**Heliothis virescens** and **Manduca sexta** Lipid Rafts Are Involved in Cry1A Toxin Binding to the Midgut Epithelium and Subsequent Pore Formation*

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Lipid rafts are characterized by their insolubility in nonionic detergents such as Triton X-100 at 4 °C. They have been studied in mammals, where they play critical roles in protein sorting and signal transduction. To understand the potential role of lipid rafts in lepidopteran insects, we isolated and analyzed the protein and lipid components of these lipid raft microdomains from the midgut epithelial membrane of *Heliothis virescens* and *Manduca sexta*. Like their mammalian counterparts, *H. virescens* and *M. sexta* lipid rafts are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol-anchored proteins. In *H. virescens* and *M. sexta*, pre-treatment of membranes with the cholesterol-depleting reagent saponin and methyl-β-cyclodextrin differentially disrupted the formation of lipid rafts, indicating an important role for cholesterol in lepidopteran lipid rafts structure. We showed that several putative *Bacillus thuringiensis* Cry1A receptors, including the 120- and 170-kDa aminopeptidases from *H. virescens* and the 120-kDa aminopeptidase from *M. sexta*, were preferentially partitioned into lipid rafts. Additionally, the leucine aminopeptidase activity was enriched approximately 2–3-fold in these rafts compared with brush border membrane vesicles. We also demonstrated that Cry1A toxins were associated with lipid rafts, and that lipid raft integrity was essential for in vitro Cry1A pore forming activity. Our study strongly suggests that these microdomains might be involved in Cry1A toxin aggregation and pore formation.

Cry proteins, major components of parasporal crystals produced by *Bacillus thuringiensis*, are specifically toxic against insect pests and widely used in agriculture as biological insecticides or in transgenic plants (1, 2). These proteins exert their toxic effects through a receptor-dependent process (1, 3, 4). Both glycosylphosphatidylinositol (GPI)-anchored aminopeptidases and cadherin-like proteins have been identified as putative Cry1A receptors. In *Heliothis virescens*, the cadherin-like protein was found to be associated with Cry1A toxin resistance and consequently plays a role in *B. thuringiensis* toxicity (5). In this insect four aminopeptidases, which differentially bind the Cry1Aa, Cry1Ab, and Cry1Ac toxins, are also putative receptors for these toxins (6–9).² Similarly, in *Manduca sexta* and *Bombyx mori*, aminopeptidases and the cadherin-like proteins bind Cry1A toxins as well (10–14). Carbohydrate modification of these proteins may also be critical in insect resistance to Cry1 toxins, because impaired glycosylation was a factor in Cry5B-resistant *Caenorhabditis elegans* (15).

Binding to membrane receptors and subsequent pore formation are critical for Cry toxicity (1). However, the process by which Cry toxin-receptor binding leads to membrane pore formation remains ambiguous. Previous studies suggested that Cry toxin aggregates on the midgut epithelial membrane (16, 17), but the mechanism of toxin aggregation is unknown. With some mammalian pore-forming toxins, lipid rafts play an essential role in toxin aggregation (18–20). These rafts function as platforms to recruit proteins from distinct classes, such as GPI-anchored proteins and palmitoylated or disaccharide transmembrane proteins (21–23). For example, the pore-forming toxin lysenin binds sphingomyelin in raft membranes (24), whereas cholera toxin requires both cholesterol and sphingolipids in rafts for its action (25). Binding to cholesterol by listeriolysin O, another pore-forming toxin, is important in triggering a conformational change required for toxin oligomerization and channel formation (19). Moreover, aerolysin of *Aeromonas hydrophila*, one of the more widely studied pore-forming toxins, functions via a GPI-anchored protein present in lipid rafts (18). In regard to Cry toxins, phospholipase C treatment of *Trichoplusia ni* brush border membrane vesicles (BBMV) cleaved GPI-anchored membrane proteins and reduced pore formation by Cry1Ac toxin (26). Binding of these toxins to their respective membrane receptors, which are preferentially associated with lipid rafts, promotes an increase in local toxin concentration within the cell membrane favoring toxin oligomerization and channel formation.

