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An Immune-Responsive Serpin Regulates the Melanization Cascade in *Drosophila*

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Summary

In arthropods, the melanization reaction is associated with multiple host defense mechanisms leading to the sequestration and killing of invading microorganisms. Arthropod melanization is controlled by a cascade of serine proteases that ultimately activates the enzyme prophenoloxidase (PPO), which, in turn, catalyzes the synthesis of melanin. Here we report the biochemical and genetic characterization of a *Drosophila* serine protease inhibitor protein, Serpin-27A, which regulates the melanization cascade through the specific inhibition of the terminal protease prophenoloxidase-activating enzyme. Our data demonstrate that Serpin-27A is required to restrict the phenoloxidase activity to the site of injury or infection, preventing the insect from excessive melanization.

Introduction

Insects have a sensitive mechanism for identifying pathogens and an array of strategies for defending themselves against microbial attack. To combat infection, the fruit fly, *Drosophila melanogaster*, relies on both constitutive and inducible defense mechanisms (Tzou et al., 2002a). However, the first line of defense that prevents microbial invasion into the hemocoel is structural. It is comprised of the external cuticle, the gut peritrophic matrix, and the tracheal lining. When pathogens breach these barriers, they activate a wide range of inducible reactions. Perforation of the cuticle by injury or by microbial infection rapidly activates proteolytic cascades that lead to blood coagulation and melanization. Upon subsequent microbial or parasitic infection, a cellular immune response, mediated by different hemocyte types, is mounted and participates in pathogen/parasite clearance by phagocytosis or encapsulation. During systemic infection, a large set of inducible effector molecules, such as antimicrobial peptides (AMP), stress-responsive proteins, and other factors required for opsonization and iron sequestration, are secreted into the hemolymph (Tzou et al., 2002a).

During the past few years, the regulation of AMP gene expression has been the focus of many studies (Hoffmann and Reichhart, 2002; Tzou et al., 2002a). AMPs are synthesized in the fat body, a functional equivalent of mammalian liver, and secreted into the hemolymph, where they directly kill invading microorganisms. Genetic analyses have demonstrated that AMP genes are regulated by the Toll and Imd pathways, which are selectively activated by different classes of pathogens. Mutations in either of these pathways block the expression of specific subsets of AMPs and increase the susceptibility to microbial infection (Hoffmann and Reichhart, 2002; Tzou et al., 2002a). A recent DNA microarray study has shown that, in addition to AMP genes, the Toll and Imd pathways regulate most of the genes induced upon microbial infection (De Gregorio et al., 2002). These two pathways share many common features with the mammalian TLR and TNF-R signaling cascades that regulate NF-κB, pointing to an evolutionary link between *Drosophila* and mammals in the regulation of the innate immune response (Hoffmann and Reichhart, 2002; Tzou et al., 2002a).

Apart from the regulation of AMP gene expression, very little is known about the other defense mechanisms that combat infection in *Drosophila*. The most dramatic and immediate immune response in *Drosophila* is the melanization reaction observed at the site of cuticular injury or on the surface of pathogens, parasites, and parasitoids invading the hemocoel. The blackening of the wounded area of the cuticle or the surface of invading organisms results from the de novo synthesis and deposition of melanin. In arthropods, melanization plays an important role in defense reactions, such as wound healing, encapsulation, sequestration of microbes, and the production of toxic intermediates, that are speculated to kill invading microorganisms (Ashida and Brey, 1998; Söderhäll and Cerenius, 1998). So far, no regulators of the melanization cascade have been identified at the molecular level in the fruit fly.

Although there is a paucity of information concerning the *Drosophila* melanization reaction, this response has been investigated in more detail in other arthropods (Ashida and Brey, 1998; Söderhäll and Cerenius, 1998).

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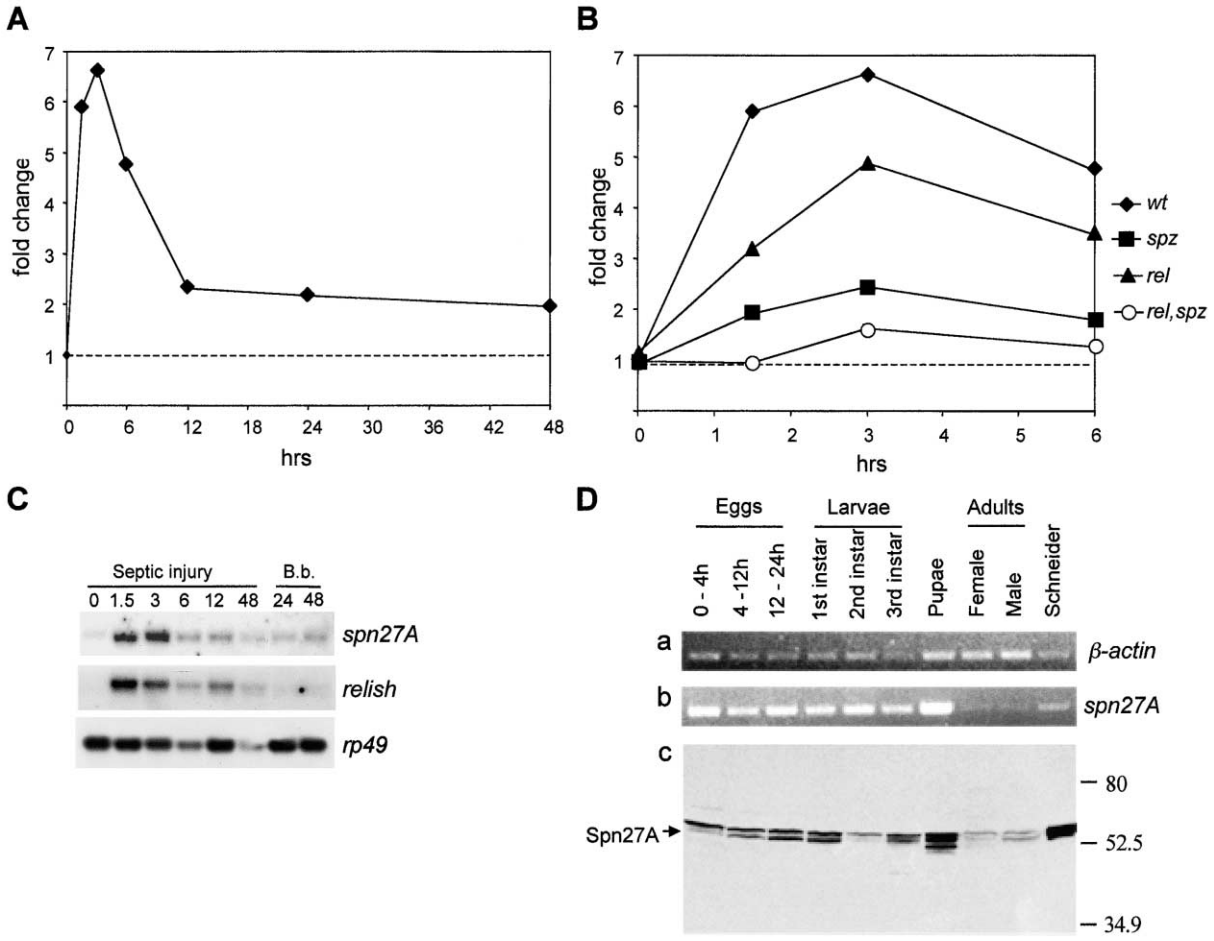

Figure 1. Expression Profile of *spn27A*

(A) The expression profile of *spn27A* in wild-type adult flies in response to septic injury with a mixture of *E. coli* and *M. luteus* was monitored by microarray analysis. The graph indicates fold change in the expression level compared with uninfected flies at specific times after infection (0–48 hr).

(B) The graph shows the expression profile (monitored by microarray) of *spn27A* in response to septic injury (time 0–6 hr) in wild-type (wt), *spz*, *rel*, or *rel,spz* double mutant flies. The expression levels are indicated as fold change relative to uninfected wild-type (wt) flies.

