to nature

GAP-43 analysis in DRG neurons

Sections of C5 and C6 DRG (10 μm) were double-immunostained for βIII tubulin (Promega, 1:8000) to identify all neurons, and for the growth-associated protein GAP-43 (gift from G. Kinnamon, 1:2000), using 7-aminoi-4-methylcoumarin-3-acetic acid and tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies. The percentage of GAP-43-positive cells was determined in four sections per animal.

Axon tract tracing in the spinal cord

Ascending dorsal column axons were labelled using the CTB tracer, injected into the left sensory motor cortex using a 0.5-mm concentric needle delivered every 2 s) of the left sensory motor cortex using a 0.5-mm concentric needle delivered every 2 s. Postsynaptic potentials evoked by the cortical stimuli were recorded with a silver ball stimulation site, located 1–2 mm lateral and 1 mm rostrocaudal from Bregma. At each recording site, axon numbers were calculated as a percentage of the fibres seen 4 mm above the lesion, where the CST was intact. To confirm a complete CST lesion, transverse lumbar spinal cord sections (20 μm) were immunostained with an antibody against the γ-subunit of protein kinase C (PKC-γ, Santa Cruz; 1:1000), visualized with a TRITC-conjugated secondary antibody.

Electrophysiology

In terminal electrophysiological experiments, the sensory motor cortex and cervical spinal cord were exposed in urethane-anesthetized (1.5 g kg−1) rats. Cortical evoked potentials were elicited by electrical stimulation (five square-wave pulses at 400 Hz, 100 μA, 200 μs, delivered every 2 s) of the left sensory motor cortex using a 0.5-mm concentric needle electrode lowered 1 mm into the cortex. For each experiment we mapped the optimal stimulation site, located 1–2 mm lateral and 1 mm rostrocaudal from Bregma. Post synaptic potentials evoked by the cortical stimuli were recorded with a silver ball electrode placed medially on the contralateral cord surface. At each recording site (1 segment above and 1–7 mm below the lesion at C4), 64 responses were averaged and for glial fibrillary acidic protein, to identify the lesion site, and for CTB, to identify the lesion site.

Behavioural assessment

After baseline testing, we tested animals once a week for 6 weeks after lesion. We averaged right and left forepaw scores, as no differences were observed between them. Experiments were blind to the treatment. A tape removal test (adapted from ref. 24) produced separate scores for sensory and motor behaviour. Adhesive tape (0.3 inch × 1 inch) was placed on the forepaw, and the time taken to sense the presence of the tape (indicated by paw shake) was determined. For animals that sensed the tape, the removal time was also scored. Rats were tested on two locomotor tasks requiring sensorimotor integration (adapted from ref. 30). Rats were trained to cross a narrow metal beam (1.25 inch × 36 inches) and a wire grid (12 inches × 36 inches with 1 inch X 1 inch grid squares). Forepaw foot slips were recorded (determined by a paw slipping off the beam or below the plane of the grid). In a footprint analysis (adapted from ref. 30), rat forepaws were covered with ink to record walking patterns during continuous locomotion across a wooden runway (4 inches × 36 inches), and stride length and width were calculated.

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The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein


* UPR 9022 du Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, F67084 Strasbourg, Cedex, France
† Exelixis Inc., South San Francisco, California 94083, USA
‡ These authors contributed equally to this work

