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The *Drosophila* Amidase PGRP-LB Modulates the Immune Response to Bacterial Infection

Anna Zaidman-Rémy,[1] Mireille Hervé,[2] Mickael Poidevin,[1] Sébastien Pili-Floury,[1] Min-Sung Kim,[3] Didier Blanot,[2] Byung-Ha Oh,[3] Ryu Ueda,[4] Dominique Mengin-Lecreulx,[2] and Bruno Lemaitre[1,*]

[1] Centre de Génétique Moléculaire du CNRS
F-91198 Gif-sur-Yvette
France

[2] Institut de Biochimie et Biophysique Moléculaire et Cellulaire
UMR 8619 CNRS
Université Paris-Sud
Bât. 430
F-91405 Orsay
France

[3] Center for Biomolecular Recognition
Department of Life Sciences
Pohang University of Science and Technology
Pohang
Kyungbuk, 790-784
Korea

[4] National Institute of Genetics
Mishima
Shizuoka 411-8540
Japan

Summary

The *Drosophila* host defense against gram-negative bacteria is mediated by the Imd pathway upon sensing of peptidoglycan by the peptidoglycan recognition protein (PGRP)-LC. Here we report a functional analysis of PGRP-LB, a catalytic member of the PGRP family. We show that PGRP-LB is a secreted protein regulated by the Imd pathway. Biochemical studies demonstrate that PGRP-LB is an amidase that specifically degrades gram-negative bacteria peptidoglycan. In agreement with its amidase activity, PGRP-LB downregulates the Imd pathway. Hence, activation of PGRP-LB by the Imd pathway provides a negative feedback regulation to tightly adjust immune activation to infection. Our study also reveals that PGRP-LB controls the immune reactivity of flies to the presence of ingested bacteria in the gut. Our work highlights the key role of PGRPs that encode both sensors and scavengers of peptidoglycan, which modulate the level of the host immune response to the presence of infectious microorganisms.

Introduction

Drosophila is devoid of an adaptive immune system and relies solely on innate immune reactions for its defense (Kaneko et al., 2005; Royet et al., 2005). One of the key features of the *Drosophila* immune response is the synthesis of several antimicrobial peptides (AMPs) with distinct but overlapping specificities by the fat body, an equivalent of the mammalian liver. AMPs are secreted into the hemolymph, where they directly kill invading microorganisms. Genetic analyses show that AMP genes are regulated by the Toll and Imd pathways. These two pathways share many common features with the mammalian TLR and TNF-α (tissue necrosis factor) signaling cascades that regulate NF-κB transcription factors. The Toll pathway is triggered by the proteolytic cleavage of the Toll ligand, the cytokine Spatzle (Spz), and leads to activation of the Rel proteins Dif and Dorsal. This pathway is activated by both gram-positive bacteria and fungi and controls to a large extent the expression of AMPs active against fungi (e.g., *Drosomycin*). In contrast, the Imd pathway mainly responds to gram-negative bacterial infection and controls antibacterial peptide genes (e.g., *Diptericin*) via the activation of the Rel protein Relish.

In *Drosophila*, bacterial recognition is achieved through the sensing of specific forms of peptidoglycan (PGN) by peptidoglycan recognition proteins (PGRPs). PGN is an essential and specific polymer of the cell wall of both gram-negative and gram-positive bacteria. It consists of long glycan chains made of alternating N-acetylglucosamine and N-acetylmuramic acid residues that are crosslinked to each other by short peptide bridges (Mengin-Lecreulx and Lemaitre, 2005). PGN from gram-negative bacteria differs from most gram-positive PGN by the replacement of lysine with *meso*-diaminopimelic acid (DAP) at the third position in the peptide chain. PGRPs are proteins that bind to and in some cases hydrolyze PGN. These molecules are highly conserved from insects to mammals and share a conserved 160 amino acid domain with similarities to the bacteriophage T7 lysozyme, a zinc-dependent N-acetylmuramoyl-L-alanine amidase (Royet et al., 2005; Steiner, 2004). Sequence analysis of the 13 *Drosophila* PGRPs points to the existence of two subgroups with either recognition or catalytic properties. The first group (PGRP-SA, SD, LA, LC, LD, LE, LF) lacks zinc binding residues required for amidase activity but still retains the ability to bind and recognize PGN. PGRP-SA and PGRP-SD are secreted proteins circulating in the hemolymph that have been shown to activate the Toll pathway in response to lysine-type PGN found in most gram-positive bacteria (Royet et al., 2005; Steiner, 2004). PGRP-LC acts as a transmembrane receptor upstream of the Imd pathway and is activated by the DAP-type PGN of gram-negative bacteria (Royet et al., 2005; Steiner, 2004). Recent studies indicate that both polymeric and monomeric gram-negative PGN mediate Imd pathway activation via various PGRP-LC isoforms (Kaneko et al., 2004; Mellroth et al., 2005; Stenbak et al., 2004). Finally, PGRP-LE, a secreted PGRP that binds preferentially to DAP-type PGN, functions synergistically with PGRP-LC in Imd pathway activation (Takehana et al., 2004). In contrast, another subgroup of PGRPs referred to as catalytic PGRPs have demonstrated (PGRP-SC1A/B) or predicted (PGRP-LB, SB1/2, SC2) zinc-dependent amidase activity that removes

*Correspondence: lemaitre@cgm.cnrs-gif.fr

peptides from the glycan chains, thereby reducing or eliminating the biological activity of PGN (Mellroth et al., 2003). The exact in vivo functions of catalytic PGRPs are not known, although their amidase activity has led to the proposal that they could either modulate the immune response by scavenging PGN or act directly as antibacterial factors (Mellroth et al., 2003). Therefore, we decided to perform an extensive biochemical and genetic analysis of PGRP-LB, a catalytic PGRP.

Results

PGRP-LB Is a Secreted PGRP Regulated by the Imd Pathway

Oligonucleotide microarray analysis performed on *Drosophila* adult males indicated that the gene *PGRP-LB* is induced 7-fold following septic injury (De Gregorio et al., 2002). The comparison of *PGRP-LB* mRNA profiles in wild-type and mutant flies deficient for Toll (*spatzle; spz*) or Imd (*Relish; Rel*) signaling revealed that *PGRP-LB* expression is controlled by the Imd pathway (Figure 1A). Time course analysis of the expression profile of PGRP-LB during the *Drosophila* immune response was performed with protein extracts from male adult flies collected at different time intervals following septic injury with gram-negative *Erwinia carotovora* (Figure 1B). Western blot analysis illustrates that PGRP-LB expression was weak in unchallenged adult males, whereas expression levels increased by 3 hr post-challenge reaching a plateau at 22 hr. PGRP-LB was abundant in protein extracts derived from the hemolymph (Figure 1C) and the fat body, the major immune-responsive tissue (data not shown). This indicates that PGRP-LB is produced by the fat body and secreted into the hemolymph. The *Drosophila* genome annotation predicts the existence of two PGRP-LB isoforms of 216 and 232 amino acids, respectively, differing by the presence or absence of a predicted N-terminal signal peptide. This is consistent with the observation of two forms of PGRP-LB in total fly extracts. In agreement with the mRNA analysis (Figure 1A), PGRP-LB was not induced by septic injury in *Rel* mutant flies lacking a functional Imd pathway (Figure 1D). Altogether, these results indicate that stimulation of the Imd pathway by gram-negative bacteria leads to the rapid synthesis and secretion of PGRP-LB by the fat body into the hemolymph.

