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Spn1 Regulates the GNBP3-Dependent Toll Signaling Pathway in *Drosophila melanogaster*▽

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The *Drosophila* genome encodes 29 serpins, most of unknown function. We show here that Spn1 is an active protease inhibitor of the serpin superfamily. Spn1 inhibits trypsin *in vitro* and regulates the Toll-mediated immune response *in vivo*. Expression of the Toll-dependent transcripts *Drosomycin* and *IM1* is increased in *Spn1* null mutants. Overexpression of Spn1 reduces the induction of *Drosomycin* upon immune challenge with fungi but not Gram-positive bacteria. Similar reductions in *Drosomycin* levels are observed in the *psh*, *spz*, and *grass* mutants of the Toll signaling pathway. These results support a role of Spn1 as a repressor of Toll activation upon fungal infection. Epistatic analysis places Spn1 upstream of Spätzle processing enzyme and Grass, in the fungal cell wall-activated side branch of the pathway. Overexpression of the pattern recognition receptor *GNBP3* activates the β-1,3-glucan-sensitive side branch of the Toll pathway. The resultant increased *Drosomycin* level is reduced by concomitant overexpression of *Spn1*, confirming that Spn1 regulates the fungal cell wall side branch. *Spn1* null mutants show altered susceptibility to fungal infection compared to the wild type, demonstrating a requirement for Spn1 in the fine regulation of the immune response.

Many physiological responses are regulated by serpins (*ser*ine *p*rotease *in*hibitors) in mammals, including the blood clotting, inflammatory, complement activation, and angiogenesis pathways (15, 48). Disorders in serpin metabolism are responsible for a wide range of human diseases, such as emphysema, cirrhosis, blood coagulation disorders, and dementia (36, 38). For this reason, the inhibitory mechanism of serpins has been extensively studied, mainly in humans and mammalian model organisms.

Serpins are large-molecular-mass protease inhibitors with a core of three β-sheets connected by short, α-helical linkers. In the native state, a reactive center loop (RCL) extends out from the serpin core and presents an ideal bait to the target protease (48). The native serpin is in a metastable (stressed) configuration. Following cleavage, the structure adopts a stable (relaxed) configuration. The rapid insertion of the RCL into the serpin β-sheet A distorts the protease's catalytic site so that the esterification step of the hydrolysis reaction cannot be completed. As a result, the protease and serpin form a covalently linked complex. The protease is translocated through 70 Å and crushed against the serpin core (25). This process denatures the serpin/protease complex, which is targeted for proteolytic destruction. This "suicide-inhibition" mechanism destroys the activity of both the serpin and target protease, both of which have a high turnover. Reduction in serpin levels results in explosive activation of signaling pathways as inhibition of the target protease is lost. Each subsequent zymogen in the proteolytic cascade is activated by its upstream protease. This mechanism gives a rapid, amplified response. With continued activation, both serpin and downstream proteases tend to be upregulated at the transcriptional level.

The number of serpins is highly variable between mammalian species, with humans having 35 and mice having 64. This compares with 29 serpin genes in *Drosophila*, which includes 12 noninhibitory serpin fold proteins (20, 43) that lack the characteristic conserved "hinge region" motif of active protease inhibitors (26). However, one of the 17 putative inhibitory serpin genes, *Spn42Da* (*Sp4*; *CG9453*), contains alternatively spliced exons. *Spn42Da* gives rise to 11 transcripts encoding eight protein isoforms, with four different RCL sequences targeted to different cellular compartments (6). On this basis, the *Drosophila* genome encodes 25 inhibitory serpin activities.

In comparison to serpins of mammals, relatively little is known about insect serpins, and those that have been studied mainly affect the immune response. The mammals have a two-stage response to immune challenge: an immediate innate response and a delayed adaptive response, mediated by antibodies. The insects lack an antibody response but mount effective innate responses, including the synthesis of antimicrobial peptides (AMPs), phagocytosis, encapsulation, and melanization of pathogens. In *Drosophila*, the humoral response involves both localized melanization (8, 41) and AMP expression in the fat body (14, 27, 52) while the cellular response is mediated by hemocytes, which phagocytose or encapsulate invading pathogens (29, 39). Expression of AMPs, including Diptericin (Dpt), Drosomycin (Drs), and Drosocin, together with several cecropins and attacins (21, 52), is mainly regulated by two signaling pathways: Imd (immune deficiency) and Toll. Drs expression is regulated by the Toll pathway while Dpt activity is controlled by the Imd pathway. An additional protein regulated by the Toll pathway is IM1 (immune-induced molecule 1) (24) although the molecular function of this protein is un-

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known. The intracellular components of the Imd and Toll pathways show similarity to the mammalian innate immune response pathways, tumor necrosis factor alpha and Toll-like receptor/interleukin-1, respectively (23). The Imd signaling pathway involves a kinase cascade activated through the peptidoglycan recognition protein LC (PGRP-LC) and PGRP-LE receptors, which recognize the meso-diaminopimelic acid-type peptidoglycans from Gram-negative bacterial cell walls (10, 18, 50), leading to expression of Dpt as a major AMP.

The Toll receptor was originally identified from its role in embryonic dorso-ventral patterning (2) and was later recognized as a component of the innate immune response to fungal and Gram-positive bacterial infections in adults (31, 33, 40). In the embryo, the Toll ligand, Spätzle (Spz), is activated by cleavage by the serine protease, Easter (Ea) (12), while in the adult Spz is activated by Spätzle processing enzyme (SPE) (28). The activated Spz ligand then binds to the Toll receptor, which initiates the intracellular immune signaling pathway (31, 33, 54). The binding of Spz to Toll at the cell membrane induces the subsequent nuclear translocation of two NF-κB family members, Dorsal (Dl) and Dorsal-related immunity factor (Dif) (16, 56). Once in the nucleus, Dl/Dif upregulates the expression of Drs and other AMPs.

The extracellular proteolytic signaling cascade which activates Spz represents a major difference between the *Drosophila* and mammalian innate immune responses. In contrast to the fly, mammalian Toll-like receptors recognize microbial determinants directly. In *Drosophila*, the lysine-type peptidoglycans of Gram-positive bacterial cell walls bind to the pattern recognition receptors (PRRs) PGRP-SA, PGRP-SD, and GNBP1 (4, 17, 40) while the β-1,3-glucans of fungal cell walls bind to the GNBP3 receptor (18). These two side branches of the Toll pathway converge on, or above, the serine protease Grass (13, 30). It has recently been proposed that Modular serine protease (ModSP) is the most apical PRR pathway protease and is common to both side branches (7). In addition, a danger-signaling side branch of the Toll pathway is activated by pathogen-secreted virulence factors, in particular proteases, from fungi and Gram-positive bacteria (13, 18) and signals through the Persephone (Psh) serine protease. All three of these side branches converge above SPE before activation of Spz signals through the Toll receptor to the intracellular pathway. This complex set of side branches of the extracellular pathway allows the response to pathogen challenges to be triggered redundantly by different inputs. This is particularly important in insects, where entomopathogenic fungal infections are a major factor limiting population growth and where the antibody-mediated adaptive response is lacking. It is not clear how many proteases might have an immune-related function in *Drosophila*, but the proteome includes 211 chymotrypsin fold serine proteases and serine protease homologues (46), whereas in humans there are 176. Thirty-seven of the *Drosophila* chymotrypsin fold genes include at least one CLIP domain, which is associated with insect immune-related proteases (46).

In *Drosophila*, five serpins are known to have immune response-related functions. Necrotic (nec, Spn43Ac, or CG1857) inhibits the danger-signaling branch of the Toll-mediated response to fungal and Gram-positive bacterial infections. Null mutants of *nec* show constitutive expression of *Drs* in adults, even in the absence of immune challenge (19, 32), and die within 24 to 48 h of hatching from the pupal case (19). Spn27A (CG11331) controls pathogen melanization, mediated via the phenoloxidase (PO) pathway (34, 41). Lack of Spn27A results in melanization of internal tissue associated with high levels of PO in the hemolymph. (In addition to its immune-related function, Spn27A regulates the formation of the dorso-ventral embryonic axis [22, 35].) Spn28D (Spn28Dc; CG7219) regulates the PO pathway in response to wounding (47) while Spn77Ba (CG6680) regulates tracheal melanization, which can trigger systemic expression of Drs via the Toll pathway (51). Spn5 (Spn88Ea; CG18525) functions in the PO and Toll cascades (1) as well as in wing inflation (9). A possible immune function for Spn1 (*CG9456*; *Spn42Dd*) is suggested by upregulation of its transcript after fungal and Gram-positive infections (27). A number of serpins are present at high levels in seminal fluid (55), and the differentially spliced transcripts of *Spn4* target four separate groups of proteases: furin (44), subtilase, chymotrypsin, and a papain-like cysteine protease (6).

In this paper we characterize the putative inhibitory serpin, Spn1. As predicted from the presence of the conserved inhibitory hinge region motif (43), transgenically expressed Spn1 forms a native serpin in the stressed conformation. Spn1 shows a narrow-range target specificity for trypsin *in vitro*, with which it forms a putative RCL-cleaved serpin/protease complex. In the fly, Spn1 modulates the activity of the fungal cell wall pattern recognition side branch of the extracellular Toll signaling pathway.

MATERIALS AND METHODS

Construction of the Spn1 protein expression construct. The *Spn1* cDNA was amplified from the Berkeley *Drosophila* Genome Project (BDGP) *Drosophila* Gene Collection (DGC) Gold (GH4125) collection and subcloned into an SpeI/EcoRI-cut *pTYB12* vector using the forward primer 5′-GCG CCA TAT GCA GAC CTC CAA AGA GAT CTA C-3′ and the reverse primer 5′-CGG CGA ATT CTC AAG GGC TTA CAA CAC GCC C-3′.

