

## REVIEW

**Specificity and signaling in the *Drosophila* immune response****N Silverman, N Paquette, K Aggarwal***University of Massachusetts Medical School, Worcester, USA**Accepted December 07, 2009***Abstract**

The *Drosophila* immune response is characterized by the rapid and robust production of a battery of antimicrobial peptides immediately following infection. The genes encoding these antimicrobial peptides are controlled by two NF- $\kappa$ B signaling pathways that respond to microbial infection. The IMD pathway is triggered by DAP-type peptidoglycan, from the cell wall of most Gram-negative and certain Gram-positive bacteria, and activates the NF- $\kappa$ B precursor protein Relish. The Toll pathway, on the other hand, is stimulated by lysine-type peptidoglycan from many Gram-positive bacteria,  $\beta$  1,3 glucans from many fungi, as well as by microbial proteases. Toll signaling leads to the activation and nuclear translocation of DIF or Dorsal, two other NF- $\kappa$ B homologs. This review presents our current understanding of the molecular mechanisms involved in microbial recognition and signal transduction in these two innate immune pathways.

**Key Words:** Toll; IMD; PGRP; peptidoglycan; antimicrobial peptides**Overview of *Drosophila* Immunity**

Insects, such as *Drosophila*, thrive in microbe-rich environments. Not surprisingly, they have evolved complex mechanisms to combat microbial infection. These defenses include structural barriers to infection, such as the cuticle and peritrophic membrane. Insects also rely on inducible responses such as phagocytosis, the production of antimicrobial compounds, and homeostatic mechanisms that help repair the damage caused by infection (Lemaitre and Hoffmann, 2007). Together, these defense mechanisms allow insects to be broadly resistant to a large range of pathogens without an acquired immune response.

The inducible humoral insect immune response has been most widely studied in the favorite model system *Drosophila melanogaster*, where microbial challenge leads to the rapid and robust production of a battery of antimicrobial peptides (AMPs). Several families of AMPs have been described in *Drosophila*, with antifungal and anti-bacterial (both anti-Gram-negative or anti-Gram-positive) activities. Some of these AMPs appear to be unique to insects, e.g., Diptericin, while others have homologs in mammals, e.g., Defensins, Cecropins and Drosomycin (Lee *et al.*, 1989; Simon *et al.*, 2008).

As best we know, production of AMPs is regulated at the transcriptional level. To date, nearly all AMP genes have been found to be controlled by NF- $\kappa$ B family transcription factors. *Drosophila* have two distinct pathways which activate NF- $\kappa$ B factors and drive transcription of AMP genes following infection. The Toll pathway responds to several different types of microbes, including fungi and many Gram-positive bacteria, and leads to the activation of the NF- $\kappa$ B family members DIF and Dorsal. On the other hand, the IMD pathway is activated by Gram-negative and certain types of Gram-positive bacteria and leads to the activation of the NF- $\kappa$ B precursor Relish. The details of the how different microbes are detected and discriminated by these two pathways is the main focus of this review. First, the basic outline of these two signaling pathways will be summarized.

**Toll and IMD Signaling**

As mentioned above, the Toll pathway is able to activate two NF- $\kappa$ B homologs, DIF and Dorsal. Both of these proteins are similar to mammalian p65, and are held in the cytoplasm of unstimulated cells by the *Drosophila* I $\kappa$ B homolog Cactus. Like mammalian I $\kappa$ Bs, Cactus is phosphorylated and degraded upon stimulation (Fernandez *et al.*, 2001). One outstanding question within this pathway is the identity of the Cactus kinase. To date, only the kinase Pelle, homologous to the mammalian IRAK family of kinases, has been shown to function in the

