

# Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity

Aya Takehana, Tamaki Yano, Shizuka Mita, Atsushi Kotani, Yoshiteru Oshima and Shoichiro Kurata\*

Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan

**In innate immunity, pattern recognition molecules recognize cell wall components of microorganisms and activate subsequent immune responses, such as the induction of antimicrobial peptides and melanization in *Drosophila*. The diaminopimelic acid (DAP)-type peptidoglycan potently activates imd-dependent induction of antibacterial peptides. Peptidoglycan recognition protein (PGRP) family members act as pattern recognition molecules. PGRP-LC loss-of-function mutations affect the imd-dependent induction of antibacterial peptides and resistance to Gram-negative bacteria, whereas PGRP-LE binds to the DAP-type peptidoglycan, and a gain-of-function mutation induces constitutive activation of both the imd pathway and melanization. Here, we generated PGRP-LE null mutants and report that PGRP-LE functions synergistically with PGRP-LC in producing resistance to *Escherichia coli* and *Bacillus megaterium* infections, which have the DAP-type peptidoglycan. Consistent with this, PGRP-LE acts both upstream and in parallel with PGRP-LC in the imd pathway, and is required for infection-dependent activation of melanization in *Drosophila*. A role for PGRP-LE in the epithelial induction of antimicrobial peptides is also suggested.**

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## Introduction

Innate immunity is a self-defense mechanism that is evolutionarily conserved throughout all metazoans (reviewed in Hoffmann and Reichhart, 2002). In *Drosophila*, a model system to study the principles of innate immunity (reviewed in Hultmark, 2003), epithelial tissues such as the epidermis, gut, and trachea (the insect respiratory organ) serve as the first line of self-defense against invading microorganisms by

functioning as structural barriers and by producing antimicrobial peptides that inhibit microbial growth (Tzou *et al*, 2000). Microorganisms that pass through the epithelial barrier are countered in hemocoels filled with the hemolymph (blood) by systemic reactions, including cellular and humoral reactions (reviewed in Tzou *et al*, 2002). The humoral reactions depend on the primary and secondary responses. The primary response is mediated by the activation of cascades of constitutive proteins present in the hemolymph, such as the prophenoloxidase (proPO) cascade leading to localized wound healing and melanization. Biochemical studies of the proPO cascade in insects and crustaceans indicate that the serine protease cascade is initiated by the recognition of microbial cell wall components (reviewed in Ashida and Brey, 1998; Söderhäll and Cerenius, 1998). The secondary response requires transcriptional activation of defense proteins such as antimicrobial peptides. In response to microbial infections, the antimicrobial peptides are synthesized in the fat body, the functional equivalent of the mammalian liver, and secreted into the hemolymph, which is mainly regulated by two distinct signaling pathways, the Toll pathway and the imd pathway (reviewed in Engström, 1999). An antifungal peptide gene, *Drosomycin* (*Drs*), is predominantly activated by the Toll pathway through Toll membrane receptor and transcriptional factors, Dorsal and Dorsal-related immunity factor (Dif), in response to fungal and some Gram-positive bacterial infections, such as *Enterococcus faecalis*. An antibacterial peptide gene, *Diptericin* (*Dpt*), however, is predominantly activated by the imd pathway through the Imd death domain adaptor protein and Relish transcriptional activator in response to Gram-negative and other Gram-positive bacterial infections, such as *Bacillus subtilis*. The induction of other antimicrobial peptide genes, such as *Attacin* (*Att*), is thought to be regulated by input from both the Toll pathway and the imd pathway. Thus, *Drosophila* possess specific mechanisms to discriminate between microbes and activate the appropriate immune reactions to these infections (Lemaitre *et al*, 1997; reviewed in Hoffmann and Reichhart, 2002).

In contrast to the relatively good understanding of the signaling cascades leading to the activation of the antimicrobial peptide genes, little is known about how the *Drosophila* immune system recognizes different groups of invading microorganisms and activates the proper immune responses. In innate immunity, the pattern recognition molecules recognize the pathogen-associated molecular patterns of repeating structural motifs, and activate immune reactions (reviewed in Medzhitov and Janeway, 2002). In fact, cell wall components containing repeated structures such as lipopolysaccharides (LPSs), peptidoglycans, and  $\beta$ -1,3-glucans strongly induce innate immune reactions. In mammals, Toll-like receptors (TLRs), mammalian homologs of *Drosophila* Toll, act as pattern recognition molecules. For example, TLR4 is a

\*Corresponding author. Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki, Aoba-ku, Sendai 980-8578, Japan. Tel.: +81 22 217 6823; Fax: +81 22 217 6821; E-mail: kurata@mail.pharm.tohoku.ac.jp

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recognition receptor for LPSs, which are components of Gram-negative bacterial cell walls (reviewed in Akira *et al*, 2001). *Drosophila* Toll, however, does not act as a pattern recognition molecule; it is activated by an endogenous ligand, Spätzle, which is cleaved by proteolytic enzymes after microbial infection (Levashina *et al*, 1999; Ligoxygakis *et al*, 2002a; Weber *et al*, 2003). Therefore, pattern recognition molecules have remained the missing pieces of the puzzle in *Drosophila* immunity. Recent reports of *Drosophila* genetic screens of loss-of-function and gain-of-function mutations highlight the important roles of peptidoglycan recognition protein (PGRP) family members as the pattern recognition molecules in *Drosophila*.

PGRP was first purified from silkworm hemolymph based on its high affinity for peptidoglycan and its ability to mediate peptidoglycan-dependent activation of the proPO cascade as demonstrated by *in vitro* reconstitution experiments (Yoshida *et al*, 1996). Subsequent cloning of PGRP genes demonstrated that PGRPs are conserved from insects to mammals, and there are four PGRPs in humans (reviewed in Dziarski, 2004). Of the 13 PGRP family members encoded by the *Drosophila* genome (Werner *et al*, 2000; reviewed in Dziarski, 2004), PGRP-SA, PGRP-LC, and PGRP-LE participate in the recognition of invading bacteria and activation of immune responses. PGRP-SA is a circulating hemolymph protein that binds to the lysine-containing peptidoglycan of *Micrococcus luteus* (Werner *et al*, 2000). A mutation of *PGRP-SA*, called *semmlweis* (*seml*), abolishes the Toll-dependent expression of *Drs* in response to *M. luteus* infection, and fails to resist Gram-positive bacterial infections (Michel *et al*, 2001). On the other hand, *seml* does not affect Toll-dependent resistance to fungi and imd-mediated resistance to bacteria, suggesting that, like the role of the TLR family in mammals, diverse PGRP members are involved in bacterial discrimination. Supporting this hypothesis, mutations in a putative transmembrane protein, PGRP-LC, affect activation of the imd pathway and resistance to Gram-negative bacteria (Choe *et al*, 2002; Gottar *et al*, 2002; Rämets *et al*, 2002). The *PGRP-LC* null mutant phenotypes have much less activation of antibacterial peptide genes and survival against Gram-negative bacterial infections than other loss-of-function mutants in the imd pathway (Gottar *et al*, 2002; Rämets *et al*, 2002). These findings suggest that there is an activator of the imd pathway in addition to PGRP-LC. Consistent with this conclusion, we identified *PGRP-LE* in a gain-of-function screen, and found that PGRP-LE also acts upstream of the imd pathway to activate antimicrobial peptide gene expression in both the systemic and local epithelial responses (Takehana *et al*, 2002). In addition to activation of the imd pathway, the overexpression of PGRP-LE also activates the proPO cascade in *Drosophila* upstream of Imd. Peptidoglycans, components of cell walls of almost all bacteria, have a diverse amino-acid composition, and the linking of stem peptides depends on the bacterial species (Schleifer and Kandler, 1972). PGRP-LE binds to the directly crosslinked diaminopimelic acid (DAP)-containing peptidoglycan, which is an extremely potent inducer of the imd pathway (Leulier *et al*, 2003), but not to the lysine-containing peptidoglycans with an interpeptide bridge of Gram-positive bacteria (Takehana *et al*, 2002). PGRP-LC also mediates the DAP-type peptidoglycan-dependent induction of *Dpt* (Leulier *et al*, 2003). In addition, a different isoform of PGRP-LC,

PGRP-LCa, participates in the LPS-dependent activation of antibacterial peptide genes, at least in cell culture systems (Werner *et al*, 2003).

