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10. In the delayed-saccade task, the monkey was first required to fixate a central fixation spot within a <1° error window. Immediately after fixation, a visual stimulus was presented within the RF of the neuron under study and it remained there until the end of the trial. The monkey was required to maintain fixation on the fixation point for a delay of 1 s, while it waited for the appearance of a saccade target (0.25°) at one of two locations distant from the RF. In two-thirds of the trials, the saccade target appeared when the fixation spot was extinguished and the monkey was required to make a saccadic eye movement to the target. In the remaining trials, the saccade target did not appear; when the fixation spot was extinguished, the monkey was required to make a saccadic eye movement to the RF stimulus. Both conditions were identical until the cue to saccade (disappearance of the fixation spot) and were pseudorandomly interleaved. RF stimuli were light and dark bars presented at one of four orientations (0°, 45°, 90°, or 135°) on a 34 × 27 cm video monitor driven by a number nine graphics board (640 × 480 resolution). Eye position was monitored at 200 Hz with a scleral search coil (*17*). Neural activity and eye position data were both stored for off-line analysis. The activity of single neurons was recorded by standard electrophysiological techniques described in K. Zipser, V. A. F. Lamme, and P. H. Schiller [*J. Neurosci.* **16**, 7376 (1996)].
11. The mean normalized response of 83 orientation-selective neurons was first assembled into a matrix of response functions in which either the preferred or the nonpreferred stimulus was presented to the RF. A running difference function was then computed from a moving average of the activity from preferred and nonpreferred trials. The moving average was computed within a 50-ms window of activity, which moved in 5-ms steps. These difference functions were constructed from both stimulus-aligned and saccade-aligned activity. A neuron was considered orientation selective if its orientation tuning index (response to best orientation–response to worst orientation)/(response to best orientation + response to worst orientation) was >0.1.
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13. We determined the angular distribution of saccade endpoints by computing the angular position of each endpoint from an axis of orientation passing through the median saccade endpoint from a set of trials. Individual saccade endpoints were taken as the eye position coordinate obtained 60 ms from the start of the movement. Because the duration of saccades of ≤10° in amplitude is typically between 30 and 50 ms (*18*), this coordinate should represent the full displacement of the movement but still exclude corrective saccades. We detected the initiation of saccadic eye movements by computing the instantaneous eye speed from the 200-Hz eye position samples. Any eye speed of ≥50° per second that displaced the eye position >0.5° was considered the initiation of a saccadic eye movement.
14. We recorded the activity of each orientation selective neuron (n = 83) during 10 to 35 trials, from which there were an equal number of saccadic eye movements made to each RF stimulus. For trials in which the preferred orientation was the RF stimulus, we sorted the presaccadic activity during each set of trials in order of response magnitude. We sorted the trials according to a 50-ms window of activity preceding the onset of the saccadic eye movement. This time window centered on the peak in the presaccadic selectivity shown in Fig. 1B, namely, −75 to −25 ms with respect to the saccade onset. We then divided the sorted sets of trials into two groups: trials with the most activity and trials with the least. We divided odd-numbered sets of trials equally into two subsets, with the middle trial being omitted. No attention was paid to the degree to which the division of trials resulted in nonoverlapping distributions of response magnitudes. However, the difference between the two trial subsets was always >0.
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Constitutive Activation of Toll-Mediated Antifungal Defense in Serpin-Deficient *Drosophila*

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The antifungal defense of *Drosophila* is controlled by the *spaetzle/Toll/cactus* gene cassette. Here, a loss-of-function mutation in the gene encoding a blood serine protease inhibitor, Spn43Ac, was shown to lead to constitutive expression of the antifungal peptide drosomycin, and this effect was mediated by the *spaetzle* and *Toll* gene products. Spaetzle was cleaved by proteolytic enzymes to its active ligand form shortly after immune challenge, and cleaved Spaetzle was constitutively present in *Spn43Ac*-deficient flies. Hence, *Spn43Ac* negatively regulates the Toll signaling pathway, and Toll does not function as a pattern recognition receptor in the *Drosophila* host defense.