(C) Northern blot analysis of *spn27A* expression in response to septic injury and to natural infection by *B. bassiana*. The times after infection are indicated in hours. Four micrograms of poly(A) has been used for each time point. The same filter was hybridized with probes directed against *spn27A*, *relish* (as an example of known immune-responsive gene [Dushay et al., 1996]) and the ribosomal protein gene *rp49* as a control.

(D) The expression of *spn27A* was examined both at the transcriptional and translational level. (a and b) RT-PCR was performed with primers specific for β-*actin* (a) or *spn27A* (b) in different developmental stages and in cultured Schneider cells. (c) Protein samples from the same developmental stages and the supernatant of Shneider cells were subjected to the Western blot analysis with the anti-Spn27A antibody. The arrow on the left indicates Spn27A protein. The molecular size markers are indicated in kilodaltons on the right.

It requires the activation of phenoloxidase (PO) (EC 1.14.18.1), an enzyme that catalyses the oxidation of mono- and diphenols to orthoquinones, which are then polymerized nonenzymatically to melanin. Insect phenoloxidase has been found to exist in the hemolymph plasma and the cuticle as an inactive form, called prophenoloxidase (PPO) (Ashida and Brey, 1998). Enzymatically inactive PPO is cleaved into active PO by a serine protease known as prophenoloxidase-activating enzyme (PPAE). PPAE also exists as an inactive zymogen that is activated through a stepwise process involving other serine proteases. PPAEs have been isolated from different insect species and other arthropods (Jiang et al., 1998; Lee et al., 1998; Satoh et al., 1999; Wang et al., 2001). Several studies indicate that the melanization cascade is triggered by injury or by recognition of microbial cell wall components, such as peptidoglycan, β-1,3 glucan, and lipopolysaccharide (LPS), through pattern recognition proteins (Yoshida et al., 1996; Yu et al., 1999; Ma and Kanost, 2000; Ochiai and Ashida, 1999). Consequently, the PPO cascade is an efficient nonself-recognition system in invertebrates, and its immediate activation suggests that it may regulate aspects of the immune response other than PPO activation.

Although vertebrates do not possess an equivalent of a PPO system, the PPO cascade in insects is reminiscent of the blood clotting reactions in vertebrates. Both reactions involve serine protease cascades that must

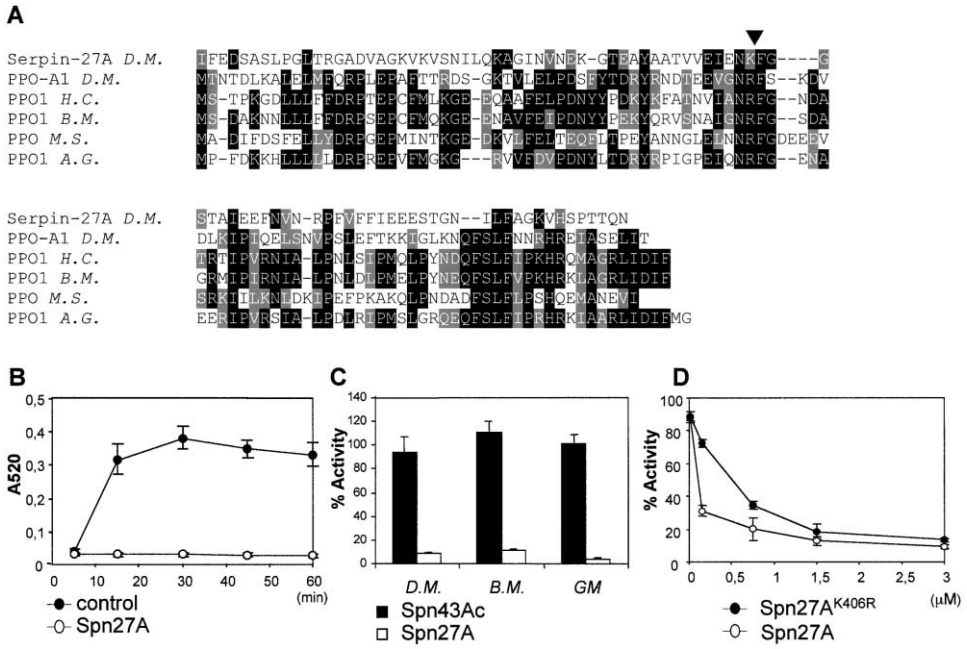

Figure 2. Amino Acid Sequence Alignment and PO Activity Tests

(A) The multiple alignment of the C-terminal portion (aa 355–447) of Spn27A with the N-terminal portion (aa 1–100) of PPOs from different insect species. The black and the gray boxes mark the identical and the similar amino acids, respectively. The protease cleavage site is indicated by the arrow. *D.M., Drosophila melanogaster*; *H.C., Hyphantria cunea*; *B.M., Bombyx mori*; *M.S., Manduca sexta*; *A.G., Anopheles gambiae*.

(B) PO activity was determined by measuring absorbency at 520 nm of the homogenate supernatant of *Drosophila* pupae after incubation with buffer only or 3 μM of rSpn27A for the indicated time.

(C) Effect of 3 μM of rSpn27A or 3 μM of rSpn43Ac on PO activity in different insect species. Values are expressed as the percent of PO activity measured in the absence of recombinant proteins. *D.M., Drosophila melanogaster*; *B.M., Bombyx mori*; *G. M., Galleria mellonella*.

(D) The inhibitory effect of increasing amounts of rSpn27A^{K406R} on PO activity in *Drosophila* pupae extract was compared with that of rSpn27A wild-type. PO activity is expressed as in (C).

be tightly regulated to avoid a systemic activation, which is often fatal. One class of serine protease regulators ubiquitous among plants, animals, and viruses is serpins (Irving et al., 2000). Serpins compose a superfamily of proteins that fold into a conserved structure and employ a suicide substrate-like inhibitory mechanism (Ye and Goldsmith, 2001). In the carboxyl terminal, they have a 30–40-residue-long "reactive center loop" (RCL), which is exposed at the surface of protein that binds to the active site of the target serine protease (Lawrence et al., 2000; Potempa et al., 1994). After cleavage of the RCL region, the P1 residue of the serpin forms a covalent acyl bond with the active site serine residue of the target protease (Cohen et al., 1977; Potempa et al., 1994). Like humans, fruit flies possess a high number of genes encoding serpins (Rubin et al., 2000), which reflects the high number of serine protease genes found in this insect. However, the serpin gene family is poorly studied in *Drosophila*. To date, a mutation in only one serpin gene, *spn43Ac*, has been studied in relation to the *necrotic* phenotype. The loss-of-function mutation in *spn43Ac* leads to constitutive activation of the Toll-mediated immune response, indicating that Spn43Ac inhibits a serine protease that functions upstream of the Toll ligand Spaetzle (Levashina et al., 1999).

The identification of genes that regulate the melanization cascade in *Drosophila* has been complicated by the high number of serpin and serine protease candidate genes in the fly genome. Recently, a DNA microarray analysis helped us to select 5 serpin genes among the 30 encoded by the *Drosophila* genome that, like *spn43Ac*, are significantly upregulated in response to infection and might control one of the serine protease cascades associated to fly immune reactions (De Gregorio et al., 2001). Here we present both the biochemical and genetic characterization of one of them, *serpin-27A* (*spn27A*, *CG11331*). Our data demonstrate that Spn27A regulates the melanization cascade through the specific inhibition of PPO processing by the terminal serine protease PPAE.

Results

Spn27A Is an Acute Immune-Responsive Gene Regulated by the Toll Pathway

Oligonucleotide microarray analysis performed on *Drosophila* adult males indicated that *spn27A* is one of the five serpin-encoding genes induced by septic injury with a mixture of gram-positive and gram-negative bacteria and, like *spn43Ac*, is also induced after natural infection by the entomopathogenic fungus *Beauveria bassiana* (De Gregorio et al., 2001). The expression profile in response to septic injury shows that *spn27A* is an acute response gene with the highest expression level at 3

hr postinfection (Figure 1A; De Gregorio et al., 2001). Northern analysis of the *spn27A* expression profile after septic injury and *B. bassiana* infection confirmed the microarray data (Figure 1C). The comparison of the expression profiles in wild-type and mutants deficient for the Toll (*spaetzle*, *spz*), Imd (*relish*, *rel*), or both (*rel,spz*) pathways revealed that the expression of *spn27A* is mainly controlled by the Toll pathway (Figure 1B; De Gregorio et al., 2002).

