A Synthetic Peptidoglycan Fragment as a Competitive Inhibitor of the Melanization Cascade*

Ji Won Park†, Byung-Rok Je‡, Shunfu Piao*, Seiichi Inamura§, Yukari Fujimoto§, Koichi Fukase§, Shoichi Kusumoto§, Kenneth Söderhäll‡, Nam-Chul Ha†, and Bok Luel Lee†*

From the †National Research Laboratory of Defense Proteins, College of Pharmacy, Pusan National University, Kumjeong Ku, Busan 609-735, Korea, the ‡Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan, and the §Department of Comparative Physiology, Evolutionary Biology Center, Uppsala University, Norbyvägen 18A, SE-752 36 Uppsala, Sweden

Melanin synthesis is essential for defense and development but must be tightly controlled because systemic hyperactivation of the prophenoloxidase and excessive melanin synthesis are deleterious to the hosts. The melanization cascade of the arthropods can be activated by bacterial lysine-peptidoglycan (PGN), diaminopimelic acid (DAP)-PGN, or fungal β-1,3-glucan. The molecular mechanism of how DAP- or Lys-PGN induces melanin synthesis and which molecules are involved in distinguishing these PGNs are not known. The identification of PGN derivatives that can work as inhibitors of the melanization cascade and the characterization of PGN recognition molecules will provide important information to clarify how the melanization is regulated and controlled. Here, we report that a novel synthetic Lys-PGN fragment ((GlcNAc-MurNAc-L-Ala-OisoGln-L-Lys-D-Ala)L2, T-4P2) functions as a competitive inhibitor of the natural PGN-induced melanization reaction. By using a T-4P2-coupled column, we purified the Tenebrio molitor PGN recognition protein (Tm-PGRP) without causing activation of the prophenoloxidase. The purified Tm-PGRP recognized both Lys- and DAP-PGN. In vitro reconstitution experiments showed that Tm-PGRP functions as a common recognition molecule of Lys- and DAP-PGN-dependent melanization cascades.

The innate immune system is a host defense mechanism that is evolutionarily conserved from insects to human and is mainly involved in the recognition and control of the early stage of infection in all animals (1). It is activated by a group of germ line-encoded receptors and soluble proteins conceptually termed pattern recognition receptors and proteins, respectively. The group recognizes microbial surface determinants that are conserved among microbes but absent in the host. These conserved motifs, called pathogen-associated molecular patterns (PAMPs), 2 include lipopolysaccharide (LPS), peptidoglycan (PGN), β-1,3-glucan, and mann. Upon recognition, these receptors activate distinct signaling cascades leading to the expression of genes that participate in innate immune responses, such as expression of cytokines and antimicrobial peptides. Recently, our understanding of the molecular mechanisms involved in the regulation of cytokines in immune cells in response to stimulation with PAMPs has increased dramatically (2, 3). Therefore, identification of novel agonists or antagonists of PAMPs that can regulate the signal pathways of innate immune reactions will be useful to elucidate the molecular mechanism of innate immunity (4–6).

The activation of the prophenoloxidase (proPO) cascade leading to melanization is a major innate immune reaction triggered by LPS, PGN, and β-1,3-glucan and is vital for the survival and development of insects (7). Melanization plays an important role in defense reactions such as wound healing, encapsulation, sequestration of invading microbes, and the production of toxic quinone intermediates that kill the microorganisms. However, melanin synthesis must be tightly controlled because systemic hyperactivation of the proPO system, excessive formation of quinones, and inappropriate melanin synthesis are also deleterious to the hosts. Only a few inhibitors of the melanization reaction have been identified from insects and crustaceans (8–10). Recently we identified a novel 43-kDa protein as a negative regulatory component of phenoloxidase (PO)-induced melanin synthesis (11). Also, it was reported that Drosophila serpin 27A specifically inhibited the proPO activating enzyme and prevented the melanin synthesis induced by activated PO and that the protease inhibitor, pacifastin, efficiently inhibited the proPO activating enzyme in a crayfish (9, 12, 13). However, a novel molecule working as a specific competitive inhibitor of PGN-dependent melanization cascade has so far not been found. The identification and characterization of a specific melanization regulatory molecule will provide important information to clarify how the melanization response is regulated and controlled.

