Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae

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In *Drosophila*, microbial infection activates an antimicrobial defense system involving the activation of proteolytic cascades in the hemolymph and intracellular signaling pathways, the immune deficiency (imd) and Toll pathways, in immune-responsive tissues. The mechanisms for microbial recognition are largely unknown. We report that, in larvae, the imd-mediated antibacterial defense is activated by peptidoglycan-recognition protein (PGRP)-LE, a PGRP-family member in *Drosophila*. Consistent with this, PGRP-LE binds to the diaminopimelic acid-type peptidoglycan, a cell-wall component of the bacteria capable of activating the imd pathway, but not to the lysine-type peptidoglycan. Moreover, PGRP-LE activates the prophenoloxidase cascade, a proteolytic cascade in the hemolymph. Therefore, PGRP-LE acts as a pattern-recognition receptor to the diaminopimelic acid-type peptidoglycan and activates both the proteolytic cascade and intracellular signaling in *Drosophila* immunity.

Insects, including *Drosophila*, respond to microbial infection by a rapid and efficient immune reaction despite their lack of adaptive immunity (1). Genetic and molecular studies of model organisms reveal a striking conservation between the mechanisms that regulate insect host defense and the mammalian innate immune response (2). In response to microbial infection, *Drosophila* secrete several antimicrobial peptides into their hemolymph from the fat body, the functional equivalent of the mammalian liver, by two distinct signaling pathways, Toll and immune deficiency (imd), which are similar to the Toll-like receptor–IL-1 receptor signaling pathways and the tumor necrosis factor receptor signaling pathway, respectively, in mammals (3). Microbial infection also activates proteolytic cascades, leading to localized wound healing and melanization (1, 4). Because the imd pathway is activated by Gram-negative bacterial infection and some Gram-positive bacterial infections, and the Toll pathway is activated by fungal infection and other Gram-positive bacterial infections, flies possess specific mechanisms to discriminate between microbes (5–8). The mechanisms for microbial recognition in *Drosophila*, however, are largely unknown. Pathogen recognition is believed to depend on the interaction between host proteins, called pattern-recognition receptors, and conserved molecular structures present on the surface of pathogens but absent in the host, such as lipopolysaccharides, peptidoglycans, and β-1,3-glucans (9). After recognition, the pattern-recognition receptors stimulate immune responses by activating proteolytic cascades in the hemolymph and intracellular signaling pathways in immune-responsive tissues. In contrast to some mammalian Toll-like receptors, Toll is activated by an endogenous ligand, Spätzle, which is cleaved by proteolytic enzymes after infection and does not appear to function as a direct receptor of the microbial component (10). Several new antibacterial defense components were identified recently in large screens of mutations that abolish the *Drosophila* immune response (3). Because of genetic redundancy, the pattern-recognition receptors were not expected to be identified in a loss-of-function screening; therefore, we used gain-of-function genetic screens to identify a gene coding a peptidoglycan-recognition protein (PGRP)-LE, which is a member of the PGRP family, which consists of at least 12 members in *Drosophila* (11). We report that PGRP-LE is capable of activating proteolytic cascades in the hemolymph, the prophenoloxidase (proPO) cascade, and the imd-mediated antibacterial response in *Drosophila*. More recently, studies based on loss-of-function mutations demonstrated key roles of PGRP-SA and PGRP-LC, other PGRP family members, in the activation of the Toll and imd pathways, respectively (12–15). The present results are consistent with the conclusion that there is an activator of the imd pathway in addition to PGRP-LC (14). Here, we also discuss the role of the PGRP family in the recognition of diversified pathogens in innate immunity.

Methods

Fly Strains. Stocks were raised on standard cornmeal–agar medium at 25°C. Oregon R flies were used as a standard WT strain. The transgenic strains *Diptericin-lacZ* (*Dpt-lacZ*) and *Drosomycin-GFP* (*Drs-GFP*) have been described (16, 17). The data of the gene search (GS) strains (18) are available through the *Drosophila* Gene Search Project (http://218.44.182.94/~dclust/index.html). *imd*, *Relish*^{E38}, and J4 have been described (6, 19, 20).