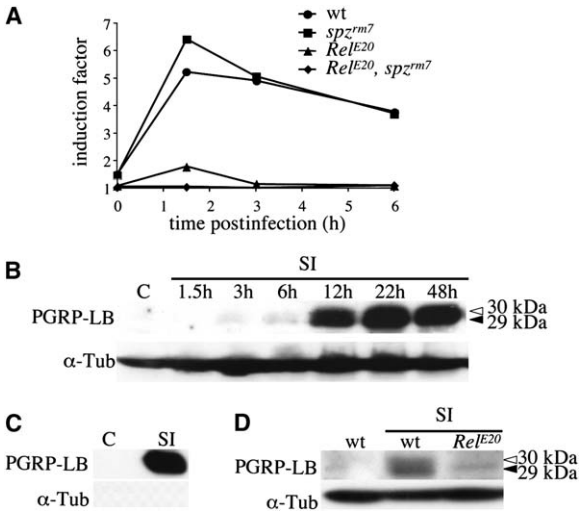


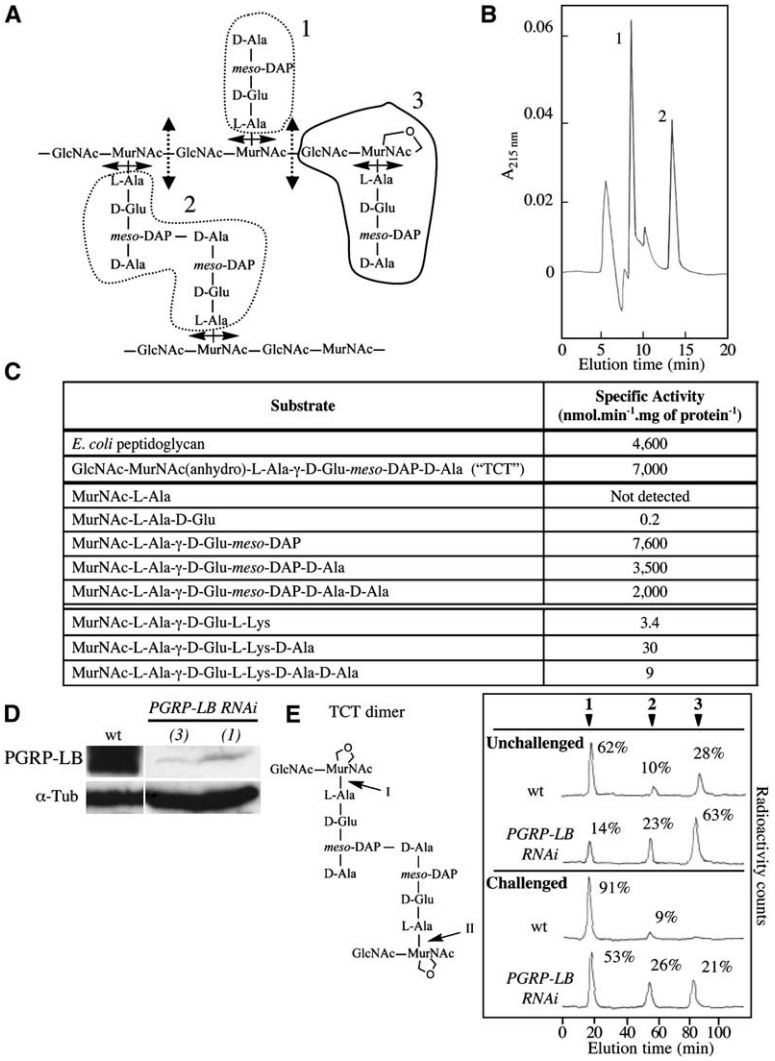
Figure 1. Expression Profile of PGRP-LB after Septic Injury

(A) The gene expression profile for *PGRP-LB* shows rapid induction at 1.5 hr, followed by sustained expression at later time points. A mutation affecting *Rel*, but not a mutation in *spz*, encoding the ligand of the Toll receptor, inhibited the expression of *PGRP-LB* after infection, demonstrating that the gene is under the control of the Imd pathway. The microarray data for this figure were extracted from De Gregorio et al. (2002). (B) This time course analysis shows that PGRP-LB expression is induced upon immune challenge. Two isoforms of about 29 and 30 kDa were detected by a mouse antiserum directed against recombinant PGRP-LB. Western blot analysis was performed with protein extracts from male wild-type flies collected at different time points after infection. (C) PGRP-LB is secreted into the hemolymph. Western blot analysis was performed with hemolymph samples extracted from female wild-type flies collected at 16 hr postinfection. The absence of a signal with α-tubulin (α-Tub) antibody indicates that the hemolymph preparations are not contaminated by cells. (D) PGRP-LB is not induced upon immune challenge in *Rel*-deficient flies. The blots obtained in independent experiments were probed with both a polyclonal anti-PGRP-LB (upper panel) and an anti-α-Tub antibody (lower panel). Wild-type (wt) flies were *da-GAL4/+* (B and C) or Oregon^R (A and D). SI, septic injury by *E. carotovora*. C, unchallenged control flies.

PGRP-LB Is an Amidase Specific of DAP-Type PGN

To determine the role of PGRP-LB, we first undertook a biochemical approach. Recombinant PGRP-LB was previously shown to bind purified PGN from various bacterial species but did not exhibit any bactericidal activity against gram-negative or gram-positive bacteria (Kim et al., 2003). However, PGRP-LB induces lysis of sensitized (EDTA-treated) *E. coli* cells, suggesting a PGN-hydrolyzing activity. More precisely, an *N*-acetylmuramoyl-L-alanine amidase activity was suspected, based on sequence homologies of PGRP-LB and catalytic PGRPs such as PGRP-SC1B with the T7 lysozyme (Mellroth et al., 2003). We therefore analyzed in more detail the enzymatic properties and substrate specificity of PGRP-LB. Incubation of pure PGN from *E. coli* with recombinant PGRP-LB resulted in a rapid and almost complete release of the tetrapeptide L-Ala-γ-D-Glu-*meso*-DAP-D-Ala and of its octapeptide dimer from the macromolecule, confirming a strong PGN-hydrolyzing amidase activity (Figures 2A and 2B). Under standard assay conditions, the specific enzymatic activity detected on the whole PGN polymer was very high, ~4.6 μmol.min^{-1}.mg^{-1}. As a control, we produced a mutated form of PGRP-LB in which the cysteine 160 is substituted with a serine. In catalytic PGRPs, Cys160 is a conserved residue, which coordinates the zinc ion required for amidase activity. The C160S mutant did not show any amidase activity (data not shown).

An anhydro form of MurNAc occurs naturally in gram-negative bacteria at the extremity of all glycan strands and is consequently present in approximately 5% of the GlcNAc-MurNAc units (Figure 2A). The GlcNAc-MurNAc(anhydro)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala monomer, also known as tracheal cytotoxin (TCT), was previously identified as the minimum PGN motif capable of efficiently inducing the Imd pathway (Kaneko et al., 2004; Stenbak et al., 2004). TCT provides an ideal "signature" of gram-negative bacteria, since this muropeptide is continuously released from PGN during cell



Substrate	Specific Activity (nmol.min^{-1}.mg of protein^{-1})
E. coli peptidoglycan	4,600
GlcNAc-MurNAc(anhydro)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala ("TCT")	7,000
MurNAc-L-Ala	Not detected
MurNAc-L-Ala-D-Glu	0.2
MurNAc-L-Ala-γ-D-Glu-*meso*-DAP	7,600
MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala	3,500
MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala	2,000
MurNAc-L-Ala-γ-D-Glu-L-Lys	3.4
MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala	30
MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala	9

Figure 2. Amidase Activity and Substrate Specificity of PGRP-LB

(A) Structure of *E. coli* PGN (DAP-type) indicating the cleavage sites for the *N*-acetylmuramoyl-L-alanine amidase activity of PGRP-LB (plain arrows). The main peptides released following treatment of crosslinked PGN with PGRP-LB are the tetrapeptide L-Ala-γ-D-Glu-*meso*-DAP-D-Ala (1) and its dimer, the octapeptide (2). Glycan chains of *E. coli* PGN all end with a 1,6-anhydro-MurNAc residue, and the terminal disaccharide-tetrapeptide motif corresponds to the structure of TCT (3). Both lytic transglycosylases and muramidases catalyze the cleavage of the β-1,4-glycosidic bond between the MurNAc and GlcNAc residues (dashed arrows). However, the bacterial transglycosylases catalyze an additional intramolecular transglycosylation reaction that results in the formation of a 1,6-anhydro MurNAc residue.

(B) HPLC analysis of soluble peptides released following digestion of *E. coli* PGN by PGRP-LB. Purified PGN (150 μg) was incubated for 30 min at 37ºC with 0.5 μg of PGRP-LB protein. Peaks corresponding to the tetrapeptide and octapeptide are indicated by 1 and 2, respectively.