The proPO activation pathway, like the vertebrate complement system, is a proteolytic cascade containing several serine proteases and their inhibitors and terminates with thezymogen, proPO. Microbial PAMPs such as LPS, PGN, or β-1,3-glucan will first react with pattern recognition proteins, which then will induce the activation of several serine proteases within the proPO system (14, 15). Previously we have characterized the biochemical properties of two β-1,3-glucan pattern recognition proteins (16, 17), three proPO-activating factors, and proPOs (18, 19) from the large beetle insects Haltotrichia diomphalia and Tenebrio molitor larvae. Recently we also reported the crystal structure of a clip domain serine protease and functional roles of the clip domains of Haltotrichia proPO-activating factor 2 (20). However, the molecular
mechanism of how PGN or β-1,3-glucan can activate proPO system and what proteins are involved in PGN or β-1,3-glucan specific proPO cascade are not fully understood at the molecular level. One reason for this is that it is difficult to obtain a fraction showing specific PO activity induced by either PGN or β-1,3-glucan. Because natural PGN and β-1,3-glucan spontaneously activate the proPO system, it is difficult to obtain a PGN or a β-1,3-glucan specific fraction from the crude hemolymph of the insect without activation of the proPO system. An important approach to understanding how natural PGN or β-1,3-glucan specifically induce the activation of proPO system is therefore to obtain a fraction from the crude hemolymph showing either a PGN or a β-1,3-glucan specific PO activity and then purify proteins involved in each pathway. To obtain the specific fraction showing PGN-dependent PO activity, we hypothesized that small PGN fragments would not activate the PGN-dependent proPO system, as is the case with small β-1,3-glucans (17), but instead could be used as a ligand in affinity chromatography to isolate a PGN recognition molecule.

PGN is a polymer consisting of glycan strands of alternating GlcNAc and N-acetylmuramic acid (MurNAc) that are cross-linked to each other by short peptide bridges (21). PGNs from Gram-negative bacteria and Bacillus species differ from other Gram-positive PGNs by the replacement of Lys with meso-diaminopimelic acid (DAP) at the third amino acid in the peptide chain. It is known that PGN stimulates the production of inflammatory cytokines, such as interleukin-6 and tumor necrosis factor α in monocytes, macrophages, and neutrophils (22). In insects, two different PGN recognition systems are present: one for the induction of antimicrobial peptides and the other for eliciting activation of the melanization cascade. A PGN recognition protein (PGRP) was first identified in the silkworm, Bombyx mori, through purification of a protein from hemolymph that binds to Lys-PGN and activates the proPO cascade system (23). Recent studies have demonstrated that several PGRPs have been shown to bind directly to PGN, each with distinct preferences for binding Lys- or DAP-PGN (24–27). This binding discrimination to Lys-PGN or DAP-PGN leads to the activation of specific immune signaling pathways (Toll and Imd, respectively, in Drosophila). Even though intensive studies of PGN ligand structures and PGN recognition proteins acting upstream of the Toll and Imd path- ways have been carried out, specific PGRPs that distinguish and recognize either Lys- or DAP-PGN during melanin synthesis have not been characterized yet.

Here, we describe the identification of a novel synthetic Lys-PGN fragment, two copies of GlcNAc-MurNAc attached to l-Ala-d-isoGln-l-Lys-d-Ala peptides (T-4P2), which functions as a competitive inhibitor of the PGN-dependent melanization cascade. Using a T-4P2-coupled column we obtained a β-1,3-glucan-specific and also a PGN-specific melanin synthesizing fraction from the hemolymph of T. molitor larvae. Also, we demonstrate that Tenebrio PGRP purified by using the T-4P2-coupled column works as a common PGN recognition molecule on both Lys- and DAP-PGN-dependent melanization responses.

**Experimental Procedures**

**Animal Collection of Hemolymph**—T. molitor larvae (mealworm) were maintained on a laboratory bench in terraria containing wheat bran. Hemolymph was collected as previously described (28). The collected crude hemolymph was centrifuged at 203,000 × g for 4 h at 4 °C. The supernatant was taken as hemolymph and then stored at −80 °C until use.

**Assay of PO Activity and Measurements of Melanin Syntheses**—An assay of PO was carried out according to our previously published method (19). Briefly, to measure PO activity, 30 µl of crude hemolymph (350 µg of proteins) or fractionated solution from T-4P2 column chromatography was preincubated in 70 µl of 20 mM Tris-HCl buffer (pH 8.0) containing 1 µg of β-1,3-glucan or soluble Lys-PGN (10 ng) or DAP-PGN (50 ng) for 10 min at 30 °C, and then 400 µl of substrate solution (1 mM 4-methyl-catechol, 2 mM 4-hydroxyproline ethylether in 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM CaCl2) was added to the reaction mixture. After incubation at 30 °C for 20 min, the increase in absorbance at 520 nm was measured using a spectrophotometer. One unit of PO activity was defined as the amount of enzyme causing an increase in absorbance of one at 520 nm/20 min of incubation (A520/20 min).

Melanin synthesis was measured according to a method published previously (28). Briefly, 30 µl of crude hemolymph (350 µg of proteins) was preincubated with 10 µl of β-1,3-glucan (1 µg) or natural soluble Lys- or DAP-PGN at 30 °C for 10 min. After incubation, 460 µl of the substrate solution (20 mM Tris-HCl, pH 8.0, containing 1 mM dopamine and 10 mM CaCl2) was added to the reaction mixture, and then incubated at 30 °C for 1 h. The increase in absorbance at 400 nm, which records melanin formation, was measured.

