THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 13, Issue of March 26, pp. 12848–12853, 2004
Printed in U.S.A.

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

Received for publication, December 5, 2003, and in revised form, January 7, 2004
Published, JBC Papers in Press, January 13, 2004, DOI 10.1074/jbc.M313324200

Sebastien Pili-Floury‡§, François Leulier‡¶, Kuniaki Takahashi‖, Kaoru Saigo, Emmanuel Samain§, Ryu Ueda‖, and Bruno Lemaitre‡ ‡‡**

*From the ‡Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette, France, the ‖Genetic Strains Research Center, National Institute of Genetics, Shizuoka 411-8540, Japan, the **Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan, and §Centre Hospitalier Universitaire, Université de Franche Comté, 3 Bd Fleming, 25030 Besançon, France*

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-LC, a putative transmembrane receptor, and PGRP-LE. 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. These 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

* This work was supported in part by grants from MITILS (to R. U.) and from the Mext of Japan (to K. S. and R. U.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a fellowship from the Fondation pour la Recherche Médicale.

‡‡ To whom correspondence should be addressed: Centre de Génétique Moléculaire, CNRS, F-91198 Gif-sur-Yvette Cedex France. Tel.: 33-1-69-82-32-27; Fax: 33-1-69-82-43-86; E-mail: lemaitre@cgm.cnrs-gif.fr.

[1] The abbreviations used are: LPS, lipopolysaccharide; PGRP, 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 were initially identified in the Silkworm *Bombyx mori* for their ability to bind Gram-negative bacteria (23). In contrast to PGRPs, GNBPs are only found in invertebrates and contain an inactive β1–3 glucanase like domain that is similar to several bacterial glucanase. This domain is also found in β-glucan recognition proteins (βGRPs) that are implicated in glucan sensing in insects and crustacae (24–27). The *Drosophila* genome encodes three *GNBPs* and two immune inducible related genes (28). The overexpression of *GNBP-1* in 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 GNBP1 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 *GNBP1* were obtained using the inducible RNAi method. A 500-bp-long cDNA fragment (nucleotide position, 122–621 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 XbaI-SfiI restriction sites. The pUAST-R57 has a 282-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.[2] Transformation of *Drosophila* embryos was carried out in w^{1118} 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 *GNBP1-IR1* and *GNBP1-IR2* insertions are located on the second chromosome. A stable line carrying *GNBP1-IR2* and *da-GAL4* was used in this study (*GNBP1-IR2; da-GAL4*)

da-Gal4, DD1 (*y, w, P(ry+, Diptericin-lacZ), P(w+, Drosomycin-GFP)*) flies were used as wild-type strains (31). spz^{rm7}, $kenny^{1}$, $PGRP\text{-}SA^{seml}$, $PGRP\text{-}LC^{E12}$, $Dredd^{B118}$, and $relish^{E20}$ alleles are described elsewhere (8, 11, 17, 19, 32). *Drosophila* 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 sporulating fungal culture (7). Bacterial and fungal strains were described previously (7). For more details on infection procedure, see Ref. 33. A highly purified solution of *Micrococcus luteus* PG was produced and injected in flies as described in (22).

Quantitative Real-time PCR—For *Drosomycin* and *Diptericin* mRNA quantification from whole animals, RNA was extracted using RNA TRIzol™. cDNAs were synthesized using SuperScript II (Invitrogen), and PCR was performed using dsDNA dye SYBR Green I (Roche Diagnostics). Primer pairs for *Diptericin* (sense, 5′-GCT GCG CAA TCG CTT CTA CT-3′, and antisense, 5′-TGG TGG AGT GGG CTT CAT G-3′), *Drosomycin* (sense, 5′-CGT GAG AAC CTT TTC CAA TAT GAT G-3′, and antisense, 5′-TCC CAG GAC CAC CAG CAT-3′), and control *Rp49* (sense, 5′-GAC GCT TCA AGG GAC AGT ATC TG-3′, and antisense, 5′-AAA CGC GGT TCT GCA TGA G-3′) were used to detect target gene transcripts. SYBR Green analysis was performed on a Lightcycler (Roche Diagnostics). All samples were analyzed in duplicate, and the amount of mRNA detected was normalized to control RP49 mRNA values. We used normalized data to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt).

