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A Serine Proteinase Homologue, SPH-3, Plays a Central Role in Insect Immunity

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Numerous vertebrate and invertebrate genes encode serine proteinase homologues (SPHs) similar to members of the serine proteinase family, but lacking one or more residues of the catalytic triad. These SPH proteins are thought to play a role in immunity, but their precise functions are poorly understood. In this study, we show that SPH-3 (an insect non-clip domain–containing SPH) is of central importance in the immune response of a model lepidopteran, *Manduca sexta*. We examine *M. sexta* infection with a virulent, insect-specific, Gram-negative bacterium *Photorhabdus luminescens*. RNA interference suppression of bacteria-induced SPH-3 synthesis severely compromises the insect's ability to defend itself against infection by preventing the transcription of multiple antimicrobial effector genes, but, surprisingly, not the transcription of immune recognition genes. Upregulation of the gene encoding prophenoloxidase and the activity of the phenoloxidase enzyme are among the antimicrobial responses that are severely attenuated on SPH-3 knockdown. These findings suggest the existence of two largely independent signaling pathways controlling immune recognition by the fat body, one governing effector gene transcription, and the other regulating genes encoding pattern recognition proteins. *The Journal of Immunology*, **2011, 186: 4828–4834.**

Genome projects have revealed that both vertebrates and invertebrates have large numbers of serine proteinase homologue (SPH) genes. For example, the fruit fly *Drosophila melanogaster* has no fewer than 63 genes encoding predicted SPHs (1), including the well-studied *masquerade* (2). Masquerade-like SPHs, which have an N-terminal clip domain (3), play a variety of roles in development (2) and immunity (see below), but most SPHs lack a clip domain. For example, in *Drosophila* there are 47 SPHs with no clip domain compared with only 16 SPHs with one or more partial or complete domains (1). However, despite the widespread occurrence of SPHs, their precise functions remain largely obscure.

In insects, many SPHs are expressed as part of the multiple immune reactions that are mounted against microorganisms. Among these immune responses is prophenoloxidase (PPO) activation, which is important for both the cellular and the humoral arm of the invertebrate immune response (4). Although it is known that cascades of catalytically active serine proteases are central to the immune-initiated activation of PPO (5–7), evidence has only recently accumulated for two clip domain-containing SPHs, SPH-1 and -2 of *Manduca sexta*, that they are involved in controlling the activation process itself (8–10). The roles of the large number of other insect SPHs are, however, largely unknown. In this study, we use systemic RNA interference (RNAi) to investigate the function of SPH-3 (a non-clip domain-containing SPH) in the immune responses of *M. sexta* to the highly entomopathogenic *Photorhabdus* bacterium. Our work on SPH-3 was initially stimulated by the finding that *Photorhabdus* produces large amounts of an RTX-like zinc metalloprotease, protease A (PrtA) (11–14), which we showed to cleave a number of specific *Manduca* hemolymph proteins (15). Among these PrtA target (PAT) proteins was PAT-41. Interrogation of the protein database revealed that the N-terminal 15-aa sequence of PAT-41 is a perfect match to a previously described non-clip domain serine protease-like protein (SPH-3) from *Manduca.* The function of *Manduca* SPH-3 is unknown, but its corresponding mRNA increases in amount following the injection into the insect of bacterial cell walls (16). We hypothesized that SPH-3 is required for host defense, and that its PrtA-mediated destruction facilitates *Photorhabdus* infection. We find that SPH-3 plays an important role in mediating the information transfer toward the regulation of immune effector genes.

Materials and Methods

Experimental organisms

Newly moulted (day 0) fifth-stage tobacco hornworm larvae, *M. sexta*, were taken from a culture maintained as described (17). Bacterial strains used were *Escherichia coli* strain DH5α and *Photorhabdus luminescens* subsp. laumondii strain TT01; they were cultured on petri dishes containing 2.5% Luria–Bertani (LB) medium and 1.5% agar, and grown for 24 h in LB liquid culture prior to injection. For insect injection, washed bacterial cells were suspended in PBS (0.15 M sodium chloride, 10 mM sodium phosphate buffer [pH 7.4]). A total of 50 μl suspension containing 2×10^7 *E. coli* or 1×10^3 primary form *Photorhabdus* cells was injected directly into the insect's hemocoel. Numbers of injected bacteria were estimated by OD at 600 nm and by plating a known volume of injected suspension on 2.5%

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Abbreviations used in this article: DMPC, dimethylpyrocarbonate; β-GRP, β-glucan recognition protein; Iml, immulectin; LB, Luria–Bertani; PAT, protease A target; PGRP, peptidoglycan recognition protein; PO, phenoloxidase; PPO, prophenoloxidase; PRP, pattern recognition protein; PRSP, pattern recognition serine protease; PrtA, protease A; RNAi, RNA interference; SPH, serine proteinase homologue.

