REVIEW

The molecular activation and regulation mechanisms of proteolytic Toll signaling cascade in insect innate immunity

KH Ryu¹, JW Park¹, K Kurokawa¹, M Matsushita², BL Lee¹

¹The National Research Laboratory of Defense Proteins, College of Pharmacy, Pusan National University, Busan, 609-735, Korea
²Department of Applied Biochemistry; Tokai University, Hiratsuka, Kanagawa 259-1292, Japan

Accepted September 2, 2010

Abstract

Recently we biochemically determined the molecular recognition and regulatory mechanisms of how beetle’s larvae recognize Gram-positive bacteria and fungi via Toll signaling cascade. The biochemical analysis of newly identified molecules provides us how beetles recognize invading pathogenic microbes and how they defend their bodies using elegant innate immunity. Here, we will focus on reviewing the biochemical analyses and biological functions of newly identified molecules involved in insect Toll signaling cascade.

Key Words: Toll cascade; innate immunity; protease inhibitor; insects

Introduction

Innate immunity is an evolutionarily conserved first-line host defense that senses pathogenic microorganisms through “pattern-recognition” molecules that recognize the conserved molecular patterns on the surface of microbes (Medzhitov and Janeway, 1997). Invertebrate’s innate immune response is a crucial host defense system to defend against microbial infection (Hoffmann et al., 1999). The ability of a host to distinguish between self and non-self remains a central hallmark of innate immunity. The pathogenic microbes possess distinct pathogen-associated molecular patterns (PAMPs), such as peptidoglycans (PGs) of Gram-positive bacteria and β-1,3-glucans of fungi. The recognition of PAMPs is achieved by a group of germline-encoded receptors and soluble proteins (Hoffmann et al., 1999).

Corresponding author:
Lee Bok Luel
The National Research Laboratory of Defense Proteins, College of Pharmacy, Pusan National University, Jangjeon Dong, Gumjeong Gu, Busan, 609-735, Korea
E-mail: brlee@pusan.ac.kr

List of abbreviation:
AMP, antimicrobial peptides; SP, serine proteinase; PG, peptidoglycan; PGRP, peptidoglycan recognition protein; GRP, β-1,3-glucan recognition protein; GNB1, Gram-negative binding protein 1; MSP, modular SP; ModSP, Modular SP; SPE, Spätzle-processing enzyme; SAE, SPE activating enzyme; HP, hemolymph protease.

In Drosophila, the synthesis of antimicrobial peptides (AMPs) in response to microbial infections is under the control of the Toll and immune deficiency (Imd) signaling pathway (Lemaitre and Hoffmann, 2007). The Toll signaling pathway responds mainly to the lysine (Lys)-type PG of Gram-positive bacteria and fungal β-1,3-glucan, whereas the Imd pathway responds to meso-diaminopimelic acid (DAP)-type PG of Gram-negative bacteria and certain Gram-positive bacilli. The biological significance of these two Drosophila signaling pathways is demonstrated by the fact that mutations of the genes involved in these pathways dramatically decrease resistance to microbial infections, e.g., Toll mutants are susceptible to fungal infections and Relish, the NF-κB protein involved in Imd pathway, mutants lose resistance to Gram-negative bacterial infections (Lemaitre et al., 1996; Hedengren et al., 1999). Toll signaling cascade is amplified in hemolymph (insect blood) by a proteolytic serine protease (SP) cascade. The amplification of Toll recognition signals results in an efficient host defense strategy in insects, which are devoid of an acquired immune system.