[2] R. Ueda and K. Saigo, in preparation.

RESULTS

Silencing of GNBP1 Confers Susceptibility to Gram-positive Bacterial Infection—In this study, we have used the inducible expression of *GNBP1* double-stranded RNA to analyze the role of *GNBP1* 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-GNBP1-IR* element. This construct consists of two 500-bp-long inverted repeats (IR) of the *GNBP1* gene, separated by an intronic DNA sequence that acts as a spacer, to give a hairpin-loop shaped RNA. Two independent *UAS-GNBP1-IR* insertions were used in this study (*GNBP1-IR1* or *GNBP1-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-GNBP1-IR* leads to significant decrease of *GNBP1* transcripts (data not shown).

To address the role of GNBP1 role in the *Drosophila* host defense, we expressed the *UAS-GNBP1-IR* transgene using the *daughterless-GAL4* (*da-GAL4*) and *Actin5C-GAL4* ubiquitous GAL4 insertions (data not shown for *Act5C-GAL4*). Flies that express *GNBP1-IR* ubiquitously through *da-GAL4* (referred to as *GNBP1-IR*) show no detectable defects, indicating that *GNBP1* is not essential for development (data not shown). We first assayed the susceptibility of *GNBP1-IR* flies and other mutant lines to infection by six micro-organisms. We pricked flies with a Gram-negative bacteria (*Erwinia carotovara carotovora*), two Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*), or the fungus *Aspergillus fumigatus*, and we naturally infected with the entomopathogenic fungus *B. bassiana*. Fig. 1(*A* and *B*) shows that *GNBP1-IR* flies rapidly succumb to infection by *E. faecalis* and *S. aureus*. The *GNBP1-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 *GNBP1-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, *GNBP1-IR* flies were resistant to fungal infection. Finally, *GNBP1-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 *GNBP1* gene product, like PGRP-SA, 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 *PGRP-SA* 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 *GNBP1-IR* also decreases the expression of the *Drosomycin* gene after infection by *M. luteus*, a Gram-positive bacteria (Fig. 2*A*). This effect was observed at the 24- and 48-h time points when the *Drosomycin* expression reaches its maximal level. Quantitative measurements show that in *GNBP1-IR* flies, *Drosomycin* is expressed to 20–40% of wild-type level at 24 h after challenge (Fig. 2*A*, and see Figs. 3*A*, 4*B*, and 5). We also determined that overexpression of *GNBP1-IR* with the *da-GAL4* driver affects the expression of a *Drosomycin-GFP* transgene in the adult fat body (Fig. 2*B*).

We next compared the effects of *GNBP1* RNAi on *Drosomycin* and *Diptericin* expression after challenge by different

