Interaction of *Bacillus thuringiensis* Vegetative Insecticidal Protein with Ribosomal S2 Protein Triggers Larvicidal Activity in *Spodoptera frugiperda*^{∇†}

Gatikrushna Singh,¹ Bindiya Sachdev,¹ Nathilal Sharma,²
Rakesh Seth,³ and Raj K. Bhatnagar^{1*}

*Insect Resistance Group, International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India*¹;
*Department of Botany, Meerut College, Meerut, Uttar Pradesh, India*²; and *Department of Zoology,*
*University of Delhi, New Delhi 110007, India*³

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Vegetative insecticidal protein (Vip3A) is synthesized as an extracellular insecticidal toxin by certain strains of *Bacillus thuringiensis*. Vip3A is active against several lepidopteran pests of crops. Polyphagous pest, *Spodoptera frugiperda*, and its cell line Sf21 are sensitive for lyses to Vip3A. Screening of cDNA library prepared from Sf21 cells through yeast two-hybrid system with Vip3A as bait identified ribosomal protein S2 as a toxicity-mediating interacting partner protein. The Vip3A–ribosomal-S2 protein interaction was validated by *in vitro* pulldown assays and by RNA interference-induced knockdown experiments. Knockdown of expression of S2 protein in Sf21 cells resulted in reduced toxicity of the Vip3A protein. These observations were further extended to adult fifth-instar larvae of *Spodoptera litura*. Knockdown of S2 expression by injecting corresponding double-stranded RNA resulted in reduced mortality of larvae to Vip3A toxin. Intracellular visualization of S2 protein and Vip3A through confocal microscopy revealed their interaction and localization in cytoplasm and surface of Sf21 cells.

Insecticidal proteins produced by strains of *Bacillus thuringiensis* can broadly be classified into two major categories based on their site of accumulation. Category I consist of proteins that are deposited as crystals in sporangia and are referred to as insecticidal crystalline proteins (ICPs). The second category consists of recently described group of insecticidal proteins, called vegetative insecticidal proteins (8). These proteins are synthesized during the vegetative growth of *Bacillus* cells and are secreted into the culture medium. Irrespective of the site of accumulation of insecticidal proteins, their ingestion by susceptible insect larvae leads to disruption and lysis of epithelial tissue from the midgut, resulting in larval death (12). The mechanism of lysis of gut epithelial tissue by ICPs has been investigated in detail in several insects (16). Ingestion of ICPs triggers a sequence of biochemical cascade that involves its solubilization and subsequent activation by gut proteases. The activated toxin interacts with specific receptors located at the midgut epithelial tissue. In this sequence of events, the interaction with the receptor is the most significant event since subsequent to interaction, pore formation is initialized, and that leads to lysis of epithelial cells. The identification and characterization of receptors from various insect larvae has led to the identification of following molecules as receptor to ICPs, such as cadherinlike protein (21), glycosyl phosphatidylinositol (GPI)-anchored aminopeptidase N (APN) (1, 9, 11, 17, 19, 20),

a GPI-anchored alkaline phosphatase (10, 14), and a 270-kDa glycoconjugate (see references 2, 7, 9, and 16 and references therein for an extensive list of receptors). In addition, certain glycopeptides have been identified as lysis-initiating receptor molecules. Although there is extensive information about the receptor-toxin interaction for ICPs, negligible work has been done toward the identification of receptors to vegetative insecticidal proteins. The ultrastructural changes induced at the midgut epithelial tissue, upon ingestion of ICPs or Vip3As, are common (12). Both ICPs and Vip3As interact at the epithelial layer of midgut, enlarging the affected cells due to osmotic imbalance and eventually causing lysis. In spite of inflicting nearly identical structural damage, the interacting receptor for the Vip3A is not identical (12). In fact, the receptor to Vip3As has not yet been characterized.

Our group has been working on the identification, cloning, and evaluation of vegetative insecticidal proteins from strains of *B. thuringiensis* held in our collection. We have characterized the Vip3A (EMBL accession no. Y17158) class of protein and evaluated its toxicity profile (2, 8, 18). Vip3A is active against larvae of *Spodoptera litura*, among several other lepidopteran pests. In a parallel series of experiments, we identified APN as a receptor to the *B. thuringiensis* protein Cry1C in *S. litura*. The heterologously expressed APN did not interact with Vip3A, suggesting that Vip3A toxicity in this insect is not through interaction with APN (1). Our preliminary results on the toxicity of Vip3A revealed that purified insecticidal protein could lyse Sf21 cells, suggesting the presence of receptors in the insect cell line. In the present study, we identified the Vip3A interacting protein in Sf21 cells and the larvae of *S. litura*. The specificity of the interaction has been examined by a com-

* Corresponding author. Mailing address: International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India. Phone: 91-11-26741358, ext. 362. Fax: 91-11-26742316. E-mail: raj@icgeb.res.in.