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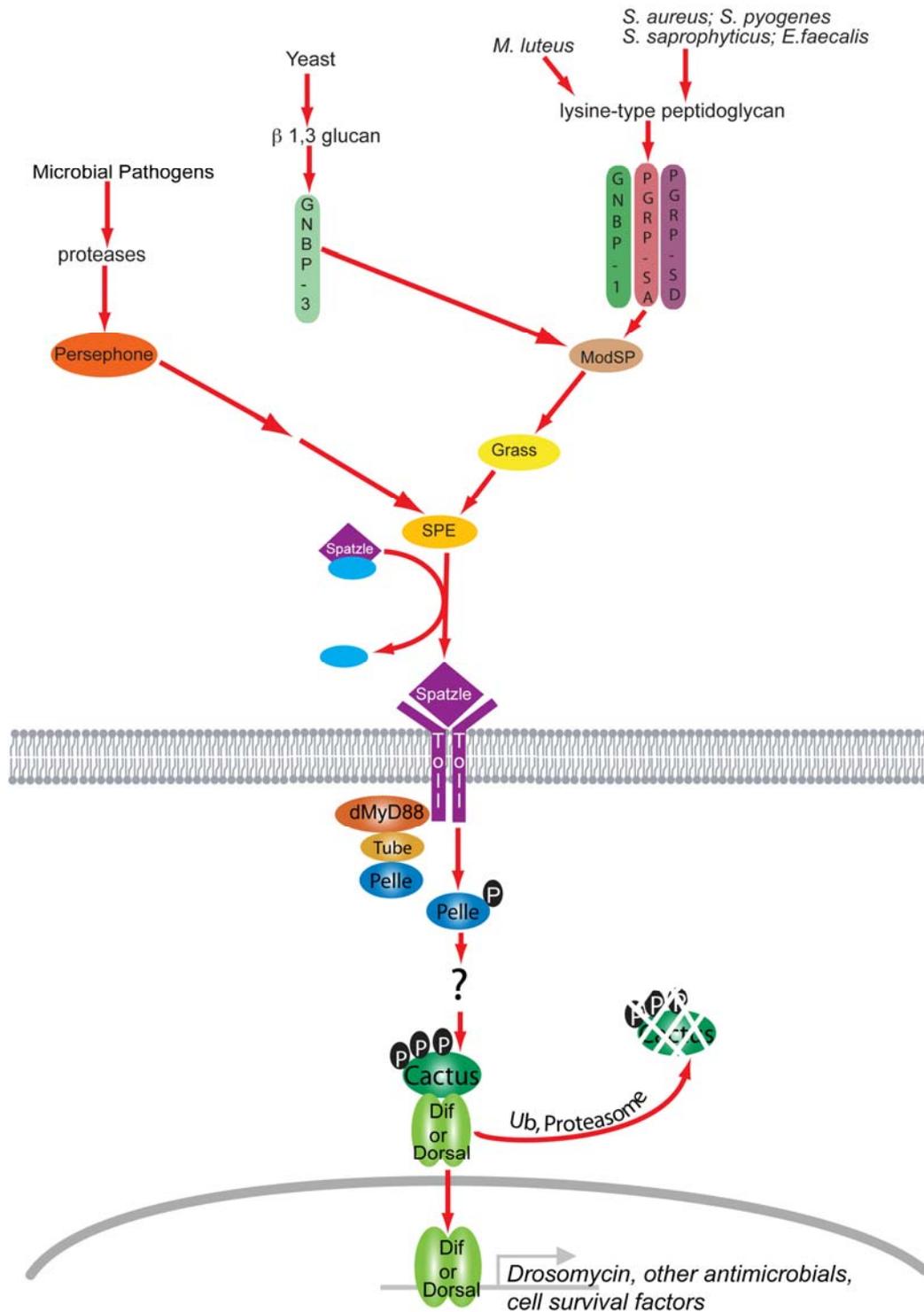
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**Figure 1** Toll signaling pathway. The Toll signaling pathway and its multiple modes of activation in the *Drosophila* immune response. Three distinct mechanisms for microbial recognition, leading to the cleavage of Spätzle and activation of Toll, are illustrated. The mechanisms include peptidoglycan detection, by PGRP-SA, PGRP-SD and GNB P1,  $\beta$ -glucan detection through GNB P3, and protease activity sensing via the serine protease Persephone. All these detection modalities lead to the activation of the Spätzle processing enzyme (SPE) which converts this cytokine into its active form, for binding and activating Toll. The intracellular signal transduction downstream of Toll is very similar to the MyD88-dependent pathway, which functions downstream of most mammalian TLRs. The key features of this pathway include a trimeric receptor associated complex, containing MyD88, Tube and Pelle, which ultimately lead to the phosphorylation and degradation of the I $\kappa$ B homology Cactus and the nuclear translocation of NF- $\kappa$ B homologs Dif and Dorsal.

Toll pathway. In mammals, IRAK kinases are indirectly involved in the phosphorylation of I $\kappa$ Bs, being required for the initiation of a kinase cascade that culminates in the activation of the I $\kappa$ B kinase (IKK) complex, which directly phosphorylates I $\kappa$ B $\alpha$  (Skaug *et al.*, 2009). In flies, a similar kinase cascade may be involved, although the components are not yet identified. Alternatively, it remains possible that Pelle directly phosphorylates Cactus on the residues necessary for its degradation. This issue remains unresolved.

It is clear that Pelle is a component of a trimeric complex that associates with the active form of the transmembrane receptor Toll. Like all the mammalian Toll-like Receptors and the mammalian IL-1 Receptor, the cytoplasmic domain of *Drosophila* Toll contains a TIR domain. This TIR domain interacts with the *Drosophila* MyD88 homolog via a homotypic TIR:TIR interaction (Tauszig-Delamasure *et al.*, 2002; Charatsi *et al.*, 2003; Kambris *et al.*, 2003). MyD88 also contains a Death domain (DD), which interacts with the protein Tube. Through its other face, the Tube DD also interacts with Pelle (Towb *et al.*, 1998; Sun *et al.*, 2002; Sun *et al.*, 2004). This trimeric MyD88/Tube/Pelle complex is thought to interact transiently with the cytosolic TIR domain of Toll. This association likely leads to the auto-phosphorylation and activation of Pelle, which in turn leads, directly or indirectly, to the phosphorylation of Cactus.

