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Knockdown of Aminopeptidase-N from *Helicoverpa armigera* Larvae and in Transfected Sf21 Cells by RNA Interference Reveals Its Functional Interaction with *Bacillus thuringiensis* Insecticidal Protein Cry1Ac*[S]

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Aminopeptidase-N (APN) and cadherin proteins located at the midgut epithelium of *Helicoverpa armigera* have been implicated as receptors for the Cry1A subfamily of insecticidal proteins of *Bacillus thuringiensis*. Ligand blot analysis with heterologously expressed and purified *H. armigera* Bt receptor with three closely related Cry1A proteins tentatively identified HaAPN1 as an interacting ligand. However, to date there is no direct evidence of APN being a functional receptor to Cry1Ac in *H. armigera*. Sf21 insect cells expressing HaAPN1 displayed aberrant cell morphology upon overlaying with Cry1Ac protein. Down-regulating expression of HaAPN1 by RNA interference using double-stranded RNA correlated with a corresponding reduction in the sensitivity of HaAPN1-expressing cells to Cry1Ac protein. This clearly establishes that insect cells expressing the receptor recruit sensitivity to the insecticidal protein Cry1Ac, and their susceptibility is directly dependent on the amount of HaAPN1 protein expressed. Most importantly, silencing of HaAPN1 in *H. armigera in vivo* by RNA interference resulted in reduced transcript levels and a corresponding decrease in the susceptibility of larvae to Cry1Ac. BIAcore analysis of HaAPN1/Cry1Ac interaction further established HaAPN1 as a ligand for Cry1Ac. This is the first functional demonstration of insect aminopeptidase-N of *H. armigera* being a receptor of Cry1Ac protein of *B. thuringiensis*.

Bacillus thuringiensis (Bt)[3] is a Gram-positive soil bacterium that produces crystalline inclusions in spore mother cells. These inclusions are composed of proteins known as Cry toxins that are toxic to the larvae of different insect orders and a few other invertebrates. Several strains of Bt are used as alternative insecticides to control the important pests of agricultural crops and also public health pests, especially mosquitoes (1). Incorporation of genes encoding these insecticidal proteins into various crops resulted in widespread cultivation of these transgenic crops. Global cultivation of these Bt-protein expressing crops resulted in economic and environmental benefits. There have been intense efforts to understand the mode of action of these insecticidal proteins. The larvae feed on the insecticidal protein, which upon solubilization in the alkaline midgut of insect is further proteolytically processed to 66-kDa mature protein that binds to specific receptors at the midgut epithelium. The activated monomeric toxin molecules oligomerize and insert into the membrane resulting in the formation of pores. The formation of the insertion pores results in osmotic imbalance of the membrane epithelium leading to collapse of the intestine and finally the death of the insect (2, 3).

Although there is an absolute agreement on the above mentioned scheme of mode of action, the identity of the receptor(s) to these insecticidal proteins and the precise mechanism of formation of the pore in the membrane are not very clear. Two membrane proteins, cadherin and aminopeptidase-N (APN), have been implicated as possible receptors to insecticidal proteins. The putative receptor molecules have been cloned, heterologously expressed, and have been shown to bind Cry1A proteins by ligand blot analysis (4- 11). Although functional proof for cadherin as a Cry1A protein receptor has been demonstrated by cytolysis of insect cells expressing lepidopteran cadherin genes on exposure to Cry1Ab/Cry1Aa (10, 12, 13), a similar characterization of APN- Cry1A interaction has not been investigated yet. The only evidence that lepidopteran APN is a functional receptor to Cry1A comes from elegant studies by Gill and Ellar (14). They expressed APN receptor of *Manduca sexta* in *Drosophila* and demonstrated that the transgenic fly acquired sensitivity to insecticidal protein Cry1Ab, a bioactive protein against *M. sexta*. We have earlier demonstrated the functional relevance of APN of *Spodoptera litura* to Cry1C protein (15) by direct gene silencing strategy using RNAi. In spite of these efforts, a complete molecular characterization of the receptor to Cry1Ac protein in the major polyphagus pest *Helicoverpa armigera* is lacking.

The insect *H. armigera* (family, Noctuidae; order, Lepidoptera) is the most important insect pest affecting a wide range of economically important crops. The insecticidal protein Cry1Ac is extremely effective against this insect. We have reported ear-

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[3] The abbreviations used are: Bt, *B. thuringiensis*; APN, aminopeptidase-N; PBS, phosphate-buffered saline; Cry, crystalline inclusion protein; AP, alkaline phosphatase; dsRNA, double-stranded RNA; RNAi, RNA interference; HaAPN, *H. armigera* APN; DEPC, diethyl pyrocarbonate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RT, reverse transcription.

lier the identification and cloning of aminopeptidases from this insect and have demonstrated HaAPN1 interaction with all the three Cry1A family of proteins (16). Now we demonstrate the specificity and functional interaction of Cry1Ac protein with HaAPN1 by insect cell line (Sf21)-based assays and by specific RNAi both in cell lines and in whole insects. Affinity studies by surface plasmon resonance also revealed tight binding of Cry1Ac with HaAPN1.

EXPERIMENTAL PROCEDURES

Membrane Preparation—The Sf21 cells infected with HaAPN1 virus (16) at a multiplicity of infection of 10 were harvested after 65 h of infection. The cells were washed twice in buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride).The cell suspension was sonicated in cold, and the resultant suspension was centrifuged at 700 × *g* for 10 min at 4 °C. The supernatant was further centrifuged at 100,000 × *g* for 60 min at 4 °C. The pellet comprising of membranes was washed twice in buffer A and finally resuspended in the same buffer, snap-frozen in liquid nitrogen, and stored at −80 °C till further use.

Ligand Blot Analysis—Membrane preparations (10 μg) of Sf-21 cells expressing HaAPN1 along with membranes from healthy uninfected cells were prepared as described earlier (16). The membranes were resolved in 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking the membrane in 3% bovine serum albumin contained in PBS, they were incubated with 200 ng/ml activated Cry1Ac toxin for 1 h. The blot was then washed thrice with PBS and overlaid with Cry1Ac monoclonal antibodies for 1 h. After three washes with PBS, they were overlaid with alkaline phosphatase-conjugated anti-mouse secondary antibodies. The blot was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate after washing three times with PBS.