**Preparation of Natural Soluble PGNs**—Insoluble Lys-PGN of Staphylococcus aureus and LPS 0111B4 were obtained from Sigma-Aldrich. Soluble Micrococcus letus Lys-PGN and curdulans were obtained from Wako Pure Chemicals. Insoluble DAP-PGN from Escherichia coli BW25113 Δipp was purified by a method previously described (26). Insoluble DAP-PGN from Bacillus subtilis was generously given by Dr. Bruno Lemaître (CNRS). Natural soluble Lys- and DAP-PGN that can induce melanin synthesis were prepared from insoluble S. aureus and insoluble E. coli PGNs, respectively, according to the method of Rosenthal and Dzierzak (29). Because the obtained soluble PGN are a mixture of active and inactive PGN fragments, we estimated the potency of proPO activation by using commercially available soluble M. leteus Lys-PGN as a reference PGN.

**Syntheses of Lys-PGN Fragments, Preparation of Synthetic PGN Fragments, Immobilized Columns, and Binding Assay**—PGN partial structures with two copies of L-β-β-linked GlcNAc-MurNAc attached to l-Ala-d-isoGln-l-Lys-d-Ala peptides (T-4P2), l-Ala-d-isoGln-l-Lys (T-3P2), l-Ala-d-isoGln-l-Lys (T-2P2), l-Ala-d-isoGln-l-Lys (T-4P2)-immo- bilized on Sepharose following the manufacturer’s instructions. The purified tetracosapeptide (TCT; Fig. 1A) was shown in Fig. 1A. The (GlcNAc-MurNAc)2 counterparts lacking peptide moieties were synthesized as reported (30). The detailed total synthesis methods of these molecules are reported elsewhere (31). T-3P2A, T-4P2A, and T-5P2A were designated the acetamido derivatives on the amino group of the lysine residue. The structures were confirmed with electrospray time-of-flight mass spectrometry (ESI-TOF MS) and NMR spectra, and the observed mass spectra were as follows (ESI-TOF MS (negative)): T-2P2, m/z 685.3 [M-2H]+; T-3P2, m/z 834.5 [M-2H]+; T-3P2A, m/z 876.4 [M-2H]+; T-4P2, m/z 905.1 [M-2H]+; T-4P2A, m/z 947.5 [M-2H]+; T-5P2, m/z 976.64 [M-2H]+; and T-5P2A, m/z 1018.5 [M-2H]+. We have made synthetic Lys-PGN fragment (T-2P2, T-3P2, T-4P2, and T-5P2)-immobilized resins by coupling synthetic PGN fragments to CNBr-activated Sepharose following the manufacturer’s instructions. The purified tracheal cytotoxin (TCT: Fig. 1B) from E. coli DAP-PGN was kindly given by Dr. Byung-Ha Oh (Pohang University, Pohang, Korea).

**Purification of Tenebrio PGRP Recognition Protein (Tm-PGRP) and cDNA Cloning of Tm-PGRP**—To purify proteins that can recognize T-4P2 from the hemolymph of T. molitor larvae, 100 ml of crude hemolymph (1300 mg of proteins) was applied to a T-4P2-immobilized...
A cDNA library from *T. molitor* larvae was constructed as previously described (16) by using a ZAP-cDNA synthesis kit (Stratagene). Three internal sequences and N-terminal sequence of the 20-kDa protein were determined. Among them, an oligonucleotide corresponding to DFLQCGVE was synthesized as follows: 5′-GAYTGYTICARTGYG-GIGTIG-3′ and it was labeled with [γ-32P]ATP. We screened 5 × 10^8 colonies and obtained six hybridization-positive clones. We analyzed two plasmids containing three internal sequences and the N-terminal sequence.

**PGN Binding Specificity of the Purified Tm-PGRP**—To examine the binding specificity of synthetic Lys-PGN fragments and insoluble DAP- and Lys-PGN against the purified Tm-PGRP, the binding assay was performed according to our previously published method (17). Briefly, 2 μg of the purified Tm-PGRP was mixed with 40 μl of 50% (v/v) suspension of the Lys-PGN fragment-coupled resins, insoluble DAP-PGN (500 μg) and insoluble Lys-PGN (500 μg) in 50 mM Tris-HCl (pH 7.0) at 4°C overnight with rocking. Unbound Tm-PGRP of the supernatant and bound Tm-PGRP recovered from the resins were analyzed by Western blot analysis using Tm-PGRP antibody. As control, agarose resin (Bio-Rad) and GlcNAc-coupled agarose (Sigma-Aldrich) were treated as described above.

