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The *Bacillus thuringiensis* Vegetative Insecticidal Protein Vip3A Lyses Midgut Epithelium Cells of Susceptible Insects

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The Vip3A protein is a member of a newly discovered class of vegetative insecticidal proteins with activity against a broad spectrum of lepidopteran insects. Histopathological observations indicate that Vip3A ingestion by susceptible insects such as the black cutworm (*Agrotis ipsilon*) and fall armyworm (*Spodoptera frugiperda*) causes gut paralysis at concentrations as low as 4 ng/cm^2 of diet and complete lysis of gut epithelium cells resulting in larval death at concentrations above 40 ng/cm^2. The European corn borer (*Ostrinia nubilalis*), a nonsusceptible insect, does not develop any pathology upon ingesting Vip3A. While proteolytic processing of the Vip3A protein by midgut fluids obtained from susceptible and nonsusceptible insects is comparable, in vivo immunolocalization studies show that Vip3A binding is restricted to gut cells of susceptible insects. Therefore, the insect host range for Vip3A seems to be determined by its ability to bind gut cells. These results indicate that midgut epithelium cells of susceptible insects are the primary target for the Vip3A insecticidal protein and that their subsequent lysis is the primary mechanism of lethality. Disruption of gut cells appears to be the strategy adopted by the most effective insecticidal proteins.

The ability of *Bacillus thuringiensis* strains to produce parasporal crystals composed of δ-endotoxins, which are insecticidal proteins, during sporulation has been the subject of numerous studies (13). Recently, a new family of insecticidal proteins produced by *Bacillus* during its vegetative stages of growth (vegetative insecticidal proteins [Vips]) has been identified (23). The Vips bear no similarity to δ-endotoxins. The *vip3A* gene encodes an 88-kDa protein that is secreted into the supernatant fluid by *B. thuringiensis* cultures (9). The Vip3A protein possesses insecticidal activity against a wide spectrum of lepidopteran insects and displays acute bioactivity towards the black cutworm (BCW; *Agrotis ipsilon*), fall armyworm (FAW; *Spodoptera frugiperda*), and beet armyworm (*Spodoptera exigua*) (9). In the case of the BCW, Vip3A affords 260-fold-higher insecticidal activity than some Cry1A proteins reported to be active against the BCW (9, 16).

An important aspect of the biology of insecticidal proteins has been the characterization of the tissue they target as well as the histopathology developed by the susceptible insect upon their ingestion. Ingestion of δ-endotoxins causes swelling and disruption of midgut epithelial cells by osmotic lysis in susceptible insects (10). The factors that may influence the insect host range of insecticidal δ-endotoxins are (i) solubilization of the crystal protein, (ii) processing by midgut proteases, (iii) the presence of specific binding sites on susceptible cells, and (iv) insertion of the bound toxin into the membranes. Immunohistological observations of tissue sections from insect midguts fed δ-endotoxins showed that the insecticidal proteins bind to the brush border membranes (22). Indeed, the ability or inability of the δ-endotoxins to bind midgut epithelium cells is considered a major factor determining their insect specificity (4, 12, 22).

Vips are newly discovered proteins with potent activities against certain target insects (23). The first step in determining their mode of action is to examine their effects on target cells in susceptible insects. In this paper, we present data in terms of behavioral symptoms displayed by the BCW and FAW, two insects highly susceptible to Vip3A, and the European corn borer (ECB), a nonsusceptible insect in the same order. We also document the histopathology experienced by susceptible insects upon ingestion of Vip3A as well as its correlation with processing in insect midguts and the in vivo binding of Vip3A to target cells.

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MATERIALS AND METHODS

Experimental insects and bioassays. Second- and third-instar larvae of three lepidoptera (ECB, BCW, and FAW) obtained from French Ag Laboratories were used in the present study. Larvae were reared on an artificial diet (F9240; Bioserv Inc.) in the presence or absence of different amounts of the Vip3A protein (see the legend to Fig. 1). For gut clearance studies, larvae were placed on small diet cubes with a surface area of about 1 cm^2 which had been previously coated with different amounts of Vip3A mixed with blue food color (Kroger). Feeding behavior and gut clearance activity were monitored by checking the depositions of blue frass by light microscopy. Selected larvae, representative of the results, were photographed. A quantitative measure of gut clearance was obtained by counting the colored frass under the different treatments.