Solubilization of the Receptor—Membrane extracts prepared from Sf21 cells expressing HaAPN1 were solubilized at 4 °C for 4 h with gentle rocking in buffer A containing anionic detergent (0.05% SDS). The undissolved materials were removed by ultracentrifugation (100,000 × *g* for 60 min) at 4 °C. The detergent from the supernatant containing solubilized HaAPN1 protein was removed by detergent removal kit (Genotechnology, Inc.) and replaced with 0.1% CHAPS. The removal of the detergent was confirmed by detecting the residual detergent using detergent estimation kit (Genotechnology) in the solubilized protein and then checked for fidelity of refolding. Aminopeptidase activity was estimated following protocols described earlier (16). Briefly, a 1-ml reaction mixture contains 67 mM Na_2HPO_4, 67 mM KH_2PO_4, pH 7.5, containing either L-alanine *p*-nitroanilide, L-leucine *p*-nitroanilide, or L-valine *p*-nitroanilide at a final concentration of 4 mM. The amount of product (*p*-nitroanilide) released was measured spectrophotometrically at 405 nm after incubating at 37 °C. The aminopeptidase activity was calculated using a molar extinction coefficient of 9.9 mM *p*-nitroaniline at 405 nm. The membranes prepared from uninfected Sf21 cells were similarly solubilized and used as a control.

Purification of Recombinant Aminopeptidase-N—Solubilized HaAPN1 was resolved through Q-Sepharose anion exchange column (4 × 20 cm). The column was equilibrated with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, and 0.1% CHAPS. A gradient of 150 mM to 1.0 M NaCl was used to elute the bound protein. All the fractions were assayed for aminopeptidase activity and profiled on SDS-PAGE. Protein fractions having HaAPN1 were pooled and dialyzed against 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, and 0.1% CHAPS (buffer B). The dialyzed protein was concentrated using Centricon concentrators (Millipore) to 2 ml. The concentrated protein was further resolved using Superose H12 size exclusion chromatography that was equilibrated with buffer B mentioned above. The purity of HaAPN1 was verified on SDS-PAGE, and the fractions containing purified HaAPN1 were concentrated again using Centricon concentrators. The sample contained a near homogenous preparation (95%) of HaAPN1 protein and was used immediately for interaction analysis with Cry1Ac protein by BIAcore. The purified protein was also visualized by Western chromatography using anti-HaAPN1 antibodies (16).

Surface Plasmon Resonance Analysis of HaAPN1-Cry1Ac by BIAcore—To study the interaction between Cry1Ac protein and HaAPN1, surface plasmon resonance was conducted on BIAcore 2000. The purified APN1 was diluted in 10 mM sodium acetate buffer, pH 4.5, and immobilized on flow cell 2 at a concentration of 20 μg/ml on the carboxymethylated dextran surface of a CM5 sensor chip by amine coupling according to the Biosensor BIA application handbook. HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P-20) was used throughout the analysis. After immobilization, the cell surface was regenerated with 1 M NaOH to remove unbound APN1. The analyte (Cry1Ac) was injected at different concentrations (0 nM-only buffer control, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, and 1600 nM) at a flow rate of 10 μl/min for both association and dissociation. After 300 s of association, analytes were replaced by HBS-EP for at least 300 s to dissociate. As a control, an unrelated protein *Plasmodium falciparum* falcipain was used as an analyte at a concentration of 400 mM and 800 nM. The data were analyzed and fitted using global analysis software available within BIAevaluation 3.1. The curves were fitted to a simple 1:1 Langmuir binding model to obtain apparent rate constants.

Purification of Cry1Ac Protein—The recombinant Cry1Ac toxin was prepared as inclusion bodies by the method reported earlier by Lee (17) from a plasmid pKK223–3 bearing the Cry1Ac gene (Bacillus Genetic Stock Centre, Columbus, OH). The amount of the toxin was quantitated densitometrically by resolving the inclusion bodies on SDS-PAGE. The inclusion bodies containing the toxin were solubilized in 50 mM sodium carbonate buffer, pH 10.4, and activated with trypsin (Sigma) at a 10:1 ratio for 30 min. at 37 °C. This activated toxin was purified by anion exchange chromatography and eluted with 0–1000 mM NaCl gradient in 50 mM sodium carbonate buffer, pH 10.4. This fraction containing Cry1Ac protein was concentrated and simultaneously dialyzed with PBS in a Centricon (Millipore) concentrator to a final concentration of 200 ng/μl.

Culturing of Sf-21 Cells—Sf-21 (Invitrogen) cells were grown and maintained at 27 °C in TNM-FH medium (Pharmingen) containing 10% fetal bovine serum and gentamycin. Cells were

grown as a monolayer up to 70–80% confluence in T-75 (Nunc) tissue culture flasks.

Toxin Overlay of Sf-21 Cells—Actively growing Sf-21 cells were harvested from a 70–80% confluent T-75 flask and transferred to a 48-well tissue culture dish (Costar) at 0.1×10^6 cells per well. After the cells settled down for 2 h, they were infected with HaAPN1 baculovirus (16) at a multiplicity of infection of 0.5 for a period of 1 h. Subsequently the virus was removed, and the cells were overlaid with TNM-FH medium and maintained at 27 °C. After 48 h, the TNM-FH medium was removed, and the cell layer was washed gently twice with PBS, pH 7.4, and overlaid with 150 μl of activated Cry1Ac insecticidal protein in PBS at 25 μg/ml. The cells were also overlaid with *P. falciparum* falcipain to act as a control. The cells were incubated with the proteins for a period of 5 h, after which they were observed under a Nikon TE-300 microscope. The total number of cells swelled in a field was counted in comparison to the total cells present in the field. Final values represent an average of four fields selected for each treatment, and each treatment was replicated three times.