To examine the temporal expression profile of *spn27A* during *Drosophila* development, we performed RT-PCR analysis and Western blot analysis (Figure 1D). *spn27A* mRNA was expressed throughout all developmental stages and also in cultured *Drosophila* Schneider cells (S2). However, it was most weakly expressed in both adult males and females (Figure 1D [b]). The calculated molecular weight of Spn27A is 48.12 kDa. However, Spn27A antiserum detected two major bands around 65 kDa in samples corresponding to the various developmental stages (Figure 1D [c]). The Western blot analysis of *spn27A*-deficient flies with the same antiserum indicates that only the lower band corresponds to Spn27A protein (see below). By mass number analysis with matrix-assisted laser desorption and ionization (MALDI) spectrometry, we determined the molecular mass of purified recombinant Spn27A to be 66.59 kDa (data not shown). Such a large difference between the observed molecular weight and the calculated mass number is most likely due to posttranslational modifications, such as glycosylation, which is often observed in the serpin family (Potempa et al., 1994). We also detected Spn27A in the culture medium of *Drosophila* Schneider cells (Figure 1D [c]), suggesting that Spn27A is a secreted protein. N-terminal amino acid sequencing with purified recombinant Spn27A showed the cleavage site of the signal peptide to be located between Gly^{25} and Asn^{26} (data not shown).

Inhibition of PPO Activation by Recombinant Spn27A

Interestingly, close examination of the deduced amino acids revealed that Spn27A contains a C-terminal region that is homologous to the region around the conserved cleavage site of insect prophenoloxidases (PPO) (Figure 2A). Previously, serine proteases have been shown to cleave the propeptide of PPO between Arg and Phe within the NRFG motif to generate active PO for melanin synthesis. Furthermore, the cleavage sites of PPO from most insects and Spn27A are very similar (NRFG versus NKFG, respectively). We hypothesize that both these proteins serve as substrates for a common serine protease. To investigate the possible involvement of Spn27A in regulating PPO activation, we performed a PPO activation assay using *Drosophila* pupal homogenate in the presence of recombinant Spn27A (rSpn27A). Time-course analysis showed that PO activity in the pupal homogenate increased over time, reaching a plateau after 30 min (Figure 2B). The addition of rSpn27A to the pupal homogenates completely blocked PO activity, regardless of incubation time (Figure 2B).

Given that insect PPO activation cascades are very similar, we postulated that the *Drosophila* rSpn27A could perhaps affect the activation of PPO in various insect systems. We used the hemolymph plasma from larvae of two lepidopterans (*Bombyx mori* and *Galleria mellonella*) and showed that Spn27A was able to significantly block PPO activation in both species (Figure 2C). As a negative control we used the only other characterized *Drosophila* serpin, Spn43Ac. Recombinant Spn43Ac was unable to inhibit the activation of PPO (Figure 2C).

Even though there is a striking amino acid sequence similarity between Spn27A and PPOs in and around the cleavage site, the cleavage site itself is not identical. Specifically, Spn27A has a Lys at the cleavage site, whereas insect PPOs have an Arg at this position. Generally, trypsin-like serine proteases are known to cleave their substrates after a basic amino acid, such as Arg or Lys (Perona and Craik, 1995). We mutated the putative P1 site of rSpn27A from Lys to Ala ($rSpn27A^{K406A}$). Following this point mutation, $rSpn27A^{K406A}$ completely lost its PO activation inhibitory effect (data not shown). In a second set of experiments, to mimic the PPO cleavage site, we mutated the lysine of the putative P1 site with arginine ($rSpn27A^{K406R}$). Interestingly, $rSpn27A^{K406R}$ inhibits PPO activation with a lower efficiency than rSpn27A wild-type in *Drosophila* pupal homogenate (Figure 2D).

The Target Protease of Spn27A Is Prophenoloxidase-Activating Enzyme (PPAE)

Serpins bind their target proteases through an RCL domain that mimics their substrate. Therefore the sequence similarity between the Spn27A RCL and the insect PPO cleavage site suggests that the target of Spn27A might be prophenoloxidase-activating enzyme (PPAE). Previously, we showed that highly purified prophenoloxidase-activating enzyme from the coleopteran insect *Holotrichia diomphalia* (HdPPAE) can convert PPO to PO (Lee et al., 1998). Because the *Drosophila* PPAE has not yet been identified, we opted to use the highly purified PPAE from *H. diomphalia*. To investigate whether HdPPAE is the target protease of Spn27A, we conducted a protease activity assay in the presence of rSpn27A and its mutants using chromogenic substrates. When HdPPAE and rSpn27A were incubated in a 1:1 molar ratio, the HdPPAE enzymatic activity was greatly inhibited (Figure 3A). A similar result was obtained using the $rSpn27A^{K406R}$ mutant. However, the $rSpn27A^{K406A}$ mutant was unable to inhibit HdPPAE enzymatic activity (Figure 3A). To verify that the inhibitory effect of rSpn27A against HdPPAE was due to specific acyl bond formation (Cohen et al., 1977), we performed a gel mobility shift assay. A specific higher-molecular weight complex was observed in the presence of both HdPPAE and Spn27A (Figure 3B).

To further demonstrate that Spn27A is a natural inhibitor of PPAE, we reconstituted the final step of the PPO activation system (HdPPO plus active HdPPAE with cofactors) in the presence of rSpn27A. The result showed that rSpn27A and the $rSpn27A^{K406R}$ mutant could almost completely inhibit de novo-generated PO activity, while the $rSpn27A^{K406A}$ mutant had no effect (Figure 3C). It is generally accepted that de novo-generated PO activity is due to limited proteolysis of PPO by the activating enzyme (PPAE). To demonstrate that the cleavage of PPO by HdPPAE is blocked by rSpn27A, we checked, by Western blot, the effect of rSpn27A variants on the conversion of PPO to PO in the reconstituted system. According to the PO activity assay (Figure 3C), we found

