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Dual detection of fungal infections in *Drosophila* through recognition of microbial structures and sensing of virulence factors

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Abstract

The *Drosophila* immune system discriminates between various types of infections and activates appropriate signal transduction pathways to combat the invading microorganisms. The Toll pathway is required for the host response against fungal and most Gram-positive bacterial infections. The sensing of Gram-positive bacteria is mediated by the Pattern Recognition Receptors PGRP-SA and GNBP1 that cooperate to detect the presence of infections in the host. Here, we report that GNBP3 is a novel Pattern Recognition Receptor that is required for the detection of fungal cell wall components. Strikingly, we find that there is a second, parallel, pathway acting jointly with GNBP3. The *Drosophila* Persephone protease activates the Toll pathway when proteolytically matured by the secreted fungal virulence factor PR1. Thus, the detection of fungal infections in *Drosophila* relies both on the recognition of invariant microbial patterns and on monitoring the effects of virulence factors on the host.

Introduction

Fungi represent a threat to insects in the wild with more than 700 entomopathogenic species described (Roberts and Humber, 1981). Insects must have evolved responses to handle these infections. We have addressed here the genetically amenable fruitfly *D. melanogaster* to decipher the mechanisms that stimulate immune responses to fungal infections.

This host response includes both cellular and humoral arms. The analysis of the humoral immune response within the framework of a septic injury model has led to the current paradigm

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in which two distinct intracellular transduction pathways, Immune deficiency (IMD) and Toll, regulate the transcription of hundreds of genes by controlling the nuclear uptake of the NF-κB transcription factors Relish and Dorsal-related Immunity Factor (DIF), respectively (reviewed in Hoffmann, 2003, and references therein). The classical effector molecules of the systemic humoral response, the antimicrobial peptides, are synthesized in the fat body, a functional analogue of the mammalian liver, and are released into the hemolymph where they kill invading microorganisms. One of these peptides, Drosomycin, exhibits fungicidal activities at micromolar concentrations and is active mainly on filamentous fungi (Fehlbaum et al., 1995; Tzou et al., 2002). Others such as Cecropins, Attacins, Drosocin, and Diptericin are active mostly on Gram-negative bacteria whereas Defensin is effective against Gram-positive bacteria.

The IMD pathway is required for the host response against Gram-negative bacteria. Mutants in this pathway fail to express antibacterial peptides and are highly sensitive to such infections, yet resist fungal and Gram-positive bacterial infections as well as wild-type flies. Toll is the receptor of the second intracellular transduction pathway and is activated by the binding of a proteolytically cleaved form of the Spätzle (SPZ) cytokine. Toll pathway mutants are susceptible to infections by the entomopathogenic fungi *B. bassiana* and *M. anisopliae* or by the opportunistic pathogen *Aspergillus fumigatus* (Lemaitre et al., 1996; Lemaitre et al., 1997; Ligoxygakis et al., 2002; Rutschmann et al., 2000a; Tauszig-Delamasure et al., 2002). Toll is also required to resist some Gram-positive bacterial infections (Gobert et al., 2003; Michel et al., 2001; Rutschmann et al., 2002).

The *Drosophila* immune response is adapted to the nature of the invading microorganism (Lemaitre et al., 1997). The Toll pathway is induced by fungi and Gram-positive bacteria, whereas the IMD pathway is predominantly triggered upon Gram-negative bacterial challenges (Lemaitre et al., 1997; Rutschmann et al., 2000a). These observations imply that several receptors mediate the discrimination between various types of microbial infections. Indeed, members of the peptidoglycan recognition protein (PGRP) family have been shown to be required for these distinct events (reviewed in Ferrandon et al., 2004; Kaneko and Silverman, 2005). PGRP-LC, a receptor of the IMD pathway, can be activated by *meso*-diaminopimelic acid peptidoglycan (PGN), a compound characteristic of the cell wall of Gram-negative bacteria and of Gram-positive bacilli. PGRP-LE, a secreted member of the PGRP family, is also involved in sensing Gram-negative bacteria (Kaneko et al., 2006). In contrast, the circulating PGRP-SA receptor activates the Toll pathway upon detection of Lysine-type PGN which is a major component of the cell wall of many Gram-positive bacterial strains. The Gram-Negative Binding Protein 1 (GNBP1) associates with PGRP-SA and this complex is both necessary and sufficient to activate the Toll pathway upon Gram-positive challenge (Gobert et al., 2003). The circulating PGRP-SA/GNBP1 complex activates a downstream proteolytic cascade that leads to the cleavage of the Spätzle cytokine, which then activates the Toll transmembrane receptor (Jang et al., 2006; Kambris et al., 2006). Thus, PGRP-SA and GNBP1 define a Gram-positive-specific branch of Toll receptor activation. PGRP-SD also belongs to this branch and is required for the detection of other Gram-positive bacterial strains (Bischoff et al., 2004).

Here, we address the existence of a second branch devoted to the detection of fungal infections, which also activates Toll. Indeed, mutants for the *persephone* (*psh*) gene, which encodes a clip-prodomain containing protease, are characterized by an increased sensitivity to natural infections with the entomopathogenic fungus *B. bassiana*, whereas they are resistant to bacterial infections (Ligoxygakis et al., 2002). The *psh* mutations had been originally isolated as suppressors of the *necrotic* (*nec*) phenotype. *nec* encodes a serine protease inhibitor of the serpin family, the absence of which leads to the constitutive, *psh*-dependent, activation of the Toll pathway (Levashina et al., 1999; Ligoxygakis et al., 2002). Thus, *psh* and *nec* define a

fungal-specific branch of Toll receptor activation. By analogy to the Gram-positive branch, it is expected that an as yet unidentified immune receptor detects fungal infections and activates in turn the *psh*-dependent proteolytic cascade.

GNBP1 belongs to the family of GNBP/β-Glucan Recognition Proteins (βGRP) (Kim et al., 2000). Members of this family have been reported to bind to β-(1,3)-glucan, a major component of the fungal cell wall (Lee et al., 2006; Ma and Kanost, 2000; Ochiai and Ashida, 2000). In *Drosophila*, three members of this family, GNBP1 to 3, have been described (Kim et al., 2000). Among these, GNBP3 shows the greatest degree of similarity to lepidopteran β-(1,3)-glucan recognition proteins and was therefore a good candidate for a fungal-specific sensor. Here, we report that GNBP3 is indeed required for Toll pathway activation in response to fungal infections. Strikingly, we also find that *psh* is required in a distinct yet complementary detection pathway that can be activated by fungal virulence factors.

Results

In this report, we have investigated the antifungal response of *Drosophila* by using two distinct models : the human opportunistic pathogenic yeast *Candida albicans* and the entomopathogenic fungi *B. bassiana* and *M. anisopliae*. We have monitored the immune response to these infections by two read-outs : kinetics of survival and expression levels of the *Toll*-dependent *Drosomycin* gene.

hades, a mutation of the *GNBP3* locus, affects the host defense against *Candida albicans*

We challenged flies by pricking with a needle dipped into a concentrated *C. albicans* solution and monitored their survival. Whereas wild-type flies were resistant to this infection, survival of the Toll pathway mutants *spz* and *Dif* was compromised, indicating that the Toll pathway is required for defense against this opportunistic yeast (Fig. 1A) (Alarco et al., 2004). We next created a null mutation in the *GNBP3* gene, which we named *hades* (for the generation of the mutation, see below) and observed that $GNBP3^{hades}$ mutants were as sensitive to *C. albicans* infection as *Dif* mutants (Fig. 1A). In contrast, $GNBP1^{osi}$ mutant flies, which are deficient for the activation branch of the Toll pathway by Gram-positive bacteria, survived *C. albicans* infection as well as wild-type flies. Flies mutant for the IMD pathway gene *kenny* (*key*) (Rutschmann et al., 2000b) also resisted this challenge (Fig. 1A), indicating that the IMD pathway is not required for the host defense against opportunistic yeast infections. The finding that both $GNBP3^{hades}$ and Toll pathway mutants succumb to *C. albicans* infection suggests that this sensitivity to yeast infection reflects a lack of activation of the Toll pathway. We next challenged wild-type, *spz*, and $GNBP3^{hades}$ mutant flies with heat-killed *C. albicans* (which cannot secrete virulence factors, see below). We found that *Drosomycin* was strongly induced in wild-type flies and that this inducibility was markedly decreased in $GNBP3^{hades}$ and *spz* mutants (Fig. 1D).

