In Vivo RNA Interference Analysis Reveals an Unexpected Role for GNBP1 in the Defense against Gram-positive Bacterial Infection in Drosophila Adults*

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The Drosophila immune system discriminates between different classes of infectious microbes and responds with pathogen-specific defense reactions via the selective activation of the Toll and the immune deficiency (Imd) signaling pathways. The Toll pathway mediates most defenses against Gram-positive bacteria and fungi, whereas the Imd pathway is required to resist Gram-negative bacterial infection. Microbial recognition is achieved through peptidoglycan recognition proteins (PGRPs); Gram-positive bacteria activate the Toll pathway through a circulating PGRP (PGRP-SA), and Gram-negative bacteria activate the Imd pathway via PGRP-LE, a putative transmembrane receptor, and PGRP-LC. Gram-negative binding proteins (GNBPs) were originally identified in Bombyx mori for their capacity to bind various microbial compounds. Three GNBPs and two related proteins are encoded in the Drosophila genome, but their function is not known. Using inducible expression of GNBP1 double-stranded RNA, we now demonstrate that GNBP1 is required for Toll activation in response to Gram-positive bacterial infection; GNBP1 double-stranded RNA expression renders flies susceptible to Gram-positive bacterial infection and reduces the induction of the antifungal peptide encoding gene Drosomycin after infection by Gram-positive bacteria but not after fungal infection. This phenotype induced by GNBP1 inactivation is identical to a loss-of-function mutation in PGRP-SA, and our genetic studies suggest that GNBP1 acts upstream of the Toll ligand Spätzle. Altogether, our results demonstrate that the detection of Gram-positive bacteria in Drosophila requires two putative pattern recognition receptors, PGRP-SA and GNBP1.

The innate immune response is activated by receptors known as pattern recognition receptors, which recognize surface determinants, such as lipopolysaccharide (LPS),1 peptidoglycan (PG), and mannan, that are conserved among microbes but absent in the host. After microbial recognition, pattern recognition receptors activate signaling cascades that regulate immune reactions (1). The Drosophila antimicrobial response has been the focus of intense study in recent years and provides a good genetic model for dissecting innate immunity (2–4). One of the landmarks of the Drosophila immune response is the synthesis of antimicrobial peptides by the fat body with distinct but overlapping specificities for different microbes. These peptides are secreted into the hemolymph, where they directly kill invading pathogens. Genetic analyses have shown that antimicrobial peptide genes are regulated by the Toll and immune deficiency (Imd) pathways. Two pathways share many common features with the mammalian Toll-like receptor (TLR) and tumor necrosis factor α receptor signaling cascades that regulate NF-κB transcription factors (2–4). The Toll and Imd pathways also activate NF-κB-like transactivators that, in turn, modulate specific transcriptional programs (5, 6). The Toll pathway is triggered by the proteolytic cleavage of the Toll ligand, Spätzle (Spz), and regulates the rel proteins dorsal immune-related factor (DIF) and Dorsal. This pathway is mainly activated by Gram-positive bacteria and fungi and controls, in large part, the expression of antimicrobial peptides active against fungi (e.g. Drosomycin) (7–10). In contrast, the Imd pathway mainly responds to Gram-negative bacterial infection and controls antibacterial peptide genes (e.g. Diptericin) via the rel protein, Relish (8, 11, 12).

Microbial recognition upstream of the Toll and Imd pathways is achieved, at least in part, through peptidoglycan recognition proteins (PGRPs) (13). PGRPs bind to PG, a component of the bacteria envelope, and are found in many species including insects and mammals (14, 15). In Drosophila, 13 PGRP genes have been identified (16), and three of them are currently implicated in the immune response; an extracellular recognition factor, PGRP-SA, activates the Toll pathway in response to Gram-positive bacterial but not fungal infection (17); PGRP-LC, a putative transmembrane protein, acts upstream of the Imd pathway (18–20); and PGRP-LE, which encodes a secreted PGRP, can activate the Imd pathway when overexpressed in flies (21). Recently, we demonstrated that the Imd pathway is activated by the recognition of DAP-type PG found in Gram-negative and Bacillus bacterial species, whereas the Toll pathway is more responsive to the lysine-type peptidoglycan recognition proteins; GNBP, Gram-negative binding proteins; Imd, immune deficiency; PG, peptidoglycan; TLR, Toll-like receptor; RNAi, RNA interference; RT-PCR, real-time PCR; Spz, Spätzle; IR, inverted repeat; GFP, green fluorescent protein.
PG found in most Gram-positive bacteria (22). Thus, the *Drosophila* immune system activates pathogen-specific immune response, at least in part, through the recognition of different forms of PG.

