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Plasmatocyte-spreading peptide (PSP) plays a central role in insect cellular immune defenses against bacterial infection

I. Eleftherianos[1,*], M. Xu[1], H. Yadi[1], R. H. ffrench-Constant[2] and S. E. Reynolds[1]

[1]Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK and [2]School of Biological Sciences, University of Exeter, Cornwall Campus, Penryn, Cornwall TR10 9EZ, UK

*Author for correspondence (Ioannis.Eleftherianos@ibmc.u-strasbg.fr)

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SUMMARY

Insect hemocytes (blood cells) are a central part of the insect's cellular response to bacterial pathogens, and these specialist cells can both recognize and engulf bacteria. During this process, hemocytes undergo poorly characterized changes in adhesiveness. Previously, a peptide termed plasmatocyte-spreading peptide (PSP), which induces the adhesion and spreading of plasmatocytes on foreign surfaces, has been identified in lepidopteran insects. Here, we investigate the function of this peptide in the moth *Manduca sexta* using RNA interference (RNAi) to prevent expression of the precursor protein proPSP. We show that infection with the insect-specific bacterial pathogen *Photorhabdus luminescens* and non-pathogenic *Escherichia coli* induces proPSP mRNA transcription in the insect fat body but not in hemocytes; subsequently, proPSP protein can be detected in cell-free hemolymph. We used RNAi to silence this upregulation of proPSP and found that the knock-down insects succumbed faster to infection with *P. luminescens*, but not *E. coli*. RNAi-treated insects infected with *E. coli* showed a reduction in the number of circulating hemocytes and higher bacterial growth in hemolymph as well as a reduction in overall cellular immune function compared with infected controls. Interestingly, RNAi-mediated depletion of proPSP adversely affected the formation of melanotic nodules but had no additional effect on other cellular responses when insects were infected with *P. luminescens*, indicating that this pathogen employs mechanisms that suppress key cellular immune functions in *M. sexta*. Our results provide evidence for the central role of PSP in *M. sexta* cellular defenses against bacterial infections.

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Key words: insect immunity, cellular response, plasmatocyte-spreading peptide, RNAi interference, *Manduca sexta*, *Photorhabdus luminescens*.

INTRODUCTION

In response to microbial infection, insects express highly efficient defense mechanisms involving both humoral and cellular immune responses (Cherry and Silverman, 2006; Lemaitre and Hoffmann, 2007). Humoral factors include molecules that regulate hemolymph clotting and phenoloxidase activation, as well as various antimicrobial peptides targeted against bacteria and fungi (Cerenius and Söderhall, 2004; Imler and Bulet, 2005). The cellular arm of insect immunity is mediated by the activity of circulating blood cells, referred to as hemocytes, which participate in multiple cellular immune responses including phagocytosis, the formation of circulating hemocyte aggregates, nodulation and encapsulation; they also may contribute to the humoral response (Lavine and Strand, 2002; Jiravanichpaisal et al., 2006). Insect hemocytes are divided into specific classes on the basis of morphology, antigenic properties, and function (Ribeiro and Brehelin, 2006). In Lepidoptera (butterflies and moths), granulocytes and plasmatocytes are the most abundant circulating hemocytes and are also the only hemocytes capable of adhering to wound sites or invading foreign organisms (Kanost et al., 2004).

Upon recognition of non-self, the response of insect hemocytes is to 'spread' (Gillespie et al., 1997). If the foreign agent is small, this spreading ability promotes the phagocytosis of the particle, whereas a larger foreign object (or many small foreign objects) would be subject to nodulation or encapsulation due to the cooperative action of a number of hemocytes. Hemocyte spreading is often used as a measure of immune fitness and can be inhibited by some insect pathogens and parasites. Inhibiting hemocyte spreading is a common strategy employed by many entomopathogens and implies that such changes in hemocyte behavior are important components of insect defense (Davies et al., 1987; Griesch and Vilcinskas, 1998; Strand and Noda, 1991; Hung et al., 1993). Many endogenous signals, thought to be released by hemocytes or wounded tissue (Clark et al., 1997), also influence hemocyte spreading.

One type of cytokine involved in this process, originally identified from the lepidopteran *Pseudoplusia includens*, is the 23-amino acid peptide PSP1 (plasmatocyte-spreading peptide 1). Its precursor protein, proPSP, is expressed by granular hemocytes and fat body cells as a pro-peptide of 142 residues, with the PSP1 sequence located at the C terminus (Clark et al., 1998). This biologically inactive precursor is secreted into the hemolymph and subsequently cleaved by an unknown protease to release the mature 23-amino acid peptide when the insect is subjected to an immune challenge (Clark et al., 1997; Clark et al., 1998). Both purified and synthetic PSP1 peptide induce plasmatocytes to rapidly adhere and spread across foreign surfaces at concentrations greater than $1\,\mathrm{nmol\,l^{-1}}$ (Clark et al., 1998; Wang et al., 1999). PSP1 homologs have been identified from other Lepidoptera and, based on the consensus sequence of their N termini, these molecules are collectively referred to as the ENF peptide family (Strand et al., 2000). ENF family members have very similar sequences (>70% identity) but their reported functions are diverse: these include growth-inhibiting and mitogenic activities (growth-blocking peptide, GBP), induction

of paralysis (paralytic peptides, PP) and regulation of hemocyte behavior (plasmatocyte-spreading peptide) (reviewed by Matsumoto et al., 2003). Among them, GBP and PSP1 have been shown to exert the multiple biological activities of larval growth retardation, plasmatocyte spreading and paralysis (Strand et al., 2000; Aizawa et al., 2001). In *Manduca sexta*, two ENF family peptides (PP1 and PP2) with potent paralytic activity were initially isolated from hemolymph (Skinner et al., 1991). Previous studies showed that synthetic *Manduca* PP1 not only had paralytic activity in whole insects but also enhanced the spreading and aggregation of plasmatocytes and prevented bleeding from wounds (Wang et al., 1999). These multiple functions suggested that *Manduca* PP1 may be important in both hemostasis and cellular immunity in this insect.

We have previously used RNA interference (RNAi) to investigate the functional role of specific pattern recognition proteins and antibacterial effector peptides in the *Manduca* humoral or cellular immune response against pathogenic and non-pathogenic bacteria. To do this, we injected double-stranded (ds) RNA specific to the targeted gene into intact insect larvae and then challenged them with the pathogen *Photorhabdus*. RNAi knock-down of any one of the recognition protein genes *hemolin*, *peptidoglycan recognition protein* or *immulectin-2* resulted in increased susceptibility following challenge with *Photorhabdus* (Eleftherianos et al., 2006b). Knock-down of *immulectin-2* was shown to interfere with the humoral response by preventing normal activation of phenoloxidase (Eleftherianos et al., 2006b). Knock-down of *hemolin* was shown to be involved in mediating hemocyte aggregation, nodule formation and phagocytosis upon *Escherichia coli* infection (Eleftherianos et al., 2007).

Here, we show that in *M. sexta* the gene encoding proPSP is transcribed in fat body, but not hemocytes, in response to injection of pathogenic (*Photorhabdus luminescens*) or non-pathogenic (*Escherichia coli*) bacteria. We find that expression of proPSP protein in both fat body and hemolymph plasma can be prevented by injecting double-stranded RNA specific for proPSP prior to bacterial infection and that RNAi-mediated knock-down of proPSP significantly increases mortality when insects are exposed to *P. luminescens*. Finally, we suggest a functional role for PSP in *M. sexta* cellular immune responses.

