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Innate immune response in insects: recognition of bacterial peptidoglycan and amplification of its recognition signal

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The major cell wall components of bacteria are lipopolysaccharide, peptidoglycan, and teichoic acid. These molecules are known to trigger strong innate immune responses in the host. The molecular mechanisms by which the host recognizes the peptidoglycan of Gram-positive bacteria and amplifies this peptidoglycan recognition signals to mount an immune response remain largely unclear. Recent, elegant genetic and biochemical studies are revealing details of the molecular recognition mechanism and the signalling pathways triggered by bacterial peptidoglycan. Here we review recent progress in elucidating the molecular details of peptidoglycan recognition and its signalling pathways in insects. We also attempt to evaluate the importance of this issue for understanding innate immunity. [BMB reports 2008; 41(2): 93-101]

Innate immunity is activated by distinct pathogen-associated molecular patterns (PAMPs)

Innate immunity is a crucial defense mechanism against microbial infection found in all animals. The ability of a host to distinguish between self and non-self remains a central hallmark of innate immunity (1). Pathogenic microbes possess distinct pathogen-associated molecular patterns (PAMPs), such as the lipopolysaccharide (LPS) of Gram-negative bacteria, the peptidoglycan (PG) of Gram-positive bacteria, and the β-1,3-glucan of fungi (1, 2). Recognition of these PAMPs is achieved by a group of germ-line-encoded receptors and soluble proteins (2). Innate immunity in invertebrates consists of humoral and cellular components (3, 4). The humoral component includes antimicrobial peptides (5), lectins (6), and the prophenoloxidase (proPO) activation cascade (7); the cellular component includes phagocytosis by circulating hemocytes (8). In *Drosophila*, the Toll signalling pathway is responsible for defense against Gram-positive bacteria and fungi, while the immune deficiency (Imd) pathway is activated primarily to defend against Gram-negative bacteria. Both of these pathways use NF-κB-like transcription factors for the induction of the expression of antimicrobial peptides (5, 9-12).

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How is Lys-type PG recognized in *Drosophila*?

Bacterial PG is a polymer consisting of glycan strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that are cross-linked to each other by short peptide bridges (13, Fig. 1). PGs from Gram-negative bacteria and *Bacillus* species differ from other Gram-positive PGs in that lysine (Lys) is replaced with *meso*-diaminopimelic acid (DAP) at the third amino acid in the peptide chain (Fig. 1B). PG is known to stimulate the production of inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α in monocytes, macrophages, and neutrophils (14). In insects, two different PG recognition systems are present, one for the induction of antimicrobial peptides and the other for activating the proPO cascade leading to melanization. A PG recognition protein (PGRP) was first identified in the silkworm *Bombyx mori* following purification of a protein from hemolymph that binds to Lys-type PG and activates the proPO cascade system (15). Recent studies have demonstrated that several PGRPs bind directly to PG, each with distinct preferences for binding Lys-type PGN or DAP-type PGN (16-18). Elegant genetic studies recently carried out in *Drosophila* revealed that *D. melanogaster* PGRP-SA (Dm-PGRP-SA) and -SD activate the Toll pathway (19, 20), while Dm-PGRP-LC and Dm-PGRP-LE serve as receptors for the Imd pathway (21-24). The immune phenotype of a loss-of-function mutant of Dm-Gram-negative bacteria binding protein1 (Dm-GNBP1) was indistinguishable from that of Dm-PGRP-SA, demonstrating that the two proteins are required to activate the Toll pathway in response to Gram-positive bacterial infection (25, 26). However, functional cross-talk between Dm-PGRP-SA and Dm-GNBP1 during a Lys-type PGN recognition signal has not been determined yet. Recognition of Lys-type PG by the PG recognition complex has been suggested to activate the serine protease (SP) cascade, leading to the processing of Spätzle (Spz) and subsequent activation of the Toll signalling pathway (27, 28).