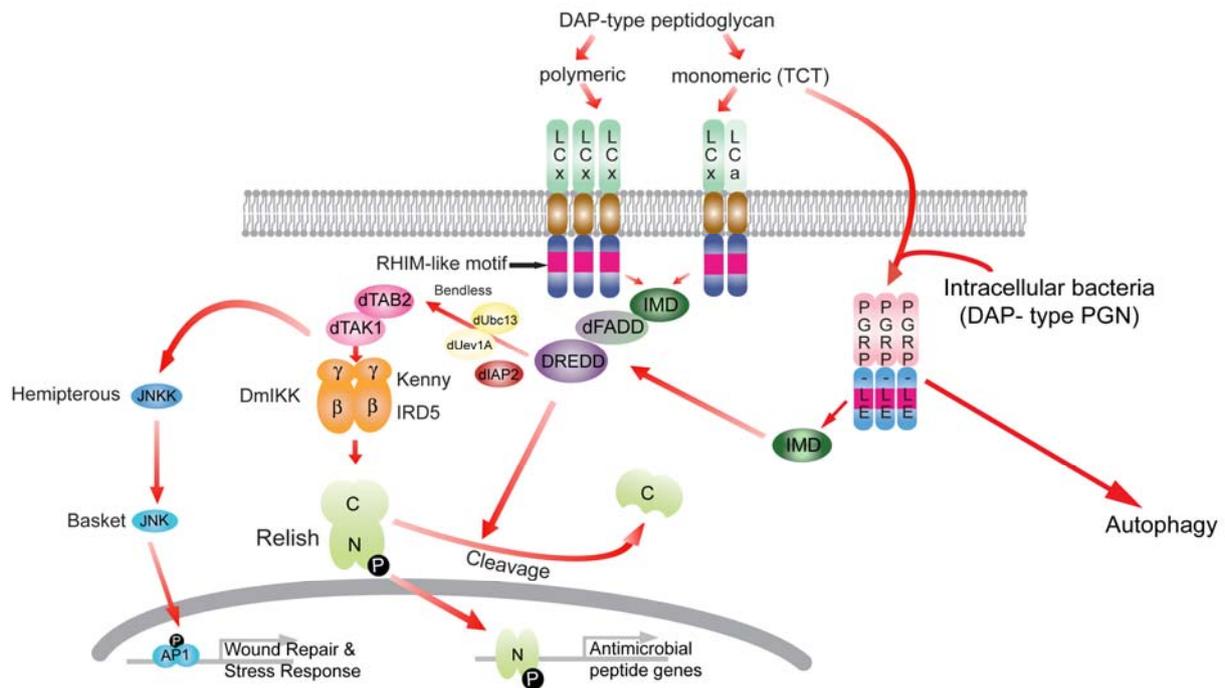
Once Cactus is degraded, the NF- $\kappa$ B factors DIF and Dorsal translocate to the nucleus where they control the transcription of target genes. In the adult fly, DIF is critical for the activation of AMP gene transcription (Meng *et al.*, 1999; Rutschmann *et al.*, 2000a; De Gregorio *et al.*, 2001, 2002; Irving *et al.*, 2001). In larvae, redundancy is observed between DIF and dorsal, and only a double mutant fails to induce AMP genes. In addition to controlling AMP gene expression, DIF and dorsal together also seem to regulate the survival of hemocytes in larvae (Qiu *et al.*, 1998; Matova and Anderson, 2006). Dorsal (and much of the rest of the Toll pathway) also plays a critical role in early embryonic development, in patterning the dorso-ventral axis. During development, phosphorylation of Dorsal is linked to enhanced nuclear localization and transcriptional activation (Drier *et al.*, 1999, 2000). DIF is not required in development but can partially substitute for Dorsal in this process when expressed in the embryo (Stein *et al.*, 1998). The role of phosphorylation of DIF, in the context of the immune response, has not been examined (for an overview of the Toll signaling pathway, see Fig. 1).

The IMD pathway culminates in the activation of a third NF- $\kappa$ B homolog, known as Relish. Like the mammalian NF- $\kappa$ B precursor proteins p100 or p105, Relish contains an N-terminal Rel Homology Domain and C-terminal I $\kappa$ B-like ankyrin repeats. In unstimulated cells, the C-terminal I $\kappa$ B-like domain is believed to hold full length Relish in the cytoplasm (Stöven *et al.*, 2000). After immune stimulation Relish is endoproteolytically cleaved, and the N-terminal transcription factor module translocates into the nucleus while the C-terminal I $\kappa$ B-like domain remains in the cytoplasm. In addition to this proteolytic cleavage, full activation of Relish also

requires phosphorylation on two residues, serines 528 and 529, (Erturk-Hasdemir *et al.*, 2009). This phosphorylation is not required for Relish cleavage, but instead seems to be crucial for the transcriptional activation of some Relish target genes. Another protein, known as Akirin, also functions in the nucleus for the induction of AMP gene expression, but how it interacts with Relish, if at all, is unclear (Goto *et al.*, 2008).

Relish cleavage and phosphorylation are controlled by two interconnected branches of the IMD signaling pathway. Serines 528 and 529 are directly phosphorylated by the *Drosophila* IKK complex. This kinase complex includes *Drosophila* IKK $\beta$  and IKK $\gamma$  homologs, also known as IRD5 and Kenny, respectively (Rutschmann *et al.*, 2000b; Silverman *et al.*, 2000; Lu *et al.*, 2001). IKK activation, in turn, requires the MAP3K TAK1 and its binding partner TAB2. Interestingly, both TAB2 and IKK $\gamma$  include conserved K63-polyubiquitin binding domains. These non-degradatory ubiquitin chains are thought to function in the IMD pathway, however the exact molecular mechanisms involved are not yet clear (Zhou *et al.*, 2005). The putative E3 ubiquitin ligase DIAP2 is also required in the IMD pathway and may promote K63-chain formation (Kleino *et al.*, 2005; Leulier *et al.*, 2006; Huh *et al.*, 2007). Further upstream, kinase activation also requires the *imd* protein, the *Drosophila* FADD homolog, and the caspase-8 like DREDD (Leulier *et al.*, 2000; Georgel *et al.*, 2001; Leulier *et al.*, 2002; Naitza *et al.*, 2002). These three proteins may form a trimeric complex as FADD can interact with both IMD and DREDD. How this complex signals the activation of TAK1 is still under investigation. In addition to a poorly defined role in the activation of TAK1, DREDD is also required for the cleavage of Relish. Interestingly, the IKK complex is also required for Relish cleavage, but its kinase activity is not involved in this function (Stöven *et al.*, 2003; Erturk-Hasdemir *et al.*, 2009). Instead, the IKK complex may function as a scaffold facilitating the cleavage of Relish by DREDD (for an overview of the IMD signaling pathway, see Fig. 2).