MATERIALS AND METHODS

Experimental organisms

Tobacco hornworm larvae, *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae), were kept individually on a wheatgerm-based artificial diet at 25°C and a photoperiod of 17h:7h light:dark (Reynolds et al., 1985). Newly moulted (day 0) fifth-stage larvae were used for all experiments. Bacterial strains used were *Escherichia coli* strain DH5α and *Photorhabdus luminescens* subsp. laumondii strain TT01. Bacteria were cultured on Petri dishes containing 2.5% Luria-Bertani medium (LB) and 1.5% agar (Difco Laboratories, Surrey, UK); prior to injection, primary-form bacterial colonies were grown for 24h at 30°C in 5ml of 2.5% LB liquid culture in the dark.

Insect infection

Insects were injected with washed bacterial cells suspended in phosphate-buffered saline (PBS; 0.15 $mol\,l^{-1}$ sodium chloride, 10 $mmol\,l^{-1}$ sodium phosphate buffer, pH 7.4). 50 μl of a suspension containing approximately 2×10^7 *E. coli* or 1×10^3 *P. luminescens* primary form cells were injected directly into the hemocoel of *M. sexta* larvae using a 100 μl disposable syringe with a 30-gauge needle. Numbers of injected bacteria were estimated by optical density at 600 nm and by plating a known volume of injected suspension on 2.5% LB agar plates. Injected larvae were kept at 28°C to determine survival. Mortality, defined as no reaction to poking with a needle, was scored at intervals up to 84h after the final injection. Where hemolymph and tissue samples were collected, insects were surface sterilized with 70% ethanol, bled and dissected to collect hemocytes and fat body, respectively. Ten insects were used for each treatment, and each bioassay was replicated three times.

RNA extraction, RT-PCR and cDNA cloning

Larvae were immobilized on ice 18h following bacterial challenge, surface sterilized with 70% ethanol and then bled and dissected in order to collect hemolymph and tissue samples, respectively. Hemocytes (30 mg) and fat body (100 mg) were homogenised in 1 ml of TRI reagent (Sigma, Gillingham, UK). RNA was then isolated as described (Eleftherianos et al., 2006a; Eleftherianos et al., 2006b). All RNA samples were treated with RNase-free DNaseI (Invitrogen, Paisley, UK) (1 U μl^{-1}), and lack of DNA contamination was confirmed in every case by performing PCR without the reverse transcriptase (RT) step. Single-step reverse transcription (RT)-PCR and cDNA cloning of *proPSP* were as previously described (Eleftherianos et al., 2006a; Eleftherianos et al., 2006b). Primers were designed from the sequence of the *M. sexta proPSP* cDNA (GenBank accession no. AF122899) (Wang et al., 1999). Sequences of the primers were: (forward) 5′-ATGAAGTTATTTTTAT-AGTT-3′ and (reverse) 5′-TCAAAATGTAAGTTTGCATCT-3′. The ribosomal subunit protein rpS3 (Jiang et al., 1996) was used as a loading control [primer sequences given by Eleftherianos et al. (Eleftherianos et al., 2006a; Eleftherianos et al., 2006b).

RNAi and western blotting

Double-stranded RNA (393 bp) corresponding to the gene sequence of *M. sexta proPSP* (ds-proPSP) was synthesized as previously described (Eleftherianos et al., 2006a; Eleftherianos et al., 2006b). A plant gene, *Manihot esculenta* catalase *CAT1* (GenBank accession no. AF170272), was used to synthesize a dsRNA control reagent (dsCON). For RNAi, 100 ng of ds-proPSP (50 μl, 2 $\mu g\,ml^{-1}$) was injected into insects 6h before challenge with *E. coli* or *P. luminescens*. Controls involving dimethyl pyrocarbonate (DMPC)-treated water without dsRNA in the primary injections and PBS without *E. coli* or *P. luminescens* in the secondary injections were also used.

After RNAi injection, treated insects were held at 28°C for 18h and then bled and dissected in order to isolate various tissues, as before. Three insects were used for each treatment. Semi-quantitative RT-PCR reactions using extracted RNA as a template and *proPSP*-specific primers were performed to determine mRNA transcription patterns of *proPSP* in each *Manduca* tissue. For survival studies, 100 ng dsRNAs (dissolved in 50 μl water) were injected 6h before challenge with *E. coli* or *P. luminescens.*

Western blot analysis was as previously described (Eleftherianos et al., 2006a; Eleftherianos et al., 2006b). In each lane, volumes of hemolymph plasma samples containing 3 μg of total protein were loaded. The hemolymph protein concentration was determined using the Bradford assay. The anti-PSP1 polyclonal antibody, which recognizes the PSP1 peptide sequence from *P. includens* (Clark et al., 1998), was used at a dilution of 1:1000. Blots were incubated in 3% Tween transblotting milk solution (TTBS; 20 $mmol\,l^{-1}$ Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.2) containing a 1:10,000 dilution of horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Upstate, Watford, UK) for 1h at room temperature. The bound antibodies were detected using the Visualizer

chemiluminescence western blotting kit (Upstate), and the membranes were exposed to Biomax X-ray film (Kodak, Cambridge, UK) for 1 min to detect the signals.

Hemocyte counts and colony forming units *in vivo*

Manduca larvae were injected with DMPC water, dsCON or ds-proPSP and PBS, *E. coli* or *P. luminescens* as before; 18 h later, insects were chilled on ice, cut at the midpoint of the dorsal horn and bled into pre-chilled sterile polypropylene tubes. A standard volume of hemolymph (500 μl) was taken from each caterpillar. Samples of hemolymph (50 μl) were immediately added to pre-chilled Grace's Insect Medium (GIM; Sigma, Gillingham, UK) (450 μl). Hemocyte numbers were counted at 100× magnification using a Neubauer hemocytometer. The average of two counts per insect of three insects per treatment was calculated. To determine the number of surviving bacteria, serial dilutions of hemolymph were plated onto Petri dishes containing 2.5% LB and 1.5% agar (Difco Laboratories), and the number of *P. luminescens* colony forming units (CFU) was recorded 24 h later, the average of five insects per treatment being calculated.

Plasmatocyte spreading assay

RNAi-treated or DMPC water control-treated *Manduca* larvae were bled 18 h after the bacteria infection, and approximately 200 μl of hemolymph was allowed to drip into 800 μl of ice-cold *Manduca*-buffered saline solution (MBS; 4 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 18 mmol l^{-1} $MgCl_2.6H_2O$, 1.5 mmol l^{-1} Na_2HPO_4, 1.5 mmol l^{-1} NaH_2PO_4, 192.8 mmol l^{-1} sucrose, pH 4.5) solution in a microcentrifuge tube. The tube was immediately inverted to mix, and then centrifuged at 200 ***g*** for 5 min at 4°C. The supernatant was replaced with 200 μl of cold GIM, and the hemocytes were gently resuspended using a cut pipette tip. Hemocyte density was adjusted to 10^6 cells ml^{-1} in GIM using a hemocytometer. Then, 10 mm coverslips were washed in 70% ethanol and placed centrally into each well of a 24-well plate. The resulting cell suspension (100 μl) was pipetted onto each coverslip and then left undisturbed for 30 min (at room temperature) to allow the hemocytes to settle and form a monolayer. The monolayers were washed gently with a few drops of GIM and removed to new wells containing 400 μl of fresh GIM. Hemocyte classes were classified according to the criteria previously described (Dean et al., 2004). Hemocyte viability was regularly assessed by Trypan Blue exclusion using 0.2% (w/v) Trypan Blue (Sigma) in PBS for 10 min at room temperature. To assess the spreading activity of hemocytes *in vitro*, monolayers were washed once in PBS and then fixed in 2% (w/v) formaldehyde in PBS (made fresh from paraformaldehyde) for 15 min at room temperature. After a further three washes in PBS, the monolayers were incubated for 10 min in 0.285 mol l^{-1} NH_4Cl. This step acted to quench the autofluorescence of the formaldehyde for subsequent fluorescent microscopy. Cells were permeabilised in 0.2% (v/v) Triton X-l00 (Sigma) for 5 min, washed and then stained with phalloidin conjugated to fluorescein isothiocyanate (FITC–phalloidin; Sigma). For each coverslip, a 60 μl drop of FITC–phalloidin solution (0.1 μg ml^{-1}, final concentration in PBS) was applied to a strip of parafilm and the coverslip was inverted onto the drop. Cells were stained at room temperature for 40 min in the dark and then washed four times in PBS and twice in distilled water. Monolayers were mounted overnight at room temperature in 5 μl Mowiol-4-88 solution (Calbiochem, Nottingham, UK) and stored at 4°C. All cell preparations were visualized using an Olympus BH-2 microscope fitted with epifluorescence optics. Individual plasmatocytes were scored as 'spread' if they assumed a flattened morphology and were greater than 15 μm along their longest axis (Miller, 2005). Estimates of plasmatocyte length were made using Image J software (http://rsb.info.nih.gov/ij/). The percentage of spread plasmatocytes and the length of at least 100 cells per monolayer were measured in a randomly chosen field of view, and the average of three monolayers per insect of five insects per treatment was calculated. Plasmatocyte spreading assays were replicated three times.