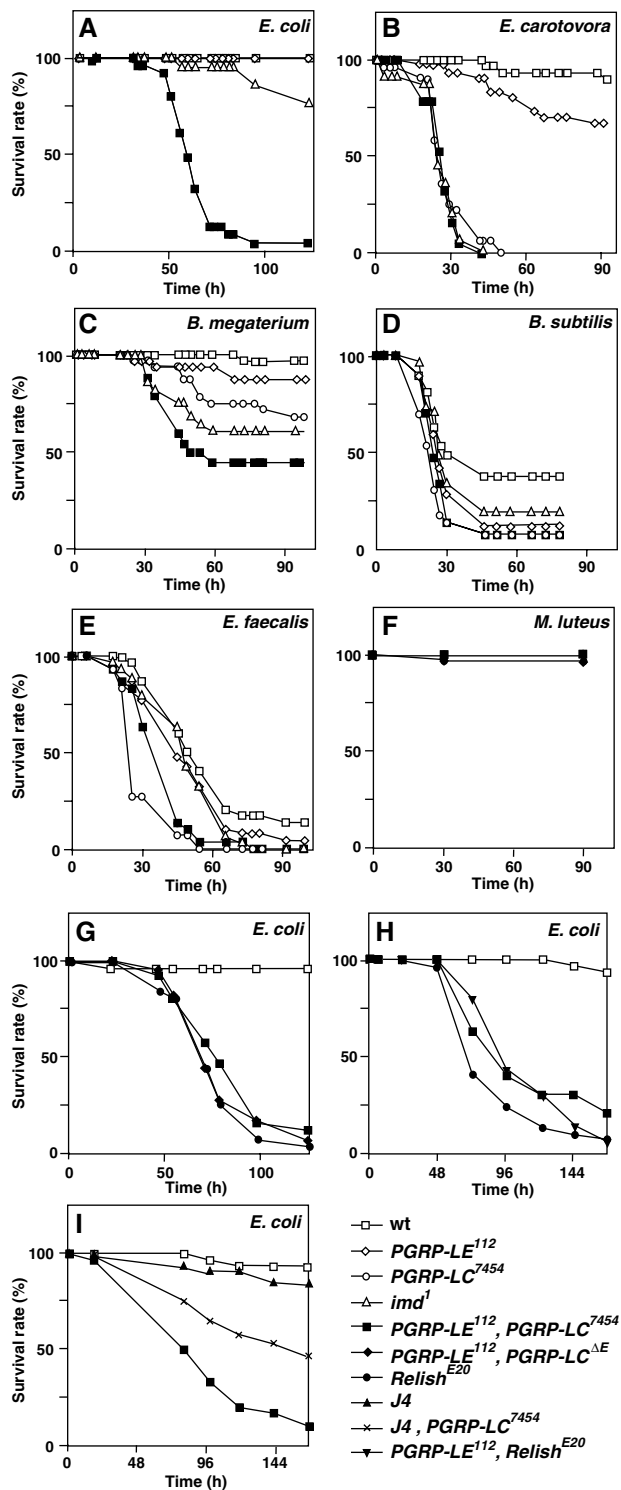
In the present paper, we generated a null mutant of *PGRP-LE*, and found that PGRP-LE functioned synergistically with PGRP-LC to produce resistance to infection by *Escherichia coli* and *Bacillus megaterium*, which have DAP-type peptidoglycans. Consistent with this finding, epistatic analyses revealed that PGRP-LE acts both in parallel and upstream of PGRP-LC in the imd-mediated induction of the antimicrobial peptides. PGRP-LE is also required for infection-dependent activation of the proPO cascade. Therefore, PGRP-LE has important roles in the activation of systemic reactions in host defense. In the epithelial reactions, PGRP-LE is localized at the luminal surface of the trachea and has non-cell autonomous effects on the activation of the *Drs* promoter in tracheal cells, suggesting a role for PGRP-LE in the recognition and subsequent activation of the signaling at the first point of contact with invading bacteria.

## Results

### ***PGRP-LE* and *PGRP-LC* synergistically induce resistance to bacterial infections**

Gain-of-function screens utilizing P element insertions indicated that PGRP-LE activates antimicrobial responses in the absence of microbial infection. To investigate the requirement of *PGRP-LE* for the antimicrobial response, we generated a *PGRP-LE*-deficient mutant by mobilizing the P element (Supplementary Figure 1A). *PGRP-LE* expression was screened by reverse transcription-polymerase chain reaction (RT-PCR) with 149 excision lines, and in one line, *PGRP-LE*<sup>112</sup>, there was no expression of *PGRP-LE* (Supplementary Figure 1B). In *PGRP-LE*<sup>112</sup>, the expression of the neighboring genes of *PGRP-LE*, CG8974, CG32581, CG15602, and CG8509, was not affected (Supplementary Figure 1B). Sequencing analysis after genomic PCR of *PGRP-LE*<sup>112</sup> revealed that a 2542-bp sequence, including the *PGRP-LE* start codon, was deleted in *PGRP-LE*<sup>112</sup>. Consistent with the molecular characterization, PGRP-LE protein was not expressed in *PGRP-LE*<sup>112</sup> (Figure 6A). *PGRP-LE*<sup>112</sup> flies were fertile and viable, suggesting that PGRP-LE is not critical for development. In wild type, the expression of *PGRP-LE* did not change after *E. coli*, *B. subtilis*, or *E. faecalis* infections, indicating that PGRP-LE is a constitutive protein (Supplementary Figure 1C).

We compared the survival rate of *PGRP-LE*<sup>112</sup> with various mutant flies including *PGRP-LC*, which also acts upstream of the imd pathway, after Gram-negative bacterial infections, *E. coli* and *Erwinia carotovora carotovora*, and Gram-positive bacterial infections, *B. megaterium*, *B. subtilis*, *E. faecalis*, and *M. luteus* (Figure 1). The survival rate of *PGRP-LE*<sup>112</sup> to bacterial infections was similar to that of wild type except for infection by *E. carotovora carotovora* and *B. subtilis*. In this experiment, *E. carotovora carotovora*, which naturally infects *Drosophila* (Basset *et al*, 2000), was injected into the flies. The susceptibility of *PGRP-LE*<sup>112</sup> to *E. carotovora* was much weaker than that of *PGRP-LC*<sup>7454</sup> and *imd*<sup>1</sup>; however, against *B. subtilis*, *PGRP-LE*<sup>112</sup> had reduced resistance, similar to *PGRP-LC*<sup>7454</sup> and *imd*<sup>1</sup> (Figure 1B and D). These results suggest that PGRP-LE contributes to sensing these bacteria. Both *PGRP-LE*<sup>112</sup> and *PGRP-LC*<sup>7454</sup> had complete resistance to *E. coli* infections, but a double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup>



**Figure 1** Survival rate of PGRP-LE mutant flies after different types of infections. (A–F) Survival rate of wild-type flies (wt), *PGRP-LE*<sup>112</sup>, *PGRP-LC*<sup>7454</sup>, *imd*<sup>1</sup>, and the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant (*PGRP-LE*<sup>112</sup>; +; *PGRP-LC*<sup>7454</sup>) infected with the indicated bacteria. In (F), the survival rate of *Relish*<sup>E20</sup> is also presented. (G) Survival rate of wild type, *Relish*<sup>E20</sup>, the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant, and the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>ΔE</sup> mutant against *E. coli* infection. (H) Survival rate of wild type, *Relish*<sup>E20</sup>, the double *PGRP-LE*<sup>112</sup>/*Relish*<sup>E20</sup> mutant, and the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant against *E. coli* infection. (I) Survival rate of wild type, J4, the double J4/*PGRP-LC*<sup>7454</sup> mutant, and the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant against *E. coli* infection. The survival experiments were performed at 25°C.