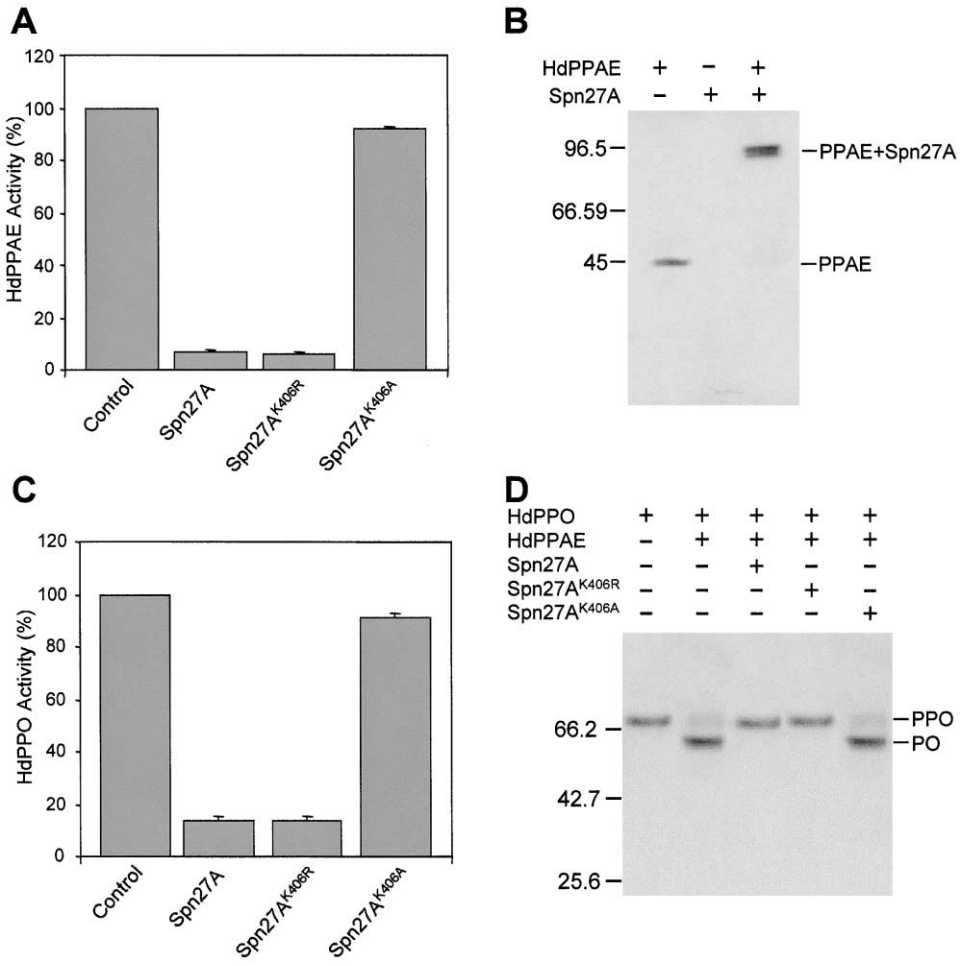

Figure 3. Inhibition of HdPPAE Activity

(A) The amidase activity of purified HdPPAE in the absence or presence of each of the rSpn27A derivatives (molar ratio 1:1) was determined with a chromogenic substrate. The HdPPAE activity measured in the absence of recombinant proteins was set to 100%.

(B) HdPPAE and rSpn27A alone or in combination were subjected to nonreducing SDS-PAGE and then to Western blot with an anti-HdPPAE-specific antibody. A specific complex formed between HdPPAE and rSpn27A is indicated by the arrow on the right.

(C) De novo-generated PO activity was determined with a reconstituted PPO activation system in the presence or absence of rSpn27A and its derivatives. The activity measured in the absence of rSpn27A variants was set to 100%.

(D) The same reaction mixtures as in (C) were subjected to Western blot analysis with anti-HdPPO-specific antibody.

that rSpn27A and the rSpn27A^{K406R} completely inhibit the cleavage of PPO, while rSpn27A^{K406A} mutant had no effect (Figure 3D). In summary, our biochemical analysis strongly suggests that Spn27A functions in *Drosophila* as a negative regulator of PPO activation through the inhibition of PPO-activating enzyme (PPAE).

Generation of an *spn27A* Mutant

In order to investigate the role of Spn27A in vivo, we constructed an *spn27A*-deficient mutant. The *spn27A* gene is nested within an intron of the *cup* gene (Figure 4A). The *cup* gene is known to be implicated in oogenesis (Keyes and Spradling, 1997). There are several reported alleles of *cup* that are all female sterile. A P element (*EP(2)2349*) located 900 bp upstream of the *spn27A* ORF and 110 bp from the last *cup* exon did not display any defect in oogenesis, suggesting that it does not interfere with *cup* expression. To generate a *Drosophila* strain deficient of *spn27A*, we mobilized the P element *EP(2)2349* and screened, by PCR, for lines presenting a deletion uncovering the *spn27A* start codon. Out of 30 lines tested, we found one (*spn27A^{1}*) bearing a deletion (*E25*), of 1175 bp downstream of the *EP(2)2349* insertion site, which included the first 275 bp of the *spn27A* ORF. In agreement with the molecular characterization, we found that *spn27A^{1}* larvae and adults neither expressed *spn27A* mRNA nor protein (Figures 4B and 4C). In addition, *spn27A^{1}* mutants were female sterile, with the embryos dying at the very early developmental stage (data not shown), further suggesting that the *cup* gene might be affected by the *E25* deletion. However, we found that *spn27A^{1}/cup* females were not sterile; therefore, we concluded that the sterility of *spn27A^{1}* female flies was a consequence of the lack of maternal *spn27A* expression during early embryogenesis. The *spn27A^{1}* homozygous larvae were viable, all reaching pupal stage. However, only 30% of *spn27A^{1}* adults emerged from the pupal stage, suggesting an implication of Spn27A during metamorphosis. *spn27A^{1}*

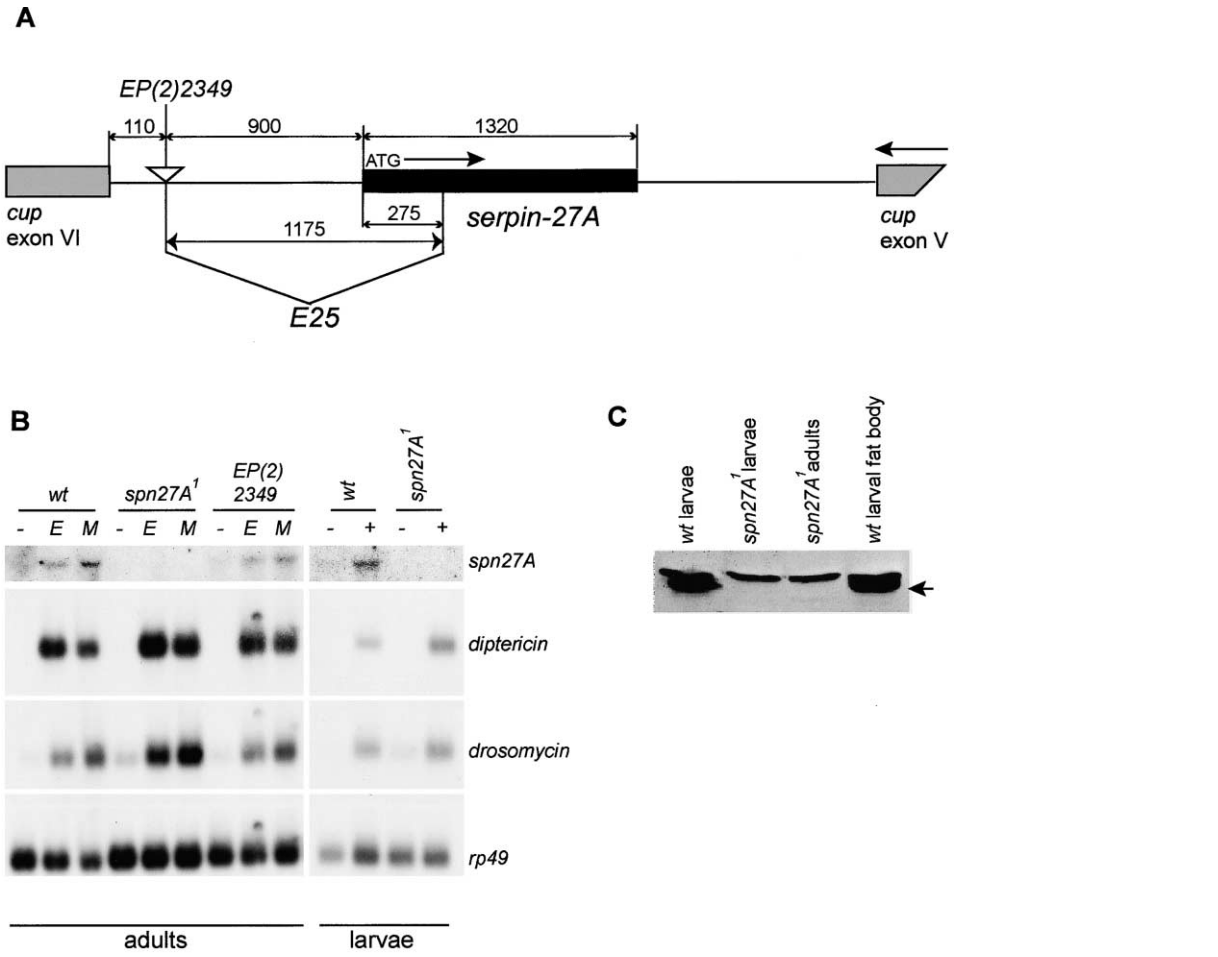

Figure 4. Generation of an *spn27A* Mutant

(A) Schematic representation of the genomic area around *spn27A*. The black box represents the *spn27A* ORF (start codon and direction of transcription are indicated), while the gray boxes represent the exon V and VI of the gene *cup*. The insertion site of the P element *EP(2)2349* and the deletion *E25* are indicated. All distances are expressed in base pairs.