Although an increasing amount is known about the functions of Lys-type PG and β-1,3-glucan recognition proteins

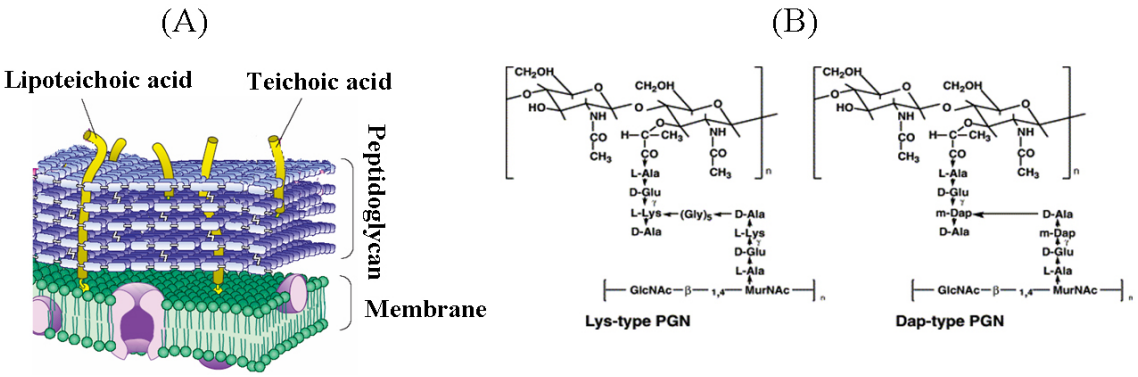

Fig. 1. Cartoon representation of the Gram-positive bacteria cell wall (A) and primary structures of Lys-type and DAP-type PG (B).

such as PGRP-SA and GNBP3, respectively, which activate the Toll cascade (27, 29), details of how the extra-cellular SP cascade in the Toll pathway is activated have not been clearly resolved. At present, several SPs and SP homologues (SPHs) have been identified as regulators of the Toll pathway (28-31). Among them, Dm-Spz-processing enzyme (SPE) was identified and reported to function as a processing enzyme to cleave Spz pro-protein to active Spz (28). Even though the biological functions of SPE have been characterized in *Drosophila* genetic studies, the identification of a direct upstream activator of SPE awaits further investigation.

Two crystal structures were recently reported: the PGRP domain of Dm-PGRP-LE in complex with tracheal cytotoxin (TCT), a naturally occurring monomer unit of DAP-type PG (32); and the ectodomains of Dm-PGRP-LCa and Dm-PGRP-LCx in complex with TCT (33). These two reports provided the first evidence that protein-protein interactions induced by the monomeric DAP-type PG from Gram-negative bacteria is likely to be essential to activation of the Imd pathway. However, the details of the upstream part of the Toll pathway for recognizing Gram-positive bacteria and the upstream part of the Imd pathway for recognizing Gram-negative bacteria remain unknown. By using *Drosophila* system, it may be difficult to provide detailed insights into what kinds of adaptor proteins are bound to the Dm-PGRP-SA/Dm-GNBP1 complex during Toll signalling.

Three essential, upstream protein components involved in Lys-type PG recognition have been obtained from a large insect, *T. molitor* larvae

Even though *Drosophila* genetic studies are very powerful for characterizing and ordering the components in the Toll pathway (5, 34), the system is limited in its ability to reveal the biochemical mechanisms that regulate this proteolytic cascade. *Drosophila* uses several alternative routes to the Toll pathway during various developmental stages and under different infection protocols, and it seems difficult to determine the clear activation mechanism of Toll pathway. For instance, Dm-Persephone is another SP linked to the Toll pathway and antifungal immunity. Its biological functions have been partially characterized through *Drosophila* genetic studies, but the proper identification of upstream or downstream factor(s) of Dm-Persephone requires further investigation (29, 35). In order to provide compelling biochemical data on how the Lys-type PG recognition signal can be sequentially transferred to Spz, it is necessary to use a larger insect, which enables the collection of large amounts of hemolymph and the purification of essential components involved in the PG recognition signaling pathway. To purify these components from the larvae of the large beetle *Tenebrio molitor*, we routinely begin our purifications with four liters of hemolymph (10 μl from one larva). This approach is certainly impossible in the *Drosophila* system. Using this approach we provided compelling biochemical data on how the PG recognition signal can be sequentially transferred to Spz, thereby leading to the production of antimicrobial peptides (36). The domain structures of the purified components from *T. molitor* larvae are shown in Fig. 2.