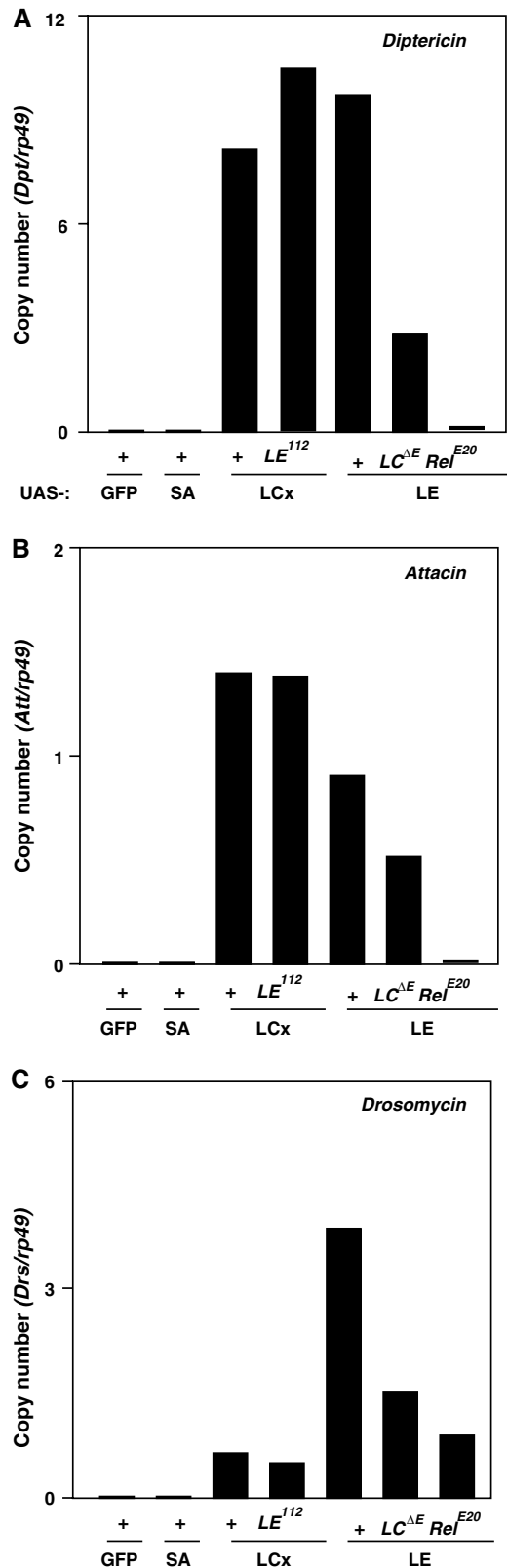
mutant had reduced resistance, and the susceptibility was much greater than that of *imd*<sup>1</sup> (Figure 1A). These results indicate that PGRP-LE and PGRP-LC function synergistically in the self-defense against *E. coli* infection. There is a similar synergy between PGRP-LE and PGRP-LC in the resistance to *B. megaterium* but not in the resistance to *E. faecalis* and *M. luteus* (Figure 1C, E, and F). *B. megaterium* and *B. subtilis* have the DAP-type peptidoglycan similar to Gram-negative bacteria such as *E. coli*, but *E. faecalis* and *M. luteus* have the lysine-containing peptidoglycan. These results are consistent with the previous conclusion that both PGRP-LE and PGRP-LC recognize the DAP-type peptidoglycan.

The double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant was much more susceptible to *E. coli* than *imd*<sup>1</sup>. To determine whether PGRP-LE and PGRP-LC regulate other host defense reactions in addition to activation of the *imd* pathway, we compared the survival rate of the double mutant to that of *Relish*<sup>E20</sup>, which is a null mutation of the transcriptional activator of the *imd* pathway (Hedengren *et al*, 1999), because *imd*<sup>1</sup> is a hypomorphic mutation (Georgel *et al*, 2001). The survival rate of the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant was similar to that of *Relish*<sup>E20</sup> (Figure 1G). The result was confirmed with a *PGRP-LC*<sup>ΔE</sup> null mutation. These results suggest that the two pattern recognition molecules are major regulators of the *imd* pathway that sense *E. coli*. Corresponding to this, the double *PGRP-LE*<sup>112</sup>/*Relish*<sup>E20</sup> mutant was not more susceptible to *E. coli* than *Relish*<sup>E20</sup> (Figure 1H). Moreover, to determine whether PGRP-LE participates in the resistance to *E. coli* infection through regulating the Toll pathway, we compared the survival rate of the double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant to that of the double J4/*PGRP-LC*<sup>7454</sup> mutant in which two transcriptional activators of the Toll pathway, *dorsal* and *Dif*, were deleted (Meng *et al*, 1999). The double *PGRP-LE*<sup>112</sup>/*PGRP-LC*<sup>7454</sup> mutant was more susceptible to *E. coli* infection than the double J4/*PGRP-LC*<sup>7454</sup> mutant (Figure 1I). These results suggest that PGRP-LE participates in host defense, mainly through regulating the *imd* pathway.

Rescue experiments using the artificial expression of PGRP-LE in the double *PGRP-LE*/*PGRP-LC* mutant could not be performed because the survival rate was decreased by the induction of PGRP-LE in the absence of bacterial infection, which is probably related to the PGRP-LE-mediated lethality described below.

### Epistatic analyses of PGRP-LE and PGRP-LC in the induction of antimicrobial peptides

It was suggested that PGRP-LE and PGRP-LC were two major regulators of the *imd* pathway in the induction of antimicrobial peptides. To determine the relation between PGRP-LE and PGRP-LC, we analyzed the epistatic relation between *PGRP-LE* and *PGRP-LC* in the activation of antimicrobial peptide genes (Figure 2). Under the control of the c564-GAL4 driver, which expresses GAL4 in the fat body and the hemocytes (blood cells) (Harrison *et al*, 1995), PGRP-LE and PGRP-LCx, an isoform of PGRP-LC from the RA transcript (Choe *et al*, 2002), similarly induced strong expression of *Dpt* and weak expression of *Att*, whereas PGRP-LE more strongly induced *Drs* than PGRP-LCx. Green fluorescent protein (GFP) and PGRP-SA overexpression did not induce expression of these antimicrobial peptide genes. PGRP-LCx-mediated activation of these antimicrobial peptide genes was not affected in the *PGRP-LE* null mutant background, indicating that



**Figure 2** Epistatic analyses of PGRP-LE with PGRP-LC on antimicrobial peptide gene expression. Under the control of c564-GAL4, GFP, PGRP-SA, PGRP-LCx, and PGRP-LE were overexpressed in the wild-type (+), PGRP-LE<sup>112</sup>, PGRP-LC<sup>ΔE</sup>, and Relish<sup>E20</sup> larvae. The amount of mRNA of Dipteracin (A), Attacin (B), and Drosomycin (C), and rp49 internal control was quantified by real-time RT-PCR in each sample.

PGRP-LE is not downstream of PGRP-LC. In the reciprocal experiments, the PGRP-LE-mediated activation of antimicrobial peptide genes was reduced but still existed in the PGRP-LC null mutant. In the Relish null mutant, PGRP-LE-mediated activation of antimicrobial peptide genes was abolished or more reduced. These results indicate that PGRP-LE acts partially upstream and partially in parallel with PGRP-LC upstream of Relish, suggesting that there is an additional receptor on the cell surface in the imd pathway other than PGRP-LC.