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LB agar plates. Injected larvae were kept at 28˚C to determine survival. In survival experiments, mortality was defined as failure to react to poking with a needle; 10 insects were used for each treatment, and each bioassay was replicated three times, giving similar results.

Purification and identification of SPH3

SPH-3 was partially purified from hemolymph, as described (15).

RNA extraction, RT-PCR, and cDNA cloning

Eighteen hours after bacterial challenge, insects were surface sterilized with 70% ethanol, bled, and dissected. Extracted hemocytes (30 mg) and fat body (100 mg) were homogenized in 1 ml TRI reagent (Sigma-Aldrich). RNA was then isolated, as previously described (18); samples were treated with RNase-free DNaseI (Invitrogen) (1 U/μl), and lack of DNA contamination was confirmed in every case by performing PCR without the reverse-trancriptase step. Single-step RT-PCR and cDNA cloning of SPH-3 and other genes were as described before (18). Amplifications were performed on a PTC-100 thermal controller (MJ Research) under the following cycling conditions: reverse transcription at 50˚C for 30 min, 95˚C for 15 min, followed by 35 cycles of 94˚C for 30 s, 45˚C for 30 s, and 72˚C for 1 min, with a final extension of 72˚C for 10 min. PCR products were visualized by agarose gel electrophoresis. Primers are listed in Table I.

RNAi and Western blotting

Cloned SPH-3 cDNA was used as a template to generate dsRNA specific to SPH-3 (dsSPH3), as described (18). The sequence of the dsRNA was from base 19 to 675 (the entire coding region) of the SPH-3 cDNA. A plant gene, *Manihot esculenta* catalase 1, *CAT1* (GenBank accession AF170272 at http://www.ncbi.nlm.nih.gov/nuccore/5759095), was used as a negative control (dsCON). For RNAi, 100 ng dsRNA (CAT1 or SPH-3, 50 μl, 2 μg/ml) in dimethylpyrocarbonate (DMPC)-treated water was injected into *Manduca* 6 h before challenge with *E. coli* or *Photorhabdus*. Controls used DMPC-treated water without dsRNA in the primary injections and PBS without bacteria in the secondary injections. After injection, treated insects were held at 28˚C for 18 h before sampling. Two insects were used for each treatment. Semi-quantitative RT-PCR reactions using extracted RNA as a template and a suitable pair of primers were performed to determine mRNA transcription patterns of each gene. For survival studies, 100 ng dsRNA (in 50 μl water at 2 μg/ml) was injected into day 0 fifth-stage *Manduca* larvae 6 h before challenge with *Photorhabdus* (2×10^3 cells). Western blotting analysis was as described (18). The anti–SPH-3 Ab was raised in rabbit against the synthetic peptide PQFKGRNTNYRNDI (corresponding to SPH-3 aa residues 135–148) and was used at 1/10,000 dilution.

Phenoloxidase activity

Total activatable phenoloxidase (PO) activity of *Manduca* hemolymph was quantified using a microplate enzyme assay, as described (19). Briefly, cell-free hemolymph plasma was used as a source of PPO, which was preactivated by exposing it in vitro to *E. coli* LPS (Sigma-Aldrich). The substrate was 4-methylcatechol (Sigma-Aldrich).

Nodule formation

Manduca larvae were injected with DMPC-treated water, dsCON, or dsSPH3, followed by PBS, *E. coli*, or *Photorhabdus*, 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 phenylthiocarbamide (Sigma-Aldrich), which prevented general postdissection darkening. Melanized nodules within the hemocoel were counted using a stereomicroscope.

Pathogen growth in vitro

To determine the ability of *Photorhabdus* to grow within the plasma of dsRNA-treated *Manduca*, insects were injected as previously and 18 h after the bacterial infection were individually bled into prechilled sterile polypropylene tubes to collect their total hemolymph fluids (~500 μl/larva). Tubes were centrifuged at 200 × *g* for 5 min at 4˚C to pellet the hemocytes, and hemolymph plasma samples were transferred to fresh tubes following the addition of 20 mM phenylthiocarbamide. All samples were inoculated with 10^3 cells (3 μl) of *Photorhabdus* and incubated at 30˚C with constant shaking for 24 h. Bacterial growth was estimated as OD at 600 nm.

Statistical analysis

Statistics were performed using the commercial computer software package GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). For data analyses, means were compared using a one-way ANOVA with a Tukey post hoc test for multiple comparisons. Comparison between survival curves (fraction death) was conducted using a log-rank (Mantel–Cox) test. The p values <0.05 were considered statistically significant.

Results

SPH-3 is required for normal immune function

We partially purified a *Manduca* hemolymph protein, PAT-41, that is cleaved by PrtA (Fig. 1*A*). The N-terminal 15 residues were sequenced and shown to be identical to that of a previously described serine proteinase homolog (GenBank accession AAO21508.1 at http://www.ncbi.nlm.nih.gov/nuccore/27733420), SPH-3 (Fig. 1*B*). The SPH3 protein found in the insect hemolymph appears to behave abnormally on SDS-PAGE, migrating with an apparent size of 41 kDa (the predicted molecular mass of mature SPH-3 after cleavage of the signal sequence is 28,434); nevertheless, the identity of the protein is confirmed by the specificity of staining by an Ab raised to a synthetic peptide from a different region of the protein to the originally identified N-terminal sequence, and the specificity of the RNAi knockdown (see below).