(B) Left panel: 20 μg of total RNA from adult wild-type (wt), *spn27A*^{1}, or *EP(2)2349* flies uninfected (−) or collected 6 hr after infection with *E. coli* (*E*) or *M. luteus* (*M*) were subject to Northern blot analysis. The same filter was hybridized with probes directed against *spn27A*, *diptericin*, *drosomycin*, and *rp49*. Right panel: a similar experiment was performed using 10 μg of total RNA from wild-type (wt) and *spn27A*^{1} larvae uninfected (−) or injected with a mixture of *E. coli* and *M. luteus* (+).

(C) Western blot analysis with anti-Spn27A antibody of wild-type larvae, *spn27A*^{1} larvae, or adults and fat body from wild-type larvae.

adults often had a defect in wing expansion but were still viable (Figure 5A). Only a modest rate of mortality was observed when the mutants were kept at 29°C (Figure 6A). Consistent with an inhibitory function of Spn27A in the melanization cascade, we observed constitutive melanization in the cuticle and wings of most of the *spn27A*^{1} adults (Figure 5A). Also, *spn27A*^{1} larvae sometimes had melanotic tumors (Figure 5B) and melanization in the cuticle and internal organs (data not shown).

Spn27A Controls the Melanization Process in Response to Injury and Infection

Injury to wild-type larvae with a needle induced a melanization, at the wound site, whose extension is usually proportional to the injury size (Figures 5C and 7D). Once the wound had efficiently healed, larvae progressed to the pupal stage and sometimes to the adult stage. Interestingly, in *spn27A*^{1} mutant larvae, integumental injury with a standard needle led to an uncontrolled hemocoelic melanization reaction visible within 2 hr of pricking (Figure 5D). Fifty percent to 70% of *spn27A*^{1} larvae died in the first 5 hr after injury, while less than 10% of wild-type larvae succumbed. In most cases, the melanization reaction diffused throughout the larval body cavity of *spn27A* mutants (Figure 5D), and dead larvae turned completely black (data not shown). It is not clear whether the *spn27A* mutant larvae die because of the toxic effect of excessive melanization or from a defect in wound healing. In agreement with the second hypothesis, a recent study indicates that flies mutated in the melanization cascade exhibit poor ability to recover from an important injury, pointing to a link between melanization and clotting (Rämet et al., 2001). Interestingly, *spn27A*^{1} larvae survived after injury with a thin tungsten needle but exhibited a more intense melanization reaction at the wound site than the wild-type (compare Figures 7F and 7D).

In adults, we observed that melanization at the wound site is more intense in *spn27A*^{1} flies than in the wild-type (Figure 5, compare panels H and G), but the difference is

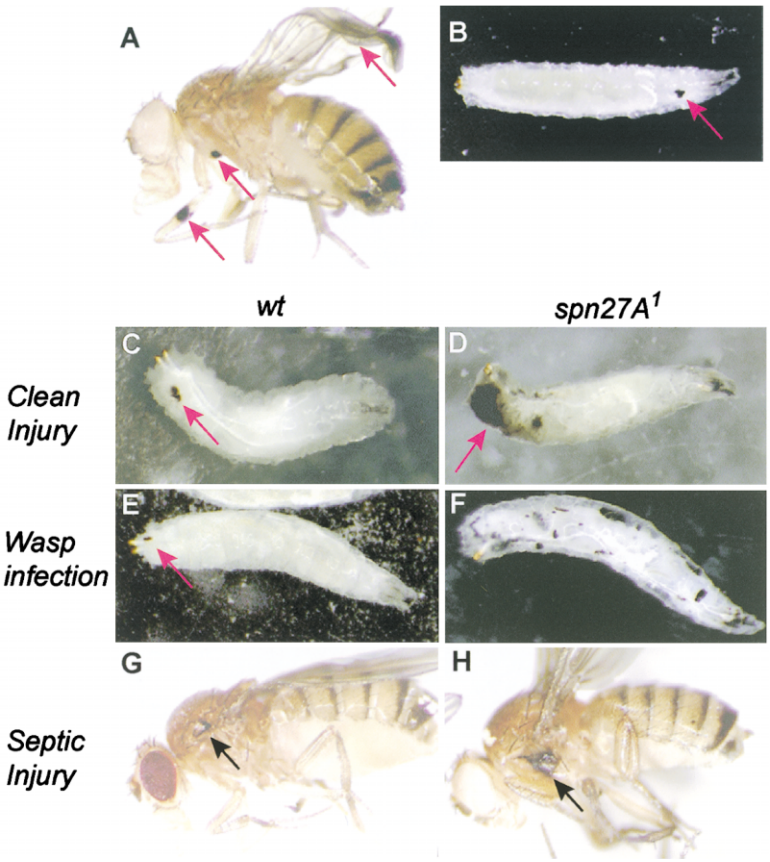

Figure 5. The Phenotype of *spn27A* Deletion Mutant

(A and B) Constitutive melanization (indicated by arrows) in uninfected *spn27A*^{1} adults (A) and larvae (B).

(C–H) Effects of injury and infection on wild-type (wt) and *spn27A*^{1} mutant.

(C and D) Larvae pricked with a clean standard needle. The arrows indicate the injury site.

(E and F) Larvae invaded by the parasitoid wasp, *L. boulardi*. The arrow in Figure 5E indicates the melanotic encapsulation in a wild-type (wt) larva.

(G and H) Adult flies pricked with a needle previously dipped in a concentrated pellet of *Erwinia carotovora*. Arrows indicate melanization at wound site.

less significant than in larvae. Interestingly, the injection of rSpn27A proteins in the thorax of *spn27A*^{1} flies blocked the melanization at the injury site (see Supplemental Figure S1 in Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/4/581/DC1). This confirms our biochemical analysis showing that the function of Spn27A is to limit PPO activation. In the adults, the uncontrolled melanization reaction only had a weak effect on the survival rate of *spn27A*^{1} flies (Figure 6A).

The invasion of *Drosophila* larvae by the parasitoid wasp, *Leptopilina boulardi*, is known to induce a melanization reaction associated with the encapsulation of the wasp's egg (Russo et al., 1996). Generally, in wild-type parasitized larvae, only one melanization spot is observed (Figure 5E). *L. Boulardi*-parasitized *spn27A*^{1} larvae induced a strong systemic hemocoelic melanization reaction, but it was not specifically associated to the encapsulation process (Figure 5F).

Our genetic analysis suggests that the role of Spn27A is to restrict the melanization reaction to the site of injury or encapsulation. These data taken together with the biochemical analysis further suggest that Spn27A inhibits *Drosophila* PPAE, thus limiting PPO activation in response to injury and parasitoid invasion.

Serpin 27A Does Not Regulate the Expression of AMP Genes but Is Required to Resist Infection by *B. bassiana*

We hypothesized that, in addition to its role in the control of melanization, Spn27A could act similarly to Spn43Ac (Levashina et al., 1999) as a negative regulator of the AMP response. However, we found that the *spn27A*^{1} mutation had no significant effect on the expression of *drosomycin* and *diptericin* in both unchallenged and bacteria-infected larvae and adults (Figure 4B). Similar results were obtained with *drosomycin-GFP* and *diptericin-LacZ* reporter genes, allowing the analysis of local AMP expression (data not shown). Because the expression of *drosomycin* and *diptericin* is controlled by the Toll and Imd pathways, respectively, we concluded that Spn27A is not a direct regulator of either of these two cascades.

