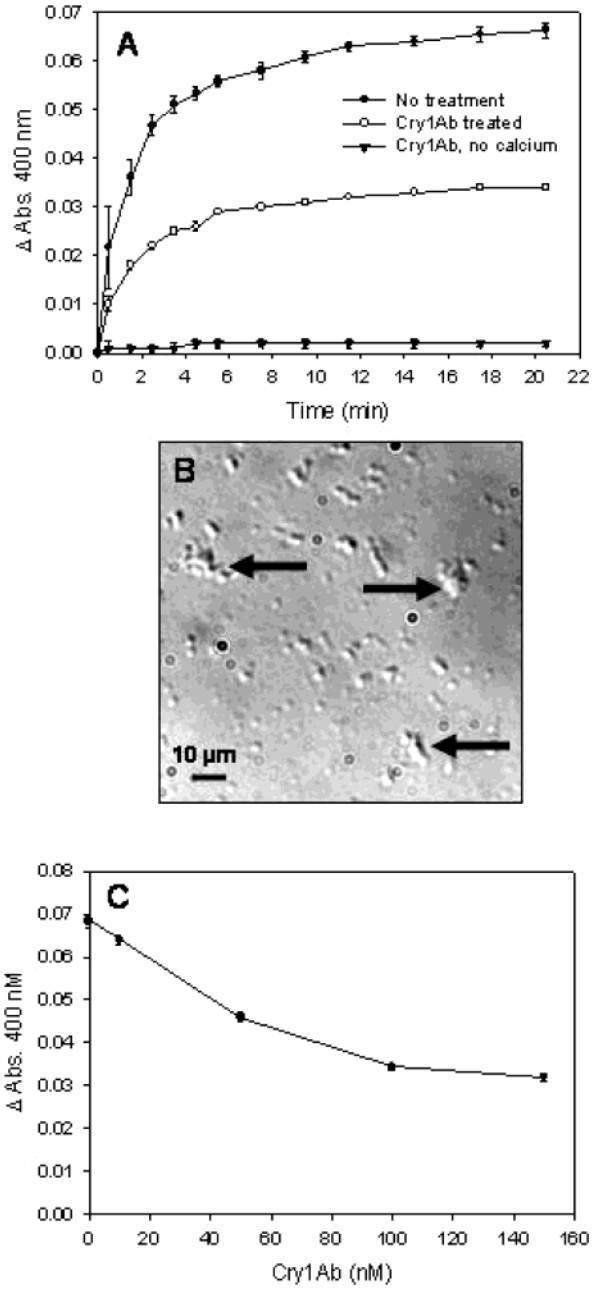

FIGURE 3: Effect of Cry1Ab on calcium-induced BBMV aggregation. The effect of Cry1Ab on aggregation of BBMVs was determined by turbidity measurements. (A) BBMVs were preincubated with Cry1Ab (100 nM). Aggregation of toxin-treated and untreated vesicles upon addition of 5 mM calcium was determined. (B) The reduced (50%) formation of BBMV aggregates in toxin-treated vesicles was examined by light microscopy. Arrows indicate the reduced clusters of BBMV aggregates. (C) Effect of various concentrations of Cry1Ab toxin on calcium-induced BBMV aggregation.

Cry1Ab but does not bind to BT-R_1 and is not toxic to *M. sexta* larvae (*26*), had no effect on BBMV aggregation (Figure 4A), demonstrating that the inhibitory effect of the Cry1Ab toxin is specifically and selectively exerted through BT-R_1. As observed for the Cry1Ab toxin, the Cry3Aa toxin also did not promote aggregation of BBMVs in the absence of calcium (data not shown). Prevention of the inhibitory action of Cry1Ab on BBMV aggregation by TBR is graphically represented in Figure 4B. Figure 4C depicts BBMV aggregation in the presence of Cry3Aa.

DISCUSSION

Formation of cell adhesion is essential for the interaction of cells with neighboring cells and with the extracellular matrix (*47*). Calcium is a key factor in this process. Calcium ions influence the structure, function, and expression of various cell adhesion receptors, including cadherins, integrins, claudins, selectins, and the Ig superfamily of proteins.