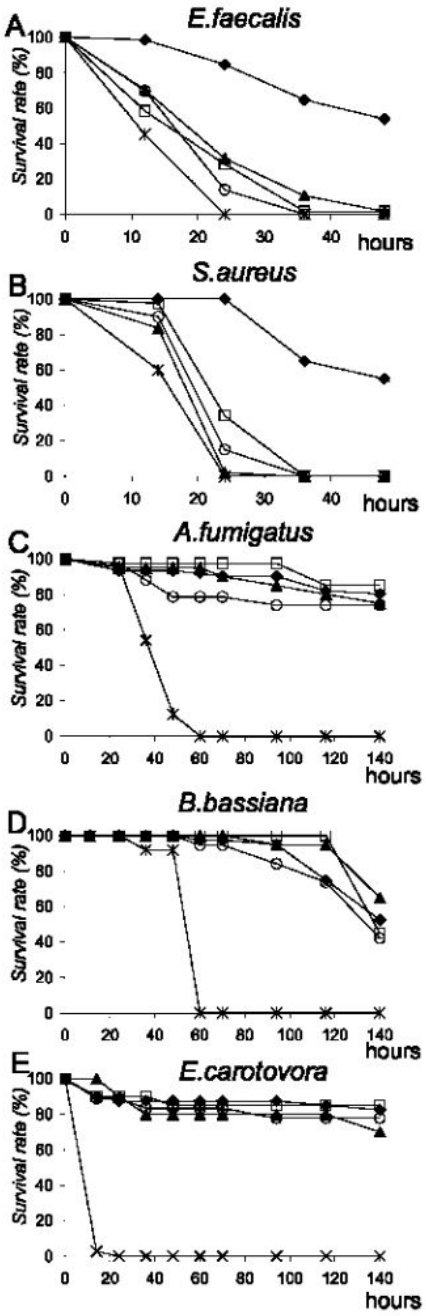

FIG. 1. GNBP1 is required for resistance to Gram-positive bacterial infection. The survival rates of *da-GAL4/+* (|), *da-GAL4; UAS-GNBP1-IR1* (M), *da-GAL4; UAS-GNBP1-IR2*(□), *PGRP-SA*^{seml} (E), *Relish*^{E20} (×), and *spz*^{rm7} (*) flies after infection by *E. carotovora carotovora 15* (Gram −), *E. faecalis* (Gram +), *S. aureus* (Gram +), *A. fumigatus* (fungi), and *B. bassiana* (fungi) are presented. 40 adults, aged 2–4 days, were pricked with a needle dipped previously into bacterial pellet (OD = 200 for *E. carotovora carotovora*, OD = 50 for *S. aureus*, and *E. faecalis*) or a concentrated spore solution of *A. fumigatus*; or naturally infected by *B. bassiana*. The infected flies were incubated at 29 °C and transferred to fresh vials every 3 days. *PGRP-SA*^{seml} and *da-GAL4; UAS-GNBP1-IR* adult flies are highly susceptible to infection by Gram-positive bacteria but resistant to Gram-negative or fungal infection (data not shown for *Act5C-GAL4*).

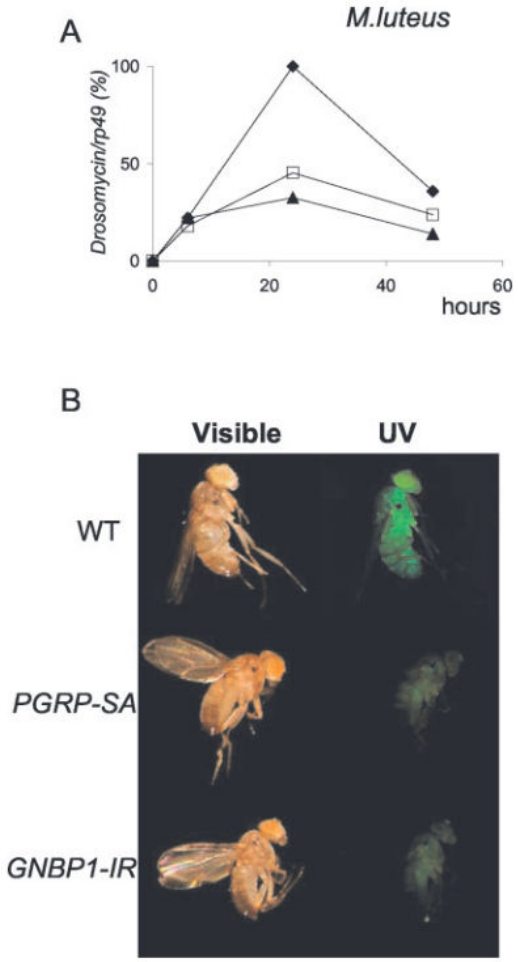

FIG. 2. GNBP1 regulates the expression of ***Drosomycin*** in response to Gram-positive bacterial infection. *A*, time course analysis of *Drosomycin* gene expression after septic injury with *M. luteus*. Quantitative RT-PCR analysis was performed on total RNA extracts from *da-GAL4/+* (|), *da-GAL4; UAS GNBP1-IR1* (M) or *da-GAL4; UAS GNBP1-IR2* (□) female flies collected at different time intervals (as indicated) after injection of *M. luteus*. This experiment was repeated twice and yielded similar results. *B*, *Drosomycin-GFP* expression in wild-type (*WT*, *top*), *PGRP-SA*^{seml} (*middle*), and *GNBP1-IR*; *da-GAL4* (*bottom*) flies collected 24 h after challenge by *M. luteus*. *Left*, bright field; *right*, GFP fluorescence.