Protein purification. Spn1 protein was expressed in *Escherichia coli* BL21(DE3) cells and purified using an Impact-CN system (New England BioLabs), following the manufacturer's recommendations. Spn1 was fused to an N-terminal intein/chitin-binding domain (pTYB12-Spn1). *E. coli* BL21 cells containing the pTYB12-Spn1 construct were induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) and grown overnight at 15°C. Bacteria were sonicated in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.5 M EDTA buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and complete inhibitors (protease inhibitor cocktail tablets; Roche); samples were centrifuged at 16,400 rpm for 30 min and loaded onto a chitin column. Protein was concentrated with a 30,000-nominal-molecular-weight-limit (NMWL) membrane Amicon centrifugal filter (Millipore) in 20 mM Tris-HCl, pH 7.5, buffer, using an ÄKTA-FLPC fast protein liquid chromatography system. Protein aggregates and low-molecular-mass proteins were removed using a Superdex 200 10/300 gel filtration column (Amersham Biosciences).

TUG-PAGE gels. Transverse urea gradient-polyacrylamide gel electrophoresis (TUG-PAGE) gels were poured between glass plates following the method of Lomas et al. (37). The urea gradient, increasing from the left to right of the gel, causes native, stressed serpins to unfold to their stable, relaxed conformation. The stressed-to-relaxed transition of active serpins gives a characteristic S-curve profile.

CD. Circular dichroism (CD) spectroscopy measurements were made in 100 mM Tris-HCl, pH 7.4, at 25°C, using a JASCO-815 spectropolarimeter. Thermal stability was assessed by monitoring the CD signal at 216 to 222 nm between 25 and 95°C using a heating rate of 1°C/min at a concentration of 0.2 mg/ml in a 0.05-cm-path-length cuvette. The thermal denaturation point (T_m) was calculated by regression analysis as described by Dafforn et al. (11).

Serpin inhibitory activity and Serpin/protease complex formation. Spn1 was incubated with different molar ratios of serine proteases (kallikrein, trypsin, chymotrypsin, thrombin, elastase, cathepsin B, cathepsin D, and cathepsin G) for 30 min at room temperature in 50 mM HEPES containing 150 mM NaCl. For

inhibitory assays, 1 mM appropriate chromogenic substrate (see above) was added, and samples were incubated for a further 30 min. The absorbance (405 nm) was determined with an Ultrospec 3100 Pro spectrophotometer (Amersham Biosciences). Spn1/protease complex formation was analyzed by running samples on a 10% SDS-PAGE gel and immunoblotting using a mouse anti-Spn1 antibody.

Proteases and chromogenic substrates. The following were purchased from Sigma: bovine pancreatic α-chymotrypsin, plasma thrombin, and spleen cathepsin D; porcine pancreatic trypsin, elastase, and kallikrein; human placental cathepsin B and leukocyte cathepsin G. Most chromogenic protease substrates were also purchased from Sigma: kallikrein (B2133; *N*-benzoyl-Pro-Phe-Arg-*p*-nitroanilide hydrochloride), trypsin and thrombin (B7632; *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride), chymotrypsin (S7388; *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide), and elastase (S8511; *N*-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide). The cathepsin B substrate (Z-Arg-Arg-pNA; 2 HCl) was purchased from Calbiochem.

Protein extraction from flies. Frozen flies were suspended in 200 μl of 50 mM Tris buffer containing 20 mM EDTA, pH 6.5, and centrifuged at 5,000 × *g* for 20 min at 4°C. An equal volume of Laemmli sample loading buffer was added to the supernatant and heated to 95°C for 4 min.

Microbial strains. The following strains were used: the Gram-positive bacterium *Micrococcus luteus* (CIP A270), the Gram-negative bacterium *E. coli* (K-12), the yeast *Candida albicans* (ATCC 36232),and the filamentous fungi *Beauveria bassiana* (strains 80.2 and CECT 20548) and *Metarhizium anisopliae* (DMS1490).

***Drosophila* strains.** Stocks were maintained on cornmeal-agar medium at 25°C. The transgenic $GNBP3^{UAS}$, $GNBP1^{UAS}$, $PGRP\text{-}SA^{UAS}$, and $Drs\text{-}GFP^{UAS}$ strains have been previously described (see references 14, 17, and 40, respectively). $Spn1^{UAS}$ and $Spn1^{oka}$ strains were constructed for this study (see below). The $Spn1^{RNAi.UAS}$ strain was provided by the National Institute of Genetics (Japan). The Dif^{1}, $grass^{Herrade}$, rel^{E20}, spz^{rm7}, psh^{4}, and $SPE^{Pasteur}$/*Df*(3R)*mbc-R1* strains were described previously (31, 33). [Note that $SPE^{Pasteur}$ is not a complete null mutation and that *Df*(3R)*mbc-R1* deletes the SPE gene. Hemizygous $SPE^{Pasteur}$/*Df*(3R)*mbc-R1* flies therefore retain less SPE activity than homozygous $SPE^{Pasteur}$ flies.] The *UAS*/*Gal4* expression system (5) was used in transgenic fly strains, with the *Gal4* driver *Gal4-YP*, *Gal4-c564* (*P*{*GawB*}*c564*), and *Gal4-ap* (*P*{*GawB*}*ap*) strains. *Gal4-Yolk Protein* (*Gal4-YP*) is expressed in the adult female fat body, *Gal4-c564* is expressed in larval and adult fat body, and *Gal4-ap* is expressed in dorsal surfaces of mesonotum and wing. Equal numbers of males and females were used in all assays except those using the *Gal4-YP* driver, for which only female flies were used. The Oregon-R wild-type strain was used for experimental controls.

Quantization of *Drs-GFP* fluorescence. To make comparisons between the Toll pathway readout using the *Drs-GFP* (where GFP is green fluorescent protein) reporter construct, adults of the same ages and grown under identical conditions were used. For each set of comparisons, adult flies were taken at 24 to 48 h posthatching and photographed together, under the same UV illumination conditions, with a Leica M216F fluorescence microscope using a GFP2 filter.

Recovery of *Spn1* null and $Spn1^{UAS}$ overexpression strains. The *Spn1* null mutation, $Spn1^{oka}$, was recovered as an intragenic deletion (of 398 bp), which includes the last two exons of the *Spn1* transcript and therefore deletes the critical RCL sequence. This chromosomal segment lies between the *PBac*(*WH*)$Spn1^{f02145}$ and *PBac*(*WH*)*f04856* insertion sites, both of which carry a yeast *FRT* recombination site and the w^{+mC} marker. The deletion was generated by FLP-mediated recombination between *PBac*(*WH*)$Spn1^{f02145}$ dp^{ov1} w^{+mC} *sp* and *PBac*(*WH*)*f04856* w^{+mC} in the male germ line. The required chromosome, *Df*(2R)*PBac*(*WH*)$f02145^{L}$ $f04856^{R}$ dp^{ov1} $Spn1^{oka}$ w^{+mC}, was identified as a dp^{ov1} sp^{+} recombinant, and the deficiency was confirmed by PCR sequencing using *PBac*-specific and genomic primers (data not shown). As no other transcripts are included within this deletion, we refer to this chromosome as simply $Spn1^{oka}$ in the text.

The *Spn1* overexpression strain, $Spn1^{UAS}$, was recovered by *P*-element-mediated germ line transformation using standard techniques. The transformation construct, *pUAST-Spn1*, was made by PCR amplification of the *Spn1* cDNA (GH4125) using the forward primer 5′-CCG CCG CGG CCG CGA CGG TCA A-3′ and reverse primer 5′-CGC GCG GTA CCA AAT ATT GTT TTT ATT TAT-3′. The amplified fragment was cloned into NotI/EcoRI-cut *pUAST* vector, and the cDNA insert was confirmed by sequencing. The recovered $Spn1^{UAS}$ strain carries a homozygous viable, $P\{w^{+mC}=Spn1^{Scer\backslash UAS}\}$ insertion on the second chromosome.

Microbial infections. For septic injury, 2- to 3-day-old adult flies were pricked with a tungsten needle dipped into concentrated microbial culture (*C. albicans*, *M. luteus*, or *E. coli*). For natural infection, flies were shaken for 30 s in a petri dish on a lawn of sporulating *B. bassiana* or *M. anisopliae*. Fly cultures were incubated at 29°C or 25°C and transferred to fresh vials every 1 to 3 days.

Survival after immune challenge. The *B. bassiana* strain used for these experiments was a recent isolate (CECT 20548; F. Granero, 1999, from dead white fly). The *B. bassiana* CECT lyophilate was subcultured once in liquid yeast extract-peptone-dextrose (YPD) medium (at 25°C for 4 days). A total of 100 ml of the resulting saturated culture containing both hyphae and conidia was sonicated, plated on potato dextrose agar (PDA), and incubated for 4 days at 25°C to produce a lawn of aerial conidia. Plates were stored at 4°C for a maximum of 3 weeks and then incubated for 1 day at 25°C just before infection. Cultures were replicated up to three times without detection of a significant reduction of the virulence of the fungus for wild-type flies. Anesthetized flies were rolled on the surface of the fungal culture plate for 30 s and then transferred to a vial containing fly food and incubated at 29°C. Flies were counted, and the survivors were transferred to fresh vials daily. Infections with *M. anisopliae* (DMS1490) were performed similarly. The fungus was grown directly on PDA plates for 10 to 13 days until black conidiophores appeared. Flies were incubated at 25°C and transferred to vials of fresh food prepared without preservatives. The survival functions of mutant strains following immune challenge with fungal pathogens, *M. luteus*, and sterile injury (see Fig. 8) were analyzed and plotted graphically using MedCalc software (www.medcalc.org/manual/kaplan-meier.php). The analysis includes data from at least three independent trials of 20 to 30 infected flies.

