THE JOURNAL OF IMMUNOLOGY

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J Immunol 2004; 173:7339-7348; ;
doi: 10.4049/jimmunol.173.12.7339
http://www.jimmunol.org/content/173/12/7339

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Peptidoglycan Molecular Requirements Allowing Detection by the *Drosophila* Immune Deficiency Pathway[1]

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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.

Innate immunity provides a first line of defense against invading organisms. This response is initiated by host pattern recognition receptors (PRRs),[5] 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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Received for publication June 21, 2004. Accepted for publication September 29, 2004.

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[1] This work was supported by the Centre National de la Recherche Scientifique and grants from Programme de Recherche Fondamentale en Microbiologie et Maladies Infectieuses et Parasitaires, Action Concertée Intégrée Microbiologie (MIC 03 33), and the Bettencourt Foundation. J.-H.R. and W.-J.L. were supported by Center for Cell Signaling Research, Korea Ministry of Science and Technology. C.R.S. and F.L. were supported by fellowships from the Fondation pour la Recherche Médicale. I.G.B. is an Institut National de la Santé et de la Recherche Médicale research associate.

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[5] Abbreviations used in this paper: PRR, pattern recognition receptor; anh, anhydro; DAP, diaminopimelic acid; GlcNAc, *N*-acetylglucosamine; GM(anh)-tetra$_{DAP}$, GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala; GM(anh)-tetra$_{Lys}$, GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-L-Lys-D-Ala; GM(anh)-tri$_{DAP}$, GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-*meso*-DAP; GNBP, Gram-negative binding protein; Imd, immune deficiency; MurNAc, *N*-acetylmuramic acid; PG, peptidoglycan; PGRP, PG recognition protein; RNAi, RNA interference.

0022-1767/04/$02.00

Bacillus-type bacteria, and it controls the expression of the antibacterial peptide gene *Diptericin* (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). GNBP1 appears to function as a secreted microbial recognition factor that, like PGRP-SA, 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 bacteria relied 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*-diaminopimelic (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/GNBP1 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

OR^R, DD1 (*y*, *w*, *P*(*ry*$^+$, *Diptericin-lacZ*), *P*(*w*$^+$, *Drosomycin-GFP*)), or the 8871A (*w*, *P*(*w*$^+$, *Drosomycin-lacZ*)) flies were used as wild-type strains (27). *Diptericin-lacZ* is a *P* transgene inserted on the X chromosome containing a fusion between 2.2 kb of upstream sequence from the *Diptericin* gene and the coding sequences from the *β-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 *β-galactosidase* gene. The *PGRP-LC*$^{\Delta E}$ 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, *Dpt-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 *Diptericin* and *Drosomycin* reporter genes; therefore, the measurements of β-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 μg of purified *Escherichia coli* PG with muramidase (mutanolysin, 50 μg) and Slt transglycosylase (10 μg) were done in 20 mM potassium phosphate buffer (pH 6.5) and 300 mM sodium acetate buffer (pH 4.5), respectively. Reaction mixtures (200 μl) 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 mbn-2 cells were maintained in Schneider medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS. In the case of immune stimulation, cells were incubated with various bacterial components at different concentrations for 6 h. Quantitative analysis of Diptericin 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 *Diptericin* 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 DNA 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* mbn-2 cells were diluted to a final concentration of 1×10^6 cells/ml in serum-free Schneider medium. dsRNA was added (15 μg) directly to the medium with vigorous agitation. The cells were incubated for 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 L-*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 β243 in minimal medium supplemented with either of the two DAP analogues, as previously described (29). Extensive replacement (~50%) of DAP by L-lysine in the PG of *E. coli* was obtained by transforming cells with the pMuSa2 plasmid that overexpresses the *Staphylococcus aureus murE* gene encoding UDP-*N*-acetylmuramic 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 (25).

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 His_6-tag at the N terminus. The *sltY* gene encoding SltY transglycosylase (31) was amplified by PCR from *E. coli* chromosome using oligonucleotides 5′-GGC GAGATCTGACTCACTGGATGAGCAGCGTAGTC-3′ and 5′-TCATA AGCTTGCGGATCAGTAACGACGTCCCC-3′ as primers. The resulting