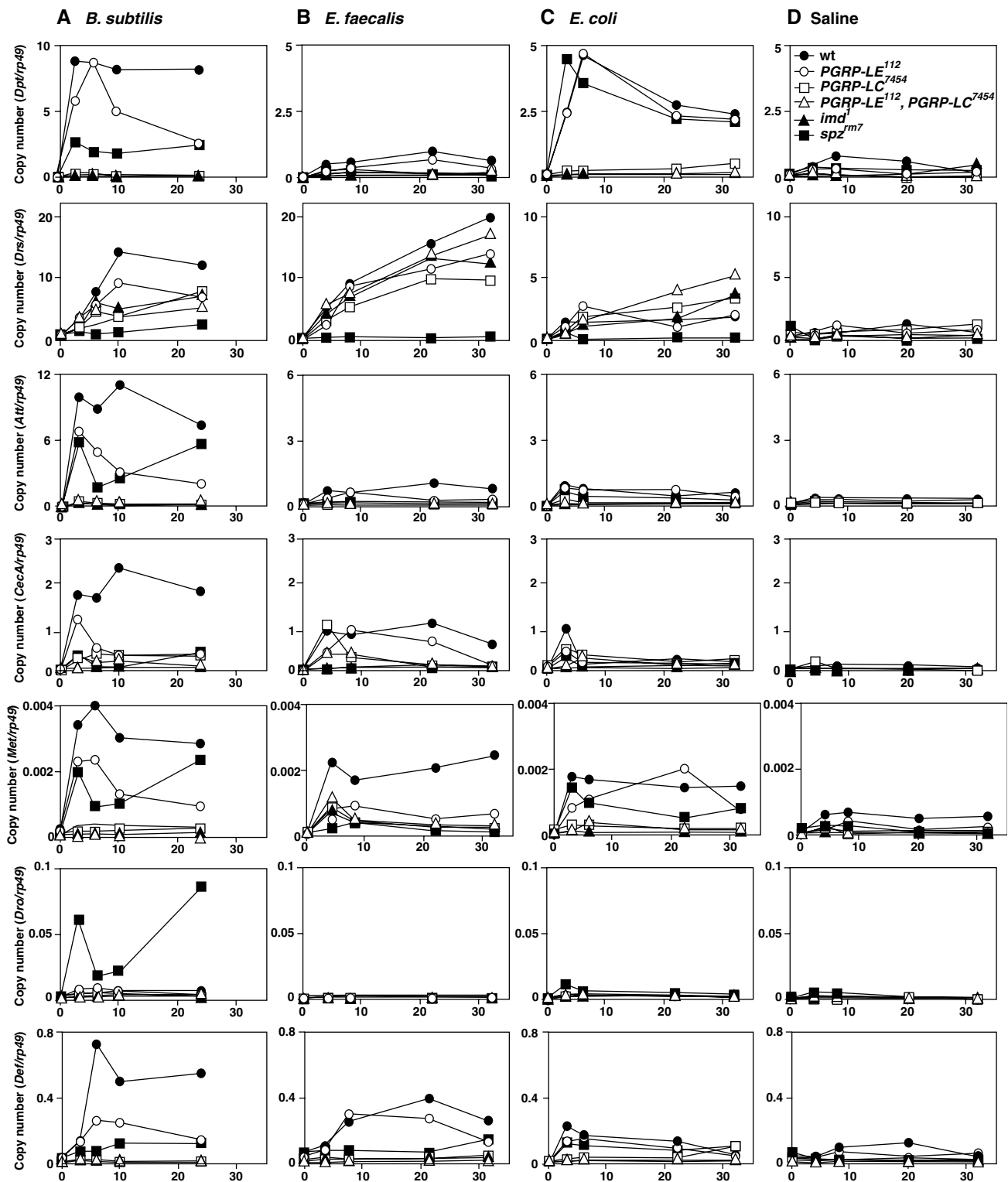
**Expression of antimicrobial peptide genes in the PGRP-LE mutant and in the double PGRP-LE/PGRP-LC mutant after bacterial challenge and natural infection**

To determine the requirement for PGRP-LE and the synergy of PGRP-LE with PGRP-LC on the induction of antimicrobial peptides, we used kinetics after bacterial challenges of *B. subtilis*, *E. faecalis*, and *E. coli* to analyze the expression of the seven classes of inducible antimicrobial peptides so far identified (Figure 3). *B. subtilis*-dependent induction of antimicrobial peptides was reduced in PGRP-LE<sup>112</sup> except for Drosocin, particularly in later stage such as 10–24 h after bacterial injection, indicating a requirement of PGRP-LE for the induction of antimicrobial peptides in response to *B. subtilis* (Figure 3A), whereas *E. faecalis*-dependent induction of antimicrobial peptides was not affected in PGRP-LE<sup>112</sup> except for Metchnikowin (Figure 3B). After *B. subtilis* infection, Drosocin expression was greatly enhanced in spätzle<sup>tm7</sup>. These results are consistent with the results of survival experiments indicating that PGRP-LE<sup>112</sup> is susceptible than wild type to *B. subtilis* but not to *E. faecalis* infections. Interestingly, there was no obvious synergy between PGRP-LE and PGRP-LC in the induction of antimicrobial peptides after *E. coli* infection (Figure 3C). Although the double PGRP-LE<sup>112</sup>/PGRP-LC<sup>7454</sup> mutant and PGRP-LC<sup>7454</sup> had similar antimicrobial peptide expression patterns after *E. coli* infection, only the PGRP-LE<sup>112</sup>/PGRP-LC<sup>7454</sup> mutant was susceptible to *E. coli* infection. These results suggest that, in addition to antimicrobial peptide induction, other self-defense reactions were affected in the PGRP-LE and PGRP-LC double mutant.

*E. carotovora* naturally infects *Drosophila* and induces local expression of antimicrobial peptides in surface epithelia such as the trachea via the imd pathway (Tzou *et al*, 2000). We investigated the effects of PGRP-LE mutation on the tracheal induction of Drs after natural infection. Compared to wild type, the number of Drs-GFP-expressing larvae was not changed in PGRP-LE<sup>112</sup>; however, Drs expression was reduced in PGRP-LC<sup>7454</sup> and was abolished in the double PGRP-LE<sup>112</sup>/PGRP-LC<sup>7454</sup> mutant (Figure 4). These results suggest that, in some contexts such as those occurring during natural infections, PGRP-LE and PGRP-LC synergistically induce antimicrobial peptides.

**Requirement of PGRP-LE for infection-dependent activation of the proPO cascade**

In a previous paper, we reported that overexpression of PGRP-LE activates the proPO cascade. We investigated the PGRP-LE requirement for the activation of the proPO cascade in response to bacterial infection. Phenoloxidase (PO) is proteolytically activated from an inactive precursor, proPO, by the proPO-activating enzyme, a terminal serine protease of



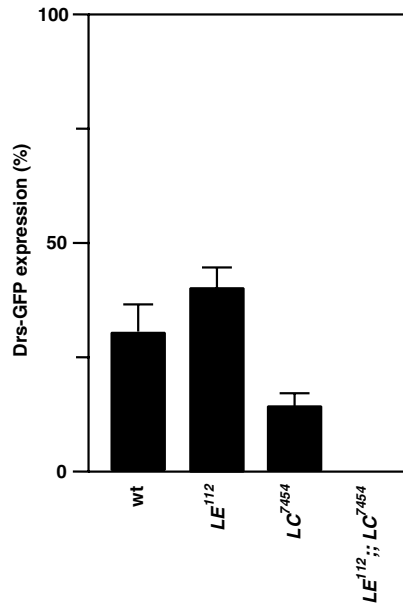
**Figure 3** Expression of seven classes of antimicrobial peptide genes in different mutants after various bacterial challenges. After *B. subtilis* (A), *E. faecalis* (B), and *E. coli* (C) infection, the amount of mRNA of seven classes of inducible antimicrobial peptides, Dipterin (Dip), Drosomycin (Drs), Attacin (Att), Cecropin A (CecA), Metchnikowin (Mtk), Drosocin (Dro), and Defensin (Def), and the *rp49* internal control in the indicated mutant flies, wild type (wt), *PGRP-LE<sup>112</sup>*, *PGRP-LC<sup>7454</sup>*, *spätzle<sup>tm7</sup>*, *imd<sup>1</sup>*, and the double *PGRP-LE<sup>112</sup>/PGRP-LC<sup>7454</sup>* mutant, was quantified by real-time RT-PCR. As a negative control, pyrogen-free saline was used (D). Each experiment is representative of at least two independent experiments.

the proPO cascade, following bacterial infections. There was significant PO activity in the hemolymph of wild-type flies after *E. coli* infection, whereas the control challenge (saline) produced only background levels of proPO cascade activation

(Figure 5A and B). The infection-dependent activation of the proPO cascade was abolished in *PGRP-LE<sup>112</sup>*. The artificial expression of *PGRP-LE*, based on *GAL4/UAS* targeted expression in *PGRP-LE<sup>112</sup>*, rescued infection-dependent activation of

