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MINIREVIEW

The multiple functions of the PGRP family in *Drosophila* immunity

A Goto, S Kurata

Graduate School of Pharmaceutical Sciences, Tohoku University, Japan

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Abstract

The innate immune system discriminates between infectious non-self and self using germ-line-encoded pattern recognition receptors (PRRs) that are highly conserved from insects to mammals. Peptidoglycan recognition protein (PGRP) is one of the hallmark pattern recognition receptors responsible for detecting unique bacteria-derived peptidoglycans. The PGRP family comprises several members (13 in *Drosophila*, 7 in *Anopheles*, and 4 in mammals) and are differentially expressed on immune-responsive organs. Some PGRPs have amidase or bactericidal activities and function as immune modulators, whereas others have lost their enzymatic activity, but still have crucial roles in the activation of innate immune signaling. Evidence from recent *Drosophila* studies suggests that PGRPs have a role in a variety of immune reactions, such as in the activation of the prophenoloxidase cascade, the production of antimicrobial peptides through the activation of the Toll and Imd pathways, intracellular bacteria recognition, and phagocytosis.

Key words: innate immunity; PGRP (peptidoglycan recognition protein); PRR (pattern recognition receptor); PAMP (pathogen-associated molecular pattern); amidase; antimicrobial peptide; phagocytosis

Introduction

Insects are diverse group of organisms and comprise more than 80 % of the animal kingdom. To prevail as such a vast majority of species among other organisms, insects have developed excellent defense mechanisms against bacterial infection. In contrast to adaptive immunity, which requires highly specific receptors created by somatic gene rearrangement, the innate immune system is a defense mechanism that exploits germ-line encoded gene products. The principal mechanisms of innate immunity are difficult to study in vertebrates largely due to the effects of the adaptive immune response. Thus, insects have become a favorite model system because, as invertebrates, they depend solely on the innate immune system to fight off infection. The powerful genetic and molecular techniques with the complete genome sequencing make *Drosophila* an attractive model for deciphering the precise mechanisms of the innate immune response (Hoffmann, 2003; Hultmark, 2003).

Corresponding author:
Shoichiro Kurata
Graduate School of Pharmaceutical Sciences,
Tohoku University,
Sendai 980-8578, Japan
E-mail: kurata@mail.pharm.tohoku.ac.jp

Although the innate immune system is composed of multiple complex processes including cellular and humoral responses, much of the attention in *Drosophila* has focused on humoral reactions. The primary response is the most immediate response (within minutes) against microorganism invasion and depends on constitutively present endogenous molecules in the hemolymph, such as coagulation factors, cell surface recognition receptors or prophenoloxidase (proPO) for wound healing, phagocytosis and melanization which confine microorganisms in small spaces, respectively (Theopold *et al.*, 2003; Goto *et al.*, 2003; Ashida, 2004; Kurata *et al.*, 2006). Shortly after the primary response, the secondary response begins and ultimately leads to the production of antimicrobial peptides (AMPs) from the fat body, the functional equivalent of the mammalian liver. To date, there are seven known distinct antimicrobial peptides in *Drosophila* and they are basically small cationic molecules with specific membrane-attackable bactericidal activity against various types of microorganisms. The transcriptional regulation of AMP expression is under the control of two distinct pathways, the Toll and immune deficiency (Imd) pathways (Kurata, 2004; Kurata *et al.*, 2006).

The Toll pathway was originally identified as a

signaling cascade involved in dorsoventral patterning in embryos (Morisato and Anderson, 1995; Roth, 2003). Subsequent genetic analysis serendipitously led to the discovery that it also controls the expression of the antifungal peptide Drosomycin, which is predominantly triggered by Gram-positive or fungal infection (Lemaitre *et al.*, 1996). Activation of the Toll pathway is initiated through the binding of the proteolytically cleaved cytokine Spaetzle to the Toll receptor, which then transduces its signal to the cells. In the cell, a Toll receptor-adaptor complex composed of *d*MyD88, Tube, Pelle, and other unidentified factors induces the phosphorylation and degradation of the ankyrin-repeat inhibitor protein Cactus. The nuclear factor NF-κB protein Dif, which is normally retained in the cytoplasm by binding to Cactus, is then translocated into the nucleus and triggers the expression of the antifungal peptide Drosomycin together with numerous effector molecules (Hoffman and Reichhart, 2002; Hoffmann, 2003). This discovery unambiguously led to the identification of the Toll-like receptor (TLR) family in mammals (Takeda *et al.*, 2003; Royet *et al.*, 2005).