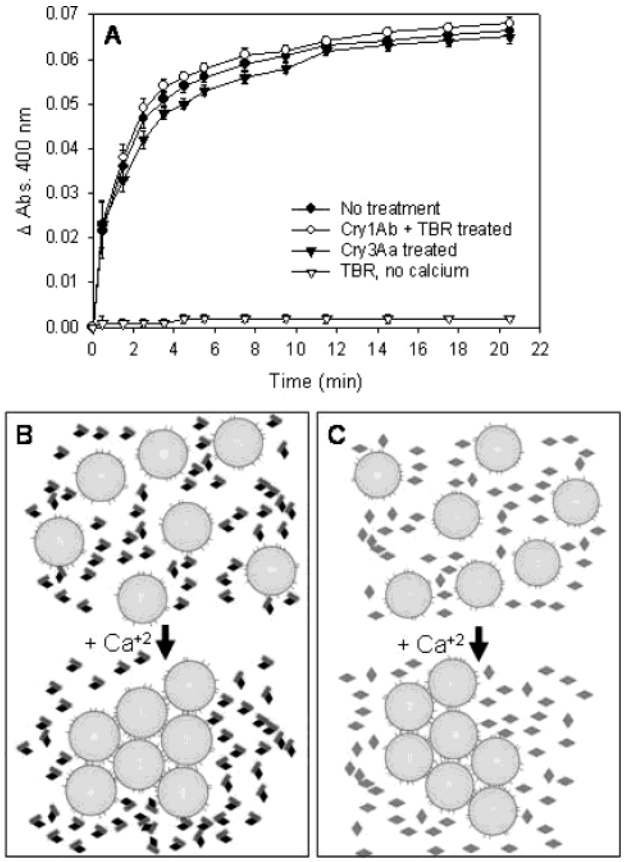

FIGURE 4: Prevention of Cry1Ab inhibition of BBMV aggregation by the TBR. Turbidity measurements were performed using BBMV suspensions to determine the effects of TBR, TBR plus Cry1Ab toxin, and Cry3Aa toxin (100 nM). (A) Aggregation of treated and untreated vesicles upon addition of 5 mM calcium was determined by measuring the change in absorbance at 400 nm. (B) Graphic illustration of TBR preventing the inhibitory effect of Cry1Ab on BBMV aggregation. Black diamonds represent the Cry1Ab−TBR complex. (C) Null effect of Cry3Aa toxin on calcium-induced BBMV aggregation. Gray diamonds represent Cry3Aa toxin molecules.

Removal of Ca^{2+} or changes in the concentration of the ion induce conformational changes in the cell adhesion receptors affecting their adhesive activity (*6*, *23*, *24*). Calcium also is an important factor in the pathways linked to cell adhesion events. Regulation of calcium concentrations in and out of cells and signaling events occasioned by changes in the concentration of the ion are critical to the dynamics of cell−cell interactions, which, in turn, are essential for the coordination of multicellular activities in tissues and organ functions (*10*, *48*−*51*).

In the present study, we show that calcium is a critical factor for the adhesion and aggregation of BBMVs. Addition of calcium promoted rapid aggregation of the BBMVs, whereas EDTA caused disaggregation (Figure 1). Aggregation of BBMVs was dependent on calcium ion concentration in the suspension (Figure 1A). Significantly, the presence of calcium at 5 mM, which is similar to the physiological calcium ion concentration of *M. sexta* midgut (*42*), sufficiently promoted BBMV aggregation (Figure 1A,B). The sensitivity of BBMV aggregation to trypsin proteolysis demonstrated that calcium-induced aggregation was mediated by proteins but not the lipid components of the BBMVs (Figure 2A). Furthermore, complete loss of BBMV aggregation as well as Cry toxin binding to the BBMVs (Figure 4) after trypsin treatment indicates that the vesicles are composed of right-side-out midgut epithelial cell membrane since the BT-R_1 ectodomain and Cry toxin-binding region are extracellular. The reversible aggregation and disaggregation of BBMVs indicate that most of the critical factors, principally those that comprise the structural and functional determinants of calcium-dependent interactions in the *M. sexta* larval midgut, are preserved on the surface of the vesicles. Therefore, we believe that BBMVs are useful for studying molecular interactions associated with the structural and biochemical characteristics of the molecules present on the larval midgut cells.

Previously, we demonstrated that calcium provides stability to BT-R_1 and that removal of calcium promotes a specific cleavage of the molecule at the N-terminus of its ectodomain (*33*). The intact BT-R_1 (M_r = 210000) contains two separate cadherin cell adhesion sequences (HAV) and two different integrin-binding sequences (RGD and LDV) on its ectodomain (*28*). The specific cleavage of the BT-R_1 ectodomain, as a result of calcium removal, leads to sequential formation of two variants of the molecule, both of which are retained on the membrane (*33*). Interestingly, the effect of calcium removal on BBMV adhesion and aggregation was reversible (Figure 1). Thus, the cleavage of BT-R_1, occasioned by calcium removal, does not necessarily abolish its adhesive function on the vesicles. It is more likely that the specific cleavage pattern of BT-R_1 is involved in modulating its interactions on the midgut epithelial cells during larval development of *M. sexta* because the concentration of calcium ions and cellular responsiveness are tightly regulated (*42*). Apparently, cleavage of BT-R_1 in response to fluctuations in calcium ion concentration not only modulates interactions between cadherins and other cell adhesion receptors but also is required for the assembly and disassembly of cell junctions as well as for triggering specific signals related to cell attachment during differentiation and development of the insect.