dsRNA Synthesis—The full-length cDNA of *haapn1* (AF521659) from the midgut of *H. armigera* larvae was already reported by us (16). A 565-bp internal fragment of *haapn1* was obtained by PCR using the primers HaAPN1–53F 5′-GTTAGCTCGAGCTGGCATT-3′ and HaAPN1–54R 5′-TGTGGTCTTGAGGCCGAGTCAT-3′. The truncated fragment of *H. armigera haapn1* was subcloned in pGEM-Te and used for the preparation of dsRNA. As a control, the gene for *falcipain* of *P. falciparum* cloned in pGEM-T was used as described earlier (18). The pGEM-Te-cloned fragments were amplified by PCR using vector-specific universal and reverse primers (Promega). The PCR product was purified (Qiagen GmbH) and used as DNA template for dsRNA preparation after the *in vitro* transcription procedure described by us (18). The T7 and SP6 RNA polymerases (MBI Fermentas) were used to generate single strand sense RNA and antisense RNA, respectively, from the DNA. To make dsRNA, equal amounts of sense RNA and antisense RNA were mixed, heated to 65 °C, and annealed by slow cooling over 4 h followed by DNase (Invitrogen) treatment for 15 min at 37 °C. The dsRNA was extracted with phenol-chloroform and precipitated overnight with ice-cold ethanol in the presence of 0.3 M sodium acetate, pH 5.4, at −20 °C. The dsRNA pellet was washed with 75% ethanol and resuspended in DEPC-treated water to a concentration of 1 μg/μl dsRNA.

RNAi in Sf-21 Cells—After the cells were infected with HaAPN1 baculovirus at an multiplicity of infection of 0.5 for 1 h, the virus-containing TNM-FH medium was removed, and the cells were washed twice gently with 500 μl of serum-free medium, Sf900II (Invitrogen) taking care not to disrupt the cells. These cells were overlaid with 10 μg of dsRNA of *haapn1* in 150 μl of serum-free medium and incubated for 2 h at 27 °C. 400 μl of TNM-FH was subsequently added to each well, and the cells were maintained at 27 °C for 48 h. Cells were harvested from each treatment for RNA transcript analysis and Western blot analysis. The other set of cells was processed for toxin overlay as described earlier for toxin overlay of Sf-21 cells. These experiments were replicated four times.

Insect Populations—Continuous population of *H. armigera* was maintained in an insectary with a controlled environment of 25 ± 1 °C, 55–60% relative humidity, a photophase of 14 h, and 10 h of scotophase. The larvae were reared on the chickpea based semisynthetic diet (19). 5th-instar larvae were used for the bioassays.

Micro-injection of dsRNA—About 6 μg (1 μg/μl) of dsRNA of *haapn1* was injected intra-hemocoelically into the 1st-day 5th-instar larvae (130 ± 10 mg). A Hamilton microsyringe attached to a programmable micro-applicator (KDS 200, KD Scientific Inc., New Hope, PA) was used for this purpose. The larvae injected with 6 μl of DEPC-treated water served as control. After 2 days of incubation, the total RNA was isolated from the midgut tissue using TRIzol reagent (Invitrogen) according to manufacturer's protocol. The mRNA was purified from the total RNA by using biotinylated oligo-dT and streptavidin magnetic particles (Roche Applied Science) and was used as template for real time reverse transcriptase-PCR (real time RT-PCR).

Western Blot Analysis of RNAi-treated Sf-21 Cells and Insect Guts—For Western blotting the cells were harvested and processed as per the procedure described earlier (34). The total cell extract was resolved in 7.5% SDS-PAGE and transferred on to nitrocellulose membranes. Whole insect midgut samples stored in Trizol were processed, and their protein fractions were extracted by following the manufacturer's (Invitrogen) instructions. After transfer, the membrane was incubated for 60 min in blocking buffer (3% bovine serum albumin in 1× PBS) and washed three times with 1× PBS. To have a rough estimate on the amount of protein loaded (loading controls), the same samples were resolved on a 10% SDS-PAGE gel and similarly processed as described above. The blot was probed with 1:2500 dilutions of anti-β actin or anti-glyceraldehyde-3-phosphate dehydrogenase antibodies for 60 min. The membranes were probed with 1:2500 dilutions of anti-APN antibodies for 60 min and washed three times with 1× PBS. AP-conjugated goat anti-rabbit secondary antibodies (Calbiochem) diluted 1:5000 in 1% bovine serum albumin was overlaid on the membrane for 60 min. After 3 washings with 1× PBS, the blot was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrates (Roche Diagnostics) in alkaline phosphatase buffer.

Cry1Ac Bioassay on H. armigera 5th-instar Larvae—Inclusion bodies of Cry1Ac toxin were prepared as reported earlier by Lee (17). The amount of the toxin was quantitated densitometrically by resolving the inclusion bodies on SDS-PAGE. Toxin amounts from 500 to 10,000 ng was diluted in 10 mM Tris, pH 7.5, and applied on 1-cm^2 artificial diet. The toxin-coated diet was air-dried and placed in a well of a 12-well tissue culture plate (Nunc Inc). One 5th-instar 3rd-day *H. armigera* larva was released on each well and exposed to the toxin treatment for 24 h. After 24 h the larva was transferred to a fresh diet (without toxin). Mortality was recorded after 4 days, and the LC_{50} value was calculated by Probit analysis using the software Indo Stat (Indostat Services, Hyderabad, India). Ten larvae were tested for each treatment, and the bioassay was replicated three times. LC_{50} values were similarly determined for 5th-instar 3rd-day larvae of *H. armigera* starved for 2 days.

