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Peptidoglycan Molecular Requirements Allowing Detection by the *Drosophila* Immune Deficiency Pathway

Carolyn R. Stenbak,†* Ji-Hwan Ryu,‡* François Leulier,§ Sebastien Pili-Floury,* Claudine Parquet,§ Mireille Hervé,¶ Catherine Chaput,§ Ivo G. Boneca,§ Won-Jae Lee,† Bruno Lemaitre,¶* and Dominique Mengin-Lecreulx‡

Innate immune recognition of microbes is a complex process that can be influenced by both the host and the microbe. *Drosophila* uses two distinct immune signaling pathways, the Toll and immune deficiency (Imd) pathways, to respond to different classes of microbes. The Toll pathway is predominantly activated by Gram-positive bacteria and fungi, while the Imd pathway is primarily activated by Gram-negative bacteria. Recent work has suggested that this differential activation is achieved through peptidoglycan recognition protein (PGRP)-mediated recognition of specific forms of peptidoglycan (PG). In this study, we have further analyzed the specific PG molecular requirements for Imd activation through the pattern recognition receptor PGRP-LC in both cultured cell line and in flies. We found that two signatures of Gram-negative PG, the presence of diaminopimelic acid in the peptide bridge and a 1,6-anhydro form of N-acetylmuramic acid in the glycan chain, allow discrimination between Gram-negative and Gram-positive bacteria. Our results also point to a role for PG oligomerization in Imd activation, and we demonstrate that elements of both the sugar backbone and the peptide bridge of PG are required for optimum recognition. Altogether, these results indicate multiple requirements for efficient PG-mediated activation of the Imd pathway and demonstrate that PG is a complex immune elicitor. *The Journal of Immunology, 2004, 173: 7339–7348.*

I
nate immunity provides a first line of defense against invading organisms. This response is initiated by host pattern recognition receptors (PRRs),§ which sense specific and highly conserved motifs found in microbes, but not in the host, such as LPS, peptidoglycan (PG), lipoproteins, and CpG DNA (1). Upon recognition, the host receptor activates signaling cascades that result in the expression of immune effectors and regulators. In vertebrates, the most widely studied class of PRRs is the TLRs, originally named based on their homology to the *Drosophila* Toll protein. TLRs are transmembrane proteins that have been shown to initiate signaling cascades that ultimately regulate the immune response via NF-κB (2). It has been shown that TLR4, in association with two cofactors, MD2 and CD14, specifically recognizes LPS, found exclusively in Gram-negative bacterial cell walls, while TLR2 recognizes lipoproteins from various bacterial cell walls (2). Nods, a newly identified class of PRRs in mammals that, unlike TLR, recognize bacterial products in the cytoplasm of cells, are activated by PG. A unique muropeptide derived from Gram-negative PG, containing a diaminopimelic acid (DAP) residue, has been shown to be specifically recognized by Nod1, while Nod2 detects a PG muropeptide found in all types of bacteria (3–6). Thus, it is thought that TLRs and Nods can detect the presence of different types of infectious agents.

*Drosophila,* in contrast to mammals, lacks adaptive immunity and therefore relies entirely on innate mechanisms of immunity for defense against invading microorganisms (7–9). Similar to mammalian innate immunity, pathogens are recognized through interactions of microbial compounds and PRRs in *Drosophila.* Toll and TLRs share structural and functional similarities; however, while TLRs interact with microbial components, Toll does not. Instead, the *Drosophila* extracellular protein Spaetzle, found in the hemolymph (blood), is proteolytically cleaved to activate the Toll receptors on the surface of the fat body (an analogue of the liver) and initiate a signaling cascade that results in the expression of antimicrobial genes via the NF-κB proteins Dif and Dorsal. Fat body cells contain a second, Toll-independent signaling cascade called the immune deficiency (Imd) pathway, which also induces the expression of antibacterial peptide genes such as *Diptericin* via the third NF-κB trans activator Relish (7–10). The Toll pathway is activated largely by Gram-positive bacteria and fungi, and it controls the expression of the antifungal peptide gene *Drosomycin,* while the Imd pathway is triggered mostly by Gram-negative and

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*Abbreviations used in this paper: PRR, pattern recognition receptor; asp, asparaginyl; DAP, diaminopimelic acid; GlcNAc, N-acetylmuramic acid; GM(anh)-tetraLys, GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-meso-DAP-L-Ala; GM(anh)-tetraLys, GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-meso-DAP-GNB, Gram-negative binding protein; Imd, immune deficiency; MurNAc, N-acetylmuramic acid; PG, peptidoglycan; PGRP, PG recognition protein; RNAI, RNA interference.*

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Bacillus-type bacteria, and it controls the expression of the anti-bacterial peptide gene Dipterericin (11). Thus, the immune system in Drosophila demonstrates how two distinct signaling pathways can modulate the expression of genes in response to different classes of microbes, and serves as a simple model of innate immune response in animals.