The WT *PGRP-LE* cDNA was obtained by PCR by using a cDNA library of *hs-GAL4*;GS1068 larvae as a template. *Eco*RI and *Xho*I sites were introduced at the 5′ and 3′ ends, respectively, of the *PGRP-LE* cDNA by using the forward primer 5′-CCGGAATTCTCCGAATCGGGAATC-3′ and the reverse primer 5′-CCGCTCGAGTCATTGTTCCTCCTC-3′ and Pyrobest DNA Polymerase (Takara Shuzo, Kyoto). This fragment was subcloned into a modified pUAST vector for FLAG-tagged protein expression, pUAS-MFLAG(RX/RI). After sequencing, the construct was injected into embryos.

Quantitative RT-PCR. Total RNA was isolated from 20 larvae by using Trizol reagent (GIBCO/BRL) and dissolved in 20 μl of RNase-free water. Total RNA (1 μg) was used in 20 μl of reverse transcription reaction by using ReverTraAce reverse transcriptase (Toyobo, Osaka) and oligo(dT)15 primer (Promega). The first-strand cDNA (0.5 μl) was used as a template for the quantitative RT-PCR. Real-time PCR was performed with a

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Abbreviations: imd, immune deficiency; PGRP, peptidoglycan-recognition protein; proPO, prophenoloxidase; GS, gene search; DAP, diaminopimelic acid.

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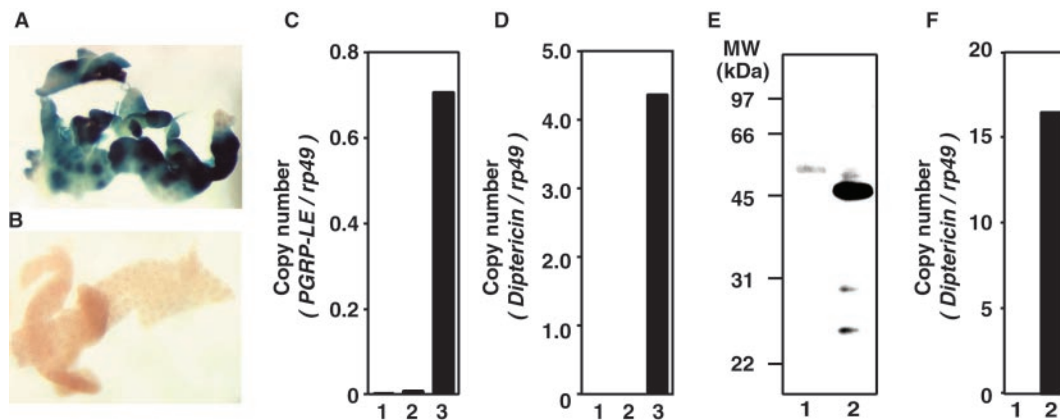

Fig. 1. Induction of *Diptericin* by forced expression of PGRP-LE in *Drosophila*. (*A* and *B*) Histochemical staining of fat-body β-galactosidase activity in third-instar larvae carrying the *Diptericin-lacZ* reporter gene. Significant expression of the *lacZ* gene is observed in the fat body of transheterozygous larvae of *hs-GAL4*;GS1068 (*A*), but not in the fat body of control larvae with *hs-GAL4* and GS insertions (*B*), in the absence of microbial infection. The transheterozygotes and control larvae were incubated at 29°C for 2 h and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside after recovery for 12 h at 25°C. (*C* and *D*) The forced expression of *PGRP-LE* and induction of *Diptericin* in GS1068 via GAL4/UAS system. The amount of mRNA of *PGRP-LE* (*C*), *Diptericin* (*D*), and rp49 internal control was quantified by real-time RT-PCR with a LightCycler (Roche Diagnostics). The RNA copy numbers were standardized against that of the RNA coding rp49 in each sample and are shown. The WT larvae (lane 1), GS1068 larvae (lane 2), and *hs-GAL4*;GS1068 transheterozygous larvae (lane 3) were incubated at 35°C for 20 min and allowed to recover at 25°C for 12 h. (*E* and *F*) Induction of endogenous *Diptericin* gene by the forced expression of PGRP-LE. The FLAG-tagged PGRP-LE was induced by the GAL4/UAS system. The *UAS-PGRP-LE* larvae and *hs-GAL4*;*UAS-PGRP-LE* transheterozygous larvae were incubated at 35°C for 20 min and allowed to recover at 25°C for 12 h. The protein (20 μg of each) of the homogenate of *UAS-PGRP-LE* larvae (lane 1) and the homogenate of *hs-GAL4*;*UAS-PGRP-LE* larvae (lane 2) were probed with anti-FLAG-tag antibody (*E*). Molecular size markers are indicated on the left. The amount of mRNA of *Diptericin* and rp49 internal control was quantified by real-time RT-PCR (*F*). Lane 1, *UAS-PGRP-LE* larvae; lane 2, *hs-GAL4*;*UAS-PGRP-LE* transheterozygous larvae. The results of real-time PCR analyses were confirmed by three independent experiments.