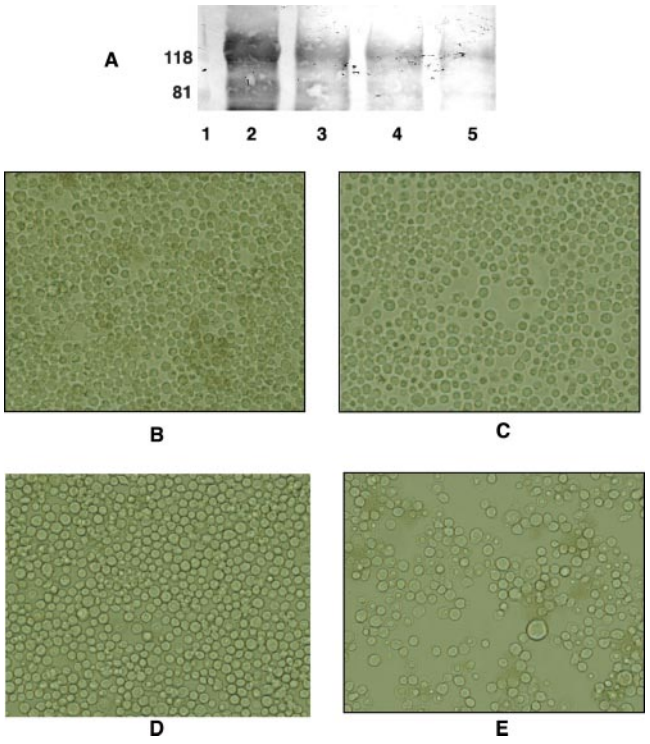

FIGURE 1. **Interaction of insect cells (Sf-21) expressing HaAPN1 with Cry1Ac.** *A*, membrane fractions of Sf-21 cells expressing HaAPN1 at various doses 10 μg (*lane 2*), 7.5 μg (*lane 3*), 5.0 μg (*lane 4*), and 2.5 μg (*lane 5*) along with protein molecular weight pre-stained marker (Bio-Rad) (*lane 1*) were separated on 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and observed for toxin binding ability by overlaying with Cry1Ac protein. The blots were then incubated with monoclonal anti-Cry1Ac antibodies followed by AP-conjugated goat anti-mouse antibodies. The blots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate for visualizing the bands. We observed an increasing intensity with increasing concentration of HaAPN1. Shown are photomicrographs of healthy uninfected cells (*B*), HaAPN1-expressing Sf-21 cells (*C*), HaAPN1-expressing Sf-21 cells overlaid with falcipain (*D*), and HaAPN1-expressing Sf-21 cells overlaid with Cry1Ac (*E*). Cells incubated with Cry1Ac show blebbing/swelling.

Real Time RT-PCR Analysis of RNAi Treatment—Total RNA was extracted from the Sf-21 cells or dissected midguts using TriZol (Invitrogen) reagent following the manufacturer's instruction and subjected to real time RT-PCR. Real time RT-PCR was performed using the Quanti Tect SYBR Green RT-PCR kit (Qiagen Gmbh) and the iCycler TM (Bio-Rad). Primers mentioned earlier and used for the cloning of the truncated fragments were used for the analysis. The real time amplification and analysis was performed in triplicate in PCR reactions of 25 μl final volume containing 7.5 pmol of each forward and reverse primers. Transcript of β-actin was used as the internal reference to normalize the transcripts of dsRNA treatment and control. The β-actin amplicon was obtained by using the primers β-actin F (5′-CAGATCATGTTTGAGACCTTCAAC-3′) and β-actin R (5′-GSCCATCTCYTGCTCGAARTC-3′). Normalized data were used to quantitate the relative levels of a given mRNA in control and dsRNA-injected larvae according to the $\Delta\Delta$Ct analysis (20).

RESULTS

Interaction of HaAPN1-expressing Sf21 Cells with Cry1Ac Toxin—HaAPN1 expressed in the membrane fractions of Sf-21 cells infected with *haapn1* baculovirus showed a positive interaction with Cry1Ac protein on ligand blot analysis (Fig. 1*A*). There was an increase in intensity with an increase in HaAPN1 concentration, suggesting that the interaction was specific and dose-dependent. Preliminary experiments of titrating membrane preparations of Sf21 cells expressing HaAPN1 with anti-HaAPN1 antibodies revealed a positive interacting protein corresponding to the expected size of HaAPN1. The biological functional significance of heterologous expression of HaAPN1 was further investigated by exposing insect cells (Sf21) expressing HaAPN1 to various concentrations of insecticidal protein Cry1Ac. Incubation of live, viable cells expressing HaAPN1 with Cry1Ac induced distinct morphological changes. A significant proportion of cells displayed swelling and lysis, and such effects were not observed in healthy Sf21 cells, suggesting that the expressing HaAPN1 directly interacts with the insecticidal protein, and as a result morphological aberrations are induced (Fig. 1, *B–E*). The number of cells with such alterations after Cry1Ac overlay was counted in four different optic fields. Nearly 32% of cells were either ruptured or were swollen and appeared abnormal (Table 1). The cells when incubated with an unrelated protein to Cry1Ac, falcipain, did not show any morphological aberration.

TABLE 1

Percentage of HaAPN1-expressing Sf-21 cells displaying morphological aberrations after incubation with 3.75 μg of activated Cry1Ac toxin for a period of 5 h

All values are the average of four sets of observations, and each treatment was replicated three times (thus, an average of $4 \times 3 = 12$ observations). All values denote % cells swollen compared to the total number of cells in a field of microscopic view according to the procedure of Kwa *et al.* (27). The assay data were analyzed statistically by a completely randomized design using the software IRRI Stat. Numbers in parentheses represent the range of values for each treatment.

Treatment	% of Sf-21 cells showing aberration with Cry1Ac	% of Sf-21 cells showing aberration with falcipain
Control cells	2.03^{a} (1–3)	1.0^{a} (0–2)
HaAPN1-expressing cells	32.34^{c} (26–41)	1.77^{a} (0.5–3)
HaAPN1-expressing cells subjected to RNAi	9.80^{b} (6–14)	2.03^{a} (1–3)

$^{a-c}$, indicate significant differences from each other at 95% confidence limits. Values superscripted with the same letters are not significantly different from each other, whereas those with different letters are significantly different from each other.

RNAi of haapn1 Virus-infected Sf-21 Cells and Its Effect on Cry Toxin Interaction—The specificity of induced morphological changes observed between HaAPN1-expressing cells and insecticidal protein Cry1Ac was analyzed by modulating the expression of HaAPN1. Sf-21 cells, after infection with *haapn1* baculovirus, were incubated for 2 h with dsRNA of HaAPN1. The abundance of *haapn1* transcript was estimated by RT-PCR, and the amount of expressed protein was estimated by Western blot analysis using anti-APN1 antibodies. Forty-eight hours after dsRNA treatment, the expression of HaAPN1 mRNA and protein was drastically reduced in dsRNA-treated cells as compared with control cells and cells treated with dsRNA unrelated to *falcipain*. The transcript reduction due to dsRNA incubation on HaAPN1-infected cells were around 70% (Fig. 2*A*). A similar reduction was observed in the HaAPN1 protein expression profile after dsRNA treatment (Fig. 2*A*, *inset*). Overlay of Cry1Ac insecticidal protein on such *haapn1* dsRNA-treated Sf21 cells resulted in a significantly low interaction with the insecticidal protein (Table 1). Reduced expression of HaAPN1 directly correlated with reduced lysis of cells (only