We also tested resistance of $GNBP3^{hades}$ mutants to Gram-positive and Gram-negative bacterial infections and observed no susceptibility as compared to wild-type flies (Fig. 1B,C). Furthermore, the induction levels of the *Drosomycin* gene by Gram-positive bacteria (Fig. 1E) or those of the *Diptericin* gene by Gram-negative bacteria (Fig. 1F) were similar in $GNBP3^{hades}$ and wild-type flies. We conclude that *GNBP3* is involved in the *Drosophila* host defense against *C. albicans* and not that against bacterial infections.

Rescue of the $GNBP3^{hades}$ mutation

We had generated the *hades* mutant of *GNBP3* mentioned above by remobilizing a P-element transposon (d01793) located 1044 base pairs upstream of the start codon ATG of *GNBP3* (Thibault et al., 2004). This resulted in an imprecise excision, a 1476 base pair deletion that

removes the promoter of the gene as well as the N-terminal region of the corresponding predicted protein up to residue 144 (Fig. 2A). As expected, the GNBP3 protein was no longer detected in the mutant with a specific antibody (Matskevich *et al.*, unpublished). We rescued the *GNBP3*^{hades} mutant phenotype by expressing in this background a wild-type *GNBP3* cDNA under the control of the ubiquitous heat-shock protein 70 (*hsp*) promoter. The low-level expression of the transgene driven by the basal activity of the *hsp* promoter using the UAS-GAL4 system was sufficient to restore the inducibility of *Drosomycin* in response to a challenge with heat-killed *C. albicans* (Fig. 2B, Supplementary Experimental Procedures), confirming that the effect of the mutation is due to the disruption of the *GNBP3* locus.

Recombinant GNBP3 binds to β-(1,3)-glucan and to polysaccharides of the fungal cell wall

The N-terminal domain of lepidopteran βGRPs, which binds directly to β-(1,3)-glucan (Ma and Kanost, 2000; Ochiai and Ashida, 2000), displays around 65% of sequence identity to GNBP3. We expressed tagged GNBP3 in bacteria and incubated the recombinant protein with curdlan, an insoluble polymer of β-(1,3)-glucan, with killed *C. albicans*, and with several polymeric glycan chains. The insoluble compounds were recovered by centrifugation (Kim et al., 2000), and assessed by SDS-PAGE and Western blot analysis for the presence of GNBP3 in the pellet. We detected an association of GNBP3 with curdlan and *C. albicans* (Fig 2C) (Lee et al., 2006). A weaker signal was detected with chitin, a polymer of N-acetyl-D-glucosamine, whereas little GNBP3 was recovered after incubation with peptidoglycan. In addition, the injection of curdlan beads into flies elicited a variable induction of *Drosomycin* that was not observed with injected cellulose, which is a β-(1,4)-glucan polymer (data not shown). Because curdlan beads form aggregates that are difficult to inject quantitatively into flies, we injected instead the alkali-insoluble fraction of the *A. fumigatus* cell wall, which consists of polysaccharides including β-(1,3)-glucans (Fontaine et al., 2000). This fraction induced reproducibly a *GNBP3*-dependent *Drosomycin* expression (Fig. S1). We conclude that *GNBP3* encodes a fungal polysaccharide-binding protein.

GNBP3 acts upstream of the Toll receptor

To provide a demonstration that GNBP3 induces *Drosomycin* expression through the Toll pathway, we overexpressed this gene in a wild-type and a *spz* mutant background. As illustrated in Fig. 3A, GNBP3 overexpression induced the challenge-independent transcription of *Drosomycin*, which was abolished in *spz* mutant flies.

The *Drosophila* infection-sensing proteins PGRP-SA and GNBP1 were recently shown to function in circulation. To probe whether this is also valid for GNBP3, we transferred hemolymph from wild-type flies into *GNBP3*^{hades} mutants. In the recipient flies, heat-killed *C. albicans* failed to induce *Drosomycin* expression (Fig. 3B). However, when flies that overexpressed GNBP3 were chosen as hemolymph donors, *Drosomycin* inducibility by heat-killed *C. albicans* was restored (Fig. 3C). Control unchallenged recipient flies did not express *Drosomycin* **(**data not shown**)**. These data suggest that a circulating form of GNBP3 can detect infection by *C. albicans*.

Entomopathogenic fungi activate the Toll pathway independently from *GNBP3*

Entomopathogenic fungi invariably induce lethality in experimental flies, whether the challenge is achieved by injection of spores or by natural infection (deposition of spores on the cuticle). Flies mutant for genes of the Toll pathway succumb significantly earlier to such infections (Fig. 4A) and exhibit a strongly decreased inducibility of Toll pathway-dependent genes such as *Drosomycin*, which is no longer expressed in these mutants (Lemaitre et al., 1997) (Fig. 4C). *GNBP3*^{hades} mutant flies succumbed to a natural *B. bassiana* infection at the same rate as *Dif* flies (Fig. 4A). Hemizygous and homozygous *GNBP3*^{hades} flies displayed the same behavior in this assay, thus indicating that the mutation is genetically null. If GNBP3

were the sole sensor of fungal infection, one would expect *Drosomycin* expression to be markedly reduced in *GNBP3^{hades}* mutant flies challenged with pathogenic fungi. To test this hypothesis, we carried out the following experiments : (i) we performed a *B. bassiana* natural infection on *GNBP3^{hades}* flies and observed a strong and persistent expression of *Drosomycin* (Fig. 4B–C); (ii) we compared the levels of expression of *Drosomycin* induced by the injection of live *B. bassiana* spores into wild-type or *GNBP3^{hades}* flies and noted a similarly strong induction of this gene in both types of flies (data not shown); (iii) however, when we injected alkali-treated spores, we detected the expression of *Drosomycin* in wild-type, but not in *GNBP3^{hades}* mutant flies (Fig. 4D). Hardly any *Drosomycin* expression was detected in *Dif* or *spz* mutant flies infected with spores, whether dead or alive (Fig. 4C–D). Thus, these two types of challenge induce the transcription of *Drosomycin* through the intracellular part of the Toll pathway. The fact that *Drosomycin* induction is blocked by the *GNBP3^{hades}* mutation only when dead spores are injected suggests that live spores can activate the Toll pathway by an extracellular pathway independent of GNBP3. A candidate for a gene belonging to this second pathway is *psh*.

Persephone is required only for the host defense against live *B. bassiana*

The PSH protease is required for activation of the Toll pathway by the entomopathogenic fungus *B. bassiana* (Ligoxygakis et al., 2002) (Fig. 4A, C). In one model, *psh* would be required downstream of a PRR that senses fungal microbial patterns such as β-(1,3) glucan. If it were to act uniquely downstream of *GNBP3*, then *GNBP3* and *psh* would be expected to share the same phenotype. In a second model, *psh* would act in the GNBP3-independent pathway that activates Toll after challenge with live fungi and would therefore have a phenotype distinct from that of *GNBP3*. In the following, we compare the *psh* and *GNBP3^{hades}* mutant phenotypes in this light.