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bination of *ex vivo* and *in vitro* assays. These assays identified ribosomal S2 protein as the interacting partner of Vip3A. The functional significance of S2-Vip3A protein interaction was examined by monitoring the reduction in Vip3A toxicity in Sf21 cells and larvae of *S. litura* by the RNA interference-induced knockdown of S2 protein. The results of these experiments are discussed in the context of colocalization of the S2-Vip3A protein interacting complex by confocal microscopy.

MATERIALS AND METHODS

Expression and purification of Vip3A protein in *Escherichia coli*. (i) The *vip3A* gene was cloned in pQE30 vector (Qiagen), resulting in a His₆ fusion, and transformed into *E. coli* M15 cells for Vip3A expression, which used for the generation of antibody and *in vitro* interaction assays. (ii) The *vip3A* gene was also cloned in pGEX4T-1 vector (GE Healthcare), resulting in a glutathione S-transferase (GST) fusion, and transformed into *E. coli* BL21(DE3) cells for Vip3A protein expression, which was used for toxin treatment of Sf21 cells.

His-tagged Vip3A protein was purified by using a Ni-NTA affinity column (Qiagen), whereas GST-Vip3A protein was purified by using a GST-Sepharose affinity column (GE Healthcare). The affinity purified His₆-Vip3A and GST-Vip3A fractions were dialyzed against 10 mM Tris (pH 8.0) containing 100 mM NaCl at 4°C with three buffer changes. The GST tag was also cleaved by thrombin from GST-Vip3A protein and purified further by GST-Sepharose affinity column (GE Healthcare). The purified proteins were quantified according to the Bradford protein quantitation method (4) and also used in subsequent experiments and for raising antibodies in rabbits.

Effect of Vip3A protein on the Sf21 cell line. One million Sf21 cells (Invitrogen) were seeded onto six-well plate. Purified Vip3A protein (500 ng/ml) was mixed with serum medium and added to the cell lines. The effect of Vip3A protein on Sf21 cells was observed and analyzed at different time points. Only buffer A (10 mM Tris [pH 8.0] containing 100 mM NaCl) was added to the cells, which served as a control. Averages of triplicate samples were determined. The experiment was performed more than three times, and the data from a single representative experiment performed in triplicates are presented.

The effect of Vip3A protein on both cell lines was visualized by using a Nikon Eclipse TE 2000-U microscope (×20). The mortality of the cells was observed by trypan blue staining, and the cells were counted with a hemocytometer.

Construction and screening of the Sf21 library. Total cellular RNA was isolated from the Sf21 cell line by using TRIzol reagent as directed by the manufacturer (Invitrogen). Genomic DNA contamination was checked by PCR using β-actin primers. Using total cellular RNA, double-stranded cDNA (ds-cDNA) was synthesized and purified by using a Matchmaker library construction and screening kit (Clontech). *Saccharomyces cerevisiae* AH109 (*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL-HIS3*) was cotransformed with 10 μg of purified ds-cDNA and SmaI-linearized pGAD-T7 vector (Clontech) by using the lithium acetate method (Clontech). The transformed cells were plated on synthetic drop-out plates (SD) lacking leucine amino acid. The SD plates were incubated at 30°C for 72 h, and the transformants were pooled in 25% sterile glycerol.

The full-length *vip3A* gene (2,370 bp) was cloned into pGBKT7 vector (Clontech), resulting in an N-terminal in-frame fusion of the GAL4 DNA-binding domain (BD). The Sf21 cDNA library was cotransformed with pGBKT7-*vip3A* construct (Bait) with herring testes carrier DNA. The cell suspension was plated on synthetic SD-agar plates deficient in leucine (Leu) and tryptophan (Trp). The growing colonies were restreaked on SD plates deficient in Leu, Trp, and histidine (His) and further on plates deficient in Leu, Trp, His, and adenine (Ade), respectively, to select cotransformants. Individual colonies were tested for β-galactosidase activity in a filter lift assay.

Generation of *in vivo* dsRNA-expressing vector. To generate double-stranded RNA (dsRNA) expression plasmid, the opIE2 promoter was amplified and introduced into pIZT-V5/His (Invitrogen) vector at AgeI and XbaI sites on reverse orientation (see Fig. S2 in the supplemental material). The pIZT-V5/His+opIE2 construct (pRBK-1/GFP-His) was transformed into *E. coli* DH5α competent cells and plated on modified LB-agar plates containing 0.5% NaCl and zeocin (30 μg/ml). The overnight-grown colonies were screened by colony PCR, restriction digestion, and DNA sequencing.