Hemocyte aggregation

These assays were conducted as previously (Eleftherianos et al., 2007). Briefly, *M. sexta* larvae were injected with dsRNAs or DMPC water and 18 h later were bled; hemocytes were centrifuged, washed and their number was adjusted to 10^6 cells ml^{-1}. Suspensions of *E. coli* and *P. luminescens* in GIM were prepared containing approximately 2×10^7 and 1×10^3 cells ml^{-1}, respectively. 100 μl of bacterial suspensions were mixed with 200 μl of hemocyte suspensions and, following a short incubation at room temperature, cell suspensions were transferred to 96-well microtiter plates. Formed microaggregates were counted using a Nikon TMS-F inverted microscope. The mean of five insects per treatment was calculated.

Nodule formation

Manduca larvae were injected with DMPC water, dsCON or ds-proPSP and PBS, *E. coli* or *P. luminescens*, as above. Nodule formation was assessed 18 h after immune challenge. Insects were immobilized on ice for 15 min before dissection under 1% (w/v) NaCl solution saturated with phenylthiourea (which prevented general post-dissection melanization). Melanized, dark nodules within the hemocoel were observed using a stereomicroscope, and a manual count of nodule number was made with the help of a tally counter. In general, it was easy to recognize individual nodules. Ten insects were used for each treatment and each experiment was replicated three times.

Phagocytosis assays

Bacterial cells were stained as before (Eleftherianos et al., 2007). Labeled *E. coli* and *P. luminescens* were injected 6 h after the RNAi treatment, as described above, and hemolymph was extracted 18 h later. Hemocyte monolayers, prepared as previously (Eleftherianos et al., 2007), were incubated overnight at 4°C in hybridoma supernatant containing the monoclonal antibody MS77 (Willott et al., 1994) followed by a second incubation with a TRITC-conjugated goat anti-mouse IgG antibody (Sigma) at 1:100 dilution in 0.3% bovine serum albumin (BSA) in PBS. Coverslips were finally mounted in 5 ml of Mowiol-4-88 solution. Control slides were incubated with the secondary antibody only. The phagocytic competence of *M. sexta* hemocytes was examined using a Zeiss LSM-510 laser scanning confocal microscope. The average number of bacteria contained in 20 hemocytes per monolayer (three monolayers per insect of five insects per treatment) was calculated.

RESULTS

Expression and RNAi of proPSP in *Manduca*

In order to determine if *Manduca proPSP* is upregulated following *E. coli* or *P. luminescens* infection, we used RT-PCR to detect *proPSP* mRNA using RNA extracted from fat body and hemocytes of infected and uninfected fifth-stage larvae as template. We found that *proPSP* was transcribed in fat body in response to *E. coli* or *P. luminescens* 18 h post-injection (Fig. 1A) but that *proPSP* mRNA was undetectable by RT-PCR in hemocytes (Fig. 1B). Controls

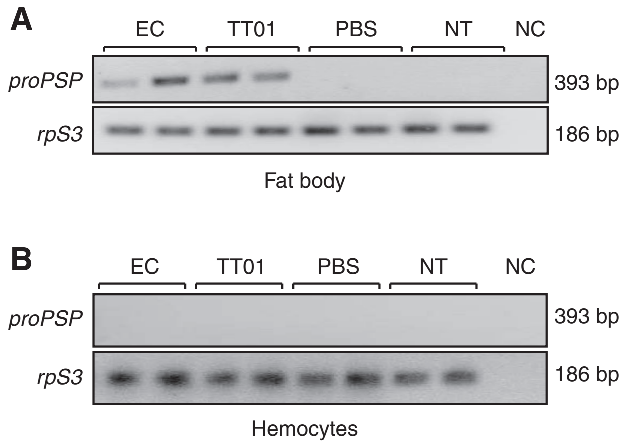

Fig. 1. Tissue-specific transcription of pro-plasmatocyte spreading peptide (PSP)-encoding gene (*proPSP*) in *Manduca sexta* is induced upon bacterial infection. (A) Insects were injected with either 2×10^7 cells of *E. coli* (EC) or 1×10^3 cells of *P. luminescens* (TT01). RT-PCR shows that mRNA levels of *proPSP* are upregulated 18 h after bacterial infection in *Manduca* fat body. (B) By contrast, *proPSP* is not transcribed in hemocytes of infected insects. Note that no transcription is seen in negative controls including *Manduca* injected with phosphate-buffered saline (PBS) or in untreated insects (NT). A template negative control of RT-PCR (NC) is also included. The ribosomal protein S3 (rpS3) was used as a loading control. The sizes of the PCR products are indicated. PCR amplifications involved 35 cycles.