Overexpression of Vip3A in *Escherichia coli*. An *Nco*I restriction enzyme site was introduced at the ATG codon of the *vip3A* gene by standard PCR methods (1) using primers with the sequences 5′-GCCCATGGACAAGAATAAT-3′ and 5′-GAACTAGTTTCTGTAGCAA-3′. The resulting 580-bp fragment was cloned into a T vector (17) and recovered by digesting it with *Nco*I-*Spe*I. This fragment and the 1.8-kb *Spe*I-*Bam*HI fragment comprising the rest of the *vip3A* gene (9) were then cloned into *E. coli* expression vector pET3d (20) by a three-way ligation. The resulting plasmid, pET-Vip3A, was transformed into *E. coli* HMS174(DE3) (20). For expression of the Vip3A protein, *E. coli* cells harboring pET-Vip3A were grown overnight in Luria-Bertani medium at 37°C. Cells were harvested and resuspended in sonication buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, a protease inhibitor. The sonicated extracts were centrifuged at 14,000 × *g* for 20 min at 4°C, and the supernatants were collected. The introduction of an *Nco*I site at the ATG codon changed the second amino acid of the Vip3A protein from Asn (AAC) to Asp (GAC).

Gut juice preparation and proteolysis of Vip3A. Fresh gut juices were collected from third- and fourth-instar larvae of the BCW, FAW, and ECB by dissecting them and extracting their gut contents by pipette as described by Baines et al. (2). The gut juices were centrifuged at 14,000 × *g* for 5 min prior to use. The protein concentration of the gut juices was measured with the Bradford assay (Bio-Rad), using bovine serum albumin as a standard. The proteolytic reactions were performed in 0.1 M glycine-NaOH buffer, pH 10.0, using different amounts of Vip3A-containing supernatants and gut juices. The reactions were

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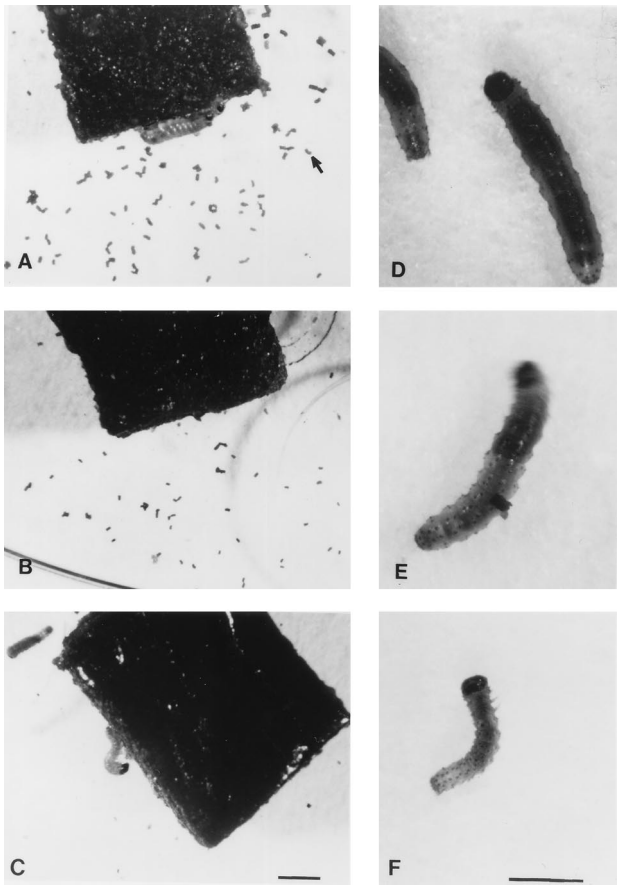

FIG. 1. Gut clearance symptoms in larvae of BCW after ingestion of the Vip3A protein. These experiments were performed twice, and 10 larvae per dose were tested in each repetition. (A, B, and C) Effects of Vip3A on gut clearance were quantified by measuring the amount of frass (arrow) resulting from a diet with no Vip3A (402 and 465 frass in experiments 1 and 2) (A), a diet with 4 ng of Vip3A per cm^2 (106 and 93 frass) (B), and a diet with 40 ng of Vip3A per cm^2 (less than 5 frass in both experiments) (C). (D, E, and F) Close-up of larvae given a diet with different amounts of Vip3A. In healthy larvae, the blue color extends evenly throughout the entire length of the midgut (D); the distribution of blue color is uneven in larvae fed a diet with 4 ng of Vip3A per cm^2 (E); and larvae are significantly smaller and the blue color is missing in the posterior part of the midgut when fed a diet with 40 ng of Vip3A per cm^2 (F). Bars, 2 mm. Scales of panels A, B, and C are identical; scales of panels D, E, and F are identical.

stopped by adding sodium dodecyl sulfate (SDS) loading buffer and boiling the samples for 5 min.