T. molitor-PGRP-SA (Tm-PGRP-SA, Fig. 2a) was purified using a novel synthetic Lys-type PG fragment [(GlcNAc-MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala)$_2$, T-4P$_2$], which functions as a competitive inhibitor of the natural Lys-type PG-induced melanization reaction (37). Using a T-4P$_2$-coupled column, Tm-PGRP-SA was purified, and the purified Tm-PGRP-SA recognized both Lys-type and DAP-type PG. *In vitro* reconstitution experiments also showed that Tm-PGRP-SA functions as a common recognition molecule in both Lys-type and DAP-type PG. It is known that insects and human PGRPs have a catalytic domain similar to that of bacteriophage T7 lysozyme (18). Among the five catalytically essential residues in the active site of T7 lysozyme, two are conserved in Tm-PGRP-SA, but three are not. Therefore it is likely that Tm-PGRP-SA does not have the same N-acetylmuramyl-L-alanine amidase activity as *Drosophila* PGRP-SA. In addition, we demonstrated that when Tm-PGRP-SA-coated insoluble Lys-type PG prepared from *Staphylococcus aureus* was co-incubated with *Tenebrio* hemolymph, Tm-GNBP1 and a modular serine protease (MSP) were recruited on the

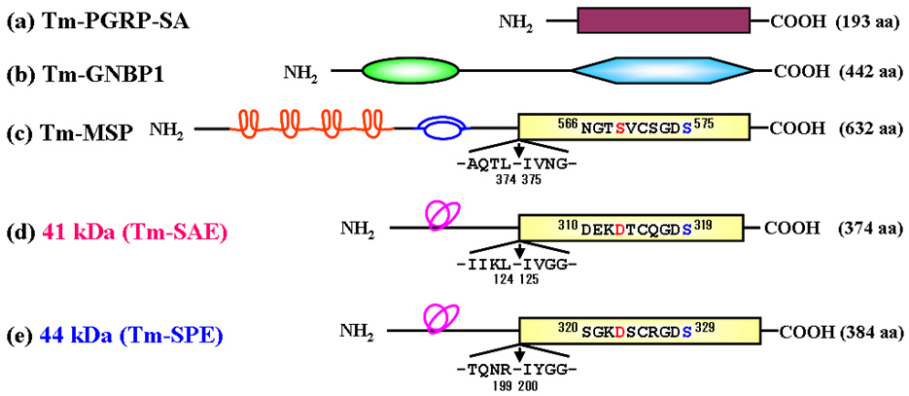

Fig. 2. The domain structures of purified five proteins. (a), Tm-PGRP-SA; (b), Tm-GNBP1, (c), Tm-MSP; (d), Tm-SAE; (e), Tm-SPE; *Purple rectangular, oval* and *diamond symbols* indicate the domains of PGRP, GNBP homology domain and glucanase-like domain, respectively. *Rabbit ears, half-double circles, yellow rectangular* and *moon symbols* indicate the domains of LDLa, CCP domain, SP domains of SPs and clip domain, respectively. The *red* and *blue* residues in the boxes indicate the specificity-related residue and catalytic triad Ser residue, respectively.

Tm-PGRP-SA-coated insoluble Lys-type PG (38). This result suggests that the Lys-type PG/PGRP-SA complex recruits Tm-GNBP1 and Tm-MSP in the beetle system, and this Lys-type PG/PGRP-SA/GNBP1/MSP complex serves as the initial activator that triggers SP cascades in the Lys-type PG recognition signalling pathway.

The deduced amino acid sequence of Tm-GNBP1 shows sequence homology with known insects' GNBP family proteins consisting of a GNBP homology domain and a β-1,3-glucanase-like domain (Fig. 2b). Tm-MSP consists of four low-density lipoprotein receptor A repeat (LDLa) domains, one complement control protein (CCP) domain, and an SP domain. This result suggests that the 82-kDa Tm-MSP zymogen functions as an initial enzyme in the Lys-type PG recognition signalling pathway mediated by Tm-PGRP-SA/Tm-GNBP1 (38). In addition, the biochemical experiments supported that the Tm-MSP zymogen was cleaved between the Leu374 and Ile375 residues located between the CCP domain and catalytic SP domain, suggesting that Tm-MSP might be cleaved by a chymotrypsin-like SP (Fig. 2c).

Three novel SP zymogens involved in PG recognition signalling also have been purified from the hemolymph of *T. molitor* larvae

Previously, we observed that when a commercially available α-thrombin fluorescence synthetic peptide substrate, Boc-Val-Pro-Arg-MCA, was co-incubated with the *Tenebrio* hemolymph in the presence of bacterial PG or fungal β-1,3-glucan, it was specifically hydrolyzed within 10 min (37-39). This result suggested that measurement of this amidase activity would be a suitable method for purifying unidentified SP zymogens from *Tenebrio* hemolymph (insect blood). Using this bioassay, three different SP zymogens and their active forms (Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteins) were purified to homogeneity from the hemolymph of *T. molitor* larvae after extensive column chromatography (36).