Drosophila genetic studies showed that Lys-type PG of Gram-positive bacteria is recognized by two different protein complex consisted of the PG recognition protein-SA (PGRP-SA) and the Gram-negative binding protein 1 (GNBP1) (Michel et al., 2001; Gobert et al., 2003; Pili-Floury et al., 2004). In contrast, fungal β-1,3-glucan is known to be recognized by GNBP3 (Gottar et al., 2006). These Lys-type PG and β-1,3-glucan recognition signals
that are mediated via PGRP-SA and GNBPs, respectively, are suggested to induce the activation of a SP proteolytic cascade, ultimately leading to the conversion of pro-Spätzle into processed Spätzle (Lemaitre et al., 1996; Weber et al., 2003; Gay and Gangloff, 2007). *Drosophila* Spätzle-processing enzyme (SPE), a typical clip-domain-containing SP, has been identified as the terminal SP that induces the cleavage of pro-Spätzle (Jiang et al., 2006; Kambris et al., 2006). The processed Spätzle functions as a native ligand for the Toll receptor, and induces the expression of AMP genes from the fat body (Levashina et al., 1999; Weber et al., 2003; Gay and Gangloff, 2007). Therefore, *Drosophila* Toll signaling pathway is an elegant example showing of that pattern recognition proteins-mediated pathogen recognition signals are amplified by a proteolytic SP cascade. In addition, recent interesting study performed by Shia et al. (2009) showed that knock-down of Toll ligand Spätzle in the *Drosophila* hemocytes (insect blood cells) induced the inhibition of the expression of AMP genes from fat body, suggesting that Toll receptor-dependent AMP responses in the fat body require Spätzle secretion from hemocytes. Also, this result indicates that the integration between humoral responses characterized by AMP production by fat body and cellular response, such as phagocytosis, mediated by hemocytes depend on the cytokine-like Spätzle production, a similar process with mammalian cytokine-mediated immune responses.

The *Drosophila* genetic studies are very powerful for characterizing and ordering the components in the *Drosophila* Toll signaling pathway (Ligoxygakis et al., 2002a). 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 by the genetic approach only. For instance, *Drosophila* Persephone 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 *Drosophila* Persephone still awaits further investigations (Levashina et al., 1999; Gottar et al., 2006). To provide compelling biochemical data on how the Lys-type PG recognition signal can be sequentially transferred to Spätzle using purified pattern recognition proteins and SPE, it is necessary to use a larger insect, which enables us to collect large amounts of hemolymph and to perform biochemical studies.

Recently, our group determined the activation and regulation mechanisms of *Tenebrio* Toll signaling cascade by approaching biochemical methods using a large beetle, *Tenebrio molitor* (Kim et al., 2008; Jiang et al., 2009; Roh et al., 2009). This large insect enabled us to collect large amounts of hemolymph for biochemical studies, allowing us to study several different *Tenebrio* SPs. We demonstrated that the recognition of Lys-type PG-mediated by the PGRP-SA/GNBPs complex induces the processing of pro-Spätzle to the cleaved Spätzle through the sequential activation of three different *Tenebrio* SPs: modular serine protease (MSP), Spätzle-activating enzyme (SAE) and Spätzle-processing enzyme (SPE) (Kim et al., 2008). *Tenebrio* SPE has been shown as a terminal SP that cleaves pro-Spätzle. Additionally, we provided another biochemical evidences of the mechanism by which the *GNBP*3-mediated fungal β-1,3-glucan recognition signal is also converted pro-Spätzle to Spätzle after activation of the same three different SPs that are involved in Lys-type PG recognition signal (Roh et al., 2009).

Therefore, it will be valuable to compare the biological diversity of Toll signaling cascades in three different insects, fruit fly *Drosophila melanogaster*, tobacco hornworm *Manduca sexta* and mealworm *T. molitor*. This will shed further insight into biological diversity of the molecular mechanisms of pathogen recognition and amplification signals in insects. Since excellent review papers focusing Toll signaling and melanin synthesis cascades are available now (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007; Cerenius et al., 2008, 2010), we mainly focus on biochemical studies of Toll signaling cascade using mealworm (*T. molitor*) and Tobacco hornworm (*M. sexta*) larvae.

**Pathogen recognition molecules of Toll cascade**

PGRPs are critical receptors in *Drosophila* immune responses that are required for the recognition of PG and for subsequent activation of AMP gene expression (Lemaitre and Hoffmann, 2007). PGRPs were first characterized in the moths *Bombyx mori* and *Trichoplusia ni* (Yoshida et al., 1996; Kang et al., 1998) and proposed to be receptors that can trigger insect’s innate immune responses. PGRPs share homology with *N*-acetylmuramoyl-L-alanine amidases, which cleave PG at the lactylamide bond between the glycan backbone and the stem-peptides (Kang et al., 1998). Some non-catalytic PGRPs, such as PGRP-LC, -LE, -SA and -SD, lack a critical cysteine residue in the catalytic pocket and are not able to cleave PG (Mellroth et al., 2003), but these PGRPs can bind PGs and are necessary for the expression of AMP genes, indicating that these PGRPs directly recognize bacteria and activate innate immune responses. In contrast, catalytic PGRPs, such as PGRP-SC1a, -LB and -SC2, include this cysteine residue in the active site and are potent enzymes that cleave PG. Post-digestion with PGRP-SC1b, *Staphylococcal* PG exhibits less activation of the AMP genes in a *Drosophila* blood cell line, so it was hypothesized that catalytic PGRPs may act as scavengers to limit an inflammatory response to free PG (Mellroth et al., 2003).

