

Dual Activation of the *Drosophila* Toll Pathway by Two Pattern Recognition Receptors

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Supporting Online Material

Material and Methods

Microbial strains

Gram-negative bacteria: *Enterobacter cloacae* (a kind gift of H. Monteil), *Erwinia carotovora carotovora* (ECC15), *Escherichia coli* 1106. Gram-positive bacteria: *Micrococcus luteus* (CIP A270), *Enterococcus faecalis*, *Streptococcus pyogenes* strepto A strain. Fungus: *Beauveria bassiana* (80.2 strain).

Fly strains

Stocks were raised on standard cornmeal-agar medium at 25°C. A5001 w flies were used as wild-type throughout the experiments since the *GNBP1^{osi}* mutant was generated in this background. In experiments involving the *Dif^d* and *key^l* mutants, the original *cn bw* stock was used as a further wild-type control (1). DD1 is a wild-type stock in which the *PGRP-SA^{seml}* mutation has been generated (2). *PGRP-SA^{seml}*, *PGRP-LC^{E12}*, *UAS-seml*, and *yolk-Gal4* stocks have been described previously (2–4). Overexpression of GNBP1 or PGRP-SA was achieved using the UAS/GAL4 system (5).

Survival experiments

Briefly, batches of 20-25 wild-type and mutant strains were challenged by septic injury using a needle previously dipped in a concentrated solution of the bacteria of interest. The vials were then put in an incubator at the desired temperature and the surviving flies counted once or twice a day. Flies were put into new vials usually every other day. For natural fungal infections, flies were shaken on a lawn of sporulating *Beauveria bassiana*.

They were then put back into vials and processed as described above. Results are expressed as percentage of infected flies at different time points after infection. Each experiment is representative of at least three independent experiments.

Quantitative reverse-transcription PCR (Q-RT-PCR)

Samples of 5 flies were frozen in liquid nitrogen and crushed with a Mixer Mill 300 (Retch) twice for 60 seconds at 25 Hz. Total RNA was then prepared using the RNeasy kit (Qiagen) or the nucleospin kit (Macherey Nagel) and RNAs eluted in 100 µl of RNase free water. 2 µl were then used in a Reverse Transcription reaction using Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen) according to the supplier's instructions. The cDNAs were then diluted to a proper concentration so that the subsequent PCR reactions would not be inhibited by components of the RT preparation. PCR reactions were set up using the qPCR kit (Eurogentec) and in a 1/50000 to 1/75,000 final concentration of SYBRGreen. Real-time PCR was then performed in 96-well plates on an i-cycler iQ (Biorad); usual PCR conditions: preincubation at 95°C, 40 cycles: 15 sec at 95°C, 1 minute at 60°C. PCR reactions were done in duplicates; to check for specificity of the PCR reaction, melting curves were analyzed for each data point. The levels of expression of the gene of interest was then normalized against the measured level of the RNA coding for ribosomal protein 49 determined in each sample. Primers were as follows: *Drosomylin*: forward 5'CGTGAGAACCTTTTCCAATATGATG3', reverse: 5' TCCCAGGACCACCAGCAT3' ; *Diptericin*: forward 5'GCTGCGCAATCGCTTCTACT3', reverse: 5'TGGTGGAGTGGGCTTCATG3'; *RP49*: forward 5'GACGCTTCAAGGGACAGTATCTG3', reverse: 5'AAACGCGGTTCTGCATGAG3'; *GNBP1*: forward: 5'CACCAAAAGGCTGTGTATCAAGAT 3', reverse: 5'TCCGCCGAGATTGCAGA 3'; *PGRP-SA*: forward: 5' CCTTCGTTGGGACTCCACTA 3', reverse: 5' CGTGTGATGGATGACCACAT 3'

Construction of the pUAS-GNBP1 transgene, generation of *GNBP1^{osi}* excision lines, and epistatic analysis

For the rescue experiment, plasmid LD15841 that carries an EST from the Berkeley Drosophila Genome Project (<http://www.fruitfly.org/>) was digested with *ApaI* and pUAST-vector with *KpnI*. Protruding nucleotides were removed by bacteriophage T4 DNA polymerase treatment and the plasmids were further digested with *EcoRI*. The cDNA fragment was then ligated to the pUAS-T vector. The cDNA construct was then checked by sequencing before injection to generate transgenic flies. The transgene was then put under the control of the appropriate driver in a wild-type or *GNBP1^{osi}* mutant background using standard genetic crosses. To excise the PiggyBac transposon, we crossed the *GNBP1^{osi}* stock with a strain expressing the PiggyBac transposase. Male progeny from flies carrying both the transposon and transposase were isogenized and tested for excision by PCR. Stocks used for epistatic analysis and overexpression analysis were generated using standard crosses. We usually checked the overexpression of a gene by Q-RT-PCR using the relevant primer sets (see above).

Hemolymph transfer experiments

Hemolymph was collected from a single fly by pricking with an empty capillary mounted on a Nanoject II (Drummond Scientific). The collected hemolymph was then directly injected into a single recipient. Recipients were left to recover for 30-60 minutes at 22°C before undergoing an immune challenge. Flies were then incubated for another 24 hours at 25°C. We cannot fully exclude that blood cells are not transferred when using this procedure. Indeed, we could detect a few cells after DAPI (4',6-Diamidino-2-phenylindole) staining of the collected hemolymph. To exclude the possibility that the rescuing activity was carried by hemocytes, we collected the hemolymph of 50 flies on 0.1% poly-L-lysine-coated PCR tubes on ice. The tube was centrifuged at 2000g for 10 minutes at 4°C. The supernatant was carefully collected. We could not detect any blood cell by DAPI staining. The cleared supernatant could rescue the *GNBP1^{osi}* mutant phenotype as well as hemolymph transferred directly into a recipient (data not shown).