In contrast, the Imd pathway is predominantly activated by Gram-negative bacterial infection. While analyzing the expression of antibacterial genes in a phenoloxidase cascade mutant, *imd* was serendipitously discovered as the first recessive mutation that impairs the inducibility of all antibacterial peptides in the *Drosophila* immune response (Lemaitre *et al.*, 1995). Six years later, the *imd* gene product was identified. It is a 25-kDa protein containing a death domain with significant sequence similarity to that of the mammalian tumor necrosis factor-receptor interacting protein RIP (Georgel *et al.*, 2001). Activation of the Imd pathway triggers the kinase cascade of *d*TAK (a MAP 3 kinase homologue) and the IKK complex, and ultimately leads to phosphorylation of the Rel protein Relish (Leulier *et al.*, 2002; Naitza *et al.*, 2002; Vidal *et al.*, 2001). Phosphorylated Relish is then cleaved by DREDD (a mammalian homolog of caspase-8), and its DNA binding domain leaving the I-κB domain in the cytoplasm is translocated into the nucleus and triggers AMP expression (Leulier *et al.*, 2000; Stoven *et al.*, 2003).

Thus, over the past decade, our understanding of *Drosophila* innate immunity has dramatically centered on the field of intracellular signaling mechanisms. There remain crucial questions, however, about how the innate immune system recognizes microorganisms, and discriminates between self and non-self infections, and how the signals from outside the cells subsequently reach the cognate innate receptors to activate the Toll or Imd pathway. The discovery of pattern recognition receptors (PRR) such as peptidoglycan recognition proteins (PGRPs) that recognize unique bacterial cell component peptidoglycans (PGNs) led to our deeper understanding of the innate immune recognition concept (Medzhitov and Janeway, 2002; Royet, 2004). Here, we review recent advances in the understanding of the multifunctional PGRP family, a representative pattern recognition molecule, in *Drosophila*.

Discovery of the PGRP family

The name ”peptidoglycan recognition protein” was first introduced by Ashida’s group (Yoshida *et al.*, 1996). They purified a 19-kDa protein from the hemolymph and cuticles of silkworm (*Bombyx mori*) and demonstrated that it binds to Gram-positive bacteria and PGN, and activates the prophenoloxidase cascade (Yoshida *et al.*, 1996). Later, its corresponding cDNA was cloned (Ochiai *et al.*, 1999). PGRP gene homologs from a moth (*Trichoplusia ni*) and subsequently from mouse and human were in turn cloned, indicating that the PGRP family is highly conserved from insects to mammals (Kang *et al.*, 1998). Completion of various genome projects (e.g., *Drosophila melanogaster*, *Anopheles gambiae*, human, mouse, etc.) also boosted the discovery of diverse PGRP family. The *Drosophila* genome encodes at least 13 PGRP family members, which are subdivided into seven short transcripts (PGRP-S; ~200 amino acids long) and six long transcripts (PGRP-L; from 200 to 600 amino acids long). The short transcripts are predicted to be secreted molecules due to the presence of signal peptides and are induced in response to bacterial infection, whereas the long transcripts are constitutively present on the cell surface or integrated into the plasma membrane, except for PGRP-LB (Werner *et al.*, 2000).