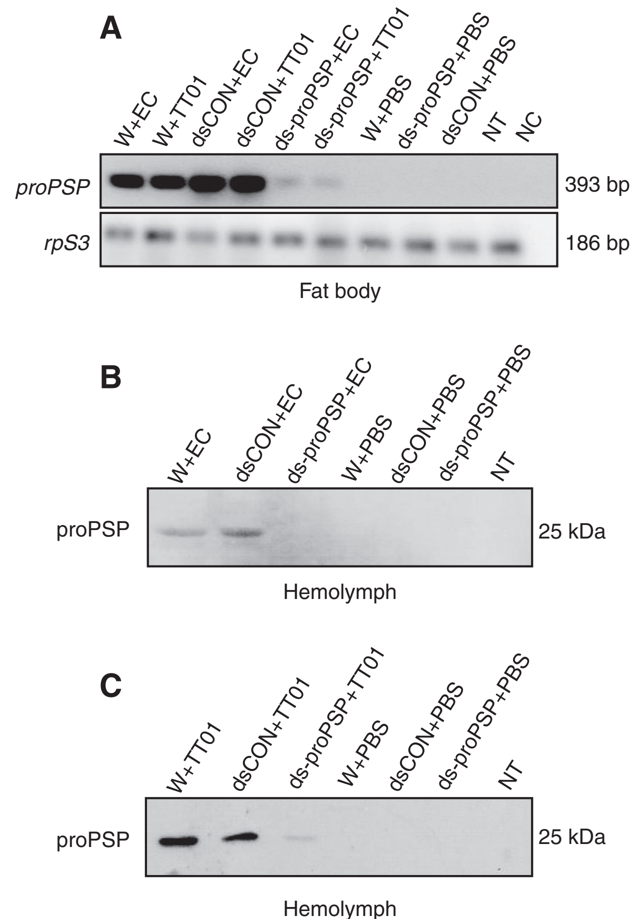

Fig. 2. RNAi-mediated knock-down of proPSP in *Manduca sexta*. (A) Injection of DMPC-water and *E. coli* (W+EC) or *P. luminescens* (W+TT01) again leads to over-transcription of *PSP* in *Manduca* fat body. However, prior injection (6 h before) of ds-proPSP downregulates *proPSP* gene transcription (ds-proPSP+EC, ds-proPSP+TT01). By contrast, the control dsRNA reagent dsCON has no effect on *proPSP* gene transcription (dsCON+EC, dsCON+TT01). Note that negative controls including injection of dsRNAs alone with no bacterial infection (dsCON+PBS, ds-proPSP+PBS), injection of DMPC-treated water and PBS (W+PBS) or no treatment (NT) produce no signal. A template negative control of RT-PCR (NC) is also included. RT-PCR amplification of the constitutively expressed ribosomal protein S3 gene (*rpS3*) is used as a loading control. The sizes of the PCR products are indicated. PCR amplifications involved 35 cycles. (B) Western analysis, using a *P. includens* anti-PSP1 antibody, shows that RNAi of proPSP also results in reduced protein levels in *Manduca* hemolymph. Injection of *E. coli* (W+EC) leads to over-expression of proPSP in *Manduca* hemolymph plasma, which in turn can be knocked-down by prior injection of ds-proPSP (ds-proPSP+EC). (C) Similarly, injection of *P. luminescens* (W+TT01) leads to proPSP over-expression in *Manduca* hemolymph, which can be reduced by RNAi (ds-proPSP+TT01). However, PSP protein expression does not change in insects injected with dsCON (dsCON+EC, dsCON+TT01). Again, note the absence of proPSP protein induction in the controls. The specificity of staining was checked by omitting the primary antibody, which led to complete absence of bands (result not shown). RT-PCR and western expression levels were assessed in duplicate for each treatment; results for single individuals are shown.

injected with PBS, or left untreated, showed that *proPSP* transcripts were absent (Fig. 1A,B).

To knock-down transcription of *proPSP*, we pre-injected caterpillars with ds-proPSP 6 h before the bacterial infection and 18 h after the RNAi treatment; *proPSP* mRNA levels, as detected by semi-quantitative RT-PCR, were strongly reduced in the fat body of *E. coli*- or *P. luminescens*-injected insects (Fig. 2A). To confirm that this decrease in *proPSP* mRNA led to a similar reduction in secreted proPSP protein, we carried out western blots and confirmed that the RNAi treatment also significantly suppressed or abolished proPSP protein levels in cell-free hemolymph plasma. No significant differences in proPSP protein expression levels were observed between hemolymph samples from *E. coli*- and *P. luminescens*-infected insects. (Fig. 2B,C). All uninfected controls showed undetectable levels of mRNA transcription or protein expression of proPSP. We also showed that the RNAi effect was gene specific. Pre-injection of a dsRNA from an irrelevant gene (dsCON) had no effect on *proPSP* mRNA (Fig. 2A) or protein levels (Fig. 2B,C). Transcription of the constitutively expressed ribosomal protein rpS3 (Jiang et al., 1996), used as a loading control, was unaffected by the ds-proPSP reagent (Fig. 2A). We also performed negative controls, including injection of DMPC-treated water instead of dsRNAs and injection of dsRNAs with no post-injection of *E. coli* or *P. luminescens*; in neither of these cases was there any detectable gene transcription or expression of PSP (Fig. 2A–C).

Knock-down of proPSP increases susceptibility of *Manduca* to *Photorhabdus* infection

To test if RNAi-mediated knock-down of proPSP could have an effect on the susceptibility to *P. luminescens*, we tested the survival of infected larvae pre-treated with dsRNAs. Insects pre-injected with ds-proPSP were significantly more susceptible to infection by *P. luminescens* and died significantly faster than insects pre-injected with the control RNAi reagent (dsCON) (Fig. 3A); the number of insects dead at 48 h was significantly different between the ds-proPSP and the dsCON treatments (χ^2=7.52, d.f.=2, P=0.003) as well as between the ds-proPSP and W (insects pre-injected with DMPC-treated water) treatments (χ^2=7.21, d.f.=2, P=0.003).

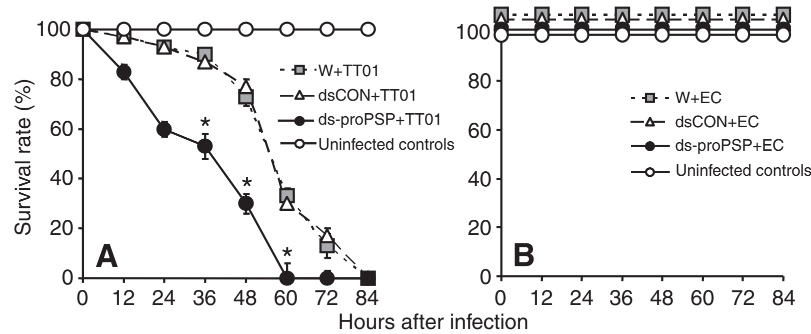

Fig. 3. RNAi-mediated knock-down of proPSP is associated with decreased insect survival following *P. luminescens* infection, but not *E. coli* infection. *Manduca sexta* larvae were injected first with DMPC water (W), dsRNA control (dsCON) or dsRNA of proPSP (ds-proPSP) and 6 h later with 1×10^3 cells of *P. luminescens* (TT01), 2×10^7 cells of *E. coli* (EC) or PBS. Insect survival was then monitored over 84 h. (A) Note that TT01 alone (W+TT01) kills all insects by 84 h and that RNAi knock-down of proPSP (ds-proPSP+TT01) speeds the rate at which *Manduca* die (60 h) following infection with the same dose of pathogen. Injection of dsCON prior to *Photorhabdus* infection (dsCON+TT01) has no effect on insect survival. Injection of dsRNAs alone without bacterial infection or injection of DMPC water and PBS (uninfected controls) results in no insect death. Values are means ± s.d. (N=3 replicates per time-point, 10 insects per replicate). Significant differences are indicated with an asterisk (χ^2 test, P<0.05). (B) By contrast, RNAi knock-down of proPSP expression has no effect on insect survival following infection with *E. coli*.

However, there was no difference in survival between the dsCON and W treatments at any of the time-points taken. Negative controls including pre-injection of water or dsRNAs not followed by *P. luminescens* injection had no impact on *Manduca* survival. Additional controls included infection by *E. coli* following pre-injection with water or dsRNAs; in each of these cases insects showed no mortality (Fig. 3B).

Effect of proPSP knock-down on circulating hemocytes and bacterial survival within *Manduca*

We first wished to look if RNAi knock-down of PSP in *Manduca* affects hemocyte numbers and bacterial survival within day 0 fifth-stage larvae. We injected ds-proPSP prior (t=–6 h) to injection (t=0 h) of non-pathogenic bacteria (~2×10^7 *E. coli*) or pathogenic (~10^3 *P. luminescens*) bacteria, and subsequently (t=+18 h) we counted both the number of hemocytes and the number of recoverable bacteria from the hemolymph of infected as well as uninfected insects. We found that hemocyte density in *E. coli*-infected insects was significantly higher compared with resting-level values from uninfected insects (one-way ANOVA, d.f.=3,20, F=13.50, P<0.0001; Tukey *post-hoc* test, P<0.05) (Fig. 4A). Control pre-injection treatments (injection of control dsRNA or water) had no effect on the number of hemocytes. However, pre-injection of ds-proPSP 6 h before the *E. coli* treatment significantly decreased hemocyte numbers 18 h after the bacteria infection (one-way ANOVA, d.f.=2,15, F=16.04, P<0.001; Tukey *post-hoc* test, P<0.05), and this effect was not observed when insects were injected with the dsRNA control reagent. Interestingly, *P. luminescens* infection of proPSP knock-down insects did not affect hemocyte numbers (one-way ANOVA, P>0.05).