To determine which components are necessary for PG-dependent amidase activity, *in vitro* reconstitution experiments were performed. When five proteins -Tm-PGRP-SA, Tm-GNBP1, Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteins- were co-incubated in the presence of Lys-type PG and Ca^{2+}, PG-specific amidase activity on an α-thrombin substrate was detected. This result suggests that these five proteins are the minimal essential factors necessary for PG recognition. To predict the biological functions and ordering of Tm-MSP, Tm-41 kDa and Tm-44 kDa proteins during Lys-type recognition signalling pathway, we first examined the amino acid sequence homology and domain organization of these SP proteins. The deduced amino acid sequence of Tm-41 kDa protease consists of an N-terminal clip domain and a C-terminal SP domain and shows greatest homology (40% identity) to Dm-Snake (40). Interestingly, the cleavage site of the Tm-41 kDa zymogen was experimentally determined to lie between the Leu124 and Ile125 residues located between the clip domain and the catalytic SP domain (Fig. 2d). The deduced amino acid sequence of the Tm-44 kDa protein also contains an N-terminal clip domain in addition to a C-terminal SP domain with trypsin-type specificity pocket residues. This SP shows 49% sequence identity to Dm-Easter (40). The cleavage site of the Tm-44 kDa zymogen was experimentally determined to lie between Arg199 and Ile200 (Fig. 2e), suggesting that a trypsin-like SP cleaves the Tm-44 kDa zymogen.

To investigate the Toll-mediated PG recognition signalling pathway in *Tenebrio* system, we needed to identify Tm-Spz and Tm-Toll proteins. Since the cDNAs of Tm-Spz and Toll proteins have not yet been determined, we expressed two *Tribolium castneum* proteins in a baculovirus culture system: Tc-Spz pro-protein (XP_975083), encoding 208 amino acids from Phe19 to Asn227; and the ectodomain of Tc-Toll

(XP_967796), encoding 757 residues from Leu20 to Leu777. Because *T. molitor* and *T. castneum* are both coleopteran beetle species belonging to the same *Tenebriodae* family, we reasoned that *Tribolium* Spz and Toll proteins would be recognized by SPs purified from *Tenebrio*. The soluble recombinant Tc-Spz and Tc-Toll ectodomains were purified to homogeneity (36). The purified Tc-Spz pro-protein was synthesized as a disulfide-bonded dimer, consistent with previous Dm-Spz studies (41, 42). The purified Tc-Toll ectodomain showed a molecular mass of 120 kDa, and its identity was confirmed by N-terminal sequencing (data not shown).

The purified Tm-44 kDa SP functions as a Tm-SPE and the cleaved Spz induces strong antibacteial activities

The key question that emerged in the course of our work was whether we could identify the Tm-SPE molecule that can convert Spz pro-protein to active Spz. As a potential candidate, we selected active form of Tm-44 kDa protein. To address whether the purified, active form of Tm-44 kDa protease cleaves Tc-Spz pro-protein *in vitro*, we incubated Tc-Spz pro-protein with trypsin or with the active forms of Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteases. Only the active Tm-44 kDa protease converted Tc-Spz pro-protein to products of 14 kDa and 24 kDa under reducing and non-reducing conditions, respectively. Under the same conditions, trypsin cleaved the Tc-Spz pro-protein non-specifically, while active forms of Tm-MSP and Tm-41 kDa protease did not cleave Tc-Spz pro-protein at all. Therefore, we named the Tm-44 kDa protease as Tm-SPE.

To examine the biological function of the cleaved form of Tc-Spz *in vivo*, the 24 kDa purified Tc-Spz protein was injected into *Tenebrio* larvae and *Tribolium* adults to check its antibacterial activities. Thirty-six hrs after injection, the hemolymph from the *Tenebrio* larvae injected with 24-kDa Tc-Spz and the extracts from the *Tribolium* adults injected with the 24-kDa Tc-Spz were collected and their bactericidal activities against *S. aureus* were estimated. The strongest bactericidal activity was induced by injecting cleaved Tc-Spz or heat-treated cleaved Tc-Spz into *Tenebrio* larvae. Using an inhibition zone assay, the extracts from the *Tribolium* adults injected with cleaved Tc-Spz or heat-treated cleaved Tc-Spz also showed antibacterial activity against *S. aureus*, indicating that cleaved Tc-Spz is biologically active *in vivo*. These results clearly suggest that Tc-Spz protein processed by Tm-SPE can activate *Tenebrio* and *Tribolium* Toll cascades *in vivo*.