Extracellular recognition by the PGRP family

PGRP roles in the immune response

A significant breakthrough with regard to the role of PGRP in the immune response came through a large-scale ethylmethane sulfonate mutagenesis screen from *Drosophila*. Michel et al isolated a mutation called *semmelweis* (*seml*), named after the Hungarian physician Ignaz Phillipp Semmelweis who is a pioneer of antiseptic treatments (Celine, 1999; Michel *et al.*, 2001). The *PGRP-SA*^{seml} mutants lead to defects in AMP expression and render flies more susceptible to several Gram-positive bacterial infections. The mutated region was found to be on a highly conserved cysteine residue that is changed to a tyrosine in PGRP-SA. Hemolymph transfer experiments revealed that PGRP-SA is secreted into the hemolymph. Surprisingly, activation of the Toll pathway by fungi and of the Imd pathway by Gram-negative bacteria is normal compared to wild-type, raising the possibility that there are other PRRs specific for fungi and Gram-negative bacteria (Michel *et al.*, 2001). Consistent with this hypothesis, three independent groups identified PGRP-LC mutations (*ird7* or *totem*) (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002). *PGRP-LC* mutant flies are highly susceptible to some Gram-negative bacterial infections and do not express Imd-mediated AMP genes (such as Diptericin and Attacin). Epistatic analysis indicates that PGRP-LC encoding a single transmembrane protein acts upstream of Imd, suggesting that PGRP-LC functions in the activation of the Imd pathway in response to Gram-negative bacterial infection. PGRP-SA binds to lysine (Lys)-type PGN

from Gram-positive bacteria, whereas PGRP-LC binds to *meso*-diaminopimelic (DAP)-type PGN from Gram-negative bacteria, reinforcing the structural basis for the recognition mechanisms (Leulier *et al.*, 2003; Kaneko *et al.*, 2004).

Cooperative roles of PGRPs for detecting pathogens

Curiously, the *PGRP-LC* mutant phenotypes are not as severe as that of *IKK* or *Relish* null mutants in terms of both AMP expression and survival rates upon some Gram-negative bacterial infections (Gottar *et al.*, 2002). Accordingly, some Gram-positive bacteria such as *Staphylococcus saprophyticus*, *Staphylococcus aureus*, or *Enterococcus faecalis* induce normal activation of the Toll pathway in a *PGRP-SA* mutant background, but this activation is completely blocked in *Dif* mutants (Bischoff *et al.*, 2004). Those findings led to the observation that the one to one PRR/ pathogen associated molecular pattern (PAMP) concept is inappropriate for innate immune sensing mechanisms. This interpretation was soon validated by the discovery of multi-functional PGRP-LE, which contains a unique acidic domain, functions in the activation of the Imd pathway and also triggers the proPO cascade when it is ectopically expressed (Takehana *et al.*, 2002). More importantly, *PGRP-LC/PGRP-LE* double mutants are more susceptible against Gram-negative bacteria (*E. coli*) infection than either single mutant alone, suggesting that these two proteins cooperate to recognize Gram-negative bacteria and lead to the activation of the Imd pathway (Takehana *et al.*, 2004). Similarly, in the case of Gram-positive bacteria sensing, other cooperative receptor complexes are required upstream of Toll. Gram-negative bacteria binding protein (GNBP), which binds Gram-negative bacteria, was first identified from silkworm (Lee *et al.*, 1996). Gobert et al (2003) subsequently reported that *GNBP1* mutants are not resistant against some Gram-positive bacterial infections, showing a similar immune defective phenotype as the *PGRP-SA* mutant. The finding that GNBP1 physically interacts with PGRP-SA in the circulation to activate the Toll pathway supports this observation (Gobert *et al.*, 2003; Pili-Floury *et al.*, 2004; Wang *et al.*, 2006). Moreover, the generation of another PGRP family member mutant, the *PGRP-SD* loss-of-function mutant, has been reported (Bischoff *et al.*, 2004). *PGRP-SD* mutants are highly susceptible to some types of Gram-positive bacterial infection, and this mutation exacerbates the PGRP-SA and GNBP1 mutant phenotypes, suggesting that PGRP-SD is another Gram-positive bacteria recognition molecule involved in the activation of the Toll pathway.

Taken together, there are four known PGRP family members, PGRP-SA, -SD, -LC, and –LE, that are crucial for microbe recognition and for activating the innate immune signaling pathways (Toll, Imd , and proPO cascades) (Fig. 1).