**Expression and Purification of Recombinant Tm-PGRP (r-Tm-PGRP)**—The cDNA encoding the mature Tm-PGRP was subcloned into BamHI and HindIII sites of pFASTBAC-Sa vector as previously described, which was modified from pFASTBAC-HTc (Invitrogen) to insert the Mellitin signal sequence for secretion (32). The recombinant baculovirus for expression of the Tm-PGRP was generated according to the manufacturer’s instructions (Invitrogen). The recombinant baculovirus was amplified using Spodoptera frugiperda 9 (SF-9; Invitrogen) cells in SF-900II serum-free medium (Invitrogen) at 27°C. For the large production of the protein, *Trichoplusia ni* BTI-TN-5B1-4 (High-five; Invitrogen) cells were grown at 27°C. The recombinant virus was amplified using *Trichoplusia ni* BTI-TN-5B1-4 (High-five; Invitrogen) cells in SF-900II serum-free medium (Invitrogen) at 27°C. For the large production of the protein, *Trichoplusia ni* BTI-TN-5B1-4 (High-five; Invitrogen) cells were grown at 27°C. The recombinant protein was eluted with a buffer containing 10 mM sodium phosphate (pH 8.0) and 150 mM NaCl. After clearing by centrifugation, the supernatant was applied to nickel-nitrilotriacetic acid affinity chromatography to purify the r-Tm-PGRP containing the N-terminal hexahistidine tag cleavable by tobacco etch virus (TEV) protease. The recombinant protein was eluted with a buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 200 mM imidazole. The eluted fractions were pooled and stored at ~80°C until use.

**In Vitro Reconstitution Experiments**—Reconstitution experiments were performed with the pass-through fraction from the T-4P_2-coupled column, which is devoid of Tm-PGRP, and the purified Tm-PGRP in the presence of DAP-PGN, Lys-PGN, or β-1,3-glucan to examine whether it was possible to obtain proPO system specific for either DAP-PGN, Lys-PGN or β-1,3-glucan. To ascertain the generation of activated Tenebrio PO from β-1,3-glucan- or PGN-specific melanization fraction, the reaction mixture after activation with PGN or β-1,3-glucan were analyzed on SDS-PAGE under reducing conditions and electrophoretically onto polyvinylidene difluoride membrane. The N-terminal sequences of the generated PO bands on polyvinylidene difluoride membrane were determined as described above.

**RESULTS**

DAP-PGN, Lys-PGN, and β-1,3-Glucan but Not LPS Activate Tenebrio Melanization Cascade—Microbial cell wall components are well known activators of the proPO system leading to melanin synthesis.
induce the activation of the proPO system and melanin synthesis (data not shown). These results suggest that LPS is not responsible for the activation of Tenebrio melanization cascade.

To exclude the possibility that melanin synthesis induced by E. coli DAP-PGN is attributed to the contaminated E. coli LPS, polymyxin B has been added into the reaction mixture to determine whether the melanin synthesis is due to LPS. Polymyxin B is a LPS-binding antibiotic that has been widely used as LPS-neutralizing reagent to avoid the possible interference of LPS contaminations (34, 35). After incubation with soluble DAP-PGN (50 ng) with polymyxin B (10 μg) and then PO activity and melanin synthesis were examined (column 9). The PO activity was not affected by the incubation of polymyxin B, indicating that DAP-PGN is not contaminated with E. coli LPS. This result suggests that DAP-PGN is the activator of the Tenebrio proPO system. However, the ability of DAP-PGN to induce proPO activation was weaker than that of Lys-PGN. Furthermore, because soluble DAP-PGN containing polymeric structures has been shown to trigger melanin synthesis, we tried to find whether the purified monomeric disaccharide tetrapeptide fragment of DAP-PGN, TCT (Ref. 36 and Fig. 1B), can activate the proPO system and melanin synthesis. TCT is a small DAP-PGN-related molecule purified from the culture supernatant of growing Bordetella pertussis that destroys the ciliated epithelial cells lining the large airways of infected individuals. The primary structure of TCT was determined as N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramyl-alanyl-γ-glutamyl-diaminopimelylalane (37). Unlike soluble polymeric DAP-PGN, TCT does not stimulate the melanization cascade (columns 13–15 in Fig. 2B). These data are consistent with a previous report that TCT did not activate silkworm larval plasma, indicating that DAP-PGN recognition by the insect proPO system requires polymeric structures of DAP-PGN (26).