In addition to playing a key role in activation of the IKK complex and Relish, TAK1 also activates *Drosophila* JNK signaling through Hemipterous (MKK7) and Basket (JNK) (Sluss *et al.*, 1996; Holland *et al.*, 1997; Chen *et al.*, 2002). Thus, TAK1 plays a crucial role at the nexus of JNK and NF- $\kappa$ B signaling in this innate immune signaling pathway. NF- $\kappa$ B (Relish) plays a critical role in the induction of AMP genes; without Relish, no AMP gene expression is detected. However, the role of the JNK pathway in AMP regulation remains controversial. Several reports have argued that JNK signaling actually down-modulates AMP gene expression (Kim *et al.*, 2005, 2007), while another report has argued that JNK signaling is required for AMP induction (Delaney *et al.*, 2006). More studies are required to resolve these conflicting conclusions. In addition to the possible inhibitory activity of JNK on NF- $\kappa$ B-responsive AMP expression, the Relish branch of the pathway also seems to generate an inhibitor of JNK signaling (Park *et al.*, 2004). The mechanism by which Relish-induced gene products interfere with JNK



**Figure 2** IMD signaling pathway. This pathway is preferentially triggered by DAP-type peptidoglycan, common to Gram-negative bacteria and certain Gram-positives, especially *Bacillus spp.* DAP-type peptidoglycan can be recognized by different receptors, depending on its location and size. Large insoluble peptidoglycan is recognized on the cell surface by PGRP-LCx, while smaller peptidoglycan fragments, like TCT, can be recognized at the cell surface by a ligand-induced dimer of PGRP-LCx and PGRP-LCa. In addition, DAP-type peptidoglycan that reaches the cytosol can trigger another receptor, PGRP-LE. Both PGRP-LC and PGRP-LE trigger a similar intracellular signal transduction pathway, as outlined here, that culminates in the activation of the NF- $\kappa$ B precursor Relish. In addition, recognition of intracellular DAP-type peptidoglycan by PGRP-LE also triggers an autophagic response, which is important in the protection against intracellular bacteria.

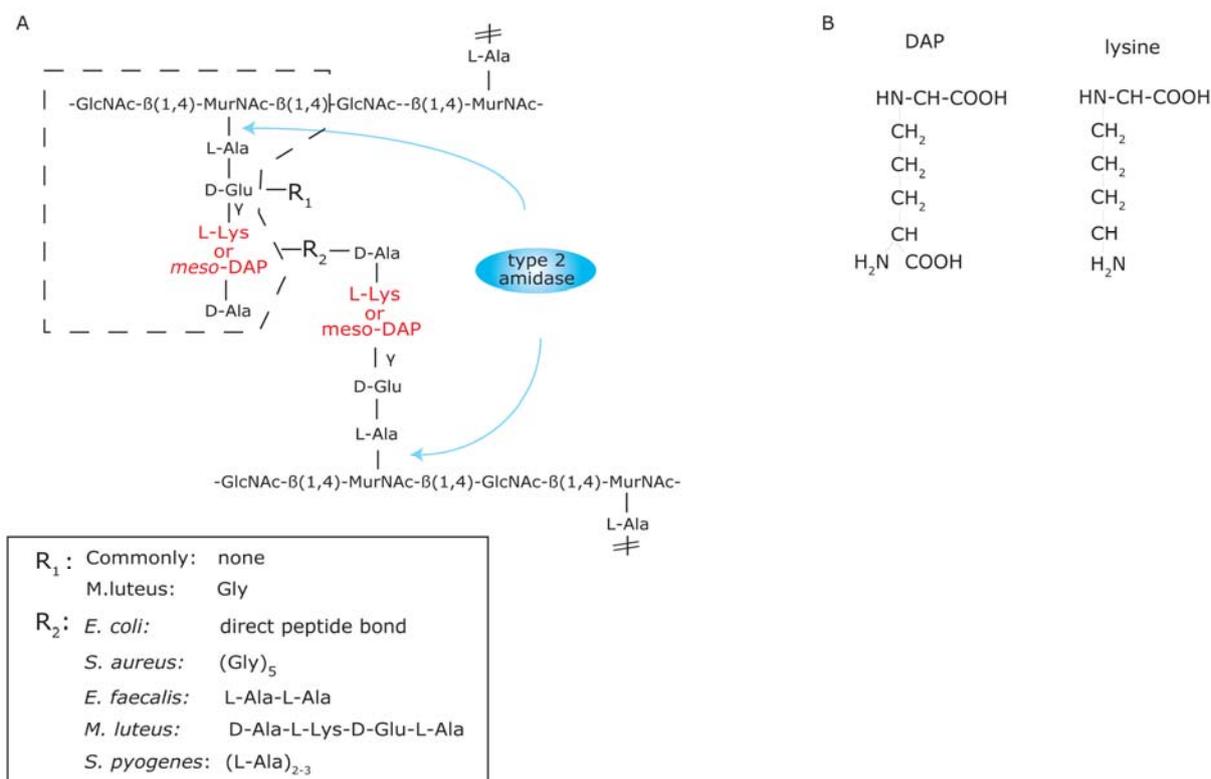
signaling has been suggested to involve the ubiquitin E3 ligase POSH. POSH is thought to target TAK1 for degradation (Tsuda *et al.*, 2005), however it is not clear how this would preferentially inhibit JNK but not Relish dependent responses. In addition to activating JNK and NF- $\kappa$ B/Relish signaling, the IMD pathway also induces the activation of the *Drosophila* p38 pathway, which requires *imd* protein but not TAK1 or any of the downstream components (Zhuang *et al.*, 2006). Little is known about p38 signaling in the *Drosophila* immune response, however it does appear to play a critical role in regulating the ROS generating enzyme DOUX in the gut, thereby controlling the intestinal microflora (Ha *et al.*, 2009).