In Drosophila, it has been shown that bacterial recognition is achieved, at least in part, through PG recognition proteins (PGRPs). PGRPs are found in many species, including insects and mammals, and have been shown to bind directly to PG (12–16). PGRP-SA, a secreted protein circulating in the hemolymph, has been shown to activate the Toll pathway in response to Gram-positive bacteria, but not fungal infection (17), while PGRP-LC acts as a transmembrane receptor upstream of the Imd pathway (18–20). In addition, PGRP-LE, which encodes a secreted PGRP, can activate the Imd pathway when overexpressed in flies. However, the exact function of PGRP-LE in the Drosophila immune response awaits loss-of-function analysis (21). A second group of newly identified pattern recognition molecules in Drosophila is the Gram-negative binding proteins (GNBPs) (22). GNBPI appears to function as a secreted microbial recognition factor that, like PGRPSA, regulates the Toll pathway in response to Gram-positive bacteria (23, 24).

Despite the identification of recognition proteins, the bacterial products recognized by the Toll and Imd pathways remain unclear. It has long been assumed that LPS is a major determinant in the specific recognition of Gram-negative bacteria, given its exclusive presence on the surface of these bacteria. Recently, using highly purified products, we have demonstrated that, in contrast to vertebrates, LPS is not the main determinant for Gram-negative bacterial recognition. Rather, we found that the ability of Drosophila to discriminate between Gram-positive and Gram-negative bacterial relying on the recognition of specific forms of PG (25). The structure of PG from Bacillus and Gram-negative bacteria differs from that of most Gram-positive PG in the third amino acid position of the peptide bridge. Gram-negative and Bacillus-type PGs are cross-linked with a peptide containing a meso-diaminopimelate (DAP) residue, whereas a lysine is found in this position in other Gram-positive bacterial PGs (26). These data suggested that PGRP-LC senses DAP-type PG from Gram-negative and Bacillus-type bacteria, while PGRP-SA/GNBPI may interact with lysine-type PG found in most Gram-positive bacteria.

During the course of bacterial infection, the structure and muropeptide composition of PG are likely to be modified through the action of host and bacterial enzymes. Therefore, to better understand the mechanisms underlying bacterial recognition in Drosophila, it is necessary to define the PG structural requirements allowing detection by PGRPs. Using cell culture and in vivo assays, we have analyzed an extensive array of PG products and derivatives, either naturally occurring or synthetically engineered, for their capacity to activate the Imd pathway. We have also tested the immunostimulatory properties of PG that have been processed by different bacterial enzymes. This work allows us to define the specific PG requirements for innate immune detection and provides new insights into Gram-negative bacterial recognition in Drosophila.

Materials and Methods

Fly stocks

OR8, DDI (w, w; Pryn-, Dipterericin-lacZ), Pw (w, Drosomycin-GFP), or the 8871A (w, Pwy Drosomycin-lacZ) flies were used as wild-type strains (27). Dipterericin-lacZ is a P transgene inserted on the X chromosome containing a fusion between 2.2 kb of upstream sequence from the Dipterericin gene and the coding sequences from the -galactosidase gene. Drosomycin-lacZ is a P transgene inserted on the X chromosome containing a fusion between 2.4 kb of upstream sequence from Drosomycin gene and the coding sequences from the -galactosidase gene. The PGRP-LC allele is described elsewhere (19). Drosophila stocks were maintained at 25°C using standard medium.

Injection and LacZ measurements

A total of 9.2 nl of solution (water or bacterial extracts) was injected into the thorax of female adults (3–4 days old) using a Nanoject apparatus (Drummond Scientific, Broomall, PA). After injection, Dp-lacZ or Drs-lacZ flies were incubated for 6 or 24 h at 25°C. LacZ measurements were previously described (11). The injection procedure creates an injury that, by itself, triggers a significant induction of the Dipterericin and Drosomycin reporter genes; therefore, the measurements of -galactosidase activity monitored after injection of microbial compounds were normalized to the value obtained with water injection using the same conditions.

Digestions of PGs

Digestions of 100 pg of purified Escherichia coli PG with muramidase (mutanolysin, 50 pg) and Slt transglycosylase (10 pg) were done in 20 mM potassium phosphate buffer (pH 6.5) and 300 mM sodium acetate buffer (pH 4.5), respectively. Reaction mixtures (200 ml) were incubated overnight at 37°C. We have controlled that the polymer was digested to almost completion (at least 95%) by isolation and quantitation of the soluble fragments generated during this process. A residual (<5%) nondegradable material is always observed following these treatments (probably highly cross-linked material). Entire digest solutions were used in our experiments.

Cell culture, immune stimulations, and quantitative real-time PCR

Drosophila mbl-2 cells were maintained in Schneider medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS. In the case of the immune stimulation, cells were incubated with various bacterial components at different concentrations for 6 h. Quantitative analysis of Dipterericin expression was performed, as described previously (25). Briefly, total RNA was extracted from cells and cDNA was synthesized by using First cDNA synthesis kit (Roche, Basel, Switzerland), according to manufacturer’s instructions. Fluorescence real-time PCR was performed using dsDNA dye SYBR Green (PerkinElmer, Boston, MA). SYBR Green analysis was performed on an ABI PRISM 7700 system (PerkinElmer) using manufacturer’s instructions. Primer pairs for Dipterericin and control Rac2 were used to detect target gene transcripts. All samples were analyzed in triplicate, and the amount of mRNA detected was normalized relative to the control Rac2 values.