LightCycler (Roche Diagnostics). PCR specificity was confirmed by the molecular weight of the PCR products and melting curve analysis at each data point. The copy numbers of RNA coding the genes of interest were standardized against that of the RNA coding rp49 in each sample. Primers were as follows: *Diptericin*, 5′-GTTCACCATTGCCGTCGCCTTAC-3′, 5′-CCCAAGTGCTGTCCATATCCTCC-3′; *Drosomycin*, 5′-TTGTTCGCCCTCTTCGCTGTCCT-3′, 5′-GCATCCTTCGCACCAGCACTTCA-3′; *Attacin*, 5′-GTGGTGGGTCAGGTTTTCGC-3′, 5′-TGTCCGTTGATGTGGGAGTA-3′; *PGRP-LE*, 5′-GATGCCGACCAAAATACCAG-3′, 5′-GTCTTCGAAATGTGTCGGAG-3′; *rp49*, 5′-AGATCGTGAAGAAGCGCACCAAG-3′, 5′-CACCAGGAACTTCTTGAATCCGG-3′. Efficiencies were as follows: *Diptericin*, 1.79; *Drosomycin*, 1.78; *Attacin*, 1.80; *PGRP-LE*, 1.87; *rp49*, 1.86. Correlation coefficients of dilution curves/*r* values were −1.00 in each experiment.

Peptidoglycan-Binding Assay. The diaminopimelic acid (DAP)-type peptidoglycan and the Lys-type peptidoglycan were purified from *Lactobacillus plantarum* ATCC 8014 and *Staphylococcus epidermidis* ATCC 155, respectively, according to the previously reported method (21, 22). The DAP-type peptidoglycan-containing cell wall was purified from *Nocardia calcarea* ATCC 17876 according to the previously reported method (23). The Lys-type peptidoglycan of *Staphylococcus aureus* (Wako) was used. Recombinant PGRP-LE with six histidine residues at the C terminus was expressed in Sf21 cells by using the BacPAK Baculovirus Expression System (CLONTECH) and was purified with DEAE-Sepharose FF chromatography (Amersham Pharmacia), followed by Ni-NTA Agarose chromatography (Qiagen, Valencia, CA). The peptidoglycan-binding assay essentially was the same as reported by Yoshida *et al.* (24), with slight modification of the washing steps. Approximately 0.5 μg of purified recombinant PGRP-LE was incubated with 0.32 mg of insoluble peptidoglycans or cell wall. Unbound protein isolated from the soluble fraction and bound protein recovered after washing the peptidoglycan with Tris-maleate buffer containing 1 mol/liter NaCl and 1 mol/liter NaCl plus 0.2% Tween 20 were analyzed by Western blot analysis using anti-penta-histidine antibody (Qiagen).

Measurements of Phenoloxidase Activity. Phenoloxidase activity was assayed with L-dihydroxyphenylalanine as a substrate according to the previously reported method (24). The third-instar larvae were homogenized in 10 mmol/liter Tris·HCl buffer, pH 7.5, containing 1 mmol/liter PMSF, a serine protease inhibitor, and the resulting supernatants were used as homogenates. The hemolymph of the larvae was recovered on ice to reduce spontaneous activation of the proPO cascade in the hemolymph *in vitro*. One unit was defined as the amount of enzyme causing an increase in the absorbency of 0.01 at OD at 450 nm for 30 min. Protein was determined by Protein Assay (Bio-Rad), using BSA as a standard.

Results

Selective PGRP-LE-Induced Activation of the imd Pathway Upstream of *imd*. To identify genes capable of activating antimicrobial responses in the absence of microbial infection, we used a modular misexpression screen based on the GAL4/UAS system (25) with *P* element-based GS vectors (18). Ubiquitous expression was induced by heat shock-inducible *hs-GAL4*. Activation of the antimicrobial responses of these larvae was monitored by the expression of two reporter genes (*Dpt-lacZ* and *Drs-GFP*), which were constructed with the promoter region of an antibacterial peptide gene, *Diptericin*, and *lacZ* (16) and the promoter region of an antifungal peptide gene, *Drosomycin*, and the GFP gene (17), respectively. In the absence of microbial infection, there was significant expression of *Dpt-lacZ* in the fat body of *hs-GAL4*;GS1068 transheterozygous larvae after heat shock (Fig. 1*A*). In the GS1068 line, a single GS vector was inserted 624 bp upstream of the transcription start site of a gene encoding PGRP-LE that drives expression of *PGRP-LE* via the GAL4/UAS system (Fig. 1*C*). The endogenous *Diptericin* gene was activated in the *hs-GAL4*;GS1068 transheterozygous larvae after heat shock (Fig. 1*D*). PGRP homologs have been identified in *Bombyx mori* (24), *Trichoplusia ni* (26), mouse, and human (27).