(C) The substrate specificity of PGRP-LB was determined by measuring its amidase activity on a series of muropeptides representing variations in the composition (DAP- or lysine-type) or length (one to five amino acid residues) of the peptide moiety. Muropeptides were tested at 0.125 mM in standard assay conditions, and substrate and products were separated by HPLC.

(D) A Western blot experiment shows that PGRP-LB was detected in wild-type but not in *PGRP-LB RNAi* flies collected 16 hr after bacterial injection. (3) and (1) represent two independent *PGRP-LB IR* insertions.

(E) The amidase activity of hemolymph samples collected from wild-type and *PGRP-LB RNAi* flies was monitored by HPLC after injection of [^{14}C]DAP-radiolabeled TCT dimer. [^{14}C] TCT dimer was injected in unchallenged flies (top) or flies collected 22 hr after a bacterial challenge (bottom) and extracted for analysis 20 min later. Peak 1, octapeptide (cleavage at both I and II sites); peak 2, TCT-octapeptide (cleavage at either I or II); peak 3, TCT dimer substrate. The radioactivity detected in peaks of these different compounds is indicated as a percentage of the total radioactivity.

growth and division. Figure 2C shows that the specific activity of PGRP-LB on TCT was quite similar to that observed on the macromolecule.

In order to more precisely define the substrate specificity of PGRP-LB, a series of muropeptides derived from the PGN structure were tested (Figure 2C). Varying the length and composition of the peptide moiety showed that PGRP-LB requires at least a tripeptide for activity, since no cleavage of MurNAc-L-Ala was detectable, and that cleavage of MurNAc-L-Ala-D-Glu occurred at a rate 10^4-fold lower compared to its tri- to pentapeptide derivatives. Furthermore, replacement of *meso*-DAP by L-lysine (as is the case in the PGN of most gram-positive species) resulted in a dramatic decrease of PGRP-LB activity, by factors ranging from 10^2 to 10^3. This finding is in agreement with the poor activity exhibited by PGRP-LB on PGN purified from gram-positive *Micrococcus luteus*, as compared to that of gram-negative *E. coli* (data not shown). This biochemical analysis demonstrates that PGRP-LB is an efficient amidase that degrades gram-negative PGN into non-immuno-stimulatory fragments.

Hemolymph of *PGRP-LB RNAi* Flies Exhibits Reduced Amidase Activity

We next analyzed the role of *PGRP-LB* in vivo using an inducible RNA interference (RNAi) strategy. We generated transgenic flies carrying the *UAS-PGRP-LB Inverted Repeat* element (referred to as *PGRP-LB IR*). In order to activate transcription of the hairpin-encoding transgene in the progeny (referred to as *PGRP-LB RNAi* flies), these transgenic flies were crossed with flies carrying the *daughterless (da)-GAL4* driver expressing *GAL4* strongly and ubiquitously.

PGRP-LB RNAi animals showed significant lethality at the pupal stage when raised at 29ºC. *PGRP-LB RNAi* adult escapers raised at 25ºC were, however, viable and showed no visible difference to wild-type flies. We confirmed that the PGRP-LB protein was almost undetectable in samples from infected *PGRP-LB RNAi* adults

(Figure 2D), whereas expression of other amidase PGRPs (SB1, SC2, SC1) was not reduced (see Figure S1 in the Supplemental Data available with this article online). In order to correlate in vitro and in vivo analyses, we compared the amidase activity in hemolymph of *PGRP-LB RNAi* flies to wild-type flies. [^{14}C]DAP-radiolabeled TCT dimer was injected into unchallenged flies and flies collected 16 hr after septic injury. HPLC analysis of soluble radiolabeled compounds from hemolymph samples collected at 20 min postinjection shows that TCT dimer was rapidly digested into free octapeptide (dimer of L-Ala-γ-D-Glu-*meso*-DAP-D-Ala, Figure 2E, peak 1), consistent with an amidase-type activity at the two cleavage sites (I and II) present in the TCT dimer (Figure 2E). An intermediate product corresponding to TCT-octapeptide (Figure 2E, peak 2) was observed that results from cleavage at only one of the two sites. This result indicates the existence of a strong amidase activity in the hemolymph of wild-type flies. A bacterial challenge increased the amidase activity of the hemolymph in wild-type flies, consistent with our observation that amidase PGRPs are upregulated by infection. Interestingly, digestion of TCT dimer was greatly reduced in both unchallenged and bacteria-challenged *PGRP-LB RNAi* flies, indicating a significant decrease in hemolymph amidase activity. At 90 min postinjection, digestion of TCT dimer was complete in wild-type samples and almost complete in *PGRP-LB RNAi* samples collected from noninfected flies (data not shown). The amidase activity detected in the latter extracts could be due to the presence of other catalytic PGRPs and/or to a residual level of PGRP-LB, since RNAi mimics a hypomorphic mutation. This study demonstrates that flies exhibit strong hemolymph amidase activity that hydrolyzes PGN into nonstimulatory fragments and that PGRP-LB contributes to this activity in vivo.

PGRP-LB Negatively Regulates the Imd Pathway Following Gram-Negative Bacterial Injection

The expression profile and activity of PGRP-LB strongly suggested a function in the immune response against gram-negative bacteria. In order to clarify its role, we analyzed the effect of PGRP-LB reduction on the systemic immune response induced upon injection of different classes of microorganisms into the hemolymph of *PGRP-LB RNAi* flies. Inactivation of *PGRP-LB* did not induce increased susceptibility to infection (Figure 3A, data not shown), as determined from fly survival rates following injection of *E. carotovora*, whereas inhibition of the Imd pathway in a *Rel* mutant had a dramatic effect upon survival (Hedengren et al., 1999). Consistent with this observation, both *Diptericin* (*Dpt*) and *Drosomycin* (*Drs*) gene expression remained inducible in *PGRP-LB RNAi* flies (see below), indicating that PGRP-LB does not function as a recognition receptor upstream of Toll or the Imd pathway.

Interestingly, a time course analysis of *Dpt* expression following septic injury with gram-negative bacteria revealed a stronger immune response in *PGRP-LB RNAi* flies compared to wild-type flies (Figure 3B). Indeed, whereas the levels of *Dpt* were similar between 3 and 6 hr postchallenge, they were 1.5- to 2-fold higher in *PGRP-LB RNAi* flies at later time points. The same phenotype was observed when flies were injected with concentrated solutions of either gram-negative PGN or TCT instead of live bacteria (Figures 3C and 3D, respectively). Since PGN and TCT are inert compounds, this result indicates that the stronger *Dpt* expression in *PGRP-LB RNAi* flies after gram-negative bacteria injection was not due to a difference in bacterial proliferation but resulted from an increase in Imd signaling stimulation. To demonstrate that the stronger *Dpt* expression was indeed due to a lack of PGRP-LB, we performed a rescue experiment using a recombinant PGRP-LB protein.