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1The abbreviations used are: GPI, glycosylphosphatidylinositol; BBMV, brush border membrane vesicle; MβCD, methyl-β-cyclodextrin; NSF, N-ethylmaleimide-sensitive factor; SM, sphingomyelin; PES, ethylamine phospholipid; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TOA, toxin overlay assay; CRD, cross-reacting determinant.

2D. I. Oltean, M. Zhuang, and S. S. Gill, unpublished data.
gomerization required for pore formation, a key step in toxin action.

Lipid rafts are detergent-insoluble microdomains enriched in cholesterol, sphingolipids, and GPI-anchored proteins (22, 27–29). Several lines of evidence show the presence of lipid rafts in vivo (30–34). In fact, the formation of the liquid ordered phase results in membrane insolubility in the nonionic detergent Triton X-100 (22, 27). Hence, insolubility in Triton X-100 has been widely used as a criterion for isolation of rafts from cellular membranes (28, 35).

Although lipid rafts have been widely studied in mammalian cells (27, 29, 35), and have been isolated from Saccharomyces cerevisiae (36, 37), Tetrahymena (38), and Drosophila melanogaster (39), their constituents differ widely between species. Currently there are no data on the nature of lipid rafts from any other insect species, including lepidopterans. In this study, we isolated and characterized lipid rafts from the midgut epithelium of two lepidopteran insects, *H. virescens* and *M. sexta*. Additionally, we investigated the role of lipid rafts in the toxicity of Cry toxins to *H. virescens* and *M. sexta*. We demonstrated that Cry1A toxin-binding molecules, including aminopeptidases and some higher molecular weight bands, and the toxin molecules themselves show differential localization in lipid rafts isolated from these insects. We also showed lipid rafts play an important role in pore formation. This is the first study that suggests a role for lipid rafts in the mechanism of action of Cry toxins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Insects, and Media**—Cry1Aa was produced from wild-type *B. thuringiensis* strain HD73 grown in nutrient broth sporulation medium for 72 h at 30 °C. Cry1Ab was produced from the acrylamidyl strain 4Q7cry transformed with pHT315-cry1Ab and grown in HCO medium (40) for 96 h at 30 °C. Both *H. virescens* and *M. sexta* were reared on artificial diet (Southland Bioproducts and Ref. 41, respectively).

**Purification of Cry1Ab and Cry1Ac Toxins and the Activated Protein Fragments**—Spores and crystals were harvested and washed with buffer containing 0.01% Triton X-100, 50 mM NaCl, and 50 mM Tris-HCl (pH 7.5). The spores were isolated by NaBr gradient as previously described (42), solubilized in 10 mM Na2CO3, pH 10, at 37 °C for 1 h, and then activated with trypsin (1.5 w/v) at 18 °C for 6 h. The proteins were further purified by anion exchange chromatography (SMART, Amersham Biosciences) as previously described (8), and the purified toxins were dialyzed against appropriate buffers.

**Toxin Biotinylation**—Cry1Ab and Cry1Ac toxins, 100 μg, were biotinylated using the protein biotinylation module kit (Amersham Biosciences) and then purified with Sephadex G25 columns. Protein concentration in the collected fractions was determined by BCA (Pierce). The biotinylated toxins were detected with horseradish peroxidase-streptavidin and ECL (Amersham Bioscience).