fragment was cut by *Bgl*II and *Hin*dIII and cloned between the compatible *Bam*HI and *Hin*dIII sites of vector pET2130, a pET21d derivative, generating plasmid pMLD204. The *ldcA* gene encoding L,D-carboxypeptidase (32) and the *nagZ* gene encoding β-*N*-acetylglucosaminidase (33) were similarly amplified, using oligonucleotides 5′-AGGAGGATCCAT GTCTCTGTTTCACTTAATTGCC-3′ and 5′-GCAGCTGCAGCGTTT TACTTAGCCCTGAAGCGTG-3′, and 5′-GGAGGGATCCGTGGGTC CAGTAATGTTGGATGTCGAAG-3′ and 5′-CATACTGCAGGGTTAGT GACCTGCTTTCTCTTCCTG-3′ as primers, respectively. The resulting fragments were cut by *Bam*HI and *Pst*I and cloned between the same sites of vector p*Trc*His30 (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 Ni^{2+}-nitrilotriacetate agarose, essentially following the manufacturer's recommendations (Qiagen, Valencia, CA). For the generation of anhydro-containing PG fragments, PG purified from *E. coli* mutant strain BW25113 Δ*lpp*::Cm^R that do not express the Lpp lipoprotein (25) was treated with *E. coli* SltY transglycosylase. The reaction mixture (0.5 ml) containing 300 mM sodium acetate buffer, pH 4.5, 500 μg of PG (briefly sonicated for homogeneization), and pure His_6-tagged SltY enzyme (40 μg) was incubated overnight at 37°C. The reaction was stopped by adding 500 μl of 50 mM sodium phosphate buffer, pH 4.45 (HPLC eluent A), and 2 μl of phosphoric acid. The main products generated were the monomer and dimer forms of *N*-acetylglucosamine (GlcNAc)-MurNAc(anhydro (anh))-tetrapeptide, but other minor tripeptide- or glycine-containing fragments were also observed that reflect the complexity of the PG structure, as reported by Glauner et al. (35). These different products were purified by HPLC on a Nucleosil $5C_{18}$ column (4.6 × 250 mm) using an isocratic elution with eluent A at 0.6 ml/min for 20 min, followed by a gradient of methanol from 0 to 25% in 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) peptides were obtained by treatment of GlcNAc-MurNAc(anh) peptides with *E. coli* NagZ β *N*-acetylglucosaminidase. The reaction mixture (200 μl) contained 20 mM HEPES buffer, pH 7.4, 50 mM NaCl, 0.5 mM substrate, and pure His_6-tagged NagZ enzyme (20 μg). GlcNAc-MurNAc(anh) tripeptides and MurNAc(anh) tripeptides were generated by treatment of the corresponding tetrapeptide compounds with *E. coli* LdcA L,D carboxypeptidase. The reaction mixture (200 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 0.5 mM substrate, and pure His_6-tagged LdcA enzyme (20 μg). GlcNAc-MurNAc(anh) was obtained by treatment of GlcNAc-MurNAc(anh) tetrapeptide with partially purified *N*-acetylmuramoyl-L-alanine amidase from *E. coli*. In all cases, incubations were performed 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* amidase, as described above. Amino acid and amino sugar compositions were determined with an Hitachi L8800 amino acid analyzer (ScienceTec, 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 PG purified from *Helicobacter pylori* strain 26695 during stationnary phase (with contains a high amount of GlcNAc-MurNAc dipeptide) and treated it with *E. coli* SltY. The reaction mixture containing 10 mM sodium acetate buffer, pH 4.5, 10 mM magnesium chloride, 200 μg of *H. pylori* PG, and pure His_6-tagged SltY enzyme (20 μg) was incubated overnight at 37°C. The reaction was stopped by boiling during 10 min. Anhydrous products and particularly 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 thuringiensis* 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 GNBP1/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. 1*A*). In contrast, the Gram-negative and *Bacillus*-type PGs showed a weak activation of *Drs* expression, while the Gram-positive PGs strongly induced *Drs* (Fig. 1*B*). 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 DAP (*P. aeruginosa*, *E. coli*, *H. pylori*, and *N. meningitidis*) all induced *Dpt* at both low and high concentrations (Fig. 1*C*). 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 contaminants (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. 1*C*). 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 mutated 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-D-Glu-*meso*-DAP, stringently requires DAP for this recognition and is weakly activated by tripeptides containing these