### Microbial recognition

Both the Toll and IMD pathways are stimulated by bacterial peptidoglycans (Leulier *et al.*, 2003; Kaneko *et al.*, 2004). In addition, the Toll pathway can be triggered by  $\beta$ -glucans, from fungal cell wall, or by proteases directly released from pathogens (Gottar *et al.*, 2006; El Chamy *et al.*, 2008). Peptidoglycan (PGN) is the major structural component of the bacterial cell wall. PGN

structures display a great deal of diversity, varying widely among different classes of bacteria. However, all PGNs include a carbohydrate backbone, usually consisting of alternating N-acetyl-Glucosamine and N-acetyl-Muramic acid residues and short stem-peptides containing both L and D amino acids. These stem-peptides are often cross-linked to each other to stiffen the cell wall; the precise structure of these cross-linking structures is highly variable. The stem-peptides also display a great deal of variation in their amino acid constituents. The carbohydrate backbone is more constant but also can be modified by various chemical substitutions, such as acetylation (Schleifer and Kandler, 1972; Mengin-Lecreux and Lemaitre, 2005) (see Fig. 3 for a diagram of PGN structures).

Both the Toll and IMD pathways rely on peptidoglycan recognition proteins (PGRPs) for sensing PGNs (Table 1). This family of proteins is structurally similar to type 2 amidases (N-acetylmuramyl-L-alanine amidases), a class of enzymes that hydrolyze the bond between the lactyl group in acetylmuramic acid and L-alanine in the stem-peptide of PGN. In fact, some PGRPs are type 2 amidases, while others lack the catalytic cysteine



**Figure 3** Peptidoglycan structure. (A) As shown, peptidoglycan has a common core structure with a great deal of inherent variation. Most notably, the constituent of the third position of the stem-peptide can vary, and are most commonly L-lysine or meso-DAP. In addition, the amount and exact chemical nature of the crosslinking bridges can vary, with some examples noted in the box below. TCT is a monomeric unit of the DAP-type peptidoglycan chain, and is indicated in the dashed box. (B) Structures of lysine and DAP.

and instead function by binding PGN (Mellroth *et al.*, 2003). *Drosophila* encode for 13 PGRP genes, making about 17 distinct proteins through alternative splicing (Werner *et al.*, 2000). Six of the *Drosophila* PGRPs (PGRP-SB1, -SB2, SC1a/b, SC2, -LB) are known or predicted type 2 amidases, that are involved in degrading PGN and dampening immune activation (Mellroth *et al.*, 2003; Bischoff *et al.*, 2006; Zaidman-Remy *et al.*, 2006). The other seven lack type 2 amidase activity but function through binding PGN. In particular, 4 PGRPs (PGRP-SA, -SD, -LC, and LE) function as receptors in the IMD or Toll pathways, as detailed below. PGRP-LF seems to function as a decoy receptor, binding PGN but not activating immune signaling (Persson *et al.*, 2007; Maillet *et al.*, 2008), while the functions of PGRP-LA and -LD remain elusive (Royet and Dziarski, 2007).

Initial studies suggested that the IMD pathway was activated preferentially by Gram-negative bacteria, which lead many to assume that LPS, the most potent activator of the mammalian innate immune response, would be the main agonist of this pathway (Samakovlis *et al.*, 1990; Werner *et al.*, 2003). However, a careful analysis of published results suggested otherwise. In addition to Gram-negatives, certain Gram-positive bacteria, e.g.,

*Bacillus spp.*, were also IMD pathway activators (Lemaitre *et al.*, 1997). Subsequently, Lemaitre's group showed that diaminopimelic acid (DAP)-type PGN, from *Escherichia coli* or *Bacillus thuringiensis*, activated the IMD pathway, while the Silverman group demonstrated that purified LPS samples were unable to trigger the IMD pathway, and IMD agonistic activity could be traced to DAP-type PGN (Leulier *et al.*, 2003; Kaneko *et al.*, 2004). Lemaitre's group also showed that the Toll pathway was activated by PGN, but in this case lysine-type PGN from Gram-positives like *M. luteus* and *E. faecalis* was more potent.