We first analyzed the survival rates of *psh* and *GNBP3^{hades}* mutant flies after natural infections with *B. bassiana* and observed that the two mutants died with similar kinetics (Fig. 4A). Interestingly, the double-mutant flies succumbed earlier to the infection than the respective single mutants. These data suggest that *GNBP3* and *psh* act in two complementary pathways upstream of *Toll* in the detection of fungal infections. We next determined whether *psh* is required for *Drosomycin* inducibility by dead fungal spores. In *psh* mutant flies, the injection of killed fungal spores still induced the expression of *Drosomycin* (Fig. 4D), in contrast to natural infections with live *B. bassiana* where *Drosomycin* inducibility was markedly reduced (Ligoxygakis et al., 2002) (Fig. 4C). Thus, live and dead fungi have distinct effects on the inducibility of the *Drosomycin* gene in *psh* and *GNBP3* mutants.

Earlier studies on the *psh* mutant phenotype had been limited to the analysis of the response to the entomopathogenic fungus *B. bassiana* (Ligoxygakis et al., 2002). We therefore asked whether *psh* is also required for the host defense against opportunistic yeast infections. In contrast to *GNBP3^{hades}* flies, we observed that *psh* mutants are as resistant as wild-type to *C. albicans* infections (Fig. 4E). These data indicate that *psh* and *GNBP3* are indeed involved in distinct branches of Toll pathway activation. Furthermore, the level of expression of *Drosomycin* induced by dead *C. albicans* was not as strongly reduced in *psh* mutants as it was in *GNBP3^{hades}* single mutant and in the *psh ; GNBP3^{hades}* double mutant (Fig. 4F). We cannot exclude however a limited role of *psh* in the defense against *C. albicans* since *psh ; GNBP3^{hades}* double mutant flies are more sensitive than the respective single mutants (Fig. 4E). In addition, only the double-mutant combination strongly blocks *Drosomycin* expression in response to a challenge with living *C. albicans* yeasts (Fig. 4G).

Taken together, our data demonstrate that GNBP3 and PSH belong to two distinct, complementary sensing pathways of fungi. Whereas GNBP3 appears to act as a classical

Pattern Recognition Receptor (able to detect microbial patterns in killed fungi), PSH may be involved in the detection of fungal factors released by live entomopathogenic fungi.

The PR1 protease from the entomopathogenic fungus *M. anisopliae* triggers *Drosomycin* expression in a *persephone*-dependent manner

Our hypothesis is that entomopathogenic fungi secrete virulence factors that can be detected by the host through activation of the Toll pathway. Entomopathogenic hyphomycete fungi are known to secrete proteases and chitinases that perforate the cuticle barrier and allow entry of the fungus into the insect body cavity (Clarkson and Charnley, 1996). This strategy is shared by *B. bassiana* and *M. anisopliae*, which both express PR1 subtilisins when spores germinate on the cuticle of insects (58.6 % identity conservation) (Bagga et al., 2004). The PR1 protease of *M. anisopliae* has been shown to be a major virulence factor of this entomopathogenic fungus (St Leger et al., 1992; Wang et al., 2002). *M. anisopliae* behaves as *B. bassiana* when infecting *Drosophila* (Fig. S2), with the notable exception that *Drosomycin* expression is not blocked in a *psh*, but is abolished in a *psh, GNBP3*^{hades} mutant background (Fig. 4H, see Discussion). To study the effects of fungal virulence factors, we generated transgenic flies that express the gene encoding the *M. anisopliae* PR1A protease under the control of the *heat shock protein* promoter using the UAS/GAL4 system. When we overexpressed *PR1A* in the transgenic flies, we observed a marked expression of *Drosomycin* in the absence of an immune challenge (Fig. 5A). Strikingly, the expression of *Drosomycin* induced by *PR1A* overexpression required the *psh* gene. In contrast, this expression was not dependent on the *GNBP3* gene (Fig. 5A). The PR1A-dependent expression of *Drosomycin* requires the proteolytic activity of PR1A since transgenic constructs expressing a catalytically-inactive form of PR1A were unable to trigger the Toll pathway (Fig. 5B). The expression of PR1A in the hemolymph leads to the progressive degradation of hemolymphatic proteins (Fig. S3). PSH is a clip domain containing protease, which is thought to be activated by the cleavage of its clip prodomain. To determine whether PSH might be activated by PR1A, we have expressed either PR1A or PSH alone and the ubiquitous *daughterless* driver and detected a mild expression of *Drosomycin* 24 hours after induction (Fig. 5C). In striking contrast, the coexpression of both *PR1A* and *psh* in the absence of immune challenge led to strong *Drosomycin* expression, which is significantly higher than the added effect of each protease alone (Fig. 5C). This experiment indicates that PR1A either activates PSH directly or that PSH is a limiting factor in the PR1A-dependent activation branch of the Toll pathway.

To discriminate between these possibilities, we first monitored the PSH protein in collected hemolymph by Western blotting with a PSH-specific antibody. As shown in Fig. 5D, PSH migrates as a 50 kD band in unchallenged wild-type flies. The overexpression of PSH leads to the appearance of an additional 33 kD band, which is also detected in flies challenged by the injection of *B. bassiana* spores. Strikingly, the 50 kD band is mostly converted to a band of approximately 28 kD (Fig. 5D) and shorter fragments (not shown). This 28 kD band is the major band observed in flies that overexpress both *PR1* and *PSH*. The 28 kD band may correspond to the active form of PSH since it is detected only in those transgenic flies that strongly express *Drosomycin*. In addition, the intensity of the band correlates with the strength of *Drosomycin* induction (compare Fig. 5C and D). Interestingly, the predicted size of the activated CLIP-domain protease PSH, which corresponds essentially to the trypsin catalytic domain, is 27.1 kD. Second, we incubated immunoprecipitated PSH collected from the hemolymph of unchallenged wild-type flies with purified preparations of fungal PR1. This digestion produced the 33 and 28 kD bands in a concentration-dependent manner (Fig. 5E). These bands were absent in the immunoprecipitate incubated with the heat-inactivated PR1 preparations. Taken together, our experiments demonstrate that the Toll pathway can be activated by a fungal protease, which likely activates PSH by direct proteolytic cleavage.

Discussion

The detection of infections is a crucial step in the timely initiation of an appropriate immune response. In *Drosophila*, the use of nonentomopathogenic bacteria such as *M. luteus* and *E. coli* has allowed the delineation of both intracellular signal transduction pathways as well as the identification of five innate receptors (PRRs), PGRP-LC and –LE for the IMD pathway, and PGRP-SA/GNBP1/PGRP-SD for the Toll pathway. To elucidate the mechanisms involved in the detection of fungi, we have first concentrated on a somewhat artificial infection system using an opportunistic human pathogenic yeast, *C. albicans*. We then refined our understanding of the system by using the entomopathogenic fungi *B. bassiana* and *M. anisopliae*.

GNBP3 serves as a receptor for fungal structures

In this study, we demonstrated that GNBP3 is a PRR dedicated to the detection of fungi since (i) recombinant GNBP3 is able to bind *in vitro* to *Candida* and to polymeric chains of β-(1,3)-glucan; (ii) it is required for the activation of the Toll pathway by polysaccharides of the fungal cell wall; (iii) *GNBP3* is required for resistance against yeast infections, including *C. albicans*, *C. glabrata*, *C. tropicalis*, and against mold infections such as *B. bassiana*, *M. anisopliae*, and *A. fumigatus* (this work, MG, DF, unpublished data); (iv) *GNBP3* triggers an adequate immune response, namely it activates the antifungal Toll pathway in a *spz*-dependent manner. We cannot formally exclude that another fungal receptor acts together with GNBP3 to activate the Toll antifungal host defense.

Of note is that fungi can induce the IMD pathway with short-term kinetics (Lemaitre et al., 1997). We have found that this induction is dependent on *PGRP-LC* and not on *GNBP3* (Fig. S4). One possibility is that a PGRP-LC coreceptor senses fungal microbial patterns. Alternatively, fungal cell wall constituents might bind directly to PGRP-LC. Interestingly, Lee and coworkers have reported that a coleopteran PGRP is able, in addition to its liaison to PGN, to bind with high affinity to tetralaminariose, a tetramer of β-(1,3)-glucan (Lee et al., 2003).

