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# A Three-step Proteolytic Cascade Mediates the Activation of the Peptidoglycan-induced Toll Pathway in an Insect*[S]

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**The recognition of lysine-type peptidoglycans (PG) by the PG recognition complex has been suggested to cause activation of the serine protease cascade leading to the processing of Spätzle and subsequent activation of the Toll signaling pathway. So far, two serine proteases involved in the lysine-type PG Toll signaling pathway have been identified. One is a modular serine protease functioning as an initial enzyme to be recruited into the lysine-type PG recognition complex. The other is the *Drosophila* Spätzle processing enzyme (SPE), a terminal enzyme that converts Spätzle proprotein to its processed form capable of binding to the Toll receptor. However, it remains unclear how the initial PG recognition signal is transferred to Spätzle resulting in Toll pathway activation. Also, the biochemical characteristics and mechanism of action of a serine protease linking the modular serine protease and SPE have not been investigated. Here, we purified and cloned a novel upstream serine protease of SPE that we named SAE, SPE-activating enzyme, from the hemolymph of a large beetle, *Tenebrio molitor* larvae. This enzyme was activated by *Tenebrio* modular serine protease and in turn activated the *Tenebrio* SPE. The biochemical ordered functions of these three serine proteases were determined *in vitro*, suggesting that the activation of a three-step proteolytic cascade is necessary and sufficient for lysine-type PG recognition signaling. The processed Spätzle by this cascade induced antibacterial activity *in vivo*. These results demonstrate that the three-step proteolytic cascade linking the PG recognition complex and Spätzle processing is essential for the PG-dependent Toll signaling pathway.**

Innate immunity is a crucial host defense mechanism against microbial infection 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 lipopolysaccharides of Gram-negative bacteria, peptidoglycans (PG)[2] of Gram-positive bacteria, and the $\beta$-1,3-glucan of fungi (2). The recognition of these PAMPs is achieved by a group of germ line-encoded receptors and soluble proteins (2). The *Drosophila* Toll signaling pathway is responsible for defense against Gram-positive bacteria and fungi, while the immune deficiency (Imd) pathway is activated primarily in defense against Gram-negative bacteria. Both of these pathways lead to the expression of antimicrobial peptides (AMPs) via NF-$\kappa$B-like transcription factors (3–6).

In insects, bacterial lysine (Lys)-type PGs are recognized by the PG recognition protein-SA (PGRP-SA) and Gram-negative-binding protein 1 (GNBP1) (7–9). These proteins are believed to mediate the activation of a serine protease (SP) cascade and ultimately, the cleavage of Spätzle (Spz). Cleaved Spz serves as a ligand for the cell membrane receptor Toll and induces the production of AMPs (6, 10). *Drosophila melanogaster* GNBP3 (Dm-GNBP3), however, is known to be required for the detection of fungal cell wall components in the Toll signaling pathway (11). These recognition signals against bacteria and fungi are amplified in hemolymph (insect blood) by a proteolytic SP cascade similar to the vertebrate complement system (6). The amplification of these recognition signals represents an efficient host defense strategy in insects, which are devoid of an acquired immune system.

Although information regarding the functions of PAMP recognition proteins, such as PGRP-SA and GNBP3, which lead to the Toll activation cascade are continuously increasing (6, 11), details of the activation mechanism of the extracellular SP cascade in the Toll pathway have not been clearly resolved. At present, several SPs and SP homologues have been identified as Toll pathway regulating factors (11–14). Among them, Dm-Spz-processing enzyme (SPE) was identified, and its biological function was determined as a processing enzyme to cleave Spz pro-protein to active Spz (13). Even though the biological func-

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*The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) Tm-SAE, AB363979; Tm-SPE, AB363980; Tm-GNBP1, AB363981; and Tm-MSP, AB363982.*

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental methods S1–S13, Figs. S1–S4, and Tables S1–S4.

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[2] The abbreviations used are: PG, peptidoglycan; PGRP, peptidoglycan-recognition protein; GNBP1, Gram-negative bacteria-binding protein 1; SP, serine protease; Spz, Spätzle; SPE, Spz-processing enzyme; MSP, modular serine protease; SAE, SPE-activating enzyme; LDLa, low density lipoprotein receptor A repeats; CCP, complement control protein; AMP, antimicrobial peptide.

tions of SPE have been characterized by *Drosophila* genetic studies, the identification of a direct upstream activator of SPE still awaits further investigation. We recently demonstrated in a large beetle, *Tenebrio molitor* that the lysozyme-mediated partial digestion of highly cross-linked Lys-type PG dramatically increases the binding of *T. molitor* PGRP-SA (Tm-PGRP-SA), which then recruits the GNBP1 homologue and an active form of modular serine protease (MSP) (9). Based on these results, we proposed a model where clustering of Tm-PGRP-SA causes the recruitment of Tm-GNBP1 and active Tm-MSP to form an initial PG recognition complex, which then leads to activation of the Toll pathway (9). However, because of the lack of molecular information regarding an immediate downstream factor(s) of the Tm-MSP molecule, the molecular details of how the PG recognition signal triggered by Tm-PGRP-SA and Tm-GNBP1 proteins remains unanswered. In addition, how many SPs are involved in the Toll signaling pathway are as yet undetermined. Therefore, purification and determination of the biological functions of unidentified SPs engaged in the Toll signaling pathway are essential for understanding the molecular host defense against bacterial and fungal infections.

The elegant *Drosophila* genetic studies are very powerful for characterizing and ordering the components in the *Drosophila* Toll pathway (3, 15). However, there is a limit for this system in terms of determining the biochemical mechanisms involved in regulating this proteolytic cascade. *Drosophila* has several alternative routes to the Toll pathway, used in various developmental stages and infection protocols, and it seems difficult to determine the clear activation mechanism. For instance, Dm-Persphone is another protease linked to the Toll pathway and antifungal immunity, yet the biological functions of this molecule have been partially characterized by *Drosophila* genetic studies. The proper identification of upstream or downstream factor(s) of Dm-Persephone still awaits further investigations (11, 12). To provide compelling biochemical data on how the Lys-type PG recognition signal can be sequentially transferred to Spz using purified pattern recognition proteins and SPs, it is necessary to use a larger insect, which enables us to collect large amounts of hemolymph. Here, we purified and cloned a novel downstream SP (Tm-41 kDa SP) of the Tm-MSP molecule, which is a link between Tm-MSP and Tm-SPE. Additionally, native Tm-GNBP1, Tm-GNBP3, Tm-MSP, and Tm-SPE proteins were purified to homogeneity from the hemolymph of *T. molitor* larvae. Also, we produced recombinant Tm-PGRP-SA, Tm-41 kDa SP, *Tribolium castneum* Spz (Tc-Spz) pro-protein and Toll ectodomain proteins using a baculovirus-insect expression cell system. By performing *in vitro* reconstitution experiments with these purified native and recombinant proteins, the sequential activation modes of these three SPs and the PG recognition signaling pathway were determined. These experiments clearly show how the PG/Tm-PGRP-SA/Tm-GNBP1 complex transfers Lys-type PG recognition signal to downstream factors through a three-step activation of a serine protease cascade in *T. molitor* larvae.