Tissue preparation and sectioning. Larvae (second and third instars) were fed an artificial diet supplemented with *E. coli* extracts containing either no Vip3A protein or 100 to 200 ng of Vip3A per cm^2 of diet cube. After 24, 48, and 72 h of exposure, the larvae were killed and fixed in Bouin Hollande's 10% sublimate fixative for 48 h (3). Following several washes in phosphate-buffered saline (PBS; 150 mM NaCl, 6 mM Na_2HPO_4, 4 mM KH_2PO_4 [pH 7.0]) solution, the larvae were dehydrated in a graded ethanol series to butanol-paraffin and embedded in paraffin. Embedded larvae were sectioned longitudinally at a thickness of ca. 8 μm. Sections were mounted on microscope slides, stained by Heidenhain's "Azan" modification (6), and prepared for photomicroscopy.

Antibody preparation and Western blot analysis. Active soluble pure Vip3A protein was injected intramuscularly into goats in order to produce anti-Vip3A antibodies (9, 11). The antiserum was fractionated by affinity chromatography utilizing staphylococcal protein A (11), and the resulting immunoglobulin G fraction was further purified by filtering it through a column containing immobilized *E. coli* lysate. For Western blot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (11). Nitrocellulose blots were blocked in 20 mM Tris-HCl (pH 7.5)–0.15 M NaCl–0.02% NaN_3–5% nonfat dry milk. Blots were developed by using goat anti-Vip3A antibodies (100 ng/ml). Immunodecorated protein bands were visualized by using a rabbit anti-goat immunoglobulin G secondary antibody (1 μg/ml) conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.).

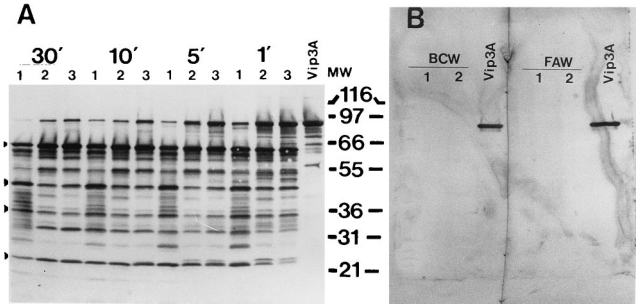

FIG. 2. Proteolytic processing of the Vip3A protein by gut fluids. (A) Gut fluids isolated from the FAW (lanes 1), ECB (lanes 2), and BCW (lanes 3). About 300 ng of Vip3A was incubated with ca. 500 ng of gut fluid proteins at room temperature at pH 10.0. The reactions were stopped after 1, 5, 10, and 30 min of incubation by adding 5× loading buffer (15) and heating at 95°C for 5 min. The samples were separated by SDS-polyacrylamide gel electrophoresis (8 to 16% polyacrylamide gels), blotted onto nitrocellulose, and processed with goat anti-Vip3A antibodies. (B) Lack of cross-hybridization of goat anti-Vip3A antibodies prepared as described in Materials and Methods with whole-insect (lanes 1) or midgut tissue (lanes 2) protein extracts from the BCW and FAW. Fifty micrograms of total protein were loaded in each lane. Vip3A, unprocessed Vip3A protein; MW, molecular weight. Arrowheads indicate the most prominent proteolytic bands derived from the Vip3A protein.

Vip3A protein quantification. Quantitative determination of the amount of Vip3A protein was obtained by enzyme-linked immunosorbent assay (ELISA) (7). Immunoaffinity-purified polyclonal goat and rabbit anti-Vip3A antibodies were used to determine the amount (in nanograms) of Vip3A per milligram of soluble protein from bacterial extracts. The sensitivity of the double sandwich ELISA is 1 to 5 ng of Vip3A per mg of soluble protein when 50 μg of total protein per ELISA microtiter dish well is used. Bacterial extracts were obtained as described previously.