Consistent with our initial hypothesis, and in accord with previous work (16), we found that SPH-3 is constitutively expressed in *Manduca* at low levels, but that mRNA levels are upregulated in both fat body and hemocytes of insects previously challenged with either of two Gram-negative bacteria, nonpathogenic *E. coli* or pathogenic *Photorhabdus* (Fig. 1*C*). To confirm its identity, and also to determine whether SPH-3 is required for normal immune function, we constructed a dsRNA reagent specific for the cDNA

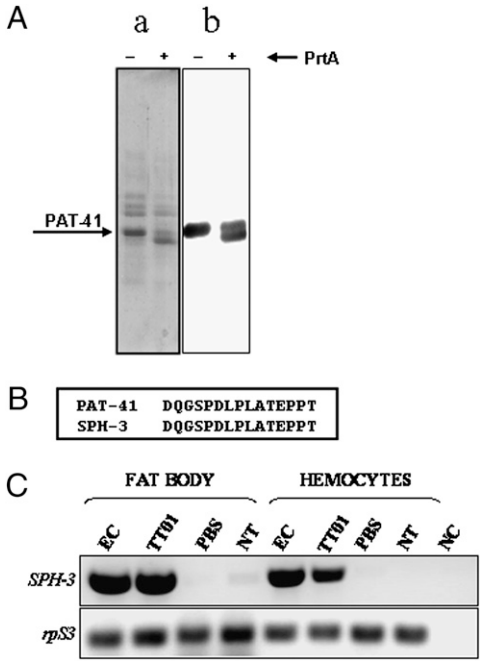

FIGURE 1. *M. sexta* SPH-3: cleavage by PrtA from *P. luminescens*, identification, and induction. *A*, A partially purified preparation of the *Manduca* hemolymph protein, PAT-41, is cleaved by PrtA (*a*, Coomassie staining; *b*, Western blot). *B*, The sequence of PAT-41 corresponds to *M. sexta* SPH-3 sequence found in the National Center for Biotechnology Information protein database. *C*, mRNA levels of *SPH-3* in fat body and hemocytes 18 h after injection of bacteria, as shown by RT-PCR. Insects were injected with *E. coli* (EC) or *Photorhabdus* (strain TT01). Controls were injected with PBS or were untreated (NT). A no-template negative control RT-PCR reaction (NC) is shown. RT-PCR amplification of the mRNA encoding the constitutively expressed ribosomal protein S3 (*rpS3*) is used to indicate RNA loading. PCR amplifications involved 35 cycles for each combination of primers and templates.

Table I. Nucleotide sequences of primers used in RT-PCR experiments

Gene	Accession No.	Primer	Sequence	Product Size (bp)
SPH3	AF413067	Forward	5′-CGTGGCACGATAATGTTGTT-3′	657
		Reverse	5′-AGTCGCTGCGTCAATGTATG-3′	
Hemolin	U11879	Forward	5′-ACAGCAACAACACAGGTGAA-3′	1273
		Reverse	5′-TTAAGCAACAATCACGAGCG-3′	
IML-2	AF242202	Forward	5′-GACTCTTGCGAGTCGTGTGA-3′	953
		Reverse	5′-GACTGTTTGGGTCCTTTTCG-3′	
PGRP	AF413068	Forward	5′-ACGGTATCACTTCCGTCCAC-3′	516
		Reverse	5′-CATTCTGGCCATCTCCTGAT-3′	
PRSP	AY380790	Forward	5′-ACGGATGGCACCCTGGTGCAGCC-3′	680
		Reverse	5′-TTTGCAACACATCAACGTAAG-3′	
β−GRP-1	AF177982	Forward	5′-CCTGACGCGAAGTTAGAAGC-3′	792
		Reverse	5′-AACGCGACCATATTTGAAGG-3′	
β−GRP-2	AY135522	Forward	5′-TCTACCCCAAAGGCTTGAGA-3′	802
		Reverse	5′-CACCACCAGAGACCCACTTT-3′	
PPO	L42556	Forward	5′-AAACAACTCCCAAACGATGC-3′	889
		Reverse	5′-TGTGCATGTTGTTGTGGATG-3′	
Attacin B	BI262658	Forward	5′-GGTCACGGCGCTACTCTTAC-3′	341
		Reverse	5′-TTGGGCATCTCGAACTTCTT-3′	
Cecropin D	BI262670	Forward	5′-TTCTTCGTCTTCGCTTGCTT-3′	161
		Reverse	5′-CCTTTGAAAATGGCGGTTG-3′	
Lebocin	BI262726	Forward	5′-ACGTGCGTAGTGTGAACGAG-3′	170
		Reverse	5′-CGCAGATTATGAGTTACGACGA-3′	
Lysozyme	BI262563	Forward	5′-TACAAACGCCACAAGTTCCA-3′	309
		Reverse	5′-AGGCTTGTAACCAAGGACGA-3′	
Moricin 2	BI262611	Forward	5′-TCGCGTTGTCGCTATTATTTT-3′	327
		Reverse	5′-CAGAAGATTCCGAAGGGAGA-3′	
rpS3	U12708	Forward	5′-CTGGCTGAGGATGGCTACTC-3′	186
		Reverse	5′-TTTCTCAGCGTACAGCTCCA-3′	