At the same time, significantly larger numbers of live *E. coli* bacteria (an increase of ~5-fold) were plated out from proPSP knock-down insects compared with insects pre-injected with DMPC-treated water or dsCON (one-way ANOVA, d.f.=2,12, F=54.05, P<0.0001; Tukey *post-hoc* test, P<0.05) (Fig. 4B). This implies that the presence of proPSP is important in the clearance of injected non-pathogenic bacteria from the circulation. However, no significant differences in recoverable *P. luminescens* were found between ds-proPSP-treated insects and the various control treatments (one-way ANOVA, P>0.05). This may indicate that *P. luminescens* is able to avoid the host insect's clearing response.

RNAi of proPSP reduces hemocyte spreading in infected *Manduca*

We next assessed the *in vitro* spreading ability of hemocytes taken from proPSP knock-down insects. In uninfected *Manduca*, most adherent hemocytes are plasmatocytes or granular cells (Dean et al., 2004). Although other hemocyte types (such as oenocytoids, spherulocytes and prohemocytes) are present in *Manduca*, these were seldom observed in our monolayers (data not shown) but were considered where appropriate in the total number of spread cells in the various treatments.

We found that the extent of spreading of hemocytes from uninfected *Manduca* was low (Fig. 5A, supplementary material Fig. S1), with no significant differences between control treatments (one-way ANOVA, P>0.05) (Fig. 5A). Similarly, hemocytes from insects that had been infected with *Photorhabdus* showed little spreading (Fig. 5A; supplementary material Fig. S1), and the mean number of spread cells was not statistically different from the uninfected controls (one-way ANOVA, P>0.05) (Fig. 5A). By contrast, more than 70% of hemocytes displayed spreading activity when these cells were from water or dsCON pre-treated larvae and subsequently infected with *E. coli* (Fig. 5A; supplementary material Fig. S1). These two control groups were not significantly different (one-way ANOVA, P>0.05) (Fig. 5A), but spreading was significantly reduced to control levels when insects were pre-injected with dsRNA specific to proPSP (one-way ANOVA, d.f.=2,6, F=300.9, P<0.0001; Tukey *post-hoc* test, P<0.05) (Fig. 5A; supplementary material Fig. S1).

We also quantified hemocyte spreading by measuring hemocyte length in the various treatments. We found that the mean length of hemocytes from uninfected *Manduca* pre-injected with PBS, dsCON or ds-proPSP was ~40 μm, which was not significantly different from the mean length of hemocytes from *P. luminescens*-infected insects pre-injected with the same reagents (one-way ANOVA, P>0.05) (Fig. 5B). By contrast, the size of hemocytes from *E. coli*-infected caterpillars pre-injected with water or dsCON increased to ~70 μm in length, which was significantly greater than the size of hemocytes estimated from larvae in which proPSP expression had been knocked-down (one-way ANOVA, d.f.=2,6, F=92.70, P<0.0001; Tukey *post-hoc* test, P<0.05) (Fig. 5B). Interestingly, mean hemocyte length in ds-proPSP pre-treated *Manduca* was not statistically different from that calculated from insects that did not

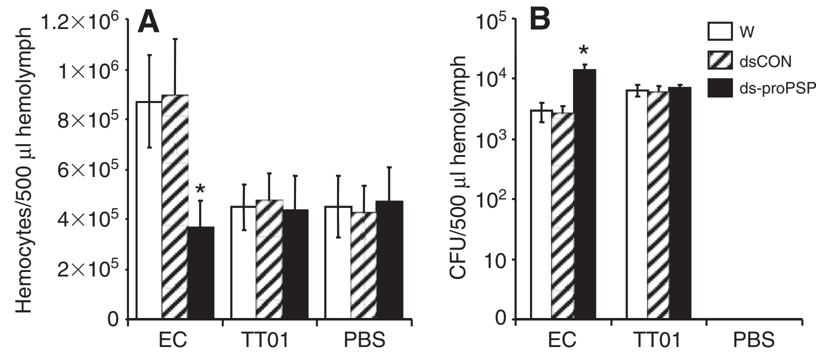

Fig. 4. Numbers of hemocytes and bacteria recovered from *E. coli*- and *P. luminescens*-infected *M. sexta* larvae. (A) Estimated free hemocyte count 18 h after bacterial infection. Insects were injected first with DMPC water (W), dsRNA control (dsCON) or dsRNA of proPSP (ds-proPSP) and 6 h later with 2×10^7 cells of *E. coli* (EC), 1×10^3 cells of *P. luminescens* (TT01) or PBS. Caterpillars were bled 18 h after bacterial infection to determine the number of free hemocytes in 500 µl of hemolymph. PBS injections were used as controls. Note the significant decrease in hemocyte density in proPSP knock-down insects when infected with *E. coli*, but not with *P. luminescens*. Bars show means ± s.d. (N=3). (B) Numbers of recoverable bacteria (CFU, colony forming units) 18 h after bacterial infection. Note that *E. coli* grows significantly better in hemolymph extracted from proPSP knock-down insects. However, RNAi of proPSP does not increase the number of recoverable *P. luminescens* bacteria in the hemolymph. Bars show means ± s.d. (N=5). Asterisks show values that are significantly different from the others (ANOVA; P<0.05).

receive *E. coli* (one-way ANOVA, P>0.05) (Fig. 5B). Thus, RNAi-mediated knock-down of proPSP prevents *E. coli*-induced hemocyte spreading.

Cellular immune defenses are reduced in proPSP knock-down *Manduca* in response to non-pathogenic bacteria

We also investigated whether RNAi-mediated knock-down of proPSP affects key cellular immune responses of *Manduca in vivo* and *in vitro*. We first examined the ability of washed hemocytes to form aggregates around bacteria when exposed to them *in vitro* (Fig. 6A; supplementary material Fig. S2). We found that hemocytes from proPSP knock-down insects formed significantly fewer microaggregates than controls when both groups were exposed to *E. coli* (one-way ANOVA, d.f.=2,12, F=13.33, P<0.001; Tukey *post-hoc* test, P<0.05) but that there was no difference between the RNAi-treated insects and the controls when they were both infected with *P. luminescens* (one-way ANOVA, P>0.05).

We then counted the number of melanotic nodules that were formed *in vivo* after experimental infection with *E. coli* or *P. luminescens* (Fig. 6B). We found that injection of bacteria into the ds-proPSP pre-treated insects resulted in the formation of significantly fewer melanotic nodules compared with the various control treatments (one-way ANOVA, d.f.=2,3, F=68.49, P<0.001; Tukey *post-hoc* test, P<0.05 for *E. coli* treatments; one-way ANOVA, d.f.=2,3, F=10.21, P<0.001; Tukey *post-hoc* test, P<0.05 for *P. luminescens* treatments). This result is illustrated visually in supplementary material Fig. S3. However, the decrease in the number of nodules was not accompanied by a reduction in the amount of prophenoloxidase in the hemolymph plasma of PSP knock-down insects (data not shown).

