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Review

The peptidoglycan recognition proteins LCa and LCx

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Abstract. Infection of bacteria triggers innate immune defense reactions in *Drosophila*. So far, the only bacterial component known to be recognized by the insect innate immune system is peptidoglycan, one of the most abundant constituents of the bacterial cell wall. Insects use peptidoglycan recognition proteins to detect peptidoglycan and to activate innate immune responses. Such specialized peptidoglycan receptors appear to have evolved from phage enzymes that hydrolyze bacterial cell walls. They are able to bind specific peptidoglycan molecules with distinct chemical moieties and activate innate immune pathways by interacting with other signaling proteins. Recent X-ray crystallographic studies of the peptidoglycan recognition proteins LCa, and LCx bound to peptidoglycan have provided structural insights into recognition of peptidoglycan and activation of innate immunity in insects.

Keywords. PGRP-LCa, PGRP-LCx, peptidoglycan, insect innate immunity.

Peptidoglycan recognition proteins and insect innate immunity

A peptidoglycan recognition protein (PGRP) of 173 amino acids was first isolated and cloned from hemolymph, or insect blood, of the silkworm *Bombyx mori* in a quest to identify molecules that were able to trigger the prophenoloxidase cascade upon binding to peptidoglycan [1]. Sequence analysis using completed genome databases of multiple organisms later concluded that PGRP genes are present in both insects and vertebrates. Insect PGRP genes outnumber their mammalian homologues, which reflects well the current understanding that PGRPs are responsible for more diverse and essential tasks in insect immunity than in the mammalian immune system. Recent comprehensive reviews of insect and mammalian PGRPs can be found elsewhere [2, 3].

PGRPs constitute a diversified family of proteins which are expressed as either secreted factors, intracellular proteins, or transmembrane receptors. Thirteen PGRP genes are present in the *Drosophila* genome and encode at least 17 proteins via alternative splicing [4]. Despite large differences in size, ranging from 200 to 600 amino acids, and in the domain organization of their primary sequences, PGRPs contain at least one conserved domain of 165 amino acids termed the PGRP domain, which shares sequence similarity to the lysozyme of bacteriophage T7 [1, 4–7]. T7 lysozyme is a single-domain protein with dual activities: it is both a zinc-coordinating amidase that hydrolyzes Gram-negative peptidoglycan (discussed below) and a T7 transcriptional regulator that

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binds and inhibits T7 RNA polymerase [8]. Given that two drastically different functions are accommodated in T7 lysozyme, it is perhaps not too surprising to also find various activities in PGRPs. For example, some PGRPs have retained an enzymatic activity within the PGRP domains to hydrolyze peptidoglycan [9, 10], whereas other PGRPs have lost catalytic residues and serve only as peptidoglycan receptors and are involved in protein-protein interactions via their PGRP domains [11–13]. As will be described later, T7 lysozyme and some PGRPs even use conserved structural elements for interacting with other proteins. Insects mount powerful innate immune defense reactions, which include synthesis of antimicrobial peptides, in response to bacterial infections. Genetics experiments using *Drosophila* as a model system have revealed diverse and essential roles for members of the PGRP protein family in modulating and activating the Toll and immune deficiency (Imd) pathways, which are the dual signaling pathways that control the expression of antimicrobial peptide genes in insects. For example, PGRP-SA and the PGRP-LCa/LCx complex are *bona fide* pattern-recognition receptors involved in recognition of Gram-positive and Gram-negative peptidoglycans, respectively. Upon binding to specific peptidoglycan ligands, PGRP-SA activates the Toll pathway, while the PGRP-LCa/LCx receptor complex triggers the activation of the Imd pathway [14, 15]. PGRP-SD is also involved in Toll activation by certain Gram-positive bacteria such as *Staphylococcus aureus* [16]. PGRP-LE has recently been demonstrated to be an intracellular receptor which can detect Gram-negative peptidoglycan and activate the Imd pathway [17]. Moreover, a fragment of PGRP-LE containing only the PGRP domain, which is secreted into the hemolymph, also functions as a soluble receptor to enhance PGRP-LC-mediated Imd signaling [17, 18]. PGRP-SC1, SC2, and LB are known to exhibit amidase activity for Gram-negative peptidoglycan. Functional studies have suggested that these amidase PGRPs serve to downregulate the Imd pathway, which is thought to modulate the level of the host immune response to bacterial infections [19, 20].