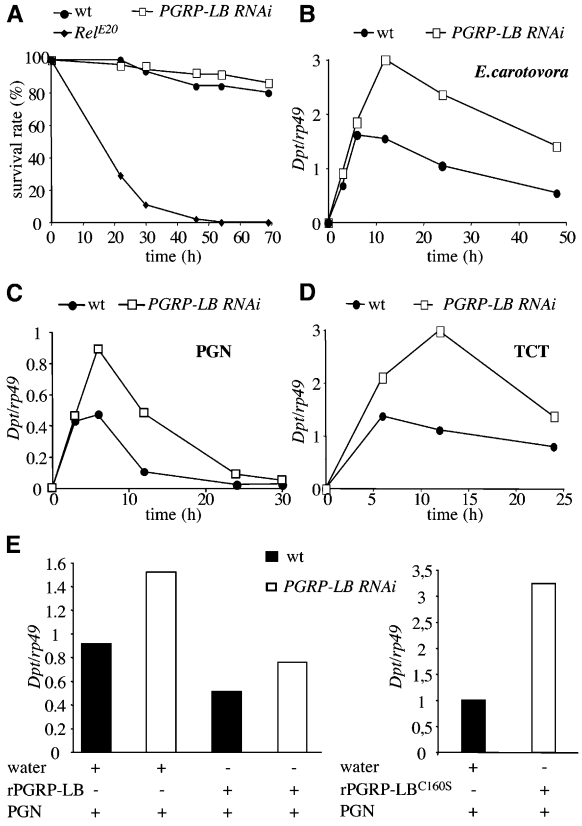


Figure 3. Role of PGRP-LB during the Systemic Response to Septic Injury

(A) A survival analysis shows that *PGRP-LB RNAi* flies were resistant to gram-negative bacterial infection. Survival tests were performed at 29ºC with 60 flies each, and the numbers of surviving flies were counted at different time points after challenge with *E. carotovora*. (B–D) PGRP-LB downregulates the Imd pathway after gram-negative bacterial infection. Time course analysis of *Dpt* gene expression in wild-type and *PGRP-LB RNAi* flies after septic injury with *E. carotovora* (B) or injection of gram-negative bacterial PGN (C) or TCT (D). RT-qPCR analyses were performed on total RNA extracts from wild-type and *PGRP-LB RNAi* female flies collected at different time points after injection of *E. carotovora*, *P. aeruginosa* PGN or TCT. (E) Injection of recombinant PGRP-LB (rPGRP-LB) restored a wild-type level of *Dpt* expression in *PGRP-LB RNAi* flies (left panel). Water (23 nl) or rPGRP-LB ([rPGRP-LB] = 1 mg.ml^{-1}) was injected into wild-type or *PGRP-LB RNAi* flies 1 hr prior to the injection of *P. aeruginosa* PGN. Note that injection of rPGRP-LB reduced the induction of *Dpt* in wild-type flies. RT-qPCRs were performed on flies collected 6 hr postchallenge. Injection of PGRP-LB C160S ([rPGRP-LB C160S] = 1 mg.ml^{-1}) did not reduce the level of *Dpt* expression in *PGRP-LB RNAi* flies (right panel). (C–E) PGN (9 nl) ([PGN] = 5 mg.ml^{-1}) or TCT ([TCT] = 0.46 mM) was injected in flies. Repeats and statistics for experiments described in (B)–(E) are provided in Figure S3.

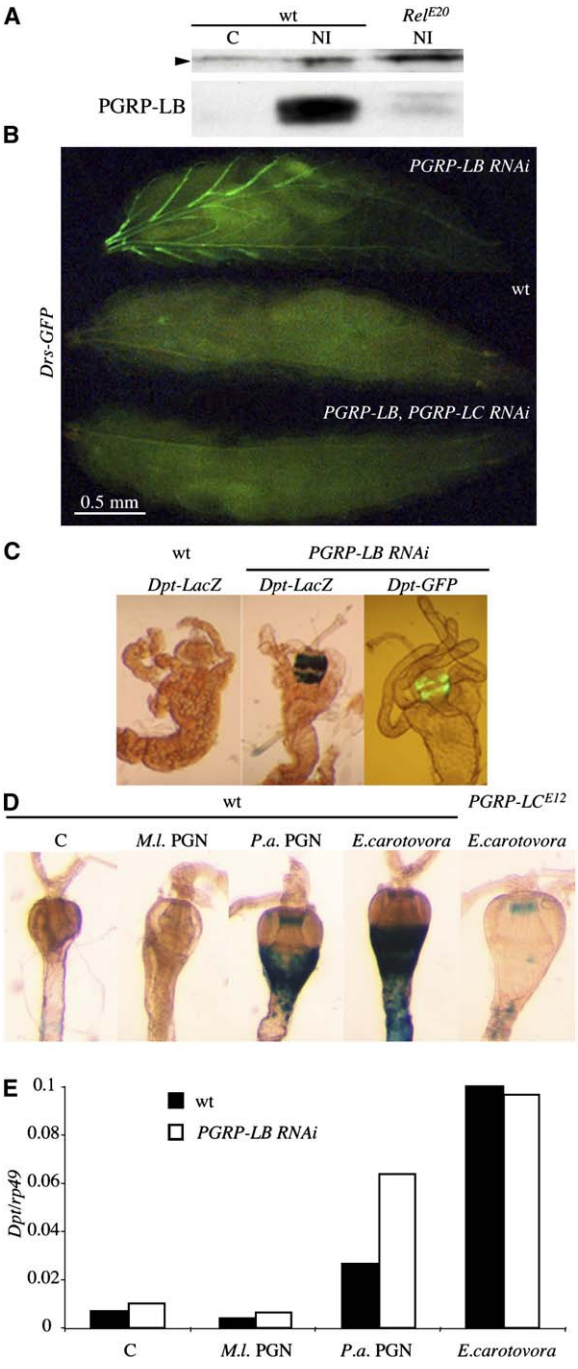


Figure 4. Role of PGRP-LB during the Local Immune Response

(A) Oral bacterial infection induced PGRP-LB in the gut of wild-type but not *Rel* flies. A Western blot was performed on gut extracts derived from flies fed for 20 hr with *E. carotovora*. An upper band (indicated by a black arrow) that crossreacts with the anti-PGRP-LB serum was used as a loading control. NI, natural infection. C, unchallenged control.

(B) In contrast to wild-type larvae, most *PGRP-LB RNAi* larvae expressed the *Drs-GFP* reporter in the trachea under normal rearing conditions. No *Drs* expression was observed in *PGRP-LB/LC* double *RNAi* larvae, confirming that *Drs-GFP* expression in the trachea is regulated by the Imd pathway. (Top panel) *Drs-GFP; da-GAL4/ PGRP-LB IR*. (Middle panel) *Drs-GFP; da-GAL4/+*. (Lower panel) *Drs-GFP; da-GAL4, PGRP-LB IR/PGRP-LC IR*.

(C) Histochemical staining of β-galactosidase activity is observed in the anterior midgut at the level of the proventriculus of most *PGRP-LB RNAi* larvae that carry the *Dpt-lacZ* reporter gene in absence of an infection. Similar results were obtained with a *Dpt-GFP* reporter gene (right panel).

(D) Oral ingestion of gram-negative PGN but not gram-positive PGN triggered *Dpt-lacZ* reporter gene expression in the proventriculus. β-galactosidase stainings were performed on guts from wild-type and *PGRP-LC* mutant flies carrying a *Dpt-lacZ* reporter gene. Flies were fed during 20 hr with sucrose alone (C, control), sucrose mixed with gram-positive PGN (*M. luteus, M.l.*), gram-negative PGN (*P. aeruginosa, P.a.*), or *E. carotovora*. After *E. carotovora* feeding, no *Dpt* expression was observed in the gut of a $PGRP\text{-}LC^{E12}$ mutant (far right panel).

(E) Oral ingestion of gram-negative PGN triggered *Dpt* expression in the gut. RT-qPCR was performed with gut extracts from wild-type and *PGRP-LB RNAi* flies that were fed during 20 hr on sucrose (control), sucrose mixed with gram-positive PGN (*M. luteus*), gram-negative PGN (*P. aeruginosa*), or *E. carotovora*. This experiment was repeated three times and yielded similar results.

Figure 3E shows that injection of PGRP-LB protein but not the C106S mutant into the hemolymph of *PGRP-LB RNAi* flies restores a wild-type expression level of *Dpt* after injection of gram-negative PGN. Interestingly, the expression level of *Dpt* after injection of gram-negative PGN was lowered in wild-type flies after a prior injection of PGRP-LB (Figure 3E). This experiment confirmed that PGRP-LB negatively regulates the Imd pathway in agreement with its capacity to scavenge gram-negative PGN.

Finally, we observed that PGRP-LB does not affect Toll pathway activation by gram-positive bacteria, consistent with the weak enzymatic activity PGRP-LB exhibits toward lysine-type PGN (Figure S2).

PGRP-LB Downregulates the Imd Pathway during Local Immune Response

In addition to the fat body, AMP genes can be expressed in several surface epithelia, which are potentially in contact with microorganisms (Tzou et al., 2000). Local expression of *Dpt* in the anterior midgut at the level of the proventriculus and *Drs* in the trachea can be induced by the Imd pathway upon natural infection by gram-negative bacteria such as *E. carotovora*.

