Activation of *Drosophila* Toll During Fungal Infection by a Blood Serine Protease

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Drosophila host defense to fungal and Gram-positive bacterial infection is mediated by the *Spaetzle/Toll/cactus* gene cassette. It has been proposed that Toll does not function as a pattern recognition receptor per se but is activated through a cleaved form of the cytokine Spaetzle. The upstream events linking infection to the cleavage of Spaetzle have long remained elusive. Here we report the identification of a central component of the fungal activation of Toll. We show that ethylmethane sulfonate–induced mutations in the *persephone* gene, which encodes a previously unknown serine protease, block induction of the Toll pathway by fungi and resistance to this type of infection.

The *Drosophila* host defense is a multifaceted process, which involves the challenge-dependent synthesis of potent antimicrobial peptides by the fat body, a functional equivalent of the mammalian liver (*1*). Two intracellular signaling pathways mediate the synthesis of these peptides: the Toll and the Imd pathway (*2*, *3*). Toll is activated during fungal and Gram-positive bacterial defenses, and the Imd pathway is predominantly activated by Gram-negative bacterial infections (*4*). *Toll* was initially identified as a gene that controls the establishment of dorsoventral polarity in early embryogenesis (*5*). A proteolytically cleaved form of the cysteine-knot growth factor Spaetzle (Spz) functions as the extracellular ligand of Toll in both embryonic development and the immune response (*2*, *6*). During embryonic patterning, Spz cleavage is achieved by the sequential activation of three serine proteases required in the germ line: Gastrulation defective, Snake, and Easter (*7*). However, in null mutants of these proteases, challenged-induced activation of the Toll pathway is not affected, as illustrated by wild-type induction of the antifungal peptide *drosomycin* (*2*, *8*). The implication is that infection may activate some other protease(s) that cleaves Spz to its active form. Immune-induced cleavage of Spz by blood serine proteases is conceptually similar to blood coagulation or complement activation, where inappropriate activation is prevented by the action of serine protease inhibitors (serpins) (*9*). We have recently reported that flies mutant for the blood serpin Necrotic (Nec) exhibit a constitutively activated antifungal host defense and that Spz is constitutively cleaved in this mutant background (*8*). The *nec* pleiotropic phenotypes include spontaneous melanization, cellular necrosis, and death in early adulthood (*10*). These changes probably all reflect a role of the *nec* serpin gene in controlling activation of one or more proteolytic cascades.

To identify components of the antifungal response cascade that activate Spz, we screened the first chromosome for ethylmethane sulfonate–induced suppressors of the *nec* phenotypes (*11*). From 9700 mutagenized male flies transheterozygous for *nec*, we isolated five suppressors that belong to the same complementation group. We named this suppressor mutation *persephone* (*psh*). Mutations in *psh* suppressed all *nec* phenotypes. In particular, *psh;nec* double mutants displayed a life-span comparable to that of wild-type (WT) flies (Fig. 1A), showed no spontaneous melanization, and did not express *drosomycin* in a constitutive manner (Fig. 1B). When challenged with fungi, *psh* mutants exhibited a severely reduced level of *drosomycin* transcription as compared with WT flies (Fig. 1C). Induction of *drosomycin* by Gram-positive bacteria was at WT levels in *psh* mutants (Fig. 1C). Finally, expression of *diptericin*, which is controlled by the Imd pathway, was not affected following Gram-negative bacterial infection (Fig. 1C).

We further noted that *psh* flies were highly susceptible to fungal infections, behaving in this respect as Toll pathway mutants (Fig. 2A). Conversely, *psh* flies showed a WT pattern of survival after immune challenge by Gram-positive bacteria (Fig. 2B). As expected, *psh* flies were resistant to Gram-negative bacterial infection, which activates the Imd pathway (*12*).

For epistasis studies, we used fly lines overexpressing a cleaved form of Spz (*11*) through the UAS/GAL4 system (*13*). These flies exhibit a challenge-independent expression of *drosomycin* (Fig. 3A). The levels of *drosomycin* transcription after overexpression of *spz* were notably similar in *psh* mutants and in WT flies (Fig.