Antibody production, purification, and Western blot analysis. Recombinant Spn1 protein was purified by 10% SDS-PAGE under reducing conditions. A mouse polyclonal antibody was raised by the Animal Service Centre of the CIC bioGUNE and affinity purified against membrane-bound Spn1. For Western blotting, proteins were transferred from SDS-PAGE gels to polyvinylidene difluoride (PVDF) membranes (Millipore), blocked in 3% bovine serum albumin (BSA)–TBS-T (0.1% Tris-buffered saline with Tween) for 1 h, and incubated with a 1:100 dilution of anti-Spn1 antibody overnight at 4°C. The secondary antibody was anti-mouse antibody–horseradish peroxidase (HRP) (1:5,000 dilution for 1 h). Blots were developed using an enhanced chemiluminescence (ECL) system.

RT-PCR and quantitative real-time PCR (qPCR). Samples of 20 flies were frozen and ground at −80°C. Total RNA was extracted using an RNeasy Kit (Qiagen) following the manufacturer's protocol. A ThermoScript reverse transcription-PCR (RT-PCR) system kit (Invitrogen) with oligo$(dT)_{20}$ primers was used for the reverse transcription reaction.

For qPCR analysis, a SYBR green One-Step Quantitative RT-PCR kit (Bio-Rad) was used with a Bio-Rad i-Cycler IQ. Preincubation was at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The specificity of amplified products was confirmed by DNA melting curve analysis. Amplified transcript levels were normalized against an internal control, ribosomal protein 49 (rp49), and expressed as a percentage of the normalized transcript levels in wild-type, noninfected, controls. Error bars indicate standard deviations for at least three independent experiments, calculated using a Student's two-tailed *t* test except for data shown in Fig. 5E, which represent five experimental replicas. For assays following immune challenge, flies were collected at 6 h postinfection for *E. coli*, at 24 h postinfection for *M. luteus*, and at 48 h or 72 h postinfection for *B. bassiana* and *C. albicans*.

Primers used were the following: for *Spn1*, 5′-GGC ATC CGA GAG CTA TTC AC-3′ (forward) and 5′-GGC CAT GAG GAA CGT AGA AA-3′ (reverse); for *Drs*, 5′-CGT GAG AAC CTT TTC CAA TAT GAT G-3′ (forward) and 5′-TCC CAG GAC CAC CAG CAT-3′ (reverse); for *Dpt*, 5′-GGC TTA TCC GAT GCC CGA CG-3′ (forward) and 5′-TCT GTA GGT GTA GGT GCT TCC-3′ (reverse); and for *rp49*, 5′-AGA TCG TGA AGA AGC GCA CCA AG-3′ (foward) and 5′-CAC CAG GAA CTT CTT GAA TCC GG-3′ (reverse).

RESULTS

Characterization of purified Spn1. Recombinant Spn1 protein was made in *E. coli* and purified as described in Materials and Methods. The recombinant protein runs as a single band on SDS-PAGE, close to the predicted size of 41.2 kDa for Spn1 (Fig. 1A). Transverse urea gradient (TUG)-PAGE shows the classic S-curve profile, typical of a native serpin; at between 5 to 6 M urea the serpin fold undergoes a stressed-to-relaxed transition (Fig. 1B). Circular dichroism measurements indicate a mixture of α-helix and β-sheet secondary structure that is

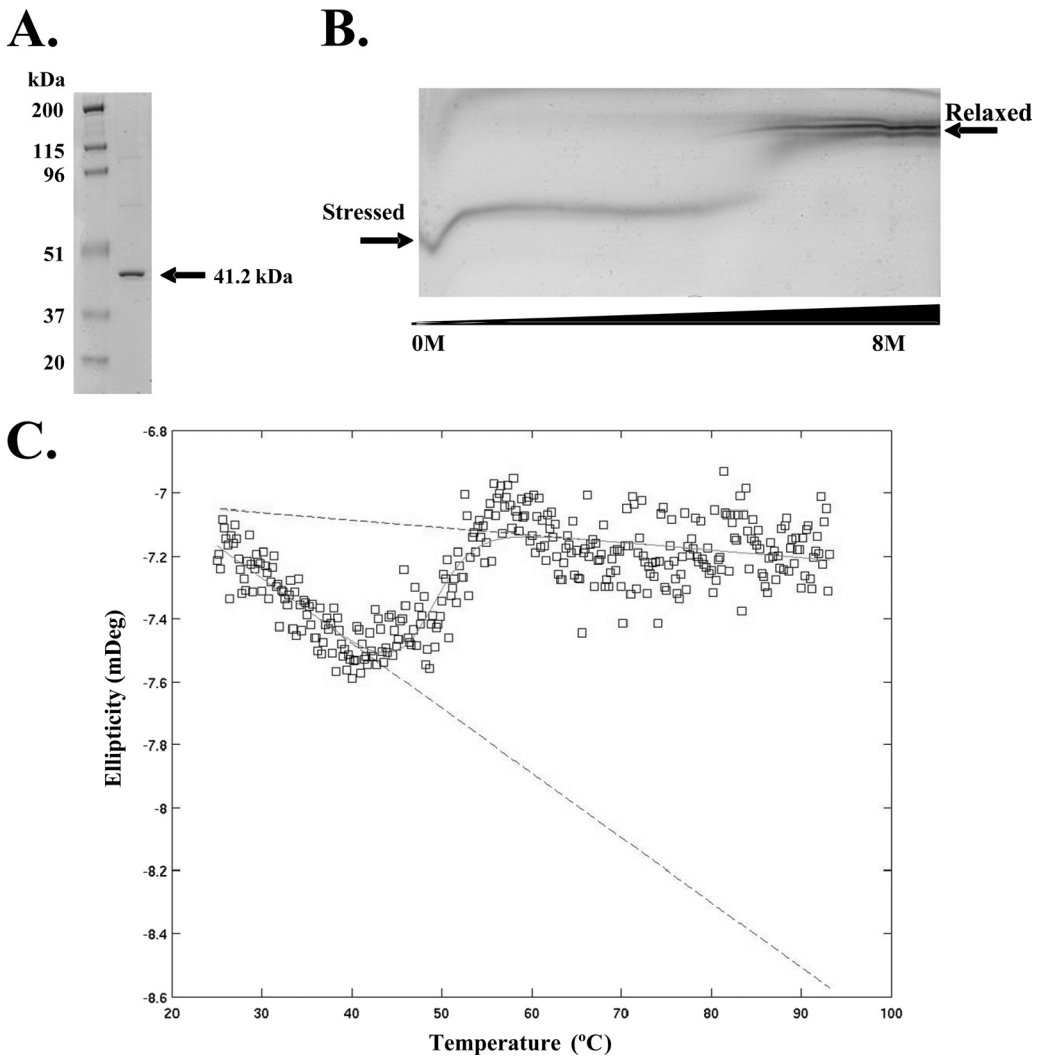

FIG. 1. Biochemical characterization and inhibitory activity of Spn1. (A) Coomassie-stained 10% SDS-PAGE gel loaded with purified Spn1 protein (lane 1, protein size marker; lane 2, Spn1). (B) TUG-PAGE gel showing the unfolding of Spn1 on a 0 to 8 M urea gradient. The curve reveals the typical S profile of a serpin in the native metastable, state, with a transition between the stressed and relaxed conformations occurring between 5 M and 6 M urea. (C) CD spectrum showing the thermal denaturation of Spn1. The CD signal at 220 nm reflects changes in protein secondary structure with respect to temperature. Spn1 shows a single transition at 48.6°C, typical for inhibitory serpins. mDeg, millidegrees.

characteristic of serpins. The thermal denaturation point (48.6°C) is typical of inhibitory serpins in the native conformation (Fig. 1C). Spn1 forms a complex with trypsin (Fig. 2A) but not with elastase, chymotrypsin, kallikrein, thrombin, or cathepsins B, D, and G (data not shown). In the case of trypsin, increasing the ratio of serpin to protease gives an increase in the 60- to 70-kDa protease/serpin complex band and a 35- to 40-kDa putative, RCL-cleaved serpin band (Fig. 2A). Chromogenic substrate assays confirm that Spn1 inhibits trypsin strongly (96% at a serpin/protease ratio of 10:1) (Fig. 2B) but not other proteases. Thus, Spn1 is an inhibitor of trypsin-like proteases *in vitro*, with a narrow substrate specificity.

These data confirm that our recombinant Spn1 protein sample consists of a native, monomeric serpin in an active, inhibitory conformation.

Activating the Toll pathway upregulates Spn1. To characterize the function of Spn1 *in vivo*, we first used quantitative real-time PCR (qPCR) to measure *Spn1* transcript levels following immune challenge. Wild-type flies infected with the Gram-positive bacterium *M. luteus*, the fungus *B. bassiana*, or the yeast *C. albicans* show increased *Spn1* transcript levels (Fig. 3A); these increases in *Spn1* transcript levels were confirmed at the protein level by Western blotting (Fig. 3B). No increase in *Spn1* transcript levels occurs after sterile injury or *E. coli* infection (Fig. 3A). To confirm that *Spn1* expression is regulated by the Toll-mediated immune response, the Toll pathway *grass*, *spz*, *SPE*, and *Dif* mutants were infected with *B. bassiana* or *M. luteus*. In all these mutant backgrounds, the upregulation of *Spn1* transcript is blocked, whereas the Imd pathway mutant, *Relish* (*Rel*), does not decrease *Spn1* transcript levels (Fig. 3C and D).