Peptidoglycan

Peptidoglycan is an essential polymer uniquely present in Gram-positive and Gram-negative bacterial cell walls. It is composed of long glycan chains made of alternating *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc) and cross-linked by stem peptides of four or five alternating L and D amino acids. For example, in *Escherichia coli*, the repeating subunit of peptidoglycan, known as muropeptide, has a sequence of GlcNAc-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala. While the glycan chain is rather conserved among different bacterial species, the composition of the stem peptides shows considerable variation. Notably, the third amino acid of the stem peptide is a lysine in most Gram-positive peptidoglycans but a diaminopimelic acid (DAP) in Gram-negative and most rod-shaped Gram-positive bacteria. In addition, the α-carboxyl group of γ-D-Glu and the ε-carboxyl group of DAP are often amidated in some Gram-negatives but not *E. coli* [21]. In Gram-negative peptidoglycan, the ε-amino group of DAP is directly cross-linked, via a peptide bond, to the carboxyl group of D-Ala at the fourth position of another stem peptide. However, the ε-amino group of Lys in Gram-positive peptidoglycan is cross-linked to D-Ala via additional interpeptides whose sequences differ from one organism to another. Free muropeptides are naturally present in bacterial cells because they are constantly synthesized *de novo* or released from peptidoglycan during the remodeling of the polymer during cell growth and division; these monomeric forms of peptidoglycan are then recycled in such a way that the sugar and peptide components are reincorporated back into polymeric peptidoglycan [21]. *Bordetella*, a Gram-negative bacterium, lacks a functional cell wall recycling component (the permease AmpG). As a result, a muropeptide fragment GlcNAc-1,6-anhydro-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala (Fig. 1) is released from growing bacterial cells. This muropeptide is known as tracheal cytotoxin, or TCT, based on the fact that it is a virulence factor responsible for epithelial cytopathology associated with pertussis infection [22].

PGRP-LCa and LCx are membrane receptors of peptidoglycan and activate the Imd pathway

The 13 *Drosophila* PGRPs have been subdivided into two groups based on the length of their RNA transcript [4]. PGRP-SA, SB1, SB2, SC1a, SC1b, SC2, and SD are the 7 short-form PGRPs of ~200 amino acids containing a single PGRP domain. Short-form PGRPs are all secreted proteins circulating in the insect hemolymph. PGRP-LA, LB, LC, LD, LE, and LF are the 6 long-form PGRPs, which contain one PGRP domain (or likely two PGRP domains for LF) plus additional polypeptide sequences of up to 400 amino acids; the structural folds of these non-PGRP regions have not yet been studied. In contrast to the short forms, long-form PGRPs exhibit a wide subcellular distribution. For example, PGRP-LB is a secreted protein [20]. Full-length PGRP-LE has recently been demonstrated to be an intracellular protein; in