## EXPERIMENTAL PROCEDURES

*Animals and Collection of Hemolymph*—*T. molitor* larvae (mealworm) were maintained on a laboratory bench in terraria containing wheat bran. Hemolymph was collected as previously described (16). Briefly, to harvest the hemolymph, a larva was pricked using a 25-gauge needle and then a 10-$\mu$l drop of hemolymph was collected in 50 $\mu$l of a modified anti-coagulation buffer (136 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 5.0). The collected crude hemolymph was centrifuged at 200,000 $\times$ *g* for 15 min at 4 °C. The supernatant was then stored at −80 °C until use.

*Purification of the Soluble Linearized Lys-type PG*—Soluble linearized Lys-type PG was prepared as previously described (9). Briefly, crude linearized Lys-type PG was obtained from *Staphylococcus aureus* insoluble peptidoglycan (20 mg) by digestion with *Achromobacter* $\beta$-lytic protease (2 $\mu$g) for 14 h at 37 °C. This was then fractionated on a size exclusion column (Toyopearl HW-55S column, 2.6 $\times$ 155 cm). The fractions that could induce phenoloxidase activity were collected and concentrated using a rotary evaporator at 4 °C. The concentrated solution was loaded again onto the same column equilibrated with distilled water at a flow rate of 0.2 ml/min. The linearized Lys-type PG-containing fractions were pooled and stored at 4 °C until use. To ascertain that the purified linearized Lys-type PG originated from *S. aureus* Lys-type PG, we analyzed the amino acid composition of linearized Lys-type PG, which had the same amino acid composition (D-Glu/L-Gly/D-Ala/L-Lys = 1:5:1:1) as reported for the *S. aureus* Lys-type peptidoglycan (17).

*Amino Acid Sequencing*—To obtain amino acid sequences from purified proteins and cleavage products generated by proteases, proteins were blotted onto a polyvinylidene difluoride membrane and stained with a solution containing 0.1% Coomassie Brilliant Blue R-250 and 50% methanol. The membrane was destained with 50% methanol containing 10% acetic acid (v/v) until the protein bands became visible. The fragments of interest were excised and analyzed on a protein sequencer (Applied Biosystems) using standard techniques.

*Antibodies and Immunoblotting*—Rabbit antisera against Tm-PGRP-SA and Tm-GNBP3 raised previously were used after affinity purification as described (16, 18). The polyclonal antibodies of Tm-GNBP1 and Tm-MSP were raised using keyhole limpet hemocyanin (KLH)-conjugated synthetic peptides based on chemically determined partial sequences present in the Tm-GNBP1 and Tm-MSP (9). The following peptides were used: LEAYEPKGFRAS is located at the N-terminal region of Tm-GNBP1 and VNGKPVKKGDYPWQ is located at the N terminus of the catalytic SP domain of Tm-MSP. KLH-conjugated synthetic peptides with more than 95% purity were purchased from Peptron, Inc. (Daejeon, Korea). Rabbits were injected subcutaneously with 500 $\mu$g of KLH-conjugated peptides in Freund's complete adjuvant. A booster dose (250 $\mu$g in Freund's incomplete adjuvant) was given after 4 weeks, and the animals were bled 2–3 weeks thereafter. The resulting antibodies were affinity-purified as previously described (9). The polyclonal antibodies against the purified Tm-41 kDa SP and Tm-SPE proteins were raised by injecting 10 $\mu$g of purified proteins into each male albino rabbit with complete Freund's adjuvant and giving two booster injections with the same amount of protein 7 and 14 days later. The resulting antibody was also affinity-purified as described above.

*Measurement of the PG-specific Amidase Activity*—To determine amidase activity in the sample, commercially available α-thrombin substrate (*t*-butyloxycarbonyl-benzyl-L-valinyl-L-prolinyl-L-arginine- 4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) was used. This substrate was dissolved in dimethylformamide according to the manufacturer's instruction. 10 μl of crude hemolymph or fractionated fractions from column chromatography were incubated with 490 μl of substrate solution, which contains 20 μM substrate in 20 mM Tris-HCl buffer (pH 8.0). After incubation of the mixture at 30 °C for 1 h, 500 μl of 17% (v/v) acetic acid was added to terminate enzyme reaction. The specific amidase activity can be detected by fluorescence spectrophotometry at $\lambda_{ex}$ = 380 nm and $\lambda_{em}$ = 460 nm. One unit of the amidase activity was defined as the amount that liberated 1 nmol of 7-amino-4-methylcoumain per min.

*In Vitro Reconstitution Experiments and Determination of Amino Acid Sequences*—To examine whether each mixture could generate Lys-type PG-dependent amidase activity, i*n vitro* reconstitution experiments were performed with the eluted fractions from a Heparin-Toyopearl column in the presence of Lys-PG and $Ca^{2+}$. During the purification of essential factors by column chromatography, the purified factors were co-incubated with other fractions or purified factors as described above. To determine the proteolytic cleavage sites, the reaction mixtures were analyzed on SDS-PAGE under reducing or non-reducing conditions after induction of activation. The generated products from zymogen were confirmed by their N-terminal sequences or by Western blot analysis after electroblotting onto polyvinylidene difluoride membranes.

*Purification and cDNA Cloning*—The detailed purification methods of *Tenebrio* active and zymogenic forms of Tm-MSP, Tm-41 kDa SP, and Tm-SPE proteins are described under supplemental methods S2–S5. Additionally, Tm-GNBP1 and Tm-GNBP3 proteins were purified to homogeneity as described under supplemental methods S6 and S7, respectively. The entire purification scheme of these proteins is shown in supplemental Fig. S2. The detailed procedures of cDNA cloning of Tm-MSP, Tm-41 kDa, Tm-SPE, and Tm-GNBP1 are described under supplemental methods S8–S11. Supplemental Tables S1–S4 list the DNA primer sequences used for this study.

*Expression and Purification of Recombinant Tm-PGRP-SA, Tm-41 kDa SP, Recombinant Tc-Spz, and Tc-Toll Ectodomain Proteins*—The cDNAs encoding the Tc-SAE zymogen, Tc-Spz (residues 19–227), and Tc-Toll ectodomain (residues 2–777) were subcloned into BamHI and XhaI sites of the pFASTBAC-SEa vector, as previously described (19). This was modified from pFASTBAC-HTc by inserting a Mellitin signal sequence for secretion. The recombinant baculoviruses to express these proteins were generated according to the manufacturer's instructions (Invitrogen). Detailed procedures are described under supplemental methods S12.

*Antibacterial Assay*—Antibacterial activity was assayed essentially as previously described (20). We monitored bactericidal activity of the collected hemolymph from the cleaved Tc-Spz-injected *Tenebrio* larvae and the whole extract from the cleaved Tc-Spz-injected *Tribolium* adults. The procedure is described under supplemental methods S13.