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. 3*A* 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*^{seml} mutations (Fig. 3*A*). On the other hand, the level of *Drosomycin* transcripts was comparable with wild-type after infection by the fungus *A. fumigatus* (Fig. 3*B*). Finally, *GNBP1* silencing did not affect the expression of the *Diptericin* gene in response to Gram-negative bacteria (Fig. 3*C*). 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. 4*A*). 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

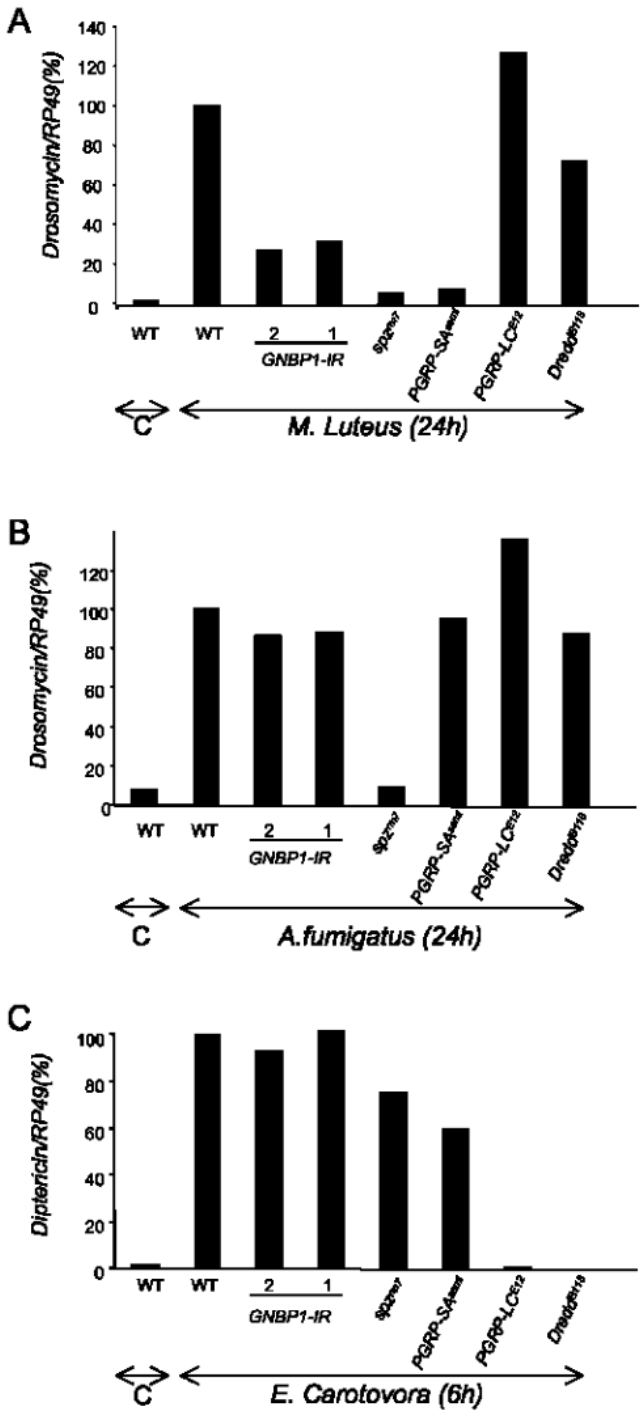

FIG. 3. GNBP1 inhibition has the same affect on ***Drosomycin*** expression as the ***PGRP-SA*** mutation. Quantitative RT-PCR analysis was performed with total RNA extracts from wild-type (*WT*, *da-GAL4/+*) and mutant females collected after septic injury with *M. luteus* (*A*), after injection of *A. fumigatus* spores (*B*), or after septic injury with *E. carotovora* (*C*). Flies carrying mutations affecting the Toll pathway (spz^{rm7} and $PGRP\text{-}SA^{seml}$) or the Imd pathway ($Dredd^{B118}$, $PGRP\text{-}LC^{E12}$) or overexpressing the *UAS-GNBP1-IR* with *da-GAL4* were collected at 24 h (*M. luteus* and *A. fumigatus*) or 6 h (*E. carotovora carotovora*) after injection. These RT-PCR analysis show that expression of *GNBP1-IR* with a *da-GAL4* driver affects the expression of the *Drosomycin* (*Drs*) gene after Gram-positive bacterial infections. Experiments were repeated twice and yielded similar results.