GNBP family, a 50-kDa protein found in hemolymph of *B. mori* and named as a Gram-negative binding protein (Lee et al., 1996). However, the biochemical studies demonstrated that GNBP belong to the family of β-1,3-glucan recognition proteins (β-GRP) that had first been purified on their ability to trigger the pro-phenoloxidase (melanin synthesizing enzyme in invertebrates) in response to fungal infections (Yoshida et al., 1986). *Drosophila*
genetic studies demonstrated that Lys-type PG is recognized by a complex comprised of the PGRP-SA and GNBP1 (Gobert et al., 2003; Pili-Floury et al., 2004), while β-1,3-glucan from yeast is recognized by GNBP3 (Gottar et al., 2006). Both the PGRP-SA/GNBP1 complex and GNBP3 are believed to mediate the activation of a SP cascade that ultimately leads to the cleavage of pro-Spätzle into processed Spätzle (Lemaître et al., 1996). Native GNBP3 proteins were purified from three insects, silk worm (B. mori, Ochiali and Ashida, 2000), tobacco hornworm (M. sexta, Ma and Kanost, 2000) and mealworm (T. molitor, Zhang et al., 2003). The purified proteins bound to 1,3-β-D-glucan but not to bacterial PG. Subsequent molecular cDNA clonings revealed that GNBP3 molecules contains a region with a sequence similar to bacterial glucanases. Interestingly, two catalytically important residues in the glucanases had been replaced with non-homologous amino acids in all three GNBP3, suggesting that GNBP3 molecules have evolved from an ancestral glucanase gene but retained only the ability to recognize β-1,3-glucan. Native GNBP1 was only purified from mealworm and it was involved in PG-recognition reaction with PGRP-SA (Kim et al., 2008).

Next, native PGRP-SA proteins were purified from three insects, silkworm, cabbage looper (T. ni) and mealworm (Yoshida et al., 1996; Kang et al., 1998; Park et al., 2006). The purified PGRP-SA recognized bacterial PGs. In vitro reconstitution experiments demonstrated that Tenebrio and Bombyx PGRP-SAs are recognition molecules of PG-dependent pro-pheno-oloxidase cascades (Yoshida et al., 1996; Park et al., 2006). Until this time, PGRP proteins from tobacco hornworm were not purified yet.