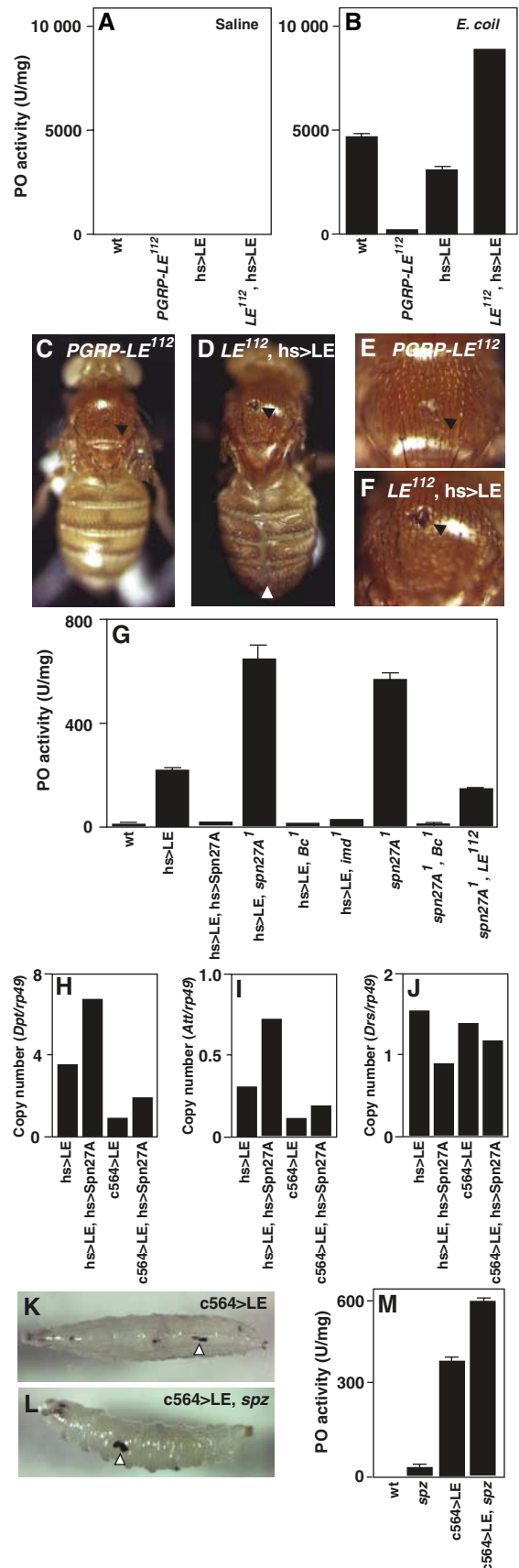
the proPO cascade. Consistent with these results, after *E. coli* infection, weak melanization was induced at the injury site of *PGRP-LE*<sup>112</sup>, whereas strong melanization was induced at the injury site of *PGRP-LE*<sup>112</sup> after forced expression of *PGRP-LE* (Figure 5C–F). These results indicate that PGRP-LE is required for activation of the proPO cascade in response to bacterial infections. As described below, PGRP-LE is produced in both hemocytes (Figure 6A) and the fat body (Figure 8G). The tissue specificity requirement for PGRP-LE function in proPO cascade activation requires further analysis. In the rescue experiments, we used UAS-*PGRP-LE* flies

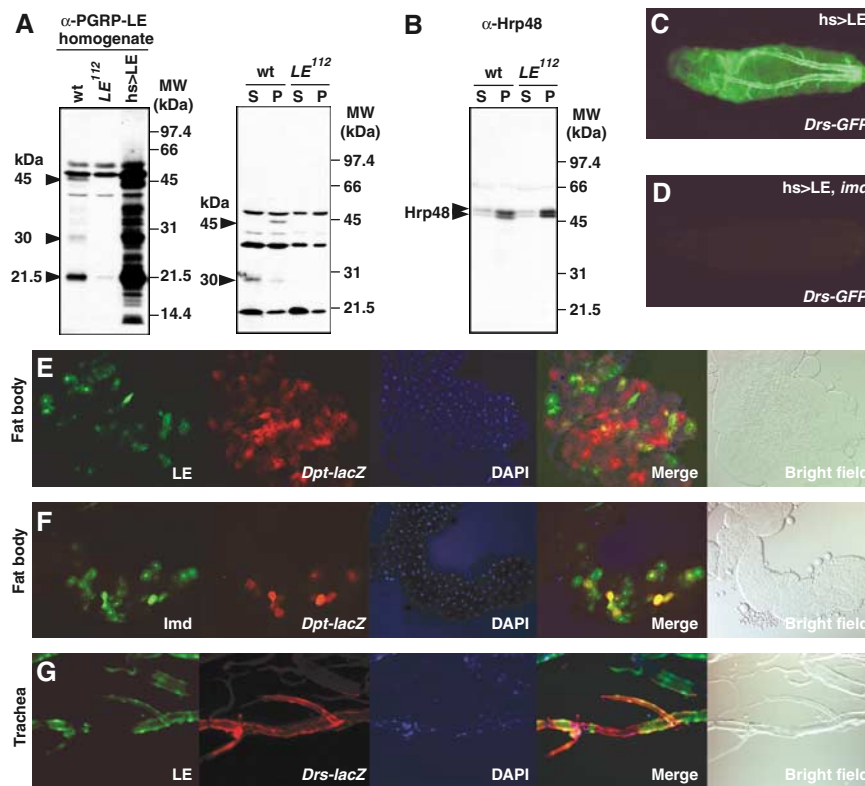
that had a low expression of *PGRP-LE* and, in combination with *hs-GAL4*, the flies had the cuticle defect at the midline of the dorsal abdomen in the absence of heat shock, suggesting



**Figure 4** Activation of *Drosomycin* promoter in various mutants after natural infection of *E. carotovora carotovora*. The number of *Drs-GFP*-expressing larvae was counted in wild type (wt), *PGRP-LE*<sup>112</sup>, *PGRP-LE*<sup>112</sup>, *PGRP-LE*<sup>112</sup>/*PGRP-LE*<sup>7454</sup>, and the double *PGRP-LE*<sup>112</sup>/*PGRP-LE*<sup>7454</sup> mutant. Bars indicate standard deviation of duplicate measurements.

**Figure 5** Requirement of PGRP-LE on the infection-dependent activation of the proPO cascade. (A, B) PO activity in the hemolymph after *E. coli* infection. The hemolymph was collected from wild-type (wt), *PGRP-LE*<sup>112</sup>, UAS-*PGRP-LE*/+; *hs-GAL4*/+, and *PGRP-LE*<sup>112</sup>; UAS-*PGRP-LE*/+; *hs-GAL4*/+ flies. (C–F) Melanization at the injury site (black arrowhead) of the indicated flies after *E. coli* challenge. White arrowhead indicates the cuticle defect (D). (E, F) Higher magnifications of (C, D) respectively. (G) PO activity in various mutant larvae. PO activity was assayed with homogenates of wild-type (wt), *GS1068*; +; *hs-GAL4*/+, *GS1068*; UAS-*Serp27A*/+; *hs-GAL4*/+, *GS1068*; *serpin27A*<sup>1</sup>; *hs-GAL4*/+, *GS1068*; *Bc*<sup>1</sup>; *hs-GAL4*/+, *GS1068*; *imd*<sup>1</sup>; *hs-GAL4*/+, *serpin27A*<sup>1</sup>, *serpin27A*<sup>1</sup>*Bc*<sup>1</sup>, *PGRP-LE*<sup>112</sup>; *serpin27A*<sup>1</sup> larvae. (H–J) The PGRP-LE-mediated induction of antimicrobial peptide genes. The amount of mRNA of *Diptericin* (H), *Attacin* (I), and *Drosomycin* (J), and *rp49* internal control was quantified by real-time RT-PCR in *GS1068*; +; *hs-GAL4*/+, *GS1068*; +; *hs-GAL4*/UAS-*Serp27A*, *GS1068*; *c564-GAL4*/+, *GS1068*; *c564-GAL4*/+; UAS-*Serp27A*/+ larvae. mRNA was recovered from the larvae at 14 h after heat shock (31 °C, 60 min). (K, L) The PGRP-LE-mediated melanization of *GS1068*; *c564-GAL4*/+ larvae (K) and *GS1068*; *c564-GAL4*/+; *spätzle*<sup>mm7</sup> larvae (L). Arrowheads indicate melanization. (M) PGRP-LE-mediated activation of the proPO cascade. PO activity was assayed with homogenates of wild-type larvae (wt), *spätzle*<sup>mm7</sup>, *GS1068*; *c564-GAL4*/+ larvae, and *GS1068*; *c564-GAL4*/+; *spätzle*<sup>mm7</sup> larvae. Bars indicate standard deviation of duplicate measurements.