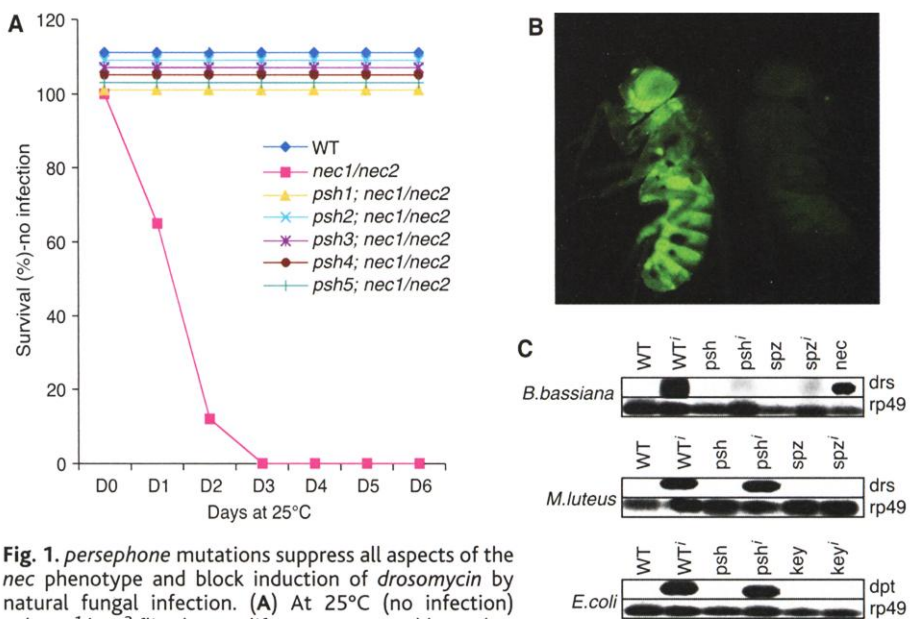

Fig. 1. *persephone* mutations suppress all aspects of the *nec* phenotype and block induction of *drosomycin* by natural fungal infection. (**A**) At 25°C (no infection) *psh;nec*^{1}/*nec*^{2} flies have a life-span comparable to that of WT flies, whereas 80% of the *nec*^{1}/*nec*^{2} flies die within 48 hours. In this graph, only 1 week of the flies' life is presented; *psh;nec*^{1}/*nec*^{2} flies lived as long as did the WT flies (*27*). (**B**) *psh;nec*^{1},*drs-GFP*/*nec*^{2} flies (right) do not express *drosomycin* in a constitutive manner and do not exhibit melanization spots on their bodies, which are two characteristics of the *nec* phenotype (*XX:=;nec*^{1},*drs-GFP*/*nec*^{2} flies, left). (**C**) Expression of antimicrobial peptides in different mutant backgrounds after infection (i) by fungi (*Beauveria bassiana*), Gram-positive bacteria (*Micrococcus luteus*), or Gram-negative bacteria (*Escherichia coli*). Northern blots were performed with total RNA from wild-type (WT), *psh*^{1}, *spz*^{rm7}, *nec*^{1}/*nec*^{2}, and *key*^{1} flies. *rp49* was used as an RNA loading control. After infection, the flies were incubated for 48 hours in the case of *drosomycin* and 6 hours in the case of *diptericin* before RNA preparation. *drs*, *drosomycin*; *dpt*, *diptericin*.

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3A). This result indicates that the *psh* mutation inactivates a gene upstream of *spz*.