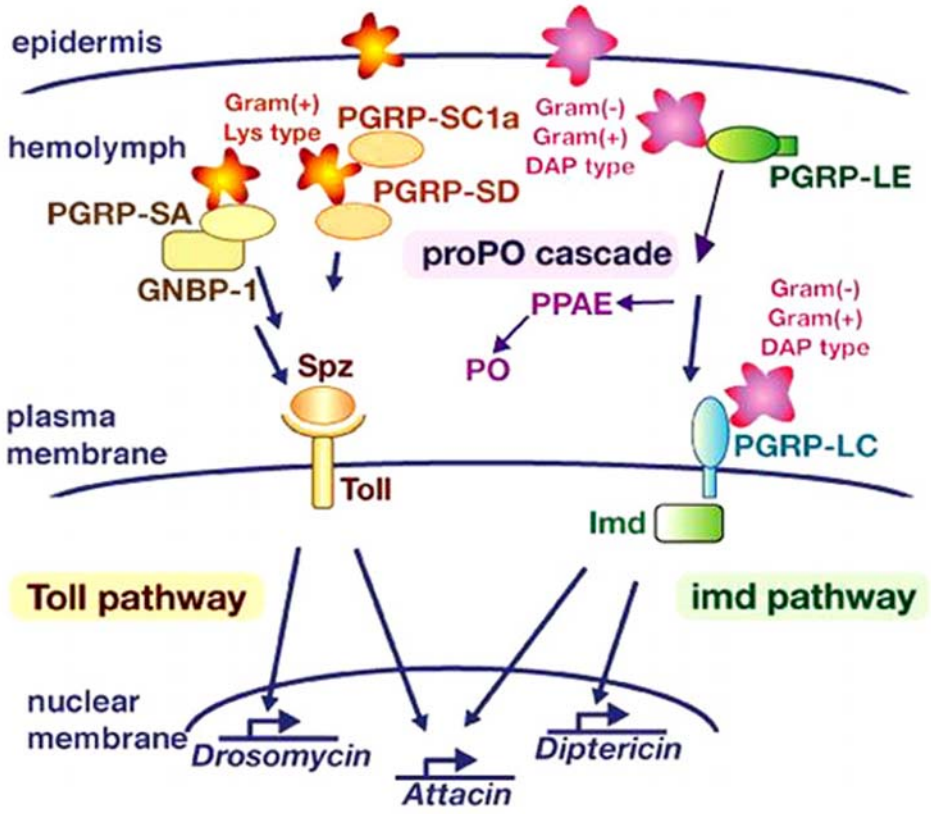

Fig. 1 *Extracellular recognition by PGRP family*. In hemolymph, *meso*-diaminopimelic (DAP)-type peptidoglycans (PGNs) from Gram-negative and some Gram-positive bacteria (*Bacillus* species) are recognized by PGRP-LC or PGRP-LE and activate the Imd pathway to trigger Diptericin or Attacin expression. In the case of lysine (Lys)-type Gram-positive bacterial infection, either PGRP-SC1a, -SD, -SA, GNBP1 alone or in combination is responsible for the activation of the Toll pathway through binding of the endogenous ligand spaetzle (Spz).

Intracellular recognition by PGRP-LE

Large-scale gain-of-function genetic screening using AMP reporters led to the identification of PGRP-LE. PGRP-LE overexpression leads to constitutive activation of the Imd pathway as well as the proPO cascade, which ultimately leads to the formation of large melanotic tumors in the hemolymph (Takehana *et al.*, 2002). *PGRP-LE* loss-of-function mutants were isolated in subsequent studies 2 years after the identification of PGRP-LE (Takehana *et al.*, 2004). The phenotypes of a single *PGRP-LE* gene mutation are fairly weak in terms of their survival rates against *E. coli* infection and AMP expression. However, when combined with the PGRP-LC mutation, the *PGRP-LE/PGRP-LC* double mutants exhibit high susceptibility to *E. coli* and to other DAP-type PGN-containing bacteria (Takehana *et al.*, 2004). This finding clearly suggests that PGRP-LE synergistically or redundantly acts with PGRP-LC to activate the Imd pathway.