Gram-negative binding proteins (GNBPs) form a second class of immune recognition proteins that are initially identified in the Silkworm *Bombyx mori* for their ability to bind Gram-negative bacteria (23). In contrast to PGRPs, GNBPs are only used in invertebrates and contain an inactive B1-3-glucanase like domain that is similar to several bacterial glucanase. This domain is also found in B-glucan recognition proteins (βGNPs) that are implicated in glucan sensing in insects and crustaceae (24–27). The *Drosophila* genome encodes three GNBPs and two immune inducible related genes (28). The overexpression of GNBPs in *Drosophila* cell culture enhanced the LPS-mediated induction of AMP genes in cultured cells, suggesting a role in the *Drosophila* antimicrobial response (28). However, the exact function of GNBPs in the *Drosophila* immune response remained unknown. Using a genetic approach, we now report that GNBPs is required to activate the Toll pathway in response to Gram-positive bacterial infection.

**MATERIALS AND METHODS**

Fly Stocks—RNA interference (RNAi) transgenic fly lines of GNBPs were obtained using the inducible RNAi method. A 500-bp-long cDNA fragment (nucleotide position, 122–282 of the coding sequence) was amplified by PCR and inserted as an inverted repeat (IR) in a modified pUAST transformation vector, pUAST-R57, which possesses an IR formation site consisting of paired KpnI-CpoI and Xbal-SfiI restriction sites. The pUAST-R57 has a 292-bp-long genome fragment of the *Drosophila* Ret oncogene, in which introns 5 and 6 are contained between two IR fragments to enhance the effect of RNAi (29). The IR was constructed in a head-to-head orientation by using a combination of tag sequences of PCR primers and restriction sites on the vector. Detailed cloning procedures will be described elsewhere. Transformation of *Drosophila* embryos was carried out in w1118 fly stock. Each experiment was repeated using two independent UAS-RNAi insertions. The GAL4 drivers have been described previously (30). In this study, we used adult flies carrying one copy of the UAS-RNAi construct combined with one copy of the GAL4 driver. The GNBPs-IR1 and GNBPs-IR2 insertions are located on the second chromosome. A stable line carrying GNBPs-IR2 and da-GAL4 was used in this study (GNBP1-IR2; da-GAL4). da-Gal4, Dd1 (y w, Piry+, Diptericin-lacZ), P[w+], Drosomycin-GFP) flies were used as wild-type strains (31). Stocks were maintained at 25 °C using standard medium.

**Infection and Survival Experiments**—Bacterial and fungal infections were performed by pricking adults with a thin needle dipped previously into a concentrated culture of bacteria. Natural infections with Beauveria bassiana were performed by shaking anesthetized flies for a few seconds in a Petri dish containing a sporeulating fungal culture (7). The young progeny. We confirmed by real-time PCR (RT-PCR) that overexpression of GNBPs-IR1 shows no detectable defects, indicating that GNBPs is not essential for development (data not shown). We first assayed the susceptibility of GNBPs-IR flies and other mutant lines to infection by six micro-organisms. We pricked flies with a Gram-negative bacteria (*E. coli*), two Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*), and the fungus *Aspergillus fumigatus*, and we naturally infected with the entomopathogenic fungus *B. bassiana*. Fig. 1A and B shows that GNBPs-IR flies succumb to infection by *E. coli* and *S. aureus*. The GNBPs-IR phenotype is similar to the phenotype induced by mutation in *PGRP-SA* and *spz*, two mutations affecting upstream components of the Toll pathway. We noticed that GNBPs-IR flies exhibit a slightly lower susceptibility to these two Gram-positive bacteria species when compared with *spz* and *PGRP-SA* mutants. In contrast to *spz* flies, GNBPs-IR flies were resistant to fungal infection. Finally, GNBPs-IR flies also resisted infection by Gram-negative bacterial infection, whereas the *relish* mutants rapidly succumbed (Fig. 1, C–E). This survival analysis demonstrates that the GNBPs gene product, like PGRPSA, is required to resist Gram-positive bacterial infection.