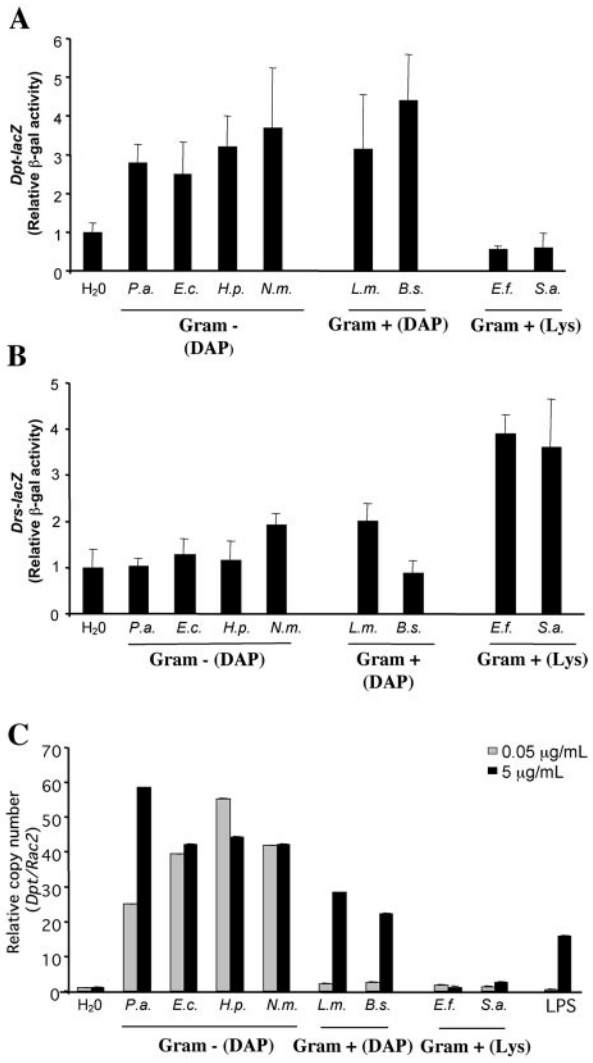


FIGURE 1. Immune induction by PG from various bacteria. *A*, Induction of *Dpt-lacZ* expression in vivo after injection of purified bacterial PGs. Adult female flies carrying the *Dpt-lacZ* reporter gene were microinjected with 9.2 nl of PG extracted from *P. aeruginosa* (*P.a.*), *E. coli* (*E.c.*), and *E. faecalis* (*E.f.*), at 5 mg/ml, or PG from *H. pylori* (*H.p.*), *N. meningitidis* (*N.m.*), *L. monocytogenes* (*L.m.*), *B. subtilis* (*B.s.*), and *S. aureus* (*S.a.*) at 10 mg/ml. β-Galactosidase activity was measured 24 h postinjection. *B*, Induction of *Drs-lacZ* expression after injection of purified bacterial PGs. Injections of PGs were performed, as described above, using adult female flies carrying the *Drs-lacZ* reporter gene. β-Galactosidase activity was measured 24 h postinjection. *C*, Induction of *Dpt* expression in mbn-2 cells 6 h after treatment with purified bacterial PGs. Cells were incubated with a final concentration of 0.05 μg/ml (▨) and 5 μg/ml (■) PG extracted from *P. aeruginosa* (*P.a.*), *E. coli* (*E.c.*), *H. pylori* (*H.p.*), *N. meningitidis* (*N.m.*), *L. monocytogenes* (*L.m.*), *B. subtilis* (*B.s.*), *E. faecalis* (*E.f.*), *S. aureus* (*S.a.*), and commercial LPS (Sigma-Aldrich) Total RNA was extracted from cells, and *Dpt* and *rac* expression was monitored with quantitative fluorescence real-time RT-PCR.

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

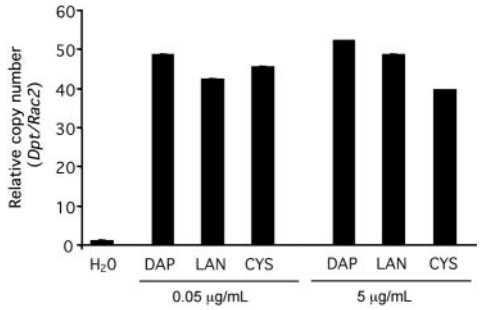


FIGURE 2. *E. coli* PG containing analogues of DAP activate the Imd pathway. Induction of *Dpt* expression in mbn-2 cells 6 h after treatment with purified *E. coli* PGs. Cells were incubated with 0.05 or 5 μg/ml PG extracted from an *E. coli* strain incapable of synthesizing DAP (*E. coli* β 243) and grown in the presence of *meso*-DAP, *meso*-lanthionine (LAN), or L-*allo*-cystathionine (CYS). Total RNA was extracted from cells 6 h after treatment, and *Dpt* and *rac* expression was monitored with quantitative fluorescence real-time RT-PCR.