To express S2 dsRNA in the Sf21 cell line, the S2 gene was amplified (877 bp) and ligated into pRBK-1/GFP-His vector at the BamHI and NotI sites. The

construct was transformed into *E. coli* DH5α competent cells, and colonies were screened as described above.

Generation of S2-silenced Sf21 cell line. Sf21 cells were grown at 27°C in TNM-FH medium supplemented with 10% fetal bovine serum and gentamicin (BD Biosciences). At 2 h prior to transfection, the Sf21 cells (80 to 90% confluent) were scraped, and 1 million cells were seeded into each well of a six-well culture plate. Transfection was carried out with 1 μg of plasmid DNA (pRBK-1/GFP-His+S2) (see Fig. S2 in the supplemental material) and Cellfectin reagent as directed by the manufacturer (Invitrogen). At 4 h posttransfection, 2 ml of serum medium was added to the culture plate. The cells were scraped and reseeded into a 100-mm plate (20% confluent) containing serum medium. Finally, the cells were selected with 750 μg of zeocin/ml. Nontransfected cells were eliminated at 1 week postselection. Only vector pRBK-1/GFP-His was transfected independently, which served as a control.

One million S2-silenced cells were used to examine the toxicity of Vip3A and/or Cry1C protein. The cells were exposed to 500 ng of Vip3A protein/ml, and the lysed cells were analyzed at different time points.

Expression and purification of S2 protein in *E. coli*. (i) The S2 gene was cloned in pET28a vector (Novagen), resulting in a His₆ fusion, and transformed into *E. coli* BL21(DE3) cells for S2 protein expression, which used for the production of antibody in BALB/c mice. (ii) The S2 gene was also cloned in pGEX4T-1 vector (GE Healthcare), resulting in a GST fusion, and transformed into *E. coli* BL21(DE3) cells for S2 protein expression that was subsequently used for *in vitro* interaction studies.

His-tagged S2 proteins were purified by using a Ni-NTA affinity column (Qiagen), while GST-S2 proteins were purified by using a GST-Sepharose affinity column (GE Healthcare). The purified His-tagged S2 protein was used for antibody generation.

Colocalization of S2 with Vip3A toxin. Sf21 insect cells (10⁶) were grown on a coverslip, and the cells were exposed to 750 ng of purified Vip3A toxin/ml for 20 h. After being carefully washed with phosphate-buffered saline (PBS) three times, the cells were fixed with 10% formaldehyde for 30 min at room temperature. After three more washes in PBS, the cells were permeabilized with 100% methanol for 20 min at room temperature. The cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h and exposed to anti-Vip3A antibody (1:1,000) and anti-S2 antibody (1:200). After 30 min of incubation, the cells were washed gently with PBS and 0.05% Tween 20, followed by two washes with 1× PBS. The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-IgG (1:200) and Cy3-conjugated mouse anti-IgG (1:500) in PBS containing 3% BSA for 20 min and then washed successively with PBS containing 0.05% Tween 20 twice, PBS, and distilled water. Coverslips were mounted with a fluorescence preserver (Bio-Rad), and the slides were analyzed by confocal microscopy (Nikon Eclipse Ai).

Vip3A (750 ng/ml)-exposed Sf21 cells were processed and incubated with anti-Vip3A antibody (1:1,000) and mouse prebleed sera (1:200) for 30 min diluted in PBS containing 3% BSA. The cells were processed for FITC and Cy3 labeling as described above. The cells were successively washed with PBS and 0.05% Tween 20, followed by PBS, and then stained with DAPI (4',6'-diamidino-2-phenylindole). These slides were analyzed by confocal microscopy (Nikon Eclipse Ai) and served as a control. In another independent experiment, Vip3A (750 ng/ml)-treated Sf21 cells not exposed to primary antibody were incubated with above-described secondary antibody. These cells were processed for confocal microscopy.

***In vitro* pulldown studies.** For *in vitro* interaction studies, the purified GST-S2 (1 μg) and purified GST protein alone (2 μg) were incubated separately with His-Vip3A (2 μg) for 45 min at room temperature. Equilibrated Ni-NTA matrix was then incubated with both reactions for 1 h at room temperature. The Ni-NTA resin was washed extensively with 10 mM Tris (pH 8.0)–300 mM NaCl (buffer A) containing 30 mM imidazole. Bound protein was eluted with buffer A containing 300 mM imidazole. All of the fractions were analyzed by SDS–12% PAGE.