The active Tm-41 kDa protease is a direct upstream activator of Tm-SPE

We next sought to identify a direct upstream activator of Tm-SPE. Since Tm-SPE has a trypsin-type cleavage site, we proposed that the upstream SP of Tm-SPE could be a trypsin-like SP. As mentioned above, the fact that the Tm-MSP and Tm-41 kDa zymogens were determined to be chymotrypsin-like and trypsin-like SPs, respectively (Fig. 2c and 2d), suggested that the active form of Tm-41 kDa protease may cleave Tm-SPE zymogen. To test this hypothesis, we incubated the activated Tm-41 kDa protein with purified, native Tm-SPE zymogen. Tm-SPE zymogen was hydrolyzed to a 35-kDa SP domain. The amino acid sequence of the 35-kDa band generated from the Tm-SPE zymogen was in complete agreement with the SP domain of Tm-SPE protein (data not shown). Therefore, based on these results, we designated the 41 kDa protease as *Tenebrio* SPE-activating enzyme (Tm-SAE).

Tm-SAE zymogen is activated by the active form of Tm-MSP

Given that the cleavage site of the Tm-SAE zymogen was determined to be Leu124-Ile125, the upstream SP of Tm-SAE should have chymotrypsin-like substrate specificity. This suggested that the activated Tm-MSP cleaved the Tm-SAE zymogen. To explore this possibility, activated Tm-MSP was incubated with recombinant Tm-SAE zymogen. As expected, the activated Tm-MSP caused a limited and specific proteolytic cleavage between the clip domain and catalytic SP domain of the Tm-SAE zymogen. This result demonstrated that the Tm-SAE protease is an immediate downstream factor of Tm-MSP. Furthermore, when we re-examined the cleavage specificities of these three proteases by incubating activated Tm-MSP with Tm-SPE zymogen or by incubating activated Tm-SPE and Tm-SAE zymogens, both zymogens remained uncleaved (36), confirming that Tm-SPE is not an immediate downstream protease of MSP.

Tm-MSP zymogen is activated after binding to Lys-type PG/Tm-PGRP-SA/Tm-GNBP1 complex in the presence of Ca^{2+}

To examine whether the Tm-MSP zymogen really acts as an initial enzyme and to address the conditions necessary for activation of the Tm-MSP zymogen, we incubated the purified Tm-MSP zymogen, recombinant Tm-PGRP-SA, and native Tm-GNBP1 in the presence of Lys-type PG and Ca^{2+}. Tm-MSP zymogen was activated after direct binding to the Lys-type PG/Tm-PGRP-SA/Tm-GNBP1 complex in the presence of Ca^{2+}. These data imply that the initial recognition signal against Lys-type PG is tightly regulated by the Tm-PGRP-SA, Tm-GNBP1, and Tm-MSP molecules, indicating that once the active form of Tm-MSP is generated, the immediate downstream SP of the active form of Tm-MSP is activated regardless of the Tm-PGRP-SA and Tm-GNBP1 proteins (Fig. 3). This result clearly shows that the whole Lys-type PG/Tm-PGRP-SA/Tm-GNBP1 complex is required for the cleavage and activation of Tm-MSP zymogen. A similar activation pattern to our study was also observed in the human MSP-like protease, mannose-binding lectin (MBL)-associated SP-2 (MASP-2) (43). Human MASP-2 is known to be activated after binding to mannose-bound MBL complex in the presence of Ca^{2+}. The subsequently activated MASP-2 hydro-