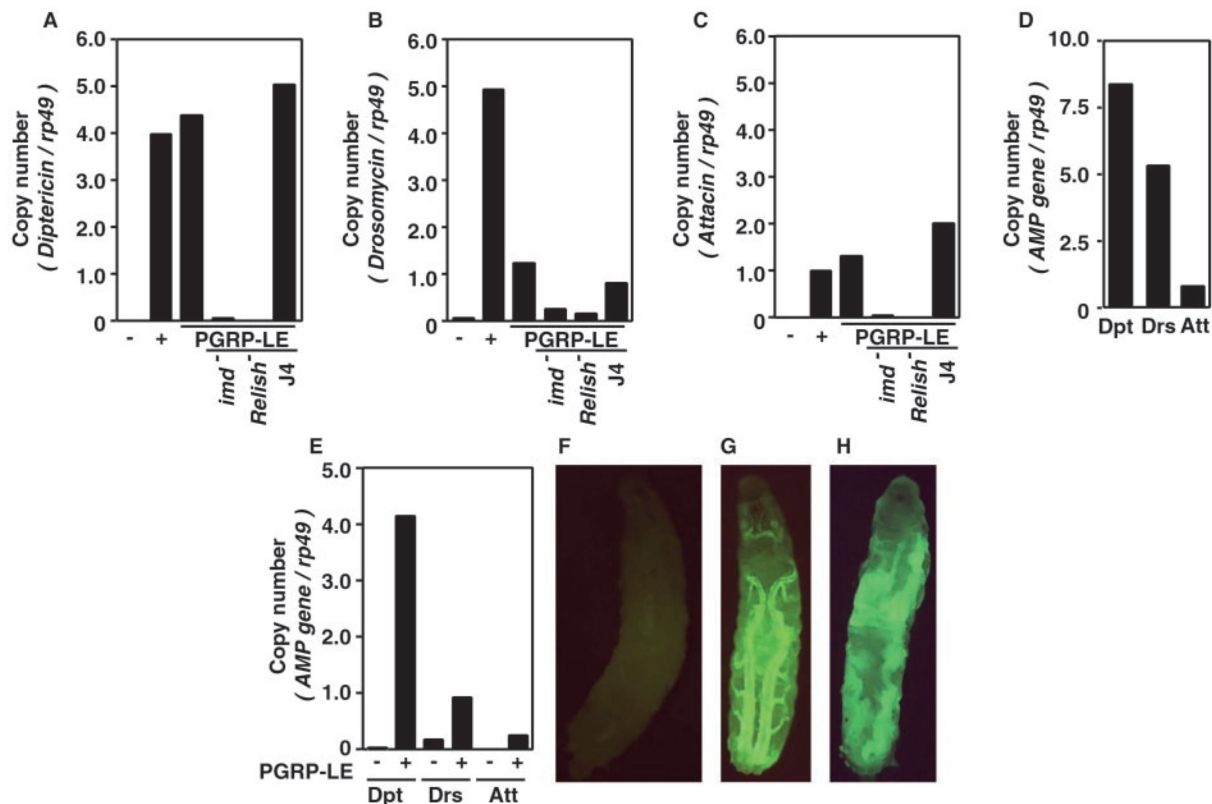

Fig. 2. Activation of imd-mediated antibacterial response by forced expression of PGRP-LE in *Drosophila*. (*A–C*) The quantitative analyses of mRNA of antimicrobial peptide genes with various mutant backgrounds. The transheterozygous larvae were incubated at 35°C for 20 min and allowed to recover at 25°C for 12 h. The amount of mRNA of *Diptericin* (*A*), *Drosomycin* (*B*), *Attacin* (*C*), and rp49 internal control was quantified by real-time RT-PCR in each sample. The copy numbers of antibacterial gene mRNA were standardized against that of rp49 in each sample. (−) Unchallenged wild-type larvae. (+) Immune challenge was performed by pricking wild-type larvae with a fine needle dipped into a concentrated culture of *Escherichia coli*, and RNA was prepared 6 h after infection. (PGRP-LE) GS1068/+;*hs-GAL4*/+. (PGRP-LE, *imd*$^-$) GS1068/+;*imd*/*imd*;*hs-GAL4*/+. (PGRP-LE, *Relish*$^-$) GS1068/+;*hs-GAL4*/+;*Relish* ^{E38}/*Relish* ^{E38}. (PGRP-LE, J4) GS1068/+; J4/J4; *hs-GAL4*/+. The results were confirmed by three independent experiments. (*D* and *E*) Differential induction of antimicrobial peptide genes by PGRP-LE in larvae and adults. The amount of mRNA of antimicrobial peptide (AMP) genes, *Diptericin* (Dpt), *Drosomycin* (Drs), and *Attacin* (Att), and rp49 in c564;GS1068 transheterozygous larvae was quantified by real-time RT-PCR (*D*). Some adults of a weak UAS-PGRP-LE line escaped from pupal lethality in combination with *hs-GAL4*. The transheterozygous adults were incubated at 35°C for 20 min and allowed to recover at 25°C for 3 h. The amount of mRNA of *Diptericin* (Dpt), *Drosomycin* (Drs), *Attacin* (Att), and rp49 was quantified by real-time RT-PCR (*E*). (−) *hs-GAL4*;*UAS-lacZ*. (+) *hs-GAL4*;*UAS-PGRP-LE* transheterozygous adults. (*F–H*) PGRP-LE-mediated induction of *Drosomycin-GFP* in the epithelial tissue. The *UAS-PGRP-LE*;*Drs-GFP* (*F*) and *hs-GAL4*;*UAS-PGRP-LE*;*Drs-GFP* (*G*) transheterozygous larvae were incubated at 35°C for 20 min and allowed to recover at 25°C for 12 h. The expression of *Drs-GFP* is induced in the trachea (*G*) and salivary glands (out of focus in *G*) by the forced expression of PGRP-LE. Immune challenge induces *Drs-GFP* expression in the fat body of *Drs-GFP* larvae (*H*).

Some of the homologs bind to peptidoglycans, which are cell-wall components of almost all bacteria (11, 24, 26, 27). There was no 5-bromo-4-chloro-3-indolyl β-D-galactoside staining detected in the fat body of the negative controls (Fig. 1*B*). Induction of the endogenous *Diptericin* gene by the forced expression of PGRP-LE was confirmed by using *UAS-PGRP-LE* transgenic larvae (Fig. 1 *E* and *F*). The expressed PGRP-LE was detected in the hemolymph fraction (data not shown).

