Peptidoglycan-recognition proteins (PGRPs) are evolutionarily conserved molecules that are structurally related to bacterial amidases. Several Drosophila PGRPs have lost this enzymatic activity and serve as microbe sensors through peptidoglycan recognition. Other PGRP family members, such as Drosophila PGRP-SC1 or mammalian PGRP-L, have conserved the amidase function and are able to cleave peptidoglycan in vitro. However, the contribution of these amidase PGRPs to host defense in vivo has remained elusive so far. Using an RNA-interference approach, we addressed the function of two PGRPs with amidase activity in the Drosophila immune response. We observed that PGRP-SC1/2-depleted flies present a specific over-activation of the IMD (immune deficiency) signaling pathway after bacterial challenge. Our data suggest that these proteins act in the larval gut to prevent activation of this pathway following bacterial ingestion. We further show that a strict control of IMD-pathway activation is essential to prevent bacteria-induced developmental defects and larval death.

Synopsis

It has long been known that the mammalian immune response needs to be kept under tight control. Responses that are delayed or of insufficient vigor can lead to a failure to control infection. However, excessive or inappropriate inflammation can be harmful or even fatal. Using the fruit fly as a model, evidence is presented that such an immune-modulation is also essential in invertebrates and is mediated by peptidoglycan-recognition proteins (PGRPs). PGRPs are evolutionarily conserved molecules derived from enzymes that cleave bacterial peptidoglycan. It has been shown previously that some PGRPs have lost this enzymatic activity and function as sensors of bacteria upstream of the Drosophila immune pathways. The contribution of PGRPs which have maintained enzymatic activity to host defense has remained elusive so far. Here, the authors investigate in vivo data on the role of Drosophila PGRPs with enzymatic activity. Their results suggest that these proteins are required in the larval gut to negatively regulate the immune response, thus preventing bacterially induced developmental defects and larval death.

microbial PGN. However, the in vivo function of PGRPs with amidase activity remains unclear, and PGRP-L, mutant mice show no immune phenotype. On the basis of in vitro experiments, it has been proposed that amidase PGRPs could act as scavenging molecules. Indeed, degradation of PGN by Drosophila PGRP-SC1b markedly reduces its immuno-stimulatory potency in cell-culture assays [27]. We report here an in vivo study on the role of Drosophila PGRPs with amidase activity. We show that two PGRPs with described amidase activity, namely PGRP-SC1 and PGRP-SC2, control the intensity of the Drosophila immune response. We also present evidence that in the absence of such a control, infection-induced IMD-pathway over-activation can cause developmental defects and larval death.

Results

Loss-of-Function Mutants for PGRP-SC1/2 Are Generated by RNA Interference

In order to address the function of NAMLAA PGRPs under in vivo conditions, we analyzed the immune response of Drosophila with reduced PGRP-SC1 and PGRP-SC2 levels. The Drosophila genome contains a cluster of two tandemly arranged PGRP-SC1 loci (named a and b) and a single PGRP-SC2 locus [25] (Figure S2A). The two PGRP-SC1 mRNAs differ by only three nucleotides and translate into a unique protein that is 70% identical to the PGRP-SC2 polypeptide (Figure S2B and S2C). Furthermore, PGRP-SC1 and PGRP-SC2 proteins form a separate cluster in the PGRP phylogenetic tree (Figure S2D). To eliminate potential problems of functional redundancy between these two homologous enzymes, we decided to simultaneously knockdown the PGRP-SC1 and the PGRP-SC2 genes in vivo. The presence of long stretches of identical sequences in their transcripts prompted us to take advantage of the RNA-interference method (Figure S2B). Using PGRP-SC1- and PGRP-SC2-specific primers, we could demonstrate that adult flies carrying a UAS iPGRP-SC construct (see Materials and Methods) together with a ubiquitous Gal4 driver (DaGal4) exhibited a 90% reduction of both PGRP-SC1 and PGRP-SC2 mRNA levels (Figure 1). The transcript levels of PGRP-SA and PGRP-SD, two closely related family members, were unaffected in these flies, demonstrating the specificity of the designed UAS iPGRP-SC construct (Figure 1).