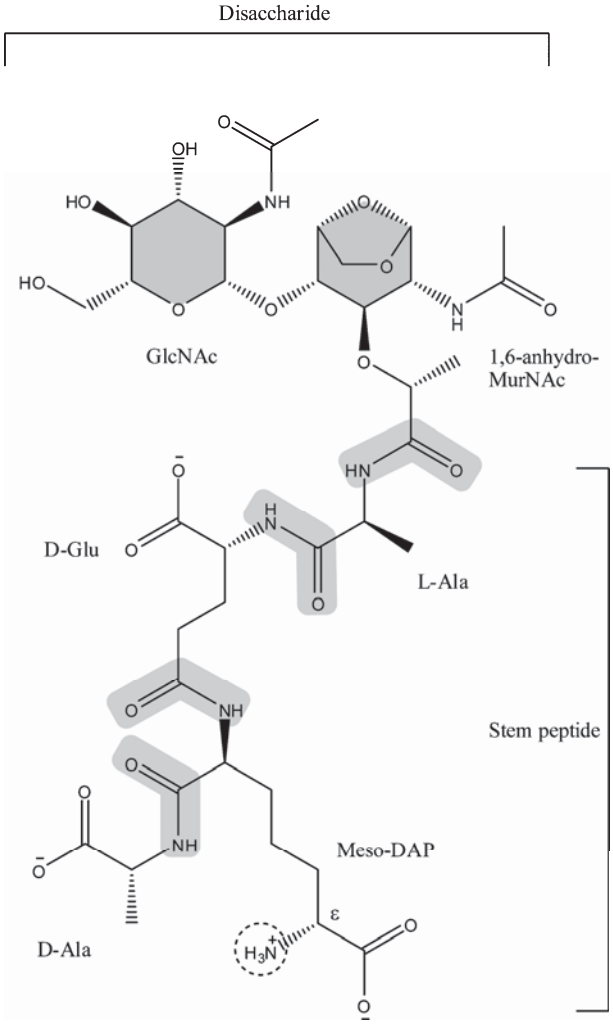

Figure 1. Chemical structure diagram of a DAP-type monomeric muropeptide, also known as tracheal cytotoxin (TCT). Glycan rings and peptide bonds are shaded. Note the anhydro bond between C1 and C6 of MurNAc, which is only made in the monomeric form and in the glycan chain termini in the polymeric form. Also note that DAP, the third residue of the stem peptide, has a carboxyl group on Cε. In lysine, the residue that occurs at the third position in most Gram-positive peptidoglycans, a hydrogen atom is attached on Cε instead. Also indicated is the ε-amino group of DAP, which would be cross-linked, via a peptide bond, to the carboxyl group of D-Ala from another stem peptide.

addition, a truncated form containing only the PGRP domain (PGRP-LE^{pg}) is secreted into hemolymph [17]. Finally, PGRP-LC is a transmembrane receptor [14, 23].

Through alternative splicing, the *PGRP-LC* gene encodes three isoforms, LCa, LCx, and LCy, which have identical intracellular domains, but unique PGRP-like ectodomains with less than 40% sequence identity [4]. Expression of PGRP-LC is required to allow activation of the *Drosophila* Imd pathway by Gram-negative bacteria [23–26], which triggers the proteolytic processing of an NF-κB-related protein Relish, which in turn controls the expression of several antibacterial peptide genes including *Diptericin* [27, 28]. Both polymeric and monomeric forms of Gram-negative DAP-type peptidoglycan are potent ligands of the Imd pathway [14, 29]. However, gene knockdown experiments using cell culture showed that recognition of polymeric peptidoglycan only requires PGRP-LCx, and that monomeric peptidoglycan recognition requires both PGRP-LCa and LCx [14]. Later, it was found that PGRP-LCx exhibited strong affinity for both polymeric peptidoglycan and monomeric peptidoglycan; however, PGRP-LCa had no affinity for either polymeric or monomeric peptidoglycan. Rather, PGRP-LCa was able to bind LCx when the latter was bound to monomeric peptidoglycan [12, 13].

The ectodomain of PGRP-LCa

PGRP-LCa possesses a PGRP-like ectodomain with a polypeptide sequence that is unique among all *Drosophila* PGRP domains [12]. The LCa ectodomain contains two dipeptide insertions, which have a profound effect on its peptidoglycan-binding ability. Before the crystal structure of the LCa ectodomain was solved, the three-dimensional structures of *Drosophila* PGRP-LB, PGRP-SA, human PGRP-IαC and PGRP-S were known; these PGRP structures all contain a similar L-shaped surface groove that was proposed to be the peptidoglycan-docking groove [10, 30–32]. Soaking of the PGRP-IαC crystal with a synthetic muropeptide analogue allowed determination of the structure of a ligand-bound PGRP, which showed that the muropeptide adopted an extended conformation and, indeed, bound to the proposed peptidoglycan-docking groove [33]. The molecular surface of the LCa ectodomain does not contain the canonical peptidoglycan-docking groove seen in all other known PGRP structures. Instead, the two dipeptide insertions of LCa effectively remodel the structural elements that would have formed the two walls lining the surface groove by inducing two short α helices not seen in other PGRPs, and create three barricade residues that disrupt the docking groove [12] (Fig. 2). Therefore, the lack of an L-shaped surface groove in the LCa ectodomain revealed by crystallographic data explains why PGRP-LCa has no affinity for peptidoglycan.