Genetic analysis has established that the Toll signaling cascade controls the antifungal host defense of flies (*1*). In particular, Toll mediates the expression of the antifungal peptide drosomycin in the fat body cells by way of Rel-Cactus complexes, which are structurally and functionally equivalent to the vertebrate NF-κB–IκB complexes (*1, 2*). *Toll* (*Tl*) was initially identified as a gene that controls dorsoventral patterning in the *Drosophila* embryo (*3*). The *Tl* gene encodes a transmembrane receptor with extracellular leucine-rich repeats and an intracellular domain exhibiting marked similarities with the cytoplasmic domain of the interleukin-1 receptor (*4, 5*). A proteolytically cleaved form of the *spaetzle* (*spz*) gene product is thought to be the extracellular ligand of Toll both in embryonic development and in the immune response (*1, 5*). In the embryo, a proteolytic cascade, involving the Gastrulation defective, Snake, and Easter proteases, cleaves Spaetzle, a cysteine-knot growth factor, cytokine-like polypeptide (*5*). The genes encoding these three proteases are dispensable for induction of a Toll-mediated immune response (*1*). A human homolog of *Toll* was recently cloned and shown to activate signal transduction by way of NF-κB, leading to the production of pro-inflammatory cytokines (*6*). Studies performed with human cell lines suggest that a lipopolysaccharide (LPS)-binding and signaling receptor complex is assembled at the cell membrane where human Toll, in association with the MD-2 protein (*7*), interacts with LPS bound to the peripheral membrane protein CD14 (*8–10*). The LPS signal is probably transduced across the membrane by Toll, as mutations in this gene in mice lead to an LPS-unresponsive state (*11*).

Here, we have addressed the activation of the Toll receptor during the immune response of *Drosophila*. For this, we have used flies carrying ethylmethane sulfonate–induced mutations in the *necrotic* (*nec*) locus (*12*). The locus, which maps at position 43A, generates three transcripts encoding putative serine protease inhibitors of the serpin family (*13*). The *nec* mutants exhibit brown spots along the body and the leg joints, corresponding to necrotic areas in the epidermis. This mutant phenotype is rescued by a single

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transgenic copy of one of the serpin genes, *Spn43Ac* (*13*).

Because the absence of a functional Spn43Ac serpin may affect proteolytic cascades involved in the host defense of *Drosophila*, we examined the level of expression of the antimicrobial peptide genes in *nec* mutants. All genes were induced 6 hours after challenge in wild-type (WT) flies; however, in *nec* mutants the gene encoding *drosomycin* was strongly expressed in the absence of immune challenge (Fig. 1A). The expression was further enhanced by immune challenge. The gene encoding the peptide metchnikowin, which has both antibacterial and antifungal activities (*14*), also exhibited constitutive expression in *nec* mutants, although the response was less marked than for *drosomycin*. In contrast, we observed no constitutive expression of the genes encoding diptericin and cecropin A1, whose expression is either independent of the Toll signaling pathway or requires a signal from an additional pathway, depending on the *immune deficiency* (*imd*) gene (*1*).