RNA interference

Linear RNA containing the PGRP-LC sequence, flanked by a T7 promoter on each side, was purchased from Open Biosystems (Huntsville, AL). The dsRNA was produced by in vitro transcription (Ribomax large scale RNA production system T7 kit; Promega, Madison, WI). For RNA interference (RNAi), Drosophila mbl-2 cells were diluted to a final concentration of 1 × 105 cells/ml in serum-free Schneider medium. dsRNA was added (15 pg) directly to the medium with vigorous agitation. The cells were incubated at 30 min at 25°C, followed by addition of 2 ml of Schneider medium containing 10% FBS. The cells were incubated for 4 days before PG stimulation.

Synthesis and purification of PGs and muropeptides

The chemical synthesis of meso-DAP, meso-lanthionine, and -allo-cystathionine has been previously described (28). Total replacement of DAP by lanthionine or cystathionine in the PG of E. coli was obtained by growing the DAP auxotrophic strain DM330 in minimal medium supplemented with both of the two DAP analogues, as previously described (29). Extensive replacement (>50%) of DAP by l-lysin in the PG of E. coli was obtained by transforming cells with the pMuSa2 plasmid that overexpresses the Staphylococcus aureus muE gene encoding UDP-N-acetylmuramatic acid (MurNAc)-l-Ala-d-Glu-l-lysine ligase (30). PG from E. coli and other bacterial species were isolated and purified, as previously described (7). Plasmids allowing overproduction of E. coli enzymes that cleave specific bonds in the PG structure were constructed. To allow a simple purification, these proteins were expressed as fusions possessing a His6-tag at the N-terminus. The slyF gene encoding Slt7 transglycosylase (31) was amplified by PCR from E. coli chromosome using oligonucleotides 5′-GGG GAGATCTGACTCACTGGATGAGCAGCGTAGTC-3′ and 5′-TCATA ACTTGCGGATCACTTTACAGCCAGGTTCCCC-3′ as primers. The resulting

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fragment was cut by BglII and HindIII and cloned between the compatible BamHI and HindIII sites of vector pET21d, a pET21d derivative, generating plasmid pMLD204. The ldA gene encoding l-D-carboxypeptidase (32) and the nagZ gene encoding β-N-acetylgalactosaminidase (33) were similarly amplified, using oligonucleotides 5′-AGAGGAGGATCATCCTGTTTCATTTTTGTAACCTCCTTGAGGTTGAATTAACGTAC3′ and 5′-GGAGGATGTTTTGCAAGGCTTCATTTTTGTAACCTCCTTGAGGTTGAATTAACGTAC3′, respectively. The resulting fragments were cut by BamHI and PvuI and cloned between the compatible sites of vector pT7Ehis30 (34), generating pMLD210 and pMLD211, respectively. These plasmids were transformed in the appropriate host strains, BL21(DE3)pLysS or DH5α, and the different proteins were overproduced (isopropyl-β-D-thiogalactoside induction) and purified from the soluble cell fraction on a DE52-nitrilotriacetaate agarose, essentially following the manufacturer’s recommendations (Qiagen, Valencia, CA). For the generation of anhydro-containing-fragmenting PGs, purified GlcNAc-MurNAc(anh) dipeptides were obtained by treatment of GlcNAc-MurNAc(anh) peptides with 4 M ammonium hydroxide for 4.5, 10 mM magnesium chloride, 200 mM sodium acetate buffer, pH 4.0. For the corresponding UDP-MurNAc peptides, Lactoyl peptides were generated by treatment of MurNAc peptides with 4 M ammonium hydroxide for 5 h at 37°C. After neutralization of the reaction mixtures with one equivalent of acetic acid, they were purified by HPLC, as described above, using an isocratic elution with eluent A applied between 20 and 80 min (peaks were detected at 215 nm). Purified compounds were lyophilized, dissolved into water, and applied on the same HPLC column for desalting, using this time 0.1% trifluoroacetic acid and a gradient of methanol for elution. MurNAc(anh) tetrapeptides were obtained by treatment of GlcNAc-MurNAc(anh) peptides with E. coli NagZ β-N-acetylgalactosaminidase. The reaction mixture (200 μl) contained 20 mM HEPES buffer, pH 7.4, 50 mM NaCl, 0.5 mM substrate, and pure His6-tagged NagZ enzyme (20 μg). GlcNAc-MurNAc(anh) tripeptides and MurNAc(anh) tetrapeptides were treated in the same way. The resulting tetrapeptides were obtained by treatment of the corresponding tetrapeptides with E. coli LdcA l-D-carboxypeptidase. The reaction mixture (200 μl) contained 50 mM Tris-Cl buffer, pH 8.0, 0.5 mM substrate, and pure His6-tagged LdcA enzyme (20 μg). GlcNAc-MurNAc(anh) dipeptides were obtained by treatment of GlcNAc-MurNAc(anh) tetrapeptides with partially purified N-acetylmuramyl-l-tanine amidas from E. coli. In all cases, the reaction mixtures were incubated overnight at 37°C and products were purified and desalted by HPLC, as described above. MurNAc peptides were generated by mild acid hydrolysis (0.1 M HCl, 10 min at 100°C) of the corresponding UDP-MurNAc peptides. Lactoyl peptides were generated by treatment of MurNAc peptides with 4 M ammonium hydroxide for 5 h at 37°C. After neutralization of the reaction mixtures with one equivalent of acetic acid, they were purified by HPLC, as described above, using 0.1% trifluoroacetic acid as eluent. Free peptides were obtained by treatment of the MurNAc peptides with partially purified E. coli amidas, as described above. Amino acid and amino sugar compositions were determined with an Hitachi L-8800 amino acid analyzer (ScienceTeс, Les Ulis, France) after hydrolysis of samples in 6 M HCl for 16 h at 95°C. The structure and purity of isolated PG fragments and synthesized compounds were also confirmed by MALDI-TOF mass spectrometry.