SP zymogens involved in the Lys-type PG-mediated Toll signaling cascade

Drosophila genetic studies provided the evidence that PGRP-SA and GNBP1 makes a complex upon recognition of Lys-type PG and then recruits down-stream SPs for the activation of Toll signaling cascade (Gobert et al., 2003). However, the identification of immediately downstream SPs of the PGRP-SA/GNBP1 complex is not easily performed in Drosophila system due to protein redundancy and a limitation of Drosophila hemolymph collection. Since Tenebrio larvae also showed high antimicrobial activities to the challenge of Gram-positive bacteria or fungi, it was supposed that Tenebrio larvae may have all essential components necessary for the activation and regulation of Tenebrio Toll signaling cascade. Therefore, we assumed that the immediate downstream SP of the PGRP-SA/GNBP1 complex can be purified using Tenebrio hemolymph by biochemical approaches (Park et al., 2007). Also, we hypothesized that GNBP1 and an SP would be recruited to the Lys-type PG/PGRP-SA complex when the soluble Lys-type PG/Tenebrio PGRP-SA complex was incubated with PGRP-SA-depleted Tenebrio hemolymph. Indeed, Tenebrio PGRP-SA/Lys-type PG complex recruited a 50-kDa protein and a 35-kDa protein. The amino acid sequencing of the N-terminal residues identified the 50-kDa protein as Tenebrio GNBP1 and the 35-kDa protein as Tenebrio MSP. Interestingly, this MSP protease does not contain a clip domain, which is commonly found in proteases upstream of the Toll and pro-phenoloxidase cascades (Piao et al., 2005). Drosophila GNBP1 was known to physically interact with PGRP-SA and activates Drosophila Toll pathway. However, an interaction between GNBP1 and PGRP-SA has not been observed in vitro (Gobert et al., 2003). Our observation suggests that the binding of PGRP-SA to PG enhanced the interaction between PGRP-SA and GNBP1, and subsequently the active form of MSP was recruited to the Lys-type PG/PGRP-SA/GNBP1 complex. Subsequent cDNA cloning demonstrated that T. molitor MSP (Tm-MSP) contains four low-density lipoprotein receptor A repeat (LDL) domains, one complement control protein (CCP) domain and a chymotrypsin-like SP domain (Kim et al., 2008). 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. MSP-like molecules was identified in other two insects, M. sexta HP (hemolymph protease)-14 (Ms-HP-14) (Ji et al., 2004) and the D. melanogaster MSP protein (Dm-MSP, CG31217, Bucheon et al., 2009). The domain organizations of these three known MSP orthologues are shown in Fig. 1A and the biological functions of these three MSP molecules are reported (Ji et al., 2004; Park et al., 2007; Bucheon et al., 2009). Also, recently, Bucheon et al reported that Drosophila MSP integrates signals originating from the circulating recognition molecules GNBP3 and PGRP-SA and connects them to the Grass-SPE-Spätzle extracellular pathway upstream of the Toll receptor (Buchon et al., 2009).

Although we found that MSP is recruited into the Lys-type PG recognition complex, the lack of information regarding the identity of the immediate downstream factor(s) of MSP limits our knowledge of the molecular details of PG recognition and the involvement of SPs in the Toll signaling pathway. Therefore, the identification and characterization of the biological functions of SPs involved in the Tenebrio Toll signaling pathway are essential for the elucidation of the molecular mechanism of innate host defense system. SPE, a terminal SP that converts Spätzle pro-protein into a processed form capable of binding the Toll receptor, was identified in Drosophila (Jang et al., 2006). We purified the SPs immediately downstream protease of Tenebrio MSP in order to obtain biochemical information regarding the Lys-type PG recognition signal-dependent activation of the Toll cascade.

Two SPs, Tenebrio 41-kDa and 44-kDa protein, were purified to homogeneity by several column chromatographies and their cDNAs were cloned (Kim et al., 2008). In order to identify the SPE molecule in Tenebrio, we initially examined the active form of the 44-kDa protease because the 44-kDa zymogen protein is similar to Drosophila SPE and Easter. Since the cDNAs of Tenebrio Spätzle and the Toll proteins have not been determined, we
expressed the recombinant Tribolium Spätzle pro-protein and the Toll ectodomain in a baculovirus system. To address whether the purified active 44-kDa protease cleaves Tribolium Spätzle pro-protein in vitro, we incubated the pro-Spätzle protein with trypsin, the active forms of Tenebrio MSP and the 41-kDa and 44-kDa proteases. Only the active 44-kDa protease cleaved the Spätzle pro-protein. Under the same conditions, trypsin cleaved the pro-Spätzle protein nonspecifically, and the active forms of Tenebrio MSP and the 41-kDa protease did not cleave the pro-Spätzle protein. After performing further biochemical experiments, we confirmed 44 kDa protease as Tenebrio SPE. Also, recombinant HP8 protease functioning as Manduca SPE was purified and its biological function was also determined (An et al., 2009).