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The antimicrobial defence of Drosophila relies largely on the challenge-induced synthesis of an array of potent antimicrobial peptides by the fat body1. The defence against Gram-positive bacteria and natural fungal infections is mediated by the Toll signalling pathway, whereas defence against Gram-negative bac-
bacteria is dependent on the Immune deficiency (IMD) pathway. Loss-of-function mutations in either pathway reduce the resistance to corresponding infections. The link between microbial infections and activation of these two pathways has remained elusive. The Toll pathway is activated by Gram-positive bacteria through a circulating Peptidoglycan recognition protein (PGRP-SA). PGRPs appear to be highly conserved from insects to mammals, and the Drosophila genome contains 13 members. Here we report a mutation in a gene coding for a putative transposable element inserted in the first exon of the Df(3L)29A6 in 67A8, a genomic region uncovered by the deficiency. Gram-negative infections in Drosophila positive infections, together with the present report, indicate that the Tolloid pathway is important in the host defense of Drosophila. Our initial results on the role of PGRP-SA in the host defence of Drosophila prompted us to screen for mutations in other members of this family of genes. From a modified P transposon (XP) insertion library, we isolated a mutant line, PGRP-LC(454), containing a P-element inserted at nucleotide 72 in the first non-translated exon of the PGRP-LC gene. This gene codes for a predicted 611 amino-acid protein of the Drosophila PGRP family. The predicted protein has no signal peptide and contains a non-canonical transmembrane domain (residues 275–294). The putative extracellular, carboxy-terminal region has three PGRP domains, one of which shows homology with N-acetylmuramyl-L-alanine amidase. The predicted intracellular region (residues 1–275) has no significant homology to established protein domains. When challenged with the Gram-negative bacteria Escherichia coli or Agrobacterium tumefaciens, the mutant flies showed a severely compromised induction in the levels of expression of the antibacterial peptide genes Diptericin (Dpt), Attacin and Cecropin (Fig. 2a). Induction of expression of the antifungal peptide gene Drosomycin (Drs) by Gram-positive or natural fungal infections was at wild-type levels in these mutant flies (Fig. 2b, c). This result contrasts markedly with our previous data on the PGRP-SA mutant semmelweis (semm) flies, in which induction of Drs expression caused by Gram-positive bacteria is blocked, whereas that of Dpt caused by Gram-negative bacteria is maintained at wild-type levels (Fig. 2). Survival experiments with PGRP-LC(454) mutant flies showed a marked susceptibility to Gram-negative infections (Agrobacterium tumefaciens, Erwinia carotovora carotovora, Enterobacter cloacae) and close to wild-type resistance to Gram-positive bacterial and natural fungal infections (Fig. 3). Again, this result contrasts with that of the PGRP-SA mutants, which are susceptible to Gram-positive infection but resistant to Gram-negative bacteria.

The results on the induction of antimicrobial peptides and the survival studies both indicate that PGRP-LC mutants have an immune response similar to that described for loss-of-function mutants of the IMD pathway. Overexpressing the imd gene has been reported to lead to a more pronounced induction of Dpt and other antibacterial peptide genes, but not to that of the antifungal peptide gene Drs. We therefore examined the effects of overexpressing the PGRP-LC complementary DNA by generating larvae that contained a heat-shock promoter and Gal4 fusion gene (hs-GAL4) and a UAS-PGRP-LC transgene in a Dpt-LacZ and Drs-green fluorescent protein (GFP) reporter genetic background. After heat-shock treatment, and in the absence of any immune challenge, we observed strong expression in cells of the fat body of the Dpt but not the Drs reporter (Fig. 4a and data not shown). PGRP-LC can also be overexpressed in the PGRP-LC(454) insertion line under the control of a UAS site present in the transposon (Fig. 1), and we observed that in heterozygous PGRP-LC(454)/hs-GAL4 flies, Dpt was constitutively expressed (data not shown). We further used the fat-body-specific yolk-GAL4 driver in place of the ubiquitous hs-GAL4 driver. In these experiments, a strong constitutive expression of Dpt was detectable by quantitative reverse transcription polymerase chain reaction (RT–PCR) in extracts of adult females (Fig. 4b). Parallel experiments with male flies yielded no constitutive expression of the Dpt gene, consistent with the absence of Yolk protein in males. This result indicates that overexpression of PGRP-LC in the fat body is sufficient to activate expression of the Dpt gene.

The data presented so far clearly point towards an involvement of

![Figure 1](nature.com)

**Figure 1** Schematic representation of the PGRP-LC locus. The PGRP-LC gene is located in 67A8, a genomic region uncovered by the deficiency Df(3L)29A6. 7454 is an XP transposable element inserted in the first exon of the PGRP-LC gene. Using two other XP transposable elements, 0693 and 4396, we generated a small deletion called PGRP-LC(454), which removed the entire PGRP-LC coding region. The XP element contains two UAS sites in opposing directions at both ends of the transposon.