IMD Pathway in PGRP-SC1/2–Depleted Flies Is Over-Activated

We first analyzed the potential role of PGRP-SC in adult flies, which are more amenable than larvae to pricking and survival experiments. For this, we infected UAS iPGRP-SC and DaGal4;UAS iPGRP-SC flies (DaGal4 is a ubiquitous driver)
with gram-negative bacteria and measured *diptericin* transcript levels as a conventional readout for IMD-pathway activation. Six hours after infection with *Enterobacter cloacae* or *Escherichia coli* (a time-point that corresponds to the peak of *diptericin* mRNA kinetics in wild-type flies), no differences were noted between the levels of *diptericin* mRNA in UAS iPGRP-SC and DaGal4;UAS iPGRP-SC flies (Figure 2A). However, whereas the *diptericin* mRNA level dropped significantly at 24 and 48 h in UAS iPGRP-SC flies (as it usually does in the wild-type condition), it remained high in DaGal4;UAS iPGRP-SC flies. Similar results were obtained with another m-DAP-PGN–containing bacteria (*Bacillus subtilis*), although the differences between UAS iPGRP-SC and DaGal4;UAS iPGRP-SC could be detected as soon as 6 h after infection (Figure 2A). Experiments performed with another ubiquitous Gal4 driver (ActinGal4) and an independent UAS iPGRP-SC insertion generated identical results (data not shown). When gram-positive bacteria were used as inducers, two of them (*Micrococcus luteus* and *Enterococcus faecalis*) did not activate the IMD pathway above clean injury levels. A third one, *Staphylococcus aureus*, triggered slightly higher *diptericin*-transcription levels in PGRP-SC–depleted flies than in controls, although the induction was mild, as expected from gram-positive bacteria (Figure 2A).

These results indicate that depletion of PGRP-SC induces an over-activation of the IMD pathway after bacterial infection. This phenotype was not observed in noninfected DaGal4;UAS iPGRP-SC flies or after pricking with a clean needle (Figure 2A). Altogether, this indicates that the observed effects are dependent on the presence of bacteria and do not correspond to a constitutive activation of the IMD pathway in PGRP-SC–depleted flies. They also demonstrate that the other putative secreted *Drosophila* amidases (PGRP-SB1 and PGRP-SB2) are not able to compensate for the absence of PGRP-SCs in vivo. In the absence of loss-of-function mutants for each of the three PGRP-SC genes, we can obviously not rule out the possibility that only one or two of them are responsible for the observed phenotype. To obtain additional proof that the effects observed in PGRP-SC–depleted flies were specific, we performed similar experiments in flies in which the non-enzymatic bacterial receptor PGRP-SA had been depleted by RNA interference. Whereas DaGal4;UAS iPGRP-SC flies showed an expected reduced ability to respond to infection by the gram-positive *M. luteus*, their response to *E. coli* remained wild-type (Figure 2B). Therefore, DaGal4;UAS iPGRP-SC flies behave as classical PGRP-SA<sup>null</sup> loss-of-function mutants [7] and not like flies with reduced PGRP-SC levels.

**PGRP-SC1/2–Depleted Flies Are Able to Clear Bacteria**

Since PGRP-SC1 has been proposed to act as a scavenger molecule [27], we tested whether the IMD-pathway over-activation observed in PGRP-SC–depleted flies could reflect the inability of these flies to clear bacteria. If this were to be proved the case, accumulation of bacteria in the body cavity of DaGal4;UAS iPGRP-SC flies could explain the over-activation of the IMD pathway. To test this hypothesis, we compared bacterial loads and survival curves of *IKK<sup>bos</sup>*<sup>mut</sup> UAS iPGRP-SC, and DaGal4;UAS iPGRP-SC flies infected with *E. cloacae* or with *E. coli* (Figure 2C and 2D). Whereas *IKK<sup>bos</sup>*<sup>mut</sup> mutant flies showed a very high bacterial load and a strong susceptibility to these bacteria, no such phenotypes were observed in DaGal4;UAS iPGRP-SC flies. Similar results were obtained with *B. subtilis* (unpublished data). This suggests that the over-response observed in PGRP-SC–depleted flies did not result from an uncontrolled bacterial growth in the hemolymph, and that the role of PGRP-SC proteins in vivo is not to scavenge bacteria from the circulating hemolymph.