**Purification of Detergent-insoluble Lipid Rafts**—BBMV were prepared from early (day 1–5th instar) *H. virescens* and *M. sexta* midguts as described (43). Purified BBMV were suspended in TNE buffer (100 mM Tris, pH 7.5, 150 mM NaCl, and 0.2 mM EDTA). An equal volume of BBMV (100 μg) and pre-chilled 2% Triton X-100 were aliquoted into a SW41 tube and mixed. After solubilization for 30 min on ice, the mixture was brought to a final concentration of 60% sucrose and then overlaid with 2 ml each of 50, 40, 30, and 15% sucrose in TNE buffer. The samples were then centrifuged at 2 °C for 18 h at 80,000 × g. Insoluble lipid rafts, present in the middle of the sucrose gradient, were collected from the top of the tube, washed twice with TNE buffer, and centrifuged at 2 °C for 30 min at 150,000 × g. Soluble fractions were collected from the bottom of the tubes. BBMV pretreatments with detergents were performed with 10 mM methyl-β-cyclodextrin (MβCD) (Sigma) at 37 °C for 30 min or with 0.2% saponin (Sigma) on ice for 30 min, as a substitute of acetylation (28). Hyperosmolar and hypotonic conditions were determined by differential centrifugation into the membrane vesicles and a fluorescence decrease, whereas depolarization has the opposite effect. Dye calibration and determinations of the resting membrane potentials were performed in the presence of valinomycin (2 μM) by successive additions of KCl to raft vesicles (10 μg) suspended in 150 mM N-methyl-N-glucamine chloride (MeGlCtti), 10 mM HEPES-HCl, pH 8.0, buffer (1.8 ml). All measurements were made at
and overnight centrifugation, the \textit{H. virescens} insoluble band (I) was collected from the interface of 50 and 40% sucrose layers, whereas the soluble fraction (S) was collected from the bottom sucrose layer. The \textit{M. sexta} insoluble band was collected from the interface of 40 and 30% sucrose layers. \textit{H. virescens} and \textit{M. sexta} Triton X-100-insoluble lipid rafts migrated to different positions in the sucrose gradient suggesting the lipid components or lipid-protein ratios of these rafts were different. Subsequent lipid analysis supported these observations (see below, and Fig. 3). BBMV subjected to sucrose gradient centrifugation alone were apparently unchanged and consistently isolated in the insoluble fraction (Fig. 1, panels A and B, lanes 2–4). However, addition of Triton X-100 solubilized some proteins, whereas others remained in the insoluble fraction (Fig. 1, panels A and B, lanes 5 and 6). The protein profiles of BBMV from \textit{H. virescens} (HB) and \textit{M. sexta} (MB), soluble fractions (S), and insoluble fractions (I) obtained upon Triton X-100 treatment were significantly different. Two isotypes of \textit{M. sexta} fasciclin, a lipid raft-marker protein (39), were recognized by the \textit{M. sexta} anti-fasciclin antibody and were present exclusively in the insoluble fractions (Fig. 1, panel C, d). Similar results were obtained with \textit{H. virescens} (Fig. 1, panel C, a).
These data confirmed that the Triton X-100-insoluble fractions isolated from sucrose gradients were lipid rafts. In contrast, the 57-kDa V-ATPase B subunit and the 84-kDa NSF homolog were exclusively partitioned into the soluble fraction (Fig. 1, panel C, b–f). In M. sexta, another 97-kDa protein, which is likely the p97/CDC48 homolog (53), was detected exclusively in lipid rafts (Fig. 1, panel C, f). Collectively, these data show that proteins are not uniformly distributed within the plasma membrane, but some are selectively localized into lipid raft microdomains.

Cholesterol Plays a Structural Role in Lipid Rafts—Both MβCD and saponin are cholesterol-depleting reagents. MβCD is an effective extracellular cholesterol acceptor that extracts cholesterol from membranes (54), whereas saponin binds cholesterol and sequesters it from other interactions, but does not extract it from the membrane (55). In our study, pretreatment of the BBMV sample with MβCD and saponin before lipid rafts isolation differentially affected the resultant lipid rafts (Fig. 2, panels A and B). Addition of MβCD resulted in BBMV becoming resistant to detergent solubilization. In addition, the protein profile of the isolated Triton X-100-insoluble fraction from MβCD-treated BBMV was similar to that of the control BBMV (without Triton X-100 treatment), but not to the profile of typical lipid rafts (lanes 4 and 5), whereas further treatment with saponin did not affect protein association with lipid rafts (lanes 6 and 7). Controls were isolated lipid rafts (C and D, lanes 2 and 3, HBLR/MBLR) and lipid rafts undergone the same process as in lanes 4–7 but without MβCD nor saponin further treatment (C and D, lanes 8 and 9). In A–D, each lane contained 10 μg of protein, except in lanes where negligible protein was present, in which case the maximum volume (40 μl) was loaded.
not extract cholesterol from the membrane. Thus, as expected, direct treatment of the isolated lipid rafts with saponin did not affect protein partitioning (Fig. 2, panels C and D, lanes 6 and 7).