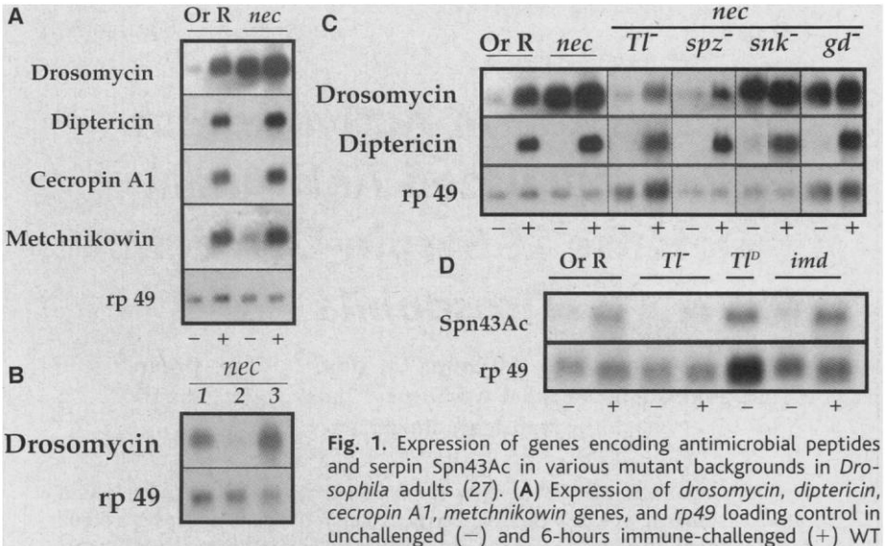

Fig. 1. Expression of genes encoding antimicrobial peptides and serpin Spn43Ac in various mutant backgrounds in *Drosophila* adults (*27*). (**A**) Expression of *drosomycin*, *diptericin*, *cecropin A1*, *metchnikowin* genes, and *rp49* loading control in unchallenged (−) and 6-hours immune-challenged (+) WT (Or R) and mutant (*nec*) flies. (**B**) The constitutive expression of *drosomycin* in unchallenged *nec* mutants (lane 1) is abolished by introducing the *Spn43Ac* (lane 2), but not the *Spn43Aa* (lane 3), transgene into this mutant background (*28*). Flies were as follows: lane 1: nec^1/nec^2; $P\{w^+$ *UAS-Spn43Ac*$^+\}/+$; lane 2: nec^1/nec^2; $P\{w^+$ *pda-GAL4*$\}/P\{w^+$ *UAS-Spn43Ac*$^+\}$; lane 3: nec^1/nec^2; $P\{w^+$ *pda-GAL4*$\}/P\{w^+$ *UAS-Spn43Aa*$^+\}$. (**C**) The constitutive expression of *drosomycin* in unchallenged *nec* mutants is abolished in *Toll* and *spaetzle* loss-of-function, but not in *snake* and *gastrulation defective*, mutants. *Diptericin* expression is induced only by immune challenge and is independent of the above mutations. The levels of *drosomycin* induction by bacterial challenge in Tl^- and spz^- flies are markedly reduced but not abolished, in agreement with previous findings (*1*). PhosphoImager (Becton Dickinson) quantification of several independent Northern blots, corrected for rp 49 loading controls, show that in *nec*;Tl^- double mutants, the level of immune induction of *drosomycin* by bacterial challenge was between 10 and 23% that of WT values. Mutant flies were as follows: *nec*: nec^1/nec^2, *nec*; spz^-: nec^1/nec^2; spz^{rm7}/spz^{rm7}, *nec*; Tl^-: nec^1/nec^2; Tl^{r632}/Tl^{9QRE} (29°C), *nec*; snk^-: nec^1/nec^2; snk^{073}/snk^{073}; gd^-, *nec*: gd^8/gd^8; nec^1/nec^2. (**D**) The expression of the serpin gene *Spn43Ac* is up-regulated by 6-hours immune challenge (+) in WT flies (Or R) and in *imd* mutants, but not in *Toll* loss-of-function (Tl^-) mutants. In *Toll* gain-of-function mutants (Tl^D), expression of Spn43Ac was five times higher than in WT, as determined by phosphoimaging, taking into account the correction for the rp49 loading control. Flies were as follows: Tl^-: Tl^{r632}/Tl^{9QRE} (29°C); Tl^D: $Tl^{10b}/+$; *imd*: *imd/imd*.

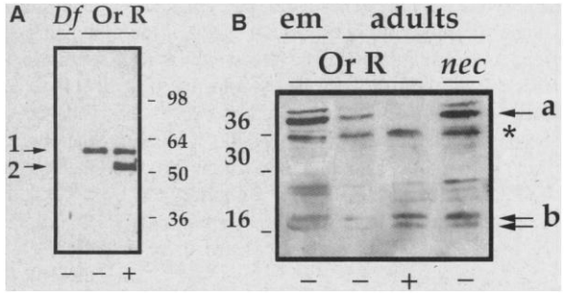

Fig. 2. Protein immunoblot analysis demonstrating the presence of the Spn43Ac protein in the hemolymph of WT flies (**A**) and the cleavage of the Spaetzle protein in WT and *nec* mutants (**B**). (A) Hemolymph was extracted from control (−) and 6-hours bacteria challenged (+) WT (Or R) flies and from unchallenged (−) flies carrying a transheterozygous combination of deficiencies, *Df(2R)sple-J1/Df(2R)nap2* (*12*), that uncovers the *Spn43Ac* gene (*Df*). Total hemolymph protein (5 μg) was probed with an antiserum directed against a GST-Spn43Ac fusion protein (*26*). Arrows 1 and 2 point to protein bands present in the hemolymph of WT flies and absent from flies deficient for the *Spn43Ac* gene. (B) Total protein extracts (40 μg) of embryos or adult flies (*29*) were probed with two independently raised antisera directed against the COOH-terminal part of the Spaetzle protein (*18*). Arrow (a) indicates band of ~40 to 45 kD detected in unchallenged (−) WT flies (Or R), embryos (em), and *nec* flies. Two additional bands corresponding to proteins of 16 to 18 kD are observed in WT flies after bacterial challenge (arrows b) and in unchallenged (−) *nec* mutants. The asterisk denotes an irrelevant band that was recognized only by one of the two antisera. Molecular size markers are indicated on the left (in kilodaltons).