To obtain GlcNAc-MurNAc(anh) dipeptide, we used purified GlcNAc-MurNAc(anh) dipeptide, which was purified using HPLC and desalted by treatment with 10 mM sodium acetate buffer, pH 4.5, 10 mM magnesium chloride, 200 μg of H. pylori PG, and pure His6-tagged SltY enzyme (20 μg) was incubated overnight at 37°C. The reaction was stopped by boiling during 10 min. Anhydrous products and partially purified GlcNAc-MurNAc(anh) dipeptide were purified and then desalted by HPLC like E. coli PG products. We confirmed the nature of GlcNAc-MurNAc(anh) dipeptide by MALDI-TOF mass spectrometry.

**Results**

**PGs containing DAP, but not lysine, induce Diptericin expression**

We have previously shown that DAP-containing PGs extracted from two Gram-negative bacterial species (E. coli and Pseudomonas aeruginosa) and two Gram-positive species ( Bacillus thurin- giensis and Bacillus subtilis) induce the Imd pathway via PGRP-LC. In contrast, PGs extracted from two Gram-positive bacteria (Enterococcus faecalis and Micrococcus luteus) strongly induce the NGBP/PGRP-SA Toll pathway, but fail to activate the Imd pathway (24, 25). A structural comparison of the different PGs revealed that those able to activate the Imd pathway have DAP present in the third position of the peptide bridge, while those that did not activate the Imd pathway contain lysine at the same position. This suggests a pivotal role for DAP and Lys in the specific induction of either the Imd or Toll pathways, respectively. To further examine this idea, a larger panel of highly purified PGs was analyzed for the ability to induce the Toll and Imd pathways. Two additional PGs from Gram-negative bacterial species (H. pylori and Neisseria meningitidis), one DAP-containing PG from a Gram-positive bacteria (Listeria monocytogenes), and one Lys-type PG from a Gram-positive species (S. aureus) were extracted and purified. This panel of bacterial PGs was analyzed in vivo using microinjection of the PG into Drosophila containing either a Diptericin (Dpt)-lacZ or Drosomycin (Drs)-lacZ reporter gene. The expression of these two antimicrobial genes is tightly regulated by the Imd and Toll pathways, respectively, and accurately reflects their specific activation (25). In agreement with our previous results, all of the Gram-negative PGs, as well as the Bacillus-type PGs, induced Dpt expression in vivo, while the Gram-positive PGs showed no induction (Fig. 1A). In contrast, the Gram-negative and Bacillus-type PGs showed a weak activation of Drs expression, while the Gram-positive PGs strongly induced Drs expression (Fig. 1B). These results confirm and extend our previous findings that DAP-containing PGs induce Dpt while Lys-containing PGs do not.

We next analyzed Dpt expression in mbn-2 cells, a Drosophila cell line derived from larval hemocytes that contains a functional Imd pathway, in response to the panel of PGs. The mbn-2 cell line strongly responds to Gram-negative PG (25), and therefore provides a more sensitive assay with which to monitor Imd pathway activity. The Diptericin gene was monitored by quantitative RT-PCR 6 h after treatment with PGs, commercial LPS, or water as an internal control. The four different Gram-negative bacterial PGs containing P. aeruginosa, E. coli, H. pylori, and N. meningitidis all induced Dpt at both low and high concentrations (Fig. 1C). The level of induction observed was significantly higher than the level observed with commercial LPS, which induces only at high concentration due to the presence of PG contaminations (25). The two Bacillus-like PGs containing DAP (B. subtilis and L. monocytogenes) also showed Dpt induction in mbn-2 cells, but only at high concentrations (Fig. 1C). In contrast, the Gram-positive Lys-containing PGs (E. faecalis and S. aureus) showed no measurable induction of Dpt, even at high concentrations. These results support the idea that the DAP residue is an important determinant of Imd pathway activation by PG, but also suggest that DAP may not be the sole determinant given that Bacillus-type PGs with DAP induced Dpt with reduced efficiency compared with Gram-negative PGs.

**PGs containing analogues of DAP still activate Diptericin expression**

To further test the role of meso-DAP in Imd-dependent detection of PG, PG preparations that differ only in the nature of the third amino acid residue were generated. E. coli β 243, a strain mutant for the dapA gene and therefore unable to synthesize DAP, was grown in the presence of either meso-DAP or one of two DAP analogues, l-allo-cystathionine or meso-lanthionine. It has been shown that Nod1, which recognizes the PG-derived tripeptide l-Ala-γ-t-Glu-meso-DAP, stringently requires DAP for this recognition and is weakly activated by tripeptides containing these analogues.
two analogues (6, 36). Mbn-2 cells were treated for 6 h with purified PGs containing either DAP or one of its analogues at the third position of the peptide bridge. Quantitative RT-PCR for Dpt expression showed that all three PGs were able to induce to similar levels at both low and high concentration (Fig. 2). The results obtained in vivo using injection of the PGs containing DAP or analogues corroborated these results (data not shown). Taken together, these data show that PGs containing analogues of DAP are able to efficiently induce Dpt expression, and demonstrate that the

Imd pathway recognition of the DAP residue in Gram-negative PG is not as discriminating as that seen for Nod1.