![Figure 2](nature.com)

**Figure 2** Expression of antimicrobial peptides in different mutant backgrounds after infection by Gram-negative bacteria, Gram-positive bacteria or fungi. Northern blots were performed with total RNA from wild-type (WT) flies, seml, PGRP-LC(454) mutant flies, or flies mutant for genes in the Toll signalling pathway (Drs) and in the IMD pathway (key). a–c, Flies were infected with E. coli and A. tumefaciens (a), M. luteus (b), or B. bassiana (c), as described in Methods. Rp49 is used as an RNA-loading control. N.I., non-induced; Drs, Drosomycin; Dpt, Diptericin; Att, Attacin; Cec, Cecropin; Df, Df(3L)29A6.
the PGRP-LC gene in the activation of the IMD pathway. This pathway has been reported to induce cleavage of the latent cytoplasmic NF-κB member Relish, allowing for nuclear translocation of the amino-terminal Rel homology domain fragment and activation of antibacterial peptide genes. Seven genes have been identified to date in the IMD–Relish intracellular signalling cascade, of which the most upstream gene is that coding for the death domain protein IMD. We performed genetic epistasis between the PGRP-LC and the imd genes using a imd plak plak null allele (D.F. et al., manuscript in preparation), and observed that the constitutive activation of Dpt in the PGRP-LC7454/hs-GAL4 flies was abolished in the imd mutant background (Fig. 4c). This result places the PGRP-LC gene upstream of imd. The mechanism by which overexpression of PGRP-LC activates the IMD pathway remains to be established. One possibility is that such an overexpression leads to the forced multimerization of a receptor complex that would bring in to close contact adapter proteins, hence initiating signalling.

Finally, to demonstrate that the PGRP-LC7454 mutant phenotype is indeed the result of the transposon insertion, we mobilized this transposon with a P-element transposase. We recovered several revertant lines that showed wild-type function with respect to Dpt inducibility and survival to E. cloacae infection (data not shown). As the overexpression of PGRP-LC from the PGRP-LC7454 transposon can be obtained with a hs-GAL4 driver (see above), we tested whether this overexpression is sufficient to rescue the PGRP-LC7454/PGRP-LC7454 homozygous mutant phenotype. To this end, we generated hs-GAL4; PGRP-LC7454/PGRP-LC7454 flies, which after heat shock became as resistant to E. cloacae infection as wild-type flies, in contrast to PGRP-LC7454/PGRP-LC7454 mutant flies (Fig. 4d).

The data presented above show conclusively that the full activation of the IMD pathway requires the presence of a wild-type PGRP-LC gene. However, in our experiments we consistently observed that the phenotypes (induction of antibacterial peptide genes, survival to Gram-negative infection) were less drastic than those reported for loss-of-function mutants of the kenny gene, which codes for the Drosophila homologue of IKKb/NEMO (compare Figs 2a and 3) active in the IMD–Relish pathway. To rule out that this discrepancy reflected a possible hypomorphic character of the PGRP-LC7454 mutant line, we engineered a null mutation (PGRP-LC7454) by promoting the recombination between two additional XP elements flanking the PGRP-LC locus, thus generating a deletion of 8.7 kilobases (kb) of genomic DNA that encompasses the full coding region of the PGRP-LC gene (Fig. 1).

Induction of Dpt and survival curves to infections in these null mutants were similar to those of the PGRP-LC7454 lines (data not shown and Fig. 3, A. tumefaciens graph). We conclude from these sets of data that PGRP-LC is not the sole upstream element

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**Letters to Nature**

**Figure 3** PGRP-LC mutant flies are highly susceptible to infection by Gram-negative bacteria. The survival rates of wild-type (WT), Dif(3L)29A6 and PGRP-LC7454 flies infected with the indicated microorganisms are presented. Each experiment was performed using 25 flies per genotype and the results shown are representative of three independent experiments. PGRP-LC7454/PGRP-LC7454 homozygous mutant flies die as fast as PGRP-LC7454/Dif(3L)29A6 heterozygous mutant flies, indicating that this mutation is genetically null.