With a deficiency kit spanning the first chromosome (*14*), we mapped the *psh* mutation to the chromosomal region 17A-17B (Fig. 3, B and C). This region contains two serine protease genes, *CG6361* and *CG6367*. We compared the genomic sequence of the *CG6367* protease in psh^5 with that of WT flies and noted the transition of a G nucleotide to an A in the sequence encoding the conserved "serine signature" sequence (Gly-Asp-Ser-Gly-Gly-Pro), which results in a Gly to Glu change at position 340 (Fig. 4A). In the sequence of psh^1 and psh^4, a transition of a C to a T was observed, which is expected to result in the change of the His of the catalytic triad to a Tyr at position 187 (Fig. 4A).

To ensure that these mutations were responsible for the observed phenotypes in *psh* mutants, we undertook rescue experiments using the UAS/GAL4 system (*13*) with the female fat body–specific *yolkGAL4* as a driver (*15*). We noted that *UAS-CG6367/yolkGAL4* flies constitutively express *drosomycin* (Fig. 4B). Furthermore, we observed that overexpression of the *CG6367* protease restores the ability of *psh* flies to respond to fungal infection (Fig. 4C). Neither an activated form of Easter (*16*) (Fig. 4C) nor the *CG6361* protease (*17*) contained in the deficiency that uncovers the *psh* locus were able to rescue the observed sensitivity to fungi. These results indicate that *CG6367* is the serine protease responsible for the *psh* phenotype.

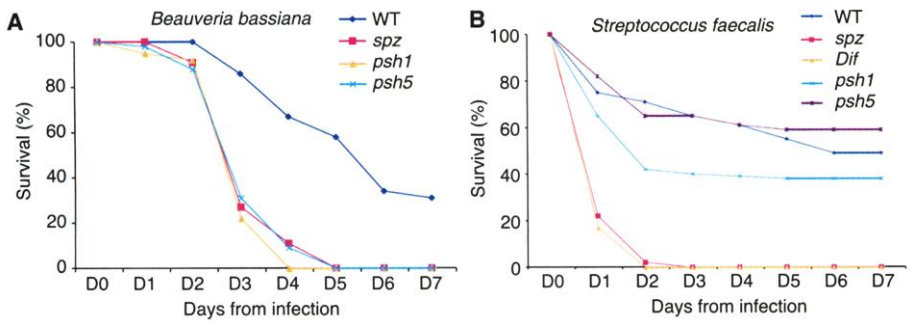

Fig. 2. *psh* mutant flies are highly susceptible to natural fungal infection. The survival rates (%) of wild-type (WT), spz^{rm7}, dif^2, and *psh* flies infected with different microorganisms are presented (see supporting online material). (**A**) Fungal infection with *B. bassiana*. (**B**) Gram-positive bacterial infection: *Streptococcus faecalis*. We observed a difference in the survival rates between psh^1 and psh^5. Because the pattern of survival for both alleles follows the WT pattern and differs significantly from that of Toll pathway mutant flies, we believe that this difference is not significant.

