ISJ 7: 181-191, 2010 ISSN 1824-307X

REVIEW

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

KH Ryu[1], JW Park[1], K Kurokawa[1], M Matsushita[2], BL Lee[1]

[1]*The National Research Laboratory of Defense Proteins, College of Pharmacy, Pusan National University, Busan, 609-735, Korea*
[2]*Department 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 germ line-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; *GNBP*, Gram-negative binding protein; *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 GNBP3, 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 (Jang *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 SPs, 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 purify several different *Tenebrio* SPs. We demonstrated that the recognition of Lys-type PG-mediated by the PGRP-SA/GNBP1 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 GNBP3-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

PGRP family proteins 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. After 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 (βGRP) 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 (Lemaitre *et al*., 1996). Native GNBP3 proteins were purified from three insects, silk worm (*B. mori*, Ochiai and Ashida, 2000), tobacco hormworm (*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-phenoloxidase 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 of 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, Buchon *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; Buchon *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

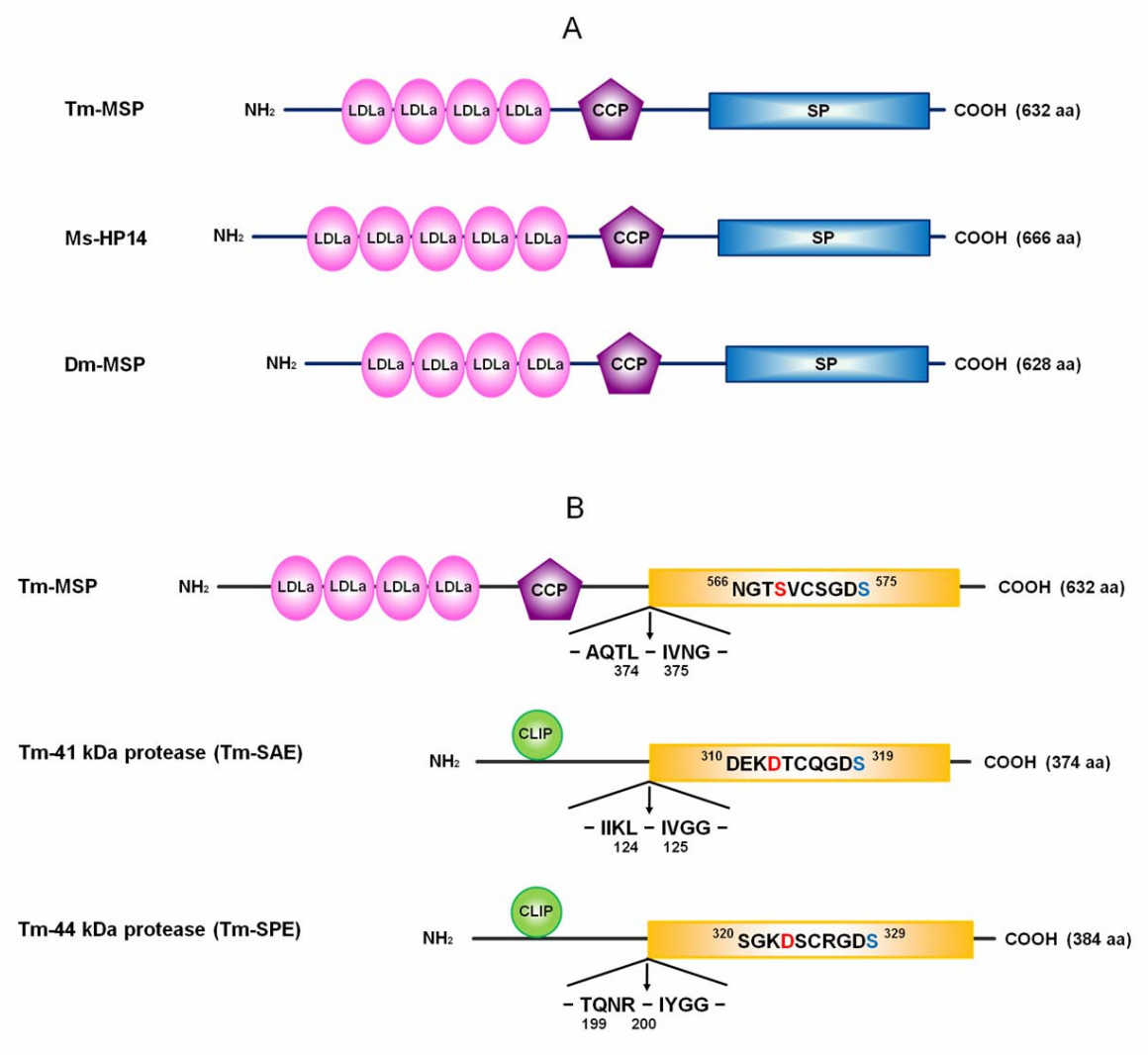

Fig. 1 The domain organizations of MSP homologues (A) and Tm-MSP, Tm-41 kDa SP (Tm-SAE) and Tm-44 kDa SP (Tm-SPE) (B). A, Comparison of domain organization between *T. molitor* MSP (Tm-MSP), *M. sexta* hemolymph proteinase 14 (Ms-HP14), and Dm-MSP homologue (CG31217). Pink circles, rectangular symbols and boxes indicate the domains of LDLa, CCP, and SP domains of MSP homologues, respectively. B, Green circles symbol indicates the clip domain. *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.

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-kDa zymogens 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). *Drosophila* SPs required for the activation of Toll signaling cascade were screened using RNAi technology by Kambris *et al*. (2006). They suggested that two *D. melanogasrter* 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 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 serine protease inhibitors (serpins) in the hemolymph and are not directly involved in the Toll pathway activation. 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.

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

Finally, to confirm whether the Spätzle pro-protein 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 (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