**Toll-Pathway Activation Is Wild-Type in PGRP-SC1/2 Mutant Flies**

It is notable that non-enzymatic PGRPs, such as PGRP-SA, PGRP-LC, or PGRP-LE are able to discriminate between Lys-type and m-DAP-type PGN [14–17] (Figure S1). Recent experiments have nevertheless demonstrated that PGRP-SC1b can act as a cleaving enzyme for both gram-positive and gram-negative bacterial PGN in vitro. We therefore asked whether reducing the endogenous levels of PGRP-SC1/2 could also have an effect on Toll-pathway activation by gram-positive bacteria. As illustrated in Figure 3, the effects were IMD-pathway–specific since Toll-dependent activation of *drosomycin* by gram-positive or gram-negative bacteria (*M. luteus*, *E. faecalis*, *S. aureus*, *E. cloacae*, *E. coli*, *B. subtilis*) were similar in UAS iPGRP-SC and DaGal4;UAS iPGRP-SC flies. This difference between the role of PGRP-SC1/2 on Toll- and IMD-pathway activation could reflect functional redundancy between amidases for Lys-type PGN which might not exist for m-DAP-type PGN-cleaving enzyme. Alternatively, this could pertain to the difference in the mode of activation of the transmembrane receptors upstream of each pathway.

**PGRP-SC1/2 Function in Larval Immune Response**

We next addressed the role of PGRP-SC proteins in larvae. Previous qualitative analyses indicated that PGRP-SC1 and PGRP-SC2 genes are transcribed in almost identical patterns and mostly in the gut cells [25]. Using quantitative RT-PCR, we confirmed that the larval gut is strongly enriched in PGRP-SC1 and PGRP-SC2 mRNA and represents the main site of PGRP-SC amidase synthesis at this developmental stage (Figure 4A). In a previously established model of infection by ingestion [31], it was observed that larvae fed with the gram-negative bacteria *Erwinia carotovora carotovora* induce *diptericin* transcription in the fat body. Surprisingly, most of the other gram-negative bacterial species tested in this assay failed to do so. We reasoned that the gut PGRP-SC amidases might act to reduce the PGN immunogenic potential of these bacteria, preventing them from activating a systemic immune response.

To test this hypothesis, UAS iPGRP-SC control larvae and DaGal4;UAS iPGRP-SC larvae, which exhibit a strong reduction of PGRP-SC1 and PGRP-SC2 mRNA levels in their gut (Figure 4A), were fed with various bacterial species. We then monitored *diptericin* expression in whole larvae or, more specifically, in the fat body. As previously reported [31], we found that ingested *E. carotovora carotovora*, but not *E. coli*, was able to activate the IMD pathway in UAS iPGRP-SC control larvae (Figure 4B). Strikingly, reducing the PGRP-SC levels induced a strong increase in the expression level of the *diptericin* mRNA at 6 and 24 h after feeding on *E. carotovora carotovora*, as compared to controls (Figure 4B). Under these conditions, *E. coli* now became a good inducer of *diptericin* expression. This increase in the level of *diptericin* transcription was totally blocked in a PGRP-LE mutant background, demonstrating that, in this process, the PGRP-SC
Figure 2. IMD-Pathway Activation Is Downregulated by PGRP-SC1/2

(A) Kinetics of diptericin mRNA induction (Dipt/RpL32) after infection by various bacteria. Each histogram corresponds to the mean value of five independent experiments (± standard deviation). Asterisks indicate that the difference between DaGal4;UAS iPGRP-SC and control UAS iPGRP-SC values is statistically significant (p < 0.05). One hundred percent corresponds to the level of activation at 6 h in control flies. In the lower panel, diptericin
amidases act upstream of the IMD-pathway transmembrane receptor (Figure 4B). As shown above for the immune response in adults, reducing the PGRP-SC levels had no effect on Toll-pathway activation in larvae (Figure 4C).