Preparation and injection of S2 dsRNA. A 500-bp S2 dsRNA was prepared according to a procedure previously described by us (13, 17). The dsRNA was extracted by using TRIzol reagent (Invitrogen). A portion (2 μg) of dsRNA was injected intrahemocoelically into early fifth-instar *S. litura* larvae by using a microapplicator (KDS 200; KD Scientific). A total of 20 larvae, each injected with dsRNA of cysteine protease of *Plasmodium falciparum* (a nonspecific dsRNA control) (13) or a saline solution or diethyl pyrocarbonate (DEPC)-water, served as controls.

Bioassay of *S. litura* larvae. Various doses of Vip3A toxin ranging from 100 ng/cm² to 5 μg/cm² were coated on both sides of a castor leaf disc (area, 3.8 cm²). On sixth-instar day 1, *S. litura* larvae were released into each well and exposed to the toxin for 40 h. The mortality was recorded after 40 h, and the 50% lethal

concentration (LC_{50}) was calculated by Probit analysis. Sixty larvae were tested for each treatment, and the bioassay was replicated three times.

To monitor the growth of the larvae injected with S2 dsRNA on Vip3A toxin, a dose of less than the LC_{50} was used. The larvae injected with S2 dsRNA, saline solution, or DEPC-water were exposed to the Vip3A toxin ($3.8 \mu\text{g}/\text{cm}^2$) for 40 h. After 40 h, the mortalities of the larvae were recorded, and *P* values were calculated.

Semiquantitative RT-PCR analysis of S2 expression in different Sf21 cell samples and the midguts of *S. litura* larvae. The relative abundances of S2 genes in the Sf21 cell line, the S2-silenced Sf21 cell line, and the Vip3A-treated Sf21 cell line and in the midguts of S2 dsRNA, DEPC-water, saline-injected *S. litura* larvae were determined by semiquantitative reverse transcription-PCR (RT-PCR). Total cellular RNA was isolated from the above-mentioned samples by using TRIzol reagent (Invitrogen). Single-step semiquantitative RT-PCR of all RNA samples were performed by using a single-step RT-PCR kit according to the manufacturer's instructions (Qiagen). β -Actin primers were used to normalize the RNA samples, as well as the loading control.

Northern hybridization of S2 small interfering RNA (siRNA) in S2-silenced Sf21 cell lines and S2 dsRNA-injected *S. litura* larvae. Total low-molecular-weight RNA was isolated from the S2-silenced Sf21 cell line, the Sf21 cell line, and the midguts of S2 dsRNA-, DEPC-water-, or saline-injected *S. litura* larvae by using TRIzol reagent (Invitrogen) and a small RNA isolation kit (Ambion). A portion (100 μg) of each low-molecular-weight RNA was resolved by electrophoresis on a 20% polyacrylamide containing 7 M urea. RNA was electrophoretically transferred for 45 min at 60 V onto a Hybond-N⁺ membrane (Amersham Biosciences) and immobilized by UV cross-linking at $1,200 \times 100 \mu\text{J}$ and baking at 80°C for 30 min. The S2 gene probe was labeled with [α - ^{32}P]dCTP by PCR using the S2 forward and reverse primers and was allowed to hybridize at 50°C overnight. After an initial wash in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and second wash in $0.2\times$ SSC–0.1% SDS at 50°C , the membrane was exposed to a phosphorimager screen (Amersham Biosciences) overnight and scanned at 200 μm in a Typhoon-9210 apparatus (Amersham Biosciences).

5' end labeling of RNA oligonucleotide. The 21- and 23-mer siRNA oligonucleotide was designed and chemically synthesized by Dharmacon and labeled with [γ - ^{32}P]ATP using T4 polynucleotide kinase (Invitrogen) as directed by the manufacturer. Labeled siRNA was purified by using a Spin-Prep column (Qiagen).

Preparation of BBMV and detection of S2 protein from the midguts of *S. litura* larvae by immunoblotting. Immunoblot analysis was done to detect the expression level of S2 protein in different *S. litura* larvae following S2 dsRNA, DEPC-water, and saline injection. Brush border membrane vesicles (BBMV) were prepared from the midguts of S2 dsRNA-, DEPC-water-, and saline-injected larvae by MgCl_2 precipitation, as described previously (5). Portions (50 μg) of the BBMV protein were resolved by SDS–10% PAGE and transferred onto a nitrocellulose membrane. After blocking with 3% BSA for 5 h, membrane was incubated with anti-mouse S2 polyclonal antibody (1:1,000 dilution). Alkaline phosphatase-conjugated secondary antibody was used for detection by chemiluminescence.