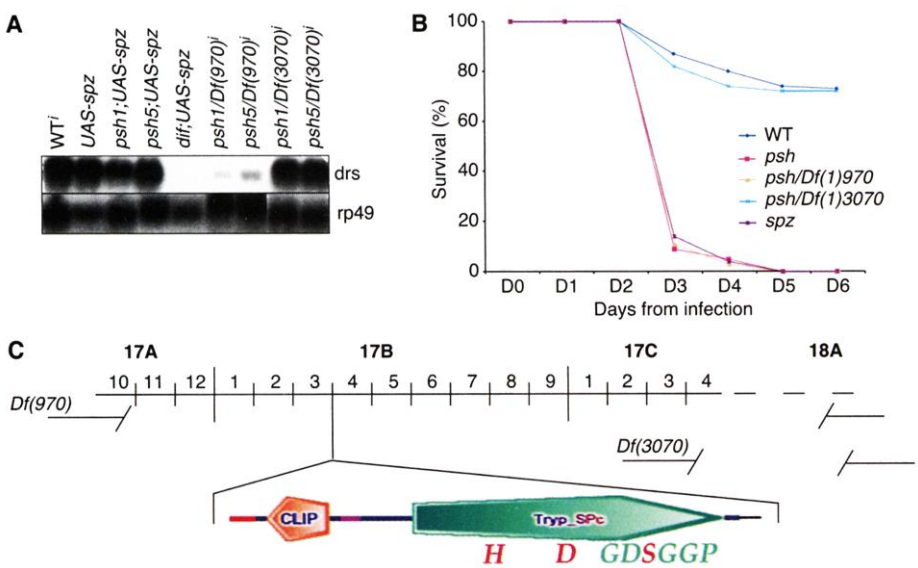

Fig. 3. Genetic mapping of the *persephone* mutation was performed with a deficiency kit spanning the first chromosome (*14*). The assay of our mapping was twofold. First, we determined a *psh*/deficiency combination that suppresses *nec*. The combination *psh/Df(1)970* mapped the mutation to the region 17A-18A. An overlapping deficiency [*Df(1)3070*], which deleted the region 17C-18A but did not suppress *nec*, narrowed the interval to 17A-17B. Second, to verify our results, we used these combinations to test for survival (**B**) and *drosomycin* expression (**A**) following natural fungal infection. Results confirmed that the mutation lies in the region 17A-17B. (A) The *psh* mutation inactivates a protein upstream of *spz* because overexpression of activated *spz* leads to induction of *drosomycin* in a *psh* genetic background. Conversely, overexpression of activated *spz* in a genetic background of a known downstream component such as *dif* does not result in infection-independent expression of *drosomycin* (*yolkGal4* was used as a GAL4 driver). We observed that flies homozygous for *psh* and *psh/Df(1)970* flies exhibit identical phenotypes, indicating that the alleles that we used for mapping (psh^1 and psh^5) are either strong hypomorphs or complete nulls. (**C**) Genomic region of the *psh* locus. The gene is located in 17B4 and has seven exons that translate in a protein of 393 amino acids. Its protein sequence contains all the characteristics of a serine protease of the S1 family of trypsin (clan SA) (*28*), with a putative signal peptide (red bar) between amino acids 1 and 20, a CLIP domain (amino acids 30 to 79), and a trypsin-chymotrypsin (Tryp-SPc) catalytic domain (amino acids 143 to 384). The catalytic triad consists of His^{187}-Asp^{234}-Ser^{338} (all three depicted in red).

The deduced sequence of the PSH protein indicates the presence of a putative signal peptide (amino acids 1 to 20), suggesting that the protein is secreted and present in the hemolymph, as has been shown for Nec (*8*). Transfer of hemolymph (table S1) from *nec* flies to WT flies carrying a *drosomycin-GFP* (*drs-GFP*) reporter resulted in expression of *drs-GFP*. In contrast, transfer of hemolymph from *psh;nec* flies did not induce *drosomycin* expression. This indicates that the blood-borne factor responsible for Toll activation observed in *nec* hemolymph transfer is suppressed in *psh;nec* flies. Finally, transfer of hemolymph from flies overexpressing WT *psh* (*UAS-CG6367/daughterlessGAL4*) to *drs-GFP* flies induced challenge-independent expression of *drosomycin*. These results confirm the presence of immune-responsive components of the system in the hemolymph.

The blood serine protease Persephone is the first identified component of the cascade, which was hypothesized to activate Toll following an immune challenge. Serine proteases are initially synthesized as inactive zymogens containing an NH_2-terminal prodomain and a COOH-terminal catalytic domain. Activation requires proteolytic cleavage of the zymogen at a defined site by a specific activating protease or a nonenzymatic ligand (*18*). The sequence of PSH also predicts an NH_2-terminal prodomain (see Fig. 3A). This prodomain contains a CLIP module most homologous to those in Easter, Snake, the horseshoe crab proclotting factor, and the *Bombyx mori* prophenoloxidase-activating enzyme. Thus, common organizing principles may direct hemolymph clotting, immune response, and developmental serine protease cascades in arthropods (*18–21*). *psh* is the first described mutation to specifically impair Toll-dependent induction of *drosomycin* by fungal infection in *Drosophila* without affecting Gram-positive bacterial induced responses. Mutations have been reported recently that affect activation of the Toll pathway by Gram-positive bacteria (*22*) and activation of the Imd pathway by Gram-negative bacteria (*23–25*). The mutated genes encode members of the family of soluble or membrane proteins referred to as peptidoglycan recognition proteins (PGRPs), in refer-