We next tried to identify the upstream activator of Tenebrio SPE. Because Tenebrio SPE has a trypsin cleavage site, we hypothesized that the upstream SP is a trypsin-like SP. As mentioned above, the MSP and the 41-kDazymogens were identified as chymotrypsin-like and trypsin-like SPs, respectively, suggesting that the active form of the 41-kDa protease may cleave the SPE zymogen. To test this hypothesis, we incubated the active form of 41-kDa protein with the purified SPE zymogen. As expected, the SPE zymogen was hydrolyzed into a 35-kDa SP domain and a 15-kDa clip domain. The 35-kDa band was identified as the SP domain of SPE. Therefore, we designated the 41-kDa protease as Tenebrio SPE-activating enzyme (SAE).
Because the sequence of the cleavage site in the SAE zymogen is Leu-124-Ile-125, the upstream SP of SAE is probably similar to chymotrypsin. This result suggests that the SAE zymogen is cleaved by the MSP. Therefore, active Tm-MSP was incubated with the recombinant SAE zymogen. The SAE zymogen was hydrolyzed into a 35-kDa SP domain and an 11-kDa clip domain. The N-terminal amino acid sequence of the 35-kDa band was Ile-Val-Gly-Gly-Thr-Asn. This sequence is identical to the amino acid sequence of the SAE zymogen from Ile-125 to Asn-130, demonstrating that the MSP induced a limited proteolytic cleavage between the clip domain and the catalytic SP domain of the SAE zymogen. Thus, the SAE protease is an immediate downstream target of MSP. The domain organization and the cleavage sites by upstream SPs were summarized in Figure 1B.

Recently, SAE orthologue from Manduca system is identified and its biochemical properties are demonstrated (An et al., 2009). Drosophilia SPs required for the activation of Toll signaling cascade were screened using RNAI technology by Kambiris et al. (2006). They suggested that two D. melanogaster catalytic SPs (Dm-Grass and Dm-Spirit) and two noncatalytic SP homologs (SPhs), such as Dm-Spheroide and Dm-Sphinx1/2, have been 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 noncatalytic 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 serine protease inhibitors (serpins) in the hemolymph and are not directly involved in the Toll pathway activation. By performing RNAI experiments against Drosophilia 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 cleaved by the released serpins leading to inhibition of the Toll pathway activation.

Lys-type PG recognition signal is amplified via a three step proteolytic cascade

Finally, to confirm whether the Spätzle proprotein is cleaved, Lys-type-PG, PGRP-SA, GNBP1, zymogens of MSP, SAE, SPE and pro-Spätzle were incubated together in the presence of Ca^{2+}, and the cleavage of pro-Spätzle was detected by western blot analysis. As predicted, a 14-kDa band corresponding to cleaved Spätzle was observed by Kim et al., 2008. However, if any one of the components was omitted from the incubation mixture, no cleavage of the pro-Spätzle occurred (Kim et al., 2008). In conclusion, these experiments demonstrated that the PGRP-SA/GNBP1-mediated Lys-type PG recognition signal is transferred by three different SPs: the initiating enzyme is the 82-kDa chymotrypsin-like MSP, and the other two enzymes are the 41-kDa SAE and 44-kDa SPE clip domain-containing trypsin-like SPs.

SP zymogens involved in the β-1,3-glucan-mediated Toll signaling cascade

To identify the immediate downstream molecule(s) that is recruited by the β-1,3-glucan/GNBP3 complex, insoluble β-1,3-glucan was incubated with the native Tenebrio GNBP3. When GNBP3/insoluble β-1,3-glucan complex was incubated with the Tenebrio hemolymph fraction, a 35-kDa band was specifically enriched in the GNBP3/insoluble β-1,3-glucan complex. The N-terminal amino acid sequence of the 35-kDa protein perfectly matched that of the catalytic SP domain of activated Tenebrio MSP, suggesting that activated MSP was recruited to the β-1,3-glucan/GNBP3 complex and that MSP is an apical SP that functions as the immediate downstream molecule of the β-1,3-glucan/GNBP3 complex (Roh et al., 2009) as like the apical downstream SP of PGRP-SA/GNBP1 in response to Lys-type PG (Kim et al., 2008). Because Tenebrio pro-MSP is activated in the presence of either the β-1,3-glucan/GNBP3 complex or the Lys-type PGN/PGRP-SA/GNBP1 complex, we assumed that the downstream SPs of the β-1,3-glucan/GNBP3 complex would be identical to Tenebrio SAE and SPE that are activated in response to Lys-type PG.

