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Silencing of Midgut Aminopeptidase N of *Spodoptera litura* by Double-stranded RNA Establishes Its Role as *Bacillus thuringiensis* Toxin Receptor*

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R. Rajagopal‡, S. Sivakumar‡, Neema Agrawal, Pawan Malhotra, and Raj K. Bhatnagar§

From the International Centre for Genetic Engineering and Biotechnology (ICGEB), P. O. Box 10504, Aruna Asaf Ali Marg, New Delhi 110 067, India

Insecticidal crystal proteins of *Bacillus thuringiensis* bind to receptors in the midgut of susceptible insects leading to pore formation and death of the insect. The identity of the receptor is not clearly established. Recently a direct interaction between a cloned and heterologously expressed aminopeptidase (*slapn*) from *Spodoptera litura* and the Cry1C protein was demonstrated by immunofluorescence and *in vitro* ligand blot interaction. Here we show that administration of *slapn* double-stranded RNA to *S. litura* larvae reduces its expression. As a consequence of the reduced expression, a corresponding decrease in the sensitivity of these larvae to Cry1C toxin was observed. The gene silencing was retained during the insect's moulting and development and transmitted to the subsequent generation albeit with a reduced effect. These results directly implicate larval midgut aminopeptidase N as receptor for *Bacillus thuringiensis* insecticidal proteins.

The bacterium *Bacillus thuringiensis* (Bt)[1] produces insecticidal crystal proteins, which upon ingestion by susceptible larvae get activated in the midgut, interact with specific receptor and form pores in the epithelium, resulting in the death of the larvae (1). Understanding the mechanism of action of Bt toxin and development of resistance in insects is fundamental in sustaining the use of Cry proteins in integrated pest management. One of the mechanisms of resistance development is an alteration in the binding ability and/or a decrease in the population of receptor molecules, which bind Bt toxin in the insect midgut (2). There have been intense efforts to characterize the nature of this receptor. As a result of several independent experiments employing ligand blot analysis and fluorescent labeling of insecticidal proteins, cadherin and aminopeptidase N (APN) have emerged as main putative receptor molecules (Ref. 3 and references there in). While the role of a receptor molecule in mediating the effect of Cry toxin is acknowledged, the identity of this receptor is still being worked out.

Aminopeptidase N from *Manduca sexta* was the first molecule to be tentatively identified as a Cry toxin-binding protein (4, 5), and APN is the most extensively studied putative receptor, having been identified and isolated subsequently from other lepidopteran insect pests. Independently, a 210-kDa cadherin-like protein from *M. sexta* was shown to interact with Cry1Ab toxin (6) and later its presence and toxin interaction was also demonstrated from another insect, *Bombyx mori* (7). Relative abundance of APN in the posterior midgut (8) and lower binding constants of Cry toxin toward cadherin as compared with APN (9) raised apprehension about the role of APN as a receptor for Bt toxin in the insect midgut. Moreover, a recent report shows that high levels of resistance to the Bt toxin, Cry1Ac, in *Heliothis virescens* is due to disruption of a cadherin superfamily gene by retroposon-mediated insertion (10). Although *in vitro* experiments such as toxin-induced increase in the $^{86}Rb^{+}$ efflux from lipid vesicles reconstituted with APN and reduction in the inhibition of short circuit current (I_{SC}) for Cry1Ac following the release of APN from midgut membrane by phosphatidylinositol phospholipase C treatment provide support for the role of APN as a receptor, the vital *in vivo* evidence for receptor characterization is lacking (5, 11).

RNA interference (RNAi) is a process of dsRNA-mediated gene silencing in which only the mRNA cognate to dsRNA is specifically degraded (12–14). Recently, we reported isolation of a 2.8-kb APN-encoding gene *slapn* (AF320764), from the midgut of the polyphagous pest, *Spodoptera litura*, and its expression in the insect cells by a baculovirus expression vector. By *in vitro* ligand blot analysis and immunofluorescence toxin binding studies, we demonstrated a direct interaction between the expressed receptor and Cry1C protein (15). Here we report that dsRNA-mediated silencing of the aminopeptidase N gene results in increased resistance of *S. litura* larvae to Cry1C protein, thereby demonstrating a functional role for this protein in Cry protein-mediated toxicity.

EXPERIMENTAL PROCEDURES

Insect Rearing, Midgut Isolation, and RNA Preparation—S. litura larvae were reared on fresh castor leaves (*Ricinus communis*) under a photoperiod of 14:10 h (light:dark), 70% relative humidity, and 27 °C. Midguts from 6th instar 1st day larvae were dissected in DEPC-treated water, snap-frozen into liquid nitrogen, and stored at −70 °C. Total RNA was isolated from the midgut tissue using TRIzol reagent (Invitrogen) according to manufacturer's protocol. The amount of RNA was quantitated spectrophotometrically at 260 nm.