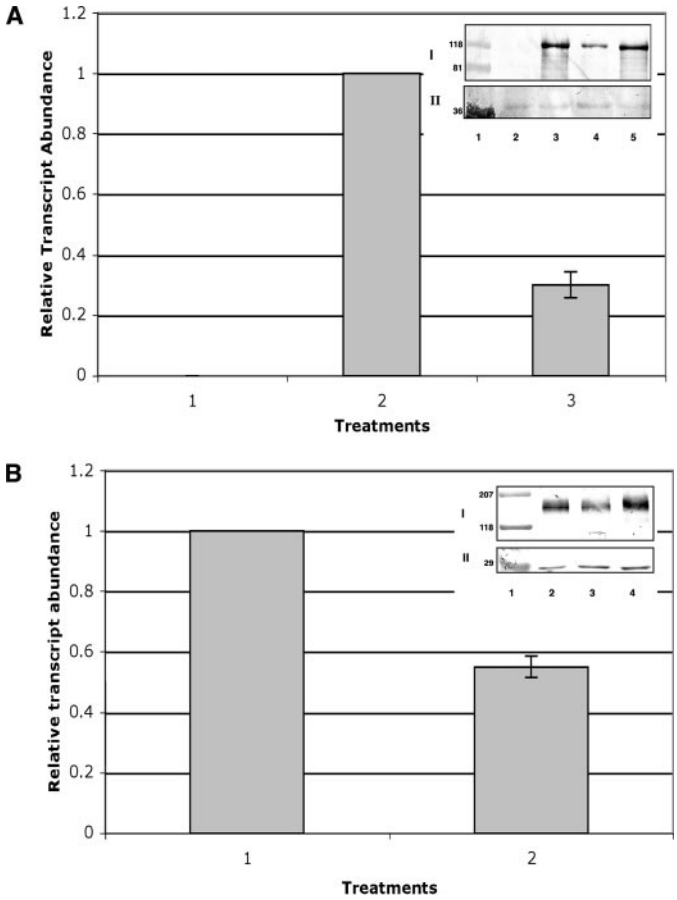

FIGURE 2. **RNAi of *haapn1* in *H. armigera*.** *A*, real time RT-PCR analysis of mRNA extracted from Sf-21 control cells (*lane 1*), cells expressing HaAPN1 (*lane 2*), and from similar cells treated with *haapn1* dsRNA (*lane 3*). Relative transcript abundance was obtained in each of the treatments after normalizing all the samples for equal amount of RNA. *Inset*, Western analysis of Sf-21 cells with HaAPN1 antibodies (*panel I*) and with β actin antibodies (*panel II*). Shown are Bio-Rad prestained protein molecular weight marker (*lane 1*), healthy cells of Sf-21 negative control (*lane 2*), Sf-21 cells infected with HaAPN1 baculovirus (*lane 3*), Sf-21 cells infected with HaAPN1 baculovirus and overlaid with *haapn1* dsRNA (*lane 4*), and Sf-21 cells infected with HaAPN1 baculovirus and overlaid with *falcipain* dsRNA (*lane 5*). *B*, real time RT-PCR analysis of *haapn1* transcripts from midgut RNA of 5th-instar 3rd-day *H. armigera* larvae (*lane 1*) and from similar larvae treated with *haapn1* dsRNA 48 h earlier. *Inset*, Western analysis of *H. armigera* 5th-instar 3rd-day larval midgets with HaAPN1 antibodies (*panel I*) and with glyceraldehyde-3-phosphate dehydrogenase antibodies (*Panel II*). Shown are Bio-Rad prestained protein molecular weight marker (*lane 1*), healthy insects negative control (*lane 2*), healthy insects injected with *haapn1* dsRNA 48 h earlier (*lane 3*), and Healthy insects injected with *falcipain* dsRNA 48 h earlier (*lane 4*).

9.8% of cells were damaged) after Cry1Ac toxin overlay as compared with 32.3% cells damaged in HaAPN1-expressing cells.

Effect of Cry1Ac on H. armigera 5th-instar Larvae—To examine the specificity of interaction between HaAPN1 and Cry1Ac and also the consequences of this interaction on Cry1Ac protein toxicity to *H. armigera* larvae, we first tried to establish the LC_{50} regimen of Cry1Ac on 5th-instar larvae. *H. armigera* 5th-instar 3rd-day larvae (320 ± 10 mg body weight) that were previously injected with 6 μl of DEPC-treated water on the 1st day (130 ± 10 mg of body weight) were exposed to varying doses of Cry1Ac toxin ranging from 1,000 to 10,000 ng/cm^2. The results (Table 2) revealed that the mortality was only 40% 4 days after toxin treatment even at the highest dose, *i.e.* 10,000 ng of Cry1Ac.

TABLE 2

Effect of different doses of Cry1Ac on 3-day-old 5th-instar larvae of *H. armigera*

Three-day-old 5th-instar larvae were used for the bioassays. Different dilutions of Cry1Ac toxin were prepared in 50 mM Tris, pH 7.5, and each concentration was coated on a small piece of semi-synthetic diet (1 cm^2). The diet was placed in a Petri dish, and pre-starved (5 h) larvae were released individually in each dish and allowed to feed on the treated diet completely for 24 h. Subsequently the larvae were reared on a normal diet. Mortality was recorded on the 4th day after toxin treatment, and the lethal concentration 50 (LC_{50}) value was calculated by Probit analysis using the software Indo Stat (Indostat Services, Hyderabad, India) and found to be 2780 ng. The LC_{50} dose was calculated as per the regression equation $y = 0.0204x - 6.7065$; fiducial limits, 2576–3139 ng of Cry1Ac insecticidal protein per larva. Ten larvae were tested for each concentration and replicated three times. The experiment was done at two different situations when the larval body weight was at 320 ± 10 mg (left) and 130 ± 10 mg (right). Numbers in parenthesis represent the range of values for each treatment.