β-1,3-glucan recognition signal is also amplified via three-step proteolytic cascade

We performed in vitro reconstitution experiments by using five purified proteins: GNBP3, pro-MSP, pro-SAE, pro-SPE, and pro-Spätzle (Roh et al., 2009). Western blot analysis revealed that processed Spätzle was generated upon incubation of the five proteins, β-1,3-glucan, and Ca^{2+} (Roh et al., 2009). Depletion of any of the components resulted in the loss of cleavage of the pro-Spätzle. Under the same conditions, the processing of pro-Spätzle to the cleaved Spätzle was also observed with PGRP-SA, GNBP1, MSP, SAE, SPE, and Spätzle in the presence of Lys-type PG and Ca^{2+} (Roh et al., 2009). These results clearly demonstrate that GNBP3, in the presence of β-1,3-glucan, induces the activation of a three-step proteolytic cascade involving MSP, SAE, and SPE sequentially. This activation leads to the processing of pro-Spätzle into the mature form that functions as a ligand for the Toll receptor. In addition, these data show that the β-1,3-glucan and Lys-type PG recognition signals are sharing a common three step proteolytic cascade to transduce their recognition signals to the Toll receptor (Fig. 2).

The effector molecules after Toll signaling cascade activation

Although we have provided biochemical evidence elucidating the mechanism by which the Lys-type PG and β-1,3-glucan recognition signals are transferred to the Toll receptor, we have not demonstrated whether this SP cascade is present in vivo. We hypothesized that if this cascade is present
in vivo, the same AMP(s) will be produced in the insect hemolymph when the pathway molecules are injected into the Tenebrio larvae since Tenebrio Toll signaling pathway is using a common proteolytic cascade. To address this hypothesis, we injected β-1,3-glucan, Lys-type PG, activated SAE and processed Spätzle into the Tenebrio larvae. The hemolymphs collected after injection of above four molecules had high antimicrobial activities against S. aureus, E. coli and S. cerevisiae. We purified two AMPs, tenecin 1 and tenecin 2, by column chromatography from these four hemolymphs. Tenecin 1 had a bactericidal activity against Gram-positive bacteria and was previously identified by our group (Moon et al., 1994). The amino acid sequence of tenecin 1 and its disulfide bond arrangement are similar to the defensin protein from Drosophila (Bulet et al., 1999). Tenecin 2 is highly homologous (65 % identity) to coleoptericin (Bulet et al., 1991), which was purified from the coleopteran insect, Zophobas atratus. Tenecin 2 showed bactericidal activity against E. coli. Taken together, tenecin 1 and tenecin 2 are induced by treatment with β-1,3-glucan, Lys-type PG, SAE and Spätzle suggesting that β-1,3-glucan and Lys-type PG activate Toll receptors by the same three-step proteolytic cascade, which results in the production of tenecin 1 and 2 (Fig. 2).
Diversity of Toll proteolytic signaling cascades in *Drosophila*, *Manduca* and *Tenebrio* system

The outline of activation mechanism of Toll signaling cascades in three different insects are summarized in Figure 3. Intensive biochemical studies performed in *T. molitor* and *M. sexta*, together with genetic analysis in *D. melanogaster*, reveal striking similarities in the mechanisms underlying SP activation by pattern recognition proteins. All involve the sequential activation of typical SPs, such as modular SP and clip-domain containing SPs. Interestingly, *Tenebrio* and *Drosophila* used MSP as an apical SP in the Toll cascade, but, *Manduca* HP14 corresponding to *Tenebrio* SAE, suggesting that another direct downstream SP of PGRP-SA/GNBP1 complex might be existing in hemolymph in *Manduca* system. In *Drosophila*, epistasis analysis has demonstrated that an *Drosophila* MSP named Modular SP (ModSP), acts down stream of PGRP-SA and upstream of the SP Grass (Buchon et al., 2009). Moreover, *Drosophila* ModSP does not participate in the Persephone-dependent branch of the Toll pathway, but is instead part of a linear pathway of SPs connecting microbe recognition by pattern recognition proteins to the activation of Spätzle by SPE. *Tenebrio* MSP, *Manduca* HP14 and *Drosophila* ModSP protease all share a common structure comprising four or five LDL domains followed by a complement control domain and a C-terminal SP domain. The upstream pathogen recognition features of insect’s Toll cascade are also reminiscent of the complement activation by the lectin pathway in mammals in which the recognition of carbohydrate by the mannose binding lectin (MBL) leads to the autoactivation of MBL-associated SPs (MASPs, Matsushita and Fujita, 1992). MASPs also showed similar domain organization with those of insect MSPs.