sequence of SPH-3 and used it in a systemic RNAi treatment that successfully reduced (knocked down) *SPH-3* mRNA levels in fat body and hemocytes (Fig. 2*A*) and also markedly reduced SPH-3 protein levels in hemolymph plasma (Fig. 2*B*).

RNAi knockdown of SPH-3 caused a drastic decrease in the ability of the treated insects to survive experimental *Photorhabdus* infections. In particular, we found highly significant differences in survival curves between SPH-3 knockdown and control treatments involving *Photorhabdus* (Mantel–Cox test, $\chi^2 = 133.4$, $p < 0.0001$; Fig. 2*C*). However, there were no differences in survival curves between the dsCON plus TT01 and W plus TT01 treatments (Mantel–Cox test, $p > 0.05$). Survival of insects injected with *E. coli* was unaffected by the RNAi treatment (Fig. 2*D*). (Insect survival was monitored for up to 7 d after injection with *E. coli*, but because there was no change in the results over time, we present data for the first 60 h following the bacterial challenge.) Consistent with this observation, the in vitro survival of *Photorhabdus* was substantially longer in hemolymph plasma from RNAi-treated insects than in plasma from untreated or *E. coli*-infected caterpillars (see below). This indicates lower levels of either immune recognition molecules or antimicrobial effectors upon SPH-3 knockdown.

SPH-3 is required for gene transcription of antimicrobial effectors, but not recognition proteins

The above results show that when SPH-3 expression is prevented by RNAi, immune responses of *Manduca* caterpillars to *Photorhabdus* are significantly compromised. This could be due to the failure of the SPH-3 knockdown insects to transcribe elevated levels of bacterial pattern recognition protein (PRP) genes, as is normally seen postinfection (20). We tested this possibility by using RT-PCR to detect mRNA encoding six separate recognition proteins, as follows: hemolin, peptidoglycan recognition protein (PGRP-1A), immulectin-2, pattern recognition serine protease (PRSP; also known as hemolymph proteinase-14), and two β-1-3-glucan recognition proteins (β-GRP-1 and -2) (see specific primers in Table I), most of which are overtranscribed following immune challenge (20). We found that RNAi knockdown of SPH-3 had no measurable effect on the transcript levels of any of these protein genes in the fat body (Fig. 3*A*, *top panel*). This shows that SPH-3 is required neither for the initial stages of immune recognition, nor for the induced transcription of any of these recognition protein genes.

We therefore tested whether RNAi of SPH-3 interferes with the antimicrobial effector functions. To this end, we examined the effect of SPH-3 knockdown on the mRNA levels of six known *Manduca* immune effectors, the antimicrobial proteins and peptides attacin, cecropin, lebocin, lysozyme, moricin, and PPO. We found that the SPH-3 RNAi treatment markedly reduced the immune-stimulated transcription of all these genes in fat body (Fig. 3*A*, *bottom panel*). This shows that the induced expression of SPH-3 is required for immune signaling upstream of events that control the transcription of a wide range of immune effector genes.

As a functional test of whether the reduction in gene transcription of immune effectors upon SPH-3 RNAi also manifests at protein level, we examined the growth of bacteria in the hemolymph of infected insects expecting an enhanced growth on knocking down of immune-related SPH-3 expression because the role of induced antimicrobial effectors is to restrict the growth of invading microbes in the insect's hemolymph (19). Therefore, we pretreated insects with SPH-3 dsRNA and then induced antimicrobial effector synthesis by infecting the insects with either *E. coli* or *Photorhabdus*, as previously. After 18 h (i.e., before any mortality had occurred), we prepared bacteria-free plasma from these insects by centrifugation, and determined whether this would support the growth of newly inoculated *Photorhabdus*. We found, as expected, that pre-exposure of insects to *E. coli* or *Photorhabdus* significantly reduced the growth of *Photorhabdus* in plasma, but this was prevented by prior knockdown of SPH-3 (Fig. 3*B*) so that *Photorhabdus* grew to the same extent in the