Synthesis of PGN Fragments—To elucidate the molecular mechanisms of how PGN or β-1,3-glucan can activate melanization cascade, it is necessary to obtain a fraction showing specific melanin synthesis by either PGN or β-1,3-glucan. To obtain this fraction, we assumed that if the PGN-coupled column traps PGN recognition molecule(s) of the crude hemolymph without causing activation of the melanization cascade, we can separate the fraction showing a melanin synthesis specifically induced by either PGN or β-1,3-glucan. For this purpose, we have prepared natural soluble Lys-PGN from insoluble Staphylococcus aureus Lys-PGN by the use of ultrasonification device, which is capable of breaking covalent bonds in insoluble Lys-PGN and releasing soluble but high molecular Lys-PGN fragments. The resulting soluble Lys-PGN fragments were then coupled to CNBr-activated Sepharose CL-6B. In this column, the crude hemolymph was passed to separate Lys-PGN recognition molecules and other proPO activation components. Unfortunately, the pass-through fraction of this column showed a strong PO activity, and the PGN recognition molecules were not trapped (data not shown). Because the prepared natural soluble Lys-PGN fraction by ultrasonification contains a mixture of PGN fragments both able to activate and unable to activate the proPO cascade, it cannot be used as ligands for purification of PGN recognition molecule(s) or for separation of other proPO regulatory molecules(s). To investigate the possibility that synthetic Lys-PGN fragments can be used as ligands to isolate PGN recognition molecules, we synthesized seven different PGN fragments (Fig. 1A). A tetrasaccharide with two copies of the key disaccharide GlcNAc-MurNAc were then coupled either with the di-, tri-, tetra-, or pentapeptide moiety.

Synthetic PGN Fragments, T-4P2 and T-5P2, Specifically Inhibit Natural Soluble Lys-PGN-induced Melanization—As shown in Fig. 2B, curdian, natural soluble Lys-PGN from S. aureus or DAP-PGN from...
E. coli induced melanin synthesis in the presence of Ca\(^{2+}\). Under the same conditions the synthetic Lys-PGN fragments were used to examine their capacity to induce PO activity and melanin synthesis. Any synthetic PGN fragments, such as T-2P\(_2\), T-3P\(_2\), T-4P\(_2\), and T-5P\(_2\), did not induce the PO activity in the presence of Ca\(^{2+}\) (columns 5–8 in Fig. 3A, respectively). Also, acetylated PGN fragments, T-3P\(_2\)A, T-4P\(_2\)A, and T-5P\(_2\)A, did not induce any PO activity (data not shown), indicating that all of the tested synthetic Lys-PGN fragments themselves cannot activate the melanization cascade.

To investigate whether these Lys-PGN fragments could function as competitive inhibitors of the Lys-PGN-induced proPO system, we examined the PO activity by co-incubation with synthetic Lys-PGN fragments and natural soluble Lys-PGN. Interestingly, T-4P\(_2\) and T-5P\(_2\) inhibited natural Lys-PGN-induced PO activity (columns 5 and 6 in Fig. 3B), whereas T-2P\(_2\) and T-3P\(_2\) did not affect the activity (columns 3 and 4). The acetylated PGN fragments, T-4P\(_2\)A and T-5P\(_2\)A, also inhibited the PO activity (data not shown). Furthermore, to explore whether PO activity and melanin synthesis induced by natural Lys-PGN also could be inhibited by T-4P\(_2\), in a dose-dependent manner, we examined the PO activity and melanin synthesis potency with different amounts of T-4P\(_2\) in the presence of soluble Lys-PGN. The PO activity and melanin synthesis are induced by 10 ng of natural Lys-PGN were completely inhibited by 1.5 \(\mu\)g of T-4P\(_2\) (Fig. 3, C and D). These results suggest that molecules with two copies of GlcNAc-MurNAc attached to L-Ala-D-isoGln-L-Lys-D-Lys-D-Ala-D-Ala (T-4P\(_2\)) and L-Ala-D-isoGln-L-Lys-D-Lys-D-Ala-D-Ala (T-5P\(_2\)) act as competitive inhibitors of natural Lys-PGN-inducible melanin synthesis but are unable to activate the proPO system. Also, these data show that it might be possible to remove the Lys-PGN recognition molecule(s) without activation of Lys-PGN-dependent proPO cascade by using T-4P\(_2\) or T-5P\(_2\)-coupled column.

**Purification, cDNA Cloning of Tenebrio-PGRP, and Binding Specificity**—When the eluted proteins from the T-4P\(_2\)-coupled column and a control column were analyzed by SDS-PAGE under reducing conditions, a 20-kDa protein was abundant in the eluate fraction compared with that of the control column (lanes 3 and 4 in Fig. 4A, respectively). The 20-kDa protein (arrowhead in lane 3) was further purified by chromatography on columns of Toyopearl HW-55S, hydroxypatite, and Mono-Q (data not shown). To characterize the biochemical properties of the 20-kDa protein, the N-terminal sequence and three internal sequences were determined as follows: N terminus, LSGSTIPRICPEII-RSTWPAGRT; F-1, DFLQXGVELGELS; F-2, NYKLFARQVSTSPGLK; and F-3, LYRELQDWHFTSRPPK. Interestingly, one partial amino acid sequence (F-1) of the 20-kDa protein showed high identity with that of Drosophila melanogaster PGRP-SA (Dm-PGRP-SA; Fig. 4C, deposited as an accession number AB219970 on DDBJ/EMBL/GenBank\(^{TM}\)). The N-terminal sequence and three partial sequences of the 20-kDa protein perfectly matched the deduced amino acid sequences in the open reading frames. Therefore, we designated this 20-kDa protein as *T. molitor* PGRP (Tm-PGRP). It is known that insect and human PGRP domain is similar in structure to N-acetylmuramoyl-L-alanine amidase, such as c\(^7\)-lysozyme (24, 38, 39). Among five amino acids (His-17, Tyr-46, His-122, Lys-128, and Cys-130) in the active site of T7 lysozyme, two residues (His-17 and Tyr-46, indicated by arrows) of T7 lysozyme are conserved in Tm-PGRP, but three residues (His-122, Lys-128, and Cys-130) of T7 lysozyme are not conserved in Tm-PGRP (changed to Ala, Thr, and Ser, respectively; boxes in Fig. 4C). Therefore, it is likely that Tm-PGRP does not have N-acetylmuramoyl-L-alanine amidase activity like the Dm-PGRP-SA.