Spn1 regulates *Drs* expression. RNA interference (RNAi) knockdown of the *Spn1* transcript activates expression of a *Drs-GFP* reporter construct in noninfected, $Spn1^{RNAi}$ flies,

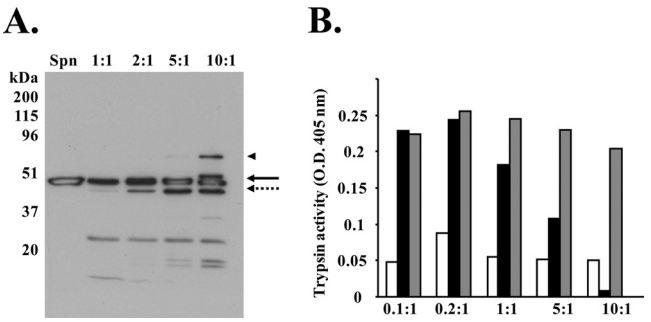

FIG. 2. (A) Immunoblot showing Spn1/trypsin complex formation (stained with anti-Spn1 antibodies). The left-hand lane contains pure Spn1, with successive lanes containing serpin-protease mixtures with successively less protease at molar ratios of 1:1, 2:1, 5:1, and 10:1. In the right-hand lane, the SDS-stable serpin-protease complex is indicated with an arrowhead, the native serpin is indicated with an arrow, and putative RCL-cleaved, but uncomplexed serpin is indicated with a dashed arrow. (Lower-molecular-mass digested serpin fragments are also present.) (B) The trypsin-inhibitory activity of Spn1, determined by a competition assay using a chromogenic substrate for trypsin. White columns represent Spn1, black columns represent incubated mixtures of Spn1-trypsin, and gray columns represent trypsin. Columns show the average of three different independent assays for each sample. OD, optical density.

whereas concomitant overexpression of *Spn1* reduces the Drs-GFP signal to wild-type values Fig. 4A. These results suggest that Spn1 is inhibiting *Drs* expression in the absence of immune challenge. However, this knockdown phenotype could be incomplete since $Spn1^{RNAi}$ flies show strongly reduced levels of Spn1, but some protein remains (Fig. 4B). To confirm and extend these results, we generated a null mutant of *Spn1*, $Spn1^{oka}$ (Materials and Methods). The expression of the *Drs* and *IM1* genes, two targets of the Toll signaling pathway, was measured by qPCR, as was expression of *Dpt*, which is induced mainly through the Imd pathway. $Spn1^{oka}$ mutants show increased (more than 2-fold) levels of *Drs* and *IM1* transcripts, whereas *Dpt* expression was not altered with respect to the wild-type fly (Fig. 4C). Overexpression of $Spn1^{UAS}$ does not reduce *Drs* transcript levels in noninfected flies. This result implies that the target protease of Spn1 is fully inhibited by the endogenous serpin levels and that additional Spn1 activity has no further effect. Thus, lack of the Spn1 inhibitor leads to constitutive activation of Toll pathway-specific gene expression in the absence of immune challenge.

Spn1 represses *Drs* transcript levels in response to fungi but not bacteria. After fungal challenge with *B. bassiana*, $Spn1^{UAS}$-

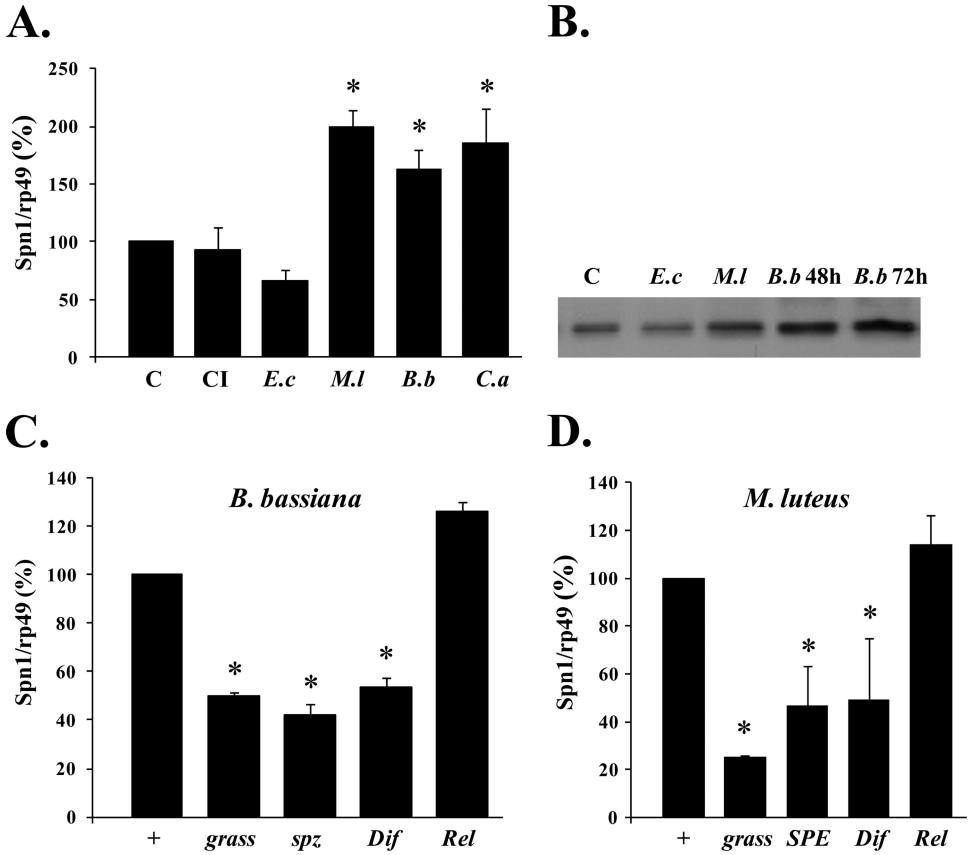

FIG. 3. *Spn1* levels in wild-type and Toll pathway mutants after microbial infection. (A) *Spn1* transcript level, measured by qPCR in the following groups: C, noninfected wild-type flies; CI, clean injury; *E.c*, 6 h postinfection with *E. coli*; *M.l*, 24 h postinfection with *M. luteus*; *B.b*, 48 h postinfection with *B. bassiana*; *C.a*, 48 h postinfection with *C. albicans*. (B) Spn1 protein levels after microbial infection, as detected by immunoblotting with anti-Spn1 at 6 h postinfection with *E. coli* (*E.c*), 24 h postinfection with *M. luteus* (*M.l*), and 48 h and 72 h postinfection with *B. bassiana* (*B.b*). (C) The *grass*, *spz*, and *Dif* mutants show reduced levels of *Spn1* transcript compared to wild-type (+) flies after fungal infection (*B. bassiana*), while the *Imd* pathway mutant, *Rel*, does not affect *Spn1* transcript levels. (D) Similarly, the *grass*, *SPE* [$SPE^{Pasteur}$/*Df*(*3R*)*mbc-R1* hemizygotes], and *Dif* mutants decrease *Spn1* transcript levels compared to wild-type (+) and the *Rel* mutant, after Gram-positive bacterial infection (*M. luteus*). *, significant difference ($P < 0.01$) with respect to the control fly strain.

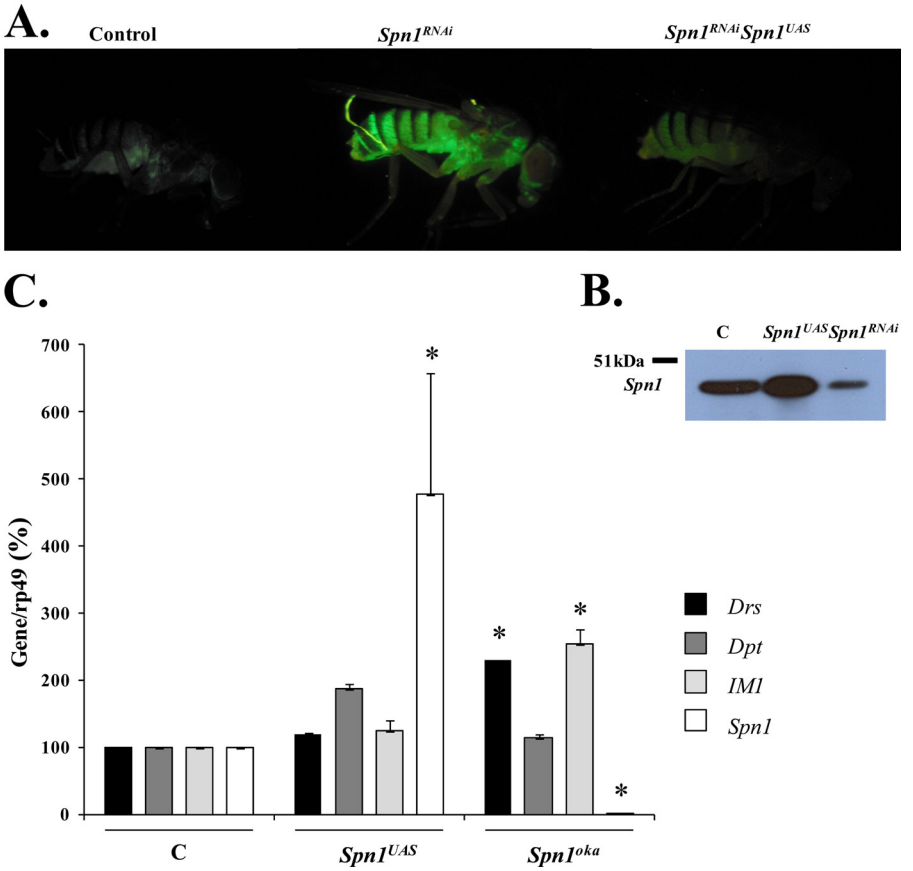

FIG. 4. Lack of *Spn1* results in increased *Drs* and *IM1* levels in unchallenged flies. Complete genotypes are given in parentheses after the abbreviated forms. (A) Fluorescent microscope images of Drs-GFP expression in control (*Drs-GFP*; *Gal4-YP*/*TM6C*), $Spn1^{RNAi}$ ($Spn1^{RNAi.UAS}$/*Drs-GFP*; *Gal4-YP*/*TM6*C), and $Spn1^{RNAi}$ $Spn1^{UAS}$ ($Spn1^{RNAi.UAS}$/*Drs-GFP*; *Gal4-YP*/$Spn1^{UAS}$) flies. $Spn1^{RNAi}$ flies show constitutive activation of Drs-GFP expression. (B) The corresponding Spn1 protein levels are increased in $Spn1^{UAS}$ ($Spn1^{UAS}$; *Gal4-YP*) flies and strongly reduced in $Spn1^{RNAi}$ ($Spn1^{RNAi.UAS}$; *Gal4-YP*) flies, as shown by immunoblotting with anti-Spn1 antibodies. (C) *Drs*, *Dpt*, *IM1*, and *Spn1* transcript levels in wild-type control, $Spn1^{UAS}$ ($Spn1^{UAS}$; *Gal4-YP*), and $Spn1^{oka}$ flies. The lack of *Spn1* transcript in the $Spn1^{oka}$ null mutant is associated with parallel increases in the *Drs* and *IM1* transcripts, while the *Dpt* transcript remains at wild-type levels. C, control. *, $P < 0.01$.