**Figure 4** PGRP-LC is genetically upstream of imd. a, Dif-GFP; Dpt-LacZ; hs-GAL4/‡; UAS-PGRP-LC7454/+; third-instar larvae were heat-shocked for 1 h at 37°C. Twelve hours later, the fat body was dissected and stained for β-galactosidase activity and checked for GFP expression (not shown). Dif-GFP; Dpt-LacZ; +/‡; PGRP-LC7454/+; sibling larvae were used as a negative control (left panel). b, c, Dipterincin (black bars) and PGRP-LC (grey bars) transcript levels were measured by quantitative RT-PCR. Results were first standardized against Rp49 (grey bars) transcript levels were measured by quantitative RT-PCR. Results were first taken to be 100 as a reference. Left y-axis scale, Dipterincin; right y-axis scale, PGRP-LC.

b, Overexpression of PGRP-LC. A, non-challenged wild-type females; B, wild-type females challenged with E. coli; C, non-challenged PGRP-LC7454/PGRP-LC7454 females; D, challenged PGRP-LC7454/PGRP-LC7454 females; E, yolk-GAL4/PGRP-LC7454 non-challenged females. c, imd is epistatic to PGRP-LC. A, non-challenged wild-type males; B, challenged wild-type males (E. coli); C, non-challenged PGRP-LC7454/PGRP-LC7454 males; D, challenged PGRP-LC7454/PGRP-LC7454 males; E, non-heat-shocked imd/‡; hs-GAL4/PGRP-LC7454 non-challenged females; F, heat-shocked imd/‡; hs-GAL4/PGRP-LC7454 non-challenged females; G, non-heat-shocked imd/imd, hs-GAL4/PGRP-LC7454 non-challenged females; H, heat-shocked imd/imd; hs-GAL4/PGRP-LC7454 non-challenged females. d, Rescue of susceptibility to E. cloacae infection by PGRP-LC overexpression. Open squares, hs-GAL4/‡; PGRP-LC7454/PGRP-LC7454; filled squares, wild type; filled triangles, non-heat-shocked PGRP-LC7454/PGRP-LC7454; filled diamonds, heat-shocked PGRP-LC7454/PGRP-LC7454.
activating the IMD pathway.

The present report together with our previous study indicates that Gram-positive and Gram-negative bacterial infections are sensed by distinct members of a diversified family of pattern recognition proteins. The name Peptidoglycan recognition protein was given to this family in reference to the initial reports of humoral proteins binding peptidoglycan in the moth Trichoplasia ni1,2, and in the silkworm Bombyx mori where they activate a defence cascade leading to melanin formation3,4,5. Ablative binding studies of PGRP family members to various microorganisms have not yet been performed; however, our data that PGRP-LC mediates the response to Gram-negative infection (this work) and that PGRP-SA is involved in the host defence against Gram-positive bacteria, suggest that recognition by these proteins is not restricted to peptidoglycan patterns. We propose that the various Drosophila PGRP genes (and corresponding splice isoforms) can provide a recognition repertoire for microbial patterns that will detect many, if not most, of the microorganisms that can invade Drosophila. Mammals, like insects, synthesize both soluble (PGRP-S) and membrane-bound (PGRP-L, -I, -L8) PGRPs. The human transmembrane PGRP-L protein is expressed in the liver, a functional homologue of the insect fat body, and binds to peptidoglycan and Gram-positive bacteria. Its functional relevance in vivo has not yet been reported. An unanswered question at present is how PGRP-LC signals to the IMD pathway. The putative intracytoplasmic region of PGRP-LC has no apparent sequence characteristics of intracellular signalling potential. We leave open the possibility that PGRP-LC acts as a co-receptor in the membrane of the fat body cell, and that activation of the death domain protein IMD during Gram-negative infection requires additional unidentified protein(s).