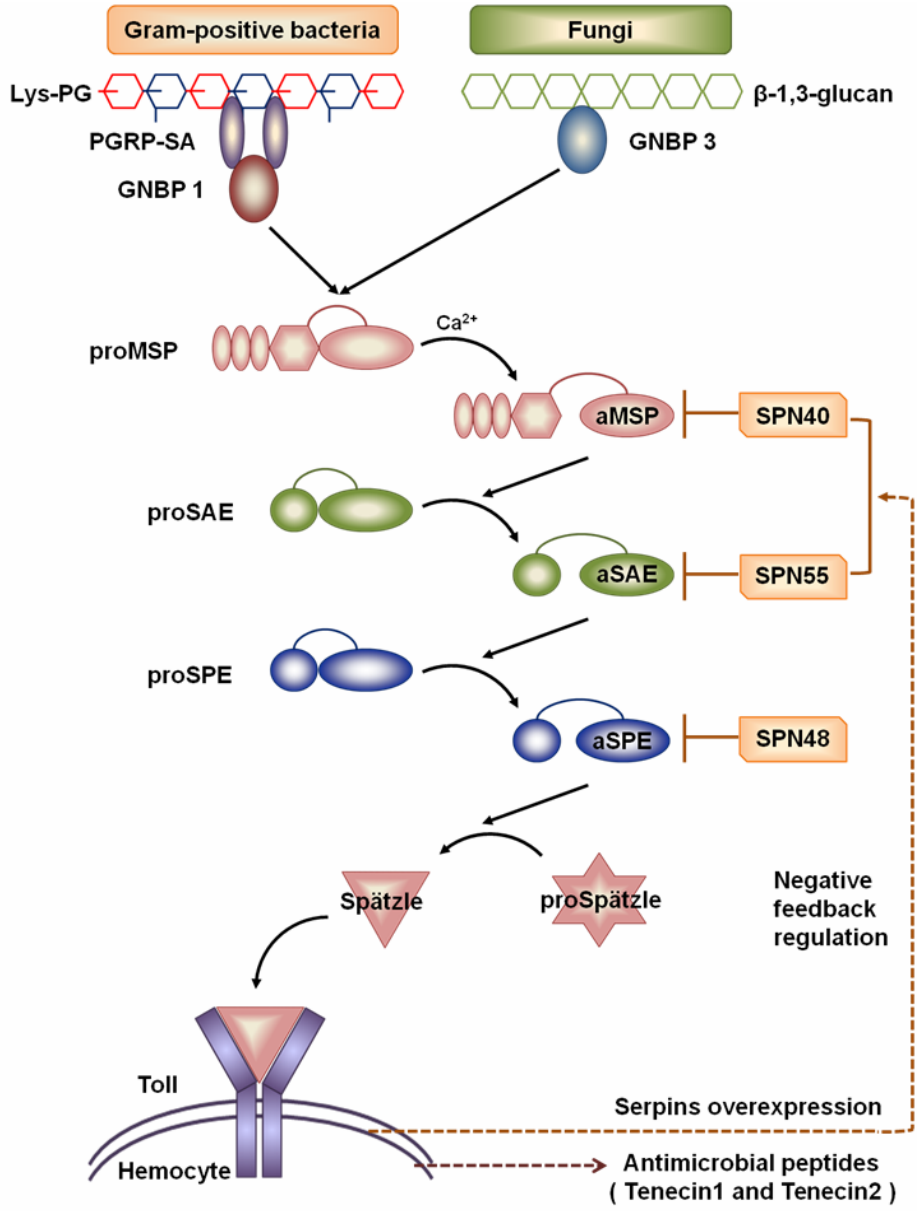

Fig. 2 Model summarizing the molecular events in the regulation of the *Tenebrio* Toll signaling cascade. The molecular activation mechanism of the *Tenebrio* Toll cascade leading to production of AMPs. Processed Spätzle induces the production of endogenous SPN40 and SPN55 as a negative feedback regulator of the Toll cascade.

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).