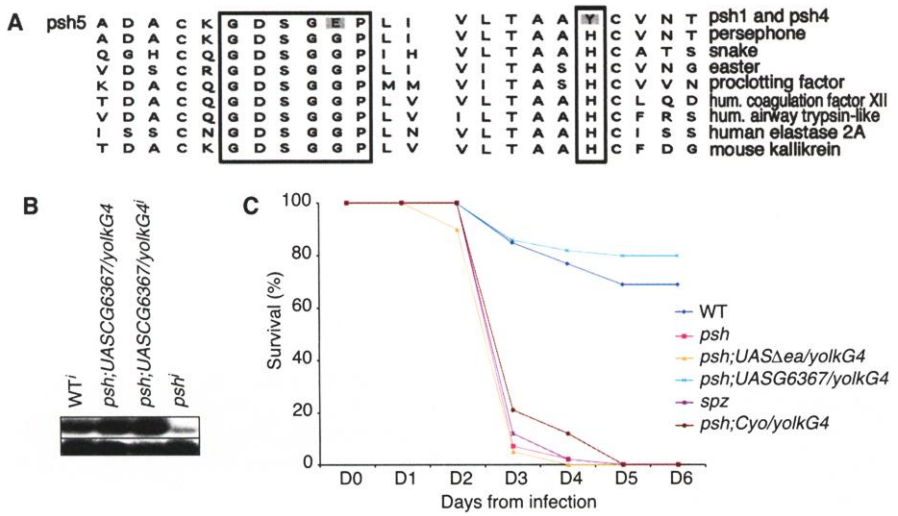

Fig. 4. *persephone* is a mutant for the *CG6367* gene. (**A**) Alignments of the regions of the serine signature (DGSGGP-boxed) and the conserved histidine (H-boxed) of the catalytic triad, two absolutely conserved features in all serine proteases of the trypsin-chymotrypsin family. Sequences used for comparison were from *Drosophila*, horseshoe crab (proclotting factor), mouse, and human. In each case, the top protein sequence corresponds to a *psh* mutant. Mutations are highlighted in gray. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) *psh;UAS-CG6367/yolkGAL4* flies constitutively expressed *drosomycin*. Infection (i) can further induce expression of *drosomycin*. (**C**) Overexpression of one WT copy of *CG6367* cDNA is sufficient to rescue the *psh* phenotype for susceptibility to fungal infections. Use of an activated form of *easter* (*16*) does not result in rescue of this phenotype. As expected, neither the GAL4 driver alone nor males that carried both the driver and the transgene but do not express *yolk* (*15*) showed rescue of the phenotype (*27*).

ence to their initial discovery as Gram-positive interacting proteins (*26*). Toll activation by Gram-positive bacteria is mediated by a soluble PGRP, whereas that of Imd by Gram-negative infection involves a putative membrane PGRP. Taken together, the results on the *psh* mutation and those on mutations in the soluble PGRP-SA and the putative membrane PGRP-LC now define three distinct upstream pathways mediating response to fungal infections and to infections by Gram-positive or Gram-negative bacteria. These data set the stage for a detailed analysis of the events leading from recognition of infection to activation of intracellular signaling pathways and consequent transcription of appropriate groups of genes concurring to fight the respective infections. Whereas PGRPs can be considered as bona fide pattern recognition receptors, *psh* has no microbial pattern recognition–binding domain. We anticipate that an as-yet unidentified, upstream fungal pattern recognition receptor functions to activate the protease function of *psh*.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/297/5578/114/DC1

Materials and Methods

Table S1

References

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