Cadherin-mediated cell adhesion processes participate in barrier formation in epithelial tissues (*4*). Previously, we showed that Cry1Ab toxin produced by the entomopathogenic bacterium *B. thuringiensis* disrupts the midgut epithelial barrier of *M. sexta* (*28*). The toxin binds with high affinity to BT-R_1 in the larval midgut epithelium and exerts cellular toxicity and lethal action on the larva through this interaction. The Cry1Ab toxin binds to a specific region (TBR) close to the membrane-proximal ectodomain of BT-R_1 (*28*). Binding of the toxin to BT-R_1 also reflects specificity of this particular toxin (*26*−*28*, *32*, *33*). At the organism level, the toxicity of the Cry1Ab toxin can be completely inhibited by blocking the toxin with a soluble peptide encompassing the TBR, demonstrating that binding of toxin to BT-R_1 in the whole insect is critical to disrupting cell adhesion and, ultimately, overwhelming the midgut epithelial barrier in the insect (*28*). Results of the present study demonstrate that the specific binding of Cry1Ab toxin to BT-R_1 inhibits calcium-induced adhesion and aggregation of BBMVs (Figure 3). Interference by the toxin may be due to high affinity (1 nM) of the toxin to BT-R_1 that could, in turn, obstruct the effect of calcium on the receptor and alter the structure of the cadherin. Alternatively, binding of the Cry1Ab toxin to BBMVs might interfere with the charge distribution on the vesicles and block or decrease the physical attraction between the vesicles

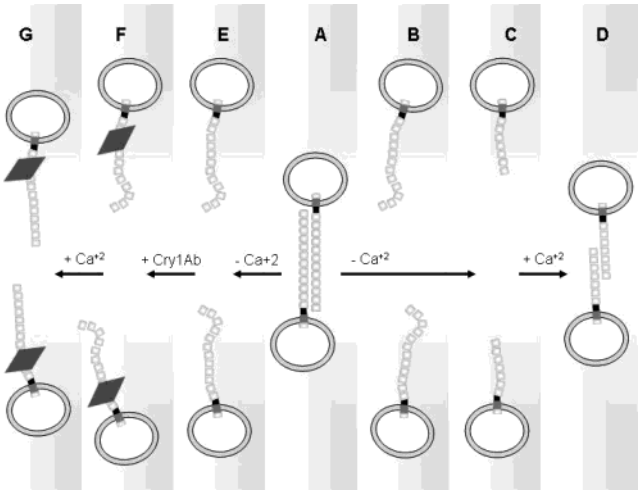

FIGURE 5: Proposed model for the effects of calcium and Cry1Ab toxin on BT-R_1 and BBMV adhesion. (A) Calcium ions stabilize the structural conformation of BT-R_1 on BBMVs and promote interaction between ectodomains and vesicle adhesion. (B) Removal of calcium leads to destabilization of the ectodomain, leading to broken interactions and loss of vesicle adhesion. (C) Absence of calcium ions for a prolonged time (24 h) leads to cleavage of BT-R_1, resulting in the formation of an ectodomain variant on the vesicles. (D) Reintroduction of calcium induces formation of interactions between the cleaved ectodomain variants and promotes BBMV adhesion. (E) Removal of calcium leads to destabilization of the ectodomain, leading to broken interactions and loss of vesicle adhesion. (F) Cry1Ab toxin binds to and occupies a critical site located close to the membrane-proximal ectodomain of BT-R_1. (G) The vesicles with the toxin bound to BT-R_1 adhere loosely or with decreased affinity to one another when calcium is introduced.

when calcium is added. However, calcium-induced adhesion of the vesicles and formation of aggregates was not affected (Figure 4) when BBMVs were preincubated with another toxin, Cry3Aa, which has a molecular structure highly similar to that of Cry1Ab but does not bind to BT-R_1 and does not kill *M. sexta*. Apparently, both the inhibitory action of Cry1Ab toxin on BT-R_1 and the involvement of BT-R_1 in calcium-induced adhesion are specific. Thus, selective, not arbitrary, binding of the Cry1Ab toxin to a specific target cadherin receptor on the vesicles results in the obstruction of calcium-induced adhesion and aggregation of vesicles. Furthermore, blocking the interaction of the Cry1Ab toxin with the target receptor BT-R_1 by preincubating toxin with the TBR hinders the inhibitory effect of the toxin on calcium-induced vesicle aggregation.