We demonstrated by Western blot analysis that PGRP-LB is induced in the digestive tract upon natural bacterial infection in a *Rel*-dependent manner (Figure 4A). This observation prompted us to test a possible implication of PGRP-LB in local AMP expression. The use of reporter genes revealed that *PGRP-LB* inactivation by RNAi leads to higher AMP gene expression: 92% of *PGRP-LB RNAi* larvae but only 5% of wild-type larvae expressed a *Drs-GFP* reporter gene in trachea in the absence of an immune challenge (Figure 4B). Similarly, almost all *PGRP-LB RNAi* larvae and adults expressed *Dpt* in the proventriculus under normal rearing conditions (Figure 4C, data not shown for adults). These observations indicate that PGRP-LB prevents the local activation of the Imd pathway in the absence of pathogenic micro-organisms. In the gut, PGRP-LB could inhibit Imd activation by hydrolyzing PGN fragments released by bacteria present in the digestive tract. To date, the nature of the bacterial elicitor(s) and host receptor(s) implicated in local immunity has not yet been investigated, and it was not known whether PGN could indeed activate this response. To address this question, we monitored the level of *Dpt* expression in the gut of

wild-type flies fed with a highly purified solution of PGN. Both the use of a *Dpt-lacZ* reporter gene and RT-qPCR show that *Dpt* was induced in the gut after ingestion of gram-negative but not gram-positive PGN (Figures 4D and 4E). The level of *Dpt* expression stimulated by gram-negative PGN was, however, weaker than the level induced after oral infection with *E. carotovora*. In agreement with our hypothesis that PGRP-LB may prevent Imd activation by hydrolyzing PGN fragments released by bacteria, we noted that *PGRP-LB RNAi* flies displayed a higher *Dpt* expression after ingestion of gram-negative PGN (Figure 4E).

We also observed that the induction of *Drs* in trachea or *Dpt* in the gut was blocked in *PGRP-LC* deficient mutants upon infection by *E. carotovora* (Figure 4D and data not shown), indicating that PGRP-LC is involved in this local activation of the Imd pathway. Consistent with this observation, the higher local AMP gene expression in gut and trachea induced by *PGRP-LB RNAi* in absence of a challenge was suppressed by coexpression of a *PGRP-LC RNAi* construct (Figure 4B and data not shown).

Altogether, these data demonstrate that the local immune response is mediated through the recognition of gram-negative PGN by PGRP-LC and that PGRP-LB plays an important role in epithelia by preventing local immune activation in the absence of a severe infection.

PGRP-LB Prevents Systemic Immune Activation after Ingestion of PGN

In addition to triggering a local response, the presence of specific gram-negative bacterial species (e.g., *P. entomophila*) in the gut can trigger a systemic immune response in the fat body without bacterial invasion of the hemolymph (Basset et al., 2000; Vodovar et al., 2005). The signals that link the bacterial persistence in the gut and Imd pathway activation in the fat body are presently unknown. Since PGRP-LB in the gut and in the hemolymph influences the immune reactivity to gram-negative bacteria, we investigated a possible role of PGRP-LB in the modulation of this response. In agreement with previous studies (Vodovar et al., 2005), oral infection by *P. entomophila* triggered a strong expression of *Dpt* in wild-type adult flies, whereas *E. carotovora* did not (Figure 5A). However, both bacterial species induced high levels of *Dpt* expression in *PGRP-LB RNAi* flies (Figure 5A). The use of a *lacZ* reporter gene and RT-qPCR experiments demonstrated that the *Dpt* gene was expressed in the fat body of almost 50% of *PGRP-LB RNAi* flies orally infected by *E. carotovora* (Figure 5B, data not shown). Taking into account the enzymatic activity of PGRP-LB, we reasoned that this phenotype could be due to the absence of degradation of the PGN released by the bacteria in the gut. PGN concentration would then increase, thus resulting in a fat body response in *PGRP-LB RNAi* flies. To test this hypothesis, we monitored systemic *Dpt* expression in wild-type and *PGRP-LB RNAi* adults fed with gram-negative PGN. Figure 5C shows that oral ingestion of gram-negative PGN induced a sustained expression of *Dpt* expression in *PGRP-LB RNAi* but not in wild-type flies. The level of *Dpt* in *PGRP-LB RNAi* flies was proportional to the concentration of the PGN solution used to feed the flies (Figure 5D). Figure 5D also illustrates that *Dpt* induction was not observed when flies were fed with PGNs derived from various gram-positive bacteria.

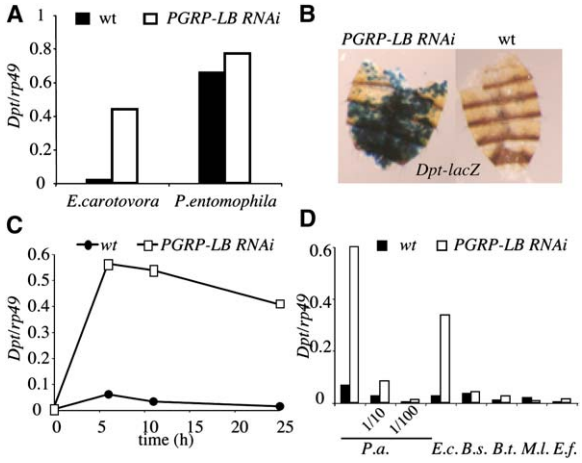


Figure 5. PGRP-LB Prevents the Systemic Immune Response after Oral Infection of Bacteria

(A) Oral infection with *P. entomophila* and *E. carotovora* induced strong *Dpt* expression in *PGRP-LB RNAi* flies, while *Dpt* was induced only by *P. entomophila* in wild-type flies. Analysis was performed on wild-type and *PGRP-LB RNAi* females collected 12 hr after oral bacterial infection. (B) Oral infection by *E. carotovora* triggers a systemic immune response in *PGRP-LB RNAi* flies. A strong β-galactosidase staining was observed in the fat body of *PGRP-LB RNAi* but not of wild-type flies carrying a *Dpt-lacZ* reporter gene. Carcasses of flies were fixed and stained 20 hr after oral infection with *E. carotovora*. (C) Ingestion of gram-negative PGN induces *Dpt* in *PGRP-LB RNAi* flies. Time course analysis of *Dpt* gene expression was performed on wild-type and *PGRP-LB RNAi* flies after ingestion of *P. aeruginosa* PGN. (D) Ingestion of gram-negative but not gram-positive PGN induces *Dpt* in *PGRP-LB RNAi* flies. Analysis was performed on wild-type and *PGRP-LB RNAi* females fed 12 hr with preparations of lysine-type PGN (*Enterococcus faecalis*, *E.f.*; *M. luteus, M.l.*), DAP-type PGN from gram-positive Bacilli (*Bacillus subtilis*, *B.s.*; *Bacillus thuringiensis, B.t.*), and gram-negative PGN (*P. aeruginosa*, *P.a.*; *E. coli, E.c.*). Dilution of *P. aeruginosa* PGN results in proportionally lower *Dpt* expression. (A, C, and D) *Dpt* expression was monitored by RT-qPCR with RNA extracts from whole flies. The levels measured in these experiments corresponded to systemic expression of *Dpt* by the fat body, since the contribution of gut to *Dpt* expression is negligible. Repeats and statistics for experiments described in (A) and (C) are provided in Figure S4.

This study shows that PGRP-LB participates in bacterial tolerance by preventing the activation of a systemic immune response by ingested bacteria.

Ingestion of TCT Induces a Potent Systemic Response in *PGRP-LB RNAi* Flies

PGN polymers can be degraded by enzymes such as muramidases or lysozymes, which catalyze the cleavage of the β-1,4 bond between MurNAc and GlcNAc in glycan strands and generate muropeptides (Figure 2A). Lysozymes, which are abundantly expressed in the gut of *Drosophila* (Daffre et al., 1994), could process ingested PGN in small fragments thereby modulating its immune activity. To address this question, we monitored *Dpt* gene expression in *PGRP-LB RNAi* and wild-type flies fed with intact or muramidase-digested PGN. Figure 6A shows that the muramidase treatment increased the ability of PGN to induce a systemic immune response in *PGRP-LB RNAi* flies.