*Manduca* Toll cascade summarized that proHP6 becomes activated in response to microbial infection and participates in two immune pathways (Fig. 3): activation of PAP1, which leads to pro-phenoloxidase activation and melanin synthesis, and activation of HP8, which stimulates a Toll-like pathway (An et al., 2009). HP8 activates Spätzle to induce AMP synthesis via Toll receptor. In melanin synthesis cascade, an initiation proteinase precursor, proHP14, is autoactivated in response to Gram-positive bacterial or fungal infection. HP14 activates proHP21; HP21 activates proPAP2 or proPAP3; PAP2 or PAP3 then cleaves pro-phenoloxidase to form active phenoloxidase in the presence of SPH1 and SPH2. Activation of pro-phenoloxidase can also be catalyzed by PAP1 when the high M, SPH complex is present simultaneously. PAP1 also activates proSPH2 directly and can indirectly lead to proHP6 activation (Wang and Jiang, 2007, 2008).

*Tenebrio* Toll cascade support a model in which Lys-type PG and β-1,3-glucan activate a common set of three SP zymogens sequentially (Fig. 3). This three-step proteolytic cascade-dependent

Fig. 3 A comparison with *Drosophila*, *Manduca* and *Tenebrio* extracellular proteolytic Toll signaling pathways involving SP cascades. Arrows indicate the activation of downstream components or steps in cascade. Dashed arrows indicate steps that have not been experimentally verified or in which components of the pathway have not yet been determined. PG, peptidoglycan; PGRP, peptidoglycan recognition protein; GRP, β-1,3-glucan recognition protein; GNBP, Gram-negative binding protein; proPO, prophenoloxidase; PO, phenoloxidase; MSP, modular SP; ModSP, Modular SP; SPE, Spätzle-processing enzyme; SAE, SPE activating enzyme; Psh, Persephone; SPH, SP homologue; HP, hemolymph protease; PAP, pro-PO activating protease.
mediated melanin biosynthesis. Also, the protein resulting in the constitutive activation of the Toll SPN43Ac extensively studied using a genetic approach. known to be involved in innate immunity (SPN43Ac, Lomas, 2009). To date, four Drosophila serpins are known to be involved in innate immunity (SPN43Ac, SPN27A, SPN77Bα and SPN28D) and have been extensively studied using a genetic approach. SPN43Ac mutant flies accumulate cleaved Spätzle, resulting in the constitutive activation of the Toll signaling pathway and expression of AMPs (Levashina et al., 1999). SPN27A and SPN28D regulate the Toll pathway during early development (Hashimoto et al., 2003; Ligoxygakis et al., 2003; Scherfer et al., 2008) and are involved in the melanin biosynthesis. (DeGregorio et al., 2002; Ligoxygakis et al., 2002b) Another serpin, SPN77Bα, was identified as a negative regulator of melanization in the Drosophila respiratory system (the trachea) (Tang et al., 2008). In Manduca system, Kanost’s group reported elegant results regarding serpin splicing: 12 different copies of Manduca serpin 1 undergo mutually exclusive alternative splicing to produce 12 putative protein isoforms, which differ in their carboxyl-terminal 39-46 residues including the P1 residue. These serpins inhibited Manduca SPs with different specificities (Jiang and Kanost, 1997; Ragan et al., 2010). These serpins were characterized and suggested as negative regulators of the pro-phenoctyriase and Toll signaling cascades (Kanost et al., 2004).

However, molecular regulatory mechanisms of how serpins regulate invertebrate’s innate immune responses are not well understood due to the uncertainty of the identity of the target SPs by the serpins. We purified three novel serpins (SPN40, SPN55 and SPN48) from the hemolymph of T. molitor (Jiang et al., 2009) (Fig. 2). These Tenebrio serpins made specific serpin-SP complexes with three Toll signaling cascade-activating SPs, such as MSP, SAE, and SPE and cooperatively blocked Tenebrio Toll signaling cascade and β-1,3-glucan-mediated melanin biosynthesis. Also, the protein expression levels of SPN40 and SPN55 were dramatically increased in vivo by the injection of a Toll ligand, processed Spätzle, into Tenebrio larvae. This increase in SPN40 and SPN55 protein levels indicates that these two serpins function as inducible negative feedback inhibitors. Surprisingly, Tenebrio SPN55 and SPN48 were cleaved after Tyr and Glu residues of reactive center loops, respectively, despite being targeted by trypsin-like SAE and SPE proteases. These unexpected cleavage patterns are also highly similar to those of unusual mammalian serpins involved in blood coagulation and blood pressure regulation, indicating that they may contribute to highly specific and timely inactivation of detrimental SPs during innate immune responses. Taken together, our results showed the specific regulatory mechanisms of innate immune responses by three Tenebrio novel serpins.

