

## CLIP-domain serine proteases in *Drosophila* innate immunity

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Extracellular proteases play an important role in a wide range of host physiological events, such as food digestion, extracellular matrix degradation, coagulation and immunity. Among the large extracellular protease family, serine proteases that contain a "paper clip"-like domain and are therefore referred to as CLIP-domain serine protease (clip-SP), have been found to be involved in unique biological processes, such as immunity and development. Despite the increasing amount of biochemical information available regarding the structure and function of clip-SPs, their *in vivo* physiological significance is not well known due to a lack of genetic studies. Recently, *Drosophila* has been shown to be a powerful genetic model system for the dissection of biological functions of the clip-SPs at the organism level. Here, the current knowledge regarding *Drosophila* clip-SPs has been summarized and future research directions to evaluate the role that clip-SPs play in *Drosophila* immunity are discussed. [BMB reports 2008; 41(2): 102-107]

### The molecular organization of clip-SPs

The clip domain, which was first described during the identification of pro-clotting enzyme found in the horseshoe crab, received its name due to the "paper clip"-like configuration as a result of the presence of unique disulfide linkages (1). The clip domain is composed of approximately 30 to 60 amino acids, contains three disulfide bonds and is always found in the amino-terminal of the SP domain or SP homologues (SPHs, inactive forms of SPs due to mutation of the enzymatic active site). The exact biological function of the clip domain is presently unknown, however, it may be responsible for mediating specific protein-protein interactions or for regulating cascades of SP activities. Some SPs/SPHs contain an imperfect form of the clip domain whereas other SPs/SPHs have two successive clip domains (2, 3). The crystal structure of clip-SPH (prophenoloxidase (PPO)-activating factor-II) found in the coleopteran,

*Holotrichia diomphalia*, was recently resolved, and the three-dimensional structure revealed a tight association with the SPH and demonstrated that its role as a protein-interacting module was essential for PPO activation (4). In the *Drosophila* genome, 24 clip-SPs (SPs containing a single perfect clip domain) have been identified (Table 1), some of which are involved in early embryogenesis and others that are involved in the innate immune response. This review focuses on current available knowledge regarding the role of clip-SPs in the innate immunity of *Drosophila*.

### The role of clip-SPs in the Toll signaling pathway

The involvement of clip-SPs in the Toll signaling pathway was initially discovered during the studies on dorso-ventral axis formation of the *Drosophila* embryogenesis (5-7). In those studies, 2 clip-SPs, known as easter and snake, were found to be involved in the spätzle cleavage, and genetic evidence showed that snake acts as an upstream SP that activates easter during a protease cascade (7). The activated-form of easter then directly cleaves the pro-form of the spätzle to generate the active-form of spätzle (8). Next, the cleaved active form of spätzle acts as a ligand for the Toll transmembrane receptor, which initiates dorso-ventral axis formation. Additionally, an initial study conducted to evaluate the role of Toll in innate immunity showed that spätzle was involved in Toll activation during the immune response whereas easter and snake were not necessary for infection-induced Toll activation (9). Unlike the Toll-like receptor in humans, *Drosophila* Toll does not function as a pattern recognition receptor that binds directly to microbes (10). Instead, circulating pattern recognition receptors, such as the gram-negative bacteria binding protein (GNBP) family and the peptidoglycan recognition protein (PGRP) family, recognize the existence of microbes and subsequently initiate the Toll activating enzymatic cascade (11-15). Taken together, these findings suggested that an easter-like clip-SP may be involved in the activation of spätzle during immune response.

The connection has been made between clip-SP cascade and immune-induced Toll activation through observation of *necrotic* (*nec*) mutant. The *nec* gene is a member of the family of serine protease inhibitors that are collectively referred to as the serpin family (10). The *nec* mutant flies have been shown to exhibit constitutive Toll signaling activation and subsequent Toll-target gene activation, such as production of the anti-

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Received 14 February 2008

**Keywords:** CLIP domain, *Drosophila*, Pro-phenoloxidase, Serine protease, Toll

**Table 1.** The functions of the clip-SPs in the *Drosophila*

Function	GC numbers or gene name	References
Toll signaling		
Dorso-ventral axis formation	cg4920/easter; cg7996/snake	(5-7)
Innate immune response	cg6367/Persephone; cg16705/SPE; cg2045/Spirit	(16, 20, 21)
PPO signaling	cg1102/MP1; CG3066/MP2/PAE1	(31, 33)
Other functions or unknown functions	cg12949, cg8213, cg5909, cg16710, cg3700, cg5896, cg7432, cg8172, cg9737, cg9372, cg11313, cg9733, cg2056, cg6361, cg16821, cg13744, cg4316/stubble	

fungal *drosomycin* peptide (10). In addition, *nec* mutant flies lack specific serpin, which indicates that a target SP of the *nec* might be constitutively activated in *nec* flies, and that this constitutive activation may lead to chronic activation of the SP cascade, thereby inducing the Toll activation.