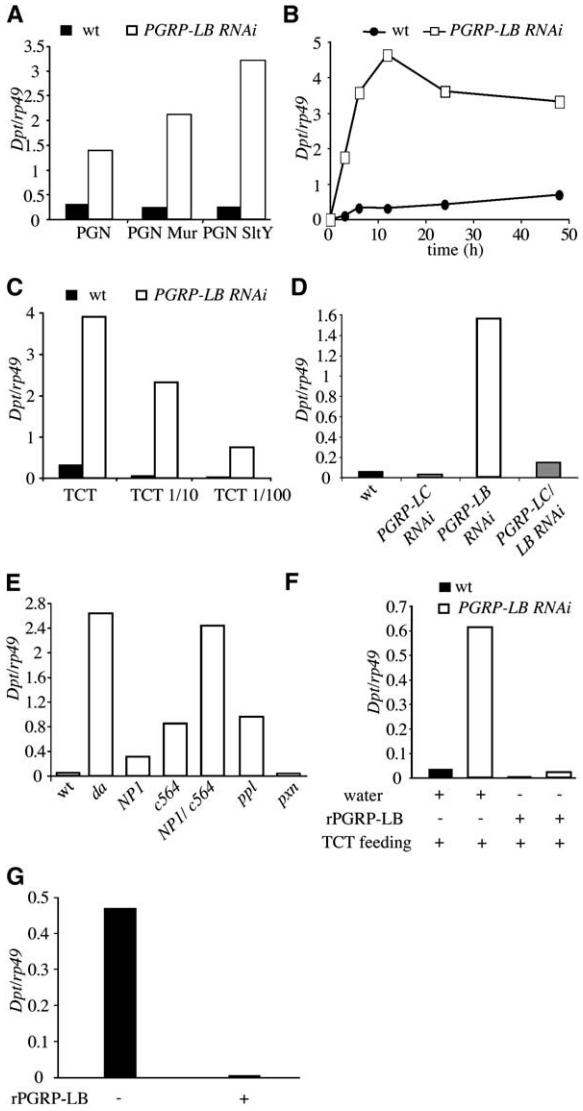


Figure 6. Ingestion of TCT Triggers Systemic Immune Response in *PGRP-LB RNAi* Flies

(A) Ingestion of gram-negative PGN digested by either muramidase or SltY increases Imd pathway activation. Female flies were fed with muramidase-treated, SltY-treated, or untreated PGN from *E. coli* ([PGN] = 0.2 mg/mL). *Dpt* expression was monitored 12 hr postinfection. (B) Ingestion of TCT strongly induces *Dpt* in *PGRP-LB RNAi* flies. Time course analysis of *Dpt* gene expression in male wild-type and *PGRP-LB RNAi* flies after ingestion of TCT ([TCT] = 0.23 mM). (C) Dilution of TCT results in proportionally lower *Dpt* expression. *Dpt* expression was monitored in wild-type and *PGRP-LB RNAi* male flies 12 hr after ingestion of serial dilutions of a solution of TCT ([TCT] = 0.23 mM). (D) *Dpt* induction by TCT requires *PGRP-LC*. *Dpt* expression was monitored in *PGRP-LC RNAi*, *PGRP-LB RNAi*, and *PGRP-LC/LB* double *RNAi* flies fed for 12 hr with TCT ([TCT] = 0.023 mM). (E) Use of fat body (*c564*, *ppl*), gut (*NP1*), and hemocyte (*pxn*) GAL4 drivers indicates a requirement of *PGRP-LB* in both gut and fat body. *Dpt* expression was monitored in flies expressing *PGRP-LB IR* with the different GAL4 drivers after 12 hr of feeding with TCT ([TCT] = 0.023 mM). (F) Injection of rPGRP-LB blocked *Dpt* expression in *PGRP-LB RNAi* flies fed with TCT. *Dpt* expression was monitored in wild-type or *PGRP-LB RNAi* flies fed 12 hr with TCT ([TCT] = 0.023 mM). Water (23 nl) or rPGRP-LB ([rPGRP-LB] = 1 mg.ml^{-1}) was injected in the hemolymph of flies 3 hr prior feeding with TCT. (G) Injection of rPGRP-LB blocked *Dpt* expression in wild-type flies after oral infection with *P. entomophila*. Analysis was performed on wild-type females collected 6 hr after oral infection by *P. entomophila*. rPGRP-LB (23 nl) ([rPGRP-LB] = 1 mg.ml^{-1}) was injected into wild-type flies 3 hr prior oral infection by *P. entomophila*. *Dpt* expression was monitored by RT-qPCR with RNA extracts from whole flies. The levels measured in these experiments corresponded to systemic expression of *Dpt* by the fat body, since the contribution of gut to *Dpt* expression is negligible. Repeats and statistics for experiments described in (C), (F), and (G) are provided in Figure S5.

The experiment illustrated in Figure 5D demonstrates that DAP-type PGN from *Bacillus* species failed to induce the Imd pathway in contrast to DAP-type PGN from gram-negative bacteria. Distinctions between these two types of PGN are the absence of an anhydro bond at the extremity of glycan strands and the presence of amidated DAP in *Bacillus* PGN. This observation raised the possibility that the elicitor of this systemic response may be small PGN fragments containing the TCT motif. To address this question, we monitored *Dpt* gene expression in *PGRP-LB RNAi* and wild-type flies fed with PGN digested with SltY. SltY is a bacterial transglycosylase that cleaves the β-1,4 bond between MurNAc and GlcNAc in glycan strands to generate muropeptides, similar to a muramidase treatment. However, unlike muramidase, SltY cleavage also results in the formation of an internal 1,6-anhydro bond in the cleaved MurNAc residue as present in TCT. Figure 6A shows that PGN treated with SltY was a more potent inducer of *Dpt* that PGN digested with muramidase. In agreement with this observation, Figures 6B and 6C show that ingestion of a concentrated solution of TCT induced an extremely potent systemic immune response in *PGRP-LB RNAi* flies in a dose-dependent manner. The level of induction was 6-fold higher than the level obtained by PGN feeding and similar to that of flies subjected to direct injection of bacteria. Interestingly, a weak induction of *Dpt* was also observed in wild-type flies when fed for a prolonged period with a concentrated solution of TCT. In addition, *Dpt* was not induced in *PGRP-LC/LB* double RNAi flies, indicating that the systemic expression of *Dpt* induced by ingestion of TCT requires *PGRP-LC* (Figure 6D). The results above indicate that both oligomerization state and presence of the anhydro bound influence the immuno-stimulatory effect of PGN.

In contrast to polymeric PGN, TCT is a small molecule that may easily cross the gut barrier. Transfer of TCT from the gut lumen to the hemolymph could then lead to activation of a systemic immune response by interacting with PGRP-LC at the surface of the fat body. Alternatively, TCT could interact directly with gut epithelial cells, which would subsequently send a signaling molecule to the fat body. According to the second hypothesis, PGRP-LB would be required only in the digestive tract to hydrolyze ingested TCT and prevent activation of gut cells. To distinguish between these two hypotheses, we determined in which tissues PGRP-LB was required by using gut (*NP1-GAL4*), fat body (*c564-GAL4*, *ppl-GAL4*), or hemocyte (*pxn-GAL4*) drivers to specifically extinguish *PGRP-LB* in these tissues. In addition to their strong expression in the fat body, both *c564-GAL4* and *ppl-GAL4* weakly expressed GAL4 in some sections of the gut and Malpighian tubules (data not shown). The inactivation of *PGRP-LB* by *NP1-GAL4*, *c564-GAL4*, or *ppl-GAL4* resulted in a modest *Dpt* expression after ingestion of TCT, while no effect was

observed with the *pxn-GAL4* driver (Figure 6E). Figure 6E shows that inactivation of *PGRP-LB* in both gut and fat body using *NP1-GAL4* together with *c564-GAL4* reproduced the immune phenotype observed with the ubiquitous driver *da-GAL4*. Collectively, this analysis reveals that flies become fully responsive to ingestion of TCT only when PGRP-LB is depleted in both gut and fat body. Hence, expression of PGRP-LB in either the gut or the fat body is sufficient to limit the systemic expression of *Dpt* after ingestion of TCT. To confirm that PGRP-LB is also required in the hemolymph, we performed a rescue experiment by injecting recombinant PGRP-LB into the body cavity of *PGRP-LB RNAi* flies. Figure 6F shows that injection of PGRP-LB into the hemolymph prevented *Dpt* induction after ingestion of TCT. Since the only known function of PGRP-LB is to hydrolyze PGN, we conclude that the systemic immune response induced by TCT ingestion is mediated by transfer of this muropeptide from the gut lumen to the hemolymph.