Binding assay and immunocytochemical localization. For immunocytochemical localization studies, the tissue was prepared as described above but the midguts were excised and the peritrophic membrane and its contents were removed prior to fixation.

The midgut sections were deparaffinated and hydrated as follows: twice for 5 min each time in HemoDe (Fisher), three times for 3 min each in 100% ethanol, and once for 1 min in distilled water. Bouin's fixative was removed by washing twice for 2 min each time in 0.5% I_2–1% KI and once for 2 min in 5% $Na_2S_2O_3$. The sections were washed twice in distilled water for 5 min each time and equilibrated in Tris-saline buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM thimerosal [pH 7.6], and 0.1% Triton X-100 [TBST]). The sections were blocked in 2% dry milk in TBST for 30 min and incubated with rabbit anti-Vip3 antibodies purified through protein A-Sepharose and immobilized *E. coli* lysate columns (9) (100 ng/ml) in 2% dry milk in TBST for 2 h. After being washed five times with TBST, the sections were incubated with goat anti-rabbit antibody coupled with peroxidase conjugate (Kirkegaard & Perry) diluted to 1 μg/ml in TBST with 2% dry milk for 60 min. Unbound secondary antibody was removed by washing, and the peroxidase was detected with a diaminobenzidine solution (Sigma Fast diaminobenzidine tablets) for 15 min. Finally, the tissue sections were dehydrated for 1 min in 70% ethanol, twice in 100% ethanol for 3 min each, and three times in HemoDe. The sections were mounted and cleared with Permount media (Fisher). Negative controls were included in all binding studies to evaluate method specificity. The specificity was checked by incubating sections with anti-Vip3A antibody that had been incubated previously with Vip3A, as well as by processing a series of immunocytochemical staining sequences in which regular steps were omitted. All negative controls resulted in the absence of a detectable signal.

RESULTS

Feeding and gut clearance studies. The temporal sequence of symptoms following the ingestion of a Vip3A-containing diet by second-instar larvae of the BCW, a susceptible insect, was recorded from the time of initial administration until larval death. Larvae given a control diet showed active feeding followed by uninterrupted gut clearance (Fig. 1A and D). In contrast, the addition of Vip3A protein to the diet had a significant effect on feeding behavior. When the protein was added at concentrations as low as 4 ng per cm^2 of diet, the larvae fed on and off during periods of 10 to 20 min. The

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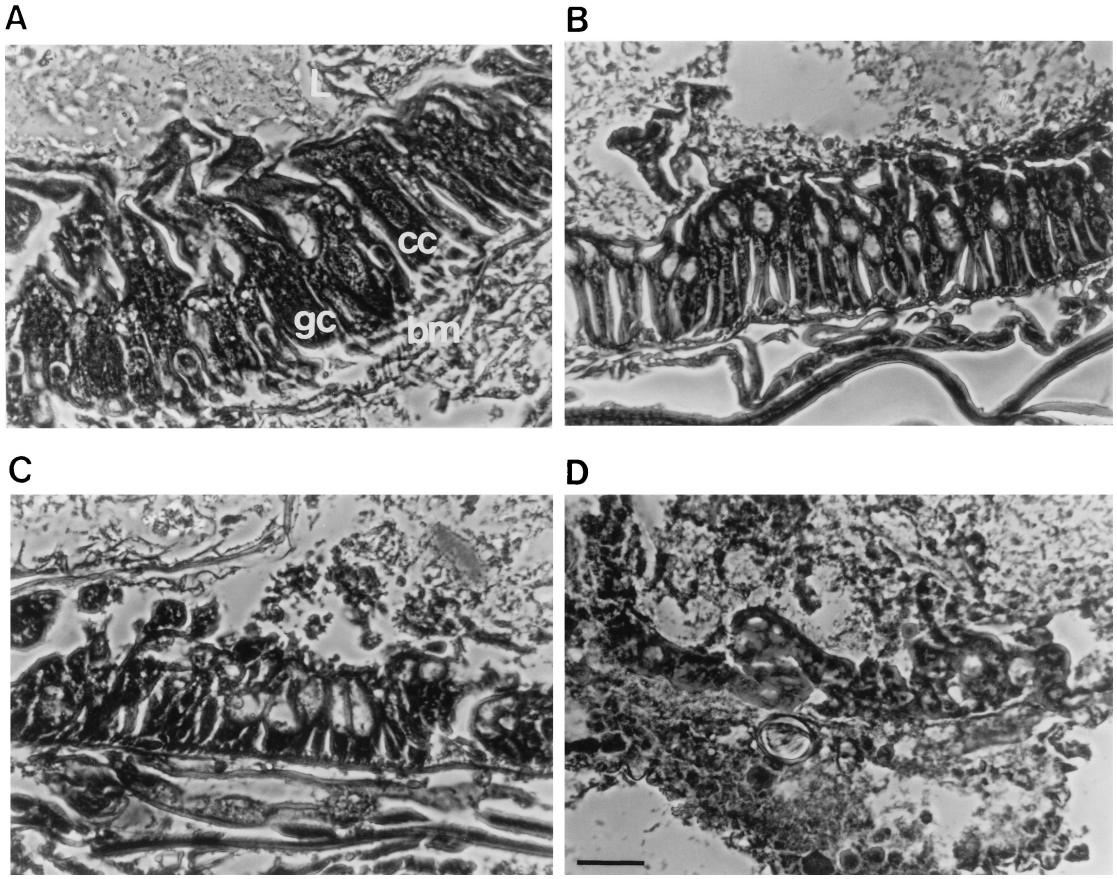