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 individual subunits by muramidase treatment prevented *Dpt* induction in vivo (25) (Fig. 4*B*). 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. 4*A*). However, in contrast to the in vivo assay (Fig. 4*B*), 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

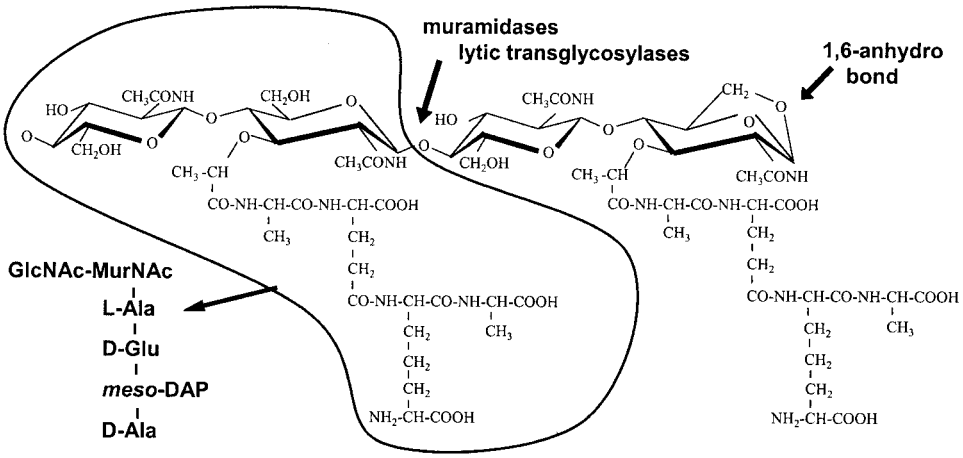


FIGURE 3. Diagram of PG structure and cleavage sites of murein hydrolases. PG is a complex heteropolymer consisting of long glycan chains of alternating GlcNAc and MurNAc residues, connected by cross-linking of short peptide bridges. All the terminal MurNAc residues ending the *E. coli* PG glycan chains have a unique internal 1,6-anhydro bond. The PG fragment circled represents one PG subunit. Both lytic transglycosylases and muramidases catalyze the cleavage of the β-1,4-glycosidic bond between the MurNAc and GlcNAc residues. However, the bacterial transglycosylases catalyze an additional intramolecular transglycosylation reaction that results in the formation of a 1,6-anhydro MurNAc residue.

concentration, the digested PG showed a level of induction similar to or greater than the undigested PG (Fig. 5*A*).

To determine whether SltY-digested PG could induce *Dpt* in vivo, the same preparations along with similar preparations of digested *P. aeruginosa* PG were microinjected into *Dpt-lacZ* flies. We observed that the SltY-digested PGs were able to efficiently induce *Dpt* expression (Fig. 5*B*). This mirrors the results from the cell culture experiments and supports the idea that the anhydro bond plays a critical role in immune recognition by the Imd pathway.

GM(anh)-tetra$_{DAP}$ 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)-tetra$_{DAP}$), 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)-tetra$_{DAP}$ from SltY-digested PG was tested for its capacity to induce the Imd pathway in mbn-2 cells and in flies. Fig. 7*A* indicates that GM(anh)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ or Gram-negative PG (Fig. 7*B*). 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)-tetra$_{DAP}$ or Gram-negative PG (data not shown). At high concentrations, GM(anh)-tetra$_{DAP}$ was also able to significantly induce *Dpt* in vivo, although to levels lower than seen with polymeric PG (Fig. 7*C*). *Dpt* expression by GM(anh)-tetra$_{DAP}$ was reduced in *PGRP-LC*-deficient flies (Fig. 7*D*). These results demonstrate that the SltY-digestion product, GM(anh)-tetra$_{DAP}$, 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)-tetra$_{DAP}$ 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)-tetra$_{Lys}$) analogue weakly activated the *Drs* gene when injected in the fly, while the GM(anh)-tetra$_{DAP}$ did not (Fig. 7*E*). 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)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ lacking the anhydro bond was generated (Fig. 6). This muropeptide, GM-tetra$_{DAP}$, 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)-tetra$_{DAP}$ (Fig. 8*A*). These results demonstrate that the anhydro bond of GM(anh)-tetra$_{DAP}$ 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)-tetra$_{Lys}$, which still contains the MurNAc