Muramidase-treated PGs show decreased capacity to induce Diptericin expression in mbn-2 cells

PG is a large polymer that consists of long glycan chains of alternating GlcNAc and MurNAc residues that are cross-linked by short peptide bridges. PG polymers can be degraded by enzymes such as muramidase and vertebrate lysozyme that catalyze the cleavage of the β-1,4 bond between MurNAc and GlcNAc in glycan strands to generate muropeptides (Fig. 3). Previous results demonstrated that cleavage of Gram-negative PG polymers into muropeptides is not as discriminating as that seen for Nod1. These studies have been extended in mbn-2 cells, and the results show that indeed the muramidase treatment of both P. aeruginosa and E. coli PG significantly reduced the level of Dpt induction (Fig. 4A). However, in contrast to the in vivo assay (Fig. 4B), muramidase-treated PG was still able to induce Dpt expression, demonstrating that muramidase treatment reduces, but does not eliminate the ability of PG to induce Dpt in cell culture. These results show that some muropeptides produced by muramidase treatment are still capable of being recognized by the PGRP-LC/Imd pathway in mbn-2 cells, but suggest that PG oligomerization is important for optimum stimulation in vivo.

SltY-treated PGs retain the capacity to induce Diptericin expression in mbn-2 cells and flies

In E. coli, SltY, a bacterial soluble lytic transglycosylase, cleaves the β-1,4 bond between MurNAc and GlcNAc in glycan strands to generate muropeptides, similar to muramidases (37). However, unlike muramidase, SltY cleavage also results in the unique formation of an internal 1,6-anhydro bond in the cleaved MurNAc residue (Fig. 3). The anhydro form of MurNAc is naturally present in Gram-negative bacteria at the extremity of all glycan strands, and consequently is present in ~5% of the GlcNAc-MurNAc repeating units (35). Thus, each E. coli PG muropeptide generated by SltY digestion will contain the 1,6-anhydro bond, while PG digested with muramidase generates muropeptides, of which only ~5% contain the anhydro bond. To further examine the importance of this MurNAc 1,6-anhydro bond in Imd activation, E. coli PG was digested with SltY and analyzed for its ability to induce Dpt in mbn-2 cells. Surprisingly, we observed that SltY-digested PG retained the capacity to induce the Dpt gene. At both low and high
GM(anh)-tetraDAP activates the Imd pathway in mbn-2 cells and in vivo

PG digestion by SltY generates a high proportion of GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-meso-DAP-D-Ala (GM(anh)-tetraDAP), a monomer also known as tracheal cytotoxin (Fig. 6). This PG fragment was originally isolated from the supernatants of Bordetella pertussis cultures, and was shown to damage hamster tracheal epithelial cells (38). HPLC-purified GM(anh)-tetraDAP from SltY-digested PG was tested for its capacity to induce the Imd pathway in mbn-2 cells and in flies. Fig. 7A indicates that GM(anh)-tetraDAP induced Dpt expression in mbn-2 cells at a high level, indicating that this muropeptide is an active compound recognized by the Imd pathway. To verify that Imd activation by GM(anh)-tetraDAP was PGRP-LC dependent, RNAi was designed to target the common region of all PGRP-LC isoforms (39). Inactivation of all PGRP-LC isoforms in mbn-2 cells prevented activation by either GM(anh)-tetraDAP or Gram-negative PG (Fig. 7B). This effect was specific of PGRP-LC because inactivation of PGRP-SA by RNAi had no effect on the induction of Dpt by either GM(anh)-tetraDAP or Gram-negative PG (not shown). At high concentrations, GM(anh)-tetraDAP was also able to significantly induce Dpt in vivo, although to levels lower than seen with polymeric PG (Fig. 7C). Dpt expression by GM(anh)-tetraDAP was reduced in PGRP-LC-deficient flies (Fig. 7D). These results demonstrate that the SltY-digestion product, GM(anh)-tetraDAP, has the ability to activate the PGRP-LC/Imd pathway and suggest that DAP in conjunction with the anhydro form of MurNAc is important for immune recognition and activation.

The third amino acid of the PG peptide bridge is important for selective Imd and Toll pathway activation

Our initial results suggested a pivotal role for the third amino acid in selective activation of either the Toll or Imd pathways. To further test this hypothesis, we produced a synthetic analogue of GM(anh)-tetraDAP in which the meso-DAP is replaced by an L-lysine using an engineered E. coli strain. This analogue is artificial because MurNAc anhydro bonds are not naturally found in Gram-positive PG (40). We found that the presence of Lys reduced the stimulatory capacity of the muropeptides in mbn-2 cells and in flies by at least 2-fold, supporting our earlier results (Fig. 7, A and C). We also observed that the GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-L-Lys-D-Ala (GM(anh)-tetraLys) analogue weakly activated the Dpt gene when injected in the fly, while the GM(anh)-tetraDAP did not (Fig. 7E). Because the two muropeptides differ only in their third amino acid residue, our results demonstrate that this residue plays a critical role in the selective activation of the Toll and Imd pathways.