Following these discoveries, a great deal has been learned about the molecular mechanisms involved in detecting various types of PGN. PGRP-SA and PGRP-SD are required for the recognition of PGN and the activation of Toll signaling (see below for more detail on the Toll pathway) (Michel *et al.*, 2001; Bischoff *et al.*, 2004), while in the IMD pathway either of two receptors, PGRP-LC or PGRP-LE, are capable of recognizing DAP-type PGN (Gottar *et al.*, 2002; Ramet *et al.*, 2002; Kaneko *et al.*, 2004, 2006; Takehana *et al.*, 2004; Choe *et al.*, 2005). PGN binding to either PGRP-LC or -LE triggers the IMD signaling pathway, described

**Table 1** Functions and specificity of the PGRPs retrieved in *D. melanogaster*

Name	Function	PGN Specificity
PGRP-SB1	Amidase	DAP- type PGN
PGRP-SB2	Amidase	DAP- type PGN*
PGRP-SC1a/b	Amidase	Lys/DAP- type PGN
PGRP-SC2	Amidase	DAP- type PGN*
PGRP-LB	Amidase	DAP- type PGN
PGRPSA SEML	Receptor for Toll Signaling	Lys- type PGN
PGRP-SD	Receptor for Toll Signaling	DAP- type PGN
PGRP-LC IRD7	Receptor for IMD Signaling	DAP- type PGN
PGRP-LE	Receptor for IMD Signaling	DAP- type PGN
PGRP-LF	Decoy receptor	DAP- type PGN
PGRP-LA	Unknown	DAP- type PGN*
PGRP-LD	Unknown	DAP- type PGN*

\* Predicted specificity, based on the presence of Arginine residue in key position for DAP recognition.

above, via a short conserved domain found in the N-terminus of both receptors (Kaneko *et al.*, 2006). This conserved signaling domain has some similarity to the RHIM domain found in the mammalian proteins RIP1, RIP3 and TRIF (Meylan *et al.*, 2004). However the molecular mechanisms involved in signaling by the PGRP-LC/LE RHIM-like domain still remain to be determined. Regardless of the mechanisms involved, activation of these receptors leads to activation of both Relish cleavage and Relish phosphorylation.

One key discovery, which enabled detailed molecular and biophysical analyses of PGRP-LC and PGRP-LE, was that a monomeric fragment of PGN from Gram-negative bacteria, known as Tracheal Cytotoxin (TCT), potently activates the IMD pathway (Kaneko *et al.*, 2004; Stenbak *et al.*, 2004). TCT is a disaccharide-tetrapeptide, featuring DAP at the third position of its stem-peptide, isolated from culture supernatants of *B. pertussis* (Fig. 3A). Synthetic lactyl-tetrapeptides (substructures of TCT) are able to serve as weak agonists of the IMD pathway only if they contain DAP at this third position, providing further demonstration that the DAP residue is key to triggering the IMD pathway (Kaneko *et al.*, 2004). PGRP-LC and PGRP-LE preferentially bind DAP containing PGN (Takehana *et al.*, 2002; Swaminathan *et al.*, 2006). The crystal structures of both PGRP-LC and PGRP-LE bound to TCT have been solved and show that a key arginine, Arg254 in PGRP-LE, provides the critical DAP-specific interaction (Chang *et al.*, 2006; Lim *et*

*al.*, 2006). Compared to lysine, DAP contains an additional carboxylate group (Figure 3B), and the guanidinium group of Arg254 forms a bidentate salt bridge with this moiety. In fact, this key arginine residue is common to all known DAP-type recognizing PGRPs and is found in the base of a deep cleft in which the PGN stem-peptide binds. In addition, DAP binding PGRPs also contain nearby glycine and tryptophan residues that are involved in other DAP-specific interactions (Swaminathan *et al.*, 2006).

PGRP-LC and PGRP-LE are found in different subcellular compartments and detect microbes in these distinct environments. PGRP-LC is a single pass, type 2 transmembrane receptor that is found primarily on the cell surface (Kaneko *et al.*, 2004; Mellroth *et al.*, 2005). Through alternative splicing, *PGRP-LC* encodes for 3 different receptors (PGRP-LCa, -LCx, and -LCy) each with an identical cytosolic domain but distinct extracellular ligand binding PGRP domains. On the other hand, *PGRP-LE* encodes for only one protein isoform, which lacks a transmembrane domain and functions as a cytosolic receptor (Werner *et al.*, 2000). While PGRP-LC is critical for recognizing extracellular bacteria (and extracellular PGN), PGRP-LE surveils the cytosol for intracellular bacteria and/or small fragments of PGN that enter cells (Kaneko *et al.*, 2006; Yano *et al.*, 2008). RNAi-based studies showed that the different splice isoforms of PGRP-LC are involved in recognizing different types of PGN. In particular, the recognition of polymeric

PGN, as isolated from *E. coli*, requires only PGRP-LCx. Moreover, *PGRP-LCx* mutants do not induce AMP genes in response to *E. coli* infection. However, the response to extracellular monomeric PGN (TCT) requires both PGRP-LCx and PGRP-LCa, while intracellular TCT triggers PGRP-LE.

The finding that polymeric and monomeric DAP-type PGNs trigger distinct PGRP-LC receptors was explained, in part, by the crystal structure of PGRP-LCa/x bound to TCT. As mentioned above, most PGRPs contain a deep cleft in which peptidoglycan fragments (sometimes referred to as muropeptides) bind. PGRP-LCa is the exception; it contains two unique dipeptide sequences that disrupt the PGN binding cleft and occlude PGN binding. Conversely, PGRP-LCx has a typical DAP-type PGN binding cleft and can avidly bind TCT or polymeric PGN. Upon binding TCT, PGRP-LCx and PGRP-LCa heterodimerize (Chang *et al.*, 2005; Mellroth *et al.*, 2005). The crystal structure of this ligand bound dimeric complex shows that the carbohydrate portion of TCT makes key contributions to the dimerization interface, providing a clear explanation for the TCT-induced dimerization (Chang *et al.*, 2006). It is reasonable to hypothesize that the ligand-induced heterodimerization of PGRP-LCa and PGRP-LCx is critical for activation of downstream signaling, although this has not yet been demonstrated. This model of dimerization-induced signaling does not explain how PGRP-LCx alone is sufficient for IMD signaling triggered by polymeric PGN. The crystal structures suggest that PGRP-LCx is unlikely to form homo-multimers upon binding polymeric PGN, because of a steric clash at the putative dimerization interface. Therefore, a distinct model must be proposed for signaling by polymeric PGN and PGRP-LCx. In this case, the ligand is polyvalent and likely binds to multiple individual PGRP-LCx receptors, perhaps creating a 'cluster' of receptors, thereby generating a density of PGRP-LCx cytosolic domains. This clustering, *per se*, may be sufficient to activate signal transduction, or perhaps the intracellular domains actually form higher order protein-protein interactions while the extracellular domains remain clustered on one large fragment of PGN, but not in direct contact with each other. Future studies are required to examine these possibilities.