**Figure 6** Non-cell autonomous effects of PGRP-LE on the activation of antimicrobial peptide genes in systemic and epithelial reactions. (A, B) Western blotting analyses using antibody against PGRP-LE (A) and Hrp48 (B). The homogenates (20 µg) prepared from wild-type (wt), *PGRP-LE<sup>112</sup>*, and *UAS-PGRP-LE/+; hs-GAL4/+* larvae after heat shock (35°C, 20 min) were applied to the analysis. The hemolymph (plasma) fraction (35 µg, S) and the hemocyte fraction (P) prepared from wild-type (wt) and *PGRP-LE<sup>112</sup>* larvae were analyzed. The arrowheads indicate 45-, 30-, and 21.5-kDa proteins (A). Molecular size markers are indicated on the right. (C, D) The *Drs-GFP* expression in GS1068; *hs-GAL4/Drs-GFP* larvae (C) and GS1068; *imd<sup>1</sup>; hs-GAL4/Drs-GFP* larvae (D) 12 h after heat shock (35°C, 20 min). (E) Non-cell autonomous effects of PGRP-LE on the expression of *Dpt-lacZ* in the fat body. (F) Cell autonomous effects of Imd on the expression of *Dpt-lacZ* in the fat body. (G) Non-cell autonomous effects of PGRP-LE on the expression of *Drs-lacZ* in the trachea. The overexpression of PGRP-LE and Imd was monitored by the expression of GFP (green). The expression of antimicrobial peptide genes was monitored by the expression of reporter genes using Cy3-labeled antibody (red). The GFP, Cy3, and DAPI (nuclear staining, blue) signals are merged.

that the leaky expression of *PGRP-LE* causes the cuticle defect (Figure 5D). Consistent with these results, *UAS-PGRP-LE* flies that had strong expression of *PGRP-LE* had pupal lethality in combination with *hs-GAL4* without any heat shock. The strong expression of *PGRP-LE* using *UAS-PGRP-LE* induces the proPO cascade activation in the larvae in the absence of bacterial challenge (Takehana *et al*, 2002), and the low level of *PGRP-LE* induction does not activate the proPO cascade in either larvae or adults.

To determine the relation between *PGRP-LE* and other components of the proPO cascade, epistatic analysis was performed with *serpin27A* and *Black cells* (*Bc*). *Serpin27A* codes a serine protease inhibitor that inhibits the terminal protease proPO-activating enzyme, and the *Bc* mutant has no detectable PO activity (De Gregorio *et al*, 2002a). *PGRP-LE*-mediated proPO cascade activation was totally inhibited by the overexpression of *Serpin27A*, a negative regulator of the proPO cascade, and was enhanced by a mutation of *serpin27A* (Figure 5G). *PGRP-LE*-mediated proPO cascade activation was also abolished in *Bc* mutants (Figure 5G). These results indicate that *PGRP-LE* activates the proPO cascade upstream of *Serpin27A* and PO. The *serpin27A* mutation causes constitutive proPO cascade activation (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). The constitutive proPO cascade activation by *serpin27A* mutation was

reduced in the *PGRP-LE<sup>112</sup>* mutant background and was abolished in the *Bc* mutant background (Figure 5G). These results are consistent with the conclusion that *PGRP-LE* is one of the activators of the proPO cascade upstream of *Serpin27A* and PO.

As described above, overexpression of *serpin27A* inhibits *PGRP-LE*-mediated activation of the proPO cascade. Overexpression of *serpin27A*, however, did not inhibit the *PGRP-LE*-mediated induction of antimicrobial peptides, except *Drs* (Figure 5H–J, data not shown for Cecropin A, Metchnikowin, Drosocin, and Defensin). *PGRP-LE*-mediated induction of *Drs* is slightly inhibited by the overexpression of *serpin27A*. These results suggest that the *PGRP-LE*-dependent pathway of antimicrobial peptide induction branched out from the *PGRP-LE*-dependent proPO cascade upstream of *Serpin27A*.

Ligoxygakis *et al* (2002b) reported that proPO cascade activation is regulated by the Toll pathway through the induction of *Serpin27A*. We investigated the relation between *PGRP-LE*-mediated activation of the proPO cascade and the Toll pathway. *PGRP-LE*-mediated melanization was also induced in the *spätzle* mutant background (Figure 5K and L). Consistent with this finding, significant PO activity was detected in the homogenate of the larvae when *PGRP-LE* was overexpressed in the *spätzle* mutant background

(Figure 5M). These results suggest that PGRP-LE-mediated proPO activation is independent of the Toll pathway. PGRP-LE-mediated proPO cascade activation was reduced by *imd* mutation (Figure 5G). We previously reported that there is proPO activation-induced melanization at the cuticle of *imd* background larvae when *PGRP-LE* is expressed under the control of a heat-shock promoter (Takehana *et al*, 2002). Therefore, further study is required to determine the involvement of the *imd* pathway in the PGRP-LE-dependent proPO cascade activation.

### **Non-cell autonomous effects of PGRP-LE in the induction of antimicrobial peptides in systemic and epithelial reactions**

PGRP-LE was required for the infection-dependent proPO cascade activation upstream of Serpin27A hemolymph protein, and PGRP-LE induced antimicrobial peptide synthesis in the fat body upstream of *Imd*, an adaptor molecule, and partially upstream of PGRP-LC, a cell surface receptor. These results suggest that PGRP-LE recognizes invading bacteria and activates subsequent signaling in the hemolymph, although PGRP-LE lacks the predicted signal peptide for secretion (Werner *et al*, 2000). To determine whether endogenous PGRP-LE is present in the hemolymph, we generated an antibody against the PGRP domain of PGRP-LE. After affinity purification of the antiserum with the antigen, antibody specificity was analyzed by Western blotting using wild-type larvae and *PGRP-LE*-deficient larvae in which the transcript of *PGRP-LE* was not detected (Supplementary Figure 1B). Bands of 45, 30, and 21.5 kDa were detected in the wild-type homogenate but not in the *PGRP-LE*<sup>112</sup> homogenate (Figure 6A). Forced expression of PGRP-LE increased band intensity. As described below, the 45-kDa protein was detected mainly in the hemocytes that express the *PGRP-LE* transcript. Although the molecular size of PGRP-LE was estimated to be 39.4 kDa, the 45-kDa band was probably the full-length PGRP-LE protein and the others were limited proteolysis products. After collecting the hemolymph from the larvae, the hemolymph (plasma) fraction and the floating hemocyte fraction were analyzed by Western blotting with antibody against PGRP-LE and antibody against Hrp48, an abundant heterogeneous nuclear RNA-associated protein (Hammond *et al*, 1997). The 45-kDa band and the 30-kDa band were detected in the wild-type hemocyte and hemolymph fractions, respectively (Figure 6A). These two bands were specific to the wild-type samples and were not detected in the *PGRP-LE*<sup>112</sup> samples. In the control experiment, the nuclear protein Hrp48 was detected mainly in the hemocyte fraction, and was observed in both the wild-type and *PGRP-LE*<sup>112</sup> samples (Figure 6B). Because the proPO cascade, a serine protease cascade, is activated spontaneously during the collection of hemolymph (Takehana *et al*, 2002), PGRP-LE was probably cleaved to the 30-kDa product in the hemolymph during preparation of the samples. These results indicate that endogenous PGRP-LE is a constitutive hemolymph protein.

We then confirmed the non-cell autonomous effects of PGRP-LE on the induction of antimicrobial peptide synthesis in the fat body. For this analysis, we applied a cell lineage tracer technique using a combination of the *flp/FRT* and *GAL4/UAS* recombinase systems (Ito *et al*, 1997). In this technique, the actin-promoter-*GAL4* is nonfunctional due to

the insertion of transcriptional termination signals with two *FRT* sequences in the initial state (*Ay-GAL4*). Flippase induces recombination at the two *FRT* sequences and removes the termination signal under the control of the *hsp70* promoter, generating a functional Actin-*GAL4* gene that drives the expression of *PGRP-LE* or *imd* under the control of *UAS*, which is monitored by *UAS-GFP*. Therefore, the PGRP-LE- or *Imd*-overexpressing cells are labeled with GFP in this system. Induction of the antimicrobial peptides was monitored by the expression of two reporter genes, *Dpt-lacZ* and *Drs-lacZ*, using an antibody against  $\beta$ -galactosidase. The double-labeling experiment revealed that *Dpt-lacZ* expression was not limited to the fat body cells overexpressing PGRP-LE (Figure 6E), whereas in the control experiments (Figure 6F), *Dpt-lacZ* expression was limited to the fat body cells overexpressing *Imd*, a cellular component of the *imd* pathway capable of inducing *Dpt* in the fat body (Georgel *et al*, 2001). These results indicate that PGRP-LE has non-cell autonomous effects on antibacterial peptide induction in systemic reactions.