To gain a better understanding of the cooperative recognition mechanisms between PGRP-LC and PGRP-LE, two independent groups recently conducted very exciting experiments using monomeric DAP-type PGN tracheal cytotoxin (TCT) (Kaneko *et al.*, 2006; Lim *et al.*, 2006). TCT is a disaccharide-tetrapeptide fragment of PGN that contains a 1,6-anhydro-arranged muramic acid and a DAP residue at the third position of the stem peptides (Cookson *et al.*, 1989), and is continuously released from Gram-negative bacteria as a turnover product of the PGN in the cell wall (Park, 2001). The presence of such a small diffusible monomeric PGN is an ideal signature for the innate immune recognition system by PRRs, as the PGN layer of Gram-negative bacteria is basically hidden under the outer lipopolysaccharide (LPS) layers. *In vitro* cell culture studies demonstrated that TCT potently activates the Imd pathway via two different PGRP-LC isoforms: PGRP-LCa and PGRP-LCx (Kaneko *et al.*, 2004). Deletion mutant studies demonstrated that the RHIM-like motif of PGRP-LC is responsible for the signaling, but is dispensable for the interaction with Imd (Kaneko *et al.*, 2006; Choe *et al.*, 2005). Whereas a single *PGRP-LC* or *PGRP-LE* mutant responds normally to TCT, *E. coli* PGN, or live *E. coli*, the response of *PGRP-LC/PGRP-LE* double mutants is completely abolished. Clonal expression studies demonstrated that PGRP-LE acts in a cell-autonomous manner on some immune responsive tissues, such as malpighian tubes. Direct delivery of TCT into the cytosol by calcium phosphate transfection triggers an enhanced AMP expression that is dependent on PGRP-LE, suggesting that PGRP-LE is a second receptor for TCT (Kaneko *et al.*, 2006). This idea is supported by analysis of the crystal structure of PGRP-LE^{pg} (PGRP domain of PGRP-LE)/TCT complex, which indicates that TCT strongly binds to PGRP-LE^{pg} with an apparent K_d of 27 nM, and induces PGRP-LE multimerization through head-to-tail dimer formation (Lim *et al.*, 2006).

Collectively, these results indicate that PGRP-LE potentially has dual functions depending on its localization; on the outside of the cells, it cooperates with PGRP-LC through its PGRP domain and triggers the proPO cascade, and inside the cells, it acts as an intracellular recognition receptor for activation of the Imd pathway (Fig. 2).

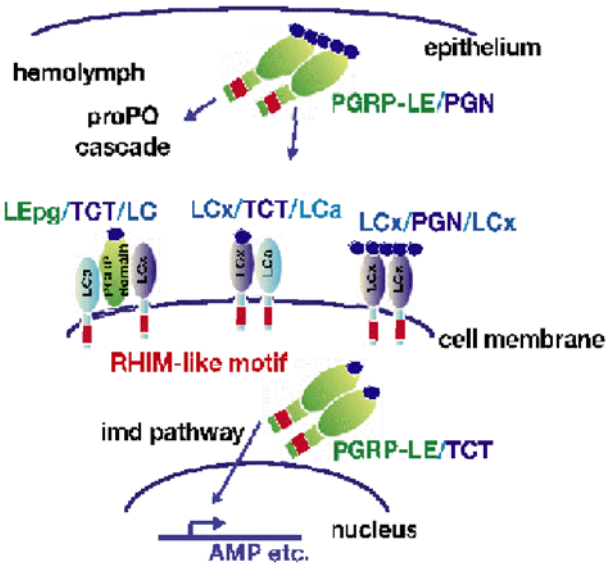

Fig. 2 *Recognition mechanisms of PGRP-LE*. Monomeric DAP-type PGNs (TCT) binds to the PGRP domain of PGRP-LE. In the hemolymph, this complex triggers the proPO cascade. TCT can also bind to PGRP-LCx to form three potential types of receptor complexes (LEpg/TCT/LC, LCx/TCT/LCa or LCx/PGN/LCx) on the cell surface. The RHIM-like motif of PGRP-LCx and LCa is responsible for the activation of the Imd pathway. The PGRP-LE/TCT complex also functions in intracellular recognition.