RESULTS

Toxicity of Vip3A protein to Sf21 cells. Our earlier results had shown that Vip3A was larvicidal to *S. litura* (18). To explore toxicity at the cellular level, we examined the cytotoxicity of Vip3A on *Spodoptera* sp.-derived Sf21 cells. The expressed recombinant Vip3A was purified to near homogeneity, quantified by densitometry, and added to exponentially growing Sf21 cells. The viability of the cells was monitored by using trypan blue staining. An increase in the lysis of Sf21 cell was observed with increasing concentrations of Vip3A (Fig. 1). Nearly 80% cells were lysed upon exposure to 500 ng of Vip3A protein/ml at 60 h (Fig. 1 and 2Ai and Bii). At a 500-ng/ml concentration of Vip3A, cell lysis was a function of time, with complete lysis occurring at 62 h of incubation (Fig. 1). In contrast, control cells not exposed to Vip3A continued to grow, suggesting that lysis in Sf21 cells is a consequence of the presence of Vip3A receptor in this cell line (Fig. 2Ai and Bi).

FIG. 1. Graph depicting cell mortality (%) at various time intervals in different concentrations of Vip3A. The mortalities of Sf21 cells exposed to different concentrations of Vip3A (100 ng, 250 ng, 500 ng, 750 ng, or 1 $\mu\text{g}/\text{ml}$) were determined for different time periods, as indicated.

Identification of interacting partner to Vip3A toxin in Sf21 cells. To identify the receptor to Vip3A, we used the yeast two-hybrid system. The Vip3A-encoding gene was used as a bait in a yeast two-hybrid vector, pGBKT7-vip3A. A cDNA library of Sf21 cells was prepared in the library construction vector pGADT7. The library was screened with pGBKT7-vip3A as bait on selective synthetic drop out media for yeast (see Fig. S1 in the supplemental material). The resulting colonies were successively grown in one (Leu^-), two ($\text{Leu}^- \text{Trp}^-$), three ($\text{Leu}^- \text{Trp}^- \text{His}^-$), and four ($\text{Leu}^- \text{Trp}^- \text{His}^- \text{Ade}^-$) dropout growth media to eliminate weak and fortuitous interactions. The fidelity and strength of interaction between Vip3A and Sf21 library members was further verified on medium containing 20 mM 3-amino-1,2,4-triazole (3-AT) and in an interaction intensity reporter assay with β -galactosidase (β -Gal; see Fig. S1i to vi in the supplemental material). The colonies, which were positive in the β -Gal assay, were processed further for identification of the inserted sequence. Plasmid DNA from 25 recombinant clones was prepared and sequenced. The DNA sequences were analyzed against the genome sequence database of *S. frugiperda* (<http://bioweb.inra.fr/spodobase/>). Analysis of the sequences of interacting clones by tblastx revealed four groups of homologous sequences. A total of 60% of the clones matched the S2 protein (accession no. AY161272), 14% clones matched the scavenger receptor SR-C-like protein (accession no. DQ289583), and 12% were identical to peritrophin (accession no. AY581894), while 8% matched α -mannosidase (accession no. AF005035). For each interacting partner a few full-length clones and some partial clones were isolated. For all subsequent experiments, full-length clones of each interacting partner were used. The full-length clones displayed absolute sequence homology among them, as well as with the sequence deposited in the *Spodoptera* data bank. Sequences generated from other clones did not display significant homology to any gene in the *Spodoptera* data bank.

FIG. 2. (A) Microscopic view of Sf21 cells at 50, 55, and 60 h of growth in TNM-FH medium containing buffer. (i) Control Sf21 cells; (ii) Sf21 cells exposed to 500 ng of Vip3A; (iii) S2-silenced Sf21 exposed to 500 ng of Vip3A. (B) Histograms showing the percentages of dead versus live cells (see description of panel A).

Validation of identified interacting partner. To ascertain the role of identified putative interacting partners that mediates Vip3A toxicity in Sf21 cells, we cloned each interacting partner (S2 protein, scavenger receptor SR-C-like protein, peritrophin, and α -mannosidase) individually into the vector pRKB1-His/GFP. The vector has an opIE2 promoter at both ends of the multiple cloning site (MCS) in opposite orientations (see Fig. S2 in the supplemental material). Consequently, any gene cloned in the MCS generates corresponding dsRNA. These constructs carrying putative receptor gene fragments were transfected individually into Sf21 cells, and the transfected cells were selected with zeocin (750 ng/ml). The zeocin-resistant cells were incubated in the presence of the Vip3A. It was expected that silencing of the putative *vip3A* gene-interacting partner would lessen the toxicity to Sf21 cells. Reversal of cell lysis due to Vip3A was observed in transfected Sf21 cell lines carrying S2 fragment dsRNA (Fig. 2Aiii and Biii, Fig. 3A). On the other hand, silencing of scavenger receptor SR-C-like protein, peritrophin, and α -mannosidase did not alter cell sensitivity to Vip3A. Thus, these putative interacting partners identified through yeast two-hybrid experiments served as controls. In another set of experiments, we evaluated the effect of Cry1C on S2-silenced Sf21 cells. These cells retained sensitivity to Cry1C and at the same time displayed reduced sensitivity to Vip3A, suggesting a lack of interaction of Cry1C with the S2 protein (Fig. 3B). The transcript levels of S2 protein in transfected cell lines were monitored by RT-PCR. The transcript of S2 was reduced by nearly 50%, correlating with reduced toxicity of Vip3A (Fig. 4A). Molecular analysis of the S2-silenced cell line revealed the presence of a 21-mer siRNA corresponding to the S2 gene (Fig. 4B).