We then investigated whether endogenous antimicrobial peptides are induced by the forced expression of *PGRP-LE* in the absence of bacterial challenge. As shown in Fig. 2 *A–C*, quantitative RT-PCR analyses revealed that the *Diptericin* gene was induced strongly by the forced expression of *PGRP-LE*, whereas there was only weak induction of *Drosomycin* and *Attacin*, an antibacterial peptide gene. The differential PGRP-LE-mediated induction of the antimicrobial peptide genes was confirmed in larvae by using another GAL4 driver, the enhancer trap GAL4 line c564, which constitutively expresses GAL4 in the fat body and hemocytes (blood cells) (28) (Fig. 2*D*). In adults, the differential activation of the antimicrobial peptide genes also was induced by the forced expression of *PGRP-LE* (Fig. 2*E*). The *Diptericin* gene is regulated almost completely by the imd pathway via the Rel transcription factor Relish (3, 19), whereas *Drosomycin* is regulated predominantly by the Toll pathway (3, 5). The induction of the *Attacin* gene is thought to be regulated by inputs from both the imd and Toll pathways (2, 3, 5, 6, 19). Therefore, the differential induction of antimicrobial genes suggests that PGRP-LE is involved in the imd pathway. In *imd* or *Relish* loss-of-function backgrounds, the PGRP-LE-mediated constitutive expression of *Diptericin* was abolished (Fig. 2*A*), indicating that PGRP-LE acts upstream of *imd* and *Relish*. The weak PGRP-LE-mediated induction of *Drosomycin* and *Attacin* also was reduced in the *imd* or *Relish* mutant backgrounds (Fig. 2 *B* and *C*), confirming that the imd pathway is partially involved in the induction of *Drosomycin* and *Attacin* (3, 6, 19). PGRP-LE-mediated activation of the *Drs-GFP* reporter was observed in larval trachea and salivary glands (Fig. 2 *F* and *G*), consistent with the fact that induction of *Drosomycin* in the epithelial tissue depends on the imd pathway (29). The Toll pathway mediates *Drosomycin* gene expression in the fat body after microbial infection (Fig. 2*H*). Moreover, the constitutive expression of antimicrobial peptides was observed in a J4 deletion (20) of Rel factor genes in the Toll pathway, *dorsal* and *Dorsal-related immunity factor* (Fig. 2 *A–C*). Therefore, PGRP-LE selectively activates the imd pathway upstream of *imd*.