Immune modulator activities of the PGRP family

A representative feature of the PGRP family is that all PGRPs in insects and mammals have in common an approximately 160 amino acid-long PGRP domain with similarity to the bacteriophageT7 lysozyme, a zinc-dependent N-acetyl-muramyl-L-alanine amidase (Steiner, 2004; Royet *et al.*, 2005). Among the 13 PGRP family members in *Drosophila*, the first group is categorized as non-catalytic PGRPs, which lack the zinc binding residues required for amidase activity, but retain the binding capacities to PGN (PGRP-SA, -SD, -LA, -LC, LD, -LE, -LF). The second group is the catalytic PGRPs, which have zinc-dependent amidase activity that either reduces or eliminates the biological activities of the PGNs (PGRP-SC1a, -SC1b, -LB, -SB1, -SC2).

The first striking discovery of the potential role of catalytic PGRPs in the *Drosophila* immune response was reported for PGRP-SC1b (Mellroth *et al.*, 2003). *In vitro* studies performed by Mellroth *et al.* (2003) demonstrated that recombinant SC1b hydrolyzes the lactylamide bond between the glycan strand and the stem peptides of PGN, and the degraded PGN has less immune stimulatory activity compared with undigested PGN, indicating that

PGRP-SC1b has scavenger activity. Unexpectedly, Chang *et al.* (2004), in the course of crystal structure analysis, demonstrated that recombinant PGRP-SA has L,D-carboxypeptidase activity that cleaves the DAP-type muropeptide, but not the Lys-type compound.

Moreover, three independent groups reported the roles of catalytic PGRPs: PGRP-SC1/2 (Bischoff *et al.*, 2006), PGRP-LB (Zaidman-Rémy *et al.*, 2006), and PGRP-SB1 (Mellroth and Steiner, 2006). First, Bischoff *et al.* (2006) used RNA interference techniques to generate *PGRP-SC1/2* loss of function mutants and reported that the mutants overactivate the Imd pathway after bacterial infection, and feeding-induced infection by *Erwinia carotovora carotovora* is enhanced compared to controls. Thus, PGRP-SC1/SC2 might modulate the activation of the Imd pathway in the gut, which is constantly threatened by bacterial infection (Bischoff *et al.*, 2006). The effects of phenotypes of recently isolated *PGRP-SC1a* mutants (*picky*) on the Toll pathway are confusing with respect to the complex immune modulator mechanisms (Garver *et al.*, 2006). Second, biochemical analysis indicates that PGRP-LB has specific amidase activity for DAP-type PGN, including TCT. Similar to the RNA-interference generated *PGRP-SC1/2* mutants, the analysis of the time course of AMP expression induced by Gram-negative bacteria or TCT infection revealed a strong immune response by the RNA-interference generated *PGRP-LB* mutants compared to wild-type controls (Zaidman-Rémy *et al.*, 2006). Therefore, they concluded that PGRP-LB negatively regulates the Imd pathway and functions as a scavenger receptor for Gram-negative bacterial PGN. Finally, recent biochemical analysis demonstrated that PGRP-SB1 is an N-acetylmuramoyl L-alanine amidase that preferentially hydrolyzes DAP-type PGN. In contrast to PGRP-LB, PGRP-SB1 lacks enzymatic activity against TCT. In addition, this report first demonstrated that PGRP-SB1 possesses bactericidal activity against *B. megaterium* (Mellroth and Steiner, 2006).

Taken together, these findings suggest that non-catalytic PGRPs preferentially function in the detection of PGN, and catalytic PGRPs serve as scavenger receptors to detoxify harmful PGN and modulate the signaling pathways, although catalytic PGRPs that specifically cleave Lys-type PGN have yet to be identified.