**RESULTS**

Silencing of GNBPs Mediates Drosomycin Expression in Response to Gram-positive Bacterial Infection—In this study, we have used the inducible expression of GNBPS-IR double-stranded RNA to analyze the role of GNBPs in the *Drosophila* immune response. This approach, which exploits the UAS/GAL4 binary system to drive expression of double-stranded RNA in a defined tissue, is a form of RNAi that has been shown previously to block the expression of defined genes without interfering with the *Drosophila* immune system (30, 34).

We have generated transgenic flies carrying the UAS-GNBPs-IR element. This construct consists of two 500-bp-long inverted repeats (IR) of the GNBPs gene, separated by an intronic DNA sequence that acts as a spacer, to give a hairpin-loop shaped RNA. Two independent UAS-GNBPs-IR insertions were used in this study (GNBP1-IR1 or GNBPs-IR2). These transgenic flies were crossed to flies carrying GAL4 drivers that express the GAL4 protein strongly and ubiquitously to activate transcription of the hairpin-encoding transgene in the progeny. We confirmed by real-time PCR (RT-PCR) that overexpression of UAS-GNBPs-IR leads to significant decrease of GNBPs transcripts (data not shown).

To address the role of GNBPs in the *Drosophila* host defense, we expressed the UAS-GNBPs-IR transgene using the daughterless-GAL4 (da-GAL4) and Actin5C-GAL4 ubiquitous GAL4 insertions (data not shown for Act5C-GAL4). Flies that express GNBPs-IR ubiquitously through da-GAL4 (referred to as GNBPs-IR) show no detectable defects, indicating that GNBPs is not essential for development (data not shown). We first assayed the susceptibility of GNBPs-IR flies and other mutant lines to infection by six micro-organisms. We pricked flies with a Gram-negative bacteria (*E. coli*) and *Aspergillus fumigatus*, the fungus *Staphylococcus aureus*, and we naturally infected with the entomopathogenic fungus *B. bassiana*. Fig. 1A and B shows that GNBPs-IR flies succumb to infection by *E. coli* and *S. aureus*. The GNBPs-IR phenotype is similar to the phenotype induced by mutation in *PGRP-SA* and *spz*, two mutations affecting upstream components of the Toll pathway. We noticed that GNBPs-IR flies exhibit a slightly lower susceptibility to these two Gram-positive bacteria species when compared with *spz* and *PGRP-SA* mutants. In contrast to *spz* flies, GNBPs-IR flies were resistant to fungal infection. Finally, GNBPs-IR flies also resisted infection by Gram-negative bacterial infection, whereas the *relish* mutants rapidly succumbed (Fig. 1, C–E). This survival analysis demonstrates that the GNBPs gene product, like PGRPSA, is required to resist Gram-positive bacterial infection.

**GNBP1 Mediates Drosomycin Expression in Response to Gram-positive Bacterial Infection**—A previous study showed that a mutation in PGRPSA strongly reduces the expression of the antifungal peptide gene *Drosomycin* during Gram-positive bacterial infection (17). Using quantitative RT-PCR, we observed that expression of GNBPs-IR also decreases the expression of the *Drosomycin* gene after infection by *M. luteus*, a Gram-positive bacteria (Fig. 2A). This effect was observed in the 24- and 48-h time points when the *Drosomycin* expression reaches its maximal level. Quantitative measurements show that in GNBPs-IR flies, *Drosomycin* is expressed to 20–40% of wild-type level at 24 h after challenge (Fig. 2A, and see Figs. 3A, 4A, and 5). We also determined that overexpression of GNBPs-IR with the da-GAL4 driver affects the expression of a *Drosomycin-GFP* transgene in the adult fat body (Fig. 2B).

We next compared the effects of GNBPs RNAi on *Drosomycin* and *Diptericin* expression after challenge by different
classes of micro-organisms with other mutations affecting the Toll or the Imd pathway. These experiments confirm that the spz mutation blocks Drosomycin expression in response to both Gram-positive bacteria and fungi, whereas mutations in the Imd pathway (PGRP-LC and Dredd) affect Diptericin expression in response to Gram-negative bacterial infection. Fig. 3A shows that the expression of GNBP1-IR inhibits Drosomycin gene expression after challenge by the Gram-positive bacterial species M. luteus, although GNBP1-IR has a slightly weaker effect than the spz and PGRP-SA^emt mutations (Fig. 3A). On the other hand, the level of Drosomycin transcripts was comparable with wild-type (WT) after infection by the fungus A. fumigatus (Fig. 3C). Finally, GNBP1 silencing did not affect the expression of the Diptericin gene in response to Gram-negative bacteria (Fig. 3C). This pattern of antimicrobial peptide gene expression in the GNBP1-IR flies is similar to the pattern displayed in the PGRP-SA mutant. Thus, our results demonstrate that, like PGRP-SA, GNBP1 regulates the Drosomycin gene, a target of the Toll pathway, in response to Gram-positive bacterial infection.