**PGRP-SC1/2 Are Required in the Larval Gut to Dampen the Immune Response**

We tested whether similar results could be obtained by reducing PGRP-SC levels specifically in the larval gut using a tissue-specific driver (CadGal4) [32]. Consistent with previous reports indicating that CadGal4 is not a very strong driver [32], we noted that the reduction of PGRP SC1/2 mRNA levels in the gut were not as pronounced in CadGal4;UAS iPGRP-SC than in DaGal4;UAS iPGRP-SC larvae (Figure 4A). However, this reduction was sufficient to trigger an activation of the IMD pathway after feeding on *E. coli* (Figure 4B). Using a DiptlacZ reporter transgene, we could show that up to 40% of the CadGal4;UAS iPGRP-SC larvae activated the IMD pathway in the fat body after feeding on *E. coli* (Figure 4D and E). In wild-type control larvae, this percentage was only 5% (Figure 4E). In parallel experiments, we tested the effects of over-expressing the PGRP-SC1b protein in the larval gut. Whereas 80% of the diptlacZ;UAS PGRP-SC1b control larvae fed with *E. carotovora* activated diptericin transcription in the fat body, this percentage dropped to 10% in larvae which specifically over-expressed PGRP-SC1b in the gut (Figure 4E). Altogether, these results are compatible with the hypothesis that an essential role of gut PGRP-SC1/2 amidases in larvae is to modulate activation of the IMD pathway. We propose that this modulation is achieved by lowering the amount of immunogenic PGN, most probably via an amidase-dependent degradation. However, we cannot rule out that it is also partly due to sequestration of the PGN.

**Larvae with Reduced PGRP-SC1/2 Levels Are Highly Susceptible to Infection**

To evaluate the consequences of the reduction of this immuno-modulatory function, we followed the fate of naturally infected wild-type and PGRP-SC–depleted larvae. We observed that mortality was three to four times higher in depleted larvae fed with *E. coli* or *E. carotovora* than in controls (Figure 5A). This increase in larval lethality was induced after gram-positive bacterial infections were compared to that obtained after infection by *E. cloacae* (100%). RpL32 is used as an internal control. ci, clean injury; ni, noninfected.

(B) Quantification of dipterac mRNA levels in UAS iPGRP-SA, DaGal4;UAS iPGRP-SA and PGRPS-A*elmt* flies shows that reduction of PGRP-SA mRNA levels does not influence IMD-pathway induction 6 h after infection by *E. coli*. Quantification of drosomycin mRNA levels 24 h after M. luteus infection indicates that PGRP-SA is efficiently knocked down by dsRNA interference. PGRPS-A*elmt* is a complete loss-of-function mutant for PGRP-SA. Each histogram corresponds to the mean value of five independent experiments (± standard deviation). Asterisk indicates that the difference between DaGal4;UAS iPGRP-SA and control UAS iPGRP-SA values is statistically significant (p < 0.05).

(C) DaGal4;UAS iPGRP-SC flies are as susceptible to infection by *E. cloacae* as control flies.

(D) *E. cloacae* and *E. coli* AmpR growth in various genetic backgrounds 24 h after infection. Flies with reduced levels of PGRP-SC1/2, unlike *IKKβ*knockdown mutants, are able to clear bacteria from their hemolymph. Each histogram corresponds to the mean value of four independent experiments (± standard deviation).