Both cholesterol and phospholipids are enriched in lepidopteran lipid rafts (22, 28, 29). However, MβCD and saponin extracted cholesterol and phospholipid differently. Under the experimental conditions used, MβCD extracted 38.7 and 48.1% of cholesterol from *H. virescens* and *M. sexta* BBMV, respectively, but it did not extract phospholipids from BBMV. In contrast, saponin extracted 11.7 and 13.2% of phospholipids from *H. virescens* and *M. sexta* BBMV, respectively, but it did not extract cholesterol from BBMV.

**Lipid Composition**—By using electrospray ionization tandem mass spectrometry, major nonsteroid lipid components of lepidopteran lipid rafts were determined. As summarized in Fig. 3 and Table I, the major non-steroid lipids of lepidopteran insect midgut epithelium lipid rafts are sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). Some of these lipid acyl chains are saturated, which facilitate formation of a liquid order phase when mixed with cholesterol (56). Our data also showed that the lipid acyl chain length from the *H. virescens* and *M. sexta* lipid rafts were shorter (Table I) than those from mammalian plasma membranes (57), but were similar to those from *D. melanogaster* lipid rafts (39).

The major nonsteroid lipid components of both *H. virescens* and *M. sexta* lipid rafts are sphingomyelins. Loss of the head group in sphingomyelins in negative mode results in ion 168, whereas loss of the head group in ethanolamine phosphosphingolipids results in ion 140. Both of these lipids were detected in *M. sexta*, but only sphingomyelins were detected in the *H. virescens*. Fragment 208, a typical derivative of the hexadec-4-sphingenine backbone, was detected in *D. melanogaster* lipid rafts (39). However, in both *H. virescens* and *M. sexta* samples, this ion was not detected. Collectively, these data showed that the lipid components of *H. virescens* and *M. sexta* lipid rafts are similar but not identical. Additionally, the amount of total lipids extracted from the same amount of *H. virescens* and *M.
M. sexta lipids were significantly different. The lipid-protein ratio (w/w, 0.55) from M. sexta lipid rafts was larger than that observed from H. virescens (w/w, 0.42) lipid rafts. Taken together, these differences explain why lipid rafts from H. virescens and M. sexta were isolated from different positions of the sucrose gradients. Differences in the lipid components from these two lepidopteran insects could arise from differences in the diet of these two insects.

**Lipid Rafts Are Enriched in GPI-anchored Proteins**—GPI-anchored proteins are enriched in mammalian lipid rafts (28, 58, 59). Similarly, Western blots showed that, in both H. virescens and M. sexta, most of the GPI-anchored proteins are partitioned into the lipid rafts (I) rather than into the soluble fraction (S). In H. virescens five GPI-anchored proteins of 170, 120, 66, 50, and 35 kDa were recognized by the anti-CRD antibody and partitioned into isolated lipid rafts (Fig. 4, panel A, lane 3), whereas a protein of 180-kDa was present in the soluble fraction (Fig. 4, panel A, lane 2). Western blot analysis with anti-APN180 antiserum suggested this protein is the 180-kDa aminopeptidase. In case of M. sexta, the 180-kDa aminopeptidase, are GPI-anchored proteins (6–9). In case of M. sexta, four GPI-anchored proteins of 170, 120, and 106 kDa were found exclusively in the lipid raft fraction, whereas the 120-kDa protein partitioned into both fractions (Fig. 5, panel A). As for Cry1Ac-binding proteins, the abundant 170- and 120-kDa proteins were preferentially partitioned into lipid rafts (Figs. 4C (a and b) and 5B). Other H. virescens lipid raft-associated Cry1Ac-binding proteins have sizes of 110, 85, and 60 kDa, whereas the 205- and 180-kDa Cry1Ac-binding proteins preferentially were partitioned into lipid rafts (Figs. 4C (c) and 5B).