## RESULTS

*The Components Involved in the PG Recognition Signaling Pathway Can Be Identified by Measuring Their Amidase Activity*—Our previous studies demonstrated that *Tenebrio* SP zymogens, Tm-PGRP-SA and Tm-GNBP proteins are known to be easily activated or rapidly degraded during the collection of hemolymph or during column chromatography (16, 18, 21). In this study, it was desirable to rapidly analyze the unstable SP zymogens, which were obtained from chromatographic procedures, for determination of their biological functions. Therefore, an easy and prompt bioassay was required rather than a time-consuming antimicrobial assay. 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 (16, 18). This result suggested that measurement of this amidase activity may be a suitable method to use during purification and characterization of the unidentified SP zymogens from *Tenebrio* hemolymph. Using this bioassay, we found that co-incubation of three different fractions (E1, E2, and E3, supplemental Fig. S1*A*) from the Heparin-Toyopearl column showed specific amidase activity against the α-thrombin substrate in the presence of PG and $Ca^{2+}$ (supplemental Fig. S1*B*, *column 1*). These results indicate that E1, E2, and E3 fractions may contain all of the essential components, such as Tm-PGRP-SA and Tm-GNBP1 and unidentified SP(s) including Tm-MSP, which are necessary for triggering the PG recognition signal. In contrast, β-1,3-glucan-dependent amidase activity was observed when E2 and E3 fractions were co-incubated with β-1,3-glucan and $Ca^{2+}$ (supplemental Fig. S1*B*, *column 9*), suggesting that Tm-GNBP3 may exist in either the E2 or E3 fractions, or in both. Prior to purification of these proteins, we determined the localization of Tm-PGRP-SA, Tm-GNBP1, Tm-MSP, and Tm-GNBP3 by immunoblotting analysis. Tm-GNBP1 was localized in fraction E1. Tm-MSP and Tm-PGRP-SA were identified in fraction E2 and Tm-GNBP3 was confirmed to be present in fraction E3 (supplemental Fig. S1*C*).

*Five Purified Proteins Are Enough to Show PG-specific Amidase Activity*—Using a combination of PG-dependent amidase activity measurements and Western blot analyses during column chromatography, two native GNBP proteins (Tm-GNBP1 and Tm-GNBP3), three different SP zymogens and their active forms (Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteins) were purified to homogeneity (Fig. 1*A*). For a detailed purification scheme and methods, see supplemental Fig. S2 and supplemental methods S2–S7, respectively. Three purified SP zymogens were each represented by a single band under both reducing and non-reducing conditions (Fig. 1*A*). However, all three purified active forms of SPs showed two bands under reducing conditions (Fig. 1*B*, *lanes 1–3*), but only one band under non-reducing conditions (*lanes 4–6*). To exclude the possibility that the generated bands (*arrows*, Fig. 1*B*) under reducing conditions were contaminating peptides, we determined their partial amino acid sequences of the these bands using LC-mass spectrometry and confirmed that the determined partial sequences

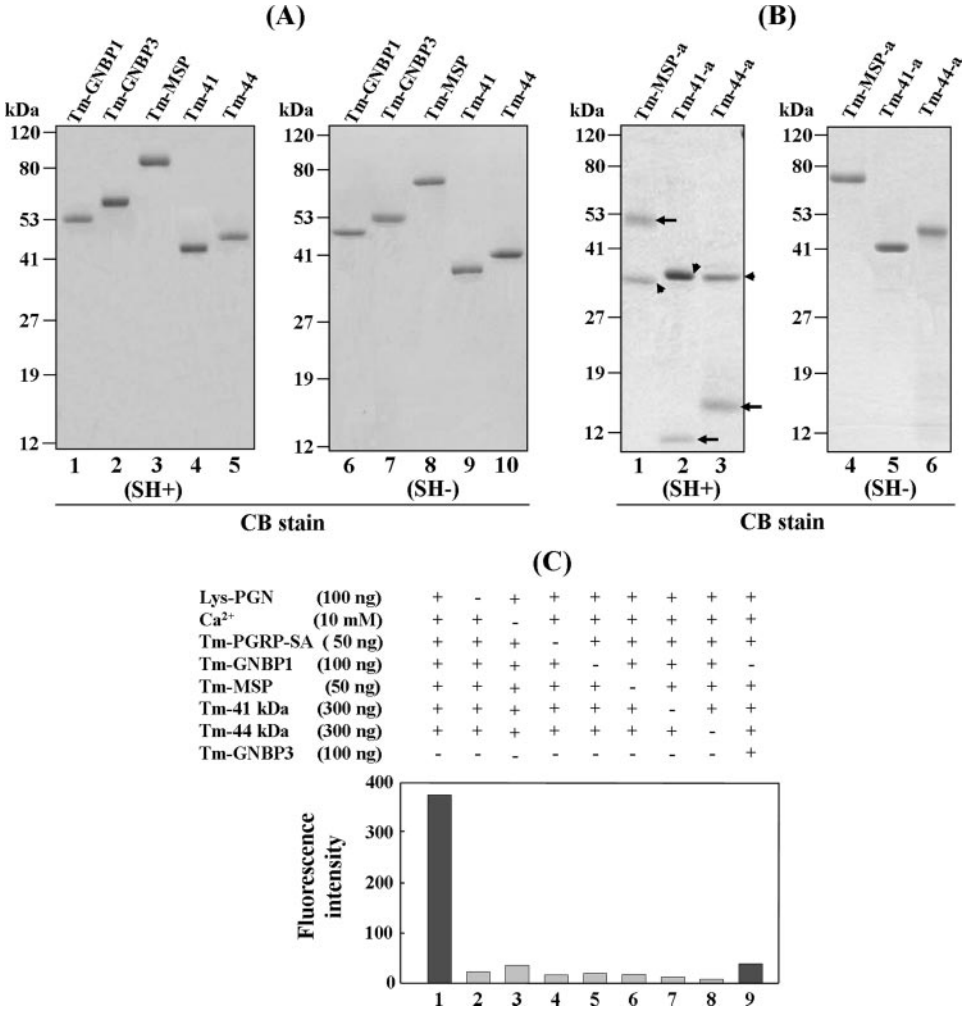


FIGURE 1. **SDS-PAGE analysis of the purified five proteins and measurements of their amidase activity.** *A*, pairs of lanes, such as *1* and *6*, *2* and *7*, *3* and *8*, *4* and *9*, *5* and *10* indicate the purities of purified Tm-GNBP1, Tm-GNBP3, Tm-MSP zymogen, Tm-41 kDa zymogen, and Tm-44 kDa zymogen under reducing and non-reducing conditions, respectively. The prestained ProSieve® color protein markers from Cambrex BioScience Rockland Inc. were used. The gel was stained by Coomassie Brilliant Blue R-250 (*CB*). *B*, pairs of lanes, such as *1* and *4*, *2* and *5*, *3*, and *6* indicate the purified active forms of Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteins under reducing and non-reducing conditions, respectively. *Arrows* and *arrowheads* indicate N-terminal and C-terminal SP domains of Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteins after cleavage, respectively. *C*, *in vitro* reconstitution experiments for measurement of amidase activity were performed using the purified five proteins, Tm-PGRP-SA, Tm-GNBP1, Tm-MSP zymogen, Tm-41 kDa, Tm-44 kDa, and Tm-GNBP3 in the presence of Lys-type PG and $Ca^{2+}$. When we co-incubated Tm-GNBP3 instead of Tm-GNBP1 with the other four proteins, namely Tm-PGRP-SA, Tm-MSP, Tm-41 kDa, and Tm-44 kDa, the PG-specific amidase activity was almost abolished (*column 9*).

originated from the N-terminal domains of the purified SPs (data not shown). The bands indicated by *arrowheads* (Fig. 1*B*) were confirmed as SP domains by immununoblot analysis (data not shown). These results suggest that the purified three SPs do not contain any other impure proteins. To determine which components are necessary for PG-dependent amidase activity, *in vitro* reconstitution experiments were performed. When five proteins except for Tm-GNBP3, (namely 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 was generated against an $\alpha$-thrombin substrate (Fig. 1*C*, *column 1*). This result suggested that these five proteins are the minimal number of essential factors necessary for PG recognition.