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**Figure 3. Toll-Pathway Activation Is Wild-Type in DaGal4;UAS iPGRP-SC Flies**

Kinetics of drosomycin mRNA induction (Drs/RpL32) after infection by gram-positive (upper panel) and gram-negative (lower panel) bacteria. Each histogram corresponds to the mean value of six independent experiments (± standard deviation). Asterisk indicates that the difference between DaGal4;UAS iPGRP-SC and control UAS iPGRP-SC values is statistically significant (p < 0.05). One hundred percent corresponds to the level of activation 24 h after infection in control flies. In the lower panel, drosomycin induction after gram-negative bacterial infections is compared to that of *S. aureus* infection which is set to 100%. RpL32 is used as an internal control.

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totally suppressed in a PGRP-LC mutant background, demonstrating that over-activation of the IMD pathway was indeed the cause of larval death. Interestingly, a small percentage of the DaGal4;UAS iPGRP-SC larvae that pupariated and eclosed as pharate adults presented developmental defects such as wing notching (Figure 5B–5D). These phenotypes were never observed in control larvae fed with bacteria. To test whether this wing phenotype could be due to

Figure 4. Reduction of PGRP-SC1/2 Levels in the Larval Gut Increases IMD-Pathway Activation after Natural Infection

(A) PGRP-SC1 and PGRP-SC2 mRNAs (PGRP-SC/RpL32) are mainly expressed in the larval gut and are severely reduced in DaGal4;UAS iPGRP-SC and CadGal4;UAS iPGRP-SC larvae. Each histogram corresponds to the mean value of four independent experiments (± standard deviation).

(B) Dipterinc mRNA induction levels (Dipt/RpL32), measured 6 h and 24 h after natural infection. Each histogram corresponds to the mean value of variable numbers (shown in parentheses) of independent experiments (± standard deviation). Asterisks indicate that the difference between DaGal4;UAS iPGRP-SC or CadGal4;UAS iPGRP-SC and control UAS iPGRP-SC values is statistically significant (p < 0.05).

(C) Drosomycin mRNA induction levels (Drs/RpL32), measured 24 h after natural infection. Each histogram corresponds to the mean value of four independent experiments (± standard deviation).

(D) Three hours after natural infection with E. coli GFP, bacteria were found to be highly concentrated in the anterior half of the larval gut. In larvae with reduced gut PGRP-SC levels (CadGal4;UAS iPGRP-SC), feeding on E. coli is sufficient to trigger IMD-pathway activation in the fat body after 24 h (visualized here by the use of a diptericin-LacZ transgene).

(E) Percentage of larvae showing β-galactosidase activity in the fat body 24 h after natural infection. For each genotype, ten larvae were dissected and stained. Each histogram corresponds to the mean value of five independent experiments (± standard deviation). Asterisks indicate that the difference between diptLacZ;UAS iPGRP-SC and diptLacZ;DaGal4;UAS iPGRP-SC or diptLacZ;CadGal4;UAS iPGRP-SC and CadGal4;UAS iPGRP-SC values is statistically significant (p < 0.05).

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increased cell death during larval development, imaginal discs from infected larvae were stained with acridine orange. Wing discs from PGRP-SC–depleted, bacteria-fed larvae showed higher levels of cell death than wing discs from control larvae fed with normal levels of PGRP-SC (Figure 5E and 5F).

Discussion

The need for a tight balance between initiation and resolution in the control of inflammation in vertebrates has been documented for a long time. Recent reports have reviewed the molecular mechanisms that are put in place to dampen inflammation and to prevent damaging effects associated with a prolonged immune response [33,34]. The data presented here suggest that immune response needs to be tightly regulated also in invertebrates.

Taken together, our data provide novel insights into the physiological roles of PGRPs in Drosophila. They show that in addition to the function as a pattern-recognition receptor of some PGRP family members, others can specifically control the level of activation of the IMD signaling pathway. Flies deficient for PGRP-SC1a, PGRP-SC1b, and PGRP-SC2 present a specific over-activation of the IMD pathway. A recent report described an effect of a PGRP-SC1 mutant (picky) on Toll-pathway activation [35], a phenotype that we did not observe in PGRP-SC1/2–depleted flies (see Figure 3). This discrepancy is not yet fully understood but could be explained by the fact that picky flies are mutant only for PGRP-SC1a and PGRP-SC1b, whereas PGRP-SC2 is also affected in our PGRP-SC–depleted flies.