FIG. 3. Histopathology of Vip3A on midgut epithelium in larvae of the BCW. (A) Untreated larval midgut showing normal midgut epithelium with columnar cells (cc) and goblet cells (gc) attached to the basement membrane (bm). The lumen (L) is free of cell debris. (B) After 24 h, Vip3A at 100 to 200 ng per cm^2 of diet induces cell swelling. (C) After 48 h, midgut cell lysis occurs, liberating cell contents into the lumen. The Vip3A-treated larvae are moribund at this stage. (D) After 72 h, the midgut epithelium is not identifiable. The treated larvae are dead. Bar, 25 μm.

presence of blue color in their guts indicated feeding, but the clearance of the gut content was dramatically affected as judged by the amount of frass (Fig. 1B and E). With 4 ng of Vip3 per cm^2 added to the diet, larval development was significantly impaired after a 48-h incubation period but no mortality was observed. At concentrations of 40 ng of Vip3A per cm^2 of diet cube, the larvae suffered gut paralysis upon ingestion of minute amounts of diet and no frass could be seen, indicating an almost complete lack of gut clearance (Fig. 1C and F). Under this condition, ca. 50% mortality was recorded after 48 h. When concentrations higher than 40 ng of Vip3A per cm^2 of diet cube were used, the larvae were moribund after only a few bites, with no frass and mortality rates approaching 100%. When similar experiments were conducted with the FAW, also a susceptible insect, similar behavioral patterns were observed (data not shown). In contrast, the ECB did not alter its feeding behavior when Vip3A was added to the diet even at concentrations as high as 400 ng of Vip3A per cm^2.

Stability of the Vip3A protein in midguts and insecticidal activity. In vitro proteolysis studies were conducted to characterize both the stability and processing of the Vip3A protein in the midgut environment of susceptible (BCW and FAW) and nonsusceptible (ECB) insects. When equal amounts of Vip3A (88.5 kDa) are incubated with BCW, FAW, or ECB gut fluids, four major Vip3A proteolysis products (bands of approximately 62, 45, 33, and 22 kDa) can be identified (Fig. 2A). Western blot analysis of the whole-insect and midgut tissue extracts from two of the insects (BCW and FAW) probed with the same purified anti-Vip3A antibody revealed no background bands (Fig. 2B). With equivalent amounts of gut fluid based on protein concentration, gut fluids isolated from the BCW and FAW seem to proteolyze Vip3A slightly faster than do those of the ECB (Fig. 2A), but the proteolytic patterns are nearly identical after 30 min of incubation. In order to study the relationship between proteolytic processing and insecticidal activity of the Vip3A protein, bioassays were conducted following the incubation of Vip3A with gut fluids isolated from the BCW, FAW, or ECB (Table 1). As expected, Vip3A processed by BCW and FAW gut fluids was active against these two susceptible insects. Interestingly, Vip3A processed by ECB gut fluid was active against the BCW and FAW (Table 1). None of the Vip3A processed forms showed acute activity against the ECB, a nonsusceptible insect.