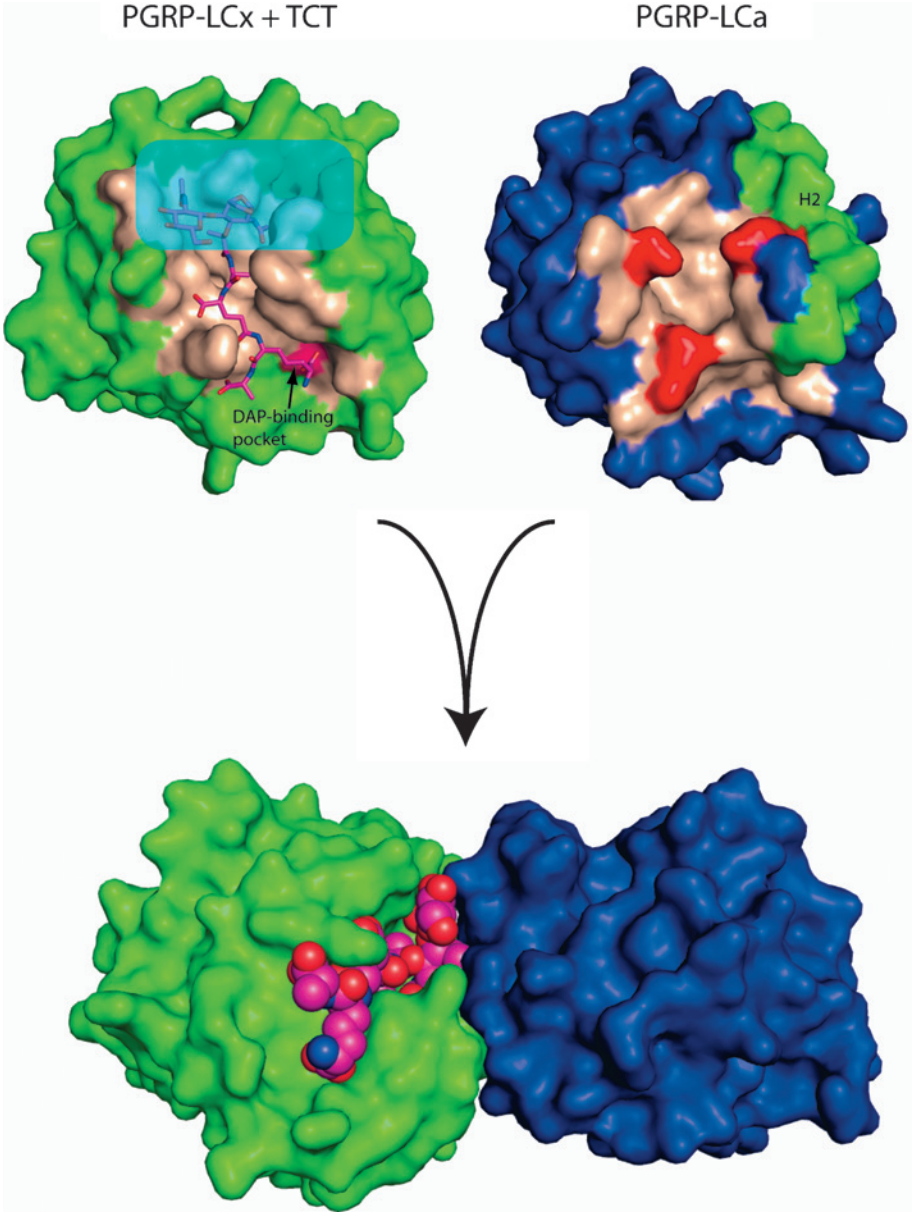

Figure 2. Surface representation of the ectodomains of PGRP-LCa and LCx and the TCT-induced heterodimerization of the LCa and LCx ectodomains. TCT is shown as both sticks (upper) and spheres (lower). The peptidoglycan-docking groove in LCx (green) and the corresponding surface region in LCa (blue) are painted in wheat. In the TCT-bound LCx ectodomain, the surface for interaction with LCa is shaded in light blue. The DAP-binding pocket in LCx is highlighted in purple. The surface groove of the LCa ectodomain is disrupted by three barricade residues highlighted in red and cannot bind peptidoglycan by itself. However, through primarily its H2 helix (indicated), LCa binds to the LCx ectodomain when the latter is bound to TCT, which is a DAP-type monomeric muropeptide, thereby forming a ternary LCa-TCT-LCx complex.

Monomeric peptidoglycan bridges PGRP-LCa and LCx receptors together

Functional analyses of PGRP-LCa and LCx have suggested that peptidoglycan-induced dimerization and/or multimerization of these receptors is essential for establishing Imd signaling [14, 23]. Recognition of polymeric peptidoglycan only requires PGRP-LCx to trigger Imd signaling; therefore, it is thought that, in order to activate signal transduction, multiple LCx receptors have to be brought into close proximity by binding to the repeating muropeptide subunits. Both PGRP-LCa and LCx, on the other hand, are required to recognize monomeric peptidoglycan; biochemical pull-down assay results have suggested that the LCa ectodomain forms a complex with the LCx ectodomain when the latter is bound to the ligand [12, 13]. But how do PGRP-LCa and LCx work together to recognize monomeric peptidoglycan, given that LCa lacks a canonical peptidoglycan-docking groove conserved in other PGRPs? The recent crystal structure of a naturally occurring muropeptide ligand bound to the LCa and LCx ectodomain complex provided a clue to the answer. The ternary complex structure showed that the muropeptide was bound to the docking groove on LCx primarily through its elongated stem peptide; the unique disaccharide GlcNAc-MurNAc(anhydro) residues of the ligand are then presented by LCx and make critical interaction with the LCa ectodomain. The LCa ectodomain interacts with the

GlcNAc-MurNAc(anhydro) moiety of the LCx-bound ligand mainly through its H2 helix (Fig. 2), a structural element which is also present in T7 lysozyme. Interestingly, T7 lysozyme also interacts with T7 RNA polymerase primarily through a similar helix [34], suggesting an evolutionarily conserved role for this helical element of T7 lysozyme and PGRPs in mediating protein-protein interaction.