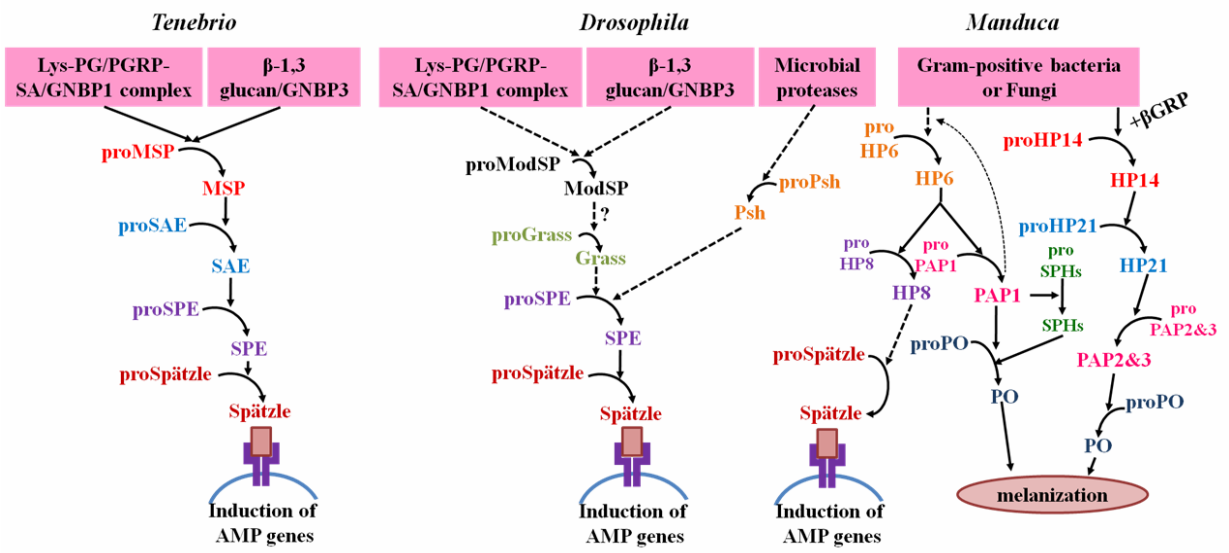

Fig. 3 A comparison with *Drosophila*, *Manduca* and *Tenebrio extracellular* proteolytic Toll signaling pathways involving SP cascades. *Arrows* indicate the activation of downstream components or steps in cascade. *Dashed arrows* indicate steps that have not been experimentally verified or in which 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.