*cDNA Cloning and Biochemical Characterization of the Newly Purified Proteins*—We previously reported the cDNA sequences of Tm-PGRP-SA (18) and Tm-GNBP3 (16) (known as $\beta$-1,3-glucan-binding protein). In this study, cDNAs encoding newly purified Tm-MSP, Tm-41 kDa, Tm-44 kDa, and Tm-GNBP1 proteins were cloned based on experimentally determined partial amino acid sequences. To predict the biological functions of the four purified proteins, we first examined the amino acid sequence homology and domain organization of the four proteins (supplemental Fig. S3, *A–D*). Tm-MSP consists of four low density lipoprotein receptor A repeats (LDLa) domains, one complement control protein (CCP) domain and an SP domain. The substrate specificity pocket residues of the SP domain, Ser-569 (c189; "c" for the chymotrypsinogen numbering), Ser-596 (c216), and Gly-610 (c226) indicate that Tm-MSP is a chymotrypsin-like SP. This SP shows 59, 36, and 33% sequence identities with Tc-MSP (XP_967486), *Manduca sexta* HP-14 (Ms-HP-14) (22), and the *D. melanogaster* MSP-like protein (Dm-MSP, CG31217), respectively (supplemental Fig. S3*A*). The domain organizations of these three known MSP-like proteases are shown in Fig. 2*A*. Furthermore, the N-terminal sequence of the purified active form of Tm-MSP was determined to be Ile-Val-Asn-Gly-Lys-Pro-Val, which was an exact match with the deduced amino acid sequence of the Tm-MSP zymogen from Ile-375 to Val-381. This result supports the hypothesis that the Tm-MSP zymogen is cleaved between the Leu-374 and Ile-375 residues located between the CCP domain and catalytic SP domain (Fig. 2*B*), suggesting that the Tm-MSP zymogen may be cleaved by a chymotrypsin-like SP. The Tm-41 kDa protease consists of an N-terminal clip domain and a C-terminal SP domain and is most homologous to the Tc-Snake-like protease (XP_973839), with 51% sequence identity. The catalytic SP domain of the 41 kDa protein shows 40, 36, and 35% identities to Dm-Snake (23), Dm-Persephone (12), and Dm-melanization protease 1 (24), respectively (supplemental Fig. 3*B*). Because the substrate specificity pocket residues of the Tm-41 kDa protease were determined to be Asp-313 (c189), Gly-345 (c216), and Gly-353 (c226), this SP is believed to be a trypsin-like SP.

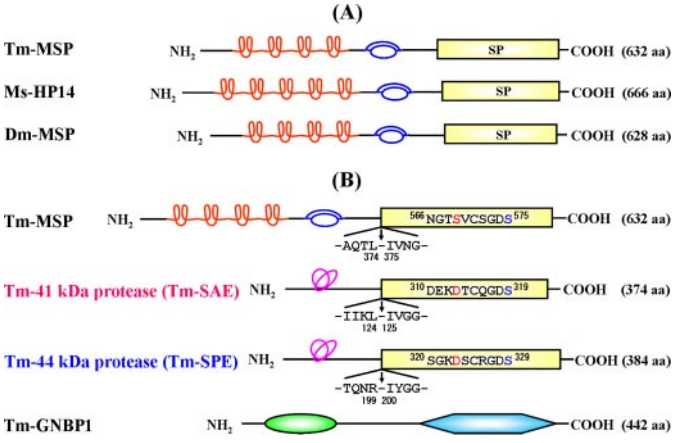


FIGURE 2. **The domain organizations of MSP homologues and the four purified proteins.** *A*, comparison of domain organization between *T. molitor* MSP (Tm-MSP), *M. sexta* hemolymph proteinase 14 (Ms-HP14) (22), and Dm-MSP homologue (CG31217). *Rabbit ears*, *half-double circle*, and *rectangular symbols* indicate the domains of LDLa, CCP, and SP domains of MSP homologues, respectively. *B*, domain organizations based on deduced amino acid sequences of cDNAs of Tm-MSP, Tm-41 kDa SP (Tm-SAE), Tm-44 kDa SP (Tm-SPE), and Tm-GNBP1 proteins. *Moon*, *oval*, and *diamond* symbols indicate the clip domain, GNBP homology domain, and glucanase-like domain, respectively. *Arrows* represent the cleavage sites of SP zymogens during activation. The *red* and *blue* residues in the boxes indicate the specificity-related residue and catalytic triad Ser residue, respectively.

Interestingly, the cleavage site of the Tm-41 kDa zymogen was experimentally determined between the Leu-124 and Ile-125 residues located between the clip domain and the catalytic SP domain (Fig. 2*B*). This cleavage pattern was also observed in Tm-MSP (as described above) and Dm-Snake protease, which is involved in controlling the dorsoventral fate of the *Drosophila* embryo (23). Dm-Snake has previously been reported to be cleaved by a chymotrypsin-like Gastrulation Defective SP (25). These results indicate that a chymotrypsin-like SP can cleave the Tm-41 kDa zymogen. 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 (Asp-323 (c189), Gly-355 (c216), and Gly-366 (c226)). This SP shows 49, 42, and 43% sequence identities with Dm-Easter (23), Dm-SPE (13), and *Holotrichia diomphalia* pro-phenoloxidase activating enzyme (Hd-PPAF1) (26), respectively (supplemental Fig. S3*C*). The cleavage site of the Tm-44 kDa zymogen was experimentally determined between Arg-199 and Ile-200 (Fig. 2*B*), suggesting that a trypsin-like SP cleaves the 44-kDa zymogen. Finally, Tm-GNBP1 shows sequence identities with Tc-GNBP1 (63%), Dm-GNBP1 (29%), Dm-GNBP3 (27%), and Tm-GNBP3 (31%) (supplemental Fig. S3*D*). These GNBP family proteins have a common domain organization, which features a GNBP homology domain and a $\beta$-1,3-glucanase-like domain (Fig. 2*B*). In addition, when we examined the amidase activities using purified active forms of the Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteases, only activated Tm-44 kDa protease showed strong amidase activity against the $\alpha$-thrombin synthetic substrate. However, activated Tm-MSP and activated Tm-41 kDa proteases did not show amidase activity (data not shown). This result suggests that the observed PG-dependent amidase activity of *Tenebrio* hemolymph is generated by the active form of the Tm-44 kDa protease.