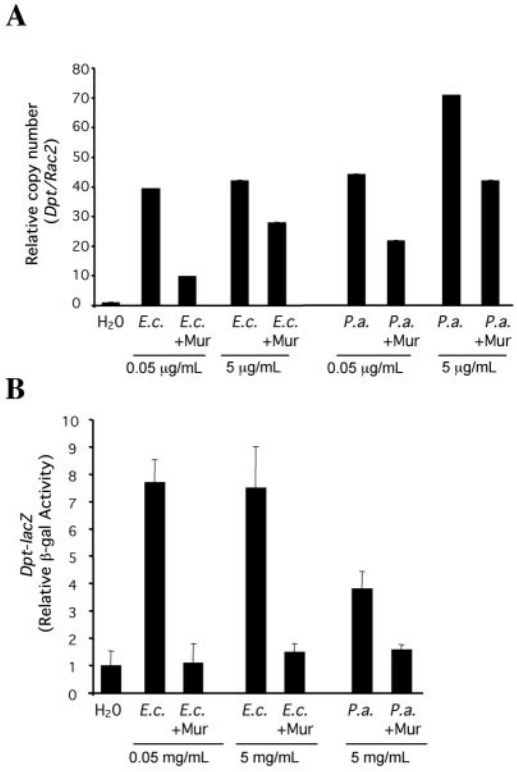


FIGURE 4. Muramidase-treated Gram-negative PG reduces, but does not abolish Imd activation in mbn-2 cells. *A*, Induction of *Dpt* expression in mbn-2 cells after treatment with untreated and muramidase-treated PGs. Purified PG from both *P. aeruginosa* (*P.a.*) and *E. coli* (*E.c.*) was digested with muramidase overnight. Mbn-2 cells were incubated with PGs at final concentrations of either 0.05 or 5 μg/ml, and quantitative RT-PCR was performed with total RNA extracted 6 h posttreatment. *B*, Induction of *Dpt-lacZ* expression in vivo after injection with untreated and muramidase-treated PGs. Adult female flies carrying the *Dpt-lacZ* reporter gene were injected with 9.2 nl of muramidase-treated or untreated PGs from *E. coli* and *P. aeruginosa* (injection solution: 0.05 or 5 mg/ml). β-Galactosidase activity was measured 6 h postinjection.

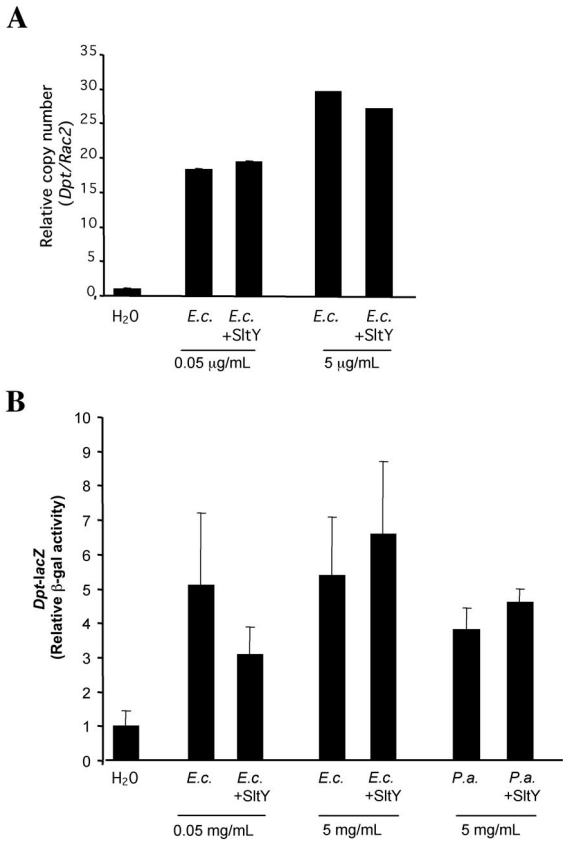


FIGURE 5. SltY-treated Gram-negative PG activates the Imd pathway in vivo and in mbn-2 cells. Induction of *Dpt* expression in mbn-2 cells and in vivo by untreated and SltY-treated PGs. *A*, Purified PG from *E. coli* was digested with SltY overnight. Mbn-2 cells were incubated with a final concentration of either 0.05 or 5 μg/ml PGs, and quantitative RT-PCR was performed with total cellular RNA extracted 6 h posttreatment. *B*, Adult female flies carrying the *Dpt-lacZ* reporter gene were injected with 9.2 nl of solutions containing 0.05 or 5 mg/ml SltY-treated or untreated *E. coli* and *P. aeruginosa* PGs. β-Galactosidase activity was measured 6 h postinjection.

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)-tetra$_{DAP}$.