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 MSP did not activate HP6 zymogen 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 proteases 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_r 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

processing of the extracellular pro-Spätzle produces active Spätzle, which then binds to the Toll receptor, resulting in the induction of AMP expression in the *Tenebrio* larval hemocytes. Each of the three SPs has unique biochemical properties to regulate activation of the *Tenebrio* Toll pathway. The initial enzyme pro-MSP is an 82-kDa SP zymogen with an N-terminal chymotrypsin-like cleavage site and a C-terminal chymotrypsin-like catalytic SP domain. Pro-SAE is a 41-kDa SP with an N-terminal chymotrypsin cleavage site and a C-terminal trypsin-like catalytic SP domain, and pro-SPE is a 44-kDa protein with an N-terminal trypsin-like cleavage site and a C-terminal trypsin-like catalytic SP domain. These three different SP combinations enhance the specificity of the proteolytic SP cascade and prevent nonspecific cleavage of these SP zymogens.

Three serpins functioning as regulatory molecules of Toll signaling cascade

Serpins act as suicide substrates by binding covalently to their target proteases and belong to a superfamily of SP inhibitors (Gettins, 2002). Serpins regulate various physiological processes and molecular defense systems, such as blood coagulation, fibrinolysis, inflammation and complement activation in mammals (Gooptu and Lomas, 2009). To date, four *Drosophila* serpins are known to be involved in innate immunity (SPN43Ac, SPN27A, SPN77Ba 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.(De Gregorio *et al*., 2002; Ligoxygakis *et al.*, 2002b) Another serpin, *SPN77Ba*, 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-phenoloxidase 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-phenoloxidase 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 proteinase and identity of *Tenebrio* or *Manduca* counter part protease of *Drosophila* Persephone proteinase, and whether really active form of

Persephone 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

An C, Ishibashi J, Ragan EJ, Jiang H, Kanost MR. Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. J. Biol. Chem. 284: 19716-19726, 2009.

Buchon N, Poidevin M, Kwon HM, Guillou A, Sottas V, Lee BL, *et al*. A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway. Proc. Natl. Acad. Sci. USA 106: 12442-12447, 2009.

Bulet P, Cociancich S, Dimarcq JL, Lambert J, Reichhart JM., Hoffmann D, *et al*, Insect immunity. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. J. Biol. Chem. 266: 24520-24525, 1991.

Bulet P, Hetru C, Dimarcq JL, Hoffmann D. Antimicrobial peptides in insects; structure and function. Dev. Comp. Immunol. 23: 329-344, 1999.

Cerenius L, Kawabata SI, Lee BL, Nonaka M, Söderhäll K. Proteolytic cascades and their involvement in invertebrate immunity. Trends. Biochem. Sci., 2010 [in press].

Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29: 263-271, 2008.

Chang CI, Chelliah Y, Borek D, Mengin-Lecreulx D, Deisenhofer J. Structure of tracheal cytotoxin in complex with a heterodimeric pattern-recognition receptor. Science 311: 1761-1764, 2006.

De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata S, *et al*. An immune-responsive serpin regulates the melanization cascade in *Drosophila*. Dev. Cell 3: 581-592, 2002.

Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. Nat. Rev. Immunol. 7: 862-874, 2007.

Gay NJ, Gangloff M. Structure and function of Toll receptors and their ligands. Annu. Rev. Biochem. 76: 141-165, 2007.

Gettins PG. Serpin structure, mechanism, and function. Chem. Rev. 102: 4751-4804, 2002.

Gobert V, Gottar M, Matskevich AA, Rutschmann S, Royet J, Belvin M, *et al*. Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors. Science 302: 2126-2130, 2003.

Gooptu B, Lomas DA. Conformational pathology of the serpins: themes, variations, and therapeutic strategies. Annu. Rev. Biochem. 78: 147-176, 2009.

Gottar M, Gobert V, Matskevich AA, Reichhart JM, Wang C, Butt TM, *et al*. Dual detection of fungal infections in *Drosophila* via recognition of glucans and sensing of virulence factors. Cell 127: 1425-1437, 2006.