The anhydro bond of GM(anh)-tetraDAP is critical for optimum activation of the Imd pathway

Both GM(anh)-tetra with DAP and with Lys contain the anhydro form of MurNAc. Interestingly, muropeptides with anhydro bonds are found in Gram-negative bacteria and at a lower level in Bacillus species during germination, but not in lysine-type PG from other Gram-positive bacteria (41). Thus, the presence of the MurNAc anhydro bond, in addition to the presence of DAP, is a signature of Gram-negative PG and correlates well with the capacity to activate the Imd pathway. To further test the importance of this bond, an analogue of GM(anh)-tetraDAP lacking the anhydro bond was generated (Fig. 6). This muropeptide, GM(anh)-tetraDAP*, which corresponds to the main monomer that is generated after muramidase treatment of polymeric PG, weakly induced the Dpt gene in mbn-2 cells, showing a 2.5-fold lower activation level as compared with GM(anh)-tetraDAP (Fig. 8A). These results demonstrate that the anhydro bond of GM(anh)-tetraDAP plays a critical role in optimal recognition and induction in cell culture, and agree with the results obtained with muramidase or SltY-digested PGs. The observation that GM(anh)-tetraDAP* still contains the MurNAc...
anhydro bond, retains a weak capacity to activate the Imd pathway in mbn-2 cells and flies (Fig. 7, A and C) while intact PG from Gram-positive bacteria does not, further supports the importance of the anhydro bond. Thus, both DAP and the MurNAc anhydro bond contribute to the stimulatory effect of GM(anh)-tetraDAP.

Multiple PG requirements for optimal Imd stimulation

To identify the minimal active PG muropeptide, we generated a larger panel of E. coli muropeptides and PG derivatives and analyzed their effect on Dpt expression in mbn-2 cells and flies (Fig. 6). The first component of the GM(anh)-tetraDAP molecule that was analyzed was the fourth amino acid of the peptide bridge. Fig. 6B shows that the GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-meso-DAP (GM(anh)-triDAP) induced the Dpt gene at levels similar to GM(anh)-tetraDAP, indicating the fourth amino acid residue is not required for optimal recognition. In agreement with this finding, a muropeptide similar to the GM(anh)-tetraDAP, but that has the fourth amino acid (D-Ala) replaced by a glycine, still retains its full activity. Thus, the GM(anh)-triDAP is competent for immune recognition and activation.

Elements of either the sugar backbone or the peptide bridge were next analyzed for their importance in Imd activation. Fig. 8A shows that a derivative of GM(anh)-tetraDAP, lacking the GlcNAc sugar residue, M(anh)-tetraDAP, induced Dpt at a level 3-fold lower than GM(anh)-tetraDAP, indicating the importance of the GlcNAc residue for optimum recognition. To examine the importance of the peptide bridge, GM(anh) without peptide was generated. This derivative was unable to significantly induce Dpt (Fig. 8C), demonstrating that the presence of the peptide bridge is important for activation. Additionally, GM(anh)-di, which lacks the third and fourth amino acids, was unable to significantly induce Dpt. Thus, a minimum of three residues, including DAP, is important for induction of the Imd pathway. Finally, we observed that PG derivatives lacking the sugar moieties, ranging from the dipeptide to the pentapeptide (Fig. 6), were not able to induce Dpt with either DAP or Lys in the third amino acid position (Fig. 8D). These results demonstrate that elements of both the sugar backbone and the peptide bridge of PG are necessary to induce the Imd pathway.

The minimal optimum motif was found to be GM(anh)-triDAP, a close analogue of GM(anh)-tetraDAP lacking the fourth amino acid. Modification of the sugar moieties, the anhydro bond, and third amino acid all resulted in decreased induction. Altogether, these results indicate multiple requirements for efficient PG-mediated activation of the Imd pathway. To further test this hypothesis, we generated an analogue of GM(anh)-tetraDAP with two modifications to determine whether the effects were additive. A GM(anh)-tetraDAP molecule lacking both the GlcNAc sugar residue and the MurNAc anhydro bond, M-tetraDAP, was tested for its ability to induce Dpt expression in mbn-2 cells. Although the absence of the

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anhydro bond alone and the absence of the GlcNAc residue alone resulted in a 2.5- and 3-fold reduction of expression, respectively, both modifications together resulted in a 7-fold reduction in Dpt expression (Fig. 8A). This confirms that the presence of both GlcNAc and the MurNAc anhydro bond is required for optimal Imd stimulation, and demonstrates that multiple PG factors can cooperatively influence recognition and Imd pathway activation.

Discussion
During the past few years, significant progress has been made toward our understanding of pathogen recognition. Microbial detection is emerging as a multistep process that ultimately requires direct contact between a host PRR and a microbial molecule. Additional host factors may be involved upstream of recognition in the degradation of cell wall compounds and/or the transport of microbial ligands to the receptors. In contrast, enzymes from the microbe may modulate the course of infection by modifying ligands or controlling their release. A major difficulty in the field of innate immunity is the complexity and diversity of the microbial compounds that are recognized by the host, and, thus, one important issue is to determine the global molecular requirements for recognition of microbial ligands. Identification of minimal microbial motifs that are recognized by the host is also prerequisite for future structural studies as well as for determining the factors that influence the outcome of microbial recognition.