In vitro binding study of Vip3A and S2. Further studies of the interaction between the Vip3A and S2 protein were performed using *in vitro* binding studies. Vip3A and S2 proteins were expressed with His tag and GST tag, respectively, and purified to homogeneity. The results of an *in vitro* pulldown assay using these purified proteins are presented in Fig. 5. It is evident from the results that His-Vip3A interacted with GST-S2 and coeluted with the Ni-NTA matrix. The GST control protein processed under an identical elution regimen did not interact with His-Vip3A. The GST protein comes in the flowthrough and His-tagged Vip3A eluted out by 300 mM imidazole from the Ni-NTA matrix.

In vivo association of Vip3A toxin with S2 protein in the Sf21 cell line. The cytotoxicity result revealed that Sf21 insect cells exposed to 750 ng of Vip3A toxin/ml resulted in 100% cell lysis after a 25-h exposure. To monitor cell lysis, the cells were exposed to Vip3A protein (750 ng/ml) for 20 h and examined microscopically. The Vip3A-exposed cells were permeabilized and treated with both anti-Vip3A and anti-S2 antibody raised in rabbits and mice, respectively. After incubation with FITC-conjugated anti-rabbit and Cy3-conjugated anti-mouse IgG antibody, the cells were analyzed. Overlaying these images revealed the colocalization of Vip3A with S2 from Sf21 insect cells, as shown in Fig. 6. The confocal view of these Sf21 cells showed that the Vip3A toxin was mainly localized in the plasma membrane and associated with the S2 protein (Fig. 6c and d, green arrows). A similar colocalization of Vip3A and S2 was also observed in the cytoplasm of Sf21 cells, which suggested the internalization of Vip3A and the subsequent interaction with S2 protein (Fig. 6g and h, red arrows). Colocalization of Vip3A-S2 at the periphery of cells is probably a prelude

FIG. 3. (A) Mortalities of Sf21 cells exposed to Vip3A at various time period. Symbols: $\blacklozenge$, mortality of Sf21 cells exposed to 500 ng of Vip3A/ml; $\diamond$, mortality of S2-silenced Sf21 cells exposed to 500 ng of Vip3A/ml. (B) Mortality of wild-type and S2-silenced Sf21 exposed to 500 ng of the insecticidal protein Cry1C/ml. Mortality was monitored by trypan blue staining at 5-h intervals for 40 h.

to cell lysis (Fig. 6). In addition, to check the possible nonspecific interaction between anti-S2 antibody and Vip3A toxin, anti-Vip3A antibodies were incubated with sera obtained prior to the injection of S2 protein for antibody generation. No

FIG. 5. *In vitro* binding study between the S2 ribosomal protein of Sf21 and Vip3A. Vip3A was expressed as His-Vip3A (lane 1), and ribosomal protein S2 was expressed as a GST fusion protein (lane 2). Lane 3 shows the purified GST. Lane 4, eluate from the Ni-NTA column with input His-Vip3A plus GST-S2; lane 5, flowthrough of input His-Vip3A plus GST-S2; lane 6, Vip3A protein eluate from the Ni-NTA column with input GST protein; lane 7, flowthrough of input His-Vip3A plus GST alone; lane M, marker.

reactivity was observed with any protein of Sf21 cells with preimmune sera. It is evident that Vip3A toxin was localized in the plasma membrane and cytoplasm (Fig. 6). The presence of Vip3A in nuclei was observed by using DAPI staining. The merge view of these cells did not reveal any Vip3A-conjugated FITC in the nuclei of toxin-exposed cells, suggesting the absence of Vip3A in the nuclear region. The cross-reactivity of both FITC- and Cy3-conjugated secondary antibodies was also examined with Vip3A-exposed cells (Fig. 6). These staining controls also clearly ruled out a cross-reaction with a nonspecific protein.