overexpressing flies show reduced levels of *Drs* transcript compared to the wild type (Fig. 5A). This reduction is similar to that observed in mutants of the Toll pathway proteases *grass* or *psh*. Since *B. bassiana* releases virulence factors as well as β-1,3-glucans, infections with this fungus should activate Toll both by the danger signaling and the PRR side branches of the pathway. *C. albicans*, however, activates mainly the fungus-sensitive PRR branch. Upon injection of *C. albicans*, the induction of *Drs* is strongly reduced in $Spn1^{UAS}$-overexpressing flies (Fig. 5B), suggesting that Spn1 represses the PRR side branch of the Toll pathway. In contrast, $Spn1^{UAS}$-overexpressing flies show no change in *Drs* transcript levels after Gram-positive challenge with *M. luteus* (Fig. 5C). The *Dipt* transcript levels (regulated via the Imd pathway) are not decreased in $Spn1^{UAS}$ flies infected with the Gram-negative bacterium *E. coli* (Fig. 5D). $Spn1^{oka}$ flies show elevated levels of *Drs* transcript in the absence immune challenge, but *Drs* transcript levels are further increased in response to infection with *B. bassiana* (Fig. 5E). These results indicate that Spn1 represses *Drs* expression specifically in response to fungal challenge.

Spn1 acts upstream of Grass. Following fungal or Gram-positive bacterial infection, the Toll ligand Spz is activated by SPE, which is common to all branches of the Toll signaling pathway. The Grass protease acts downstream of circulating pattern recognition receptors and upstream of SPE. To localize Spn1 function upstream of Toll, we determined the epistatic relationships between Spn1 and both the SPE and Grass proteases.

Overexpression of the activated form of SPE (SPE*) upregulates *Drs* expression (13). The upregulated level of Drs-GFP protein in SPE^{*UAS} flies is not blocked by expression of $Spn1^{UAS}$ (Fig. 6A). On the other hand, the upregulated expression of *Drs* transcript in $Spn1^{oka}$ mutants is suppressed in *SPE* mutant flies (Fig. 6C). Taken together, these data indicate that Spn1 acts upstream of SPE.

Similarly, the upregulated level of Drs-GFP protein in $grass^{UAS}$ flies is not blocked by expression of $Spn1^{UAS}$ (Fig. 6B), showing that Spn1 is not downstream of Grass. As with SPE, the *Drs* activation in $Spn1^{oka}$ mutants is reduced in a *grass* mutant background (Fig. 6D). Therefore, Spn1 functions upstream of Grass.

Spn1 acts downstream of the GNBP3 receptor but not the PGRP-SA and GNBP1 receptors. The increased level of *Drs* expression in $GNBP3^{UAS}$ flies is reduced by $Spn1^{UAS}$ overex-

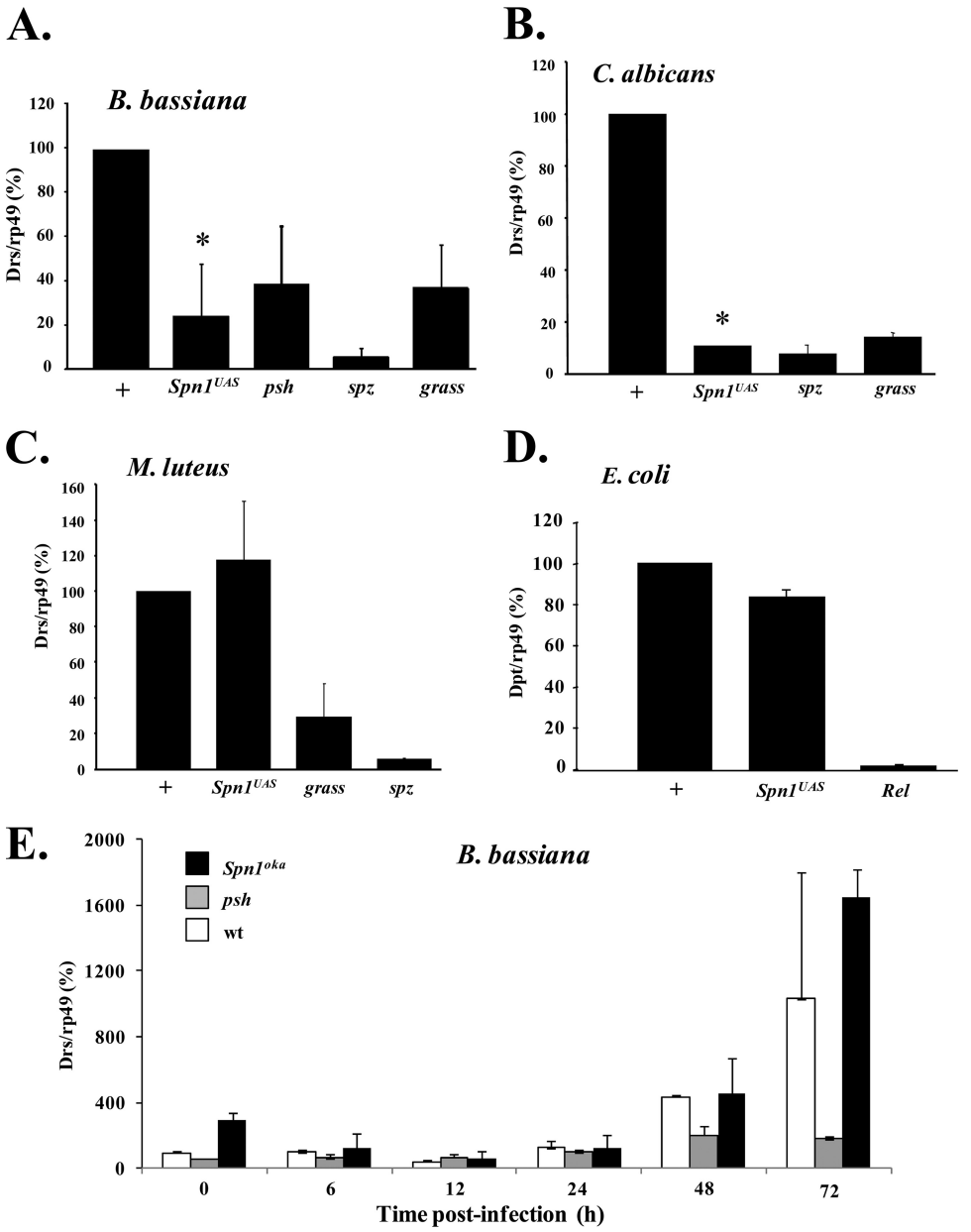

FIG. 5. AMP transcript levels in $Spn1^{UAS}$ flies following immune challenge with fungi and Gram-positive and Gram-negative bacteria. *Drs* transcript levels in wild-type (+), $Spn1^{UAS}$ (*Gal4-c564*/+; $Spn1^{UAS}$/+), *psh*, *spz*, and *grass* flies at 72 h postinfection with *B. bassiana* (A), at 48 h postinfection with *C. albicans* (B), and at 24 h postinfection with *M. luteus* (C). (D) *Dpt* levels in $Spn1^{UAS}$ and *Rel* flies at 6 h postinfection with the Gram-negative bacterium *E. coli*. $Spn1^{UAS}$ overexpression reduces the elevated *Drs* transcript levels in flies challenged with *B. bassiana* and *C. albicans* (*, $P > 0.01$) but not those of flies challenged by *M. luteus*. The *Dpt* transcript levels following *E. coli* infection are unaffected in $Spn1^{UAS}$-overexpressing flies, but *Dpt* expression is blocked by the Imd pathway mutant *Rel.* (E) Time course of *Drs* transcript levels following immune challenge with *B. bassiana* in wild-type, *psh*, and $Spn1^{oka}$ flies. qPCR measurements were taken at 0 to 72 h postinfection.

pression, as monitored by Drs-GFP fluorescence and qPCR (Fig. 7A and B). These results indicate that Spn1 functions downstream of the fungal recognition receptor GNBP3. In contrast, activation of Drs expression by the Gram-positive bacterial recognition receptors $PGRP\text{-}SA^{UAS}$ and $GNBP1^{UAS}$ is unaffected by $Spn1^{UAS}$ overexpression (Fig. 7C and D).

***Spn1* is independent of *modSP*.** We confirm that the *Drs* transcript level is strongly reduced in an *modSP* background (7), but the elevated *Drs* level in $Spn1^{oka}$ flies is only partially reduced by the *modSP* mutation (in $Spn1^{oka}$; *modSP* double mutants) (Fig. 7E). Thus, ModSP activity is not required for upregulation of the *Drs* transcript in an $Spn1^{oka}$ background. On the other hand, the Drs-GFP fluorescence of an $ModSP^{UAS}$ overexpressing fly is not attenuated by the overexpression of Spn1 (Fig. 7F). It follows that Spn1 does not repress ModSP activity.