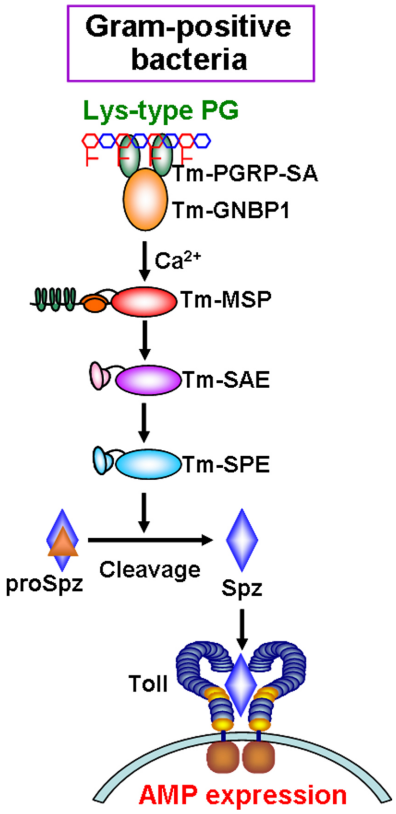

Fig. 3. A model summarizing the three-step activation cascade of the Lys-type PG recognition signalling pathway. When the host is exposed to processed Lys-type PG from Gram-positive bacteria, Tm-PGRP-SA binds to Lys-type PG and then recruits Tm-GNBP1 and the Tm-MSP zymogen. In the presence of Ca^{2+}, the PG/Tm-PGRP-SA/Tm-GNBP1 complex induces activation of the Tm-MSP zymogen to activated Tm-MSP. The active form of Tm-MSP activates the conversion of Tm-SAE zymogen to activated Tm-SAE, which subsequently converts the Tm-SPE zymogen to activated Tm-SPE protease. The active Tm-SPE cleaves Tc-Spz pro-protein to yield processed Tc-Spz, leading to the production of AMP(s).

lyzes the downstream complement factor C4 to C4a and C4b in the human lectin complement pathway.

A three-step proteolytic cascade mediates the activation of the Lys-type PG-induced Toll pathway in *T. molitor* larvae

The *in vitro* determination of the sequential biochemical functions of these three SPs suggests that the activation of a three step-proteolytic cascade is necessary and sufficient for Lys-type PG recognition signalling. To confirm further whether the Tm-SPE zymogen is really cleaved by the combination of the seven components, Lys-type-PG, Tm-PGRP-SA, Tm-GNBP1, and zymogens of Tm-MSP, Tm-SAE, and Tm-SPE were co-incubated in the presence of Ca^{2+}. The specific cleavage of Tm-SPE zymogen was detected by Western blot and by direct measurement of amidase activity. As expected, a band of 35 kDa corresponding to the SP domain of Tm-SPE was specifically observed with amidase activity. Omitting any one of the components from the incubation mixture resulted in no cleavage of the Tm-SPE zymogen (36). These experiments demonstrate that Lys-type PG recognition signalling mediated by Tm-PGRP-SA/Tm-GNBP1 involves a relay of three different SPs: the initial enzyme is the 82-kDa, chymotrypsin-like Tm-MSP, and the second Tm-SAE and third Tm-SPE are the Tm-41 kDa and the Tm-44 kDa clip domains containing trypsin-like SPs, respectively. Based on these our results, we propose that this PGRP-SA/GNBP1/MSP/SAE/SPE/Spz cascade is an essential unit that triggers the Lys-type PG recognition signalling pathway in response to Gram-positive bacteria infection in insects (Fig. 3).

A linearized Lys-type PG activates the Toll and prophenoloxidase (proPO) pathways

To determine the minimal concentration of Lys-type PG required for activation of the PG-dependent innate immune reactions, such as the proPO or Toll activation cascades, we prepared biologically active, soluble Lys-type PG. Because it was quite difficult to estimate the minimal concentration of the insoluble, natural Lys-type PG, we used *Achromobacter* β-lytic protease (blp), which is a lysostaphin-like enzyme that hydrolyzes the peptide bonds in the penta-Gly bridge present in *S. aureus* Lys-type PG (44). Using blp, we solubilized *S. aureus* Lys-type PG by cleaving the penta-Gly bridge between the stem peptides, generating a linearized PG containing a long glycan chain with stem peptides. We reasoned that this linearized PG would provide multiple binding sites for Tm-PGRP-SA, since it contained many copies of the muropeptide units. As expected, we confirmed that both the recombinant Dm-PGRP-SA and Tm-PGRP-SA proteins bound to the linearized PG (38).