Silencing of S2 reduces the toxicity of Vip3A. To validate the role of S2 in the insecticidal activity of Vip3A toxin, 2 μ g of purified S2 dsRNA was injected into fifth-instar *S. litura* larvae (17), followed by exposure to 3.8 μ g of purified Vip3A toxin/

FIG. 4. (A) Relative abundances of S2 transcripts in Sf21 cells and S2-silenced Sf21 cells exposed to 500 ng of Vip3A/ml. (i) RT-PCR analysis of S2 transcript at various time points; (ii) RT-PCR analysis of β -actin used as a control for corresponding samples; (iii) histogram depiction of the relative abundances of S2 transcript under conditions described above. (B) Northern blot analysis for S2-specific siRNA. Lane M, siRNA (21- and 23-mer) marker; lane 1, S2-silenced Sf21 cell line; lane 2, Sf21 cells.

FIG. 6. Confocal microscopy images for the localization of Vip3A toxin with S2 protein in Sf21 insect cells. Images a, e, i, and m show views of the localization of Vip3A toxin from different fields labeled with FITC-conjugated anti-rabbit IgG. Images b, f, j, and n show views of the localization of S2 protein from different fields labeled with Cy3-conjugated anti-mouse IgG. Images c and g show the bright-field image. Images d, h, l, and p show the colocalization of Vip3A toxin and S2 by merging the three different images. The green arrows points to the colocalization of Vip3A toxin and S2 in the plasma membrane of the cell, and the red arrow points to the internalization of Vip3A toxin into the cell and colocalization at the cytoplasm.

cm². The larvae were observed for growth for 48 h in the control set; ca. 85% of the larvae died within 48 h of Vip3A ingestion. On the other hand, in the experimental set (S2 dsRNA-injected larvae) only 20% mortality was observed. The mortality data were subjected to chi-square analysis, and the values for control and dsRNA-injected larvae were significantly different ($P < 0.0001$; Table 1). Larvae injected with DEPC-water, saline solution, or nonspecific *Plasmodium falciparum* dsRNA (2 μ g) were used as a control. It is clear from the results (Fig. 7) that the transcript of S2 and its expression were reduced in the dsRNA-injected larvae. The reduction in the expression of S2 protein correlates well with the observed reduced toxicity of larvae against Vip3A.

TABLE 1. Percent mortality of larvae after injection of S2 dsRNA (2 μ g) into *S. litura* larvae exposed to Vip3A toxin

Sample	Total no. of larvae	% Mortality ^a
Naive larvae	60	88.33 ^A
Larvae injected with DEPC-water	60	83.33 ^A
Larvae injected with saline	60	86.67 ^A
Larvae injected with nonspecific dsRNA (2 μ g)	60	88.33 ^A
Larvae injected with S2 dsRNA (2 μ g)	60	20.0 ^B

^a The mortality data was subjected to chi-square analysis, and values indicated by superscript letters A and B are significantly different ($P < 0.0001$).

FIG. 7. (A) (i) Histogram showing the levels of S2 transcripts in different *S. litura* larva midguts after the injection of S2 dsRNA. (ii) RT-PCR analysis of the S2 transcript in the midguts of *S. litura* larvae. Lane 1, DEPC-water injected; lane 2, saline injected; lane 3, nonspecific dsRNA injected (2 µg); lane 4, S2 specific, dsRNA injected (2 µg). (iii) RT-PCR of β-actin used as a control of the respective samples. (B) Histogram showing the levels of S2 expression in different *S. litura* larvae midgut. (ii) Immunoblot analysis of S2 protein expression using anti-S2 antibody. Lane 1, DEPC-water injected; lane 2, saline injected; lane 3, nonspecific dsRNA injected (2 µg); lane 4, S2-specific dsRNA injected (2 µg). (C) Northern blot analysis for S2-specific siRNA in the midguts of *S. litura* larvae. Lane M, siRNA (21- and 23-mer) marker; lane 1, DEPC-water injected; lane 2, saline injected; lane 3, S2-specific dsRNA injected (2 µg).

Analysis of S2 protein abundance in S2 dsRNA-injected larvae of *S. litura*. To examine the profile of S2 transcript after the injection of S2 dsRNA, the total RNA was isolated from the midgut of injected and control larvae and normalized by β-actin amplification. RT-PCR analysis of the S2 transcript showed reduction of the specific transcript by nearly 65% (Fig. 7A).

The level of expression of S2 protein in S2 dsRNA-injected larvae was also examined. BBMVs were prepared from the midguts of control and dsRNA-injected larvae. Portions (50 µg) of the total BBMVs proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Probing BBMVs proteins with anti-S2 antibodies revealed a nearly 65% reduction in S2 expression in S2 dsRNA-injected larvae (Fig. 7B).