*The 44-kDa Protease Is Tm-SPE*—The key question raised in our current work is the nature of the Tm-SPE molecule, which can convert Spz pro-protein to active Spz. As Tm-44 kDa zymogen showed high identities with Dm-SPE and Dm-Easter as described above, we first selected active Tm-44 kDa protease as a potential candidate for Tm-SPE. To investigate this possibility, we expressed Tc-Spz pro-protein (XP_975083) encoding from Phe-19 to Asn-227 (208 amino acids) and Tc-Toll (XP_967796) ectodomain encoding from Leu-20 to Leu-777 (757 amino acids) in a baculovirus culture system, since the cDNAs of Tm-Spz and Toll proteins have not yet been determined. *T. molitor* and *T. castneum* are both coleopteran beetle species belonging to the same family, Tenebriodae. The soluble recombinant Tc-Spz and Tc-Toll ectodomains were purified to homogeneity (Fig. 3*A*). The purified Tc-Spz pro-protein was synthesized as a disulfide-bonded dimer, which is consistent with those of the previous Dm-Spz studies (27, 28). The purified Tc-Toll ectodomain showed a molecular mass of 120 kDa, and its identity was confirmed by N-terminal sequencing (Fig. 3*A*). To address whether the purified active Tm-44 kDa protease really cleaves Tc-Spz pro-protein *in vitro*, we incubated Tc-Spz pro-protein with trypsin, the active forms of Tm-MSP, Tm-41 kDa, and Tm-44 kDa proteases (Fig. 3*B*). Only the active Tm-44 kDa protease converted Tc-Spz pro-protein to 14 kDa (*arrow (a)*) and 24 kDa (*arrow (b)*) molecular mass products under reducing and non-reducing conditions, respectively (*lanes 5* and *10*). Under the same conditions, trypsin cleaved the Tc-Spz pro-protein nonspecifically (*lanes 2* and *7*), and active forms of Tm-MSP and Tm-41 kDa protease did not cleave Tc-Spz pro-protein (*lanes 3*, *4*, and *8*, *9*). In addition, we determined the N-terminal amino acid sequence of the cleaved 24 kDa Tc-Spz (*band (b)*) as Phe-Asn-Thr-Asp-Glu-Met-Ser-Leu (data not shown), showing a perfect match with Tc-Spz from residues Phe-125 to Leu-132. Therefore, we named the Tm-44 kDa protease as Tm-SPE.

*Injection of Cleaved Tc-Spz into Insects Induces Strong Antimicrobial Activities*—To examine the biological function of this cleaved form of Tc-Spz *in vivo*, the 24-kDa purified Tc-Spz protein (Fig. 3*C*) was injected into *Tenebrio* larvae and *Tribolium* adults to check its antimicrobial activities. 36 h after injection, the hemolymph from the 24-kDa Tc-Spz-injected *Tenebrio* larvae, and the extracts from the 24-kDa Tc-Spz-injected *Tribolium* adults were collected, and then their bactericidal activities against *S. aureus* were estimated. The strongest bactericidal activity was induced by injection of cleaved Tc-Spz and heat-treated cleaved Tc-Spz into *Tenebrio* larvae (Fig. 3*D*, *columns 3* and *4*). Using an inhibition zone assay, the extracts from the cleaved Tc-Spz-injected and heat-treated cleaved Tc-Spz-injected *Tribolium* adults also showed antibacterial activity against *S. aureus* (Fig. 3*E*), indicating that cleaved Tc-Spz is biologically active *in vivo*. These results clearly suggest that Tc-Spz protein processed by Tm-SPE is able to activate *Tenebrio* and *Tribolium* Toll cascades *in vivo*. Finally, to confirm whether cleaved Tc-Spz can form a complex with the soluble Tc-Toll ectodomain *in vitro*, we mixed purified