Bioassay—Inclusion bodies (IB) of Cry1C toxin were prepared as reported earlier by Lee (16). The amount of the toxin was quantitated densitometrically by resolving the IB on SDS-PAGE. Toxin amounts from 1000 to 10,000 ng was diluted in 10 mM Tris, pH 7.5, and 10 μl of each concentration was coated on both the sides of a castor leaf disc (area = 3.8 cm^2). The toxin-coated leaf disc was air-dried and placed in a well of a 12-well tissue culture plate (Nunc Inc). One 6th instar 1st day larva was released on each well and exposed to the toxin treatment

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‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed: Insect Resistance Group, ICGEB, P. O. Box 10504, Aruna Asaf Ali Marg, New Delhi 110 067, India. Tel.: 91-11-6195007 (or 91-11-6177357, 91-11-6189358, 91-11-6189360, or 91-11-6189361); Fax: 91-11-616-2316; E-mail: raj@icgeb.res.in.

[1] The abbreviations used are: Bt, *Bacillus thuringiensis*; APN, aminopeptidase N; BBMV, brush border membrane vesicles; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate toluidine; Cry, crystalline inclusion protein; dsRNA, double-stranded RNA; RNAi, RNA interference; LMW, low molecular weight; IB, inclusion bodies; siRNA, small interfering RNAs; sRNA, sense RNA; asRNA, antisense RNA; DEPC, diethyl pyrocarbonate; RT, reverse transcriptase; nt, nucleotide(s).

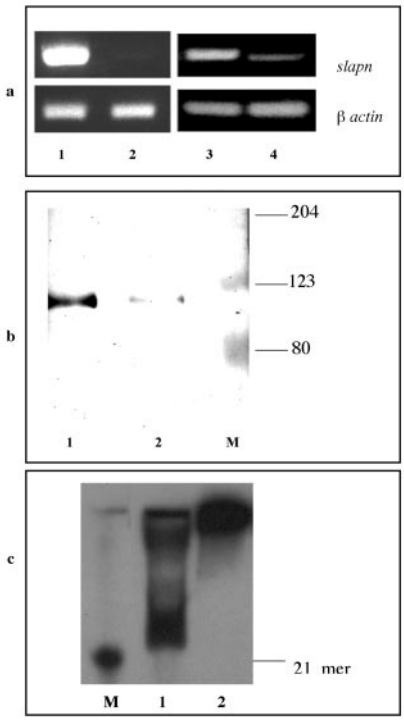

FIG. 1. **Introduction of dsRNA corresponding to *slapn* gene into *S. litura* larvae results in its silencing.** *a*, relative RT-PCR with β-actin (β-actin F, 5′cagatcatgtttgagaccttcaac3′; β-actin R, 5′gsccatctcytgctcgaartc3′) and *S. litura apn* (APN F, 5′agcactttcatcgtactga3′; APN R, 5′agcttcaccaccatatgtag3′) primers on total RNA isolated from: midguts of control 6th instar larvae (*lane 1*), midgut of dsRNA injected 6th instar larvae (*lane 2*), neonate from untreated parentage (*lane 3*), and neonate of F1 generation whose parents were injected with dsRNA (*lane 4*). *b*, the Western blot of BBMV proteins (1 μg) prepared from 6th instar control larvae (*lane 1*) and dsRNA-injected larvae (*lane 2*) was probed with polyclonal anti-SLAPN antibodies and developed with NBT/BCIP. *c*, generation of 25-mer small interfering RNA in dsRNA injected larvae (*lane 1*) and dsRNA used for injection (*lane 2*). *Lane M* indicates marker. Bands were analyzed for their net intensity by spot densitometry using the software Kodak 1D, version 2.0.

for 24 h. After 24 h the larva was transferred to fresh castor leaves (without toxin). Mortality was recorded after 4 days and the LC_{50} value calculated by Probit analysis using the software Indo Stat (Indostat Services, Hyderabad, India). Ten larvae were tested for each treatment and the bioassay replicated three times. LC_{50} values were also determined for neonate larvae of *S. litura* by coating castor leaf discs with different doses of Cry1C toxin and scoring the mortality in each treatment after 72 h. 10 neonate larvae were released on each leaf disc, and each treatment was replicated five times.