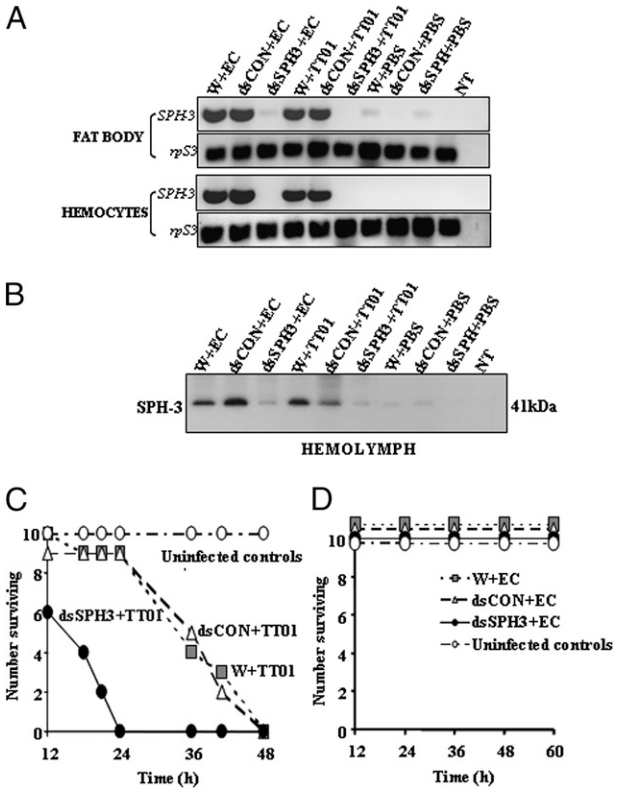

FIGURE 2. RNAi-mediated knockdown of SPH-3. *A*, RT-PCR analysis of mRNA levels of *SPH-3* following systemic RNAi. Insects were pre-injected ($t = -6$ h) with dsRNA specific to SPH-3 (dsSPH3), control dsRNA (dsCON), or water (W). At $t = 0$ h, they were challenged with bacteria, and sampled at +18 h, as previously. PCR amplifications involved 35 cycles for each combination of primers and templates. *B*, SPH-3 protein in hemolymph plasma is shown by Western blot analysis using anti-SPH-3 Ab. Treatments and controls designated as above. *C*, Survival of *Photorhabdus*-infected *Manduca* larvae. Groups of 10 insects were pretreated with dsRNA 6 h before administering a lethal inoculum of *Photorhabdus.* Treatments are designated as before. Control groups (all shown together) included W plus PBS, dsCON plus PBS, dsSPH3 plus PBS, and no treatment. The experiment was repeated three times with similar results. *D*, Survival of *E. coli*-infected *Manduca* larvae. Groups of 10 insects were pretreated with dsRNA 6 h before administering *E. coli.* Treatments and control groups were as above. The experiment was repeated three times with same results. Comparison between survival curves (fraction death) was conducted using a log-rank (Mantel–Cox) test.

plasma of RNAi-treated insects as in that of insects that had not been pre-exposed to bacteria.

SPH-3 is required for PO activation and nodule formation

To test further the effect of knocking down SPH-3 expression on immune effectors at the level of protein function, we measured PO activity in cell-free hemolymph of insects because PO activation in hemolymph plasma is an important and easily observed component of immune defense. RNAi knockdown of SPH-3 followed by infection with *E. coli* caused hemolymph of treated insects to remain unmelanized after bleeding. By comparison, hemolymph from infected insects given control dsRNA or water turned black within 1 h (Fig. 4*A*). We also examined hemolymph from insects infected with *Photorhabdus*. In the case of infected controls, the result is complicated by the fact that *Photorhabdus* produces molecules that inhibit plasma PO (21, 22). Consequently, the extent of PO-mediated darkening observed in hemolymph from these insects was less than in the case of *E. coli*-infected insects. Nevertheless, hemolymph from *Photorhabdus*-infected insects pretreated with SPH-3 dsRNA darkened much less than that of infected controls. Under the conditions of our assay, hemolymph from uninfected controls did not darken at all.

These visual observations were in accordance with separate measurements of PO activity in hemolymph exposed in vitro to the PO-activating agent, bacterial LPS. PO activity was higher in hemolymph from *E. coli*-infected caterpillars than in uninfected individuals, but this increase was prevented in insects pretreated with SPH-3 dsRNA (Fig. 4*B*). *Photorhabus* infection led to lower overall PO activity than *E. coli*, for the reasons stated above, but SPH-3 knockdown caused a further reduction in PO activity compared with the control pretreatments.

PO also participates in the formation of melanotic nodules around invaders in the insect body and is required for the production of the dark-colored antimicrobial compound, melanin, and the formation of cross-links between protein molecules (23). Following injection of bacteria into SPH-3 knockdown insects, we observed significantly fewer melanotic nodules (Fig. 4*C*) in the body cavity. This may have been due to the production of fewer nodules, or because the nodules that were produced were too small or insufficiently dark to detect. Both are consistent with the previously found lower hemolymph PO activity in SPH-3 knockdown insects, but it cannot be excluded that the effect is due either to a direct or indirect requirement for SPH-3 in other aspects of nodule formation (e.g., the production of opsonins or cell adhesion factors).