We wondered whether the uncontrolled melanization reaction displayed by *spn27A*^{1} homozygous flies affects the survival rate after different types of microbial infections. We found that *spn27A*^{1} adults resisted injection of gram-negative bacteria (*Escherichia coli*), gram-positive bacteria (*Micrococcus luteus*), and fungi (*Aspergillus fumigatus*) (the survival rate 6 days after infection is the same as that observed after clean injury) but were more susceptible than the wild-type to *B. bassiana* natural infection (Figure 6A). However, the graph in Figure 6B shows that *spn27A*^{1} flies resist better than *spaetzle*-deficient flies (*spz*) to *B. bassiana* infection, in agreement with the finding that *spn27A*^{1} mutation did not affect the Toll pathway. Interestingly it has been reported that natural fungal infections are often associated with intense melanization reactions (Braun et al., 1998; Gotz and Vey, 1986). Therefore, we propose that the lethality observed in the *spn27A*^{1} strain after *B. bassiana* natural infection is linked to an uncontrolled melanization reaction.

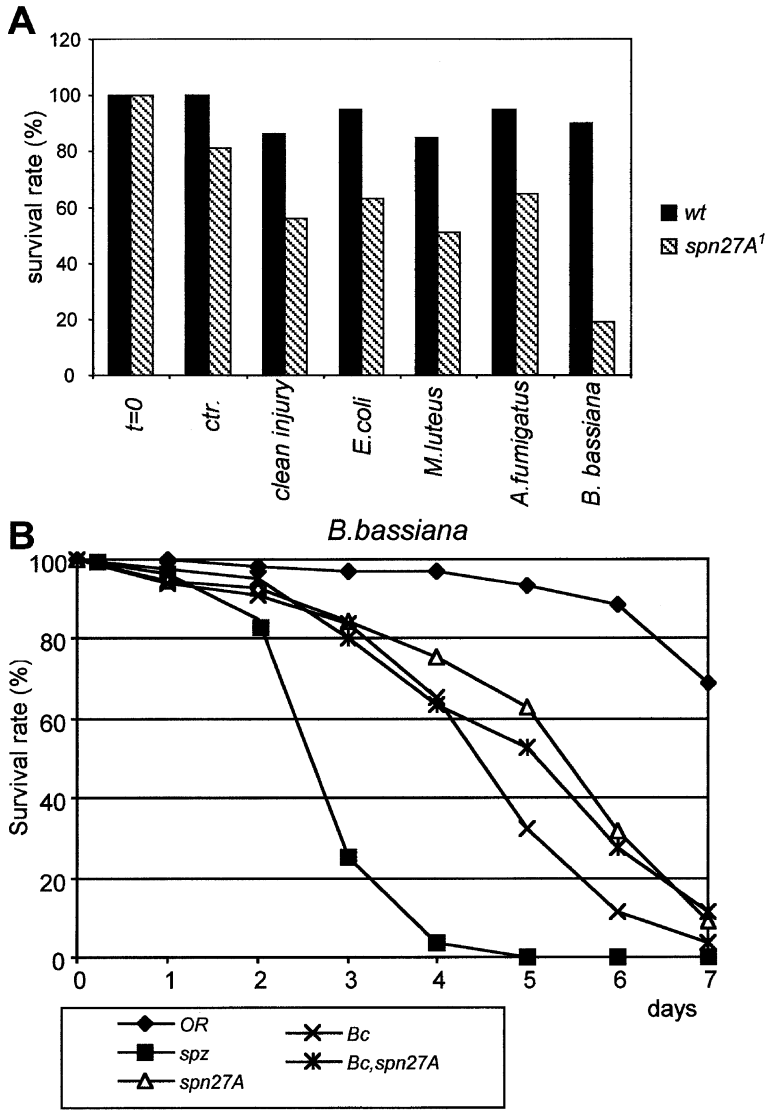

Figure 6. Survival Rate of *spn27A*^{1} Mutants after Different Types of Infection

(A) Wild-type and *spn27A*^{1} adult flies were subjected to clean injury, septic injury using *E. coli*, *M. Luteus*, or *A. fumigatus*, and natural infection by *B. bassiana*. ctr, noninjured flies. The graph shows the survival rate 6 days after infection.

(B) Survival rate of wild-type (wt), *spz*, *spn27A*^{1}, *Bc*, and *Bc,spn27A*^{1} adults 1–8 days after *B. bassiana* infection. The original P line *EP(2)2349* showed a survival rate identical to the wild-type Oregon strain (data not shown).

The *Black cells* Mutation Suppresses the *spn27A* Phenotype

A mutation in the *Drosophila* uncharacterized gene, *Black cells* (*Bc*), affects crystal cells and blocks the melanization reaction in the hemolymph (Rizki et al., 1980). To confirm that the zygotic phenotype of *spn27A* is due to a misregulation of the melanization cascade, we generated *Bc,spn27A*^{1} homozygous double mutant. Interestingly we observed that all the phenotypes induced by the *spn27A* mutation except for female sterility were suppressed in a *Bc* mutant background. *Bc,spn27A*^{1} double mutants, like *Bc*, show a better viability at pupal stage compared with *spn27A*^{1} single mutants (Figure 7A). Adults have normal wings (Figures 7B and 7C). Interestingly, mutation in *spn27A* did not enhance the weak melanization reaction observed in *Bc* homozygous larvae after injury (Figure 7, compare panels G and E).

We observed that *Bc* adult flies, like *spn27A*^{1} mutants, were more susceptible than wild-type to infection by *B. Bassiana*, confirming a role of the melanization reaction to resist this fungus (Figure 6B). *Bc,spn27A*^{1} double mutants showed the same survival curve as *Bc* flies. The absence of additive effect in the double mutant suggests that Spn27A contributes to fungal resistance through the control of the melanization cascade.

Discussion

A surprising feature of the *Drosophila* genome is the high number of trypsin-like serine protease (199) and serpin (30) genes (Rubin et al., 2000). It has been speculated that the existence of so many serine proteases in *Drosophila* might reflect the presence of a primitive, yet sophisticated, immune system. Insects, although lacking an acquired immune system, may rely on concerted networks of serine protease cascades to orchestrate a highly specific and efficient defense system capable of self-/nonself-recognition, wound healing, microbial killing, and parasite/pathogen sequestration. We previously identified five serpin-encoding genes that are

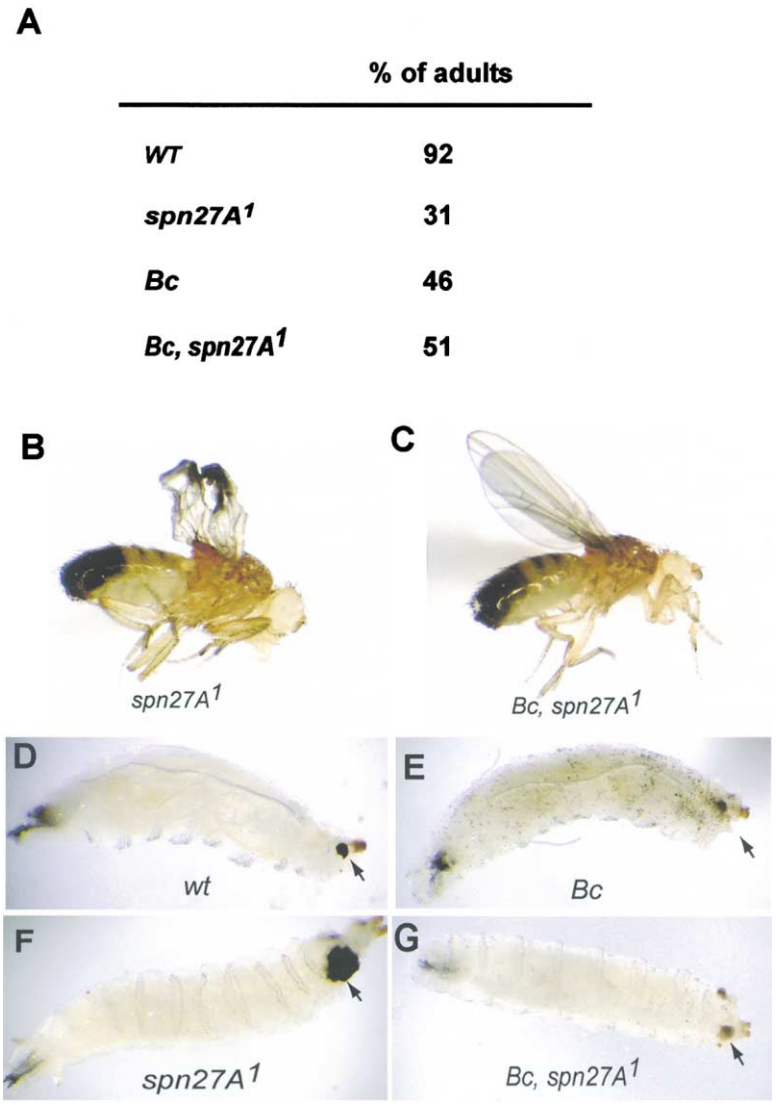

Figure 7. *Bc* Suppresses the *spn27A* Phenotype

(A) *spn27A* induces mortality at the pupal stage, which is reduced in the *Bc* background. One hundred wild-type and mutant third instar larvae were selected, and the numbers of emerging adults were counted (indicated as percentage).