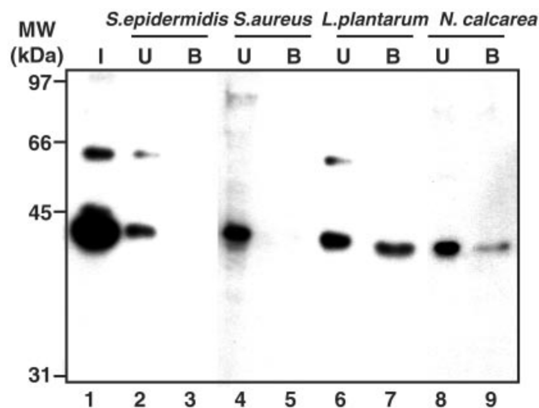

Fig. 3. Specific binding of PGRP-LE to the DAP-type peptidoglycan. The DAP-type peptidoglycan of *L. plantarum* ATCC 8014, the Lys-type peptidoglycans of *S. epidermidis* ATCC 155, the Lys-type peptidoglycan of *S. aureus* (Wako), and the DAP-type peptidoglycan-containing cell wall of *N. calcarea* ATCC 17876 were used for binding assay. Approximately 0.5 μg of purified recombinant PGRP-LE (lane 1) was incubated with 0.32 mg of insoluble peptidoglycans of *S. epidermidis* (lanes 2 and 3), *S. aureus* (lanes 4 and 5), and *L. plantarum* (lanes 6 and 7) and the same amount of the DAP-type peptidoglycan-containing cell wall of *N. calcarea* ATCC 17876 (lanes 8 and 9). Unbound protein (lanes 2, 4, 6, and 8) isolated from the soluble fraction and bound protein (lanes 3, 5, 7, and 9) recovered after washing the peptidoglycans and the cell wall with Tris-maleate buffer containing 1 mol/liter NaCl and 1 mol/liter NaCl plus 0.2% Tween 20 were analyzed by Western blot analysis by using anti-penta-histidine antibody (Qiagen). Molecular size markers are indicated on the left.

Specific Binding of PGRP-LE to the DAP-Type Peptidoglycan. Peptidoglycans have a diverse amino acid composition, and the linking of stem peptides depends on the bacterial species (30). *Diptericin* is induced by some species of Gram-positive bacteria, such as *Bacillus thuringiensis*, which all have directly cross-linked DAP-containing peptidoglycans, and is not induced by Gram-positive bacteria with an interpeptide bridge and lysine instead of DAP in the peptidoglycan stem peptide (7, 30). Many Gram-negative bacteria have the DAP-type peptidoglycan and induce the imd pathway (7, 30). Consistent with the selective PGRP-LE-induced activation of the imd pathway, PGRP-LE binds selectively to the DAP-type peptidoglycan (Fig. 3). PGRP-LE was incubated with DAP-type and Lys-type insoluble peptidoglycans and the DAP-type peptidoglycan-containing purified cell wall. After extensive washing, PGRP-LE was retained in the DAP-type peptidoglycan of *L. plantarum* and the DAP-type peptidoglycan-containing cell wall of *N. calcarea* but not in the Lys-type peptidoglycans of *S. epidermidis* and *S. aureus*, indicating a high affinity of PGRP-LE that was selective to the DAP-type peptidoglycan. Therefore, PGRP-LE acts as a pattern-recognition receptor to the DAP-type peptidoglycan, which is a cell-wall component of the bacteria capable of activating the imd pathway in *Drosophila*.