The $Spn1^{oka}$ mutant shows altered susceptibility to fungal infections but not to *M. luteus*. The increased *Drs* levels ob-

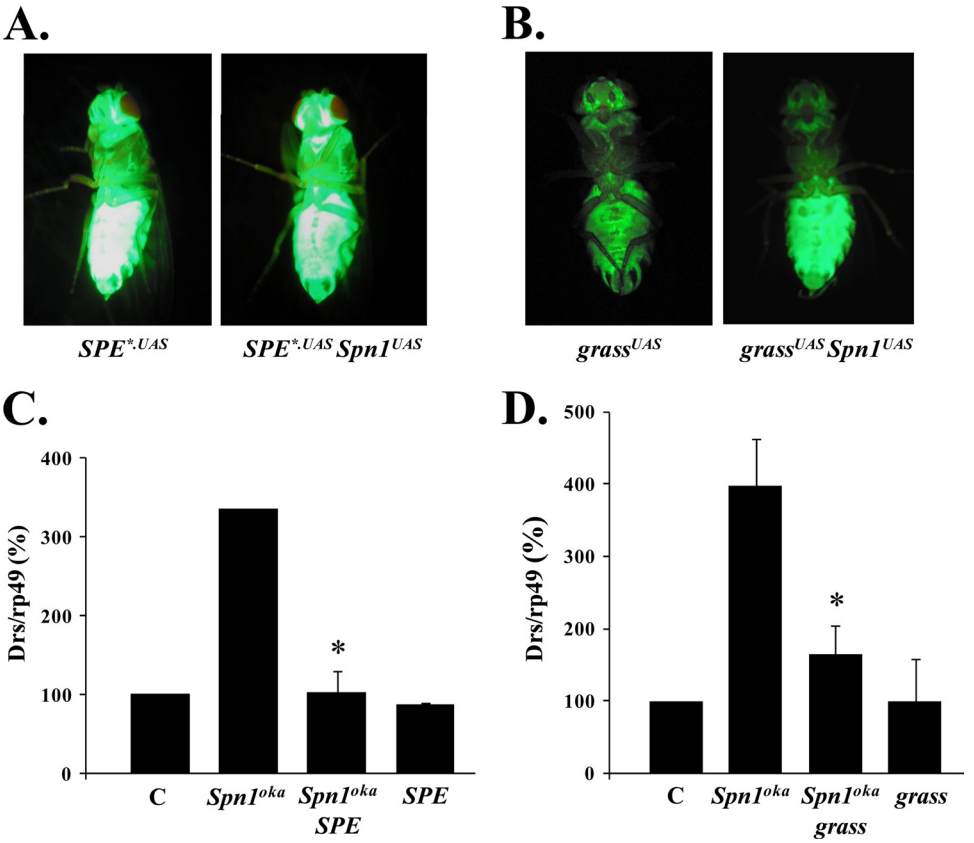

FIG. 6. Spn1 acts upstream of SPE and grass. Complete genotypes are given in parentheses after the abbreviated forms. (A) Drs-GFP expression is upregulated by activated *SPE*∗ (*SPE*∗^{UAS}/*Drs-GFP*; *Gal4-YP*/+). *Spn1*^{UAS} overexpression does not block this upregulation in *SPE*∗ *Spn1*^{UAS} (*SPE*∗^{UAS}/*Drs-GFP*; *Gal4-YP*/*Spn1*^{UAS}) flies, indicating that *Spn1*^{UAS} is acting upstream of SPE, in the common branch of the extracellular Toll pathway. (B) Similarly, overexpression of *Spn1*^{UAS} does not block Drs-GFP expression in *grass*^{UAS} (*grass*^{UAS}/*Drs-GFP*; *Gal4-YP*) and *grass*^{UAS} *Spn1*^{UAS} (*grass*^{UAS}/*Drs-GFP*; *Gal4-YP*/*Spn1*^{UAS}) flies. The increased *Drs* transcript levels in *Spn1*^{oka} flies are reduced in SPE (C) and *grass* (D) mutant backgrounds. Genotypes are abbreviated as follows: *Spn1*^{oka}; *SPE* denotes *Spn1*^{oka}; *SPE*^{Pasteur}/*Df*(*3R*)*mbc-R1* flies *SPE* denotes *SPE*^{Pasteur}/*Df*(*3R*)*mbc-R1* flies. C, uninfected wild-type controls. For panels C and D, the asterisk indicates a significant difference ($P < 0.01$) between the double mutant strain and the *Spn1*^{oka} single mutant.

served in *Spn1*^{oka} flies might be expected to correlate with increased resistance to fungal infections. However, while *Drs* expression acts as a useful marker of Toll pathway activation, Drs is not the only AMP, nor do secreted AMPs form the only defense mechanism following immune challenge. As a direct test of the effect of lack of Spn1 activity on the immune response, the viability of *Spn1*^{oka} mutants was determined after infection with two species of entomopathogenic fungi, a Gram-positive bacterium, and clean injury.

The survival probability of the *Spn1*^{oka} mutant strain was compared with that of *psh* in the danger-signaling and *DIF* in the intercellular Toll pathway (Fig. 8A, top panels) and with *GNBP3*, *modSP*, and *grass* mutants in the PPR side branch of the Toll pathway (Fig. 8A, bottom panels) following natural infection with *B. bassiana*. The survival rate of noninfected controls indicates any nonspecific effects on the viability of the different mutant strains (Fig. 8A, right panel). *Spn1*^{oka} mutants show a rapid decrease in survival postinfection, with only 85% surviving the first 24 h, whereas 85% of wild-type flies survive for 120 h. After 264 h, the survival probability of *Spn1*^{oka} flies is 25%, compared to 75% for wild-type flies. These differences in survival are statistically significant ($P < 0.0001$, log rank analysis). The survival of *Spn1*^{oka} flies is reduced compared to that of *psh* mutants during the first 72 h postinfection, but thereafter the mortality of *psh* flies is higher, with only 15% surviving to 168 h and none to 264 h. The survival rates of *psh* and *Dif* mutants are reduced to similar degrees with respect to wild-type flies, as previously reported (18). Surprisingly, *psh*; *Spn1*^{oka} double mutants die more rapidly than *psh* mutants. Given that the *Spn1*^{oka} and *psh* mutants have an additive effect on susceptibility to infection, Spn1 must be involved in a different branch of the Toll signaling pathway to Psh. In contrast, the susceptibility of *Spn1*^{oka} mutant is similar to that of mutants in the fungal PRR branch, being somewhat lower than that of the GNBP3, very similar to that of the *modSP*, and a little higher than that of the *grass* mutants. Unlike the *psh*; *Spn1*^{oka} double mutant, the combination of the *Spn1*^{oka} and *modSP* mutations does not have an additive effect on susceptibility. Indeed the *Spn1*^{oka}; *modSP* double mutant is slightly more resistant to infection than either single mutant alone. (We were unable to test the *GNBP3*; *Spn1*^{oka} and *grass*; *Spn1*^{oka} double mutant combinations as these flies have strongly reduced viability.)