Preparation of dsRNA—The dsRNA was prepared following the procedure prevoiusly described by us (17). A 756-bp fragment, from amino acid 609 to 861, was amplified from the *S. litura* APN gene and subcloned in pGEM-Te. The cloned fragment was amplified using vector-specific universal and reverse primers (Promega). The PCR product was purified using PCR purification kit (Qiagen GmbH) and used as a template to generate sense RNA (sRNA) and antisense RNA (asRNA) using T7 and SP6 RNA polymerases (Ambion), respectively. To make dsRNA, equal amounts of sRNA and asRNA 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 at −20 °C. The pellet was washed with 75% ethanol and resuspended in 50 μl of DEPC-treated water.

Injection of dsRNA and Quantitative RT-PCR—Varying doses of dsRNA were injected intrahemocoelically into early 5th instar *S. litura* larvae using a microapplicator (KDS 200, KD Scientific Inc., New Hope, PA). After 48 h, the insect midguts were dissected, and total RNA was extracted. The quantity of RNA in each treatment was normalized by amplifying the β-actin gene in each treatment to equal intensity after 20 cycles of RT-PCR using the Titan one-tube RT-PCR kit (Roche Diagnostics Gmbh). The amplification regimen was as follows: reverse transcription at 43 °C for 35 min followed by 20 PCR cycles of denaturation at 94 °C for 30 s, re-annealing at 51 °C for 30 s, and extension at 72 °C for 30 s followed by a final extension of 10 min at 72 °C. Using this normalized amount of RNA as template, the *slapn* transcript in different treatments was compared by amplifying a 756-bp part of the *slapn* gene. Before loading on 1% agarose gel the RT-PCR products were treated with 1 μg of RNase (Qiagen GmbH) at 37 °C for 10 min to eliminate template RNA, since it hinders the correct estimation of the β-actin gene product in the gel. The gels were photographed with Polaroid 667 black and white print film and scanned for net intensity of each RT-PCR product using the software Kodak 1D, version 2.0.

TABLE I
Reduction in S. litura's toxicity to Cry1C by dsRNA

slapn dsRNA was injected into 5th instar, 1st day larvae at 4 μg/larvae. Control larvae were injected with DEPC water of equal volume. On moulting to 6th instar, the larvae were transferred to individual leaf discs (3.8 cm^2) coated with 6.0 μg of Cry1C protein. After 24 h, the larvae were transferred to fresh leaves. The experiment was replicated on three different occasions with 50 larvae each. Mortality was recorded 4 days after toxin application. In a separate experiment, 50 larvae injected with *slapn* dsRNA were allowed to grow without being exposed to Cry1C, pupate, and mate among themselves. The neonates from this treatment were transferred to castor leaf discs (3.8 cm^2) coated with 250 ng of Cry1C and compared with neonates emerging from normal insects. Ten larvae of each treatment were released on toxin-coated leaf discs and replicated 15 times. The mortality data were subjected to Students *t* test and values superscripted by *a* and *b* are significantly different at 95% confidence limits. Values in parentheses indicate percent reduction in mortality over control.

Treatment	Effect on 6th instar larvae		Effect on neonate larvae in next generation, mortality %
	Mortality	Pupation	
	%		%
Control	61.0[a]	00.0[a]	65.0[a]
dsRNA injected	15.0 (75.0)[b]	70.0 (70.0)[b]	45.0 (30.0)[b]

BBMV Preparation and Western Blotting—BBMVs were prepared from 6th instar midgut by following the protocol of Wolfersberger (18). One microgram of the BBMV protein was resolved by 7.5% SDS-PAGE and electrotransfered to nitrocellulose membranes at 50 mA for 2 h at 4 °C. The blot was blocked in 3% bovine serum albumin in 1× phosphate-buffered saline and then incubated with 1:50,000 dilution of anti-APN antibodies. Subsequently, the blot was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG and developed with NBT-BCIP substrate.

Low Molecular Weight (LMW) RNA Extraction and Detection—Low molecular weight RNA was isolated from midgut of insects injected with α-^{32}P-labeled dsRNA following the protocol of Hamilton and Baulcombe (19) and resolved on 12% acrylamide 7 M urea gel in 1× TBE buffer (10.8 g of Tris, 5.5 g of boric acid, 4.0 ml of 0.5 M EDTA, pH 8.0, made up to 1 liter with water). The gel was dried and exposed to a Kodak Bio-Max MR autoradiographic film overnight at −70 °C. The film was developed to detect the occurrence of radiolabeled siRNA.