We finally examined whether RNAi knock-down of proPSP affects the ability of *M. sexta* hemocytes to phagocytose bacteria *in vivo*. We counted engulfed stained *E. coli* or *P. luminescens* cells within hemocytes from either dsRNA or water pre-treated insects

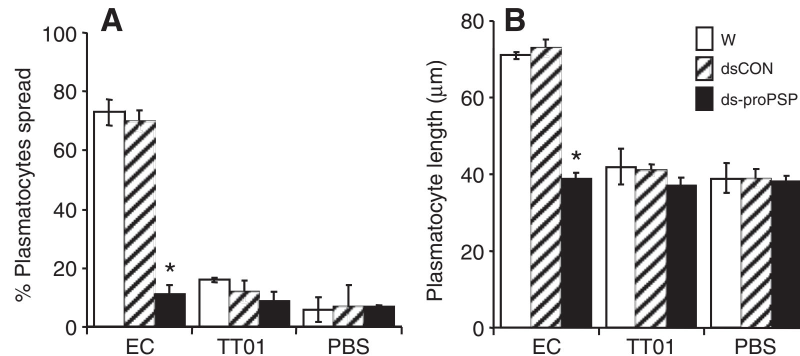

Fig. 5. RNAi of *Manduca* proPSP decreases hemocyte spreading *in vitro*. (A) Histogram showing spreading of *Manduca* hemocytes in various treatments. Day 0 fifth-stage caterpillars were injected with DMPC water (W) or dsRNAs and 6 h later with PBS, 2×10^7 cells of *E. coli* (EC) or 1×10^3 cells of *P. luminescens* (TT01). Individual hemocytes were scored as 'spread' if they assumed a flattened morphology and were greater than 15 µm along their longest axis. Note, first, that *P. luminescens* suppresses hemocyte spreading in all treatments and, second, the reduced spreading in proPSP knock-down insects infected with *E. coli*. Bars show means ± s.d. (N=5 insects, in each of which hemocyte spreading was estimated for 100 hemocytes). (B) Histogram showing quantification of hemocyte spreading in insects treated as previously. Again, note that the mean length of hemocytes from *P. luminescens*-infected insects (W+TT01, dsCON+TT01, ds-proPSP+TT01) was significantly reduced compared with that of the hemocytes from *E. coli*-infected individuals (W+EC) and that this effect can further be reduced by knocking down proPSP (ds-proPSP+EC) but not when injecting dsCON (dsCON+EC). Bars show means ± s.d. (N=5 insects, in each of which the length of 100 hemocytes was measured). Asterisks show values that are significantly different from the others (ANOVA; P<0.05).

(Fig. 6C). We found that dsPSP injection before infection with *E. coli* significantly reduced the number of bacteria within the phagocytic cells (one-way ANOVA, d.f.=2,12, F=17.35, P<0.001; Tukey *post-hoc* test, P<0.05) but that other treatments, including RNAi knock-down of proPSP before infection with *P. luminescens*, did not (one-way ANOVA, P>0.05). Confocal microscopy of TRITC–phalloidin-stained hemocytes containing engulfed labeled bacteria allowed us to confirm that these bacteria were indeed located within the hemocytes (supplementary material Fig. S4).

DISCUSSION

The detection of 'non-self' invaders in the insect hemocoel results in a range of defensive responses including induction of antimicrobial peptides and proteins, activation of the prophenoloxidase cascade, changes in the hemocyte population, and changes in the state and behavior of the hemocytes (i.e. spreading, aggregation, nodulation and phagocytosis) (Gillespie et al., 1997). In terms of the cellular immune response, hemocyte spreading is a commonly assessed parameter in response to a microbial or parasitic infection and provides an indication of hemocyte activity and thus the fitness of the immune system (Dean et al., 2004).

Insects produce several types of hemocytes with diverse functions in immunity and development (Lackie, 1988; Gillespie et al., 1997; Lavine and Strand, 2002). In Lepidoptera, the main hemocyte classes are plasmatocytes and granulocytes, which normally circulate freely in the hemocoel, but upon immune challenge rapidly attach to wound sites, foreign targets or one another (Ribeiro and Brehelin, 2006). Plasmatocytes are normally non-adhesive and must be activated by cytokines in hemolymph in order to bind and spread onto foreign surfaces. The most potent known activator of plasmatocytes is the plasmatocyte-spreading peptide (PSP) (Clark et al., 1997), homologs of which have been identified from a number of other moth species, including *M. sexta* (Clark et al., 2004). PSP and related homologs are the only molecules identified to date that activate plasmatocytes (Clark et al., 1997; Strand et al., 2000; Miura et al., 2002; Nakahara et al., 2003), although circumstantial evidence suggests that several other molecules besides PSP mediate plasmatocyte function (Lackie, 1988; Gillespie et al., 1997; Lavine and Strand, 2002). However, there has been no detailed investigation of the participation and role of PSP in hemocytic reactions responsible for bacterial clearance *in vivo*. In the current paper, we attempt to investigate the contribution of PSP in regulating the spreading ability of hemocytes as well as other cellular immune functions during infection of the lepidopteran model insect *M. sexta* with pathogenic and non-pathogenic bacteria. We first measured the levels of proPSP mRNA in *M. sexta* fat body and hemocyte tissues following *E. coli* or *P. luminescens* challenge. We also used western blots to follow the expression of proPSP protein. Our results confirmed previous findings that *M. sexta* fat body cells, but not circulating hemocytes, are induced by bacteria to produce proPSP, which is then secreted into hemolymph plasma (Wang et al., 1999; Yu et al., 1999). Interestingly, the restriction of proPSP expression to fat body cells is not true in other lepidopterans, and proPSP1 mRNAs have been previously detected by northern blot in both fat body and hemocytes of *P. includens* (Clark et al., 1998), where detection of additional larger hybridizing RNAs in fat body, hemocytes and nervous tissue was also reported. Larger mRNAs hybridizing to the GBP cDNA probe have also been observed in nervous tissue from *P. separata* (Hayakawa et al., 1995). In the present work, we did not examine expression of *M. sexta* proPSP in nervous tissue. However, it is evident that in this insect a major site of proPSP synthesis is the fat body, as is well established for most *M. sexta* immune pattern

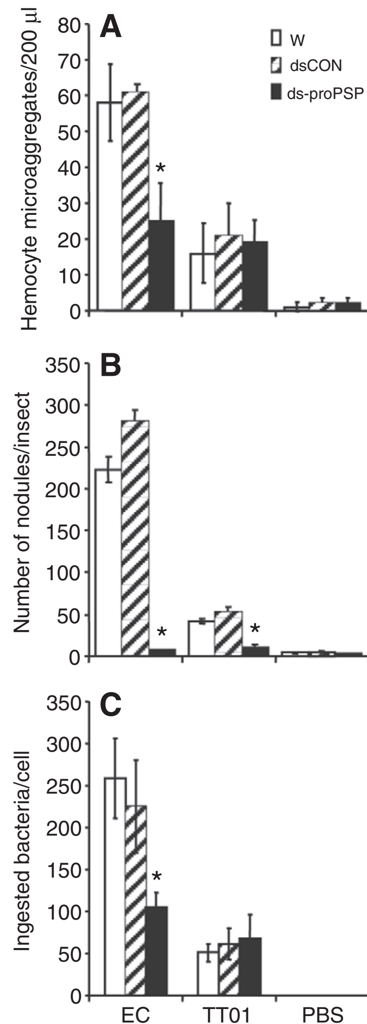

Fig. 6. RNAi of *Manduca* proPSP decreases cellular immune defenses against non-pathogenic bacteria. (A) Number of hemocyte microaggregates in hemolymph taken from uninfected *Manduca*, to which *E. coli* (EC), *P. luminescens* (TT01) or PBS were added *in vitro*. Day 0 fifth-stage caterpillars were injected with DMPC water (W) or dsRNAs; 18 h later they were bled and hemocytes were collected. A suspension of 2×10^7 *E. coli* or 1×10^3 *P. luminescens* cells ml^{-1} was added to 10^6 hemocytes in a 1:2 ratio and, following incubation for 15 min, the number of microaggregates was counted using an inverted microscope. Bars show means ± s.d. (N=5). (B) Histogram showing number of melanotic nodules in *M. sexta* larvae pre-injected with proPSP dsRNA (ds-proPSP), unrelated dsRNA control (dsCON) or water (W) and 6 h later injected with 2×10^7 cells of *E. coli* (EC), 1×10^3 cells of *P. luminescens* (TT01) or PBS. Insects were dissected 18 h after the second injection. Bars show means ± s.d. (N=10). (C) Histogram showing numbers of *E. coli* (EC) or *P. luminescens* (TT01) cells ingested by *M. sexta* phagocytic cells during 18 h of bacterial infection, which was initiated 6 h after DMPC water or dsRNAs injection. PBS treatments were used as controls. Bars show means ± s.d. (N=5 insects, in each of which engulfed bacteria in 20 hemocytes were counted). Asterisks show values that are significantly different from the others (ANOVA; P<0.05).