**GNBP1 Functions Upstream of the Toll Ligand Spz—**Overexpression of a mature form of the Toll ligand spz leads to the constitutive transcription of the Drosomycin gene (35) (Fig. 4A). Fig. 4 shows that expression of GNBP1-IR did not reduce Drosomycin expression in flies that overexpress spz, indicating that GNBP1 does not function downstream of Spz. This observation and the report that GNBP1 is present in the culture medium of...
mbn-2 cells (25) strongly suggest that GNBP1 functions as a secreted microbial recognition factor upstream of Spz.

**PGRP-SA and GNBP1 Function in the Same Pathway**—We next tested whether GNBP1 and PGRP-SA function in a synergistic fashion; i.e., do flies carrying a mutation affecting both GNBP1 and PGRP-SA have a stronger phenotype than either single mutant? For this experiment, we expressed GNBP1-IR...
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Fig. 5. Gram-positive bacterial lysine-type PG-mediated Drosomycin expression requires both GNBP1 and PGRP-SA. Quantitative RT-PCR analysis was performed with total RNA extracts from wild-type (WT, da-GAL4/+ ) and mutant females collected 24 h after injection with 9 nl of M. luteus PG (IPG = 5 mg/ml). In flies carrying mutations affecting the Toll pathway (spz−/− and PGRP-SA−/−) or overexpressing the GNBP1-IR construct, Drosomycin expression by M. luteus PG is blocked. Mutations affecting the Imd pathway (henny1, PGRP-LCE12) did not affect Drosomycin expression after injection of lysine-type PG.

with da-GAL4 in PGRP-SA−/− flies. Fig. 4B shows that inactivation of GNBP1 by gene silencing did not significantly increase the PGRP-SA phenotype. Both GNBP1-IR; PGRP-SA−/− flies and PGRP-SA flies died at the same rate after infection by the Gram-positive bacteria E. faecalis and failed to express the Drosomycin gene (Fig. 4, A and B). No additional phenotype was observed in GNBP1-IR; PGRP-SA−/− flies. This points out that PGRP-SA and GNBP1 do not work in synergy but are both required to regulate Drosomycin expression. Altogether, our genetic analysis indicates that GNBP1 and PGRP-SA are both required to regulate the Toll pathway in response to Gram-positive bacterial infection and probably function upstream of Spz.

GNBP1 Is Required for Toll Activation in Response to Lysine-type PG—GNBP1 was initially identified as a pattern recognition receptor for LPS or β-3 glucan (25); however, our present results demonstrate a role of GNBP1 in the response to Gram-positive bacterial infection. Recently, we have reported that lysine-type PG, a PG form found in most Gram-positive bacteria, is a very potent inducer of Drosomycin, suggesting that PG is one of the main bacterial determinants of Gram-positive bacteria recognized by the Toll pathway (22). We also demonstrated that Drosomycin expression by lysine-type PG is mediated through PGRP-SA and Spz (22) (Fig. 5). To determine whether the Drosomycin induction by lysine-type PG also requires GNBP1, we injected GNBP1-IR flies with 9 nl of a solution of highly purified PG extracted from the Gram-positive bacterial species M. luteus and monitored the level of Drosomycin expression by RT-PCR. Fig. 5 clearly shows that overexpression of GNBP1-IR blocks the induction of Drosomycin in response to lysine-type PG as observed in PGRP-SA and spz mutants. This experiment demonstrates that GNBP1 is required either for the direct recognition of lysine-type PG or in a step downstream of the recognition event.