Dose per larvae	Mortality after 4 days (larval body weight, 320 ± 10 mg)	Dose per larvae	Mortality after 4 days (larval body weight, 130 ± 10 mg)
ng	*%*	*ng*	*%*
1,000	0 (0)	500	10 (10)
2,000	0 (0)	1,000	23 (20–30)
3,000	0 (0)	1,500	36 (30–40)
4,000	20 (10–30)	2,000	40 (40)
5,000	20 (20)	2,500	46 (40–50)
6,000	30 (30)	3,000	60 (50–70)
7,000	40 (40)	3,500	66 (60–70)
8,000	40 (30–50)	4,000	70 (70)
9,000	40 (40)	4,500	86 (80–90)
10,000	40 (40)	5,000	100 (100)

To clearly focus on the consequences of HaAPN1 as a receptor for Cry1Ac, we had to get a clear LC_{50} dose for the larvae to be exposed 2 days after dsRNA injection, and this required a modification of the bioassay protocol. The 5th-instar 1st-day larvae (130 ± 10 mg body weight) after injection with 6 μl of DEPC water were made to starve for 2 days (which resulted in no weight gain) and on the 3rd day (130 ± 10 mg body weight) fed on the toxin containing diet having Cry1Ac, ranging from 500 to 5000 ng/cm^2. By adopting this strategy, the larval growth and development was restricted to the same stage as 1-day-old 5th instar, thereby making the larvae more susceptible to lower doses of Cry protein. Mortality observations recorded after 4 days of treatment resulted in a dose-dependent mortality of the larvae (Table 2). The LC_{50} dose was calculated as 2780 ng (regression equation, $y = 0.0204x - 6.7065$; fiducial limits, 2576–3139 ng) of Cry1Ac insecticidal protein per larva.

RNAi of haapn1 on H. armigera 5th-instar Larvae and Its Effect on Cry1Ac Interaction—Having identified the LC_{50} range for Cry1Ac on 5th-instar larvae of *H. armigera*, we injected these larvae with 6 μg of dsRNA of HaAPN1. Analysis of midgut RNA of such insects after 2 days showed a 50% reduction in transcript levels of *haapn1* when compared with control insects injected with DEPC water (Fig. 2*B*). Analysis of the HaAPN1 protein expression profile in these insects revealed an ~30% reduction of HaAPN1 expression in *haapn1* dsRNA-injected insects when compared with control and *falcipain* dsRNA-injected insects (Fig. 2*B*, *inset*). We studied the response of such dsRNA-injected 1st-day larvae to Cry1Ac. These larvae were starved for 2 days and on the 3rd day were exposed to two different doses of Cry1Ac, *viz*. 3000 ng (LC_{50} dose) and 1500 ng (sub-lethal dose). A significant difference in mortality was obtained between the control and dsRNA-injected larvae (Table 3). At a 1500-ng dose, 40% mortality was noticed in control, whereas only 22% of the larvae died that were injected with

TABLE 3

Effect of Cry1Ac on dsRNA injected 3-day-old 5th-instar larvae of *H. armigera*

Two days after dsRNA injection the larvae were fed with Cry1Ac toxin, and after 24 h the larvae were reared on a normal diet. Ten larvae were used per treatment, and the assays were repeated on four different occasions. Mortality was recorded on the 4th day after toxin application. The bioassay data were statistically analyzed by Student's *t* test, and their significance was calculated at 95% confidence limits. Values superscripted with [a] and [b] are significantly different from each other.

Treatment	Mortality after 4 days (initial larval body weight, 130 ± 10 mg)	
	1500 ng/larva	3000 ng/larva
	%	
Control	40[a]	60[a]
dsRNA *haapn1*	22[b]	33[b]

TABLE 4

Aminopeptidase activity (nmol of *p*-nitroanilide formed /min/mg of protein) of solubilized HaAPN1 protein from Sf-21 cells

The APN assay was performed with total protein prepared by solubilizing HaAPN1-expressing Sf21 cell membranes. The assay for aminopeptidase was done by calculating the production of *p*-nitroaniline after 30 min of incubation of the solubilized protein with 4 mM concentrations of either alanine *p*-nitroanilide, leucine *p*-nitroanilide, or valine *p*-nitroanilide. The absorbance was recorded at 405 nm. Absorbance of similarly processed uninfected healthy Sf21 cell membranes was subtracted from HaAPN1-expressing cell membranes. The molar concentrations of product formed was calculated by an absorption coefficient of *p*-nitroanilide of 0.0099 M. All values represent an average of three replications with S.D. Values in parenthesis in bold indicate the range of values. Values in parenthesis in % indicate the percentage inhibition of APN activity in the presence of APN inhibitor amastatin.

	Alanine *p*-nitroanilide	Leucine *p*-nitroanilide	Valine *p*-nitroanilide
		nmol min^{-1} *mg*$^{-1}$	
Solubilized HaAPN1 from Sf21 cells	273.5 ± 10.3 (**265–285**) (100%)	259.5 ± 4.58 (**254.5–263.5**) (100%)	92.8 ± 6.9 {**85.2–98.7**} (100%)
Purified HaAPN1	1781.7 ± 68.25 (**1704–1832**) (100%)	1529.2 ± 35.48 (**1496–1562**) (98%)	1473 ± 26 {**1443–1493**} (95%)

haapn1 dsRNA. Similarly, at 3000 ng, the larvae that were injected with dsRNA of *haapn1* could resist the toxin dose and incurred only 33% mortality against 60% in control.

Solubilization and Purification of HaAPN1 Receptor—The *haapn1* baculovirus-infected cells express HaAPN1 as a transmembrane protein anchored via glycosylphosphatidylinositol and, thus, necessitating its solubilization for purification. Non-ionic detergents, *viz.* Triton X-100, Triton X-114, Nonidet-P 40, octylglucoside, digitonin, Tween 20, were used initially but failed to release the protein in a soluble fraction of cell free extract. Zwitterionic detergent CHAPS, cationic detergent cetyltrimethylammonium bromide, and anionic detergents like sodium lauryl sarcosine and sodium deoxycholate also failed to release the protein in the soluble fraction. Finally sodium dodecyl sulfate, a strong anionic detergent, at the minimal concentration of 0.05% was able to solubilize the protein in appreciable amount. Before evaluating protein for enzyme assay and toxin interaction, the detergent was removed from the protein using an SDS removal kit, and the residual SDS was found to be below detection level. Aminopeptidase assay indicated that the protein was properly refolded and catalytically active. The substrate utilization by this protein is summarized in Table 4. Alanine was metabolized more efficiently followed by leucine and valine, similar to the results obtained earlier with whole cells expressing HaAPN1 (16).