After observing these binding activities, we wondered whether the linearized PG would also stimulate the insect innate immune system. To address these points, we studied two innate immune responses: the well-characterized *in vivo Drosophila* Toll pathway to examine antibacterial peptide expression, and the *Tenebrio* proPO cascade system leading to synthesis of melanin pigment in the large beetle *in vitro*. We found that the linearized PG consistently induced a strong phenoloxidase (PO) activity when the fractionated, linearized PG was added to *Tenebrio* hemolymph solution, which indicated that linearized PG can activate PG-dependent proPO cascade. Next, we injected the linearized PG into wild-type and *PGRP-SA*^{seml} mutant flies and then monitored expression of the gene encoding drosomycin in order to test whether the linearized PG could activate the *Drosophila* Toll pathway *in vivo*. Indeed, the wild-type flies injected with linearized PG induced normal drosomycin expression, but *PGRP-SA*^{seml} mutant flies were defective in the induction of the antimicrobial peptide, demonstrating that the linearized PG activates the Toll pathway in a Dm-PGRP-SA-dependent manner. Likewise, the

linearized PG strongly induced melanin synthesis when injected into the larvae of *Tenebrio*, most likely by activation of the proPO. These results suggest that the PG fragment containing multiple binding sites for Tm-PGRP-SA can induce both the Toll and proPO pathways *in vivo*.

Clustering of Tm-PGRP-SA is needed for activation of the Lys-type PG signalling pathway

We initially tried to determine the minimal concentration of the linearized PG for activating the PG-dependent proPO cascade. The PO activity was measured by incubation of the hemolymph solution with different amounts of linearized PG. Unexpectedly, high concentrations of the linearized PG were found to inhibit PO activity down to baseline levels; the dose-response curve showed a classic bell shape (38). Moreover, addition of exogenous *Tenebrio* PGRP-SA protein to the reaction mixture could significantly shift the concentration of the linearized PG where maximal PO activity was produced and thereby produce stronger PO activity. This shift in optimal concentration of linearized PG by addition of Tm-PGRP-SA protein suggests that the molar ratio between Tm-PGRP-SA and Lys-type PG is important in the activation of the proPO cascade. These observations imply that excess linearized Lys-type PG acts as a competitive inhibitor by sequestering Tm-PGRP-SA molecules, thereby impairing the initial activating complex composed of clustered PGRP-SA molecules bound to one linearized PG molecule. Similar observations were reported in the recognition of β-1,3-glucan by Factor G in horseshoe crab, and in the recognition of LPS by the proPO system of crayfish (45, 46).

Lys-type PG fragments that accommodate at least two Tm-PGRP-SA molecules activate the proPO system

To determine how many molecules of Tm-PGRP-SA constitute the initial activating complex for the proPO system, various lengths of sugar chains in Lys-type PG were generated by partial lysozyme digestion of linearized PG. The digested products were fractionated according to their length on a size-exclusion column. Three active fractions showed PO activity when each fraction was incubated with hemolymph solution depleted of Tm-PGRP-SA in the presence of Tm-PGRP-SA protein and Ca^{2+}. We added the fraction with the smallest PG fragments to an excess of *Tenebrio* PGRP-SA protein, and analyzed how many Tm-PGRP-SA molecules bound to the fragments by monitoring the apparent molecular weight on a size-exclusion column. The apparent molecular weight of the complex between the Lys-type PG fragments in the fraction and Tm-PGRP-SA was determined to be ~40 kDa, which indicates that the Lys-type PG fragment binds to two molecules of PGRP-SA. When the complex of PG fragment and Tm-PGRP-SA was isolated from the second size-exclusion column, it was incubated with the hemolymph solution depleted of Tm-PGRP-SA and found to induce PO activity even without adding Tm-PGRP-SA (38). This result clearly demonstrates that two molecules of Tm-PGRP-SA are sufficient to induce this activity. We therefore conclude that the PG fragment that accommodates two PGRP-SA molecules is the minimum unit that can induce downstream events and lead to activation of the Toll and prophenoloxidase pathways.