To further confirm the binding specificity of the purified Tm-PGRP for synthetic Lys-PGN fragments, we prepared synthetic Lys-PGN fragment-coupled Sepharose resins and a control Sepharose resin. Tm-PGRP were detected both in the eluate fraction of T-4P\(_2\) and T-5P\(_2\) resins (lanes 8 and 10 in Fig. 5A) but not in the control, T-2P\(_2\) and T-3P\(_2\) resins (lanes 2, 4, and 6, respectively). Although the binding of Tm-PGRP was detected only in the presence of Ca\(^{2+}\), the extent of this binding was concentration-dependent.
PGRP to T-4P2 is apparently stronger than that to T-5P2, these results further confirmed that T-4P2 and T-5P2 are competitive inhibitors of Lys-PGN-specific melanization cascade as shown in Fig. 3. As Tm-PGRP strongly binds to T-4P2 and T-5P2, we tested the possibility of binding ability of Tm-PGRP against DAP-PGN. Surprisingly, Tm-PGRP also recognized two kinds of DAP-PGNs from E. coli and B. subtilis (lanes 8 and 10 in Fig. 5B). However, the binding ability to DAP-PGNs is weaker compared with natural Lys-PGN (lanes 8, 10, and 6, respectively). Under the same conditions, Tm-PGRP did not bind to agarose and GlcNAc-agarose (lanes 1 and 3). Recently, it was reported that Dm-PGRP-SA binds strongly to DAP-PGN from E. coli and Lactobacillus plantarum (40), even though Dm-PGRP-SA was known as a Lys-PGN recognition molecule (41). This result suggests that Tm-PGRP may function as a common recognition molecule of the DAP- and Lys-PGN-dependent melanization cascade.

In Vitro Reconstitution Experiments—To ascertain the biological function of the Tm-PGRP on proPO system and melanin synthesis, we purified native Tm-PGRP and r-Tm-PGRP to homogeneity from the eluate fraction of T-4P2 column and in a baculovirus expression system, respectively (lanes 1 and 2 in Fig. 6A). To determine the function of the Tm-PGRP on the β-1,3-glucan- and PGN-dependent proPO system, we first performed in vitro reconstitution experiments by using the pass-through fraction from the T-4P2-coupled column, which is devoid of the Tm-PGRP, with purified Tm-PGRPs. As shown in Fig. 6B, both the native Tm-PGRP and r-Tm-PGRP specifically induced PO activity in the presence of soluble Lys-PGN and the pass-through fraction from the T-4P2-coupled column (columns 3 and 7), suggesting that the native Tm-PGRP and r-Tm-PGRP can recognize soluble Lys-PGN and induce Lys-PGN-dependent PO activity in the presence of the pass-through fraction. PO activity was not shown when pass-through solution and Lys-PGN were incubated in the presence of Ca2+ (column 5). Surprisingly, β-1,3-glucan-dependent PO activities could be specifically induced in the pass-through fraction (column 2), indicating that all essential components necessary for the activation of the β-1,3-glucan-dependent proPO system are present in the pass-through fraction. Next, to examine whether Tm-PGRP can work as common DAP- and Lys-PGN recognition molecule on proPO system, we incubated with native or r-Tm-PGRPs, pass-through fraction, and soluble DAP-PGN as described above and then tested PO activities. As expected from our binding experiments, DAP-PGN-dependent PO activities were also induced as those of Lys-PGN (columns 4 and 9). These results are summarized and shown in Fig. 7A. Thus the pass-through fraction of T-4P2-coupled column is designated as a β-1,3-glucan-dependent melanin.
synthesis system, and the mixture of the pass-through fraction and Tm-PGRP is as a PGN-dependent melanin synthesis system.