The expression of the *Tl* gene and that of the downstream genes in the signaling cascade is up-regulated by immune challenge (*1*). We similarly found that the transcription of the *Spn43Ac* gene is up-regulated by immune challenge (Fig. 1D). This up-regulation is not observed in a *Tl* loss-of-function background. Conversely, *Tl* gain-of-function mutants exhibit a constitutive expression of *Spn43Ac*. In *imd* mutants, the up-regulation of *Spn43Ac* by immune challenge is similar to that in wild-type flies. Thus, *Spn43Ac* is an immune-responsive gene, and its expression is under the positive control of the Toll pathway. This could represent a negative feedback mechanism to shut down the activation of Toll by inhibiting the upstream proteolytic cascade.

To function as a negative regulator of the Toll pathway upstream of Spaetzle and Toll, Spn43Ac should be present in the hemolymph of adult flies. Indeed, immunoblotting with an antiserum directed against recombinant Spn43Ac revealed a band of ~60 kD in the blood of WT flies. This band was absent from the hemolymph of flies deficient for the *Spn43Ac* gene (Fig. 2A). The size of the mature Spn43Ac protein

predicted from the cDNA sequence (*13*) is smaller (52 kD) than the size of the immunoreactive protein, possibly reflecting post-translational modifications (because serpins are generally glycoproteins) (*16*). After immune challenge, a band of ~50 kD was observed (Fig. 2A), which may correspond to the Spn43Ac serpin that had undergone cleavage by activated protease or proteases.

During dorsoventral patterning of the embryo, the 382-residue Spaetzle protein is cleaved to a 106-residue COOH-terminal active ligand form (*17, 18*). Experiments on the putative proteolytic cleavage of Spaetzle in the host defense have not been reported so far, and we therefore analyzed protein extracts from naïve and immune-challenged flies by protein immunoblotting, using two polyclonal antisera directed against recombinant COOH-terminal Spaetzle (*18*). In extracts of embryos these antisera recognize the full-length Spaetzle protein and a smaller COOH-terminal fragment of 16 to 18 kD (Fig. 2B). In experiments with unchallenged flies, a band corresponding to a protein of 40 to 45 kD was detected in denatured extracts (Fig. 2B). It was also present in extracts of hemolymph (*19*). One hour after immune challenge, the 40- to 45-kD band had disappeared, whereas an immune-reactive doublet of ~16 to 18 kD was apparent (Fig. 2B), which we assume to correspond to the processed form of Spaetzle protein. The Spaetzle protein has glycosylation sites, which may account for slightly larger molecular sizes than predicted from the cDNA sequences. The 16- to 18-kD doublet was detected in unchallenged *nec* flies, together with the 40- to 45-kD protein corresponding to uncleaved Spaetzle. This result is in agreement with our working hypothesis that in *nec* mutants the absence of the functional serpin leads to the constitutive cleavage of Spaetzle. Finally, the strong signal of the 40- to 45-kD form of Spaetzle together with that of the 16- to 18-kD form in *nec* mutants confirms at the protein level that the expression of the *spz* gene is regulated by a positive-feedback loop.

Our data indicate that in the absence of a functional product of the *Spn43Ac* serpin gene in the blood of adult flies, the Spaetzle protein is spontaneously cleaved, leading to constitutive activation of the Toll signaling pathway. This phenotype can be rescued, either by a functional *Spn43Ac* transgene or by a *spz*- or *Tl*-deficient background. It is not known whether the protease, which cleaves Spaetzle, is a direct target of the serpin.

Conceptually, the activation of Spaetzle by blood protease zymogens is similar to the coagulation cascade in the horseshoe crab, which can be activated by binding of LPS to an upstream multidomain recognition protein (*20*). Several serpins, which fall into the same class as Spn43Ac, can specifically inhibit the proteases of the coagulation cascade.