(B and C) The *spn27A*^{1} wing phenotype is suppressed in *Bc* mutant.

(D–G) These panels show the effect of injury by a thin tungsten needle in wild-type, *spn27A*^{1}, *Bc*, and *Bc,spn27A*^{1}. Excessive melanization is observed in *spn27A* mutant, while *Bc* and *Bc,spn27A*^{1} larvae show reduced melanization. Arrows indicate melanization at wound site. *Bc* larvae show melanized crystal cells (E and G).

upregulated after septic injury, suggesting that they function in the immune response (De Gregorio et al., 2001). One of them, *spn27A*, shows an acute phase transcriptional profile and is regulated by the Toll pathway (De Gregorio et al., 2002, and this study). Using a biochemical and genetic approach, we now demonstrate a key role of Spn27A in the regulation of the melanization reaction in *Drosophila*.

Spn27A Controls the Activity of PPAE

Expression of *spn27A* in *Drosophila* Schneider cells results in the accumulation of an active, correctly processed Spn27A protein in the supernatant. This finding demonstrates that Spn27A is a secreted protein and allowed us to map the signal peptide. In addition, Western analysis showed that *spn27A* is expressed in the fat body (Figure 4C). Therefore, we believe that Spn27A, like the AMPs, is synthesized in the fat body tissue and secreted in the hemolymph. Biochemical assays show that rSpn27A inhibits the activation of PPO in different insect species including *Drosophila*, pointing to a remarkable conservation of the melanization mechanisms. This finding is consistent with the sequence homology of PPO genes from the same species and allowed us to investigate the role of Spn27A in heterologous systems for which the melanization cascade is better characterized than in *Drosophila*. The similarities between the PPO cleavage site and the RCL of Spn27A prompted us to test whether PPAE was the target protease of Spn27A. We showed that rSpn27A, but not a recombinant variant mutated in the RCL, inhibits the PPAE purified from *H. diomphalia*. In agreement with the current models of serpin-serine protease interaction, the inhibition occurs through the formation of a specific covalent link between the two proteins (Figure 3B). These findings strongly suggest that, in *Drosophila*, the role of Spn27A is to modulate PPO processing through the inhibition of a still-unidentified PPAE. The role of Spn27A in melanization is supported by our genetic analysis. *spn27A*^{1} mutant flies frequently exhibit melanotic tumors and

darkening of the cuticle, which may be the consequence of natural injury during the fly lifecycle. The most dramatic phenotype of *spn27A*^{1} is, however, the excessive melanization reaction observed after injury or wasp infection (Figure 5). These observations indicate that the role of Spn27A is to limit the PPO cascade at the site of injury to prevent systemic melanization. The acute phase expression profile of *spn27A* in response to septic injury is probably critical for a tight spatial and temporal regulation of PPO activity.

The observation that some of the *spn27A*-deficient mutants do not present any form of constitutive melanization indicates the existence of multiple levels of regulation of the melanization reaction. An Spn27A-independant mechanism may limit the activation of PPO in the absence of stimulus. Although this has not been firmly demonstrated in *Drosophila*, this step is probably regulated by the availability of PPO in the hemolymph. At least in *Drosophila,* hemolymphatic PPO is localized in a hemocyte cell type called crystal cell (Rizki and Rizki, 1984; Rizki et al., 1980), which belongs to the oenocytoid linage of hemocytes, known for their capacity to synthesize PPO. Crystal cells can be freely circulating in the hemolymph or sessile. Sessile crystal cells have been shown to be grouped in densely packed clusters along with plasmatocyte type hemocytes, in direct contact with the cuticular epithelial layer (Lanot et al., 2000). In response to different stimuli, like integumental injury or microbial invasion, crystal cells rupture and release their PPO content into the hemolymph plasma, especially around the area where stimulation occurs. The PPO may then be subjected to cleavage by PPAE in a process tightly regulated by Spn27A.

Melanization Reaction and the *Drosophila* Immune Response

The melanization reaction is considered as an important facet of the insect host defense. Melanotic encapsulation can prevent completion of the malaria parasite lifecycle in *Anopheles gambiae* (Collins et al., 1986), while, in *Drosophila*, it plays an important role against infection by parasitoid wasps (Vass and Nappi, 2000). However, little is known about the exact role of melanization during *Drosophila* immune response. The *Bc* mutation affects crystal cells and blocks the melanization reaction in the hemolymph but does not alter the inducibility of AMP genes (Lemaitre et al., 1995; Rizki et al., 1980). Interestingly, this mutation induces a higher susceptibility to microbial infection when combined with mutations affecting the Toll and Imd pathways (Braun et al., 1998; Lemaitre et al., 1995). Our experiment, showing that both *Bc* and *spn27A*^{1} mutants are more susceptible to infection by the entomopathogenic fungus *B. bassiana* (Figure 6B), indicates that a tight control of melanization is required for a proper antifungal response. The analysis of *Bc* and *spn27A*^{1} mutants clearly shows that the terminal components of the melanization reaction (PPO, PPAE, and Spn27A) are not involved in the regulation of the antimicrobial peptide response. Hence, the PPO cascade and AMPs provide two uncoupled defense systems that cooperate to fight microbial infection.

Pleiotropic and Distinct Phenotypes of Serpins

In mammals, a variety of proteolytic cascades, including blood coagulation, fibrinolysis, inflammation, and complement activation are regulated by serpins. In addition, serpins are also involved in various kinds of physiological processes, such as angiogenesis, apoptosis, neoplasia, and viral pathogenesis (Silverman et al., 2001). Several serpin genes have been inactivated by targeted mutation in the mouse. Some of these mutations have failed to reveal an overt phenotype, whereas others show various physiological and developmental alterations (Silverman et al., 2001). Spn27A and Spn43Ac are two serpins characterized by genetic studies in *Drosophila* (Green et al., 2000; Levashina et al., 1999; this study). Interestingly the phenotype induced by the deletion of these serpins shows similarities and differences. Both *spn27A*^{1} and *necrotic* mutations result in a similar pleiotropic phenotype: female sterility, low viability at the pupal stage, and ectopic melanization in unchallenged animals. In addition, these two genes are both induced upon infection and are mainly regulated by the Toll pathway. Mutation in *spn43Ac*, however, induces a constitutive activation of the Toll pathway (Levashina et al., 1999), whereas mutation in *spn27A* causes an uncontrolled melanization reaction. Our biochemical study clearly indicates a difference in target specificity between these two serpins: recombinant Spn43Ac was unable to inhibit the PPO cascade. It would be interesting to know whether the phenotype of each serpin mutant is mediated by more than one target protease. The observation that *Bc* is epistatic to *spn27A* indicates that the pleiotropic phenotype of *spn27A*^{1} mutation can be explained by the multiple functions of melanization reaction in *Drosophila*. The role of Spn27A in early embryogenesis, which is not *Bc* dependent, remains to be investigated.