As is the case for members of the PGRP family, the GNBP/βGRP proteins have evolved to recognize distinct carbohydrate chains that form the cell wall of microorganisms. Given their distribution in the arthropod lineage, it is likely that these two families form an essential part of their immunity repertoire. Whereas PGRP homologs exist in mammals, βGRP members have not been reported in vertebrates. However, the phagocytic and signalling receptor Dectin-1 detects β-(1,3)-glucans (Brown and Gordon, 2001) and may to some extent fulfill in mammals a primary function that is similar to that of GNBP3 in insects, *i. e.*, the sensing of fungal infections.

Since *spz* is required for Toll activation by GNBP3, we propose that the binding of GNBP3 to its microbial ligand leads to the activation of a proteolytic cascade that ultimately processes proSPZ into a functional Toll ligand (Fig. 6). Because *psh* and $GNBP3^{hades}$ have distinct phenotypes as regards Toll pathway activation (Fig. 4C, D, F) and because the double mutant *psh;* $GNBP3^{hades}$ displays a stronger phenotype than either mutant alone when challenged with live fungi (Fig. 4G, H), PSH cannot belong exclusively to a proteolytic cascade activated by GNBP3. However, epistatic analysis reveals that the *spz*-dependent expression of *Drosomycin* induced by *GNBP3* overexpression partly requires *psh* function (Sup. Fig. 5). Taken together, these data indicate the existence of an alternative, *psh*-independent proteolytic cascade that mediates the *GNBP3*-dependent maturation of the Toll ligand Spätzle. This cascade is distinct from the one that activates Toll signalling during early embryogenesis (not shown).

The host defense against entomopathogenic fungi does not solely rely on the Toll pathway

An unexpected finding of this study is that the Toll pathway is normally induced in GNBP3^{hades} mutants undergoing a *B. bassiana* infection. Yet, these mutants are more susceptible to this pathogen than wild-type flies. These observations suggest that GNBP3 fulfills other functions required in the host defense against fungal pathogens that are independent of its role in triggering the Toll pathway. Indeed, we have some biochemical evidence that GNBP3 is involved in other aspects of host defense (AM, DF, unpublished).

PSH is involved in the sensing of fungal virulence factors

Many pathogens have adapted to their hosts and developed specific strategies to defeat their defenses. Fungi such as *B. bassiana* and *M. anisopliae* are able to infect insects following deposition of spores on the surface of the cuticle. To penetrate this physical barrier, they secrete several virulence factors such as chitinases and proteases. We found that the PR1A protease is able to activate *Drosomycin* expression in the absence of infection when overexpressed in flies. This effect on Toll pathway activation is specific since it can be blocked in a *psh* background and depends on the proteolytic activity of PR1A (Fig. 5). These data establish the proof of concept that a virulence factor can be detected by the innate immune system. Interestingly, our data indicate that PR1 can directly process PSH into its active form.

PR1A is one of ten proteases in this subtilisin family and is expressed only during cuticle penetration (Bagga et al., 2004; Wang et al., 2005). We have found that a PR1A/PR1B-deficient strain is still able to induce *Drosomycin* expression in a *GNBP3^{hades}* mutant background, presumably through other fungal PR1 proteases (Fig. S2). Thus, further work will be required to understand the multiple pathogenic mechanisms taking place during a natural fungal infection.

Our data show that the detection of fungal infections relies on a two-pronged sensor system that constitutes a partially redundant recognition system. The *psh; GNBP3* double mutant strain consistently yields a stronger phenotype than that of the respective single mutants. Since only *GNBP3* is strictly required in the defense against opportunistic yeasts, it is likely that the recognition of fungal patterns represents an ancestral, basal mode of infection sensing. The *psh*-dependent system that monitors virulence factors may have evolved secondarily in response to the selective pressure exerted by entomopathogenic fungi. Indeed, if the *psh*-based and the *GNBP3*-based sensing systems were perfectly redundant, it would be expected that the deletion of one of these systems would not prevent the activation of the Toll pathway. This is indeed what we observe when infecting flies with live *C. albicans* or with *M. anisopliae*. In contrast, *Drosomycin* inducibility is abolished in *psh* mutants, but not in *GNBP3* mutants, infected by *B. bassiana*. These data indicate that *B. bassiana* has evolved a strategy that allows it to escape or to block GNBP3 surveillance.

Future studies will reveal whether similar systems of virulence factor detection exist also to sense infection by entomopathogenic bacteria.

A general mechanism of pathogen detection ?

We surmise that some pathogens have developed strategies to inactivate the GNBP basal sensor system of *Drosophila* and that this led to the selection of a novel host counter-strategy : the surveillance of virulence factor activity. This theme is a central tenet of the current understanding of plant innate immunity. In plants, basal sensor systems detect the presence of microbial elicitors and trigger an immune response. Some virulence factors of the plant pathogen inhibit the elicitor-induced signaling by manipulating host proteins that regulate the host basal response (Kim et al., 2005). In some plant cultivars, a surveillance system based on R proteins “guards” the targets of virulence factors (coded by microbial *avirulence* (*avr*) genes)

and triggers a strong immune response when under attack (reviewed in Dangl and Jones, 2001). One example is provided by *Arabidopsis* where the cleavage of the endogenous PBS1 kinase by the *Pseudomonas syringae* type III effector AvrPphB, a cysteine protease, leads to the activation of the hypersensitive response by the R protein RPS5 (Shao et al., 2003). A case possibly more relevant to fly immunity is provided by the tomato where the host protease Rcr3 is required for the recognition of the pathogen virulence factor Avr2 by the Cf-2 transmembrane receptor (Rooney et al., 2005 and references therein).

Fungal proteases secreted by entomopathogenic fungi have to cross the structurally invariant cuticular barrier of the insect host that thus conditions the type of proteolytic activity required to degrade the cuticular proteins. This phenomenon may have been exploited by *Drosophila* to detect entomopathogenic infections in a mechanism that is hence conceptually related to the guard hypothesis of plants, although in this case, PSH would monitor indirectly a passive defense mechanism, the protection provided by the bodily armor. To date, the analysis of the immune response in *Drosophila* has been largely limited to the study of laboratory strains in a controlled environment. By analogy to plant-pathogen interactions that involve *avr* genes and their cognate plant *R* resistance genes, a major challenge for the coming years will be to determine whether the insect-pathogen interactions in a natural environment involve several distinct virulence factors and their associated host detection systems.

The discovery of a host sensor system dedicated to the detection of virulence factor activity begs the question of the relevance of such a system to mammalian innate immunity. It has been reported that virulence factors such as the cholesterol-dependent cytolysin or pertussis toxin are able to induce immune responses through TLR4 (Kerfoot et al., 2004; Malley et al., 2003; Park et al., 2004). In these cases, the possibility remains open that TLR4 functions as a co-receptor needed for intracellular signal transduction and that the actual recognition is mediated by unknown receptors. A second class of interest is that of the Protease Activated Receptors. Indeed, PAR2 has been implicated in the induction of the HB2 defensin by bacterial proteases in epithelial cells (Chung et al., 2004). Similarly, *Citrobacter rodentium* induces the intestinal release of host proteases that activate the PAR2 receptor and subsequent colonic inflammation (Hansen et al., 2005). Finally, virulence factors from *Salmonella* and *Yersinia* have been shown to inhibit NF-κB and MAPK signaling (Mota and Cornelis, 2005; Viboud and Bliska, 2005). Thus, it is legitimate to ask whether receptors dedicated to the perception of virulence factor activity have been selected during the evolution of the mammalian innate immune system.

Experimental Procedures

Microbial strains

Gram-negative bacteria : *Enterobacter cloacae* (a kind gift of H. Monteil), *Escherichia coli* 1106. Gram-positive bacteria : *Micrococcus luteus* (CIP A270), *Enterococcus faecalis*. Fungi : *Beauveria bassiana* (80.2 strain), *M. anisopliae* (V275), *C. albicans* : a pathogenic strain isolated in Patient #3 by Pr. M. Koenig (CHU Strasbourg-Hautepierre).