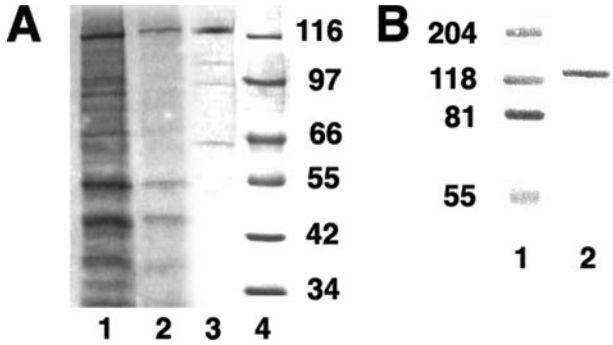

FIGURE 3. **Solubilization and purification of HaAPN1.** *A*, heterologously expressed HaAPN1 on Sf21 cells were solubilized (*lane 1*) and purified by anion exchange chromatography (*lane 2*) and finally fractionated by gel filtration chromatography (*lane 3*) protein molecular weight marker NEB (*lane 4*). *B*, the final purified protein was electroblotted and probed with HaAPN1 antibody (1:5000 dilution) (*lane 2*) Bio-Rad prestained protein molecular weight marker (*lane 1*).

The solubilized HaAPN1 protein was purified using Q-Sepharose anion exchange chromatography which resulted in HaAPN1 eluting between 300 and 450 mM NaCl concentration (fractions 9–18) (Fig. 3, *A*, *lane 2*, and supplemental Fig. *A*). This fraction was further concentrated to 2 ml and resolved using Superose H-12 size exclusion chromatography (Fig. 3, *A*, *lane 3*, and supplemental Fig. *B*). HaAPN1 eluted in fractions 9–12 ml, which were pooled and concentrated to 0.5 ml, finally resulting in a near homogenous HaAPN1 preparation (Fig. 3*B*). The purified protein was immediately used for toxin interaction and real time assays.

Affinity Estimation of Cry1Ac Insecticidal Protein and HaAPN1 Receptor—To study real time binding kinetics of Cry1Ac toxin protein to purified HaAPN1, the receptor (HaAPN1) was immobilized on a CM5 biosensor chip surface, and the toxin receptor complex formation was analyzed by surface plasmon resonance. Simple bimolecular binding of Cry1Ac was observed with HaAPN1 (Fig. 4). The apparent rate constants for Cry1Ac toxin protein and HaAPN1 are summarized in Table 5. The final equilibrium dissociation rates of Cry1Ac was estimated as 0.465 nM. As a control, we also studied the binding of HaAPN1 with an unrelated protein to Cry1Ac, falcipain. There was no binding of falcipain to HaAPN1, thus showing no interaction between them.

DISCUSSION

H. armigera is the most important agricultural insect pest in the developing world as it is widespread (occurring in south and middle of Africa, South, Southeast Asia, China, and North and Central Australia) and attacks many important crops like cotton, groundnut, chickpea, soybean, sunflower, and vegetables like tomato, brinjal, and potato. The insecticidal crystal protein Cry1Ac produced by the bacterium *B. thuringiensis* is the most potent of all the insecticidal proteins produced by this bacteria against the polyphagus insect pest *H. armigera*. More than 90% of the transgenics deployed in modern agriculture for resisting insect pests harbors this gene. All the cotton transgenic lines growing in the developing world harbors this protein, and it has proved very efficient in controlling the damage of *H. armigera*, resulting in more than 50% reduction in chemical pesticide

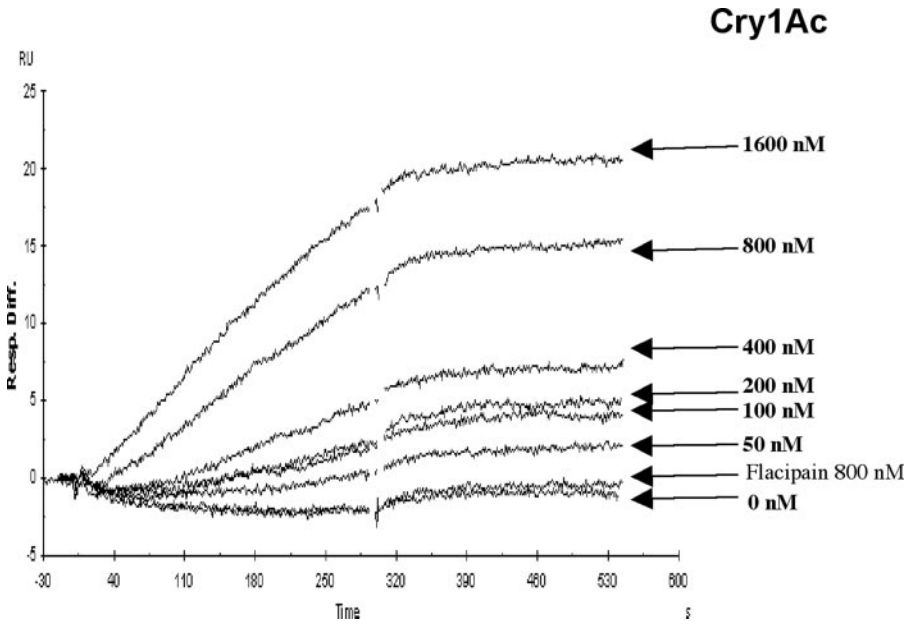

FIGURE 4. **BIAcore sensograms of HaAPN1 with different concentrations of Cry protein.** The purified HaAPN1 protein (20 μg/ml) was coated on a CM-5 chip and analyzed for interactions by determining its affinity coefficients with Cry1Ac protein. *Resp. Diff.*, response differece.

TABLE 5
Affinity estimations of HaAPN1 receptor protein against Cry1Ac protein

K_a	4.47×10^3 M^{-1} s^{-1}
K_d	2.08×10^{-6} s^{-1}
K_D	4.65×10^{-10} M (0.465 nM)
χ^2	0.625

usage (21). Hence, it is very critical for us to understand the exact mechanism by which Cry1Ac kills *H. armigera*.