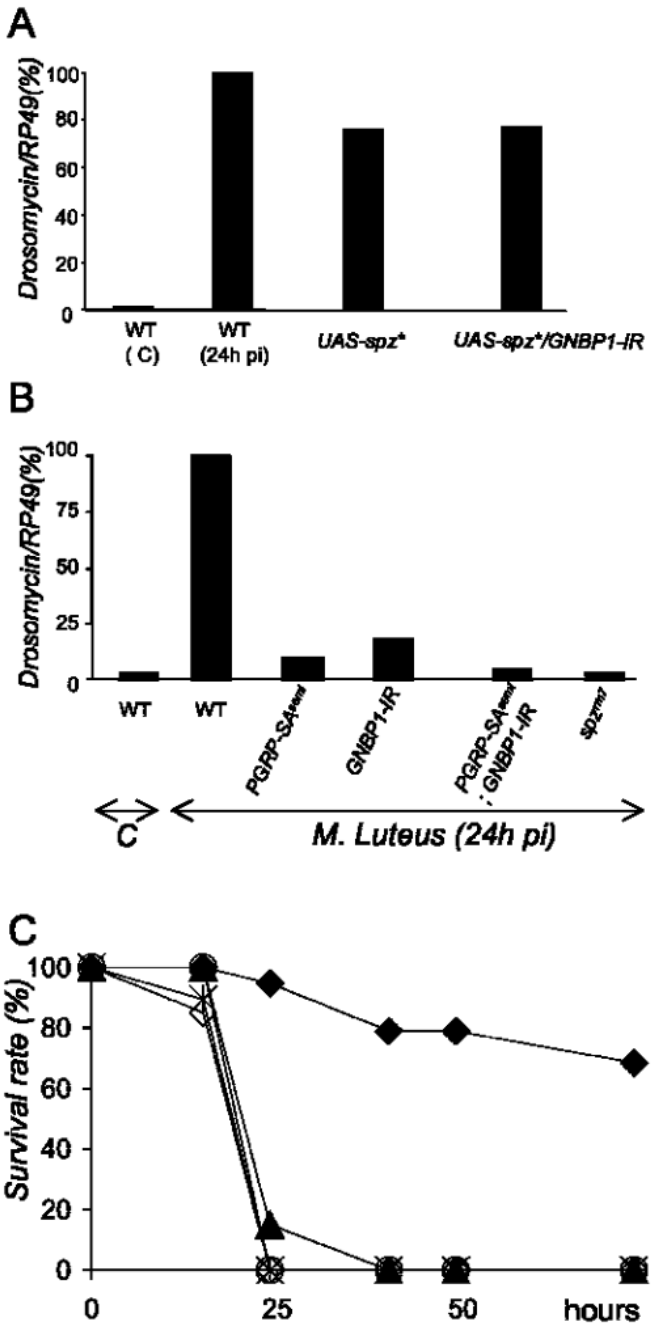

FIG. 4. GNBP1 functions upstream of Spz and does not synergize with PGRP-SA. *A*, overexpression of *UAS-spz** (encoding an active form of Spz) by the *da-GAL4* driver induced *Drosomycin* expression in the absence of challenge and independently of the *GNBP1* gene. The genotypes of the tested flies are UAS-*spz**/+; *da-GAL4/+* and UAS-*spz**/*GNBP1-IR*; *da-GAL4/+*. Noninfected flies (*C*) and flies collected 24 h after bacteria challenge with *M. luteus* were used for control of the *Drosomycin* expression range. *WT*, wild type. *B* and *C*, *Drosomycin* expression (*B*) and survival analysis (*C*) in $PGRP\text{-}SA^{seml}$ mutant flies overexpressing the *GNBP1-IR* construct. Overexpression of *GNBP1-IR* did not significantly enhance the PGRP-SA phenotype. *B*, RT-PCRs were performed on flies collected 24 h after septic injury with *M. luteus* as in Fig. 3*A*. *C*, the survival analysis was performed as follows: *da-GAL4/+* (❙), *da-GAL4; UAS-GNBP1-IR2* (□), $PGRP\text{-}SA^{seml}$; *UAS-GNBP1-IR2; da-GAL4* (●), $PGRP\text{-}SA^{seml}$ (E) and spz^{rm7} (×), flies as in Fig. 1*A*.