It has been demonstrated earlier that, after the injection of dsRNA into larvae, the RNA interference pathway is activated, cleaving dsRNA into 21- to 25-mer siRNA (17). To confirm the activation of RNA interference and the formation of S2-specific siRNA in the midguts of S2 dsRNA-injected *S. litura* organisms, low-molecular-weight RNAs were isolated and resolved in urea denaturing gel. The total RNAs were transferred onto nylon membrane and hybridized with a [α - 32 P]dCTP-labeled S2 gene. A radiochromatogram scanning image of these blots revealed the formation of S2-specific 21-mer siRNA only in the dsRNA-injected larvae (Fig. 7C).

DISCUSSION

Insecticidal proteins from *B. thuringiensis* have been extensively used to control the predation of crops by insects. These proteins are generally regarded safe because of their specificity toward the target pest and the lack of activity against mammals. The specific activity of these proteins is a consequence of interaction with receptors that are structurally unique in the targeted pests. A diverse array of housekeeping molecules located in the insect gut have been identified as targets of the crystalline inclusion protein of *B. thuringiensis* (16).

The lack of any specific information on the mode of action of Vip3A prompted us to identify and characterize the interacting partner in a susceptible pest, *S. litura*. We examined molecular activity of Vip3A in larvae of *S. litura* and in the *S. frugiperda*-derived cell line Sf21. Earlier it has been reported that Vip3A is larvicidal against *S. frugiperda* and *S. litura* (8, 18). To elucidate the mode of action of Vip3A in *Spodoptera* species, we used a combination of *in vitro* and *in vivo* investigative mechanisms. Our results on the *ex vivo* interaction of S2 and Vip3A and the reduced toxicity of Sf21 cells with Vip3A upon S2 knockdown and our findings from pulldown assays with S2 and Vip3A indicate that S2 protein is the toxicity-mediating partner of Vip3A. These observations have been further strengthened by data obtained from larvae that had been injected with S2 dsRNA. These S2-silenced larvae dis-

played reduced toxicity against Vip3A exposure and concomitant reduction in the abundance of S2 protein expression.

One of the common features of the toxicity of bacillary insecticidal proteins is that their ingestion by susceptible insect results in the lysis of epithelial tissue of the midgut. Ultrastructural visualization of Vip3A-ingested midgut tissue of susceptible larvae also revealed extensive disintegration of epithelial tissue as a consequence of pore formation (12).

In the recent times, concern has been raised about the possible emergence of resistance in insects against insecticidal proteins. One of the possible strategies suggested to overcome such a threat is the pyramiding of insecticidal toxin proteins that are active against same target insect. Since *Spodoptera* species are sensitive to the insecticidal proteins Cry1C and Vip3A, we examined the cross-reactivities of these proteins with corresponding receptor proteins. The retention of sensitivity to the insecticidal protein Cry1C in S2-silenced Sf21 cells suggested that Cry1C does not interact with the S2 protein. This finding is in agreement with our earlier results, wherein we demonstrated interaction of Cry1C with APN (1, 2, 17). Thus, the divergence of receptors for Cry1C and Vip3A offers a potentially useful combination of insecticidal proteins for integration into strategies for delaying the onset of resistance to insecticidal proteins by *Spodoptera*.

Although Vip3A interacts with S2 protein, the precise mechanism of access of Vip3A to the S2 protein and consequent cell lysis is difficult to speculate. Nevertheless, results obtained by confocal microscopy demonstrate that Vip3A toxin at a non-lethal concentration preferentially localizes on the cell surfaces of Sf21 cells. Most importantly, S2 protein is also visualized on the surfaces of Sf21 cells. Thus, S2-Vip3A interaction is evident on the surfaces of insect cells initially and subsequently in the cytoplasm of cells that are beginning to lyse. That this interaction is mainly localized at the cell surface and in the cytoplasm and is absent in the nuclei is evident from the results of nuclear DAPI staining. In Sf21 cells that are exposed to a lethal concentration of Vip3A or are incubated longer in a sublethal concentration of Vip3A, a direct interaction of Vip3A with S2 can be seen at all surfaces of cells and in the cytoplasm. This Vip3A-S2 protein interaction triggers the lysis of Sf21 cells. The subsequent action of the S2-Vip3A adjunct in the disruption of the membrane and the formation of the pore is not clear. The precise cellular function of S2 protein is not clearly defined; nevertheless, it has been speculated to modulate diverse pathways, including the methylation of the laminin-like receptor, the control of oogenesis in *Drosophila melanogaster*, and the suppression of ribosomal protein synthesis to trigger apoptosis (3, 6, 15, 20). It will be interesting to unravel the subsequent action of S2-Vip3A in disrupting the membrane structures of susceptible insect larvae.

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