Discussion

Photorhabdus bacteria are highly virulent specialist pathogens of insects; in the laboratory, only 10–100 bacterial cells are sufficient to kill the host (24). It would be easy to suppose that no effective immune defenses are mounted by the host. However, we have previously shown that this is not the case (18). Infection of *Manduca* with *Photorhabdus* elicits enhanced expression of several different immune-related genes, and when these genes are knocked down using RNAi, the insects succumb significantly more quickly to *Photorhabdus* infection than they normally would. We reasoned that *Photorhabdus* must possess genes that enable it to avoid or overcome host defenses, and that these virulence genes should therefore be able to reveal the identities of the host insect's important, but still unknown, defense mechanisms (25).

One of the most highly expressed genes in *Photorhabdus* is *PrtA*, which encodes an RTX-like metalloprotease (11). We have shown elsewhere that PrtA is a selective endoproteinase that targets only a small number of *Manduca* hemolymph proteins, of which all of the identified ones are immune related (15). Among these is SPH-3, a serine proteinase homolog that was originally identified in a survey of immune-induced mRNAs in *Manduca* fat body cells (16). In this protein the catalytic triad's serine residue is mutated to glycine, thus inactivating the enzyme's hydrolytic function.

Four SPH protein-encoding, immune-inducible genes have to date been found in the genome of *Manduca*. Whereas functions have been proposed for two SPHs, SPH-1 and SPH-2 (see below), nothing is at present known for the role of SPH-3. This protein differs from other known members of the *Manduca* SPH family, in that it lacks an N-terminal clip domain, and its sequence is unlike the other known *Manduca* SPHs. It is thus likely that SPH-3 functions significantly differently from these proteins.

We investigated the function of SPH-3 by using a specific dsRNA to suppress its expression through systemic RNAi and found that knocking down SPH-3 drastically compromised the resistance of *M. sexta* to *Photorhabdus*. In similar experiments, we

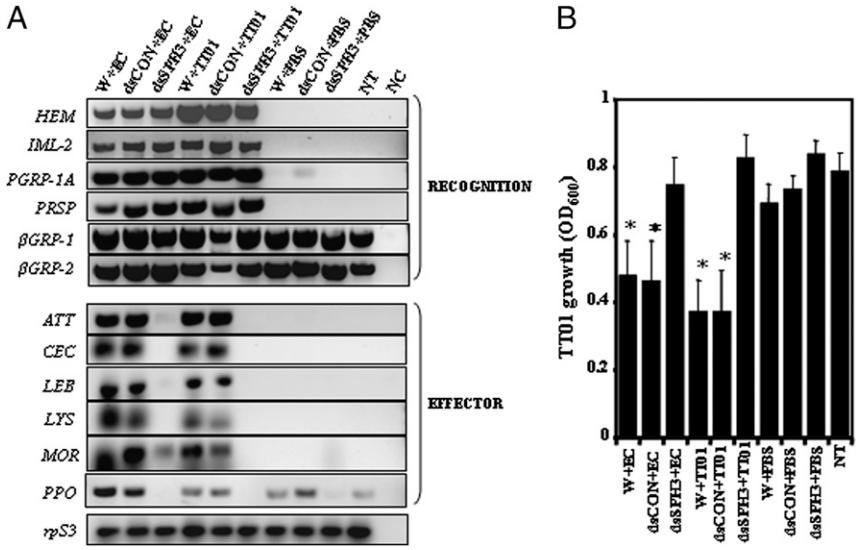

FIGURE 3. RNAi knockdown of SPH-3 has no effect on bacteria-induced transcription of recognition genes, but decreases transcription of antibacterial effector genes, thus allowing bacteria to grow in hemolymph. *A*, RT-PCR results indicating mRNA levels in fat body of six microbial PRP genes, as follows: *hemolin* (*HEM*), *IML-2*, *PGRP-1A*, *PRSP*, and *β-GRP-1* and *-2*, and six antibacterial effector peptide genes, as follows: *attacin* (*ATT*), *cecropin* (*CEC*), *lebocin* (*LEB*), *lysozyme* (*LYS*), *moricin* (*MOR*), and *PPO*. Treatments and controls were as described before. Loading is indicated by RT-PCR of *rpS3*. PCR amplifications involved 35 cycles for each combination of primers and templates. *B*, Final cell density (OD at 600 nm, OD_{600}) of *Photorhabdus* after 24-h growth in cell-free hemolymph plasma collected from *Manduca* larvae following different treatments (designated as before). Scale bars show means ± SE ($n = 5$). Asterisks show values that do not differ from each other, but that differ significantly from all other values (one-way ANOVA). $p < 0.01$.