### Table I

| m/z | Species | Acyl chain |
|-----|---------|------------|
| 659 | SM      | C22:1      |
| 687 | SM      | C24:1      |
| 713 | SM      | C26:2      |
| 715 | SM      | C26:1      |
| 717 | SM      | C26:0      |
| 742 | PE      | C18:0/C18:2|
| 742 | PC      | C16:0/C18:2|
| 753 | SM      | C28:1      |
| 757 | SM      | C28:1      |
| 770 | PC      | C18:0/C18:2|
| 786 | PS      | C18:0/C18:2|
| 837 | PI      | C18:0/C18:0|
| 863 | PI      | C18:0/C18:1|

M. sexta proteins partitioned into both lipid rafts and the soluble fraction (Figs. 4C (c) and 5B). In M. sexta, the 210-kDa Cry1Ac-binding proteins partitioned into both lipid rafts and the soluble fraction, whereas the 120-kDa protein partitioned exclusively into lipid rafts (Figs. 4C (c) and 5C). The rest of the Cry1A-binding proteins, including the 195-, 140- and 130-kDa proteins, were partitioned into the soluble fraction (Fig. 5, panel C, lane 2). Similarly, the M. sexta Cry1Ac-binding proteins of 210, 190, 140, and 106 kDa partitioned into both fractions, whereas the 120-kDa protein was exclusively lipid raft-associated (Fig. 5, panel D).

**Cry1A Toxin Is Associated with H. virescens Lipid Rafts**—Our data showed that several of the Cry1Ac-binding proteins are associated with H. virescens and M. sexta lipid rafts. Cry1Ab does not bind to M. sexta brush border membrane uniformly, but preferentially binds the tip of the microvilli (60). These results suggested that distribution of receptors is not uniform in these membranes and specific microdomains in microvilli could be involved in Cry toxin binding. To determine whether Cry toxin itself is associated with lipid rafts, biotinylated Cry1Ac was incubated with BBMV prior to Triton X-100 treatment. Fig. 6 shows that, at low toxin concentrations (1 nM), most of the Cry1Ac toxin fractionated with lipid rafts (Figs. 6, panel A, lane 4). At 10 nM, higher levels of Cry1Ac toxin were detected in lipid rafts than in the soluble fraction (Fig. 6, panel A, lanes 5 and 6). At even higher toxin levels (40 nM), the toxin was present in both fractions (Fig. 6, panel A, lanes 7 and 8). These data suggest Cry1Ac associated with H. virescens lipid rafts specifically, and the toxin association in lipid rafts was saturated between 1 and 10 nM. Similar experiments performed with M. sexta membrane samples produced the same results (data not shown). Pretreatment of BBMV-Cry1Ac with MβCD and saponin, before Triton X-100 treatment, affected the distribution of Cry1Ac as expected. Pretreatment with saponin caused the lipid raft-associated Cry1Ac to partition into the soluble fraction (Fig. 6, panel B, lanes 3 and 4). However, no change in the association of the Cry1Ac toxin was observed if BBMV were pretreated with MβCD (Fig. 6, panel B, lanes 5 and 6).

**MβCD Disrupts Cry1A Pore Formation Activity**—Cry toxins have been shown to alter K⁺ permeability in liposomes and BBMV (17). We thus tested whether lipid rafts have a role in pore formation. We have shown in Fig. 2 (panels C and D) that MβCD treatment disrupted the isolated lipid rafts; thus, MβCD was used in this experiment. Cry1Ab-dependent K⁺ permeability was determined in lipid rafts isolated from H. virescens and M. sexta before and after extraction of cholesterol by MβCD. As a control to determine vesicle integrity after...
lipid rafts, even though these rafts were iso-

densities of 120 and 106 kDa, which were preferentially associated

with lipid rafts. However, as noted with mam-

M. sexta

fibroblasts, lymphoma, endothelial cells, muscle cells, thymo-

cytes, epithelium, neuron, and T cells (22, 23, 28, 35, 61, 62).