The PGRP family is a conserved class of proteins found in both insects and invertebrates that can function as either PRRs or amidases (42–44). The PGRP/PG system in Drosophila is one of the best-characterized mechanisms of bacterial detection by the innate immune system. In this study, we have undertaken a detailed analysis of the PG requirements, allowing activation of the PGRP-LC/Imd pathway in flies and in the hemocytic cell line mbn-2. We found that multiple requirements participate in the stimulatory effects of PG.

Importantly, this study confirms and extends our previous work showing that the Imd pathway is activated by Gram-negative PG, but not by Lys-type PG from Gram-positive bacteria. Using GM(anh)-tetraDAP and an analogue that differs only in the third amino acid (GM(anh)-tetraLys), we now clearly demonstrate the critical role of this amino acid. Interestingly, DAP-type PG from Bacillus species showed a capacity to induce the Imd pathway only at high concentration. An anhydro bond at the extremity of the glycan strand is not usually found in PG from Bacillus species except as a minor compound during the germination process (41). The observation that Bacillus PG is a less potent inducer of the Imd pathway might be explained by the fact that Bacillus PGs contain a high proportion of amidated DAP, the fact that they lack GM(anh)-tetraDAP-like muropeptides containing an anhydro bond, or both. Our results show that the capacity of the fly to discriminate between Gram-negative and Gram-positive bacteria involves the ability to distinguish between DAP-type PG and Lys-type PG. The recognition of GM(anh)-tetraDAP, a PG derivative specific to Gram-negative bacteria, may further accentuate the discriminatory capacity of the fly.

In our previous studies, we have shown that PG digested by muramidase loses its activity when injected into flies, suggesting that monomeric PG is not active. However, by using a cell culture assay that is more sensitive, we now demonstrate that muramidase treatment reduces, but does not abolish the capacity of PG to activate the Imd pathway. These results clearly indicate that: 1) polymeric PG has a more potent stimulatory activity compared with digested PG, and 2) PG fragments retain some activity. The muramidase treatment of Gram-negative PG generates predominantly the monomer GM-tetraDAP, and a low proportion of GM(anh)-tetraDAP, the terminal monomer of the glycan chain that contains a 1,6-anhydro MurNAc residue. Using HPLC-purified compounds, we now demonstrate that the terminal subunit GM(anh)-tetraDAP is
a potent inducer of the Imd pathway in mbn-2 cells and in flies, while the main monomer GM-tetraDAP is poorly recognized. This is also supported by our observation that PG treated with SltY, an enzyme that generates only GM(anh)-tetraDAP fragments, shows the same stimulatory ability as intact PG. The observation that monomers without anhydro bond and blocked configuration do not efficiently activate the Imd pathway clearly explains the differential stimulatory capacities of PG treated with muramidase and SltY.

The anhydro bond locks the MurNAc residue of the GM(anh)-tetraDAP into a fixed configuration, while other monomers generated by muramidase treatment have a flexible configuration, with the hydroxyl group on C1 of MurNAc oscillating between α and β forms. Our results suggest that the hydroxyl group in the β configuration, and possibly the fact that the MurNAc residue is in a fixed position, is important for PGRP recognition. We have shown that polymeric PG, in addition to GM(anh)-tetraDAP muropeptide, is efficiently recognized by PGRP-LC despite the presence of only a small percentage of subunits containing the MurNAc anhydro bond. Furthermore, DAP-type PG polymers from Bacillus can activate the Imd pathway even though under our growth conditions they do not contain any PG subunits containing MurNAc with an anhydro bond. This clearly indicates that both GM(anh)-tetraDAP and muropeptides joined together in polymeric PG have stimulatory capacities. Interestingly, the process of PG polymerization results in a chain of alternating GlcNAc and MurNAc residues, in which the hydroxyl groups on C1 of sugars are all fixed in the β configuration. This suggests that having a fixed configuration of the MurNAc residue may be sufficient to allow recognition, and may explain why only polymeric Gram-negative PG and GM(anh)-tetraDAP, but not fragments generated by muramidase treatment, have the capacity to activate strongly the Imd pathway.

According to this model, the absence of anhydro bonds in the muropeptides of Gram-positive PG would result in the fact that only polymeric PG, but not muropeptides from Lys-type PG could be recognized by PGRP. This is supported by our previous and current observations showing that muramidase-treated Lys-type PG does not induce the Toll target Drosomycin even though the Toll pathway in vivo assay that we use shows much greater sensitivity than the assay used to monitor Imd pathway activity (25). Also, a previous report demonstrated that the minimum structure