DAP-binding pocket

All peptidoglycans in bacteria share the same glycan chain. However, the stem peptides linked to the carbohydrate backbone vary, depending on bacterial species, in the amino acid composition at the third position. In most Gram-positive bacteria, the third amino acid is L-Lys. In all Gram-negative bacteria and most rod-shaped Gram-positive bacteria, the third residue is *meso*-DAP, which differs from Lys only by the presence of a carboxyl group on the Cε with D chirality. Several PGRPs have been shown to bind to or digest specific peptidoglycan ligands with apparent selectivity [10, 13, 17, 20, 35, 36]. Therefore, the third amino acid of the peptidoglycan stem peptide is thought to be a major specificity determinant for recognition by PGRPs. Indeed, the crystal structure of TCT-bound PGRP-LCx, as well as a recent structure of TCT-bound PGRP-LE, have revealed that the ε-carboxyl group of the *meso*-DAP residue is engaged in a key electrostatic interaction with the guanidinium side chain of an Arg residue (Fig. 2) [11, 37]. This Arg residue, located within a small surface pocket in PGRPs, is conserved in *Drosophila* PGRP-SB1, SC1/2, SD, LA, LB, LC/a/x/y, LE, and LF. The presence of the DAP-binding pocket in most of these PGRPs is consistent with their preferred recognition of DAP-type peptidoglycan (no functional or binding data are available for LA and LF). However, PGRP-SD, which possesses a DAP-binding pocket, appears to recognize Lys-type peptidoglycan based on functional assay results [16]. We hypothesize that all PGRPs with a DAP-binding pocket are likely to bind DAP-type peptidoglycan *in vitro*. However, for some PGRPs such as PGRP-SD, the significance of such binding *in vivo* may not be uncovered yet by functional/genetics assays. On the other hand, although the positively charged Arg within the DAP-binding pocket would prevent it from interacting with monomeric lysine-type peptidoglycan due to electrostatic repulsion, a PGRP with the DAP-binding pocket may still be able to bind polymeric lysine-type peptidoglycan because the ε-amino group of Lys in the polymeric form will not retain its basic charge due to its peptide linkage to the carboxyl group of D-Ala of another stem peptide [11]. PGRP-SA and human PGRP-IαC have been demonstrated to bind only lysine-type peptidoglycan. Accordingly, they lack the DAP-interacting Arg residue; the corresponding residues in SA and IαC are threonine and valine, respectively.