Hashimoto C, Kim DR, Weiss LA, Miller JW, Morisato D. Spatial regulation of developmental signaling by a serpin. Dev. Cell 5: 945-950, 2003.

Hedengren M, Asling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, *et al*. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Mol. Cell 4: 827-837, 1999.

Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. Science 284: 1313-1318, 1999.

Jang IH, Chosa N, Kim SH, Nam HJ, Lemaitre B, Ochiai M, *et al*. A Spatzle-processing enzyme required for toll signaling activation in *Drosophila* innate immunity. Dev. Cell 10: 45-55, 2006.

Ji C, Wang Y, Guo X, Hartson S, Jiang H. A pattern recognition serine proteinase triggers the prophenoloxidase activation cascade in the tobacco hornworm, *Manduca sexta*. J. Biol. Chem. 279: 34101-34106, 2004.

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

sexta. J. Biol. Chem. 272: 1082-1087, 1997.

Jiang R, Kim EH, Gong JH, Kwon HM, Kim CH, Ryu KH, *et al*. Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. J. Biol. Chem. 284: 35652-35658, 2009.

Kambris Z, Brun S, Jang IH, Nam HJ, Romeo Y, Takahashi K, *et al*. *Drosophila* immunity: a large-scale *in vivo* RNAi screen identifies five serine proteases required for Toll activation. Curr. Biol. 16: 808-813, 2006.

Kan H, Kim CH, Kwon HM, Park JW, Roh KB, Lee H, *et al*. Molecular control of phenoloxidase-induced melanin synthesis in an insect. J. Biol. Chem. 283: 25316-25323, 2008.

Kang D, Liu G, Lundstrom A, Gelius E, Steiner H. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. Proc. Natl. Acad. Sci. USA 95:10078-10082, 1998.

Kanost MR, Jiang H, Yu XQ. Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol. Rev. 198: 97-105, 2004.

Kim CH, Kim SJ, Kan H, Kwon HM, Roh KB, Jiang R, *et al*. A Three-step Proteolytic Cascade Mediates the Activation of the Peptidoglycan-induced Toll Pathway in an Insect. J. Biol. Chem. 283: 7599-7607, 2008.

Kurata S. Extracellular and intracellular pathogen recognition by *Drosophila* PGRP-LE and PGRP-LC. Int. Immunol. 22: 143-148, 2010.

Lee WJ, Lee JD, Kravchenko VV, Ulevitch RJ, Brey PT. Purification and molecular cloning of an inducible gram-negative bacteria-binding protein from the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. USA 93: 7888-7893, 1996.

Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25: 697-743, 2007.

Lemaitre B, Nicolas E, Michaut L, Reichhart, JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell 86: 973-983, 1996.

Levashina EA, Langley E, Green C, Gubb D, Ashburner M, Hoffmann JA, *et al*. Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila.* Science 285: 1917-1919, 1999.

Ligoxygakis P, Pelte N, Hoffmann JA, Reichhart JM. Activation of *Drosophila* Toll during fungal infection by a blood serine protease. Science 297: 114-116, 2002a.

Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, Belvin M, *et al.* A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. EMBO J. 21: 6330-6337, 2002b.

Ligoxygakis P, Roth S, Reichhart JM. A serpin regulates dorsal-ventral axis formation in the *Drosophila* embryo. Curr. Biol. 13: 2097-2102, 2003.

Lim JH, Kim MS, Kim HE, Yano T, Oshima Y, Aggarwal K, *et al*. Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. J. Biol. Chem. 281: 8286-8295, 2006.

Ma C, Kanost MR. A beta1,3-glucan recognition protein from an insect, Manduca sexta, agglutinates microorganisms and activates the phenoloxidase cascade. J. Biol. Chem. 275: 7505-7514, 2000.

Matsushita M, Fujita T. Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J. Exp. Med. 176: 1497-1502, 1992.

Medzhitov R, Janeway CA. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91: 295-298, 1997.

Mellroth P, Karlsson J, Steiner H. A scavenger function for a *Drosophila* peptidoglycan recognition protein. J. Biol. Chem. 278: 7059-7064, 2003.