had previously observed that ablation of expression of several microbial PRPs (e.g., hemolin, IML-2, and PGRP) had a stronger effect on immunity than the ablation of some antimicrobial effector proteins and peptides (19). Thus, in this regard, SPH-3 functionally resembles a PRP rather than an antimicrobial effector. Consistent with such a function, which is supposed to precede effector action, we found in the fat body that inhibition of SPH-3 expression had no effect on the mRNA levels of any of six PRPs, but strongly repressed the immune-related transcription of all six tested antimicrobial effectors. Therefore, the impaired ability of insects, lacking induced SPH-3 expression, to defend themselves against *Photorhabdus* infection is due at least in part to interference with effectors downstream of SPH-3. The observation of an enhanced ability of *Photorhabdus* to grow in *Manduca* hemolymph plasma in vitro on SPH-3 knockdown supported this conclusion because the most plausible explanation of better bacterial growth conditions might be a reduced antimicrobial effect due primarily to a lower content of antimicrobial peptides and proteins. Indeed, we found reduced plasma levels of PO (LPS-activatable PO) and absence of melanization in infected insects when they had been pretreated with dsRNA of SPH-3. Several causal factors may contribute to the lower level of PO activity. The

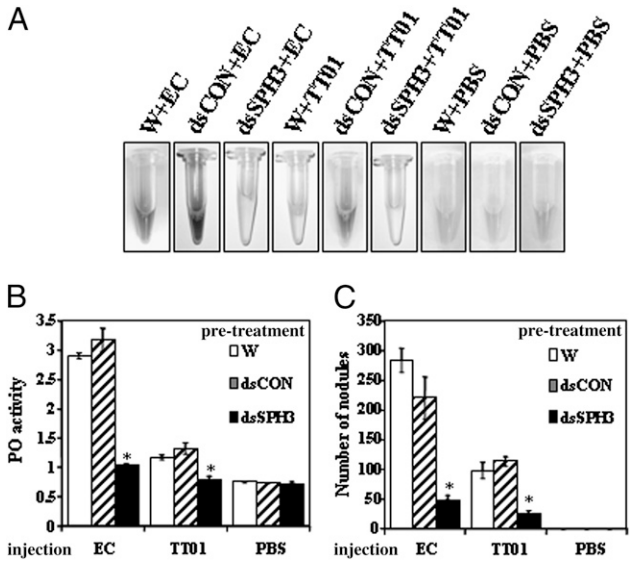

FIGURE 4. RNAi of SPH-3 reduces PO activity in hemolymph plasma and number of melanotic nodules. *A*, PO activity as shown by visual examination of hemolymph exposed to air for 1 h after bleeding. Treatments and controls were as described above. *B*, Quantitative PO activity measurements in hemolymph plasma samples subjected to the same treatments as before. *C*, Number of melanotic nodules formed in vivo in *Manduca* larvae following various treatments. Scale bars show means ± SE. Five insects were used for each treatment, and each assay was replicated three times. Asterisks indicate significant differences between the SPH-3 knockdown insects and the two control groups (one-way ANOVA). $p < 0.05$.

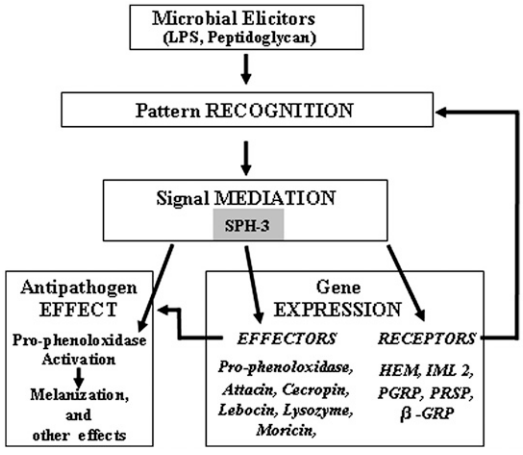

FIGURE 5. Proposed model for the role of SPH-3 in *Manduca* immune pathways. SPH-3 acts downstream of the PRPs and is involved in signal mediation from pathogen recognition toward the gene regulation of immune effectors, but not of recognition proteins.

simplest interpretation is the lack of induction of *PPO* gene transcription and a concomitant depletion of hemolymph PPO protein, although the complexity of the PPO activation process and PPO production by hemocytes also (in which we did not investigate mRNA levels other than that of SPH-3) leaves open alternative explanations and implies possible future experiments. However, because a control dsRNA treatment (corresponding to an irrelevant gene from a plant) did not have any effect on SPH-3 silencing and because the immune-induced overtranscription of PRSP, which has a considerable sequence similarity with the C-terminal part of SPH-3 (see below), was completely unaffected by the SPH-3 RNAi, the observed effects can be attributed (ultimately) only to SPH-3–specific gene inactivation.