Since the *Drosophila* genome encodes five serine proteases for every serpin, it is very likely that serpins can inhibit multiple target proteases (Rubin et al., 2000). Our previous microarray analysis has shown that about 19 serine proteases are upregulated upon infection in *Drosophila* (De Gregorio et al., 2001). Among them, new targets of Spn27A and Spn43Ac activities might be identified.

Concluding Remarks

In this study we combined postgenomic, genetic, and biochemical approaches to identify a regulator of the PPO pathway in *Drosophila*. This finding opens the way for the molecular characterization of the insect melanization cascade that plays a central role in host defense. The dramatic phenotype of *spn27A*^{1} mutant flies underlines the critical role of serpin molecules in the tight regulation of proteolytic cascades activating host defense reactions. Furthermore, the high number of serine protease and serpin genes encoded in the fruit fly genome suggests that *Drosophila* is a remarkable model to understand the role of proteolytic cascades in the integrated host defense.

Experimental Procedures

Insect Stocks and Cells

G. mellonella was reared on beeswax and pollen at 30°C. *B. mori* was reared on the artificial diet Silkmate 2M (Kyodo Shiryo, Tokyo)

at 24°C under a 12 hr photoperiod. *Drosophila* Schneider cell line was grown at 25°C in Schneider medium (Life Technologies) supplemented with 10 % fetal bovine serum (Life Technologies) heat inactivated at 56°C for 30 min.

Drosophila Stocks

D. melanogaster strains were reared on a standard cornmeal-yeast medium at 25°C. Oregon R was used as wild-type *Drosophila* stock. *Bc* and spz^{rm7} stocks are described elsewhere (Rizki et al., 1980; De Gregorio et al., 2002). The deletion of *spn27A* was generated by P element mobilization of the *EP(2)2349* insertion by standard protocols. Experiments were done on homozygous *spz*, *Bc*, or $spn27A^{1}$ mutants. To characterize the $spn27A^{1}$ mutation, we subjected the genomic DNA from $spn27A^{1}$ homozygous adult flies to PCR using several oligonucleotides, and the PCR products obtained were sequenced. The presence of a *CyO actin-GFP* balancer in the $spn27A^{1}$ mutant strain allows the rapid identification of homozygous larvae (Reichhart and Ferrandon, 1998).

RT-PCR

Total RNA was extracted from *D. melanogaster* at different developmental stages and from the *Drosophila* Schneider cell line with Trizol reagent (SIGMA) according to manufacturer's instructions. RT-PCR was performed as described before (Kim et al., 2000) with the following primers: for *spn27A*, sense (5′-AAGAGCGCTCAGTGGGCCA-3′) and antisense (5′-CGTTCCCTTCTCGTTCACAT-3′); for β-*actin*, sense (5′-GATCACCATTGGCAACGA-3′) and antisense (5′-TCTTGATCT TGATGGTCG-3′).

Expression and Purification of Recombinant Proteins in *Drosophila* Schneider Cell Line

The open reading frame corresponding to the full length of Spn27A or Spn43Ac, which have hexahistidine tags in the C-terminal end, were subcloned into pMT/V5 vector (pMT/V5-Spn27A or pMT/V5-Spn43Ac) under control of the metallothionein promoter (Invitrogen). The mutant Spn27A derivatives, in which the lysine residue in the P1 site is substituted with arginine ($Spn27A^{K406R}$) or alanine ($Spn27A^{K406A}$), were generated by site-directed mutagenesis, with pMT/V5-Spn27A as template. The primers for point mutation were as follow: 5′-ACCGAACCTGTTTTCGATC-3′ for $Spn27A^{K406R}$ and 5′-ACCGAACG CGTTTTCGATC-3′ for $Spn27A^{K406A}$. Stable cell lines expressing rSpn27A, $rSpn27A^{K406R}$, $rSpn27A^{K406A}$, or rSpn43Ac were generated by transfection of each pMT/V5 clone into *Drosophila* Schneider cells. Then the recombinant proteins were purified from the supernatant with Ni-NTA resin according to the manufacturer's instructions (QIAGEN). Purified recombinant proteins were concentrated and buffer exchanged with buffer A (100 mM Tris [pH 7.2]) wtih a centrifugal filter device (Millipore) and then kept in the refrigerator or freezer (−80°C) until being used.

PO Activity Assay

About 20 mg of *D. melanogaster* pupae was ground in liquid nitrogen, immediately resuspended with 100 μl of buffer A only for control test or for the same volume of each recombinant protein (indicated concentration in buffer A), and centrifuged, and the supernatant was transferred to new tube. After incubation at room temperature for the indicated time (when the time is not indicated, a 30 min incubation period was employed), 10 μl aliquot was subjected to PO assay. Larvae of *B. mori* (fifth instar) or *G. mellonella* (final instar) were bled by cutting their posterior legs with fine scissors, and, immediately, 10 μl for *B. mori* or 5 μl for *G. mellonella* of hemolymph was mixed with the same volume of buffer A or recombinant Spn27A (6 μM in buffer A). After incubation at room temperature for 30 min, the total reaction mixture was subjected to PO assay. PO assay was performed according to the method of Pye, with slight modification (Pye, 1974). Briefly, each testing solution was added to a PO assay mixture: 1 ml of 100 mM phosphate buffer (pH 6.0), 200 μl of 50 mM 4-hydroxyproline ethyl ester, and 50 μl of 100 mM 4-methylcatechol as substrate, and, then, after 5 min of incubation at 30°C, the reaction was terminated by the addition of 20 μl of 1 M thiourea. Phenoloxidase activity was estimated by measuring the absorbency at 520 nm.

HdPPAE Activity Assayes

Amidase Activity Test

The same molar concentration of HdPPAE and rSpn27A derivatives were mixed in buffer B (20 mM Tris and 5 mM CaCl2 [pH 8.0]). After 30 min of incubation at 37°C, 10 μl of the reaction mixture was added to 990 μl of prewarmed buffer B containing chromogenic substrate (100 μM of boc-Phe-Ser-Arg-MCA). Further incubation was performed at 37°C for 10 min, and the reaction was terminated by adding 20 μl of 10% acetic acid. The intensity of fluorescence was measured with excitation at 380 nm and emission at 440 nm.

Gel Mobility Shift Assay

The same molar concentration of HdPPAE and rSpn27A was mixed in buffer B. After 30 min of incubation at 37°C, 10 μl of the reaction mixture was subjected to SDS-PAGE in nonreducing condition. Western blot analysis was performed using anti-HdPPAE-specific antibody.

In Vitro Reconstituted PPO Activation Test

A total of 0.5 μg of active purified HdPPAE with cofactors and 1 μg of HdPPO (Kwon et al., 2000) were mixed with buffer B only or with 2 μg of each Spn27A derivative. After 10 min of incubation at room temperature, PO activity was measured as described above. Reaction mixtures were also subjected to SDS-PAGE in nonreducing condition and then to Western blot analysis using anti-HdPPO-specific antibody.

Microbial or Parasitic Infection Tests

Septic injury of adult flies was performed by pricking the thorax with a standard sharp steel needle (0.2 mm diameter) previously dipped into a concentrated culture of *E. coli*, *M. Luteus*, or *E. carotovora* or in a suspension of *A. fumigatus* spores. Natural infection was initiated by shaking anesthetized flies in a petri dish containing a sporulating culture of the entomopathogenic fungus *B. bassiana* (strain 802). Larvae were infected by pricking the posterior side with a standard needle. For septic injury, the needle was previously dipped in a mixed culture of *E. coli* and *M. luteus*. To obtain a very thin needle employed in the experiment described in Figures 7D–7G, we use 0.1 mm diameter tungsten wire that has been sharpened in 0.1N NaOH solution by electrolysis. For survival experiments 60 adult flies were infected and incubated at 29°C. *L. boulardi* G486 (Eucoilidae) were reared at 25°C on second instar larvae of the Oregon R strain (Russo et al., 1996). More information on infection procedures can be found in Tzou et al. (2002b). For Northern analysis larvae or adult flies were incubated at 25°C after infection. The Northern blot was performed as previously described (Tzou et al., 2002b).

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