To test a possible role of PGN transfer in a more physiological condition, we next monitored *Dpt* expression in wild-type flies orally infected by *P. entomophila* after a prior injection of PGRP-LB into the hemolymph. Figure 6G shows that injection of PGRP-LB prevented *Dpt* induction after oral *P. entomophila* infection, indicating that transfer of PGN is the basis of *P. entomophila* capacity to trigger a systemic response.

Discussion

PGRPs are conserved in both vertebrates and insects, and combined genetic and biochemical approaches have suggested that they play various roles in immunity linked to their capacity to bind PGN. Noncatalytic PGRPs have been implicated as sensors of bacterial infection, initiating host defenses in insects. PGRP-S, a noncatalytic PGRP present in the neutrophil granules of mammals, has antibacterial activity (Cho et al., 2005; Dziarski et al., 2003). To date, the actual physiological role of catalytic PGRPs has not been identified in vivo, although their amidase activity has led to the hypothesis that they could modulate the immune response by scavenging PGN (Mellroth et al., 2003). Out of the four mammalian PGRPs, only PGRP-L, a secreted protein circulating in the serum, has amidase activity (Dziarski, 2004). The absence of an immune phenotype of *PGRP-L* deficient mice did not provide an indication as to its precise role (Xu et al., 2004). The present study is the first report that demonstrates the contribution of an amidase PGRP to the host defense in vivo. We show that PGRP-LB specifically controls the level of Imd pathway activity in *Drosophila*. Our observations that PGRP-LB is secreted and scavenges DAP-type PGN in vitro and in vivo strongly suggest that PGRP-LB reduces the Imd pathway by degrading PGN. Although the *Drosophila* genome encodes four other catalytic PGRPs, the phenotype induced by *PGRP-LB RNAi* in flies suggests the absence of functional redundancy between them. However, it is probable that other catalytic PGRPs act together with PGRP-LB in different tissues and/or developmental stages. Indicative of this possibility is the residual amidase activity detected in the hemolymph of *PGRP-LB RNAi* flies. In addition, RNAi extinction of *PGRP-SC1/2* leads to a higher *Dpt* expression after injection but not after oral ingestion of PGN in adults (our unpublished data). We assume that combination of inducible (PGRP-LB) or constitutive (PGRP-SC) PGRPs would ensure an adequate level of immune reactivity of each fly tissue. In addition, the high specificity of PGRP-LB toward DAP-type PGN suggests that other amidases with activity against lysine-type PGN may modulate the response to gram-positive bacteria upstream of the Toll pathway. Analyzing the contribution of each of these catalytic PGRPs is now essential to understand the regulation of the fly immune response.

Adjustment of Immune Response to Bacterial Load through a Negative Feedback

Our data suggest that the level of immune response to gram-negative bacteria involves a balance between PGN sensing by recognition PGRPs and PGN degradation by amidase PGRPs. The basal level of PGRP-LB would determine a threshold below which the immune response is not induced. Furthermore, our study shows that *PGRP-LB* is transcriptionaly regulated by the Imd/PGRP-LC pathway. Bacterial infection induced a rapid increase of PGRP-LB that resulted in higher amidase activity, effectively blocking the activation of PGRP-LC by PGN. This negative feedback loop, illustrated in Figure 7, would ensure an appropriate level of immune activation in response to bacterial infection. This mechanism provides a good explanation for why infection by nonpathogenic bacteria only induces a transient induction of the Imd pathway. This pathway would be activated only in the case of severe infection, when bacterial proliferation overcomes the scavenging capacity of PGRP-LB. This negative control provides a sensitive mechanism by which the immune response is repressed once bacteria are under control and not simply following their complete elimination. This mechanism economizes host resources by anticipating the termination of the immune response and may also prevent potentially severe consequences to host tissues through prolonged immune activity. The observation that overexpression of *imd* results in cell death by apoptosis indicates that high Imd activity is deleterious to the fly (Georgel et al., 2001). In this context, the PGN scavenger function of PGRP-LB could limit the noxious effect of excessive Imd pathway activation in a manner reminiscent of the mechanisms that detoxify LPS in vertebrates. It is possible that in mammals, the amidase PGRP-L may downregulate the immune response by scavenging PGN present in the serum in a way similar to PGRP-LB in *Drosophila*.

PGRP-LB Prevents Local Immune Activation by Gut Bacteria

Considering that the molecular motifs that activate the innate immune response are present in both commensal and pathogenic microorganisms, it is important to understand why commensal microorganisms do not generate a state of permanent intestinal immune activation (Sansonetti, 2004). Our study reveals a central role for amidase PGRPs in bacterial tolerance at epithelium surfaces of *Drosophila*.

We show for the first time that the activation of the Imd pathway in the gut is mediated upon sensing of DAP-type PGN by PGRP-LC. This observation is, at first sight,

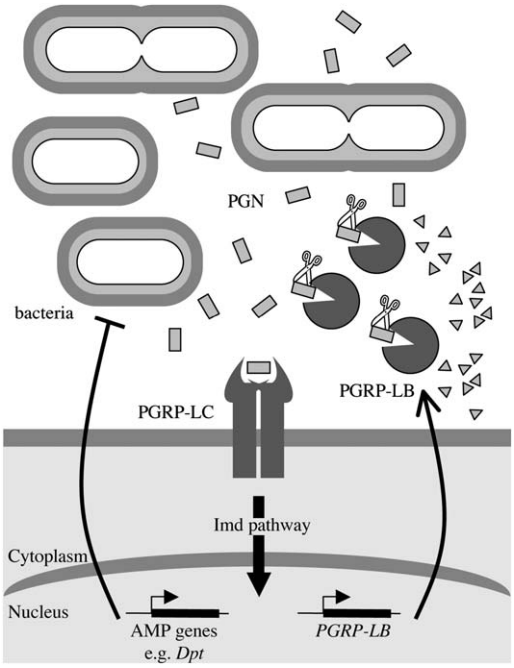


Figure 7. A Model of Imd Pathway Activation by Gram-Negative Bacteria

During bacterial infection, the presence of actively dividing gram-negative bacterial cells results in the release of PGN fragments that overcome the scavenger capacity of PGRP-LB, thus provoking an induction of the Imd pathway through PGRP-LC. Concomitant inductions of AMP genes and of *PGRP-LB* result in the reestablishment of an equilibrium by active destruction of bacteria by AMPs and an increase in free circulating PGRP-LB protein and consequent elimination of immune activating PGN. This model is based on our observations that PGRP-LB (1) is induced by the Imd pathway, (2) scavenges gram-negative PGN, and (3) downregulates the Imd pathway.

intriguing, since DAP-type PGN is found in all gram-negative bacteria, including those present in the alimentary canal. However, our study shows that local Imd immune activation in trachea and gut by resident bacteria is prevented by PGRP-LB. This is illustrated by our observation that AMP genes are upregulated in these tissues in *PGRP-LB RNAi* but not in wild-type flies grown under normal rearing conditions. An important role of PGRP-LB in the gut is also supported by our immunoblot analysis showing that PGRP-LB is expressed in this tissue and is regulated by the Imd pathway. Although epithelia are in contact with PGN ingested with the food or released by the bacterial flora, our data indicate that the mechanisms that regulate the local activation of Imd in these tissues are similar to those previously described for the systemic immune response (Figure 7). There are two main reasons that might explain how a single sensing mechanism permits the differentiation between bacteria of the natural flora and potentially pathogenic microorganisms. First, the feedback loop at the level of PGRP-LB, discussed above, prevents immune reaction except when the scavenger capacity of PGRP-LB is overcome by bacterial proliferation. A low level of Imd pathway activity is probably induced in the gut by resident bacteria, resulting in the synthesis of both PGRP-LB and antimicrobial factors that together maintain control of both the immune system and the microbial flora. This basal level of immune activation would be surpassed only when invading bacteria multiply in the gut. The second reason is that gram-negative PGN is a cell wall component hidden in the periplasmic space underneath the outer membrane of LPS. PGN fragments are released from bacteria as a consequence of PGN structure remodeling occurring during cell growth and division. Therefore, recognition of released PGN allows the direct measurement of bacterial proliferation rather than the detection of total bacterial load. It is probable that most gram-negative bacteria residing in the gut have a low division rate and therefore release low levels of PGN that can be hydrolyzed by amidase PGRPs.