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 mutation affecting both *GNBP1* and *PGRP-SA* have a stronger phenotype than either single mutant? For this experiment, we expressed *GNBP1-IR*

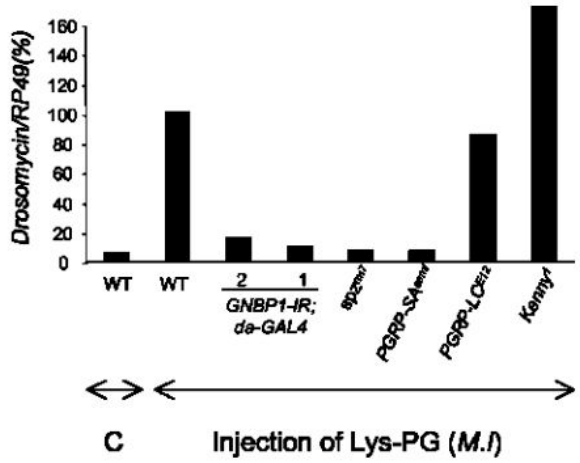

FIG. 5. Gram-positive bacterial lysine-type PG-mediated *Drosomycin* expression requires both GNBP1 and PGRP-SA. Quantitative RT-PCR analysis was performed with total RNA extracts from wild-type (*WT*, *da-GAL4*/+) and mutant females collected 24 h after injection with 9 nl of *M. luteus* PG ([PG] = 5 mg/ml). In flies carrying mutations affecting the Toll pathway (spz^{rm7} *and PGRP-SA*^{seml}) or overexpressing the *GNBP1-IR* construct, *Drosomycin* expression by *M. luteus* PG is blocked. Mutations affecting the Imd pathway ($kenny^1$, *PGRP-LC*^{E12}) did not affect *Drosomycin* expression after injection of lysine-type PG.

with *da-GAL4* in *PGRP-SA*^{seml} flies. Fig. 4*B* shows that inactivation of *GNBP1* by gene silencing did not significantly increase the PGRP-SA phenotype. Both *GNBP1-IR*; *PGRP-SA*^{seml} 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, *B* and *C*). No additional phenotype was observed in *GNBP1-IR*; *PGRP-SA*^{seml} 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 GNPB1 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 β1–3 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 GNPB1, 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 *GNBP-IR* flies have a slightly weaker phenotype than *PGRP-SA*^{seml} flies that carry a genetically 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 GNBPs contain a mutated β1–3 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 prophenoloxydase cascade by PG but not by β1–3-glucan.[3] 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 GNBPs 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 a putative downstream serine protease. Additional biochemical studies are required to elucidate the relationship 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 for GNBP1 demonstrates the absence of redundancy among the three *Drosophila* GNBP members as observed previously in other large families of recognition proteins such as TLRs and PGRPs. This study also confirms the power of the inducible expression of the double-stranded RNA technique to address the *in vivo* function of genes that mediate the *Drosophila* antimicrobial response. Disruptions of the *GNBP2* and *GNBP3* genes using a similar approach are promising projects to determine the exact function of the GNBP family.

Acknowledgments—We thank Jean Marc Reichhart for providing *uas-spz* flies, Won-Jae Lee for the gift of the GNBP1 cDNA, and our colleagues Brigitte Maroni and Marisa Vinals for assistance and stimulating discussions. The laboratory of B. L. was funded by the Association pour la Recherche contre le Cancer, the Fondation Schlumberger, and Programme Microbiologie (PRMMIP00).