DISCUSSION

In this study, we have identified GNBP1, a putative pattern recognition receptor, as a regulator of the Drosophila antimicrobial response to Gram-positive bacteria. GNBP1 inactivation by RNAi induces a high susceptibility to infection by Gram-positive bacterial species and reduces the expression of the Drosomycin gene after challenge by Gram-positive bacteria and lysine-type PG. The GNBP1 phenotypes we observed are identical to the phenotypes induced by a mutation in PGRP-SA. We observed, however, that GNBP1-IR flies have a slightly weaker phenotype than PGRP-SA−/− flies that carry a genet-ically null mutation in PGRP-SA. It is generally assumed that RNAi mimics partial loss-of-function mutations of the target gene. Thus, we cannot exclude that a null mutation in GNBP1 may induce a stronger phenotype than those described here.

Several studies have already demonstrated that Drosomycin expression is tightly regulated by the Toll pathway in response to Gram-positive bacterial infections. Therefore, our results strongly suggest that, like PGRP-SA, GNBP1 regulates the Toll pathway in response to Gram-positive bacterial infection. Our observation that GNBP1-IR does not interfere with the constitutive expression of Drosomycin induced by the overexpression of a mature form of Spz suggests that GNBP1 acts upstream of the Toll ligand. A role for GNBP1 in the extracellular compartment is supported by the observation that GNBP1 is secreted into the culture medium of mbn-2 cells (25).

The implication of GNBP1 in the response to Gram-positive bacteria was unexpected since GNBP1 contains a mutated β-3 glucanase domain that is present in β-glucan recognition protein of other insects (24–27). However, our data are supported by a recent study in the silkworm B. mori showing that a specific anti-GNBP antibody blocks the PGRP-mediated activation of the prophenoloxidase cascade by PG but not by β1-3-glucan. Although there is no evidence for direct interaction between GNBP1 and a Gram-positive bacterial compound, this biochemical study and our genetic results point to a clear implication of some members of the GNBP family in the activation of immune response by PG. The complexity of pattern recognition receptor/microbial ligand interactions was recently underlined by the implication of a PGRP from the beetle Holotrichia diomphalia in the activation of the prophenoloxidase cascade in response to β1,3-D glucan (36). Therefore, it is not surprising that GNBP1s may also be involved in the recognition of distinct classes of micro-organisms.

The similarities between the phenotypes induced by the PGRP-SA mutation and GNBP1 RNAi and our observation that GNBP1 inactivation did not block Drosomycin induction by Spz expression suggest that both proteins function in the same extracellular pathway that links Gram-positive bacterial recognition to activation of Spz by serine protease(s). It has already been reported that in vitro, PGRP-SA binds to lysine-type PG found on Gram-positive bacteria cell walls. Our observation that the activation of the Toll pathway by lysine-type PG requires both PGRP-SA and GNBP1 indicates that the two proteins cannot function independently. The implication of two putative pattern recognition receptors in sensing Gram-positive bacteria is reminiscent of the situation observed in mammals, where protein complexes rather than a single recognition receptor participate in LPS and Glucan recognition. In vertebrates, it is proposed that CD14 transfers LPS to the co-receptor MD2/TLR4 (37). GNBP1 may play a similar function by acting upstream of PGRP-SA. However, we believe that this is not likely because PGRP-SA is a secreted protein that binds to lysine-type PG by itself in vitro (16). Alternatively, GNBP1 may be part of a recognition complex with PGRP-SA. Under this hypothesis, GNBP1 could facilitate PG sensing by PGRP-SA or could bind to another factor from Gram-positive bacteria (for example, lipotechoic acid or techoic acid). Finally, we cannot exclude that GNBP1 is not directly involved in microbial recognition but functions as a downstream adaptor.

3 M. Ochiai and M. Ashida, personal communication.
that links PGRP-SA to the serine protease that processes Spz. The crystal structure of PGRP-LB has revealed the presence of a hydrophobic groove in the PGRP domain that may be involved in protein-protein interactions (38). GNBP1 is a good candidate for the factor that interacts with a similar domain in PGRP-SA to establish a link between PGRP-SA and GNBP1 in Gram-positive bacteria sensing and to determine whether GNBP1 interacts directly with microbial ligands. In conclusion, using an RNAi approach, we demonstrate that GNBP1 plays a critical role in the antibacterial defense against Gram-positive bacteria. The existence of a specific phenotype that links PGRP-SA to the serine protease that processes Spz. Additional biochemical studies are required to elucidate the relationship between PGRP-SA and GNBP1 in Gram-positive bacteria sensing and to determine the exact function of the GNBP family.

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