Activation of ProPO Cascade by PGRP-LE. In the silk moth, PGRP participates in peptidoglycan-mediated activation of the proPO cascade leading to melanization (24). Melanization is a common defense mechanism among invertebrates (4). In the process of catalytic conversion of dopamine into melanin by phenoloxidase, cytotoxic reactive oxygen species and melanin are produced and are thought to be toxic to microorganisms (4). In the present study, the forced expression of *PGRP-LE* led to melanization in the absence of microbial infection in *Drosophila*. As shown in Fig. 4*C*, there was localized melanization in the cuticle of the larvae when the ubiquitous expression of *PGRP-LE* was induced by heat shock-inducible GAL4. *PGRP-LE* expression was induced in the fat body and hemocytes, and the pigmented particles were

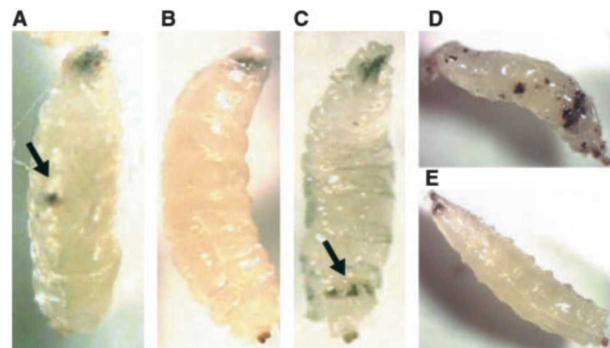

Fig. 4. Induction of melanization by forced expression of PGRP-LE in *Drosophila*. Localized melanization (arrow) is induced in immune-challenged, WT larvae (*A*) but not in unchallenged larvae (*B*). When ubiquitous *PGRP-LE* expression was induced by heat shock-inducible GAL4, localized melanization (arrow) was observed in the cuticle of the larvae (*C*). After the appearance of the localized melanization, the larvae died of melanization in the whole body. The transheterozygous larvae of *hs-GAL4*;GS1068 were incubated at 37°C for 1 h and allowed to recover at 25°C. Pigmented particles were observed in the hemolymph of transheterozygous larvae of c564;GS1068 (*D*) but not in the hemolymph of control larvae of c564;*UAS-lacZ* (*E*).

induced in the hemolymph of the larvae (Fig. 4*D*). There were no pigmented particles in the hemolymph of control larvae when β-galactosidase was induced by the same GAL4 driver, c564 (Fig. 4*E*). PGRP-LE-mediated melanization was observed in the *imd* loss-of-function background larvae (data not shown), consistent with the fact that melanization does not depend on the *imd* gene (31). These results suggest that PGRP-LE is capable of activating the proPO cascade *in vivo*. To confirm that PGRP-LE activates the proPO cascade *in vivo*, we quantified the phenoloxidase activity of homogenates of these larvae under conditions in which the proPO-activating enzyme is inactivated. Phenoloxidase is proteolytically activated from an inactive precursor, proPO, by proPO-activating enzyme, a terminal serine protease of the proPO cascade (4). Significant phenoloxidase activity was detected in the homogenate of the larvae when *PGRP-LE* was expressed in the fat body and hemocytes, whereas there was no activity detected in the homogenate of the negative control larvae in which β-galactosidase was induced by the same GAL4 (Table 1). Similar results were obtained with the hemolymph of these larvae. These results indicate that, in *Drosophila*, PGRP-LE is capable of activating the proPO cascade, a proteolytic cascade present in the hemolymph.

Table 1. Activation of the proPO cascade by forced expression of PGRP-LE *in vivo*

Samples	Activity of phenoloxidase, units/mg
c564;GS1068 homogenate	224 ± 2.4
c564;*UAS-PGRP-LE* homogenate	282 ± 1.5
c564;*UAS-lacZ* homogenate	−4.9 ± 1.9
E. coli-infected larvae homogenate	−15.1 ± 12.8
c564;GS1068 hemolymph	700 ± 111
c564;*UAS-lacZ* hemolymph	149 ± 37

Phenoloxidase activity was assayed with L-dihydroxyphenylalanine as a substrate according to the previously reported method (24). There was no phenoloxidase activity detected in the homogenate of wild-type larvae 1 h after *E. coli* infection, consistent with localized activation of the proPO cascade *in vivo* (Fig. 4*A*). One unit was defined as the amount of enzyme causing an increase in the absorbency of 0.01 at OD at 450 nm for 30 min. Protein was determined by Protein Assay (Bio-Rad) by using BSA as a standard. The average and SD of duplicate experiments are shown.