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)-tetra$_{DAP}$ molecule that was analyzed was the fourth amino acid of the peptide bridge. Fig. 8*B* shows that the GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-*meso*-DAP (GM(anh)-tri$_{DAP}$) induced the *Dpt* gene at levels similar to GM(anh)-tetra$_{DAP}$, indicating the fourth amino acid residue is not required for optimal recognition. In agreement with this finding, a muropeptide similar to the GM(anh)-tetra$_{DAP}$, but that has the fourth amino acid (D-Ala) replaced by a glycine, still retains its full activity. Thus, the GM(anh)-tri$_{DAP}$ 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. 8*A* shows that a derivative of GM(anh)-tetra$_{DAP}$ lacking the GlcNAc sugar residue, M(anh)-tetra$_{DAP}$, induced *Dpt* at a level 3-fold lower than GM(anh)-tetra$_{DAP}$, 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. 8*C*), 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. 8*D*). 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)-tri$_{DAP}$, a close analogue of GM(anh)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ with two modifications to determine whether the effects were additive. A GM(anh)-tetra$_{DAP}$ molecule lacking both the GlcNAc sugar residue and the MurNAc anhydro bond, M-tetra$_{DAP}$, was tested for its ability to induce *Dpt* expression in mbn-2 cells. Although the absence of the

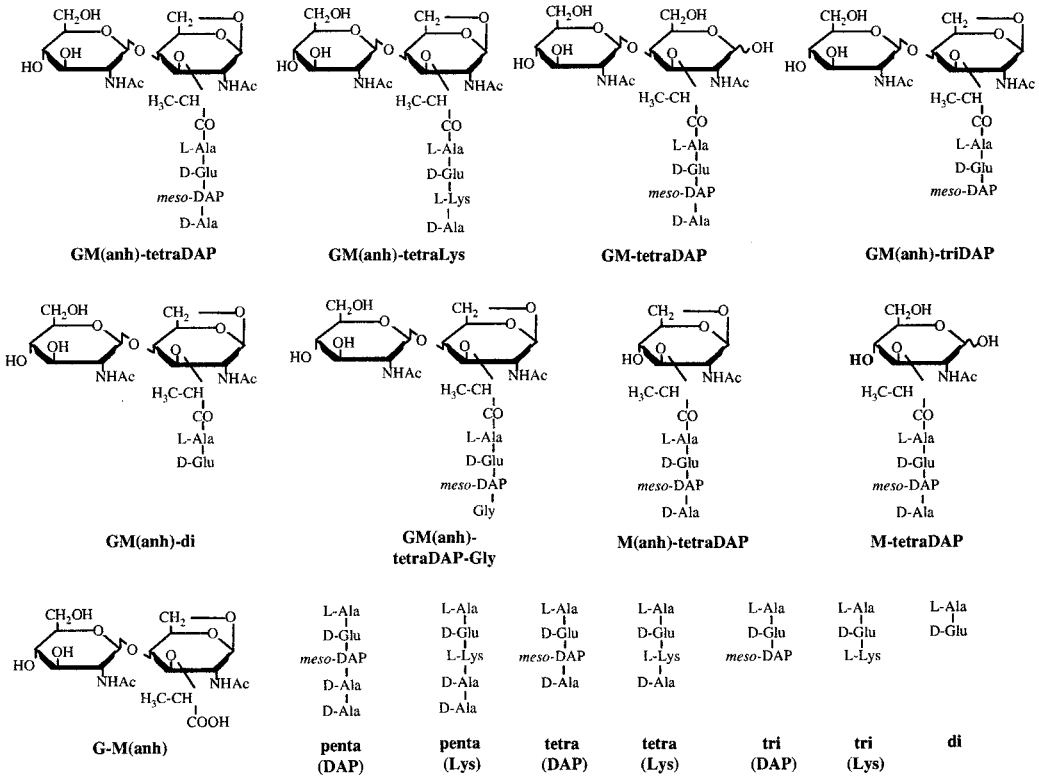


FIGURE 6. Schematic representation of the *E. coli* PG-derived muropeptides used in this study.

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. 8*A*). 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.