Polyacrylamide gel electrophoresis and Western blotting under native conditions

Batches of 10 flies from each experimental group were homogenized in PBS (pH 6.8) plus protease inhibitors for 10 minutes at +4°C. After centrifugation, equal protein amounts of the supernatant were separated on 8% polyacrylamide gels under non-denaturing conditions. The proteins were transferred to a nitrocellulose membrane under native conditions. Membranes were dried out for 15 minutes at room temperature, blocked using 5% fat dry milk in PBST (PBS, 0.1% Tween 20) for two hours at 21°C. The blots were then incubated with antiPGRP-SA antibodies in PBST+5% fat dry milk overnight at +4°C, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibodies (one hour at 21°C) and detected by enhanced chemiluminescence according to the manufacturer's instructions. Bound antibodies were removed from the membrane by incubation in 25 mM glycine-HCl, pH 2.2, 0.7% SDS for 30 minutes at 21°C and probed again with anti-GNBP1 polyclonal antibodies. The PGRP-SA-specific antibody has been raised against a C-terminal peptide (QQGELSEDYALIAGS) in rabbit. It recognizes a band of the expected size (22 kD) after SDS-PAGE electrophoresis and Western blotting of whole flies or hemolymph extracts.

Supporting Text

Members of the GNBP/ β (1,3)-Glucan Recognition Proteins (β GRP) were originally isolated biochemically from lepidopterans (6–9). *Bombyx mori* GNBP was identified as a protein binding Gram-negative bacteria whereas β GRPs from *B. mori* and *Manduca sexta* were purified as proteins binding β -(1,3)-glucan. Members of this family are characterized by an N-terminal glucan binding domain (8, 9) and a C-terminal half domain that bears homology to bacterial glucanases. However, essential catalytic residues have not been conserved in many insect GNBP/ β GRP proteins, suggesting that this domain has been selected for its recognition function (10). However, the ligand of this domain in insects has not yet been identified. More divergent members have been found in several protostome species, some of which may be functional glucanases (10–13). In contrast, only one member of the family has been isolated, on the basis of its enzymatic

glucanase activity, in a deuterostome species, namely the sea urchin *Strongylocentrotus purpuratus* (14).

In striking contrast to the reported abilities of GGBP1 to bind to cell wall components of Gram-negative bacteria or fungi (15), we have demonstrated that *GNBP1* is required specifically for the activation of the Toll pathway by some Gram-positive bacterial strains. The precise molecular patterns carried by Gram-positive bacteria to which the *Drosophila* GGBP1/PGRP-SA complex binds have not yet been experimentally defined, although LYS-type peptidoglycan and lipoteichoic acid are prime candidates (16, MG, unpublished data). This finding suggests that members of the GGBP/βGRP family may have evolved to recognize distinct patterns of carbohydrate chains characteristic of different microbial classes. Alternatively, it could be that members of this family display a large array of binding to various microbial elicitors *in vitro*, when not complexed with other pattern recognition receptors.

Legends to Supplementary Figures

Fig. S1. *Drosomycin* induction after a Gram-positive bacterial immune challenge.

The results of 12 independent experiments are presented. The bar to the right corresponds to the average expression detected in *GNBP1^{osi}* mutants. The standard deviation is indicated. Experiment 2 is presented in Fig. 1A.

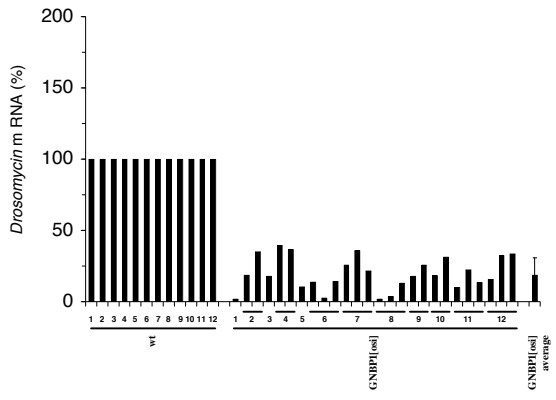
Fig. S2. *Drosomycin* induction after a natural fungal infection. The results of 6 independent experiments are presented. The bar to the right corresponds to the average expression detected in *GNBP1^{osi}* mutants. The standard deviation is indicated. Experiment 5 is presented in Fig. 1C.

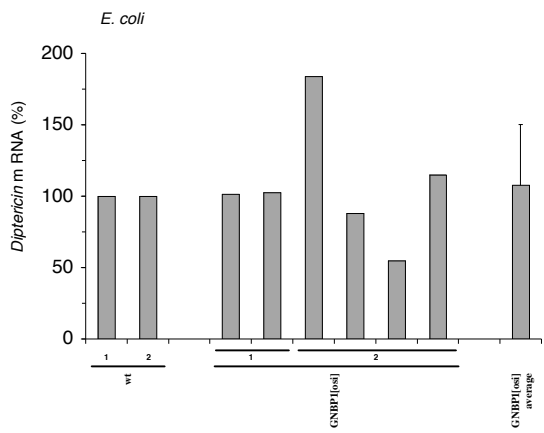
Fig. S3. *Diptericin* induction after a Gram-negative bacterial immune challenge. The results of two independent experiments are presented. The bar to the right corresponds to the average expression detected in *GNBP1^{osi}* mutants. The standard deviation is indicated. Experiment 1 is presented in Fig. 1E.

Supporting References

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M. luteus





B. bassiana