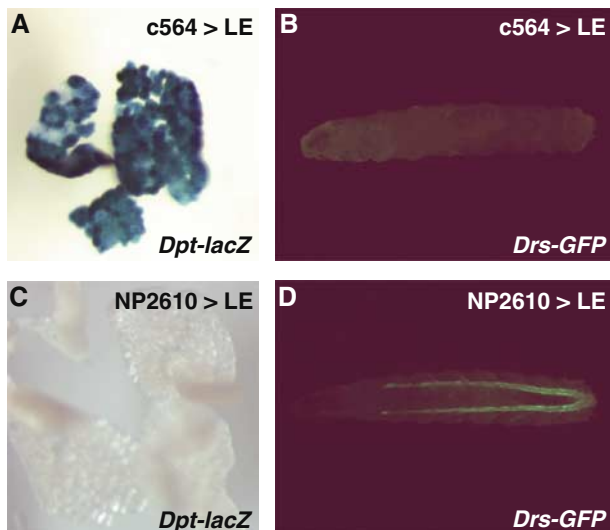
A local epithelial reaction, activation of the *Drs* promoter in the trachea, is also induced by the overexpression of PGRP-LE (Takehana *et al*, 2002). The PGRP-LE-mediated expression of *Drs-GFP* in the trachea was abolished in an *imd* mutant background (Figure 6C and D). This result indicates that the PGRP-LE-mediated tracheal induction of *Drs-GFP* is *imd* dependent, consistent with the fact that induction of antimicrobial peptides in epithelial tissues depends on the *imd* pathway (Tzou *et al*, 2000). As PGRP-LE activates the epithelial reaction as well as the systemic reaction via the *imd* pathway, we examined whether PGRP-LE has a non-cell autonomous effect on the epithelial reaction using the same technique. In the trachea, *Drs-lacZ* expression was not limited to the cells overexpressing PGRP-LE (Figure 6G), whereas *Drs-lacZ* expression was limited to the cells overexpressing *Imd* (data not shown). These results indicate that PGRP-LE has a non-cell autonomous effect on the local epithelial reaction as well as on the systemic reaction.

### **Involvement of epithelial PGRP-LE in the epithelial immune reaction**

PGRP-LE is present in the hemolymph and has non-cell autonomous effects on antimicrobial peptide synthesis in systemic and epithelial reactions. We investigated whether the epithelial *Drs* activation is mediated by the epithelial induction of PGRP-LE (Figure 7). As reported before, *hs-GAL4*-mediated ubiquitous expression of *PGRP-LE* activates *Dpt-lacZ* and *Drs-GFP* in the fat body and trachea, respectively (Takehana *et al*, 2002). The *c564-GAL4*-mediated induction of PGRP-LE activated *Dpt-lacZ* expression in the fat body, but did not activate *Drs-GFP* in the trachea. In the reciprocal experiments using NP2610-*GAL4* as a driver that expresses *GAL4* in the trachea, malpighian tubule, and gut but not in the fat body (Hayashi *et al*, 2002), *Drs-GFP* was activated in the trachea, whereas *Dpt-lacZ* was not activated in the fat body. These results suggest that the epithelial induction of PGRP-LE is sufficient to activate the epithelial response but not the systemic response.

Because it was suggested that there is a role for epithelial PGRP-LE in the epithelial immune reaction, we investigated whether PGRP-LE was present in the trachea by Western blotting (Figure 8G). The 45-kDa band was detected in the



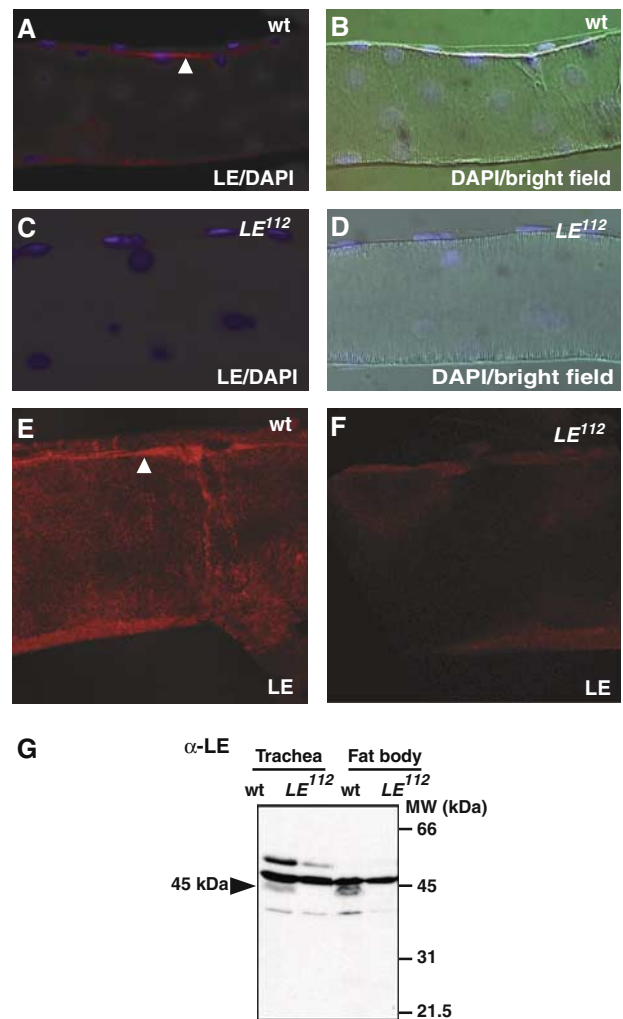


**Figure 7** The epithelial induction of PGRP-LE is sufficient to activate the epithelial response. (A, B) Expression of *Dpt-lacZ* in the fat body (A) and *Drs-GFP* (B) in *Dpt-lacZ*, *Drs-GFP*; *c564-GAL4/UAS-PGRP-LE* larvae. (C, D) Expression of *Dpt-lacZ* in the fat body (C) and *Drs-GFP* (D) in *Dpt-lacZ*, *Drs-GFP*; *NP2610-GAL4/UAS-PGRP-LE* larvae.

trachea of wild-type larvae but not in that of *PGRP-LE<sup>112</sup>*, indicating the presence of PGRP-LE in the trachea. The 45-kDa band was also detected in the wild-type fat body but not in the *PGRP-LE<sup>112</sup>* fat body. Because the affinity-purified antibody against PGRP-LE crossreacted with three proteins that are present in the *PGRP-LE<sup>112</sup>* trachea, the antibody was absorbed with *PGRP-LE<sup>112</sup>* larval tissues. Immunostaining using absorbed antibody revealed the localization of PGRP-LE at the luminal surface of the trachea (Figure 8A–D). The trachea is a respiratory organ with a tube-like structure that can be visualized by DAPI nuclear staining. The anti-PGRP-LE antibody signal was detected at the surface of the wild-type trachea lumen. The signal was not detected at the surface of the tracheal lumen of *PGRP-LE<sup>112</sup>*, indicating the specificity of the PGRP-LE signal. Confocal microscopic analysis confirmed this observation (Figure 8E and F). These results imply that PGRP-LE recognizes invading bacteria and activates subsequent signaling at the luminal surface of the trachea, which is the first site of contact between the pathogens and the host.

## Discussion

In the present paper, we report that two members of the PGRP-family, PGRP-LE and PGRP-LC, function synergistically to induce resistance to *E. coli* and *B. megaterium* infections. Consistent with these findings, PGRP-LE and PGRP-LC are two major upstream regulators of the imd pathway in response to *E. coli* infection, and PGRP-LE acts both in parallel and upstream of PGRP-LC in imd-mediated antibacterial peptide induction. Moreover, survival experiments suggest that PGRP-LE participates in host defense against *E. coli*, mainly through regulating the imd pathway of antimicrobial peptide induction. There is, however, no obvious synergy between PGRP-LE and PGRP-LC in the induction of the antibacterial peptides in response to *E. coli* infection,



**Figure 8** Localization of PGRP-LE in the trachea. (A–F) Immunostaining was performed in wild type (wt, A, B, E) and *PGRP-LE<sup>112</sup>* (C, D, F) using anti-PGRP-LE antibody. The antibody staining is merged with DAPI staining (A, C). The bright-field micrograph is merged with DAPI staining (B, D). Confocal analysis in wild type (E) and *PGRP-LE<sup>112</sup>* (F) using anti-PGRP-LE antibody. Arrowheads indicate PGRP-LE signal. (G) Western blotting analysis was performed with trachea and fat body homogenates prepared from wild type (wt) and *PGRP-LE<sup>112</sup>* using anti-PGRP-LE antibody. Arrowhead indicates 45-kDa protein.

although, in some contexts such as natural infection, PGRP-LE and PGRP-LC synergistically induce antimicrobial peptides. A possible reason that the double *PGRP-LE<sup>112</sup>/PGRP-LC<sup>7454</sup>* mutant has reduced resistance and *PGRP-LC<sup>7454</sup>* has complete resistance to *E. coli* infection, although both mutants have similar antimicrobial peptide induction patterns, is that *PGRP-LE<sup>112</sup>* lacks the infection-dependent proPO cascade activation. The lack of both antimicrobial peptide induction and proPO cascade activation probably leads to susceptibility to *E. coli* infection. Synergy between two pattern recognition molecules in *Drosophila* probably reflects the nature of innate immune systems in that, in innate immunity, pattern recognition molecules with a different pattern specificity, such as PGRP family members, are recruited to expand the immune repertoire and to establish an effective defense network with limited factors in response to new challenges during evolution.