Like the PGRP-LCa/x heterodimers, PGRP-LE also multimerizes upon binding TCT. However, the PGRP-LE-TCT complex forms very high order multimers in a 'head to tail' fashion. Like the PGRP-LC structures, the PGRP-LE structure was solved with the isolated PGRP domain, and we cannot be certain what quaternary structure the holo-receptor forms upon TCT binding. However, these biophysical studies clearly demonstrate that TCT causes PGRP-LE to multimerize into large complexes (Lim *et al.*, 2006). As mentioned above, PGRP-LE detects DAP-type PGN that enters the cytosol. PGN may enter the cytosol from infection with intracellular bacteria, like *Listeria monocytogenes*, or small PGN fragments, such as TCT, appear to directly enter into cells. Upon binding these PGNs, PGRP-LE likely forms higher order multimers and triggers IMD signaling. In addition, PGRP-LE activation can also induce an

autophagic response that helps protect against intracellular microbes (Yano *et al.*, 2008). The role of ligand-induced receptor multimerization in the activation of IMD signaling pathway, via PGRP-LCs and PGRP-LE, requires further study. As mentioned above, these receptors signal through a RHIM-like domain in their N-terminal domains. The mechanism by which the RHIM-like domain functions in the context of dimerized, multimerized or clustered PGRP receptors is unknown.

### Toll activation by PGN and beyond

Unlike the IMD pathway, which is activated in a fairly specific manner by DAP-type PGN, Toll activation occurs *indirectly* by a wider array of immune stimuli. Toll functions more like a cytokine receptor, binding a processed form of the cytokine Spätzle, a member of the cysteine knot family of growth factors and cytokines (Weber *et al.*, 2003; Hu *et al.*, 2004; Hoffmann *et al.*, 2008). Spätzle is made as a pro-protein that is found circulating in the hemolymph. Upon immune activation (or developmental cues), serine protease cascades are triggered that culminate in the cleavage of Spätzle. Once processed, mature Spätzle binds to and dimerizes the transmembrane receptor Toll, initiating the intracellular signaling pathway described above. Four different serine protease cascades appear to converge on the cleavage of Spätzle. In early development, the protease Easter is responsible for cleaving Spätzle. During the immune response, bacterial PGN, fungal  $\beta$ -glucans, and microbial proteases are sensed by three distinct mechanisms, but converge upon activation of one serine protease, known as the Spätzle processing enzyme (SPE), which in turn cleaves and activates Spätzle (Fig. 1).

The serine protease Persephone appears to function as a sensor for proteases secreted by both fungal and bacterial pathogens (Gottar *et al.*, 2006; El Chamy *et al.*, 2008). Persephone is likely activated by cleavage after a histidine residue, unlike the other proteases involved in the Toll signaling pathways, and maybe a good target for subtilisin-like proteases produced by microbial pathogens (El Chamy *et al.*, 2008). The activation of the Persephone-Toll pathway by microbial proteases occurs independently of recognition of microbial cell wall material, which can also stimulate the Toll pathway through more classical receptor-mediated recognition.

For example,  $\beta$ -glucans from the cell wall of yeast are recognized by Gram-negative binding protein 3 (GNBP3) (Gottar *et al.*, 2006), while two secreted PGRP receptors and GNBP1 are involved together in PGN recognition (Gobert *et al.*, 2003; Bischoff *et al.*, 2004; Wang *et al.*, 2006, 2008). [Despite their name, none of the GNBP3s have been linked to the response to Gram-negative bacteria, but GNBP1 and GNBP3 are involved in the recognition of fungal or Gram-positive bacterial cell walls]. The N-terminus of GNBP3 binds to long  $\beta$ -1,3 glucans, common to the cell walls of many types of fungi, especially yeast (Mishima *et al.*, 2009). GNBP1, on the other hand, is involved in PGN recognition, although its role is controversial.

Lysine-type PGNs, common to many Gram-positive bacteria, are potent agonist of the Toll pathway. As in the IMD pathway, PGRP receptors are critical for the recognition of lysine-type PGN. In particular, two secreted PGRPs, PGRP-SA and -SD, are involved in the Toll pathway. Genetic studies have shown that some Gram-positive bacteria and the PGN from these same species, such as *M. luteus*, are sensed through PGRP-SA (Michel *et al.*, 2001). In fact, the structure of PGRP-SA bound to a lysine-containing muropeptide has been solved. PGRP-SA can also bind DAP-type PGN, albeit to a lesser degree. However, PGRP-SA appears to be able to specifically cleave DAP-type muropeptides, removing the final amino acid in the stem-peptide. It has been postulated that this carboxypeptidase activity prevents DAP-type PGN from stimulating the Toll pathway via PGRP-SA (Chang *et al.*, 2004).