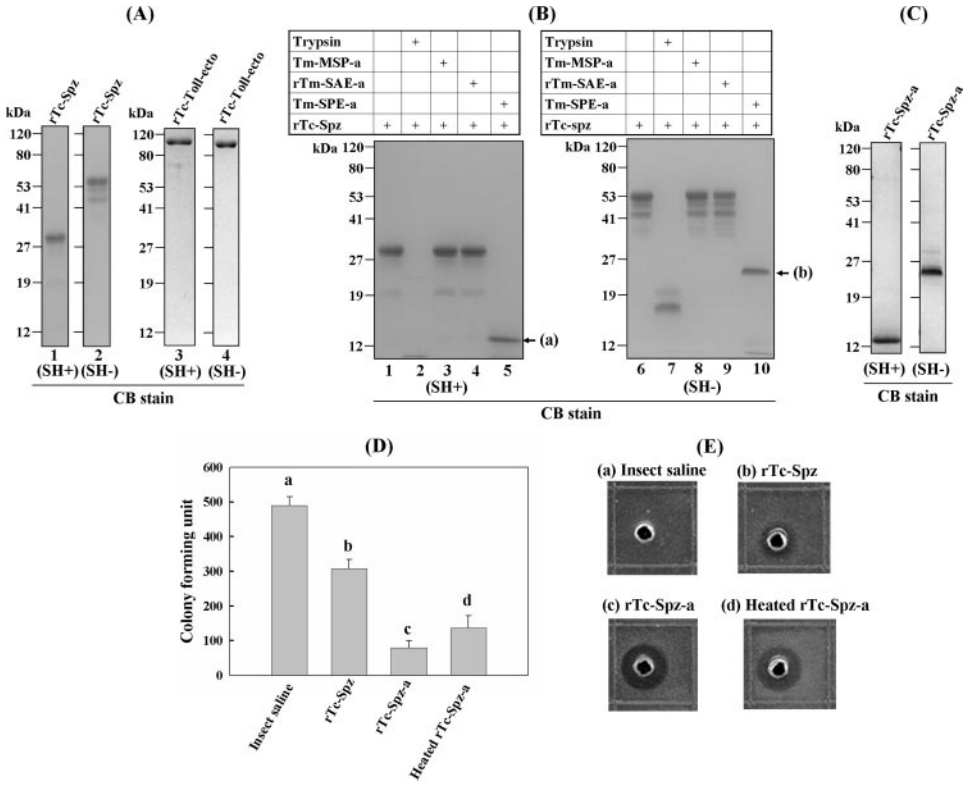


FIGURE 3. **Identification of Tm-SPE and antibacterial activity by injection of processed Tc-Spz into insects.** *A*, pairs of lanes, such as *1* and *2*, *3* and *4*, indicate the SDS-PAGE analysis pattern for the purified recombinant Tc-Spz pro-protein (*r-Tc-Spz*) and Tc-Toll ectodomain protein (*r-Tc-Toll*) under reducing and non-reducing conditions, respectively. *B*, pairs of lanes, such as *1* and *6*, *2* and *7*, *3* and *8*, *4* and *9*, *5* and *10* indicate purified r-Tc-Spz pro-protein (2.5 $\mu$g), the mixture of trypsin (50 ng) and r-Tc-Spz pro-protein (2.5 $\mu$g), the mixture of activated Tm-MSP (50 ng) and r-Tc-Spz pro-protein (2.5 $\mu$g), the mixture of activated r-Tm-SAE (50 ng) and r-Tc-Spz pro-protein (2.5 $\mu$g), and the mixture of activated Tm-SPE (50 ng) and r-Tc-Spz pro-protein (2.5 $\mu$g), respectively. *Band (a)* indicates cleaved 24-kDa Tc-Spz by active r-Tm-SPE. *C*, pattern of SDS-PAGE analysis for purified cleaved r-Tc-Spz. *Lane 1*, 1.5 $\mu$g per lane under reducing conditions; *lane 2*, non-reducing conditions. *D*, each column indicates the colony numbers after incubation with *S. aureus* cells with hemolymph collected from insect saline (*column a*)-, r-Tc-Spz pro-protein (*column b*)-, cleaved r-Tc-Spz (*column c*)-, and heat-treated cleaved r-Tc-Spz (*column d*)-injected *Tenebrio* larvae after 36-h injection. The bacterial cells ($2 \times 10^4$ cells/100 $\mu$l) were incubated with 100 $\mu$l of collected hemolymph for 1 h and then serially diluted. Each mixture was spread on nutrient broth agar plates. The plates were incubated at 37 °C overnight, and the resulting colonies were counted. *E*, antibacterial susceptibility disc tests against the *S. aureus* cells were performed with lyophilized hemolymph from 100 $\mu$l of whole extract from insect saline (*panel a*)-, Tc-Spz pro-protein (*panel b*)-, cleaved Tc-Spz (*panel c*)- and heat-treated cleaved Tc-Spz (*panel d*)-injected *Tribolium* adults after 36-h injection. The zones of inhibition were estimated after overnight incubation. Data represent means ± S.E. for three experiments.

cleaved Tc-Spz with Tc-Toll ectodomain and then separated the complex on a calibrated gel filtration column. Cleaved Tc-Spz is able to form a high molecular mass complex with the Tc-Toll ectodomain (supplemental Fig. S4), suggesting that cleaved Tc-Spz recognizes the Tc-Toll ectodomain and forms a stable complex *in vitro*.

*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. Because 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 Tm-MSP and Tm-41 kDa zymogens were determined as chymotrypsin-like and trypsin-like SPs, respectively, 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 (Fig. 4*A*, *lanes 1* and *4*) with purified native Tm-SPE zymogen (*lanes 2* and *5*). Tm-SPE zymogen was hydrolyzed to a 35-kDa SP domain (*band (a)*) and a 15-kDa clip domain (*band (b)*). The amino acid sequence of the 35-kDa band (*bands (a)* and *(c)*) 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*—Because the cleavage site of the Tm-SAE zymogen was determined as Leu-124—Ile-125, the upstream SP of Tm-SAE should have a chymotrypsin-like substrate specificity. This suggests that it is possible that the Tm-SAE zymogen will be cleaved by the activated Tm-MSP. To confirm this possibility, activated Tm-MSP (Fig. 4*B*, *lanes 1* and *4*) was incubated with recombinant Tm-SAE zymogen (*lanes 2* and *5*). Tm-SAE zymogen was hydrolyzed to a 35-kDa SP domain (*band (d)*) and an 11-kDa clip domain (*band (e)*). The N-terminal amino acid sequence of the 35-kDa band generated from Tm-SAE zymogen was determined as Ile-Val-Gly-Gly-Thr-Asn (*bands (d)* and *(f)*). This sequence perfectly matches the amino acid sequence of the Tm-SAE zymogen from Ile-125 to Asn-130, demonstrating that activated Tm-MSP had caused a limited and specific proteolytic cleavage between the clip domain and catalytic SP domain of the Tm-SAE zymogen. This result demonstrates 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 were not cleaved (Fig. 4, *C* and *D*, *lane 3*), confirming that Tm-SPE is not an immediate downstream protease of MSP.

*Tm-MSP Zymogen Is Activated after Binding to the Lys-type PG/Tm-PGRP-SA/Tm-GNBP1 Complex in the Presence of $Ca^{2+}$*—Recently, we proposed that the Tm-MSP zymogen may function as an initial enzyme in the Tm-PGRP-SA/Tm-GNBP1-mediated Lys-type PG recognition signaling pathway (9). 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 $Ca^{2+}$. The catalytic SP domain generated from the Tm-MSP