Roles of PGRPs in phagocytosis

Phagocytosis is a phenomenon whereby invading microbes or altered self-like apoptotic or infected cells are engulfed and, eventually, digested by host cells called phagocytes (Aderem and Underhill, 1999). In *Drosophila*, most humoral immune responses leading to Toll and Imd pathway activation are mainly mediated by the fat body, whereas cellular responses such as phagocytosis, encapsulation, or melanization are mediated by blood cells called hemocytes (Hoffmann and Reichhart, 2002). There are three known hemocyte types in *Drosophila*. Plasmatocytes account for approximately 90 % of hemocytes and are the main executors of phagocytosis. Crystal cells, approximately 5% of the hemocyte population, contain a set of substrates and enzymes responsible for proPO-mediated humoral melanization. Lamellocytes, the third hemocyte type, show a large flattened morphology. Under non-infected conditions, lamellocytes are absent, but once large invaders, such as wasps, are infected, lamellocytes are differentiated from the lymph glands, the main hemocyte-producing organs in larvae (Meister and Lagueux, 2003). In addition to several phagocytic receptors, such as Croquemort (“catcher of death”) (Franc *et al.*, 1996, 1999; Stuart *et al.*, 2005), scavenger receptor (SR) -CI (Ramet *et al.*, 2001; Ulvila *et al.*, 2006), Peste (Philips *et al.*, 2005), Eater (Kocks *et al.*, 2005), and Dscam (Watson *et al.*, 2005), PGRP family members are also involved in phagocytosis.

PGRP-LC was the first PGRP family member discovered to be involved in phagocytosis. A double-stranded (ds)RNA interference-based screen of the *Drosophila* macrophage cell line S2 identified 34 genes, including PGRP-LC, that are essential for phagocytosis. Fluorescence Activated Cell Sorting analysis indicated that S2 cells with PGRP-LC knock-down had substantially decreased phagocytic activity towards Gram-negative bacteria *E. coli*, but not Gram-positive bacteria *S. aureus*. They also demonstrated that long transcripts of the same types of PGRP family, PGRP-LA or PGRP–LD, are not involved in phagocytosis (Ramet *et al.*, 2002). Conversely, another group recently reported that *PGRP-SC1a* mutation (*picky*) impairs phagocytic ability *S. aureus*, but not *E. coli* or *S. cerevisiae* (yeast), based on the results of a forward genetic ethylmethane sulfonate screen. Moreover, activation of the Toll pathway is significantly impaired in *PGRP-SC1a* mutants. Transgenic rescue experiments indicated that the amidase activity of PGRP-SC1a does contribute to Toll pathway activation, but is essential for the uptake of *S. aureus* (Garver *et al.*, 2006).

Conclusion

Over the past 5 years, the concept of the PRR/PAMP theory originally introduced by Charles Janeway has been verified experimentally using versatile molecular and genetic strategies (Janeway and Medzhitov, 2002). In mammals, functional characterization of Toll-like receptor family has considerably deepened our understanding of the recognition mechanisms of innate immunity (Takeda and Akira, 2005). In contrast, it seems that insect Tolls are not involved in immune functions as pattern recognition receptors, but are more likely to have other functions. *Drosophila* Toll is a kind of cytokine receptor that is activated by the endogenous proteolytically cleaved ligand spaetzle (Imler and Hoffmann, 2002). Now, the PGRP family members are coming to the forefront as molecular sensors against microbial infection in insects. Recent functional characterization of the PGRP family members in *Drosophila* has enabled us broaden the partial picture of pattern recognition mechanisms: 1) Specific types of PGN (Lys-type or DAP-type) can be detected by different PGRPs; 2)

Catalytic amidase PGRPs act as immune modulators; 3) Microbe detection by PGRPs triggers the activation of innate immune signaling pathways; and 4) PGRPs possess receptor functions for phagocytosis. Still, a fundamental question remains as to how the signals are transmitted into the cells to kill microbes after PGNs are recognized by PGRPs. Further studies will address this issue to complete the picture of the PRR/ PAMP mechanisms.

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