Histopathological observations of the effects of the Vip3A protein. Histopathological observations of the effects of the Vip3A protein on the BCW were conducted on second- and third-instar larvae which had been fed a diet containing Vip3A. Analysis of BCW gut cross sections showed extensive damage to the midgut epithelium, indicating that the midgut tissue is a primary site of action of the Vip3A protein (Fig. 3). No damage was discernible in the foregut and hindgut (data not shown). Midgut epithelial cells from untreated larvae were closely associated with one another, showing no evidence of damage (Fig. 3A). Sections from larvae that had been fed for

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TABLE 1. Insecticidal activity associated with processed Vip3A protein[a]

Treatment[b]	Mortality (%)[c]		
	BCW	FAW	ECB
E. coli extract	5	3	5
E. coli-Vip3A	100	95	7
BCW gut fluids	7	5	5
FAW gut fluids	5	5	5
ECB gut fluids	7	7	5
Vip3A (BCW processed)	100	100	5
Vip3A (FAW processed)	100	100	10
Vip3A (ECB processed)	100	95	5

[a] Diet incorporation assays contained supernatants from sonicated *E. coli* extracts (1 mg of total protein per ml). The amount of Vip3A added to the bioassays was about 200 ng per cm^2.

[b] Processed samples were incubated with gut fluids (about 10 μl) from each of the insects for 60 min under the conditions described in Materials and Methods. Vip3A processing was assessed by Western blot analysis and was complete in all cases. As a control, about 10 μl of gut fluids was added to 2 ml of the diet.

[c] Mortality was scored 6 days after insect larvae were applied to the diet. The results are the averages of at least two trials with a minimum of 20 larvae per trial.

24 h with a diet containing Vip3A showed that distal ends of the epithelium columnar cells had become distended and bulbous (Fig. 3B). Although the goblet cells exhibited some morphological alterations, they did not show signs of damage at this stage (Fig. 3B). Degeneration of the epithelium columnar cells continued such that after 48 h of ingestion of a Vip3A-containing diet, the lumen was filled with debris of disrupted cells (Fig. 3C). The goblet cells also exhibited signs of damage after 48 h, but both types of cells were still attached to the basement membrane. BCW larvae were dead at 72 h, and desquamation of the epithelial layer was complete (Fig. 3D). While a similar histopathology was observed for the FAW, the ECB did not exhibit any tissue damage under similar experimental conditions (data not shown).

In vivo immunolocalization of the Vip3A protein. Third-instar larvae of the BCW and ECB fed an artificial diet supplemented with 100 to 200 ng of Vip3A per cm^2 were used for immunocytochemical characterization of Vip3A binding to midgut sections (Fig. 4). The bound Vip3A was visualized with rabbit anti-Vip3A antibodies previously purified through protein A-Sepharose and immobilized *E. coli* lysate columns (9, 11). Vip3A binding was detected in midgut epithelia of the BCW (Fig. 4A and B), while no binding to ECB midguts was found (Fig. 4C and D). Midgut sections from BCW larvae fed a control diet showed no Vip3A binding (data not shown). Vip3A binding seems to be specifically associated with the apical microvilli (Fig. 4B) and mostly associated with columnar cells, with no detectable signal in goblet cells.

DISCUSSION

The Vip3A protein is a novel insecticidal protein with activity against a broad spectrum of lepidopteran insects (9). In this study we report on gut clearance and histopathological effects