Michel T, Reichhart JM, Hoffmann JA, Royet J. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414: 756-759, 2001.

Moon HJ, Lee SY, Kurata S, Natori S, Lee BL. Purification and molecular cloning of cDNA for an inducible antibacterial protein from larvae of the coleopteran, *Tenebrio molitor*. J. Biochem. 116: 53-58, 1994.

Ochiai M, Ashida M. A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, *Bombyx mori.* J. Biol. Chem. 275: 4995-5002, 2000.

Park JW, Je BR, Piao S, Inamura S, Fujimoto Y, Fukase K, *et al*. A synthetic peptidoglycan fragment as a competitive inhibitor of the melanization cascade. J. Biol. Chem. 281: 7747-7755, 2006.

Park JW, Kim CH, Kim JH, Je BR, Roh KB, Kim SJ, *et al*. Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. Proc. Natl. Acad. Sci. USA 104: 6602-6607, 2007.

Piao S, Song YL, Kim JH, Park SY, Park JW, Lee BL, *et al.* Crystal structure of a clip-domain serine protease and functional roles of the clip domains. EMBO J. 24: 4404-4414, 2005.

Pili-Floury S, Leulier F, Takahashi K, Saigo K, Samain E, Ueda R, *et al. In vivo* RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in *Drosophila* adults. J. Biol. Chem. 279: 12848-12853, 2004.

Ragan EJ, An C, Yang CT, Kanost MR. Analysis of mutually-exclusive alternatively spliced serpin-1 isoforms and identification of serpin-1 proteinase complexes in *Manduca Sexta* hemolymph. J. Biol. Chem. 2010 [in press].

Roh KB, Kim CH, Lee H, Kwon HM, Park JW, Ryu JH, *et al.* Proteolytic cascade for the activation of the insect toll pathway induced by the fungal cell wall component. J. Biol. Chem. 284: 19474-19481, 2009.

Scherfer C, Tang H, Kambris Z, Lhocine N, Hashimoto C, Lemaitre B. *Drosophila* Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. Dev. Biol. 323; 189-196, 2008.

Shia AK, Glittenberg M, Thompson G, Weber AN, Reichhart JM, Ligoxygakis P. Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spatzle secreted by haemocytes. J. Cell. Sci. 122: 4505-4515, 2009.

Tang H, Kambris Z, Lemaitre B, Hashimoto C. A serpin that regulates immune melanization in the respiratory system of *Drosophila*. Dev. Cell 15: 617-626, 2008.

Wang Y, Jiang H. Reconstitution of a branch of the *Manduca sexta* prophenoloxidase activation cascade *in vitro*: Snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenoloxidase-activating proteinase-2 precursor. Insect Biochem. Mol. Biol. 37: 1015-1025, 2007.

Wang Y, Jiang H. A positive feedback mechanism in the *Manduca sexta* prophenoloxidase activation system. Insect Biochem. Mol. Biol. 38: 763-769, 2008.

Weber AN, Tauszig-Delamasure S, Hoffmann JA, Lelievre E, Gascan H, Ray KP, *et al.* Binding of the *Drosophila* cytokine Spatzle to Toll is direct and establishes signaling. Nat. Immunol. 4: 794-800, 2003.

Yano T, Mita S, Ohmori H, Oshima Y, Fujimoto Y, Ueda R, *et al*. Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat. Immunol. 9: 908-916, 2008.

Yoshida H, Kinoshita K, Ashida M. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori.* J. Biol. Chem. 271: 13854-13860, 1996.

Yoshida H, Ochiai M, Ashida M. Beta-1,3-glucan receptor and peptidoglycan receptor are present as separate entities within insect prophenoloxidase activating system. Biochem. Biophys. Res. Commun. 141: 1177-1184, 1996.

Zhang R, Cho HY, Kim HS, Ma YG, Osaki T, Kawabata S, *et al.* Characterization and properties of a 1,3-beta-D-glucan pattern recognition protein of *Tenebrio molitor* larvae that is specifically degraded by serine protease during prophenoloxidase activation. J. Biol. Chem. 278: 42072-42079, 2003.