Fly strains

Stocks were raised on standard cornmeal-agar medium at 25°C. *w* A5001 flies were used as wild-type throughout the experiments since the $GNBP3^{hades}$ mutant was generated in this background. In experiments involving the Dif^{1} and key^{1} mutants, the original *cn bw* stock was used as a further wild-type control (Jung et al., 2001; Rutschmann et al., 2000a; Rutschmann et al., 2000b). $GNBP1^{osi}$, $PGRP\text{-}LC^{E12}$, psh^{4}, *UAS-psh*, and *hsp-Gal4* stocks have been described previously (Gobert et al., 2003; Gottar et al., 2002; Ligoxygakis et al., 2002). The *hades* deficiency was generated by crossing the modified P-element-containing strain d01793

to a strain carrying the Delta2–3 transposase. Revertants that had lost the dominant mini-*white* marker were tested by PCR for imprecise excision events. The exact extent of the deletion was determined by sequencing of PCR products. Stocks used for epistatic analysis and overexpression analysis were generated using standard crosses. We checked the overexpression of *GNBP3* by Q-RT-PCR using the relevant primer set (see Supp. Mat). Heat-shocks were performed as follows : 20 minutes at 37°C, 20 minutes at 18°C, 20 minutes at 37° C, 1 hour at 29°C and then 24 hours incubation at 25°C. Two large deficiencies that remove about 40 kb of the genomic region were generated by crossing d01793 flies to either d08034 or d01127 flies. These strains carry modified P-elements that contain FRT recombination sites and selected progeny of the cross also carried a transgene expressing the yeast flippase. The offspring was screened by PCR to isolate the desired recombinants.

Binding assay

Curdlan (insoluble polymeric β-(1,3)-glucan), a kind gift of JP. Latgé, peptidoglycan from *S. aureus*, a kind gift of Y. G. Boneca (Paris), chitin (β-(1,4)-N-acetyl-D-glucosamine) from crab shells, Aldrich Chem. Comp, Inc, and paraformaldehyde-treated *Candida Albicans* yeast were used for *in vitro* binding assay of GNBP3. All preparations were resuspended in pyrogen-free water (ACILA GMN). 100 μg of each insoluble polysaccharide or 10 μl of PFA-fixed yeast was added to 5 μg of purified GNBP3 and incubated in 200 μl of binding buffer (10mM Tris-HCl, pH 7.5, 500mM NaCl) at room temperature with mild agitation for 1 hour. The mixture was centrifuged (14,000g for 5 min) and the pellet was washed three times with 0.5 ml of washing buffer (10mM Tris, pH 7.5, 500 mM NaCl, 0.02% Tween 20). The proteins bound on insoluble polysaccharide or yeast cells were detached by adding SDS-PAGE sample buffer and analyzed by Western blot using affinity-purified mouse monoclonal anti-His antibody coupled to horseradish peroxidase following the manufacturer's instructions (Penta-His HRP Conjugate Kit, Qiagen).

The PGN and PBS (phosphate buffer saline, 10 μl, as a control) binding assay was carried out by essentially the same method as described above except the binding mixture was centrifuged at 14,000g for 30 min at each step to precipitate small particles of PGN. Further experimental information is found in the Supplementary online Material section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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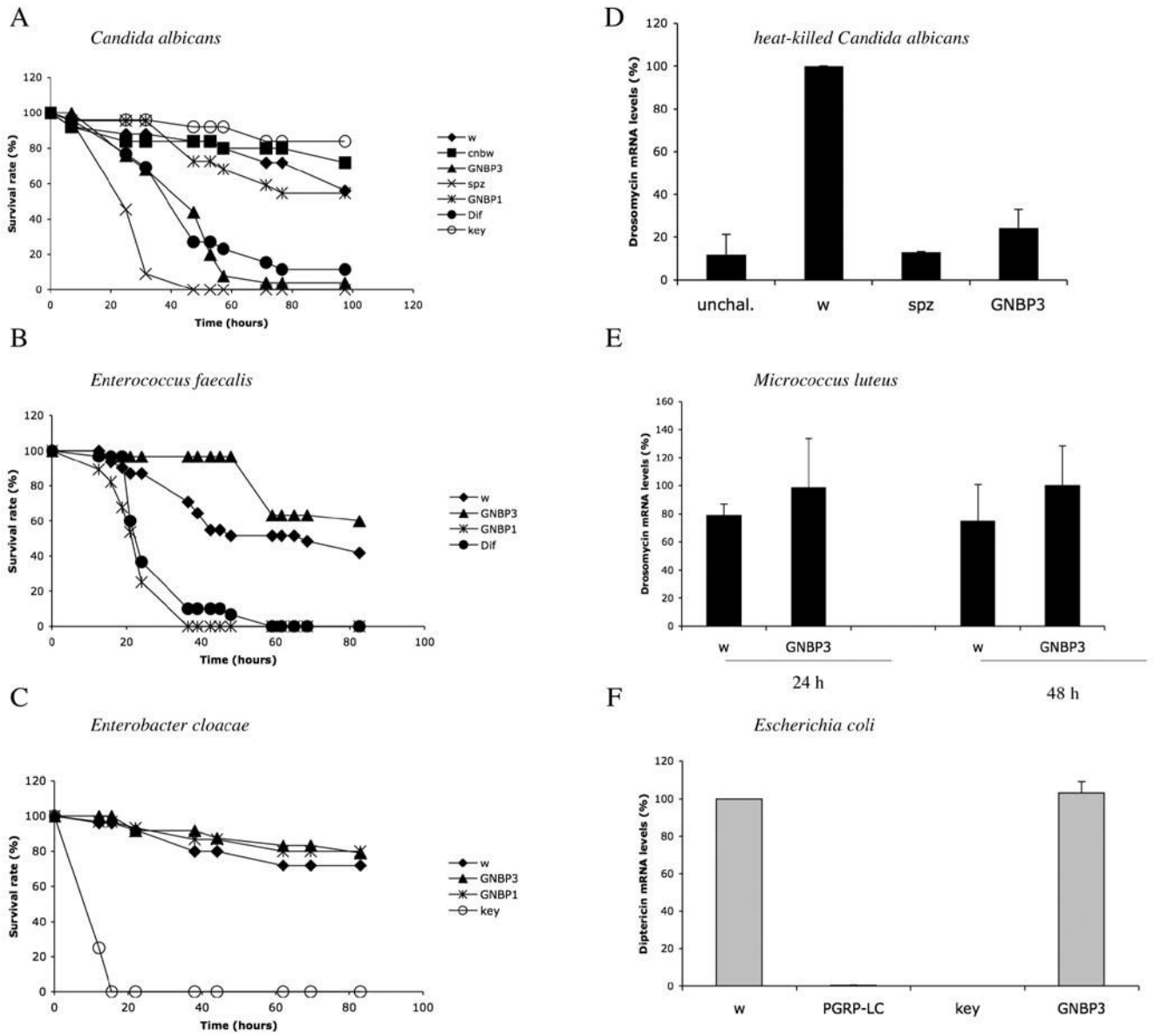

Figure 1 . GNBP3 is required in the host defense against yeast infections

A–C : survival experiments were performed at 29 °C (*C. albicans* : A) or 25°C (*E. faecalis* : B, *E. cloacae* : C). The survival rate expressed in percentage is shown. *w : white* A5001 and *cn bw* flies were used as wild-type controls. D–F : expression of antimicrobial peptide genes determined by real-time PCR. Results are expressed as a percentage of the induction observed in *w* control flies. *Drosomycin* expression was measured 24 hours after a challenge with heat-killed *C. albicans* (D; unchal. : unchallenged control flies). *Drosomycin* RNA levels were monitored 24 and 48 hours after a challenge with *M. luteus* (E). *Diptericin* inducibility was checked 6 hours after a septic wound with a needle dipped into a concentrated *E. coli* solution. *GNBP1*, *Dif*, *spz* (*spätzle*) are mutants of the Toll pathway whereas *key* (*kenny*) and PGRP-LC are mutants of the IMD pathway.