Lipid rafts occur in a variety of mammalian cells, including

MβCD treatment, the effect of the ionophore, valinomycin, on

K+ permeability was analyzed. Fig. 7 shows that the valino-

mycin dependent K+ permeability was only slightly affected

with MβCD treatment in vesicles obtained from lipid rafts

(Table III). In contrast, Cry1AB-dependent K+ permeability

was severely affected by MβCD treatment (Fig. 7 and Table

III). These results show that lipid rafts integrity is essential for

Cry1Ab pore formation activity. At the levels of Cry1Ab used in

these experiments, this toxin was associated with lipid rafts

(data not shown).

DISCUSSION

Lipid rafts occur in a variety of mammalian cells, including

fibroblasts, lymphoma, endothelial cells, muscle cells, thymo-

cytes, epithelium, neuron, and T cells (22, 23, 28, 35, 61, 62).

Their presence was also reported in

cytes, epithelium, neuron, and T cells (22, 23, 28, 35, 61, 62).

fibroblasts, lymphoma, endothelial cells, muscle cells, thymo-

cytes, epithelium, neuron, and T cells (22, 23, 28, 35, 61, 62).

Their presence was also reported in

H. virescens and M. sexta Lipid Rafts

TABLE II

Leucine aminopeptidase activities in isolated lipid rafts obtained from

H. virescens and M. sexta midgut BBMVs

| Sample                  | Aminopeptidase activities | H. virescens | M. sexta |
|-------------------------|---------------------------|-------------|----------|
|                         | µmol/mg/min of protein    |             |          |
| Midgut homogenate       | 0.39 ± 0.08               | 0.53 ± 0.09 |          |
| Unsolubilized BBMVs     | 3.82 ± 0.04               | 3.93 ± 0.05 |          |
| Lipid rafts             | 7.36 ± 0.07               | 10.66 ± 0.11|          |
| Soluble fraction        | 0.031 ± 0.01              | 0.047 ± 0.01|          |

lipids, and a certain level of cholesterol is required to form a

liquid-ordered phase, which is known as lipid rafts (63, 64).

Cholesterol-depleting reagents including MβCD and saponin

disrupt lipid rafts, but they affect the rafts differently. Al-

though saponin caused lipid raft-associated proteins to solubi-

lize upon Triton X-100 treatment, pretreatment with MβCD

casted more membrane proteins to become Triton X-100-insol-

uble, and the resultant insoluble fraction was no longer typical

lipid rafts. We also showed that saponin caused lipid raft-

associated Cry1Ac to partition into the soluble fraction, whereas

MβCD did not affect the distribution of Cry1Ac.

Abrami et al. (20) also observed similar effects with MβCD.

Using immunofluorescence to detect probeolysin distribution,

they showed MβCD treatment did not affect the ability of the

toxin to bind microdomains, whereas treatment with saponin

affected toxin association with lipid rafts. Therefore, the toxins

were highly enriched in lipid rafts after MβCD treatment, but

not after saponin treatment. It is likely that saponin causes the

receptors and their bound toxin molecules to become evenly

distributed in the plane of the plasma membrane by preventing

any clustering.

Interestingly, direct treatment of isolated H. virescens and

M. sexta lipid rafts with saponin had no effect on protein-raft

association, whereas MβCD caused some of the lipid raft-asso-

ciated proteins to become soluble. This solubilization could

occur because MβCD extracts cholesterol nonspecifically from

the membrane, resulting in insufficient cholesterol levels to

maintain the liquid-ordered structure of lipid rafts. Because

saponin does not extract cholesterol from the membrane, the

lipid raft structure was weakly maintained even with saponin

treatment. But addition of Triton X-100 did disrupt the sapon-

in-treated rafts (data not shown).