To further confirm whether proPO can be converted to PO in the β,1-3-glucan- or PGN-dependent proPO system in the presence of β,1-3-glucan, Lys-PGN or DAP-PGN, the reaction mixtures showing the specific PO activities by β,1-3-glucan, Lys-PGN or DAP-PGN were analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 7, β,1-3-glucan, Lys-PGN, or DAP-PGN-dependent proPO fractions generated new protein bands on the gel (bands A, B, and C in lanes 3, 5, and 7, respectively) after 30 min of incubation. When we determined their N-terminal sequences of these three bands, we could confirm that all three N-terminal sequences were perfectly matched with Tenebrio PO (Fig. 7C), which are generated from proPO as we previously reported (42). These results suggest that these proPO system fractions contain all of the essential components necessary for activation by either β,1-3-glucan or Lys-PGN and DAP-PGN, and hence it will be possible to purify and characterize melanization-regulatory proteins involved in these three different melanization cascades as we have demonstrated with Tm-PGRP here.

**DISCUSSION**

Although recent studies strongly imply that pattern recognition proteins, serine proteases, serine protease homologues, and serine protease inhibitors (serpins) are involved as regulatory proteins in the melanin synthesis of invertebrates (9, 12, 19, 43), the molecular mechanisms of the melanization response are not fully understood. In this study, we have demonstrated two novel findings regarding the melanization cascade. One is that T-4P₂, a synthetic fragment of Lys-type PGN can bind to soluble PGRP and can inhibit induction of melanin synthesis by competing with natural PGNs. The other is that Tm-PGRP showing high homology with Dm-PGRP-SA functions as a common PGN recognition molecule of DAP- or Lys-type PGN-dependent melanization cascade. Previously, Ashida and co-workers (23, 44) in 1986 also obtained β,1-3-glucan- and PGN-specific fractions showing PO activity by passing hemolymph of the silkworm by using β,1-3-glucan polysaccharide beads or passing hemolymph over a M. luteus Lys-PGN-coupled Sepharose column, respectively. This pioneering research led to the discovery of the first insect PGRP. They used a soluble M. luteus Lys-PGN generated by a treatment of egg white lysozyme to prepare ligand mixture for preparing the Lys-PGN-coupled column. Recent reports support that such lysozyme treatment might produce a mixture of inactive and active PGN fragments, because lysozyme can induce the degradation of polymeric PGN to monomeric PGN, inducing the loss of capacity of induction for Drosophila immune responses (45) and of the activation of the proPO system (27). This is probably the reason that they were able to obtain a PGN-specific fraction without a subsequent activation of proPO system, because the inactive fragments in their preparation might function as competitive inhibitors of the PGN-dependent proPO system. In contrast, we screened the exact structure of the negative regulator of Lys-PGN-dependent melanization cascade and then prepared the PGN-coupled column by using T-4P₂. Ashida and co-workers did not distinguish the difference between the DAP- and Lys-PGN-dependent proPO pathways because they could not at that time characterize which molecule was working as a DAP-PGN recognition molecule during DAP-PGN-dependent proPO activation pathway. Our study clearly shows that Tm-PGRP functions as a common PGN recognition molecule in the DAP- and Lys-PGN-dependent proPO system.
Competitive Inhibitor on Melanization Cascade

![Diagram of melanization cascade](https://via.placeholder.com/150)

**FIGURE 7.** PGN and β-1,3-glucan-specific melanization pathways, SDS-PAGE pattern of in vitro reconstitution experiments for the generation of active Tenebrio PO, and sequence comparison between bands A, B, and C with Tm-proPO. A, the preparation scheme of PGN and β-1,3-glucan-specific melanization pathways. B, the reaction mixture was analyzed on SDS-PAGE under reducing conditions. Lane 1, the pass-through fraction of the T-4P2-coupled column (10 μg); lane 2, the purified Tm-PGRP (indicated by arrowhead); lane 3, after co-incubation with pass-through fraction (10 μg) of the T-4P2-coupled column and curdian (1 μg) for 1 h, and then the mixture was analyzed by SDS-PAGE under reducing conditions; lane 4, co-incubation with pass-through fraction and soluble Lys-PGN (10 ng) without Tm-PGRP for 1 h, and then this mixture was treated as that in lane 1; lane 5, co-incubation with pass-through fraction, Lys-PGN (10 ng), and Tm-PGRP (0.2 μg); lane 6, co-incubation with pass-through fraction and soluble DAP-PGN (50 ng) without Tm-PGRP for 1 h; lane 7, co-incubation with pass-through fraction, DAP-PGN (50 ng) and Tm-PGRP (0.2 μg). C, the generated bands A, B, and C were transferred to polyvinylidene difluoride membrane, and their N-terminal sequences were determined. The sequences are compared with that of Tm-proPO.

Furthermore, it will be essential to identify and characterize unknown adaptor molecule(s) linking PGN recognition signal between Tm-PGRP and downstream proPO activating factor(s) for determination of the molecular activation mechanism of the proPO system. Also, the determination of the exact agonist structures of Lys- and DAP-PGN is important in understanding the upstream part of the PGN-dependent proPO activation pathway.