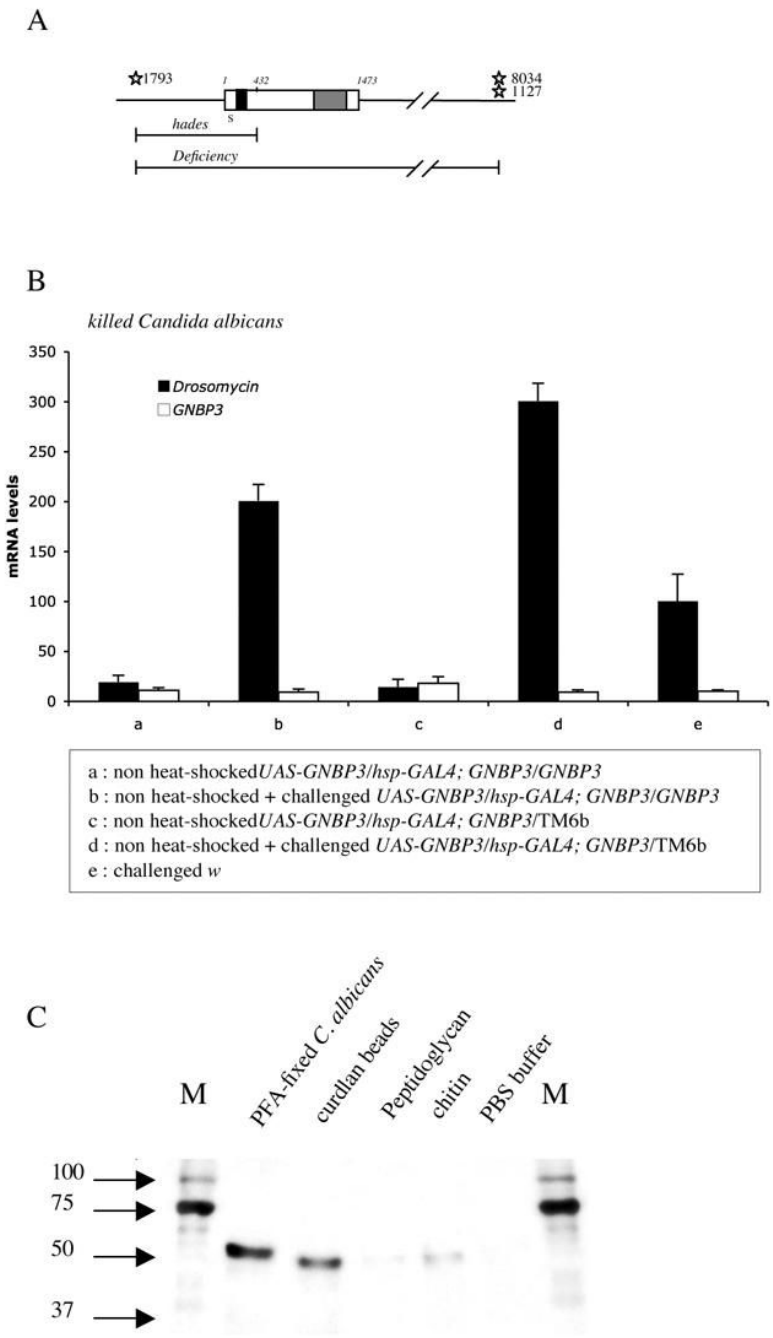

Figure 2 . the *hades* mutation affects the *GNBP3* locus that encodes a glucan recognition protein

A : scheme of the *GNBP3* locus. The *GNBP3* gene does not contain any intron. The upper line represents the genomic organization. The open bar shows the structure of the GNBP3 gene (nucleotides 1–1473) that encodes an N-terminal β-(1,3)-glucan binding domain (black domain) and a C-terminal β-glucanase homology region (grey domain). The signal peptide (S) is indicated. The sites of insertions of modified P-elements present in the strains d01793, d08034, and d01127 are shown (stars). The extent of the *hades* deficiency (up to nucleotide 432 of *GNBP3*) as well as that of a large deficiency are depicted (lower lines). The latter deficiency was used in genetic tests to check that $GNBP3^{hades}$ is a null mutation.

B : rescue experiment of the *GNBP3* mutant phenotype by a UAS-*GNBP3* transgene after a challenge with heat-killed *C. albicans*. The *Drosomycin* RNA steady-state levels (black bars) were measured in flies of the indicated genotypes. White bars : level of expression of *GNBP3* placed under the control of the *hsp* promoter using the UAS GAL4 system. The flies were not heat-shocked so as to avoid the constitutive activation of the Toll pathway by overexpressed GNBP3 (see Fig. 3A). TM6b is a balancer of the third chromosome; heterozygous *GNBP3*/TM6b sibling flies are used as controls.

C : Recombinant His-tagged GNBP3 protein was incubated with several insoluble oligosaccharides found in the cell walls of various microorganisms. After centrifugation and washing steps, the proteins associated with the precipitated glycan chains were recovered in Laemmli buffer and analyzed by Western blotting with a poly-His-specific antibody. GNBP3 binds to paraformaldehyde-fixed (PFA) *C. albicans* blastospores or curdlan beads (long chains of β-(1,3)-glucan) and hardly to peptidoglycan from the Gram-positive bacterium *Staphylococcus aureus*, or to chitin. PBS : phosphate buffer alone, M : marker (kDa).

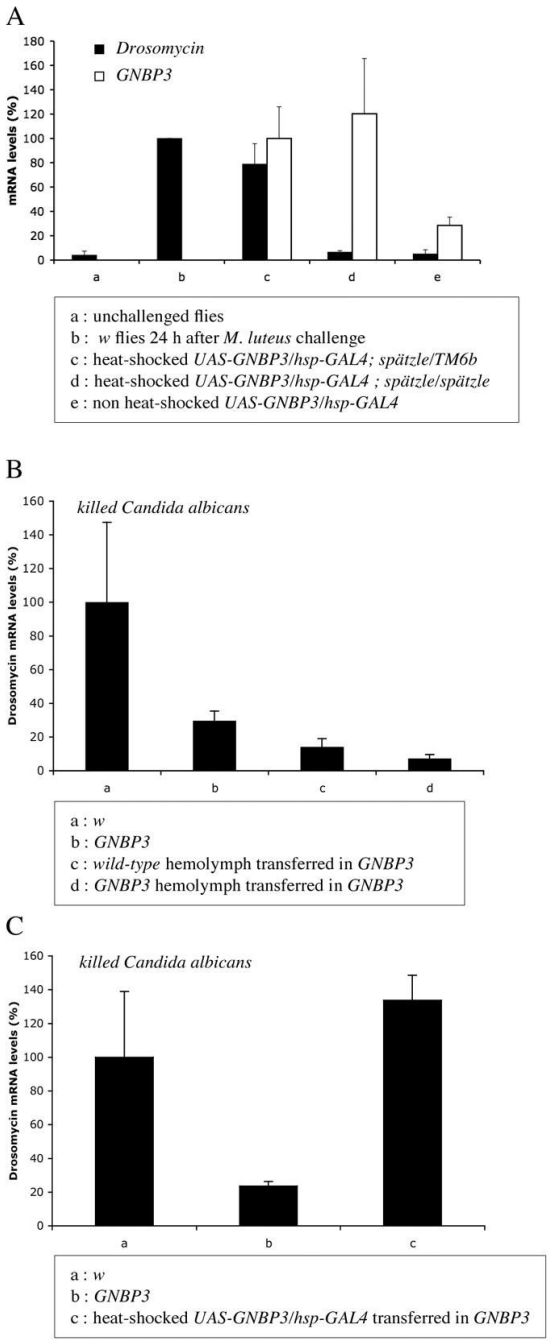

Figure 3 . Fungal detection can be mediated by secreted GNBP3, which functions upstream of the Toll ligand Spätzle

Drosomycin transcript levels as measured by quantitative RT-PCR are shown as black bars. A : Epistatic analysis of the relationship between *GNBP3* and *spz*. The expression of *Drosomycin* induced by *GNBP3* overexpression under *hsp* promoter control is blocked in a *spz* mutant background. White bars represent *GNBP3* mRNA levels as measured by quantitative RT-PCR.