Our results indicate that the gut is the main tissue in which the regulation by PGRP-SC proteins is taking place. However, the fact that IMD-pathway over-activation was also detected when bacteria were introduced directly into the circulating hemolymph suggests that these secreted proteins could also be present in the blood or in the circulating hemocytes. We further show that in the absence of a control of the immune response, infection can lead to developmental defects or death by over-activation of the immune pathway. Interestingly, recent reports indicate that other immune-induced pathways can have a harmful effect on fly survival. Salmonella typhimurium–infected flies produce a tumor necrosis factor (TNF)–like cytokine which has been shown to be damaging for the host [36]. In addition, flies in which the gut catalase level is experimentally reduced show high mortality rates after ingestion of microbe-contaminated foods. This has been interpreted as evidence that infection-mediated induction of reactive oxygen species (such as H₂O₂) must be tightly balanced to avoid larval lethality [32]. In this respect, our data indicate that PGRP-SC1/2 may act as detoxifying proteins for bacterial PGN in flies. Although we did not demonstrate that the amidase function of these PGRPs is required for this effect, in vitro biochemical data strongly suggest that it is the case. Similar functions have recently
been attributed to enzymes which reduce the immunogenic potential of lipopolysaccharide during vertebrate immune response [37].

The results presented here are consistent with previous data showing that over-expression of some components of the IMD pathway are larval lethal. However, the molecular mechanisms by which over-activation of the IMD pathway leads to lethality remain unknown. A number of observations may provide clues about this issue: (i) several components of the IMD pathway are homologous to mammalian proteins involved in signaling through the TNF receptor, a pathway known to trigger apoptosis [38]; and (ii) the MAP3 kinase Tak1, which is an essential component of the IMD pathway, has been shown to function both as an Ikk kinase (regulating dipterin expression) and as a JNK (c-Jun N-terminal kinase) kinase [39]. It is significant in this context that inappropriate activation of the JNK signaling cascade in the wing disc leads to apoptotic pathway-dependent morphological defects [40]. Further investigations will be needed to clarify the molecular links existing between the activation of the Drosophila IMD pathway and the developmental defects which we observed; in particular, a role of the apoptosis pathways in this process should be considered. Finally, it will be of interest to investigate whether amidases or other PGN-modifying enzymes are involved in modulating bacteria-induced immune response in mammals. In this respect, it is intriguing that one human PGRP family member (PGRP-Iβ) is expressed in the esophagus [21], which evokes the gut expression of PGRP-SC.

Materials and Methods

Bacterial strains. The following microorganisms were used: E. coli, M. luteus, E. carotovora carotovora 15B, S. subtilis, E. cloacae, E. faecalis, S. aureus, and E. coli AmPr.

*Drosophila strains. IKK*Δ*31* is a loss-of-function mutation allele. *PGRP-LE*Δ*3* is a complete deletion of the *PGRP-LC* locus. Flies carrying either of these mutations are unable to activate the IMD pathway. *PGRP-SA*Δ*5* is a point-mutation null allele which prevents Toll-pathway activation by some bacteria. All these alleles have been generated by crossing flies carrying *UPI* or *UPIA* with flies carrying *EPIC* or *EPIC2*.

Accession Numbers

The FlyBase (http://flybase.bio.indiana.edu) accession numbers for the *Drosophila* strains produced include: *PGRP-SC*, *PGRP-SA*, *PGRP-LC*, *PGRP-LE*, *IKK*, and *Rpl32*. The Swiss-Prot Enzyme Nomenclature database (http://www.expasy.org/enzyme) accession number for NAMLAA is EC3.5.1.28.

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Supporting Information

**Figure S1.** Schematic Representation of the PGN Structure

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**Figure S2.** PGRP-SC Alignments and Phylogeny

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Competing interests

The authors have declared that no competing interests exist.
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