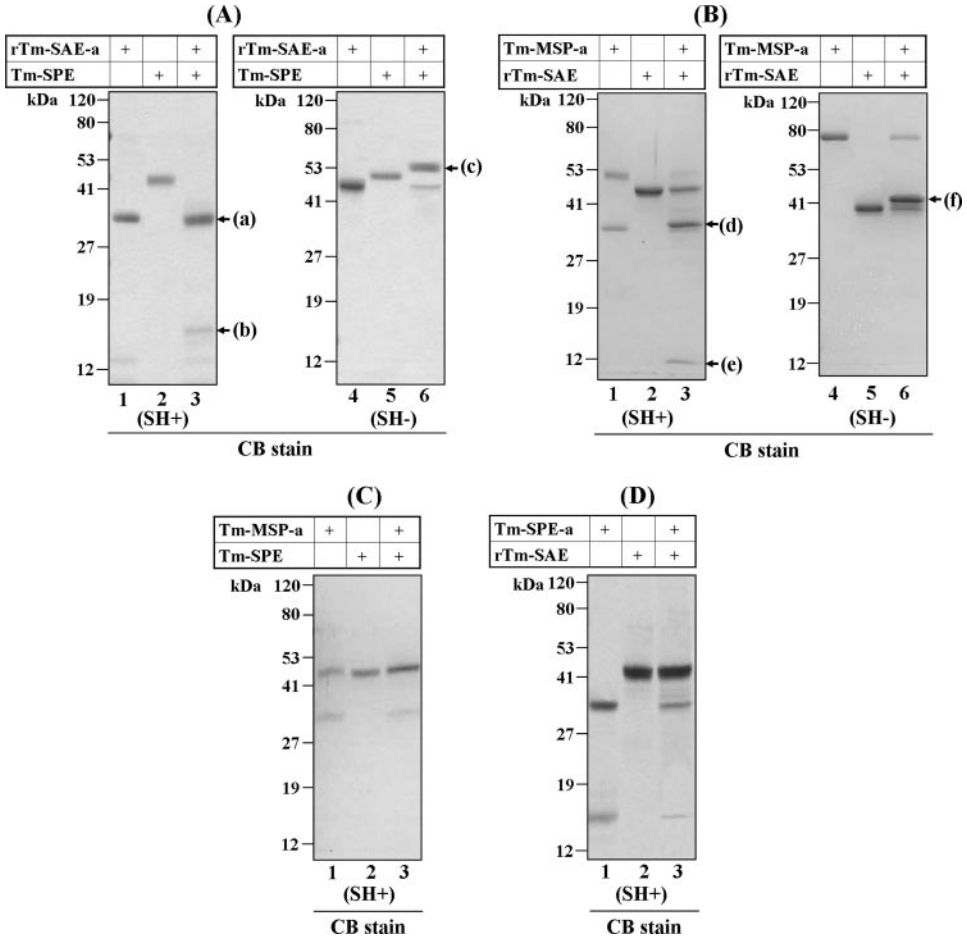


FIGURE 4. **Determination of molecular activation mechanisms between Tm-MSP, Tm-SAE, and Tm-SPE.** *A*, cleavage patterns between activated Tm-SAE and Tm-SPE zymogens. Pairs of lanes, such as *1* and *4*, *2* and *5* and *3* and *6*, indicate the activated Tm-SAE (1 $\mu$g), Tm-SPE zymogen (1 $\mu$g), and the mixture of active forms of Tm-SAE and Tm-SPE zymogens, respectively. *Lanes 1–3* and *4–6* show the gel mobility of these proteins under reducing conditions and non-reducing conditions, respectively. *Bands a* and *b* represent the catalytic domain and clip domain of Tm-SPE generated after cleavage, respectively. *Band c* represents the cleaved Tm-SPE protein. *B,* cleavage pattern between activated Tm-MSP and Tm-SAE zymogen. Pairs of lanes, such as *1* and *4*, *2* and *5* and *3* and *6*, indicate the activated Tm-MSP (1 $\mu$g), Tm-SAE zymogen (1 $\mu$g) and the mixture of active forms of Tm-MSP and Tm-SAE zymogen, respectively. *Lanes 1–3* and *4–6* show the gel mobility of these proteins under reducing conditions and non-reducing conditions, respectively. *Bands d* and *e* represent the catalytic domain and clip domain of Tm-SAE protease generated after cleavage. *Band f* represents the cleaved Tm-SAE protein. *C*, *lanes 1*, *2*, and *3* indicate the activated Tm-MSP (1 $\mu$g), Tm-SPE zymogen (1 $\mu$g), and the mixture of activated Tm-MSP and Tm-SPE zymogen, respectively. *D*, *lanes 1*, *2*, and *3* indicate the activated Tm-SPE (1 $\mu$g), Tm-SAE zymogen (2 $\mu$g), and the mixture of activated Tm-SPE and Tm-SAE zymogens, respectively.

zymogen was monitored by Western blot analysis. The Tm-MSP zymogen is almost entirely converted to the active form of Tm-MSP in the presence of Tm-PGRP-SA, Tm-GNBP1, Lys-type PG, and $Ca^{2+}$ (Fig. 5*A*, *band a* and *lane 4*). The N-terminal amino acid sequence of the generated 35-kDa band (a) perfectly matched the catalytic SP domain of activated Tm-MSP (data not shown). However, in the absence of any one of these components, the Tm-MSP zymogen cannot be converted to the active form of Tm-MSP (Fig. 5*B*, *lanes 2–5*). These results imply that the 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 also 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. A recent *Drosophila* genetic study demonstrated that Dm-GNBP3 is required for Toll pathway activation in response to fungal infections (11), suggesting that Dm-GNBP3 is a pattern recognition protein for fungal $\beta$-1,3-glucan. To examine the effects of purified Tm-GNBP3 upon the Lys-type PG recognition signal, Tm-GNBP3 was substituted for Tm-GNBP1 and incubated with Tm-PGRP-SA, Tm-MSP zymogen, Lys-type PG, and $Ca^{2+}$, and the cleavage pattern of the Tm-MSP zymogen was analyzed. As expected, the Lys-type PG/Tm-PGRP-SA/Tm-GNBP3 mixture did not cleave the Tm-MSP zymogen (Fig. 5*B*, *lane 6*). Therefore, this result clearly shows that the whole 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) (29). 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 hydrolyzes the downstream complement factor C4 to C4a and C4b in the human lectin complement pathway. Finally, to confirm whether the Tm-SPE zymogen is really cleaved by the combination of the seven components; Lys-type-PG, Tm-PGRP-SA, Tm-GNBP1, zymogens of Tm-MSP, Tm-SAE, and Tm-SPE in the presence of $Ca^{2+}$, all these components were co-incubated, and then the specific cleavage of Tm-SPE zymogen was detected by Western blot analysis and measurement of amidase activity. As expected, a band of 35 kDa corresponding to the SP domain of Tm-SPE was specifically observed (Fig. 5*C*, *column 1*) with amidase activity (data not shown). However, if any one of the components was omitted from the incubation mixture, no cleavage of the Tm-SPE zymogen occurred (*columns 2–9*). Taken together, these experiments demonstrate that the Tm-PGRP-SA/Tm-GNBP1-mediated Lys-type PG recognition signal is transferred by three different SPs: the initial enzyme is the 82 kDa chymotrypsin-like Tm-MSP and the second Tm-SAE and the third Tm-SPE are the 41 kDa and the 44 kDa clip domain-containing trypsin-like SPs, respectively.

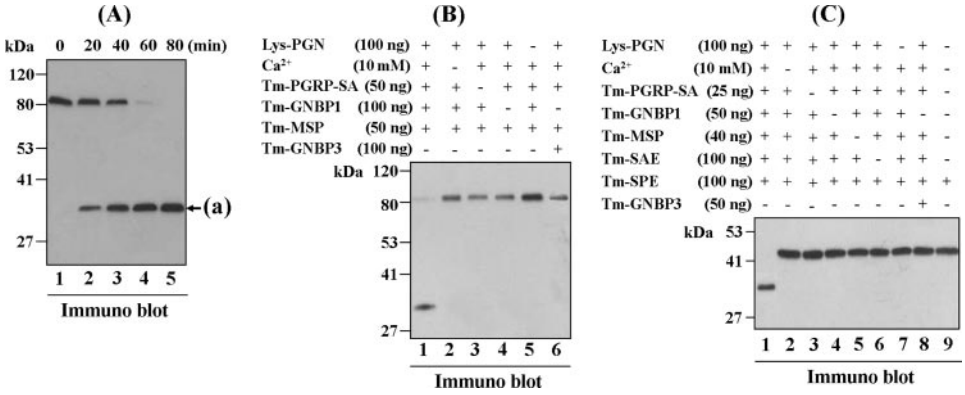


FIGURE 5. **Activation mechanisms of Tm-MSP zymogen and *in vitro* reconstitution experiments using purified components.** *A*, mixture of Tm-MSP zymogen, Tm-PGRP-SA, and Tm-GNBP1 in the presence of Lys-type PG and $Ca^{2+}$ was incubated for 0 min (*lane 1*), 20 min (*lane 2*), 40 min (*lane 3*), 60 min (*lane 4*), and 80 min (*lane 5*) and analyzed by Western blot analysis with an affinity-purified anti-Tm-MSP antibody. *Arrow (a)* represents the position of the catalytic domain of Tm-MSP zymogen after cleavage. *B*, in the absence of any one of these components, Tm-MSP zymogen cannot be converted to its active form (*lanes 2–5*). The Lys-type PG/Tm-PGRP-SA/Tm-GNBP3 complex did not cleave Tm-MSP zymogen (*lane 6*). *C*, when seven components were incubated in 20 mM Tris-HCl, pH 8.0, for 60 min, the Tm-SPE zymogen was cleaved (*lane 1*). If any one of the components was not included in the incubation mixture, no cleavage of the Tm-SPE zymogen occurred (*columns 2–9*).