Interestingly, not all lysine-type PGN requires PGRP-SA to trigger the Toll pathway. In particular, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, and *Staphylococcus saprophyticus* infections still produce strong AMP gene responses in PGRP-SA mutant (*semi*) flies. Response to these bacteria or their PGNs instead requires either PGRP-SA or PGRP-SD (Bischoff *et al.*, 2004). The mechanism of PGRP-SD-mediated recognition of some, but not all, lysine-type PGN producing bacteria remains unclear. One possibility is a structural difference common to those PGNs sensed by PGRP-SD, which prevents detection by PGRP-SA, or vice versa. However, biochemical studies of PGRP-SD do not support the notion that it is involved in recognizing lysine-type PGN. Crystallographic studies show that PGRP-SD has a deep PGN binding cleft, typical of all PGRPs, and this binding cleft includes an arginine (Arg90) in the key position typical of DAP-PGN specific recognition. In fact, binding studies confirm that PGRP-SD binds DAP-type PGN, from *B. subtilis*, but not Lys-type from *S. aureus* (Leone *et al.*, 2008). In addition, the moderate induction of *drosomycin* observed following either *B. subtilis* or *E. coli* infection, which is Toll dependent (Leulier *et al.*, 2003), required both PGRP-SA and PGRP-SD. So, somehow PGRP-SA and PGRP-SD function together in the recognition of DAP-type PGN, for moderate Toll induction, but function in a more redundant manner for the recognition of certain Lys-type PGN, for robust Toll induction. However, the recognition of *M. luteus* PGN appears to more simply require only PGRP-SA. The molecular mechanisms involved in the recognition of various PGNs by PGRP-SA and/or PGRP-SD remain to be determined.

As mentioned above, GGBP1 also functions in PGN recognition and Toll signaling. In fact, PGRP-SA, -SD, and GGBP1 form a trimeric complex together in the presence of PGN fragments (Wang *et al.*, 2008). Some groups have reported that GGBP1 provides a critical PGN processing activity to this complex, required to generate small PGN fragments which are bound by PGRP-SA and/or PGRP-SD for Toll activation (Filipe *et al.*, 2005; Wang *et al.*, 2006). However, another group has reported that they do not observe a similar PGN digesting activity associated with GGBP1, in

*Drosophila* or *Tenebrio molitor* (Buchon *et al.*, 2009). Instead, this group proposes that GGBP1 serves to link the PGRPs with the downstream serine protease cascade, described below. Thus, it appears that GGBP1 functions in a complex with the PGN sensing receptors PGRP-SA and PGRP-SD, but the biochemical mechanism by which it contributes to immune recognition or Toll signaling are not yet clear.

Both the GGBP3-mediated recognition of  $\beta$ -glucans and the PGRP-SA/SD/GGBP1-mediated recognition of bacterial PGNs trigger Toll signaling through the same serine protease cascade. This cascade involves the modular serine protease (ModSP), which is probably directly activated by these microbial sensing receptor complexes, and at least two downstream CLIP-domain serine proteases - Grass and SPE. As mentioned above, SPE cleaves and thereby activates Spätzle, the ligand for Toll. Another protease, known as Spirit may function between Grass and SPE, and other non-catalytic serine-protease homologs, Sphinx 1/2 and Spheroid, were also implicated this pathway by RNAi based studies (Kambris *et al.*, 2006). However, the assignment of these factors to this pathway requires further genetic and biochemical characterization. The pathways presented in Fig. 1 suggest a protease cascade that is consistent with the genetic analysis of mutants in *Drosophila* and the biochemical analysis of the cascade from the hemolymph of *Tenebrio*. However, biochemistry of the *Drosophila* serine protease cascade still requires further study, as several issues remain unresolved, including the role of Spirit. In addition, the predicted specificity of the *Drosophila* ModSP does not match the predicted cleavage site of the downstream serine protease Grass, and ModSP does not cleave Grass *in vitro* (Buchon *et al.*, 2009). Thus, it remains possible that other factors may be involved. Currently, it is not clear how (or even if) PGN binding to PGRP-SA/SD/GGBP1 leads to the activation of ModSP, and, as mentioned above the exact biochemical role of GGBP1 remains controversial.

## Concluding remarks

The goal of this review is to summarize recent work on the Toll and IMD pathways, two important innate immune signaling pathways in *Drosophila*, with an emphasis on the molecular mechanisms involved in microbial recognition in this model system. This review is not meant to be a comprehensive analysis of all aspect of the insect immune response. Notably, many very exciting studies have been published recently on phagocytosis, anti-viral immunity, melanization and clotting - all topics not covered here. Instead, significant detail is presented on the biochemistry and genetics of bacterial recognition by the PGRP family of innate immune receptors. Over the past 5 years, much progress been made in this area, and the basic underpinnings of how bacteria are recognized by PGRP receptors, which bind the bacterial cell-wall derived compound peptidoglycan, has been resolved. In addition, the preferential binding of DAP-type PGN by some of these

receptors, notably PGRP-LC and PGRP-LE, provides a firm explanation on the specific activation of the IMD pathway by Gram-negative and certain Gram-positive bacteria. However, the specificity, or lack thereof, in activating the Toll pathway by different types of peptidoglycans still requires further investigation. Moreover, in both IMD and Toll signaling, it remains unclear how peptidoglycan binding by these PGRP receptors leads to the activation of downstream signaling events. These questions will be the focus of future study.

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