Our results, and the parallels with the horseshoe crab coagulation cascade, imply that non-self recognition is an upstream event. Toll does not qualify as a bona fide pattern recognition receptor in *Drosophila*, in contrast to what has been proposed for Toll-like receptors in mammals (*9, 10*). The actual pattern recognition receptor, which initiates the cascade leading to the cleavage of Spaetzle and activation of Toll, remains to be identified.

Genetic aberrations and deficiencies of mammalian serpin genes have been correlated with clinical syndromes, such as pulmonary emphysema, angioedema, and coagulopathies, as a result of inappropriate inhibition of their respective target proteases (*21*). Our demonstration that a serpin functions in the regulation of the *Drosophila* immune response highlights the similarities between innate immunity in insects and mammals and reinforces the idea of a common ancestry of this system (*8*).

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26. A chimeric protein composed of glutathione S-transferase (GST) fused to the *Spn43Ac* gene product was produced with the use of a GST-Spn43Ac expression vector as follows: A Sac I (filled in)–Xho I 1200-bp fragment of *Spn43Ac* cDNA was subcloned into the Sma I and Xho I sites of the pGEX2T expression vector (Pharmacia). The GST-Spn43Ac fusion protein was expressed in *Escherichia coli* strain LE 392 and purified according to Pharmacia standard procedures on a glutathione Sepharose 4B column. Antibodies were obtained by inoculating the recombinant protein (100 to 200 μg for each innoculation) into a rabbit according to standard methods. Hemolymph was extracted from flies with a Nanoject apparatus (Drummond Scientific) and recovered in phosphate-buffered saline. Hemolymph extract (5 μg) was loaded on a 10% SDS-polyacrylamide gel. After SDS–polyacrylamide gel electrophoresis, proteins were blotted onto Hybond ECL nitrocellulose membranes (Amersham). Blots were incubated overnight at 4°C with a 1/5000 dilution of antiserum to GST-Spn43Ac. After washing with tris-buffered saline, the blots were incubated for 1 hour at 37°C with a 1/5000 dilution of horseradish peroxidase–conjugated donkey antibody to rabbit immunoglobulin G (Amersham). The blots were developed with the use of an ECL system (Amersham).
27. Immune challenge was performed by pricking adult flies with a fine needle dipped into a concentrated culture of *E. coli* and *Micrococcus luteus* as described in (*1*); see also (*22*). Northern blotting experiments were performed as in (*1*); for (A) to (C), total RNA was extracted from whole flies; for (D), polyadenylated RNA was prepared as in (*1*). The cDNA probes used were as follows: drosomycin, diptericin, cecropin A1, rp 49, as described in (*1*); metchnikowin, as in (*14*); Spn43Ac, an Eco RI fragment of ~750 base pairs (bp) corresponding to the 3′ region of Spn43Ac cDNA (*13*).
28. Rescue experiments were based on the *GAL4/UAS* system (*23*). Using the Eco RI site, filled in by the DNA polymerase Klenow fragment, and the Xho I site of the pUAST transformation vector, we inserted an ~1600-bp fragment corresponding to the *Spn43Ac* coding sequence 3′ to the *GAL4 UAS* control element. In a similar cloning experiment, the Eco RI site, filled in by the DNA polymerase Klenow fragment, and the Xba I site of the pUAST transformation vector were used to insert the *Spn43Aa* coding sequence 3′ to the *GAL4/UAS* control element. Transformant flies were obtained by microinjection (*24*) with a *w* recipient strain. Three and seven different transformant lines were obtained, respectively, for the $P\{w^+$ *UAS-Spn43Ac*$^+\}$ and $P\{w^+$ *UAS-Spn43Aa*$^+\}$ transgenes. Lines 933 and 932 carrying, respectively, $P\{w^+$ *UAS-Spn43Ac*$^+\}$ and $P\{w^+$ *UAS-Spn43Aa*$^+\}$ transgenes on chromosome 3 were used in all experiments. The nec^1/nec^2 allele combination was chosen for rescue. A third-chromosome *daughterless-GAL4* transgene [$GAL4^{daG32}$ (*25*)] was used as a ubiquitous driver line. Both serpins and $GAL4^{daG32}$ transgenes were crossed into the nec^1/CyO and nec^2/CyO backgrounds. The resulting stocks were crossed with each other to yield a heteroallelic *nec* combination.
29. Protein extracts from embryos and whole flies were prepared as in (*17*). Extracts (40 μg) were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel, and immunoblotting was performed as in (*26*).

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