recognition protein and antimicrobial effector genes (Kanost et al., 2004; Eleftherianos et al., 2006a; Eleftherianos et al., 2006b). Previous studies suggest that sensitivity of hemocytes to PSP varies both between and within the larval stages of the insect and that plasmatocyte sensitivity in *P. includens* is most variable during

critical periods as larvae undergo the transition from feeding to preparation for molting or metamorphosis (Clark et al., 2005; Lavine and Strand, 2002). Interestingly, we have recently observed that small age changes within the fifth larval stage of *Manduca* play a critical role in bacterial immune challenge. We compared several aspects of the humoral and cellular immune response of newly ecdysed (day 0) and pre-wandering (day 5) fifth-stage *M. sexta* caterpillars and found a dramatic decrease in immune function in the older larvae which led to faster *P. luminescens* colonization in the host's tissues and rapid insect death (Eleftherianos et al., 2008). The experiments described here all involved larvae of identical age; however, it would be worth testing whether proPSP expression changes within the fifth larval stage of *M. sexta* or even between the various larval stages and how this correlates with host resistance to *P. luminescens* infection.

The key experimental technique used in this paper to investigate PSP function is systemic RNAi to silence the expression of the *proPSP* gene. This was achieved by injecting dsRNAs specific to the targeted gene into newly molted final-stage *M. sexta* larvae. We have previously used the same technique to suppress the expression of hemolymph recognition proteins and antibacterial peptides, which are expressed at only very low levels in unchallenged insects but which are strongly induced upon infection with *E. coli* and *P. luminescens* (Eleftherianos et al., 2006a; Eleftherianos et al., 2006b). In a similar fashion, in the present study we have shown that ds-proPSP injection 6 h prior to bacterial infection is effective in silencing the expression of PSP in *M. sexta*. RNAi treatment strongly repressed the expression of proPSP, as shown by reductions in levels of both mRNA in the fat body and protein in the cell-free hemolymph plasma. We also performed several additional control treatments (e.g. a control dsRNA; injecting dsRNAs without subsequent infection, or no treatment), all of which were consistent with specific knock-down of the targeted gene. Once more, we did not observe any case in which administration of ds-proPSP alone elicited expression of proPSP, as previously reported for hemolin in response to the corresponding dsRNA reagent in *Antheraea pernyi* pupae (Hirai et al., 2004).

We used the proPSP knock-down insects to investigate the role of proPSP in *M. sexta* immune capacity with particular emphasis on cellular defenses. RNAi knock-down of induced proPSP expression caused significant decrease in hemocytic responses against *E. coli*, but not against *P. luminescens*. The number of *E. coli* CFU recovered from the hemolymph of ds-proPSP pre-treated larvae was increased by approximately fivefold, and the increase in the total number of hemocyte population, which is observed in DMPC water pre-treated insects, was reduced by approximately 60%. Interfering with the induced expression of proPSP also significantly reduced plasmatocyte spreading activity and hemocyte aggregation *in vitro* in response to the injection of *E. coli*, as well as depressing nodule formation and phagocytosis of *E. coli* bacteria by hemocytes *in vivo*. These findings highlight the key role of this protein in *M. sexta* cellular immune reactions. We suggest that the negative effects on immune function of reducing proPSP availability are a consequence of a lack of PSP itself. Without proPSP, we presume that circulating titers of PSP peptide will be reduced. However, we did not directly measure PSP peptide levels, and currently no technique has been established to do this.

Strikingly, we found that all cellular responses (with the exception of nodulation) in *P. luminescens*-infected insects were significantly suppressed compared with those estimated when insects were challenged with non-pathogenic *E. coli*, regardless of whether proPSP expression was knocked-down or not, and in most cases they were not significantly different from the responses of uninfected control insects. Our results also indicate that the pathogenic *P. luminescens* TT01 bacteria do not kill host hemocytes, but instead have an adverse effect on hemocyte spreading. In previous work, we found that *P. luminescens* strains W14 and K122 actively killed hemocytes by mechanisms that differ between strains (Au et al., 2004). It is well documented that *Photorhabdus* is armed with a variety of pathogenicity factors and toxins, many of which interfere with host immune defenses (ffrench-Constant et al., 2007). For example, the *P. luminescens* genome encodes a type III secretion system (TTSS), and one of the effectors, LopT (similar to the YopT effector secreted by *Yersinia pestis*), represses nodule formation and prevents phagocytosis in *Spodoptera littoralis* and *Locusta migratoria* (Brugirard-Ricaud et al., 2004; Brugirard-Ricaud et al., 2005). Our results further suggest that *P. luminescens* TT01 produces anti-spreading factors, which may have direct pathological effects on *M. sexta* hemocytes. It has previously been established that proteases and toxic metabolites produced by *Beauveria bassiana* cause reduced hemocyte spreading in the greater wax moth, *Galleria mellonella* (Griesch and Vilcinskas, 1998), and the beet armyworm, *Spodoptera exigua* (Hung et al., 1993; Mazet et al., 1994).

In order to assess the effect of proPSP silencing on the cellular immune defenses of infected *M. sexta*, we injected insects with a standard dose of *P. luminescens* or *E. coli* following a previous RNAi treatment and monitored their survival over four days. We found that proPSP knock-down insects survived *Photorhabdus* infection significantly less well than controls. However, RNAi silencing of proPSP had no effect on survival when insects were infected with non-pathogenic *E. coli*. This is in agreement with our previous results that showed that RNAi-mediated knock-down of specific recognition or antibacterial effector genes had a profound effect on the susceptibility of *M. sexta* larvae to pathogenic *P. luminescens*, but not to the non-pathogenic *E. coli*. This also shows that the induced expression of proPSP is an important, although ultimately unsuccessful, component of the insect's immune defenses against this pathogen. It is worth noting that current survival curves for proPSP knock-down *M. sexta* challenged with *P. luminescens* are similar to those previously obtained for hemolin knock-down insects (Eleftherianos et al., 2006b). Hemolin has also been recently shown to play an important role in mediating cellular immune reactions of *Manduca* against bacteria. RNAi-mediated knock-down of hemolin also significantly reduced the ability of hemocytes to engulf bacteria through phagocytosis, form melanotic nodules *in vivo* and aggregate around bacteria *in vitro* (Eleftherianos et al., 2007). However, it needs to be stressed that previously we examined the effect of hemolin knock-down on hemocyte properties of *M. sexta* larvae infected with a non-pathogenic strain of *E. coli* and not with a potent insect pathogen such as *P. luminescens* (Eleftherianos et al., 2007). Given that both hemolin and PSP play important roles in *M. sexta* hemocyte-based antibacterial defenses, it is currently not known whether there is an interaction between these two immune proteins upon synthesis and secretion into the hemolymph and whether this affects hemocyte behavior and/or immune function. It would also be of particular interest to test whether silencing the expression of hemolin also downregulates the expression of PSP, and *vice versa*, or whether systemic RNAi of both immune proteins leads to greater deterioration of *M. sexta* cellular responses to bacteria and faster insect death. These various possibilities remain to be determined.