Could PGRP-LCa and LCx be cross-linked by PGRP-LE^{pg} in a TCT-dependent manner ?

Recently, PGRP-LE has been demonstrated to be an intracellular receptor for TCT. Furthermore, PGRP-LE^{pg}, a truncated version of PGRP-LE containing only the PGRP domain, was found to be expressed extracellularly and acted to enhance PGRP-LCa/LCx-mediated TCT recognition on the cell surface [17]. Interestingly, the cocrystal structure of PGRP-LE^{pg} complexed with TCT showed formation of an infinite array of PGRP-LE^{pg} dimers induced by the peptidoglycan ligand (Fig. 3) [37]. In the structure, TCT is bound by the two PGRP-LE^{pg} molecules in a very similar way that the same ligand mediates the association of the LCa and LCx ectodomains [11]. The major difference lies in the fact that the LCa ectodomain does not bind TCT, therefore perpetuation of LCa-TCT-LCx interaction is not possible. By contrast, not only does each PGRP-LE^{pg} bind TCT via the peptidoglycan-docking groove, but it can also interact, via its LCa-like H2 helix, with another LE molecule that has already loaded with TCT, to form an infinite array of LE-TCT-LE-TCT multimers. Because both LCa and LE share similar H2 helix residues that are able to mediate protein-protein interaction [37], it appears possible that LCa may be able to associate with TCT-loaded LE^{pg}, in a way similar to its complex with TCT-loaded LCx. Likewise, LE^{pg} may also be able to interact with TCT-bound LCx. Hence, we hypothesize that PGRP-LE^{pg} may enhance LCa-TCT-LCx-mediated Imd signaling by inducing more cross-linked LCa-LCx and LCx-LCx receptors, which may be too far away from each other to be linked together by TCT, through the TCT-PGRP-LE^{pg} array bridge (Fig. 3). The validity of this model awaits testing by future biochemical and functional assays.

Conclusions

The structural studies of PGRP-LC and LE in complex with TCT have provided insights into activation of the Imd pathway by monomeric and polymeric peptidoglycan ligands. Also, the structural