Lysozyme presents a processed form of Lys-type PG for PG recognition signalling pathways

Most natural Gram-positive Lys-type PG is highly cross-linked between the glycan chains (Fig. 1A), which is a different structure from that of linearized PG. We anticipated that Tm-PGRP-SA would have limited access to natural Lys-type PGs because of the highly cross-linked structures of PG. Moreover, we previously observed that insoluble Lys-type PGs disrupted by sonication induced a strong PO activity *in vitro*, whereas intact insoluble Lys-type PGs did not induce prophenoloxidase activation under the same conditions (37). In order to loosen the PG structure using an enzyme present in insect hemolymph, we chose lysozyme because it can hydrolyze almost all types of intact bacterial PG (47). We performed partial lysozyme digestion of Lys-type PG from both *S. aureus* and *Micrococcus luteus in vitro*. Indeed, the partially-digested Lys-type PGs induced a rapid and strong PO activity in the *Tenebrio* hemolymph *in vitro*. Moreover, when the partially-digested insoluble Lys-type PGs were injected into *Tenebrio* larvae, stronger and faster melanin synthesis was observed in all of the injected larvae compared with larvae injected with intact, insoluble Lys-type PGs (38). However, when the lysozyme inhibitor N, N′, N″-triacetylchitotriose (48) was co-injected with intact Lys-type PG, no melanin synthesis could be observed. These observations strongly suggest that prior partial degradation of Lys-type PG by lysozyme is necessary for activation of the innate immune response, such as the proPO cascade.

To ascertain the role of lysozyme in the recognition of Lys-type PG by PGRP-SAs *in vitro*, we examined the ability of PGRP-SAs to bind to the partially digested Lys-type PGs using Dm-PGRP-SA and Tm-PGRP-SA (38). To our surprise, the partial lysozyme digestion of Lys-type PG dramatically increased the binding of both Dm-PGRP-SA and Tm-PGRP-SA to Lys-type PG. The enhanced interaction between PGRP-SAs and Lys-type PG is expected to result in clustering of PGRP-SAs in PG, leading to activation of the Toll and proPO pathways. Our study presents *in vitro* biochemical evidence that lysozyme plays a crucial role in enhancing the access of Dm-PGRP-SA or Tm-PGRP-SA to insoluble Lys-type PG in the Toll and proPO pathways. Nevertheless, the data so far do not allow us to exclude the possibility that other proteins showing lysozyme-like activity process PGs for PGRP-SA binding *in vivo*.

Concluding remarks

We purified and cloned three different upstream SPs from the hemolymph of a large beetle *T. molitor* larvae; we named the enzymes Tm-MSP, Tm-SAE, and Tm-SPE. By *in vitro* reconstitution experiments, we demonstrated that the activation of three-step proteolytic cascade is necessary and sufficient for induction of Lys-type PG recognition signalling pathway. Spätzle processed by this cascade induces antibacterial activity *in vivo*. These experiments provide the first biochemical insights into how the PGRP-SA-mediated Lys-type PG recognition signalling pathway modulates Spätzle activity, leading to antimicrobial activity *in vivo*.

Our results do not seem to coincide with those recently reported by Kambris *et al*. (31). They suggested that two *Drosophila* catalytic SPs (Dm-Grass and Dm-Spirit), as well as two non-catalytic SPHs, such as Dm-Spheroide and Dm-Sphinx1/2, are involved in the PG-dependent *Drosophila* Toll pathway. Homology searches against known SPs indicate that Dm-Grass and Dm-Spirit are trypsin-like SPs containing clip domains. On the other hand, Dm-Spheroide and Dm-Sphinx1/2 have a non-catalytic SP domain with no-clip domains at the N-terminus, and they have Gly and Ile residues, respectively, in lieu of the Ser residue normally found in the catalytic site of SPs. Why we do not identify these similar SPs and SPHs in our studies is unclear and further studies are needed to answer this question. Nevertheless, one plausible explanation may be that *Tenebrio* SPHs exist with serpins in the naïve hemolymph but are not directly involved in Toll pathway activation. Horseshoe crab factor D, an SPH-like molecule in *Limulus*, was co-purified with a horseshoe crab serpin (49), suggesting that SPH may form a complex with serpin *in vivo*. RNAi experiments against *Drosophila* SPs and SPHs support the possibility that serpin may be released into hemolymph in the absence of SPH, and that the released serpins may trap catalytic SPs like Dm-Grass and Dm-Spirit, leading to inhibition of Toll pathway activation.

Additional work is needed to completely understand the molecular events of Gram-positive bacteria infection in insects. We demonstrate that three SPs are sufficient to mediate Spz maturation, but we have not established whether they are required *in vivo*. Monitoring the effect of the inactivation of these SPs by RNAi or other approaches may help to verify the conclusions of our work. In addition, determining how serpin(s) regulate this cascade could provide clear answers on how insects defend their bodies against infection by Gram-positive bacteria infection and how innate immune responses are tightly regulated so that they do not become over-activated and thereby damage the host.

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