Moreover, quantification of cholesterol and phospholipid lev-

els in detergent-treated BBMV revealed significant differences

of these two cholesterol-depleting reagents. MβCD extracts

cholesterol from BBMV, but saponin prevents interactions be-

between cholesterol and other lipids/proteins without extracting

it from BBMV membranes, and these interactions have profound

effects on formation of lipid rafts (21, 29). Both chole-

sterol and phospholipids are enriched in lipid rafts. However,
cholesterol levels in lipid rafts vary widely because its role in maintenance of lipid raft structure is remediable by saturated long fatty acyl chains of phospholipids and sphingomyelins (55, 56). Thus, partial depletion of membrane cholesterol by MζCD pretreatment of BBMV does not completely disrupt the isolation of lipid rafts. On the other hand, pretreatment of BBMV with saponin not only prevents interactions between cholesterol and other lipids/proteins, it also extracts phospholipids from BBMV, which accordingly disrupts further isolation of lipid rafts with Triton X-100.

Clustering of sphingolipids and cholesterol in the form of lipid rafts can recruit a specific set of membrane proteins and exclude others (21, 22, 65). These lipid rafts could act as platforms for increased concentration of receptors to interact with

**FIG. 5.** TOAs of *H. virescens* (A and B) and *M. sexta* samples (C and D) with Cry1Ab and Cry1Ac. Each lane contains 10 µg of protein. HB, *H. virescens* BBMV; MB, *M. sexta* BBMV; S, soluble fractions; I, insoluble fractions isolated from the sucrose gradient. A, TOA of *H. virescens* samples with Cry1Ab. B, TOA of *H. virescens* samples with Cry1Ac. C, TOA of *M. sexta* samples with Cry1Ab. D, TOA of *M. sexta* samples with Cry1Ac. Anti-Cry1 antibody detected the bound toxin.

**FIG. 6.** Association of biotinylated Cry1Ac toxins with *H. virescens* lipid rafts. Each lane contains 10 µg of protein. A, at 1 nM, Cry1Ac was only associated with *H. virescens* lipid rafts (lane 4). With increasing toxin concentrations, some of the toxins partitioned to the soluble fraction (lanes 5–8). B, association of Cry1Ac with *H. virescens* lipid rafts was disrupted by saponin. With saponin pretreatment prior to isolation of lipid rafts by Triton X-100, all the Cry1Ac toxins partitioned to the soluble fraction (lane 3). MζCD-pretreated sample showed that Cry1Ac remained in the insoluble fraction (lane 6).
H. virescens and M. sexta Lipid Rafts

Fig. 7. Effect of MβCD on the K+ permeability of raft membrane vesicles isolated from H. virescens (A) and M. sexta (B). Upper panels show the K+ permeability induced by valinomycin, and lower panels show that induced by activated Cry1Ab. Changes in the distribution of a fluorescent dye (3,3′-dipropylthiodicarbocyanine) sensitive to changes in membrane potential were recorded as described under “Experimental Procedures.” Midgut juice-activated Cry1Ab toxin (50 nM) was added to membrane vesicles, which were previously loaded with 150 mM KCl, 10 mM HEPES-HCl, pH 8.0, and suspended in 150 mM MeGluCl, 10 mM HEPES-HCl, pH 8.0, buffer (1.8 ml). The arrow on top of the traces corresponds to the time of valinomycin or toxin addition. An upward deflection indicates a membrane potential depolarization, whereas a downward one indicates a hyperpolarization. AFU, arbitrary fluorescence units. Final K+ concentrations (mM) were as follows: 4 (1), 8 (2), 12 (3), 16 (4), 32 (5), and 64 (6).

TABLE III
Pore formation by Cry1Ab in membrane vesicles of lipid rafts isolated from H. virescens and M. sexta midgut

|                         | Slope arbitrary fluorescence units/K+ equilibrium potential |
|-------------------------|-----------------------------------------------------------|
|                         | −MβCD                                                     | +MβCD                                                |
| Valinomycin-dependent K+ permeability |                                                           |
| H. virescens lipid rafts | 0.13 ± 0.02                                               | 0.13 ± 0.02                                          |
| M. sexta lipid rafts    | 0.22 ± 0.01                                               | 0.19 ± 0.03                                          |
| Cry1AB-dependent K+ permeability |                                                           |
| H. virescens lipid rafts | 0.043 ± 0.01                                              | 0.015 ± 0.004                                        |
| M. sexta lipid rafts    | 0.091 ± 0.02                                              | 0.010 ± 0.002                                        |