In summary, the present study describes the cellular immune responses of *M. sexta* towards pathogenic and non-pathogenic bacteria and provides firm evidence that the PSP precursor protein, proPSP, is required for these responses. The effects of RNAi

suppression of proPSP availability are consistent with the hypothesis that PSP peptide functions as a cytokine that mediates the spreading properties of *M. sexta* hemocytes. However, the final story may not be that simple. Several other factors (e.g. soluble peptides containing the amino acid sequence RGD) also affect hemocyte spreading, encapsulation, aggregation and nodulation (Johansson, 1999). Future work will focus on the contribution of PSP to overall *Manduca* humoral and cellular immune response against pathogenic microbes or endoparasitoid wasps and whether PSP mediates the induction of antimicrobial effector genes.

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CFU	colony forming units
DMPC	dimethyl pyrocarbonate
dsCON	double-stranded control
ds-proPSP	double-stranded pro-plasmatocyte spreading peptide
dsRNA	double-stranded RNA
FITC	fluorescein isothiocyanate
GBP	growth-blocking peptide
GIM	Grace's insect medium
MBS	*Manduca*-buffered saline
PBS	phosphate-buffered saline
PP	paralytic peptide
PSP	plasmatocyte-spreading peptide
RNAi	RNA interference
rpS3	ribosomal protein S3
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
TRITC	tetramethyl rhodamine iso-thiocyanate
TTSS	type III secretion system

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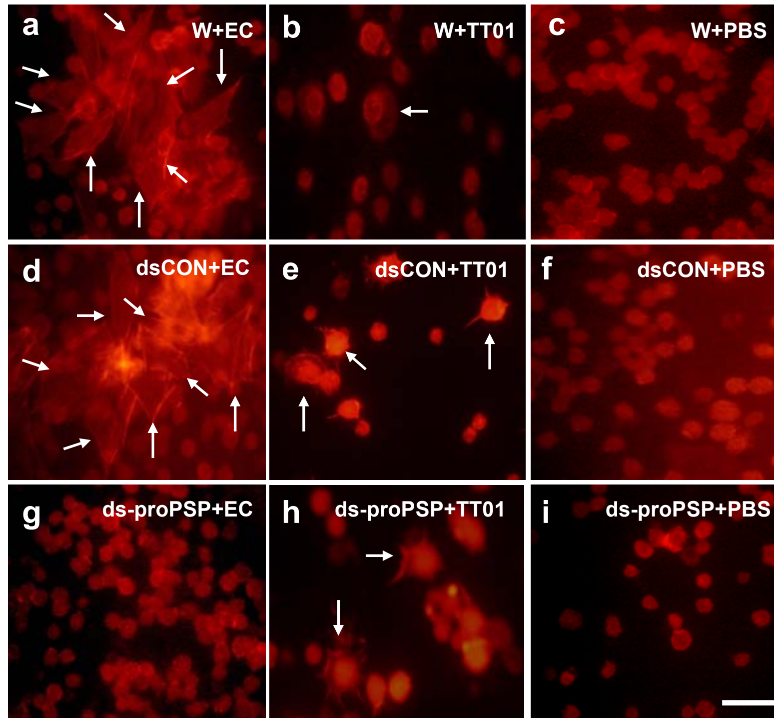
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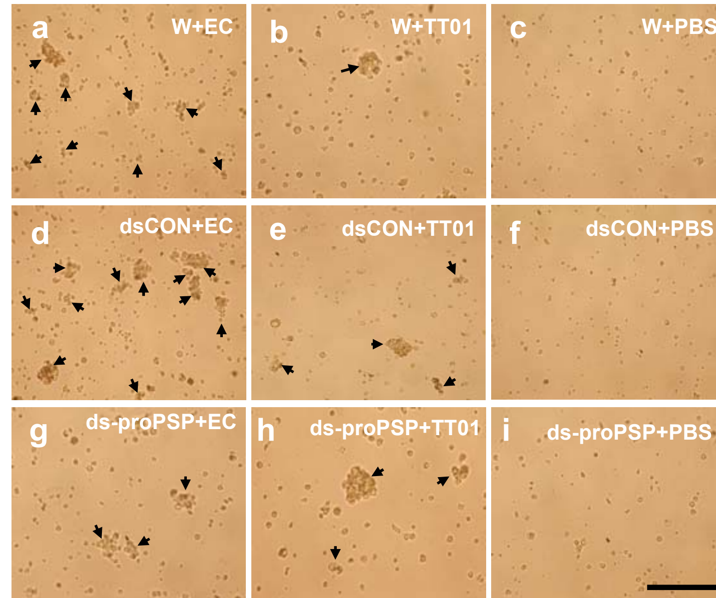
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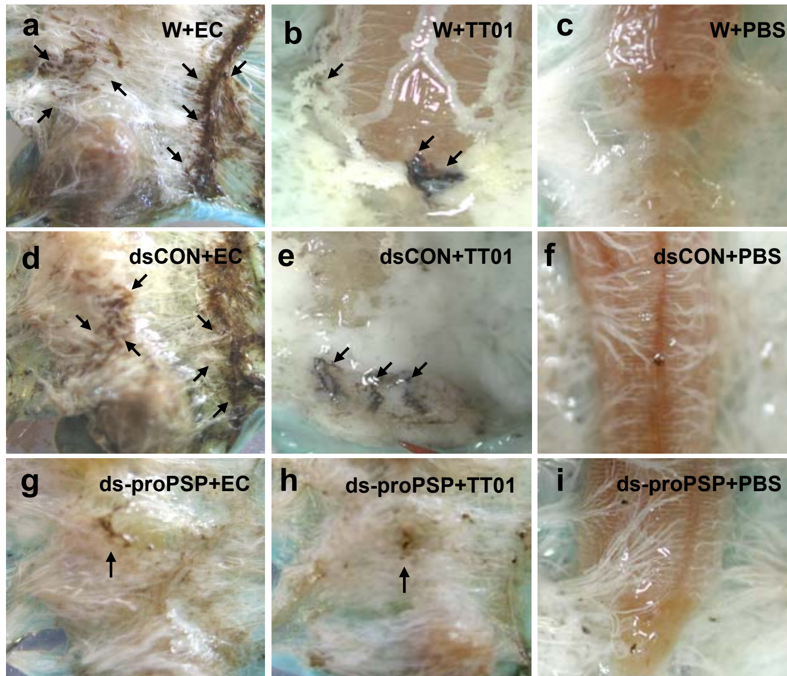
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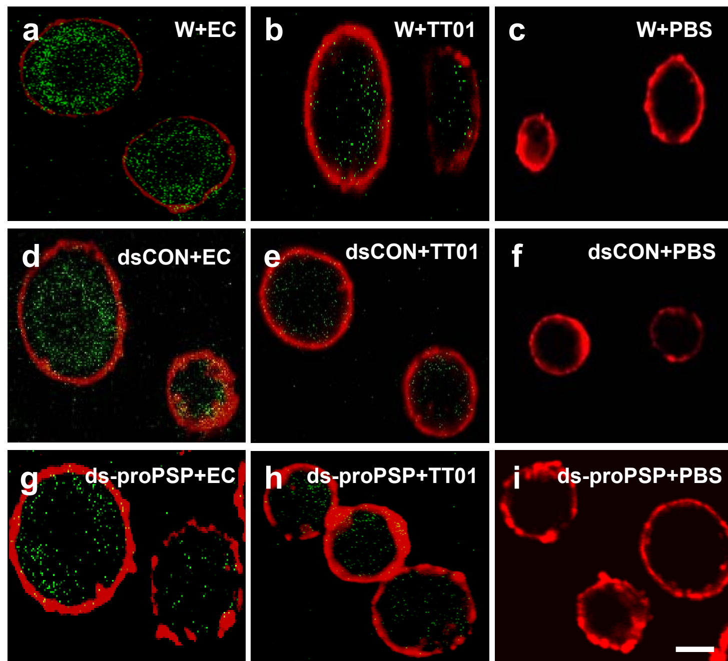

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