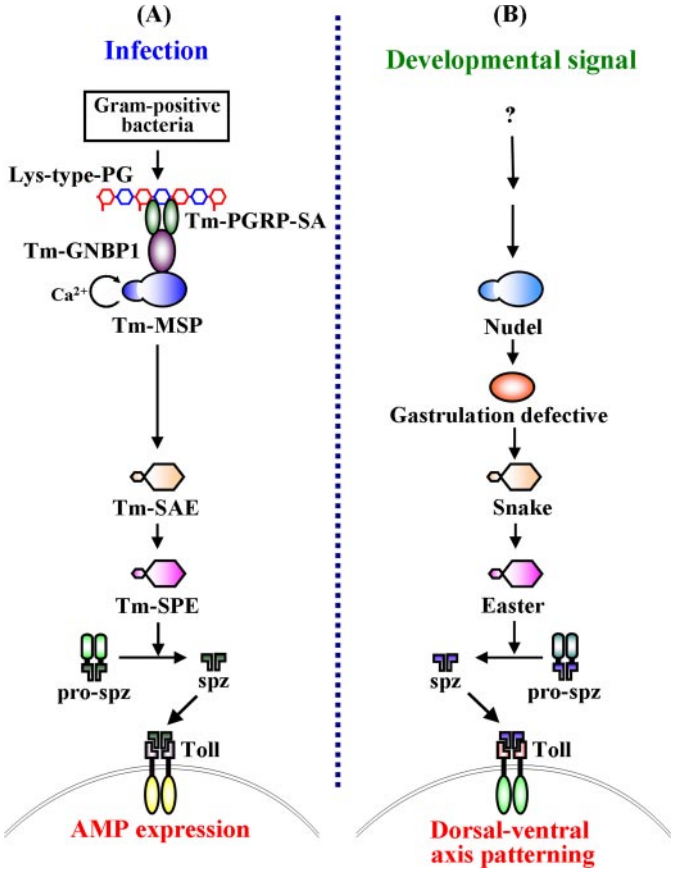


FIGURE 6. **A model summarizing the three-step activation cascade of the Lys-type PG recognition signaling pathway.** *A*, when the processed Lys-type PG from Gram-positive bacteria is exposed to the host, Tm-PGRP-SA binds to Lys-type PG and then recruits Tm-GNBP1 and the Tm-MSP zymogen. In the presence 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 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 processed Tc-Spz, leading to the production of AMP(s). *B*, known SP cascade in the *Drosophila* developmental Toll signaling pathway (23).

## DISCUSSION

In this study, we have described three novel findings regarding the Lys-type PG recognition Toll signaling pathway: 1) biological functions of a novel Tm-SAE have been determined; 2) the sequential molecular activation mechanisms of three SPs involved in the Toll signaling pathway have been determined; and 3) the first biochemical evidence of how the Tm-PGRP-SA-mediated Lys-type PG recognition signal is transferred to Spz, leading to antimicrobial activity *in vivo*, is provided. Based on these results, we propose that this PGRP-SA/GNBP1/MSP/SAE/SPE/Spz cascade may be an essential unit that triggers the Lys-type PG recognition signaling pathway in response to Gram-positive bacteria infection in insects (Fig. 6).

Our results do not seem to correspond with those recently reported by Kambris *et al.* (14). They suggested that two *Drosophila* catalytic SPs (Dm-Grass and Dm-Spirit) and two non-catalytic SP homologs (SPHs), such as Dm-Spheroide and Dm-Sphinx1/2, are involved in the PG-dependent *Drosophila* Toll pathway. By homology research with known SPs, it turned out that Dm-Grass and Dm-Spirit are clip domain-containing trypsin-like SPs. Dm-Spheroide and Dm-Sphinx1/2 each have a non-catalytic SP domain but no clip domains at the N terminus. They also have Gly and Ile residues, respectively, instead of a Ser residue in the catalytic site of SPs. The reason why we do not identify similar SPs and SPHs in our studies is unclear, and further studies are necessary to answer this question. However, one plausible explanation for this can be that *Tenebrio* SPHs may exist with serpins in the hemolymph and are not directly involved in the Toll pathway activation. A horseshoe crab factor D, a SPH-like molecule in *Limulus*, was co-purified with a horseshoe crab serpin (30), suggesting that SPH might make a complex with serpins *in vivo*. By performing RNAi experiments against *Drosophila* SPs or SPHs, there is a possibility that serpins can be released to the hemolymph by lack of SPH and that catalytic SPs, such as Dm-Grass and Dm-Spirit might be trapped by the released serpins leading to inhibition of the Toll pathway activation.

Similarly, our current data may provide a clue for the screening of Dm-MSP and Dm-SAE-like *Drosophila* counterparts in the Toll pathway. A plausible candidate for a Dm-MSP is the protease encoded by CG31217, which has the same domain organization as *Tenebrio* and *Tribolium* MSPs (Fig. 3*A*). Therefore, a valuable study would be one which addresses whether flies with reduced CG31217 expression can transfer Lys-type PG recognition signals to Dm-SPE or whether they will become susceptible to a Gram-positive bacteria infection. However, when we examined the amino acid residues of the tentative cleavage site and the substrate specificity pocket residues of the

CG31217 protease, their amino acid residues were quite different from those of beetle MSPs. Notably, the CG31217 zymogen has a putative chymotrypsin-like cleavage site between Phe-368 and Ser-369 and the elastase-like substrate specificity pocket residues of CG31217 protease were identified as Leu-557 (c189), Ala-593 (c216), and Thr-604 (c226, see supplemental Fig. S3*A*). These data suggest that there is a possibility that an SP other than the CG31217 protease may function as an initial SP in the *Drosophila* Toll pathway. If our model can be applied to the *Drososphila* system, the direct downstream SP of the CG31217 protein may have the same cleavage site as the CG31217 protease, as we have shown for the Tm-MSP and Tm-SAE zymogens. We found 24 different *Drosophila* clip-domain-containing SPs in our Blast search of the *Drosophila* genome sequence (data not shown). To find out which of these 24 *Drosophila* clip domain SPs correspond to the Tm-SAE, further genetic analysis studies are necessary. However, we cannot exclude the possibility that as yet unidentified isoforms of Dm-SAE and Dm-MSP-like protease might induce the activation of an alternative Toll pathway in *Drosophila*.

In summary, our biochemical studies shed further light on the molecular mechanism of how the Lys-type PG recognition signal is transferred to the Toll receptor. Our work supports a model in which a PG recognition complex activates three different SPs zymogens sequentially. This three-step proteolytic cascade-dependent processing of the extracellular protein Spz and then the binding of the processed Spz to Toll receptor are required for the induction of antimicrobial peptides expression in this innate immune pathway. A greater understanding of this cascade will also facilitate the development of a kit to rapidly and sensitively detect bacterial PG in blood and food products.

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