Following immune challenge with *M. anisopliae*, the survival

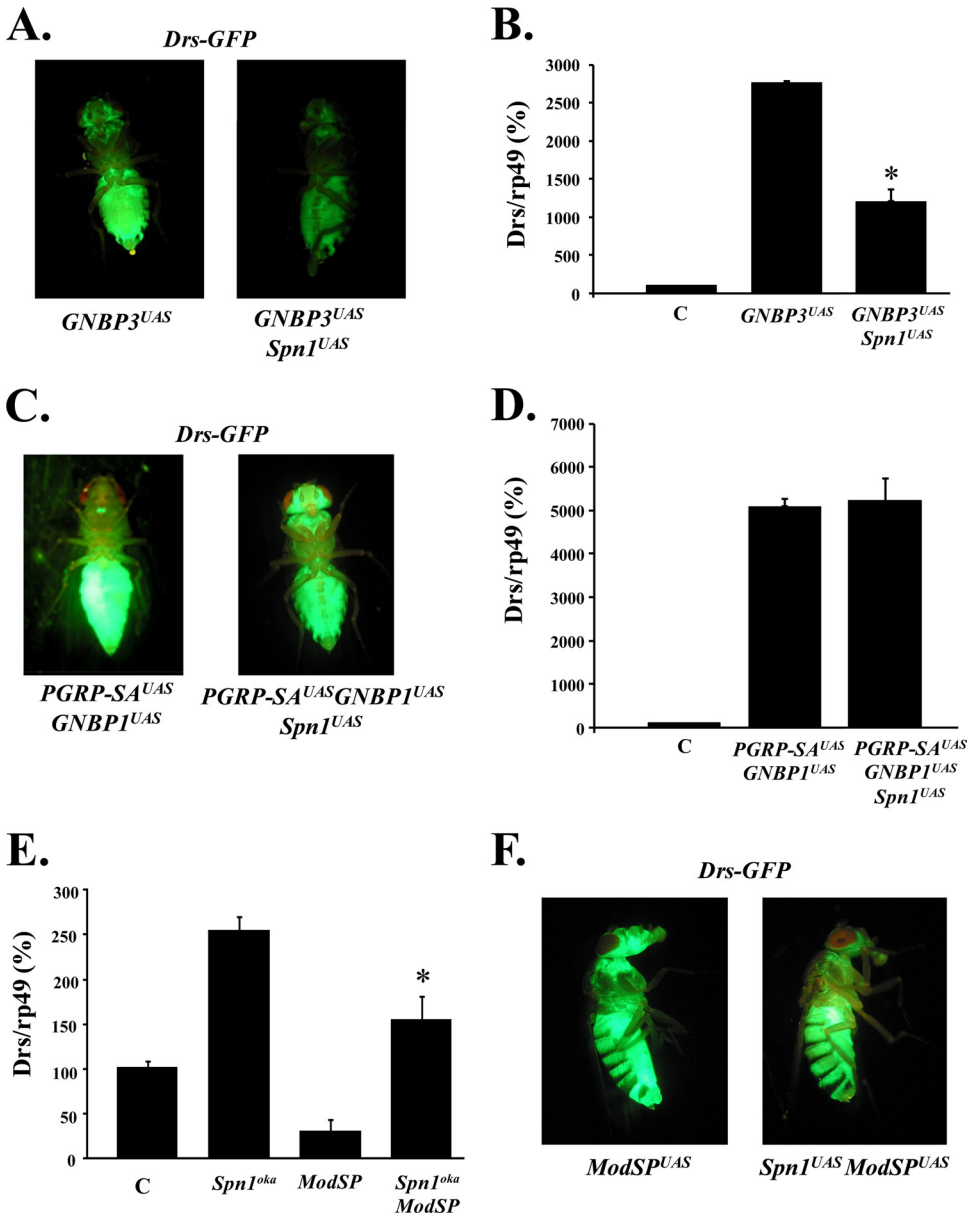

FIG. 7. Spn1 acts in the GNBP3-dependent Toll pathway but not in the PGRP-SA/GNBP1-dependent pathway. Complete genotypes are given in parentheses after the abbreviated forms. (A) The Drs-GFP fluorescence in *GNBP3*^{UAS} (*GNBP3*^{UAS}/*Drs-GFP*; *Gal4-YP*/+) flies is reduced in *GNBP3*^{UAS} *Spn1*^{UAS} (*GNBP3*^{UAS}/*Drs-GFP*; *Gal4-YP*/*Spn1*^{UAS}) flies. (B) The elevated *Drs* transcript levels in *GNBP3*^{UAS} (*GNBP3*^{UAS}/+; *Gal4-YP*/+) flies are reduced in *GNBP3*^{UAS} *Spn1*^{UAS} (*GNBP3*^{UAS}/+; *Gal4-YP*/*Spn1*^{UAS}) flies. C, uninfected wild-type control. *, $P < 0.01$, between *GNBP3*^{UAS} and *GNBP3*^{UAS} *Spn1*^{UAS} flies. (C) Drs-GFP fluorescence in *PGRP-SA*^{UAS} *GNBP1*^{UAS} (*PGRP-SA*^{UAS} *GNBP1*^{UAS}/*Drs-GFP*; *Gal4-YP*) flies remains activated in *PGRP-SA*^{UAS} *GNBP1*^{UAS} *Spn1*^{UAS} (*PGRP-SA*^{UAS} *GNBP1*^{UAS}/*Drs-GFP*; *Spn1*^{UAS}/*Gal4-YP*) flies. (D) The elevated *Drs* transcript levels in *PGRP-SA*^{UAS} *GNBP1*^{UAS} (*PGRP-SA*^{UAS} *GNBP1*^{UAS}/*Drs-GFP*; *Gal4-YP*) flies remains unchanged in *PGRP-SA*^{UAS} *GNBP1*^{UAS} *Spn1*^{UAS} (*PGRP-SA*^{UAS} *GNBP1*^{UAS}/*Drs-GFP*; *Spn1*^{UAS}/*Gal4-YP*) flies. C, uninfected wild-type control. (E) The elevated levels of *Drs* transcript in the *Spn1*^{oka} mutant contrasts with the reduced levels in *modSP* mutants. The double mutant *Spn1*^{oka}; *modSP* shows intermediate levels of *Drs* transcript, implying that while ModSP activates *Drs* transcription, it is not required for Spn1 function. C, wild-type control. *, $P < 0.01$, between the *Spn1*^{oka}; *modSP* and the *Spn1*^{oka} single mutant. (F) The Drs-GFP fluorescence in *modSP*^{UAS} (*modSP*^{UAS}/*Drs-GFP*; *Gal4-YP*/+) flies is not reduced in *modSP*^{UAS} *Spn1*^{UAS} (*modSP*^{UAS}/*Drs-GFP*; *Gal4-YP*/*Spn1*^{UAS}) flies. This result confirms that the Spn1 inhibitor is not downstream of the ModSP protease.

of *Spn1*^{oka} mutants is higher than that of wild-type flies (Fig. 8B). At 40 h postinfection 72% of *Spn1*^{oka} mutants survive, compared to 52% of wild-type flies. At 94 h postinfection, 33% of *Spn1*^{oka} flies survive, but only 10% of wild-type flies survive. These differences are statistically significant ($P = 0.0012$, log rank analysis). The increased resistance of *Spn1*^{oka} mutants to *M. anisopliae* infection is consistent with a role of Spn1 as a specific repressor of the immune response to this fungus. The differential response to *B. bassiana* and *M. anisopliae* apparently reflects the different pathogenic mechanisms of each fun-

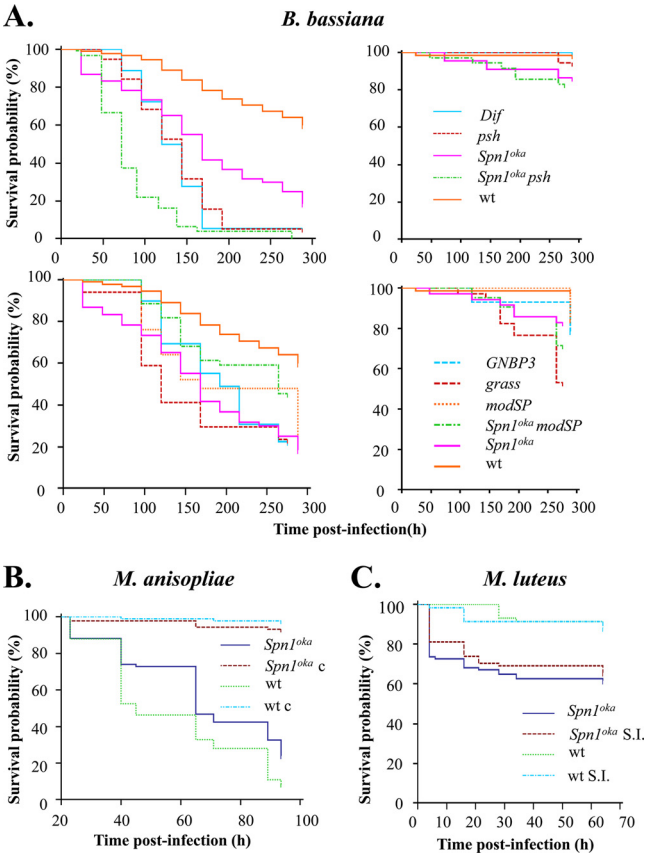

FIG. 8. Viability of *Spn1*^{oka} mutants after immune challenge with fungi, a Gram-positive bacterium, or clean injury. Kaplan-Meier plot of the survival functions. (A) Natural infection with the fungus *B. bassiana* CECT 20548. The top left graph shows data for the survival of *Spn1*^{oka} flies compared to mutants in the danger-signaling pathway (*psh* and *psh*; *Spn1*^{oka}) and the intracellular Toll pathway (*Dif*). The survival of wild-type and *Spn1*^{oka} mutant flies is significantly different according to the log rank test (inverse density function χ^2 [χ^2_{IDF}] = 32.658; $P < 0.0001$). The top right graph shows data for the noninfected controls. In the bottom left graft, survival of *Spn1*^{oka} flies is compared to that of mutants in the PRR signaling pathway (*GNBP3*, *grass*, *modSP*, and *Spn1*^{oka}; *modSP*). The bottom right graph shows survival data of noninfected, control flies. (B) Natural infection with the fungus *M. anisopliae* in wild-type and *Spn1*^{oka} mutant flies. Control noninfected flies (wt C and *Spn1*^{oka} C) are represented on the same graph. The survival of wild-type and *Spn1*^{oka} mutant flies is significantly different according to the log rank test ($\chi^2_{IDF} = 10.552$; $P = 0.0012$). (C) Survival after injection of a saturated culture of *M. luteus* or sterile injection (S.I) in wild-type and *Spn1*^{oka} flies. The differences in survival between flies injected with *M. luteus* and the sterile injection controls are not significant.

gus (see Discussion). In contrast to fungal challenge, injection of *M. luteus* does not cause significant changes in the viability of *Spn1*^{oka} flies compared to a clean, mock injection (Fig. 8C) although both treatments cause a transient, rapid lethality, with 20% of the flies dying within the first 10 h.

DISCUSSION

We report here the biochemical and functional characterization of the *Drosophila* Spn1 serpin. We initially determined the biochemical properties of Spn1 *in vitro*, followed by a genetic analysis of its role in the innate immune response. The recombinant Spn1 protein exhibits a stressed-to-relaxed transition between 5 and 6 M urea and a thermal denaturation point at 48.6°C. Both these values are typical of inhibitory serpins in the native conformation. Recombinant Spn1 protein is a potent inhibitor of trypsin but is inactive with the chymotrypsin, elastase, thrombin, kallikrein, or cathepsin proteases. The putative P1/P1′ site for protease cleavage within the RCL falls 17 residues C-terminal to the start of the hinge region of the RCL (26). On this basis, the P1/P1′ site of Spn1 is R/A, which is an ideal target for trypsin (3) and is consistent with our inhibitory assays. Taken together, these data establish that Spn1 is an inhibitor of trypsin-like proteases. In contrast, the Nec serpin (which also regulates the immune response) does not inhibit trypsin but is a broad-range inhibitor of elastase, thrombin, and chymotrypsin-like proteases (45). In the case of Nec, the serpin core carries an N-terminal peptide of 73 amino acids (after cleavage of the putative export signal peptide) (42) that is cleaved on immune challenge (32). Both the full-length serpin (Nec-fl) and the truncated core serpin (Nec-ΔN) are active protease inhibitors, but Nec-fl shows a 13-fold increase of specificity for porcine pancreatic elastase compared to Nec-ΔN (42). These two inhibitors of the Toll pathway show very different ranges of protease target specificities *in vitro*.