The field of study concerning the recognition of microbial pathogens in innate immunity has experienced a marked progression, both in insects and mammals, in the last few years. Whereas most studies to date have focused on Toll/Toll-like receptors26–28, PGRPs are now coming to the forefront. In Drosophila, their functions as putative pattern recognition receptors appear to overshadow those of Toll: the only Toll family member whose role in the immune response is clearly documented—Toll itself—requires activation through a PGRP sensing microbial infection.

Methods

Microbial strains

We used the following microbial organisms in this study: Agrobacterium tumefaciens, Erwinia carotovora carotovora, Enterobacter cloacae, Escherichia coli 1106 (Gram-negative bacteria); Micrococcus luteus, Streptococcus faecalis (Gram-positive bacteria); Beauveria bassiana (fungus).

Fly strains

Stocks were raised on standard cornmeal-agar medium at 25°C. We used white and yw Drosophila flies as controls. Def, key, yw Dros-GFP Dpt-LacZ flies were described elsewhere.25,26 The Df(3L)29A6 stock was obtained from the Bloomington stock centre.

Septic injury and survival experiments

Bacterial infections were performed by challenging adult flies with a thin tungsten needle previously dipped into a concentrated culture of the appropriate bacterial strains. To test antifungal response, anaesthetized flies were manually shaken for 30 s on a Petri dish containing a sporulating Beauveria bassiana culture. Infected animals were incubated at 25°C (bacteria) or at 22°C (fungus). Survival experiments were carried out with 25 flies for each tested genotype. Surviving flies were transferred daily into fresh vials and counted. Results are expressed as percentage of infected flies at different time points after infection. Each experiment is representative of at least three independent experiments. For northern blot analysis, flies were collected 24 h after infection and stored at −80°C. For rescue experiments, flies were heat-shocked for 30 min at 37°C. After 6 h incubation at 25°C, flies were infected as described above.

Cloning and transformation of UAS-PGRP-LC

The wild-type PGRP-LC cDNA was obtained by PCR using expressed-sequence-tag (EST) clone (SD26236, ResGen) as a template. EcoRI and Xhol sites were introduced 5′ and 3′ respectively of the PGRP-LC cDNA using the primers 5′-GAATTCACAAAAAGCTTTTA GCCAATGAAAGG-3′ and 5′-GCTCTAGACTCACCAGCAATGACGTTCAGT-3′, and the plasmid pGEM3Zf(+) (Promega) was used as a vector.

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The recognition and phagocytosis of microbes by macrophages is a principal aspect of innate immunity that is conserved from insects to humans. *Drosophila melanogaster* has circulating macrophages that phagocytose microbes similarly to mammalian macrophages, suggesting that insect macrophages can be used as a model to study cell-mediated innate immunity. We devised a double-stranded RNA interference-based screen in macrophage-like *Drosophila* S2 cells, and have defined 34 gene products involved in phagocytosis. These include proteins that participate in haemocyte development, vesicle transport, actin cytoskeleton regulation and a cell surface receptor. This receptor, Peptidoglycan recognition protein LC (PGRP-LC), is involved in phagocytosis. These authors contributed equally to this work.

![Figure 1](image1.png)

**Figure 1** Quantification of phagocytosis of *E. coli* (**a**, **c**, **e**) and *S. aureus* (**b**, **d**, **f**) by FACS. S2 cells were incubated with FITC-labelled bacteria for 30 min at 4 °C followed by 15 (E. coli) or 20 (S. aureus) min incubation at 26 °C to allow internalization. Gate M1 represents the background autofluorescence of S2 cells; gate M3 in **a**, **b**, represents brightly fluorescent S2 cells that have bound and/or internalized FITC-labelled bacteria; **c**, **d**, Representative FACS scans after Trypan blue quenching. The M3 peak represents S2 cells that have internalized FITC-labelled bacteria, and the M2 peak represents S2 cells that have bound but not internalized FITC-labelled bacteria; **e**, **f**, RNAi treatment (β-Cop) causing decreased phagocytosis. The amount of phagocytosis was quantified as the percentage of cells within the gate M3 multiplied by the mean fluorescence intensity of these cells.