Our findings point to a central role for SPH-3 in immune signaling in *Manduca*. Such a role is not known yet, in the case of SPH proteins, although several of the numerous SPHs that have been investigated in both invertebrates and vertebrates proved to be important regulators of immune responses. For example, masquerade-like clip domain SPHs have been proposed to modulate PPO activation in arthropods (26–30). In *M. sexta*, the clip domain-containing SPH-1 and SPH-2 are required for the full activity of the PPO-activating clip domain serine protease, PAP-2 (8–10). Other known immune-related functions of invertebrate clip domain-containing SPHs include antimicrobial activity (31), cell adhesion (32–35), and opsonization (33, 34). The roles of the SPHs that do not contain a clip domain are, however, much less well studied. The known ones include mammalian haptoglobin, which regulates Langerhans cell function and is toxic to *Plasmodium* trophozooites (36–38); the hepatocyte growth factor that suppresses dendritic cell function (39, 40); and the human neutrophil granule protein, azurocidin, which has both signaling and antimicrobial activity (41). In insects, in addition to SPH-3, the two non-clip domain SPHs suggested to play immune roles are an azurocidin-like protein in *Trichoplusia ni* (another lepidopteran) (40) and ISP15 in the mosquito *Anopheles gambiae* (41). Thus, our observations regarding SPH-3 are a significant step forward to explore the role(s) of similar proteins in invertebrate immunity for several reasons, as follows: 1) SPH-3 regulates gene expression, whereas other SPH-s are not known to do this; 2) the range of affected immune-related genes is wide; 3) SPH-3 function is specific, it affects antimicrobial effector genes, but not those encoding PRP genes.

The last point is of particular significance because it suggests that SPH-3 is a component in a signaling pathway that lies between the recognition of microbial patterns and the initiation of antimicrobial effector synthesis (Fig. 5). Further experiments will investigate whether SPH-3 regulation influences the immune response to infections other than those by Gram-negative bacteria. We suppose it does, at least as much as the SPH-3–regulated effectors participate in those processes. Similarly, our results make reasonable to expect that the function of SPH-3 is important in the production of other immune molecules, for example, those that modulate PPO activation, nodule formation, or cytokines that are needed for hemocyte function. At the same time, the fact that SPH-3–depleted insects did not succumb to the infection with a large number of nonpathogenic bacteria indicates, on one hand, a sufficient capacity of the host's defense system even without induction of important immune effectors and, on the other, capabilities of the pathogen, *Photorhabdus*, other than inhibition of SPH-3 function, which are also crucial to evade and/or suppress the immune response of the host.

The obvious selectivity of SPH-3 regulation for immune effector encoding genes, inferred from our knockdown experiments, is of particular interest, for it implies the existence of separate immune signaling pathways in *M. sexta*. In response to infection, *Drosophila* initiates the synthesis of numerous antimicrobial effectors, which are activated via two distinct NF-κB signaling pathways, Toll and Imd. Regulation of the immunity effector genes is also influenced by the JNK and JAK–STAT signaling pathways, which can act in a competing or cooperative mode (42, 43). However, a signaling cascade that is specific according to whether the target gene encodes a receptor or an effector of the immune system has not to date been found. In addition, because SPH-3 is an extracellular protein, the signaling toward the controlled genes must somehow be distinct from other pathway(s) from somewhere outside the cell all the way down to the transcription factors involved. Alternatively, the input from the SPH-3 pathway is someway indispensable to those others that are specifically toward immune effector genes.

As a nonfunctional serine proteinase homolog, SPH-3 cannot be an enzymatically active proteolytic component in a signaling cascade. However, thinking in a signaling pathway akin to that toward Toll family receptor in *Drosophila*, SPH-3 can play a similar role to, for example, Spätzle, which is a ligand of Toll. Alternatively, SPH-3 might be a key adaptor in signal-mediating events that are upstream to the receptor. To investigate whether a relationship is found at amino acid sequence level, which can be informative about the function or the origin of SPH-3, we performed pairwise amino acid sequence comparisons first between SPH-3 and the cascade components toward Toll receptor. These showed no significant similarity between SPH-3 and Spätzle. The highest values were obtained for Easter, a clip–domain serine proteinase (22% identity, 33% similarity), but even these values were not exceptional because we found similar ones for several other proteins in additional 20 sequence comparisons with serine proteinases and SPHs of immune-related function in *M. sexta*, *Bombyx mori*, and some other invertebrates. It might, however, be interesting that the closest similarity (51% at the amino acid level) was between the amino acid sequence of the C-terminal region of SPH-3 and that of the PRSP, also designated hemolymph protease 14, a *Manduca* immune-related protein. PRSP, which has twice as long a polypeptide chain as SPH-3, has a full complement of canonical active site residues in its C-terminal region, and is catalytically active (44).

Taken together, our observations indicate an unusually central significance of SPH-3 in the insect innate immune system: it participates in the control of a large number of molecular effector functions. This makes its destruction an ideal strategy for a highly virulent pathogenic bacterium, like *Photorhabdus*. We anticipate that the exploration of the mechanism of SPH-3 might reveal fundamental processes of the innate immune response in insects.

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Disclosures

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