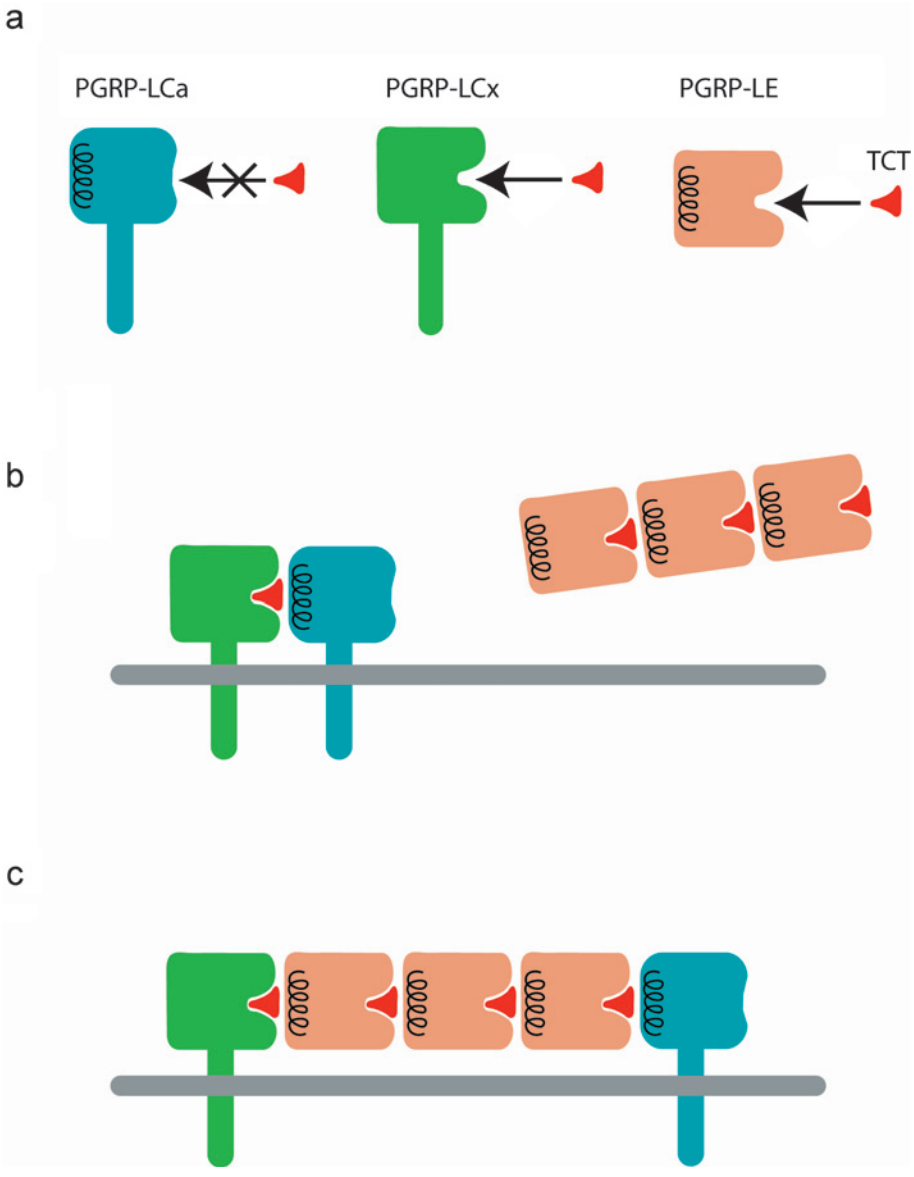

Figure 3. Schematic illustration of monomeric peptidoglycan (ex. TCT) recognition by PGRP-LCa, LCx, and LE^{pg}. (*a*) PGRP-LCa lacks a peptidoglycan-docking groove and cannot bind TCT. However, its H2 helix (indicated as a coil) can interact with PGRP-LCx when the latter is docked with a peptidoglycan ligand. PGRP-LE^{pg} not only contains a peptidoglycan-docking groove, but also an LCa-like H2 helix that can interact with another TCT-bound PGRP-LE^{pg}. (*b*) Therefore, binding of TCT induces heterodimerization of PGRP-LCa and LCx. TCT also induces infinite head-to-tail homodimerization of PGRP-LE^{pg}. Here only an array of triple dimers is shown. (*c*) A hypothetical model of how PGRP-LE^{pg} enhances TCT-triggered Imd signaling by cross-linking more PGRP-LCaLCx receptor complexes.

data have revealed the structural basis of discrimination of Lys versus DAP-type peptidoglycan by PGRPs. Recent data have indicated that the *Anopheles* Imd pathway regulates malaria parasite infection [38] and therefore may have medical implications. Sequence analysis showed that *Drosophila* PGRP-LC and its *Anopheles* orthologue share similar architectures; however, their PGRP domains appear to have evolved independently [39] and thus might exhibit different recognition properties. It is interesting to note that *Anopheles* PGRP-LC receptors appear to contain DAP-binding pockets as well. *Drosophila* PGRP-LE has no *Anopheles* orthologue; it would be interesting to find out whether any of the *Anopheles* PGRP-LC proteins carries out the functionality of PGRP-LE in *Drosophila*. Future X-ray structural and functional studies of *Anopheles* PGRP-LC receptors with their ligands may aid the discovery of potent agonists that can disrupt the *Plasmodium* life cycle by preventing its infection in *Anopheles* through enhanced Imd signaling.

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