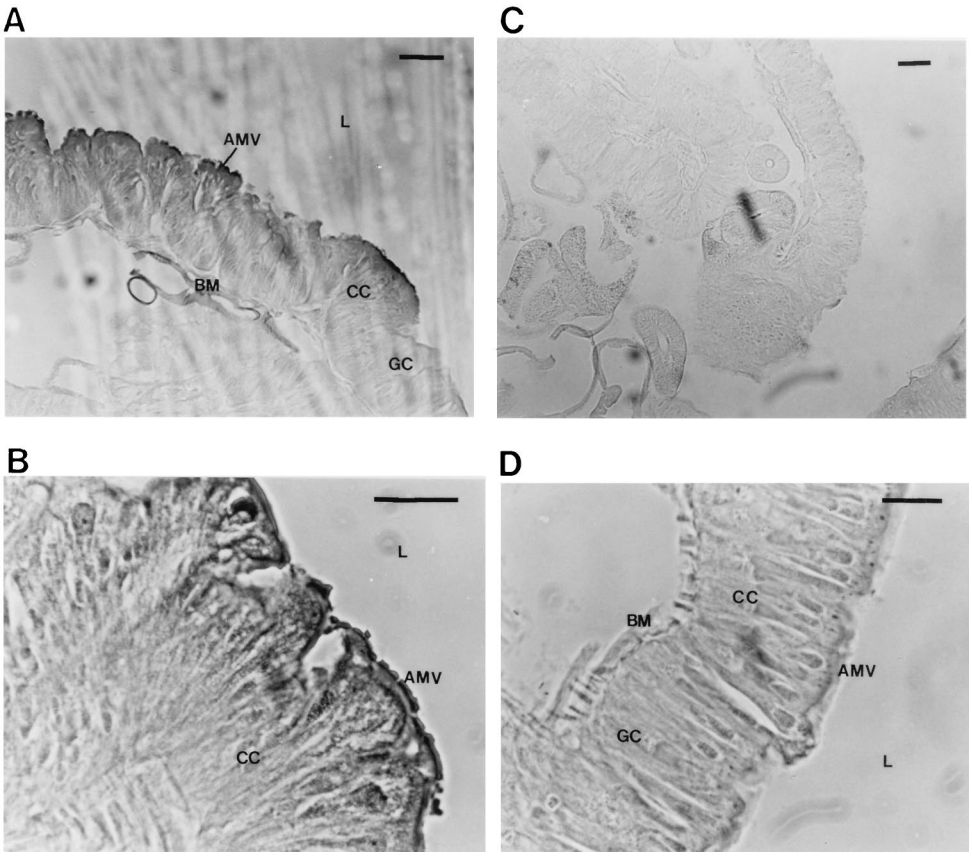

FIG. 4. In vivo immunolocalization of Vip3A bound to midgut tissue sections of BCW (A and B) and ECB (C and D) larvae. Larvae were fed a Vip3A-containing diet for 8 h. L, lumen; AMV, apical microvilli; BM, basement membrane and connective tissue; CC, columnar cells; GC, goblet cells. Bars, 60 μm (A and C) and 25 μm (B and D).

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on insects susceptible to Vip3A following its ingestion, with a particular emphasis on the BCW. Although the symptomatology developed upon Vip3A ingestion resembles that caused by δ-endotoxins (i.e., cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death), its timing seems to be delayed (8, 14, 21). The symptoms provoked by ingestion of Vip3A develop over a period of 48 to 72 h, whereas consumption of δ-endotoxins may cause death of susceptible insects in 16 to 24 h. Nevertheless, the Vip3A protein produces an acute biotoxicity comparable to that afforded by δ-endotoxins.

Numerous studies on the mode of action of δ-endotoxins have shown that solubilization of crystals and processing of δ-endotoxins in the midgut environment followed by binding to the gut cells occurs prior to manifestation of their insecticidal properties (10, 13). Solubilization and/or proteolytic processing seems to be a relatively ubiquitous process occurring in most of the lepidopteran insects that ingest δ-endotoxins (10). In contrast, a close association between the toxicity of a given δ-endotoxin and its ability to bind to the midgut of the insect has been established for insects such as *Manduca sexta*, *Heliothis virescens*, and *Spodoptera littoralis* (4, 12, 22). Although the solubility of the Vip3A protein is compromised at a pH below 7.5, the protein is expected to be in a soluble form in the alkaline midgut environment of lepidopteran insects (19). We have shown that the proteolytic products of the Vip3A protein from gut fluids isolated from the BCW and FAW, two susceptible insects, and those from the ECB, a nonsusceptible insect, are comparable (Fig. 2). Furthermore, in vivo immunolocalization studies have revealed binding of the Vip3A protein only to gut cells of susceptible insects (Fig. 4). These observations are consistent with a correlation between the ability of the Vip3A protein to bind to the midgut cells and its toxicity to the insects.

In conclusion, the histopathology associated with the ingestion of Vip3A resembles the well-documented series of events that follow the ingestion of δ-endotoxins by susceptible insects (8, 14, 21). Of particular interest, midgut cell lysis seems to be the underlying mechanism of toxicity associated with cholesterol oxidase, another insecticidal protein with acute toxicity (18). Considering that the midgut is the organ in insects where critical functions like digestion and absorption take place (5), interference in midgut function by disrupting the gut cells seems to be the strategy adopted by the most effective insecticidal proteins, including Vip3A.

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