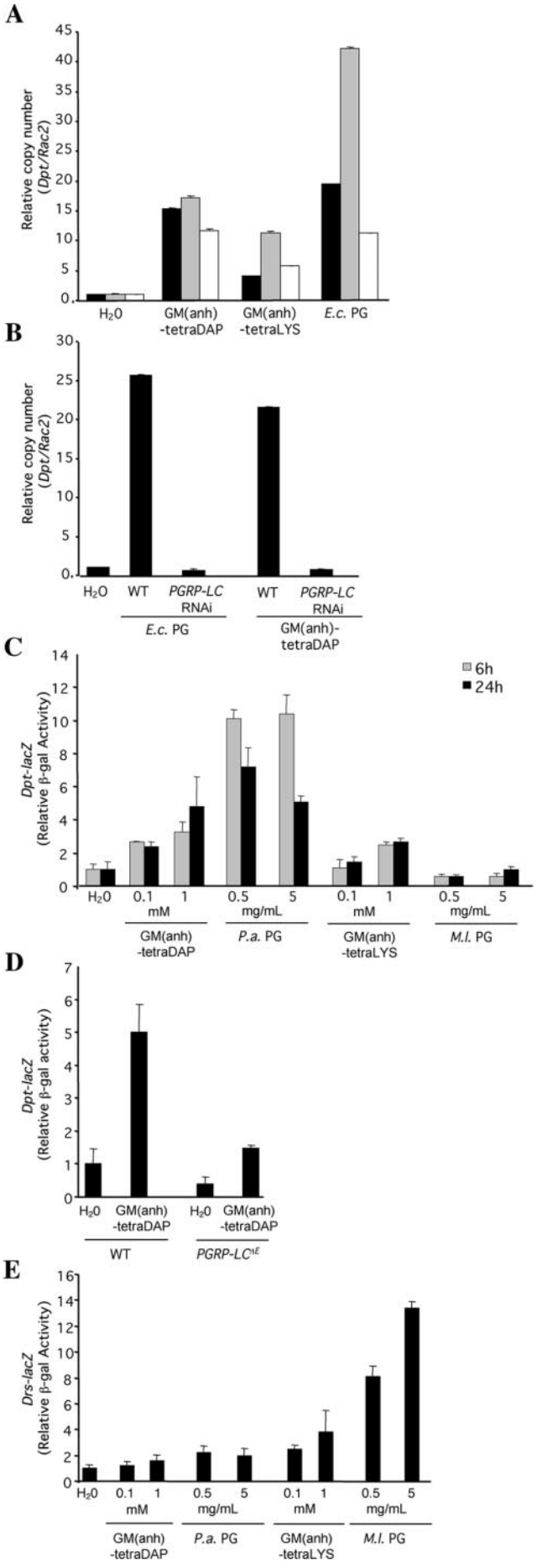
Discussion

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)-tetra$_{DAP}$ and an analogue that differs only in the third amino acid (GM(anh)-tetra$_{Lys}$), 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)-tetra$_{DAP}$-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)-tetra$_{DAP}$, 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-tetra$_{DAP}$ and a low proportion of GM(anh)-tetra$_{DAP}$, 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)-tetra$_{DAP}$ is



a potent inducer of the Imd pathway in mbn-2 cells and in flies, while the main monomer GM-tetra$_{DAP}$ is poorly recognized. This is also supported by our observation that PG treated with SltY, an enzyme that generates only GM(anh)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ into a fixed configuration, while other monomers generated by muramidase treatment have a MurNAc residue with 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. This is also in agreement with our data showing that the sugar backbone is essential for optimum recognition. We have shown that polymeric PG, in addition to GM(anh)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ 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)-tetra$_{DAP}$, 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)-tetra$_{DAP}$ activates the Imd pathway. Induction of *Dpt* expression in mbn-2 cells after treatment with GM(anh)-tetra$_{DAP}$, GM(anh)-tetra$_{Lys}$, 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)-tetra$_{DAP}$ and PG. Mbn-2 cells were pretreated with dsRNA to inactivate the *PGRP-LC* gene, followed by incubation with either GM(anh)-tetra$_{DAP}$ 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-LC*$^{\Delta E}$ flies after injection with GM(anh)-tetra$_{DAP}$ and PG. Wild-type flies and flies lacking the *PGRP-LC* gene were injected with 9.2 nl of GM(anh)-tetra$_{DAP}$ (1 mM) or *P. aeruginosa* PG (5 mg/ml). β-galactosidase activity was measured 24 h postinjection.