Discussion

Insect humoral immunity depends on primary and secondary responses. The primary response is mediated by the activation of cascades of constitutive proteins present in the hemolymph, such as the proPO cascade, whereas the secondary response requires transcriptional activation of defense proteins, such as induction of antimicrobial peptides (1–4). The present study demonstrated that PGRP-LE is capable of activating both the proPO cascade and imd/Relish-mediated antibacterial peptide induction, showing PGRP-LE as an example of an immune factor that activates both primary and secondary insect humoral immunity responses. PGRP-LE-mediated activation of the proPO cascade does not depend on intracellular imd signaling. Therefore, the pathways promoting melanization and antibacterial peptide expression are thought to be coupled upstream of *imd* in the hemolymph. Because PGRP-LE is a constitutive hemolymph protein, PGRP-LE probably constitutes the first line of self-defense involving pathogen recognition and transmission of signals downstream of the defense reactions. In the present paper, we demonstrated a role of PGRP-LE in insect immunity by using overexpression studies. It cannot be excluded, however, that the observations are linked to the overexpression procedure. The use of loss-of-function mutations might clarify this point.

In *Drosophila*, the secondary humoral responses consist of two distinct signaling pathways, the Toll and imd pathways. A loss-of-function mutation in a *Drosophila* serine protease inhibitor, Spn43Ac, causes constitutive processing of Spätzle and constitutive activation of the Toll pathway, suggesting the involvement of some serine proteases in the induction of the Toll-mediated defense (10). The proteolytic cascade controlled by Spn43Ac is not involved in the imd-mediated antibacterial defense (10). In the present paper, we demonstrated that PGRP-LE-mediated a proteolytic cascade, the proPO cascade, which is linked to the imd-mediated antibacterial defense. Therefore, proteolytic cascades have a major role in insect humoral immunity. In innate immunity, proteolytic cascades triggered by non-self-recognition also have an important role in the coagulation cascade of the horseshoe crab (32) and in mammalian complement activation (33, 34). These findings imply some evolutional conservation of a link between recognition of microbial molecular patterns, proteolytic cascades, and activation of host defenses.

In *Drosophila*, PGRPs constitute a highly diversified family of at least 12 members. Of these, many short PGRPs, including PGRP-SA, are induced after bacterial challenge, whereas some long PGRPs are constitutive proteins, including PGRP-LE (11). Recently, a PGRP family consisting of short and long PGRPs that bind to the Lys-type peptidoglycan of *S. aureus* with different affinity was identified in humans (27). During the preparation of this manuscript, it was reported that *PGRP-SA* and *PGRP-LC* are required for Gram-positive bacterial activation of the Toll pathway and for Gram-negative bacterial activation of the imd pathway, respectively (12–15). PGRP-SA binds to the Lys-containing peptidoglycan of *Micrococcus luteus* (11), and *PGRP-LC* participates in lipopolysaccharide recognition (13, 15), although there is no evidence for the direct binding of PGRP-LC to lipopolysaccharides. The findings of the present study indicate that PGRP-LE selectively activates the imd pathway and has strong affinity to the DAP-type peptidoglycan, which is a cell-wall component of bacteria capable of activating the imd pathway in *Drosophila*. These results are consistent with the conclusion that PGRP-LC is not the sole upstream element activating the imd pathway (14). In innate immunity, the diverse members of PGRP might be required to distinguish between invading bacteria.

Although predicted signal peptide is lacking (11), PGRP-LE has humoral functions, such as activation of the proPO cascade in the hemolymph and activation of the imd pathway upstream of *imd*. PGRP-LE is similar to proPO, a protein that is functionally related to PGRP-LE. ProPO also lacks a signal peptide and has humoral functions after release from producing hemocytes into the hemolymph by cell rupture (35, 36). Further investigation is necessary, however, to understand the export mechanisms of PGRP-LE. Overexpression of PGRP-LE activates the *Diptericin* and *Drosomycin* promoters in the fat body (Fig. 1*A*) and the trachea (Fig. 2*G*), respectively, via the imd pathway. Because the imd pathway mediates *Diptericin* expression in the fat body and *Drosomycin* expression in the trachea, circulating PGRP-LE probably activates the imd pathway in the fat body and in the trachea through a putative cell-surface receptor that is activated by the PGRP-LE-mediated upstream cascade. In the cuticle, melanin formation is induced by the ubiquitous induction of PGRP-LE but not by the tissue-specific induction of PGRP-LE in the fat body and hemocytes (Fig. 4 *C* and *D*). Cuticular PGRP-LE might be required for the activation of the proPO cascade in the cuticle.

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