In the absence of infection, both *Spn1*^{oka} and *nec* mutants show constitutive expression of the Drs antifungal peptide (19). If nec and Spn1 inhibit a single, linear pathway, both would need to be inactivated to allow Drs expression in response to infection. If, however, the two serpins inhibit separate pathways, lack of function of either of them could activate Drs expression. Recently, it has become clear that the extracellular proteolytic cascade that activates the Toll receptor is not a simple linear zymogen cascade but, instead, is split into three separate side branches (13, 18), allowing the two serpins to act independently. An additional phenotype shown by *nec* mutants is that they develop melanotic spots, with an associated cellular necrosis phenotype, shortly after eclosion from the pupa and die within 24 to 48 h (19). This phenotype is completely suppressed in *psh*; *nec* double mutant flies, together with the activation of Drs expression (33). In contrast, the *Spn1*^{oka} mutant shows no cellular necrosis phenotype.

The three separate side branches of the Toll signaling pathway can be activated by either Gram-positive or fungal cell wall pattern recognition receptors or by microbial proteases (which correspond to pathogen-encoded virulence factors, secreted after contact with the host) (4, 13, 17, 18, 40). The three side branches converge on the Spätzle processing enzyme (28), giving a multiply triggered sensing mechanism. It is the microbial protease-sensitive, danger-signaling side branch of the Toll pathway that is regulated by Nec (32) and includes the serine protease Psh (33). As we show here, Spn1 acts downstream of the fungal cell wall pattern recognition receptor, GNBP3. The inhibition of these two side branches is independent so that lack of either serpin function will activate Drs expression (Fig. 9).

Spn1 transcript levels are upregulated following immune challenge with the fungi *B. bassiana* and *C. albicans* unless the Toll pathway is blocked (in *SPE*, *grass*, *spz*, or *Dif* mutant flies). In unchallenged flies, the *Drs* transcript levels of *Spn1*^{oka} mutants are increased to between 2.5 to 3.5 times that of wild-type

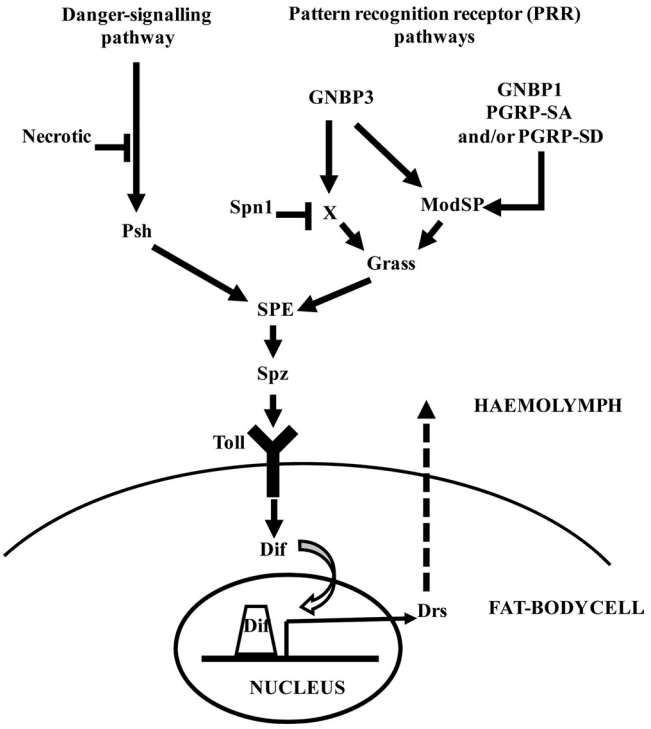

FIG. 9. Model of Spn1 regulation of the Toll-mediated innate immune response. Three separate side branches of the Toll signaling pathway are activated either by microbial cell wall PRRs (GNBP3, for fungi and yeasts; GNBP1, PGRP-SA, and PGRP-SD for Gram-positive bacteria) or danger-signaling (virulence factors). These three side branches of the pathway converge on SPE, which activates Spz (the ligand of Toll). The intracellular Toll pathway signals trough Dif to activate the expression of *Drs* and other antimicrobial peptides in the fat body. The GNBP3 and danger-signaling branches of the Toll signaling pathway are regulated independently by Spn1 and nec. Both of these serpins represent nonredundant genetic functions, and the lack activates *Drs* expression.

flies. The levels of the Toll-responsive transcript *IM1* are also increased, in parallel with the *Drs* transcript, in $Spn1^{oka}$ mutants while the Imd pathway-responsive transcript, *Dpt*, remains unaffected (Fig. 4C). Overexpression of $Spn1^{UAS}$ does not reduce *Drs* or *IM1* transcript levels in unchallenged flies (Fig. 4C). Similarly, when *Drs* transcription is activated by overexpressing two Gram-positive PRRs (in $GNBP1^{UAS}$ $PGRP\text{-}SA^{UAS}$ flies), increasing the endogenous levels of *Spn1* does not reduce the elevated *Drs* transcript levels (Fig. 7D). In contrast, *Drs* transcription levels are reduced by *Spn1* overexpression in flies overexpressing $GNBP3^{UAS}$ (the receptor for fungal cell walls) (Fig. 7B) or infected with *B. bassiana* or *C. albicans* (Fig. 5A and B). The levels of *Spn1* can be rate limiting for *Drs* transcript expression but only when the *GNBP3*-regulated side branch of the Toll signaling pathway is activated. This is a striking result; in general, regulatory serpin activity is in excess of that of the target proteases so that overexpression has no physiological consequences. In addition to facilitating epistatic analysis, the GNBP3 pathway-dependent requirement for the levels of Spn1 activity to be rate limiting is consistent with *Spn1* having a specific role in the Toll-mediated response to fungal infections (see below).

Further analysis with lack-of-function mutations shows that the activation of *Drs* expression in the $Spn1^{oka}$ mutant is blocked in both *grass* and *SPE* mutant flies (Fig. 6). In this context, the Grass protease was originally assigned to the Gram-positive pattern recognition pathway (30), activated by the GNBP1 and PGRP-SA receptors (17). Subsequent study, however, has shown that Grass is downstream of both the Gram-positive bacterial and fungal PRRs (13). Our analysis confirms that Grass is common to both the PRR-regulated branches; Spn1 acts between the GNBP3 receptor and Grass but is independent of the GNBP1 and PGRP-SA receptors. We show, however, that *Drs* upregulation in the $Spn1^{oka}$ background does not require ModSP activity, which would be unexpected if ModSP were the apical protease in both PRR-regulated pathways. The simplest model consistent with our data is that Spn1 regulates a side branch of the fungal cell wall pattern-sensing pathway (Fig. 9). GNBP3 can activate the Grass zymogen either via the target protease inhibited by Spn1 or via ModSP. Even complete removal of the *Spn1* inhibitor, however, allows only partial activation of Grass and a moderate increase in *Drs* transcription. These results are consistent with the results of Buchon et al. (7): while the *modSP* mutant completely blocks *Drs* activation in $PGRP\text{-}SA^{UAS}$ $GNBP1^{UAS}$-overexpressing flies, in $GNBP3^{UAS}$-overexpressing flies *Drs* transcript levels are only partially blocked by the *modSP* mutant and remain about 3-fold higher than levels in the wild type. Apparently, Grass can be partially activated via the GNBP3 pathway in the absence of ModSP activity. In addition, we show that the activation of Drosomycin in *ModSP*-overexpressing flies is not blocked by *Spn1* overexpression (Fig. 7E); by this criterion, the Spn1 inhibitory activity does not act downstream of the ModSP protease. This result strongly supports the model presented in Fig. 9.

The increased susceptibility of Spn^{oka} flies to immune challenge with *B. bassiana* is the opposite of what would be expected from increasing expression of the Drs AMP. It is unlikely, however, that the moderate increase in Drs activity in unchallenged Spn^{oka} flies would be sufficient in itself to confer significant resistance to fungal infections. In contrast to infection with *B. bassiana*, resistance to infection by *M. anisopliae* is increased in Spn^{oka} flies, in agreement with a role of *Spn1* as a repressor of the immune response. Although the response of *Drosophila* to *M. anisopliae* has been less well characterized than that to *B. bassiana*, mutants which block the Toll pathway do show increased susceptibility to *M. anisopliae* infections (21). Significantly, the PR1 protease of *M. anisopliae* has been shown to be a major virulence factor (49, 53). Following immune challenge with *M. anisopliae*, *Drs* expression is not blocked in either *psh* or *GNBP3* single mutant flies, but it is abolished in the *psh*; *GNBP3* double mutant background. In contrast, with *B. bassiana* infection *Drs* expression is very strongly reduced in *psh* mutants but is unaffected in *GNBP3* mutants (18). These results suggest that *B. bassiana* has evolved a mechanism which allows it to escape *GNBP3* surveillance (18). Thus, the opposite effects on the viability of $Spn1^{oka}$ mutants by these two fungal pathogens may reflect differential contributions of the danger signaling and GNBP3-dependent mechanisms for detecting immune challenge. As suggested by Gottar et al. (18), it seems likely that the original mechanism for sensing fungal pathogens was via PPRs and that the surveillance of virulence factors represents a host counter-

strategy to detect pathogens that have adapted to avoid the GNBP detection mechanism. It is not unlikely that the adaptive pressure to respond to pathogens that can avoid the PPR sensor system would lead to the additional cross-regulatory interactions in the GNBP3-dependent activation of the Toll pathway.

In summary, we have characterized the serpin family inhibitor Spn1. This protein forms covalently linked complexes with trypsin *in vitro*, indicating that the natural target protease of Spn1 is a trypsin-like protease. Spn1 regulates the innate immune response against fungal pathogens in *Drosophila*. In particular, Spn1 acts downstream of the GNBP3 receptor and upstream of the Grass protease in the fungal cell wall recognition branch of the Toll pathway.

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We declare that we have no conflicts of interest.

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