Our study shows that PGRP-LB amidase plays an important role in epithelia by determining the immune reactivity of the host to environmental bacteria. The immune regulation by PGRP-LB provides a flexible mechanism for an immediate adaptation of flies in various ecological niches that are either poor or enriched in microbes, thereby ensuring an immune response primarily tailored to bacterial proliferation.

PGN Is the Mediator between Gut Infection and Fat Body Immune Response

Some bacterial species are able to trigger a strong systemic immune response in *Drosophila* after oral infection, indicating the presence of an as-yet-unidentified signaling pathway from the gut to the fat body. This immune reaction correlates with the capacity of these bacterial species to persist and multiply inside the gut and does not appear to rely upon physical crossing of the gut wall (Vodovar et al., 2005). The analysis of the *PGRP-LB RNAi* phenotype reveals new insights in the nature of this process. Our study shows that *PGRP-LB RNAi*, but not wild-type flies, activate a systemic immune response after ingestion of nonpathogenic bacteria or DAP-type PGN. This is consistent with the above discussion that PGRP-LB establishes a threshold below which no immune response is triggered. Moreover, our study provides strong evidence that this systemic immune response is mediated by the translocation of small PGN fragments from the gut lumen to the hemolymph. We first observed that ingestion of muramidase-treated PGN induces a higher systemic immune response than polymeric PGN, consistent with the idea that small molecules cross the gut barrier easier than large molecules. Furthermore, injection of purified PGRP-LB into the hemolymph of *PGRP-LB RNAi* flies prevents the induction of a systemic immune response following TCT ingestion. This result can only be explained by the presence of immunologically active TCT in the hemolymph that is hydrolyzed and thus inactivated by PGRP-LB. Collectively, our data suggest a model in which bacterial persistence in the gut leads to a local increase of DAP-type PGN fragments small enough to cross the intestinal epithelium and activate PGRP-LC at the surface of fat body cells. Production of small PGN fragments could be amplified through the action of gut lysozymes (Daffre et al., 1994) that, by contrast to amidase PGRPs, can cleave PGN into small fragments that retain their immune activity as shown in our study. Importantly, transfer of PGN provides an indirect mechanism for recognition of gram-negative bacteria that does not require a physical

contact between PGRP-LC and the bacteria. We speculate that, under the pressure of recurrent gram-negative oral infections, transport of PGN into the hemolymph has evolved as a proper signal to anticipate possible breaching of the gut barrier. Such a mechanism gives a biological sense to the existence of PGRP-LC isoforms specifically devoted to the detection of monomeric PGN (Kaneko et al., 2004; Mellroth et al., 2005). It is interesting to note that gram-positive bacteria or fungi that induce the Toll pathway are recognized by molecules exposed at their cell surface, while gram-negative bacteria have their ligands hidden under an LPS layer. This difference between direct or indirect sensing could explain the difference in organization between the Toll pathway, which is activated by secreted sensors (e.g., PGRP-SA, -SD, and GNBP-1) and the Imd pathway, which is activated by recognition receptors present at the surface of the fat body.

Furthermore, the threshold level of immune sensitivity to PGN imposed by PGRP-LB would explain in part the apparent oral nonresponsiveness of *Drosophila* to most ingested bacteria since they normally do not persist in the gut. Determining the nature of the transport mechanism, facilitated or passive, and the identity of epithelial cells involved in PGN translocation will be a future challenge.

Conclusions

The present study demonstrates how a simple recognition mechanism based on a competition between sensing and degradation of bacterial elicitors can be deployed under three distinct situations involving either systemic, local, or dual immune activation. The data demonstrate a key role for PGN and associated degradation products for bacterial recognition by flies. The specific differences in structure between gram-positive and gram-negative PGN, combined with host enzymes including lysozymes and amidases, permit the subtle discrimination between microorganisms and relative quantification of bacterial proliferation. It is perhaps not surprising that PGRP and NOD proteins, in flies and vertebrates, respectively, use this complex polymer to dynamically anticipate and modulate interactions with native commensals and potentially pathogenic microbial flora.

Experimental Procedures

Fly Stocks

Oregon^R flies or flies carrying one copy of *daughterless* (*da*)-*GAL4* were used as wild-type controls. *RNAi* transgenic fly lines of *PGRP-LB* or *PGRP-LC* were obtained using an inducible in vivo RNAi approach. A cDNA fragment corresponding to the first 500 bp of the coding sequence was amplified by PCR and inserted as an inverted repeat (*IR*) in a modified pUAST transformation vector, pUAST-R57 (Leulier et al., 2002). Each experiment was repeated using two independent *UAS-PGRP-LB IR* insertions. In the present study, we used larvae and adult flies carrying one copy of the *UAS-RNAi* construct combined with one copy of the *GAL4* driver except in Figure 6C in which two *UAS-PGRP-LB IR* insertions were combined with one *GAL4* driver. The *pumpless* (*ppl*) and *peroxidasin* (*pxn*) *GAL4* drivers are described in Stramer et al. (2005) and Zinke et al. (2002). A recombinant line carrying both the *da-GAL4* driver and the *PGRP-LB IR* construct was used to obtain *PGRP-LB*, *PGRP-LC* double *RNAi* flies. *Drosophila* stocks were maintained at 25ºC using standard fly medium. The F1 progeny carrying both the *PGRP-LB IR* and the *GAL4* driver were transferred to 29ºC at late pupal stage for optimal GAL4 efficiency. The transgenic strains *Dpt-lacZ*, *Dpt-GFP*, and *Drs-GFP* were previously described (Tzou et al., 2000). *PGRP-LC^{E12}*, *spz^{rm7}*, *Rel^{E20}*, *and TAK1^1* alleles are null mutations in *spz*, *PGRP-LC*, *TAK1*, and *Relish*, respectively (Leulier et al., 2003).

Bacterial Strains and Infection Experiments

P. entomophila is an entomopathogenic bacteria that can trigger a systemic immune response after oral infection in both larvae and adults (Vodovar et al., 2005). *E. carotovora* is a gram-negative bacterium that induces a systemic immune response after oral infection in larvae but not in adults (Basset et al., 2000). Bacterial infections were performed by pricking adults in the thorax with a thin needle previously dipped into a concentrated pellet of a bacterial culture (OD = 200). For oral infection, flies were first incubated 2 hr at 29ºC in an empty vial and then placed in a fly vial with food solution. The food solution was obtained by mixing a pellet of an overnight culture of bacteria, PGN (5 mg/ml) or TCT (0.046 mM) with a solution of 5% sucrose (50/50) and added to a filter disk that completely covered the agar surface of the fly vial. For injection, 9.2 nL of solution water, PGN (5 mg.ml^{-1}), TCT (0.46 mM), or 23 nl recombinant PGRP-LB (1 mg.ml^{-1}) was injected into the thorax of female adults (3–4 days old) using a Nanoject apparatus (Drummond).

Additional Experimental Protocols

For purification of PGN and muropeptides, assay for amidase activity in fly hemolymph, assay for *N*-acetylmuramoyl-L-alanine amidase activity, Western blot analysis, and quantitative real-time PCR (RT-qPCR), see Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and five figures and can be found with this article online at http://www.immunity.com/cgi/content/full/24/4/463/DC1/.

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