A balance between activation and inhibition of the SP-mediated innate immune response must be maintained to avoid damage to the host (Ferrandon et al., 2007). As described above, Tenebrio three serpins targeting three Toll cascade-activating SPs act as negative regulators of Toll signaling, indicating that each SP in a cascade may be regulated by a specific serpin. This is first biochemical evidences that SP-serpin pairs directly regulate the pattern recognition protein-dependent Toll signaling cascade. To date, several cascade reactions including Drosophila Toll signaling cascade have been hypothesized to be regulated by a single “bottleneck” protease. However, our data show that the control of Tenebrio Toll proteolytic signaling cascades is more precisely regulated. Furthermore, we have demonstrated that SPN40 and SPN55 function as inducible negative feedback regulators in vivo. These results, in combination with our other reports (Kan et al., 2008; Kim et al., 2008; Roh et al., 2009) support a model in which the Lys-type PG- and β-1,3-glucan-dependent Toll signaling and pro-phenoctyriase cascades are negatively regulated by three endogenous serpins (Fig. 2). Finally, our studies highlight the elaborate regulatory mechanism of invertebrate innate immune defense systems.

Conclusion

The developments of genetics and molecular biology enable us to screen the Drosophila mutants that had deficient immune responses against bacterial and fungal infection, subsequently to discover two conserved innate immune signaling cascades-Toll and Imd-both of which lead to the activation of nuclear factor kB (NF-kB) transcription factors (Lemaitre and Hoffmann, 2007). In this review, the activation and regulation mechanisms of Toll cascade were discussed. However, molecular mechanism by microbe-derived protease-mediated Toll cascade (so called danger signaling pathway) is not determined yet. The exact molecular activation and regulation mechanism of microbial protease-mediated Toll pathway should be determined, such as how pro-Persephone is cleaved by microbial protease and identity of Tenebrio or Manduca counter part protease of Drosophila Persephone proteinase, and whether really active form of
Persphone specifically cleave pro-SPE for the activation of Spätzle in Tenebrio or Manduca system. Also, DAP-type PG-mediated Imd signal pathway is unclear. Even though the identities of pattern recognition receptors of Drosophila Imd pathway and recognition mechanism between tracheal cytotoxin (TCT) and PGRP-LE or PGRP-LC were determined (Chang et al., 2006; Lim et al., 2006), TCT will not be generated naturally by all Gram-negative bacteria and bacilli species. Therefore, we should find a natural ligand of Imd pathway and also should elucidate the DAP-type PG recognition mechanism and extracellular signaling pathway of Imd pathway in future. Another unexploited area is how mycoplasma bacteria, which are deficient of PG in cell membrane, are recognized in insects. Understanding the signaling pathway triggered by mycoplasma and how their recognition activates invertebrate innate immune responses will be big homework for invertebrate immunologist and biochemists.

Also, further challenging theme in insect immunity is the determination of recognition and activation mechanisms against intracellular pathogenic microbes, such as Listeria monocytogenes. Recently, Kurata’s group nicely discussed the relationship between autophagy and insect innate immunity (Yano et al., 2008; Kurata, 2010). Drosophila PGRP-LC and -LE are known to sense the DAP-type PG of extracellular and intracellular-infective bacteria and then to induce several innate immune responses, such as AMP production, via activation of Imd pathway. Yano et al observed PGRP-LE-dependent autophagy when Drosophila adults were infected with L. monocytogenes, which is independent of the Imd pathway (Yano et al., 2008). It will be worthwhile to investigate the identification of agonist molecule that induces PGRP-LE-mediated autophagy and to determine the molecular mechanism of DAP-type PG-dependent autophagy.

Acknowledgements
This work was supported for two years by Pusan National University Research Grant.

References


Jiang H, Kanost MR. Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from Manduca