The extent of inhibition of BBMV aggregation by the Cry1Ab toxin was approximately 50% (Figure 3). This finding suggests that other factors besides BT-R_1 are involved in calcium-dependent vesicle aggregation. Alternatively, it is possible that the binding of Cry1Ab toxin to BT-R_1 hinders the effects of calcium on BBMV adhesion and aggregation. Nevertheless, the results indicate that BT-R_1 is a major factor in the adhesion and aggregation of BBMVs because interference with BT-R_1 impedes (50%) the calcium-induced aggregation process. The results also strongly suggest that the TBR of BT-R_1, which is located close to the membrane-proximal ectodomain, is important for adhesion. As depicted in the proposed model (Figure 5), calcium provides structural integrity to BT-R_1, promoting the interaction of intact ectodomains between BBMVs and contributing to vesicle adhesion (Figure 5A). Removal of calcium induces structural alterations on BT-R_1, resulting in the loss of interactions and detachment of vesicles (Figure 5B,E). Prolonged absence of calcium ions eventually leads to cleavage of the BT-R_1 ectodomain (*46*; Figure 5C). However, when calcium is reintroduced, the portion of the ectodomain that is retained on the vesicles can afford efficient interaction and adhesion (Figure 5D). Binding of the Cry1Ab toxin to BT-R_1 (Figure 5F) hampers the effect of calcium on adhesion (Figure 5G), which may be due to decreased affinity of the ectodomains or weakening of interactions between the ectodomains when toxin is bound to the molecule, or both. Apparently, toxin binds and occupies a critical site on BT-R_1 that interferes with both the structure and function of its ectodomain and diminishes its calcium-induced adhesive properties. Indeed, binding of the toxin to BT-R_1 most likely lessened the attraction between the BBMVs, resulting in weak adhesion and increased distance between the vesicles, as evidenced by the 50% decrease in light absorbance (increased transmittance) in the BBMV suspension that contained vesicles bound to toxin (Figure 3).

All cellular activities involving structural and functional organization of cells are accompanied by cell−cell adhesion events, and these events are manifested by adhesion receptors that enable cells to contact and link their cytoskeleton to neighboring cells and to extracellular matrix via transmembrane connections (*52*−*54*). Cadherins are cardinal in various kinds of cell adhesion events including formation of gap junctions, tight junctions, and juxtacrine interactions. Both the structure and function of cadherins depend on calcium ions, which induce conformational changes in their ectodomain and promote adhesive activity (*13*, *48*, *55*). Cadherin-mediated, calcium-dependent cell adhesion events are critical to differentiation and survival of cells during establishment of cell polarity, morphogenesis, tissue patterning, organ formation, growth, and development, and wound repair (*56*−*58*). The disruption of calcium-dependent cell contacts with either a chelating agent or an anti-cadherin antibody results in cell death and substantiates that cadherin-mediated cell contacts are crucial to cell adhesion processes (*17*).

The results of the present study suggest that BT-R_1 is involved in calcium-dependent interactions and that the binding of the Cry1Ab toxin interferes with the structural or functional properties, or both, of BT-R_1. However, the mechanism by which the Cry toxin disrupts the calcium-dependent structural and functional aspects of BT-R_1 in the midgut epithelium of *M. sexta* is not known. Toxic action in vivo involves swelling of midgut epithelial cells upon toxin binding to BT-R_1, which leads to detachment of the cells from the basal lamina (*28*). Other studies have shown that interference with cadherin function can lead to pathological conditions such as loss of contact inhibition during cell proliferation and cancer (*59*). Cells that lose contact with other cells or become detached at intercellular junctions undergo apoptosis. Recent studies show that exposure to Cry toxins, indeed, leads to increased apoptosis of epithelial cells in the midgut tissue of toxin-susceptible insect larvae (*60*). Therefore, it is important to understand whether the cytotoxicity associated with binding of Cry1Ab toxin to BT-R_1 involves apoptosis. Identification and characterization of pathways and components of these pathways involved in Cry toxin action are critical to understanding the function of BT-R_1. BT-R_1 has structural features that usually are characteristic of protocadherins, which constitute a major subfamily

of the cadherin superfamily (*61*, *62*). Little is known about the functions and intracellular signal transduction activities of protocadherins. Elucidating the structural features of the BT-R_1 ectodomain and determining the adhesive properties of the molecule should contribute to a better understanding of the overall function of this particular insect cadherin. Determining how the Cry1Ab toxin interacts with BT-R_1 and knowing how the toxin interferes mechanistically with the calcium-induced adhesion phenomenon are essential to identifying the molecular and cellular components of midgut epithelial cell adhesion in *M. sexta*.

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