Recently, two studies clearly showed that Gram-negative DAP-PGN is the most potent inducer of the IMD pathway, and the Toll pathway is predominantly activated by Gram-positive Lys-PGN, indicating that the ability of *Drosophila* to discriminate between Gram-positive and Gram-negative bacteria relies on the recognition of specific forms of PGN (26, 27). In addition, *Drosophila* PGRP-LC loss-of-function mutations affect the IMD-dependent induction of antibacterial peptides and resistance to Gram-negative bacteria, whereas PGRP-LE binds to the DAP-PGN, and a gain of function mutation induces constitutive activation of both the IMD pathway and melanization (43). In silkworm, the minimum structure of PGN for the induction of antibacterial protein was determined to be two repeating GlcNAc-MurNAc with side chains (45). But the possibility that this structural unit of PGN can work as an agonist or antagonist on the proPO activation cascade was not determined. Interestingly, TCT worked as an activator of the *Drosophila* immune responses but not in the melanization cascade of the insect. These studies indicate that polymer chain size, stem peptide length, and the three-dimensional organization of PGN molecules may be crucial factors in recognition of PGN recognition molecules.

Recently, the crystal structures of human PGRP-S and PGRP-1α with muramyltripeptide were reported (46, 47). In this complex, the tripeptide stem of muramyltripeptide was held in an extended conformation at the deep end of the PGRP-1α binding groove, whereas the MurNAc moiety lies in a pocket in the middle of the groove, with the pyranose ring oriented perpendicular to the base of the pocket, indicating that the protein can accommodate a fourth d-Ala residue making contact with Gln-261, Tyr-266, and Asn-269. On the contrary, fifth d-Ala residue of muramyl Lys-type pentapeptide was expected to extend beyond the binding groove. Another recent surface plasmon resonance study showed that human PGRP-S showed significantly higher affinity for the both the DAP-type muramyl tetrapeptide and Lys-type muramyl tetrapeptide with dissociation constants of $K_d = 62 \mu M$, whereas a much lower affinity for Lys-type muramyl tripeptide was shown (48). Under the same conditions, Lys-type muramyl dipetide, stem pentapeptides of Lys-PGN and DAP-PGN exhibited no binding to human PGRP-S. These data agree with our major two results of this work. First, T-4P2 and T-5P2 can bind to Tm-PGRP, whereas T-3P2 and T-2P2, cannot bind to Tm-PGRP. Second, Tm-PGRP can function as a common recognition molecule of Lys- and DAP-PGN on pro-PO activation cascade system.

Also, it was reported that recombinant human PGRP-S (rhPGRP-S) binds to and inhibits the growth of both *S. aureus* (Lys-PGN) and *E. coli* (DAP-PGN) (49). When they examined the molecular requirement for Lys-PGN binding to rhPGRP-S by using the same PGN fragments (T-2P$_6$, T-3P$_6$, and T-4P$_6$) as used in this study, they observed that T-3P$_2$ showed high affinity binding to rhPGRP-S, whereas muramyl-tripeptide and T-2P$_2$ did not bind to rhPGRP-S, indicating that at least three amino acids in the stem peptide are required for rhPGRP-S binding to T-3P$_2$ ($K_d = 5.50 \times 10^{-8} \pm 3.13 \times 10^{-7} \text{M}$). Interestingly the binding affinity was decreased over 70-fold by T-4P$_2$ ($K_d = 3.69 \times 10^{-9} \pm 2.65 \times 10^{-9} \text{M}$). They suggested that the terminal/fourth d-Ala residue of Lys-type PGN may act to hinder optimal interactions between rhPGRP-S and the L-Lys residue at position three in the peptide stem. These data are different from those obtained in this study. Namely, Tm-PGRP does not bind to T-3P$_2$, but rhPGRP-S binds to T-3P$_2$, and Tm-PGRP strongly recognizes T-4P$_2$ and T-5P$_2$, but rhPGRP-S bind to T-4P$_2$, weakly than T-3P$_2$. The reasons for these differences should be examined in future studies. Finally, because natural bacterial PGNs are well known as strong immunity stimulators (50–52), novel inhibitors of PGN can be useful for preventing excess release of inflammatory cytokine or chemo-kine. Based on the results of this study, it will be interesting to examine the immunostimulatory or immunoinhibitory activities of novel synthetic PGN agonists or antagonists to the natural PGNs in the mammalian immune cell system.

The early events of the melanization cascade can be divided into two parts: the recognition reaction of invading pathogens by pattern recognition proteins and signal transfer to the downstream parts of the proPO activation. Now it will be possible to purify proteins specifically involved in the two melanization pathways as we have done in this study where a PGN-specific PGRP has been characterized and where we demonstrate that the two pathways induced either by PGN or β-1,3-glucan can be separated. Furthermore, this study may help to design insecticidal agents preventing the melanin synthesis that is vital for the survival of insects.