RESULTS AND DISCUSSION

To evaluate the functional role of aminopeptidase N in the insecticidal activity of Cry1C *in vivo*, we sought to specifically inhibit the expression of *slapn* by its corresponding dsRNA. The reported routes of dsRNA delivery in lower eukaryotes such as *Caenorhabditis elegans* are soaking, feeding, or injection of dsRNA solution into the worm (12, 20), while dsRNA in *Drosophila melanogaster* was introduced by injecting into eggs. *D. melanogaster* embryos hatched from dsRNA-injected eggs displayed nearly 75% gene-silencing, which reduces to 3% with maturity (21). Preliminary experiments to introduce dsRNA into neonate larvae of *S. litura* by soaking them in dsRNA solution or by feeding through diet were unsuccessful, since no reduction in transcript levels was detected. Subsequently, varying amounts of dsRNA were injected into hemolymph of 5th instar larvae (2 μl/insect) using a microapplicator (KDS 200, KD Scientific Inc.). After 48 h of injection of dsRNA the transcript abundance was estimated by relative RT-PCR. An injection of 4 μg of dsRNA resulted in a 95% reduction over

control of midgut *slapn* transcript levels (Fig. 1*a*, *lanes 1* and *2*).

The consequence of decrease in *slapn* transcript on the susceptibility of *S. litura* larvae against Cry1C protein was examined by performing toxicity assays. Initially, the assays were performed on freshly moulted, 6th instar larvae (injected with 2 μl of DEPC-treated water in 5th instar), a developmental stage corresponding to the growth status of 5th instar larvae after 48 h of injection. These larvae were released on 3.8-cm^2 castor leaf discs coated with various concentrations of Cry1C protein (1–10 μg). Mortality was recorded after 4 days and LC_{50} (50% lethal concentration) value calculated by probit analysis. The calculated LC_{50} value was 5.642 μg/disc with the regression equation, $y = 5.1921x$-14.478, and the fiducial limits lie between 5.355 and 6.025 μg. Larvae injected with dsRNA and released on castor leaf discs coated with 6 μg of Cry1C displayed reduction in the mortality rate. The increase in tolerance for dsRNA-injected larvae to Cry1C protein correlated with a comparable (70%) increase in the larvae that pupated. On the other hand, none of the control larvae could reach pupation (Table I).

To examine the effect on the expression of APN upon dsRNA delivery, BBMVs were prepared by the differential $MgCl_2$ method from the midgut of 6th instar larvae and resolved by 7.5% SDS-PAGE. Probing the BBMV proteins with anti-APN antibodies revealed nearly 80% reduction in APN expression in dsRNA-injected larvae as compared with control larvae (Fig. 1*b*). Thus the reduction in the expression of aminopeptidase correlates well with the reduction of *slapn* transcript levels and the decreased sensitivity to Cry1C proteins (Fig. 1*a*).

Fifth instar larvae injected with *slapn* dsRNA were reared up to pupation and bred into the next generation. Analysis of neonate larvae for abundance of *slapn* transcript revealed 60% reduction in *slapn* levels (Fig. 1*a*, *lanes 3* and *4*). Neonate larvae of the F1 generation with *slapn* silencing displayed resistance to Cry1C toxin compared with untreated neonate larvae (Table I). The LC_{50} value of Cry1C on untreated neonate larvae was 200 ng/3.8 cm^2.

An important step in RNAi-mediated gene silencing is the formation of 21–25-nt siRNAs, which target and degrade mRNA of the target gene (22). In the present study, the generation of siRNA was investigated by extracting LMW RNA (19) from the midgut of larvae injected with α-P^{32}-labeled dsRNA. By resolving the LMW RNA preparation on PAGE, a distinct band corresponding to 25 nt is observed (Fig. 1*c*). Occurrence of a 25-mer oligonucleotide establishes that RNAi pathway in larvae is similar to that observed in other organisms.

Thus in the present study, we have demonstrated that the dsRNA-mediated silencing of *slapn* in whole *S. litura* larvae resulted in the decrease in the amount of APN expressed in the epithelial membrane of midgut cells, which in turn enabled the larvae to tolerate lethal concentrations of the Cry1C protein. By performing *in vivo* experiments we are able to provide a direct evidence for the role of APN as a Bt toxin receptor in the midgut of insects. The silencing of the *S. litura apn* by introducing cognate dsRNA into larvae demonstrates that RNAi functions in whole larvae and also that the midgut columnar cells can take up dsRNA molecules injected in the hemocoel. This result shows that like in the plant kingdom, there is a "systemic" effect of RNAi in animals too, in organs away from the point of delivery of dsRNA. Also, we show that RNAi effect by dsRNA administration is heritable in the next generation, as comparable with the interference obtained by using "hairpin-loop" RNA (23). Here, we have used RNAi as a technique to probe the function of a protein as a toxin receptor, thereby implying that it can be used to explore the functional role of different proteins involved in various host-pathogen interactions.

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