lrigands and effectors on both sides of the membrane. This would allow for efficient and rapid coupling of receptors to the effector system and prevent inappropriate cross-talk between pathways (22). Our data showed that proteins associated with lipid rafts differently. Most of GPI-anchored proteins from H. virescens and M. sexta were preferentially partitioned into the lipid raft fractions, as observed in mammalian lipid rafts.

Lipid rafts also play critical roles in the action of several pore-forming toxins, including aerolysin (18), cholera toxin (25), lysenin (24), and thiol-activated toxins (19, 66–68), such as streptolysin O, lysteriolysin O, and perfringolysin O. Toxin association with lipid rafts probably triggers a conformational change necessary for pore formation. At the juncture between lipid rafts and liquid phase phosphoglyceride domains, unfavorable energetic effects probably locally weaken the lipid bilayer and might favor membrane penetration (18, 20, 27, 64). By providing receptor-binding sites for these toxins, lipid rafts appear to play multiple roles: targeting, promotion of oligomerization, triggering a membrane insertion-competent form, and stabilizing the toxin-induced pore (64, 69).

The currently accepted mode of action of B. thuringiensis Cry toxins suggests that binding of activated Cry toxin to membrane receptors is crucial for pore formation (1). Cry toxin pores are likely to be composed of four to six toxin molecules (52), which suggests that Cry toxin aggregation occurs before the membrane pore is formed, but it is not clear how the Cry toxin aggregates. However, it is clearly established that many of the processes that lead to toxic action of Cry proteins involve the insect midgut epithelium, including activation, toxin insertion, aggregation, and pore formation (17). In this report we show that detergent-insoluble lipid rafts are present in the midgut epithelium of insects susceptible to the Cry1A toxins, and several of the Cry1Ac-binding proteins, including the 120- and 170-kDa aminopeptidases from H. virescens and the 120-kDa aminopeptidase from M. sexta, were preferentially associated with lipid rafts. Interestingly, the H. virescens 180-kDa aminopeptidase, which binds the Cry1Ab toxin is not enriched in lipid rafts. Specific antibodies to the cadherin-like proteins of H. virescens are needed to determine whether these proteins partition into the lipid rafts or the soluble fraction. Our data suggest that Cry1A toxicity is likely mediated via lipid rafts.

Indeed, at very low toxin concentrations, at which toxicity occurs, most of the Cry1Ac toxin was detected in lipid rafts. This toxin-lipid raft association is specific because the toxin partitioned into the lipid raft fraction upon Triton X-100 treatment. When the toxin concentration was increased to 10 nM, some of the toxin was detected in the soluble fraction, suggesting Cry1Ac-lipid raft binding is saturated between 1 and 10 nM.

Lipid rafts also play critical roles in the formation of toxin pores, which are required for insect toxicity. Our study revealed that the integrity of lipid rafts is important for Cry1Ab pore formation activity, because MβCD inhibited toxin-induced K+ permeability in isolated lipid rafts. The effect observed with MβCD was not the result of membrane vesicle disruption because K+ permeability induced by valinomycin was not affected by MβCD treatment. MβCD’s effect on the Cry1Ab pore formation activity could be caused by disruption of lipid rafts that would alter the local toxin concentration and, thus, toxin oligomerization as proposed for other pore-forming toxins (20). Alternatively, we cannot exclude the possibility that cholesterol could have a role in triggering Cry1Ab conformational change that renders the toxin capable of oligomerization and membrane insertion as suggested for listeriolysin O (19). These data collectively suggest that lipid rafts are important elements in the mode of action of Cry1Ab toxin. This study provides supplemental biochemical information to the mode of action of B. thuringiensis Cry toxins.

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Lipid Rafts Are Involved in Cry1A Toxin Binding to the Midgut Epithelium and Subsequent Pore Formation

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