B : *Drosomycin* induction by the injection of heat-killed *C. albicans* is not restored by the transfer of wild-type hemolymph into $GNBP3^{hades}$ mutant flies.

C : The transfer of hemolymph from flies overexpressing *GNBP3* leads to a restored inducibility of *Drosomycin* expression upon the same challenge as in (B).

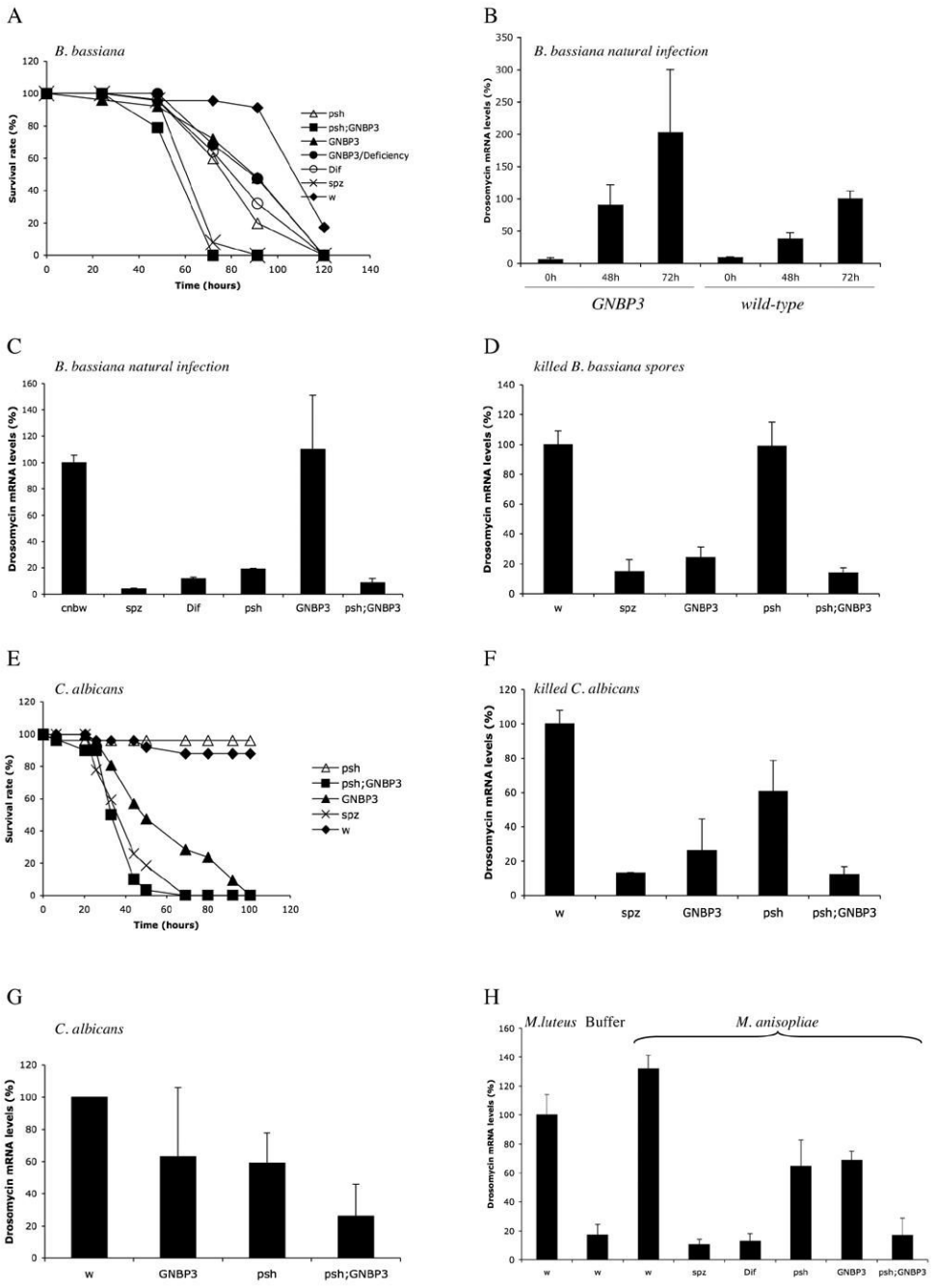

Figure 4 . Distinct phenotypes of *psh* and *GNBP3^{hades}* in response to fungal challenges

A : Survival of *psh* and *GNBP3^{hades}* flies to natural *B. bassiana* infections. The genotypes of the infected flies are indicated.

B : Expression of *Drosomycin* as determined by quantitative RT-PCR after a natural fungal infection at 29°C with *B. bassiana* in wild-type and *GNBP3^{hades}* flies. The expression was measured 0, 48, and 72 hours after coating the flies with fungal spores. The difference between wild-type and mutant flies at 72 hours is not statistically significant.

C : The expression of *Drosomycin* induced by a natural *B. bassiana* infection (48 hours) is blocked in *psh* and other mutants of the Toll pathway, but not in *GNBP3^{hades}* mutants. Similar results were obtained when *B. bassiana* spores were injected.

D : The expression of *Drosomycin* induced by the injection of 9.2 nl of killed alkali-treated *B. bassiana* spores (48 hours) is blocked in *GNBP3*^{hades} and *spz* mutants, but not in *psh* mutants.
E : Survival of *psh* and *GNBP3*^{hades} flies to infections with *C. albicans : psh* is not required in the host defense against this infection.
F : *psh* does not have a major effect on *Drosomycin* inducibility by heat-killed *C. albicans.* Similar results were obtained with paraformaldehyde-treated *C. albicans*.
G : The expression of *Drosomycin* induced by living *C. albicans* yeasts is decreased significantly only in *psh ; GNBP3*^{hades} double-mutant flies. The intermediate reduction of *Drosomycin* expression in *GNBP3*^{hades} mutants in response to live *C. albicans* may be due to *Candida* proteases, which, like entomopathogenic virulence factors, may trigger partially the PSH pathway and thus bypass the GNBP3 pathway.
H : *psh* and *GNBP3*^{hades} mutations do not block the induction of *Drosomycin* expression by injected *M. anisopliae* spores (24 hours). M. l : flies infected with *M. luteus* were taken as a 100% reference. Similar results were obtained in natural infections.