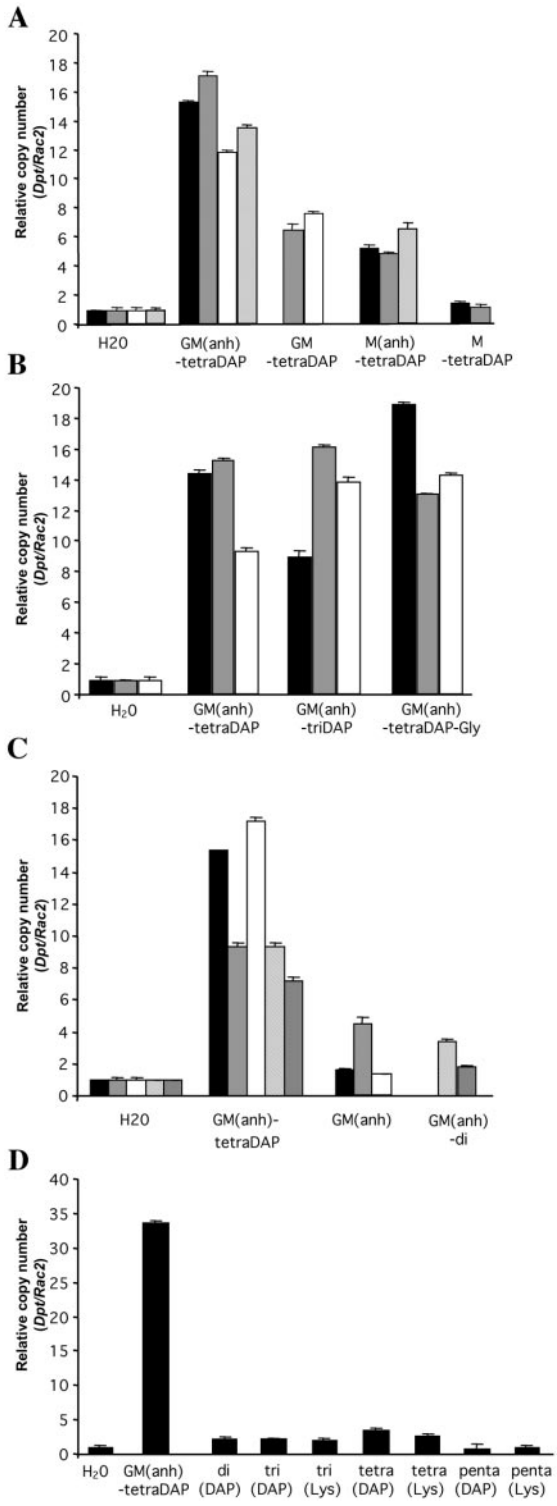


FIGURE 8. Characterization of the minimal PG motifs detected by PGRP-LC. Induction of *Diptericin* was monitored in mbn-2 cells by RT-PCR 6 h after treatment with GM(anh)-tetra$_{DAP}$ and related muropeptides (1 μM). Independent experiments, each representing an average of three samples, are represented by single bars of different colors. *A*, Cells were treated with GM(anh)-tetra$_{DAP}$, GM-tetra$_{DAP}$, M(anh)-tetra$_{DAP}$, and M-tetra$_{DAP}$. *B*, Cells were treated with GM(anh)-tetra$_{DAP}$, GM(anh)-tri$_{DAP}$, and GlcNAc-MurNAc(anh)-L-Ala-γ-D-Glu-*meso*-DAP-Gly (GM(anh)-tetra$_{DAP\text{-}Gly}$). *C*, Cells were treated with GM(anh)-tetra$_{DAP}$, GM(anh), and GlcNAc-MurNAc(anh)-L-Ala-D-Glu (GM(anh)-di). *D*, Cells were treated with GM(anh)-tetra$_{DAP}$, 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 L-Lys).

of Lys-type PG required for antibacterial peptide expression in the silkworm *Bombyx mori* was two repeating GlcNAc-MurNAc subunits with peptide chains (45). This structure has the first MurNAc residue with the hydroxyl group in the β configuration, and is therefore rigid. Thus, it is possible that a fixed MurNAc configuration is important for efficient recognition by both PGRP-LC and PGRP-SA, and may influence the recognition of PGs from various bacterial origins.

By comparing the activity of a large panel of natural and synthetic muropeptides, some of them being generated for the first time, we were able to demonstrate that the minimum active muropeptide is GM(anh)-tri$_{DAP}$. Additional deletion or modification of 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 subtleties than those required to activate the Nod system. The use of PGs containing analogues of DAP also suggests that the PGRP-LC/PG 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)-tetra$_{DAP}$ 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 10^6 times more lactyl-tetrapeptide than GM(anh)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ 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)-tetra$_{DAP}$ may be a way to efficiently recognize Gram-negative bacteria, even though these bacteria contain less PG 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 *Dpt* 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 bacterial ligands are opening the way to structural studies and to further detailed studies of the host and bacterial factors influencing recognition.

Acknowledgments

We thank Geneviève Auger for amino acid analyses, and Didier Blanot for mass spectrometry experiments.

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