Studies on midgut brush border membrane vesicles of lepidopteran insects to identify the putative receptor for Cry1Ac have reported them to be aminopeptidases. Such aminopeptidases have been cloned and expressed from *M. sexta*, *Plutella xylostella*, *Bombyx mori*, and *H. armigera* and have been shown to interact with Cry1A toxins (4–6). Toward this effort, we have cloned two different aminopeptidases from *H. armigera*, HaAPN1 and HaAPN2, and have shown that both these proteins interact with Cry1Ac. All such toxin-ligand interactions have been demonstrated by ligand blot analysis under a denaturing environment where many of the epitopes would be exposed, which may not hold true under native/natural conditions (22).

A direct functional assessment of putative receptor has also been employed by various other strategies. Insect cell lines have earlier been used to study Bt insecticidal protein membrane insertion and channel formation (23, 24). Various types of insect cell lines have also been studied for their toxicity response to different Cry proteins (25, 26), and it was found that Sf-9 cells showed very less toxicity to Cry1Ab and showed maximum toxicity with Cry1C (27). The effectiveness of using live insect cells expressing putative receptor proteins and demonstrating the ability of the protein to function as a receptor to Bt toxin has been shown by Nagamatsu (10). They have been able to demonstrate swelling and lysis of Sf-9 cells expressing cadherin protein of *B. mori* after overlay with Cry1Ab toxin. *Drosophila* S-2 cell line constitutively expressing *M. sexta* cadherin gene showed heavy damage and lysis when overlaid with Cry1Ab (13).

Overlaying Sf21 cells expressing HaAPN1 with Cry1Ac protein resulted in their swelling and expansion of cytoplasm (Fig. 1). Control cells overlaid with Cry1Ac did not show any such effects. This shows very clearly that the Cry1Ac binding ability of HaAPN1 seen in a ligand blot can be functionally related to swelling of cells expressing this protein under natural conditions on addition of Cry1Ac. Hence, HaAPN1 can be a functional receptor of Cry1Ac.

The dissociation constants obtained during the course of this study are the maiden observations for a heterologously expressed and purified aminopeptidase receptor from any insect against Cry1Ac. Earlier studies have reported overall binding affinities using the surface plasmon resonance protocol against insect aminopeptidases purified from brush border membrane vesicles of the midgut (28–30). The on rates for association of HaAPN1 with Cry1Ac falls in the range of 4.47×10^3 M^{-1} s^{-1} and is comparable with the values obtained in other studies employing APN purified from brush border membrane vesicles of *B. mori*, *M sexta*, and *Lymantria dispar* (31, 32). The off rates for dissociation calculated in this study for HaAPN1 with Cry1Ac works out to 2.08×10^{-6} s^{-1}, which is the lowest recorded for any APN/Cry protein interaction. Earlier studies observed off rates in the range of 1.5×10^{-3} s^{-1}. Thus, the overall binding affinity of Cry1Ac to HaAPN1 works out to 0.465 nM, a very tight binding interaction due to the very low dissociation of the toxin-APN complex. We are rather intrigued by the differences in the observed APN-Cry1Ac dissociation constant and those reported from other insect APNs. One possible contributory factor could be the different purification protocols employed. Although we have used a strong detergent, SDS, for solubilization from membranes, other studies have employed CHAPS as detergent. In both instances the catalytic aminopeptidase activity has been taken as a detrimental marker for correct conformational release of aminopeptidase. In the present situation the strong detergent SDS could have exposed all hidden motifs that may not be normally exposed. Also, the preliminary data of catalytic and Cry1Ac-interfering domain mapping suggests that the two domains do not overlap and may reside at different positions on the APN polypeptide. The lower values of dissociation obtained might be due to the fact that the APN employed in this study has been heterologously expressed and purified, whereas earlier studies relied on APN purified from brush border membrane vesicles of the host insect midgut (32).

RNA interference has become an effective and important tool to study the functional relevance of various proteins and genes in an organism. As described in detail in various reviews,

RNAi by dsRNA results in sequence-specific post-transcriptional degradation of the target mRNA (33). Our results also show a reduction of HaAPN1 transcript levels after dsRNA incubation. This treatment also resulted in a corresponding decrease in the protein expression levels. Such dsRNA-treated HaAPN1 Sf21 cells when tested for their reaction with Cry1Ac toxin protein showed reduced damage when compared with non-dsRNA-treated HaAPN1-expressing cells (Table 1). Similar results for toxin ligand interaction has been demonstrated with *S. litura* APN and Cry1C toxin in Sf-21 cell lines (34). This clearly demonstrates the functional relevance of HaAPN1 as a receptor for Cry1Ac protein, and the specificity of the interaction has been shown by the reduction in the response of these cells after RNAi treatment to Cry1Ac toxin protein.

Assaying Cry1Ac dose response in *H. armigera* 5th-instar larvae was not straightforward, and we could not get a clear LC_{50} value (Table 2). Previous studies on the larvae of *Ostrinia nubilalis* and *Danus plexippus* had reported that the matured larvae were more resistant to Cry toxins than their younger counterpart (35, 36), but younger larvae of *H. armigera* are not amenable to dsRNA injection. Although effects of RNAi can be followed into the next generation, albeit with a lesser effect, such treatments with *haapn1* on *H. armigera* resulted in less than 20% transcript reduction (data not shown), making biological responses difficult to compare statistically. Hence, starving the insect for 2 days after injection resulted in no increase in body weight (130 ± 10 mg body weight), and mortality responses of such insects when assayed against Cry1Ac showed a LC_{50} dose of 2780 ng. Functional knockouts of *haapn1* in *H. armigera* showed increased tolerance at the LC_{50} dose as well as the sublethal dose when compared with control. These results corroborate well with our earlier-mentioned results with Sf21 cells expressing HaAPN1 showing reduced responses to Cry1Ac after RNAi. Thus, although these results unambiguously demonstrate a functional role for HaAPN1 in the toxicity of the insecticidal protein Cry1Ac to *H. armigera*, they do not rule out involvement of other Cry protein-interacting moieties at the midgut. Specifically, there are overwhelming genetic data implicating cadherin as a receptor to Cry1Ac in *Heliothis virescens* (37). It is likely that both midgut-associated protein ligands for Cry1Ac and the lysis of the midgut of susceptible insect are a consequence of multiple interacting partners (38). How the insecticidal protein positions into these receptors is a subject of intense investigations now.

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Knockdown of Aminopeptidase-N from *Helicoverpa armigera* Larvae and in Transfected Sf21 Cells by RNA Interference Reveals Its Functional Interaction with *Bacillus thuringiensis* Insecticidal Protein Cry1Ac

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