FIGURE 7. GM(anh)-tetraDAP activates the Imd pathway. Induction of Dpt expression in mbn-2 cells after treatment with GM(anh)-tetraDAP, GM(anh)-tetraLys, and purified Gram-negative PG. A. Mbn-2 cells were treated with 1 μM purified muropeptide fragments or 5 μg/ml purified PG, total RNA was extracted 6 h later, and Dpt expression was quantified using fluorescence real-time RT-PCR. Independent experiments, each representing an average of three samples, are represented by single bars of different color. B. Induction of Dpt expression in mbn-2 cells with and without RNAi of PGRP-LC by GM(anh)-tetraDAP and PG. Mbn-2 cells were pre-treated with dsRNA to inactivate the PGRP-LC gene, followed by incubation with either GM(anh)-tetraDAP or PG. Total RNA was extracted 6 h later and analyzed, as described above. C and E. Induction of Dpt-lacZ and Drs-lacZ expression in vivo after injection with muropeptides and PGs. Adult female flies were injected with 9.2 nl of solutions of purified muropeptides (100 μM and 1 mM) or purified PG (5 mg/ml), and β-galactosidase activity was measured 6 (□) and 24 h (△) postinjection. D. Induction of Dpt-lacZ expression in PGRP-LCΔ flies after injection with GM(anh)-tetraDAP and PG. Wild-type flies and flies lacking the PGRP-LC gene were injected with 9.2 nl of GM(anh)-tetraDAP (1 mM) or P. aeruginosa PG (5 mg/ml). β-galactosidase activity was measured 24 h postinjection.
Characterization of the minimal PG motifs detected by PGRP-LC. Induction of Diptericin was monitored in mbn-2 cells by RT-PCR 6 h after treatment with GM(anh)-tetraDAP and related muropeptides (1 μM). Independent experiments, each representing an average of three samples, are represented by single bars of different colors. A. Cells were treated with GM(anh)-tetraDAP, GM-tetraDAP, M(anh)-tetraDAP, and M-tetraDAP. B. Cells were treated with GM(anh)-tetraDAP, GM(anh)-triDAP, and GlcNAc-MurNAc(anh)-tetraDAP-Gly (GM(anh)-tetraDAP-Gly). C. Cells were treated with GM(anh)-tetraDAP, GM(anh), and GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-mesoDAP. D. Cells were treated with GM(anh)-tetraDAP, dipeptide γ-D-Glu-meso-DAP, tripeptides L-Ala-γ-D-Glu-X, tetrapeptides L-Ala-γ-D-Glu-X-d-Ala, and pentapeptides L-Ala-γ-D-Glu-X-d-Ala-d-Ala (in which X is meso-DAP or t-Lys).

The observation that GM(anh)-tetraDAP is a potent activator of the Imd pathway supports this second hypothesis because this muropeptide resulted in a lower stimulating capacity. The importance of the complexity of PG architecture is underscored by the additive effect of some of the modifications. Recently, the mammalian intracellular bacterial-sensing proteins Nods have also been shown to recognize small PG fragments (36). The minimal motif sensed by Nod1 was the tripeptide l-Ala-γ-D-Glu-meso-DAP, which contains the first three residues of a Gram-negative PG peptide bridge, without the sugar backbone. In contrast, the minimal motif recognized by Nod2, which can be found in all bacteria, was MurNAc-L-Ala-d-Glu, a compound including the MurNAc residue from the backbone and the first 2 aa of the peptide bridge. Our study indicates that the minimum active PG motif required for optimum activation of the PGRP-LC/Imd pathway is larger and involves more subunits than those required to activate the Nod system. The use of PGs containing analogues of DAP also suggests that the PGRP-LC/PGL recognition is less stringent compared with the Nod system. This is consistent with our finding that there are multiple requirements for optimal PGRP-LC-mediated PG recognition.

Very recently, another group has also analyzed in further detail which components from Gram-negative bacteria can induce an immune response. Kaneko et al. (46) confirmed our previous study showing that Gram-negative PGs, but not LPS, stimulate the Imd pathway. Using another Drosophila cell line, S2 treated with ecdysone, they also showed that GM(anh)-tetraDAP can activate the Imd pathway. In contrast to our study, they observed that lactyl-tetrapeptide can induce an immune response. However, the observation that they need 100 times more lactyl-tetrapeptide than GM(anh)-tetraDAP indicates that lactyl-tetrapeptide is not likely to be a physiological ligand. This is supported by our results, which clearly show that the sugar backbone is important for optimum recognition.

It remains surprising that flies can detect Gram-negative bacteria on the basis of a microbial component that is present at the surface of the inner membrane and is therefore hidden by the LPS-containing outer membrane. This suggests that Gram-negative bacteria may be degraded by humoral or cellular mechanisms that release PG and elicit an antimicrobial response. Alternatively, PGRP-LC may recognize the PG fragments that are continuously released from Gram-negative bacteria as a consequence of PG structure remodeling occurring during cell growth and division processes. The observation that GM(anh)-tetraDAP is a potent activator of the Imd pathway supports this second hypothesis because this muropeptide is known to be continuously released from the PG structure by lytic transglycosylases and reused for de novo synthesis of PG in Gram-negative bacteria (47). GM(anh)-tetraDAP is also a signature of Gram-negative bacteria given that this muropeptide is not present in Lys-type PG from Gram-positive bacteria. Therefore, recognition of GM(anh)-tetraDAP may be a way to efficiently recognize Gram-negative bacteria, even though these bacteria contain LPS and this cell-wall component is hidden underneath an
outer layer of LPS. To date, it is not known whether other components of bacterial cell walls are recognized by the IMD pathway. In our hands, we found that a commercially available flagellin, another proposed elicitor of the IMD pathway, did not activate the Dpr gene in mbn-2 cells (data not shown).

In the last few years, we have learned more about the mechanisms used by Drosophila to recognize bacteria. The identification of the recognition proteins acting upstream of the Toll and IMD pathways as well as the determination of the respective microbial ligands are opening the way to structural studies and to further detailed studies of the host and bacterial factors influencing recognition.

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References
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