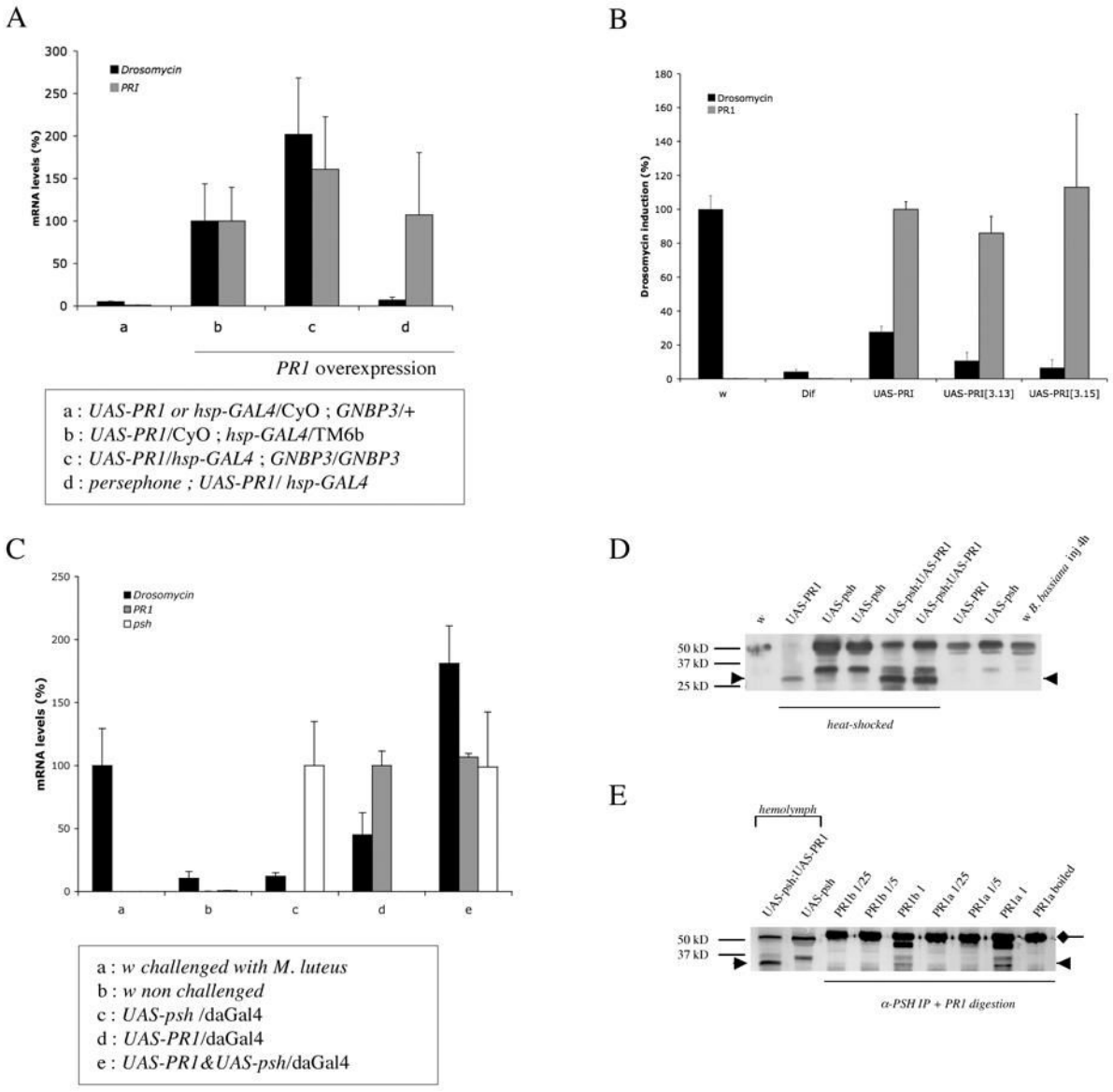

Figure 5 . The proteolytic activity of *Metarhizium anisopliae* PR1A triggers the *psh*-dependent expression of *Drosomycin*

A : The overexpression of *M. anisopliae PR1* in unchallenged wild-type (b) or *GNBP3*^{hades} mutant flies (c) induces the expression of *Drosomycin* 24 hours after heat-shock as measured by quantitative RT-PCR (black bars). However, this induction is blocked in a *psh* background (d). Background levels of *Drosomycin* expression are observed in wild-type unchallenged siblings of the *hsp-Gal4* to *UAS-PR1* cross (a). The level of expression of the *PR1* transcript is shown (grey bars). mRNA levels measured in (b) are taken as reference. The expression of the Drosomycin peptide has also been checked by MALDI-TOF mass spectrometry and coincides with the level of expression of the transcript.

B : The catalytic activity of PR1A is required for the induction of *Drosomycin* expression. Two transgenic lines expressing a mutated version of the PR1A (S to A mutation of the catalytic triad), [3.13] and [3.15] fail to induce *Drosomycin* expression. The induction observed in *M. luteus*-challenged *w* flies is taken as 100%. The difference of *Drosomycin* expression observed between UAS-PR1 and UAS-PR1[3,13 (or3,15)] overexpressing flies is statistically significant

whereas that between *M. luteus*-challenged *Dif* flies and UAS-PR1[3,13 (or 3,15)] overexpressing flies is not.

C : Synergistic activation of the Toll pathway by the joint overexpression of PR1A and PSH. The transgenes are expressed under the control of a *tub*-Gal80^{ts}; *daughterless*-GAL4 driver for 24 hours at 29°C, the restrictive temperature for the thermosensitive mutant GAL80, which represses GAL4 at permissive temperatures. This strategy enables the obtention of flies of the desired phenotype that would otherwise die during development as a result of PR1 overexpression.

D : Hemolymph was collected from wild-type (*w*) or *hsp-GAL4* transgenic flies carrying also the indicated UAS transgenes and analyzed by SDS-PAGE and Western blotting with a polyclonal antibody raised against PSH. The overexpression of *psh* from two distinct *psh* trangenes leads to the appearance of a 33 kD band while the expression of fungal PR1 induces the formation of a 28 kD band (arrowhead), although a faint 33 kD band could also be observed in some experiments. Interestingly, the *Bombyx mori* clip protease BAEEase is processed first into a 33 kD band, and second, into a 29.5 kD band, the latter processing event being essential for activation (Jang et al., 2006). The two lanes with flies overexpressing both *psh* and *PR1* correspond to two distinct sets of *psh* and *PR1* transgenes.

E : Hemolymph was collected from wild-type flies and immunoprecipitated with a PSH-specific antibody. The proteins were then digested with PR1 purified preparations and analyzed by Western blotting with the PSH antibody. The 28 (arrowhead) and 33kD proteins obtained by *PR1* and *psh* overexpression are shown on the left. The unprocessed 50 kD PSH band is masked by the Ig heavy chain.

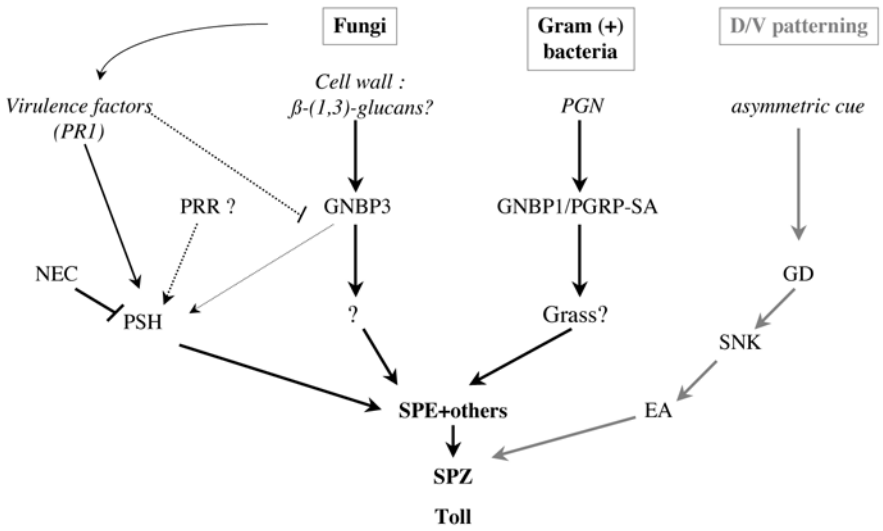

Figure 6 . Model of Toll pathway activation

We hypothesize that at least four distinct proteolytic cascades converge to process the Toll ligand Spätzle (SPZ) (see text). Dorso-ventral (D/V) patterning occurs during early embryogenesis and involves the proteases Gastrulation Defective (GD), Snake (SNK), and Easter (EA) : this proteolytic cascade is unlikely to be involved in the activation of *Drosomycin* expression by fungi (MG, unpublished data). In addition to sensing virulence factors, PSH might function downstream of an unknown Pattern Recognition receptor (PRR). Indeed, epistatic analysis indicates that PSH partially functions downstream of GNBP3. PGN : peptidoglycan; SPE : Spätzle processing enzyme.