Activation of the proPO cascade is the most immediate response following microbial infection or septic injury, and it produces cytotoxic reactive oxygen species and melanin by the catalytic conversion of dopamine into melanin. Biochemical *in vitro* reconstitution experiments indicate that the proPO cascade is triggered by the recognition of microbial cell wall components through pattern recognition molecules. We provide the first *in vivo* evidence that a pattern recognition molecule is required for bacterial infection-dependent activation of the proPO cascade. The lack of infection-dependent activation of the proPO cascade in *PGRP-LE*<sup>112</sup> does not affect the survival of the host against *E. coli* infection. These results suggest that the proPO cascade is activated immediately after microbial infection and has an important role in the initial stage of host defense; however, for survival of the host extending to several days after infection, secondary responses that are mediated by transcriptional activation, such as antimicrobial peptide induction, are important.

The proPO cascade is regulated by Serpin27A, which is linked to the Toll pathway (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). PGRP-LE-mediated activation of the proPO cascade is suggested to be independent of Toll activation, because PGRP-LE overexpression induces proPO cascade activation and melanization in the *spätzle*<sup>mm7</sup> mutant background. The *spätzle*<sup>mm7</sup> is not a null allele, but a strong allele in which activation of the Toll pathway is severely affected (Lemaitre *et al*, 1996; De Gregorio *et al*, 2002b). Moreover, PGRP-LE activates the proPO cascade upstream of Serpin27A. Therefore, PGRP-LE participates in the primary activation of the proPO cascade upstream of Serpin27A and not in the secondary regulation, which depends on the regulation of Serpin27A by the Toll pathway.

Epistatic analyses suggest the existence of a cell surface receptor that acts downstream of PGRP-LE in the imd pathway in addition to PGRP-LC. This is consistent with the results that PGRP-LE is a constitutive hemolymph protein and has non-cell autonomous effects on activation of the antibacterial peptide gene. Because PGRP-LE-mediated activation of the antimicrobial peptide genes was reduced in the PGRP-LC mutant background, the putative receptor might make a functional complex with PGRP-LC, which is required for the full activation of PGRP-LE-mediated signaling. PGRP-LE overexpression had stronger activity to induce the Relish-dependent activation of *Drs* than that of PGRP-LCx. Thus, the putative receptor is also suggested to preferentially mediate the Relish-dependent activation of *Drs*. The identification of the putative receptor will provide further understanding of the details of signaling activation.

The epithelial induction of antimicrobial peptides is a highly conserved immune reaction observed in humans, insects, and plants, and is suggested to be the true ancestral antimicrobial defense (reviewed in Hoffmann and Reichhart, 2002). In contrast to the relatively good understanding of the systemic induction of antimicrobial peptides, the mechanisms of epithelial induction of the antimicrobial peptides are largely unknown in *Drosophila*. Thus, an understanding of the mechanisms of the epithelial responses will aid in the advancement of this field. In this paper, we demonstrated that the epithelial induction of PGRP-LE is sufficient to activate imd-mediated tracheal expression of *Drs-GFP*, but not to activate the systemic activation of antimicrobial pep-

tide genes. Moreover, PGRP-LE is localized at the surface of the tracheal lumen and has non-cell autonomous effects on the induction of *Drs-GFP* in tracheal cells. Therefore, PGRP-LE is suggested to recognize invading bacteria and activate subsequent signaling at the front line of the epithelial barrier.

## Materials and methods

### Fly strains

Stocks were raised on a standard cornmeal-yeast agar medium at 25°C. Oregon R flies were used as a standard wild-type strain. *UAS-LCx*, *PGRP-LC*<sup>7454</sup>, *PGRP-LC*<sup>ΔE</sup>, *c564-GAL4*, *NP2610-GAL4*, *Relish*<sup>E20</sup>, *Ay-GAL4*, *imd*<sup>1</sup>, *spätzle*<sup>mm7</sup>, *J4*, *PGRP-SA*<sup>semi</sup>, *GS1068*, *UAS-PGRP-LE*, *UAS-Serpin27A*, *serpin27A*<sup>1</sup>, and *Bc*<sup>1</sup> are described elsewhere (Harrison *et al*, 1995; Lemaitre *et al*, 1995, 1996; Ito *et al*, 1997; Hedengren *et al*, 1999; Meng *et al*, 1999; Michel *et al*, 2001; Choe *et al*, 2002; Gottar *et al*, 2002; Hayashi *et al*, 2002; Takehana *et al*, 2002; De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). The deletion of *PGRP-LE* was generated by P element mobilization of the *GS1068* using standard protocols. To characterize the *PGRP-LE*<sup>112</sup> mutation, genomic PCR was performed with two primers (LE loss F-1, 5'-CTGGCCCAACTCGCTTCAGT-3', and LE loss R-2, 5'-GAGAGACGTCTGCGACTCT-3', in Supplementary Figure 1A) after preparation of genomic DNA from adults, and the obtained PCR products were sequenced with a primer (PGRP-1, 5'-GTTCTCC TCCTCGATATTG-3'; Supplementary Figure 1A).

### Infection experiments

Bacterial infections were performed by challenging adult flies with a thin tungsten needle previously dipped into a concentrated culture of the bacterial strains. Survival experiments were performed with 30 flies for each genotype tested at 25°C. Surviving flies were transferred daily into fresh vials. *E. coli* K-12, *E. carotovora carotovora* 15, *M. luteus* (IFO13867), *E. faecalis* (IFO12964), *B. subtilis* (IFO3134), and *B. megaterium* (IFO13498) were used. For natural infection experiments, approximately 200 second instar larvae were placed into a 2-ml tube containing 200 μl of overnight cultured *E. carotovora carotovora* 15 pellet and 400 μl of crushed banana. The larvae, bacteria, and banana were mixed well, incubated at room temperature for 30 min, and transferred to standard fly medium. After incubation at 18°C for 5 days, observation of GFP expression was performed. Each experiment is representative of at least three independent experiments.

### Measurements of PO activity

Hemolymph was collected 4 h after bacterial challenge from 50 flies for each genotype tested into 20 μl phosphate-buffered saline (PBS) containing protease inhibitors (Complete Cocktail, Roche, Germany) using glass capillaries with a handmade mouthpiece. Protein was determined by Protein Assay (Bio-Rad, Hercules, CA), using BSA as a standard. PO activity of 5-μl samples (ca 5 μg) was assayed as described previously (Takehana *et al*, 2002). For the rescue experiments, hemolymph was recovered from *PGRP-LE*<sup>112</sup>; *UAS-PGRP-LE/+*; *hs-GAL4/+* flies at 12 h after heat shock (29°C, 2 h). Larval homogenate was prepared as described previously (Takehana *et al*, 2002). Each experiment is representative of at least three independent experiments.

### Clonal analyses

For the clonal analyses, four kinds of *trans*-heterozygous larvae were used as follows: *Dpt-lacZ/hs-flp*; *Ay-GAL4,UAS-GFP/UAS-PGRP-LE*, *Drs-lacZ/hs-flp*; *Ay-GAL4,UAS-GFP/UAS-PGRP-LE*, *Dpt-lacZ/hs-flp*; *Ay-GAL4,UAS-GFP/GS9049* and *Drs-lacZ/hs-flp*; *Ay-GAL4,UAS-GFP/GS9049*. In *GS9049*, the *GAL4*-dependent activation of *imd* is induced through a *GS* vector (unpublished data). Heat shock (37°C, 1 h) was applied twice at 24–48 h (the first instar larval stage) and at 48–62 h (the early second instar larval stage) after egg deposition.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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