REFERENCES

1. Girardin, S. E., Philpott, D. J., and Lemaitre, B. (2003) *EMBO Rep.* 4, 932–936
2. Tzou, P., De Gregorio, E., and Lemaitre, B. (2002) *Curr. Opin. Microbiol.* 5, 102–110
3. Hoffmann, J. A., and Reichhart, J. M. (2002) *Nat. Immunol.* 3, 121–126
4. Hultmark, D. (2003) *Curr. Opin. Immunol.* 15, 12–19
5. De Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M., and Lemaitre, B. (2002) *EMBO J.* 21, 2568–2579
6. Boutros, M., Agaisse, H., and Perrimon, N. (2002) *Dev. Cell* 3, 711–722
7. Lemaitre, B., Reichhart, J., and Hoffmann, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 14614–14619
8. Leulier, F., Rodriguez, A., Khush, R. S., Abrams, J. M., and Lemaitre, B. (2000) *EMBO Rep.* 1, 353–358
9. Rutschmann, S., Jung, A. C., Hetru, C., Reichhart, J. M., Hoffmann, J. A., and Ferrandon, D. (2000) *Immunity* 12, 569–580
10. Rutschmann, S., Kilinc, A., and Ferrandon, D. (2002) *J. Immunol.* 168, 1542–1546
11. Hedengren, M., Asling, B., Dushay, M. S., Ando, I., Ekengren, S., Wihlborg, M., and Hultmark, D. (1999) *Mol. Cell* 4, 827–837
12. Vidal, S., Khush, R. S., Leulier, F., Tzou, P., Nakamura, M., and Lemaitre, B. (2001) *Genes Dev.* 15, 1900–1912
13. Kurata, S. (2004) *Dev. Comp. Immunol.* 28, 89–95
14. Yoshida, H., Kinoshita, K., and Ashida, M. (1996) *J. Biol. Chem.* 271, 13854–13860
15. Kang, D., Liu, G., Lundstrom, A., Gelius, E., and Steiner, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 10078–10082
16. Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., and Hultmark, D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 13772–13777
17. Michel, T., Reichhart, J. M., Hoffmann, J. A., and Royet, J. (2001) *Nature* 414, 756–759
18. Choe, K. M., Werner, T., Stoven, S., Hultmark, D., and Anderson, K. V. (2002) *Science* 296, 359–362
19. Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J. A., Ferrandon, D., and Royet, J. (2002) *Nature* 416, 640–644
20. Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R. A. (2002) *Nature* 416, 644–648
21. Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki, T., and Kurata, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 13705–13710
22. Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J. H., Caroff, M., Lee, W. J., Mengin-Lecreulx, D., and Lemaitre, B. (2003) *Nat. Immunol.* 4, 478–484
23. Lee, W., Lee, J., Kravchenko, V., Ulevitch, R., and Brey, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 7888–7893
24. Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Van Dyck, E., Revets, H., Brys, L., Gomez, J., De Baetselier, P., and Timmermans, M. (1998) *J. Biol. Chem.* 273, 24948–24954
25. Lee, S. Y., Wang, R., and Soderhall, K. (2000) *J. Biol. Chem.* 275, 1337–1343
26. Ochiai, M., and Ashida, M. (2000) *J. Biol. Chem.* 275, 4995–5002
27. Ma, C., and Kanost, M. R. (2000) *J. Biol. Chem.* 275, 7505–7514
28. Kim, Y. S., Ryu, J. H., Han, S. J., Choi, K. H., Nam, K. B., Jang, I. H., Lemaitre, B., Brey, P. T., and Lee, W. J. (2000) *J. Biol. Chem.* 275, 32721–32727
29. Kalidas, S., and Smith, D. P. (2002) *Neuron* 33, 177–184
30. Leulier, F., Vidal, S., Saigo, K., Ueda, R., and Lemaitre, B. (2002) *Curr. Biol.* 12, 996–1000
31. Manfruelli, P., Reichhart, J. M., Steward, R., Hoffmann, J. A., and Lemaitre, B. (1999) *EMBO J.* 18, 3380–3391
32. Rutschmann, S., Jung, A. C., Zhou, R., Silverman, N., Hoffmann, J. A., and Ferrandon, D. (2000) *Nat. Immunol.* 1, 342–347
33. Tzou, P., Meister, M., and Lemaitre, B. (2002) *Methods Microbiol.* 31, 507–529
34. Reichhart, J. M., Ligoxygakis, P., Naitza, S., Woerfel, G., Imler, J. L., and Gubb, D. (2002) *Genesis* 34, 160–164
35. Ligoxygakis, P., Pelte, N., Hoffmann, J. A., and Reichhart, J. M. (2002) *Science* 297, 114–116
36. Lee, M. H., Osaki, T., Lee, J. Y., Baek, M. J., Zhang, R., Park, J. W., Kawabata, S. I., Soderhall, K., and Lee, B. L. (2003) *J. Biol. Chem.*
37. Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., Kosugi, A., and Miyake, K. (2003) *J. Exp. Med.* 198, 1035–1042
38. Kim, M. S., Byun, M., and Oh, B. H. (2003) *Nat. Immunol.* 4, 787–793