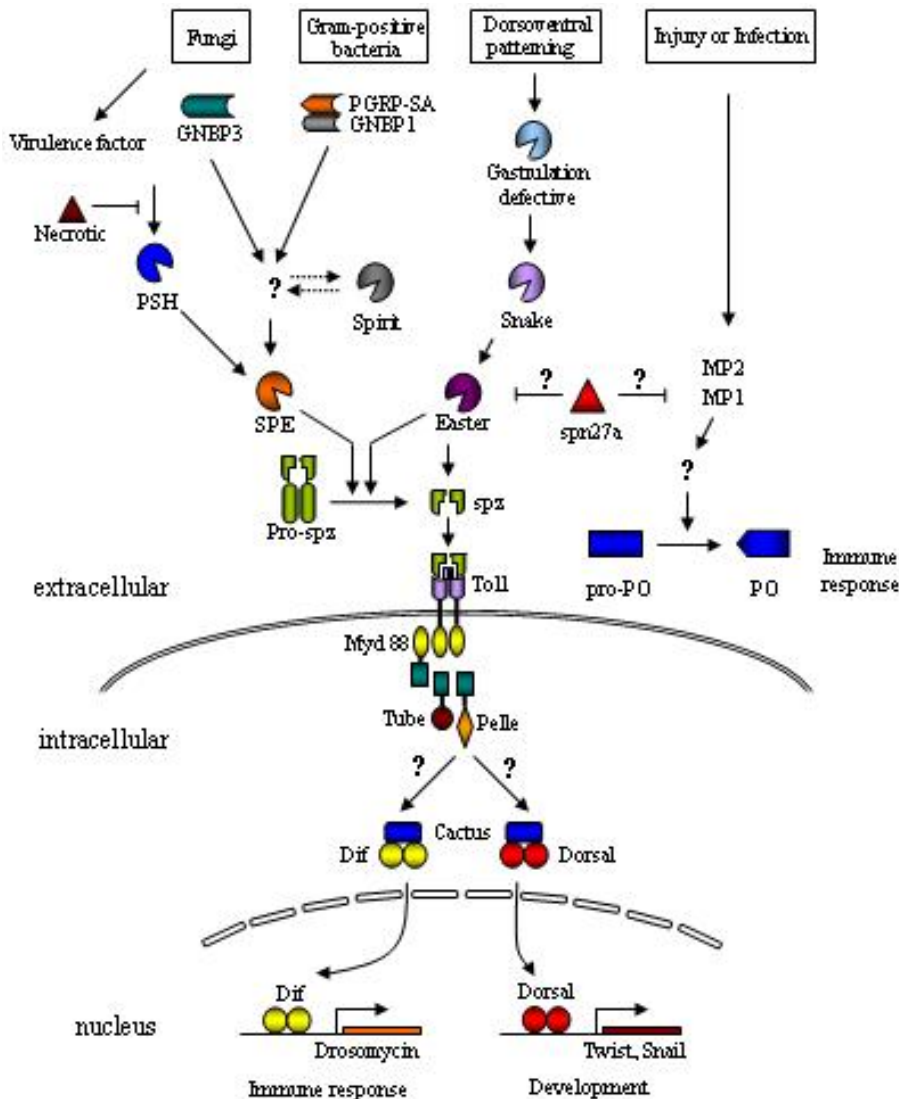
Suppression screening of *nec* mutant (16) allowed the discovery of the clip-SP involved in the immune-activated Toll signaling. This clip-SP, which is known as *persphone* (*psh*) (16), mediates fungi-induced Toll activation but not Gram-positive (G+) bacteria-induced Toll activation. This also suggests that at least two independent SP cascades exist in the immune-induced activation of Toll. Although the *psh* gene has been found to suppress aspects of the *nec* phenotype such as constitutive Toll activation, it is not yet clear whether the *psh* is a direct target clip-SP of the *nec*.

Recently, it has been shown that an enzyme secreted by a cuticle-penetrating fungus is capable of activating *psh* for the Toll activation (12). However, in the case of G+ bacterial infection, Toll pathway can also be activated in a *psh*-independent manner, suggesting that another protease cascade must be involved in Toll activation (11, 15). Because both the fungi- and G+ bacteria-induced protease cascades would presumably lead to the conversion of pro-Spätzle to its active form through limited proteolysis, they must share a downstream protease that cleaves pro-Spätzle. However, the identification of this easter-like pro-Spätzle processing protease has been difficult possibly due to limitations that are inherent in the *Drosophila* model, which is a powerful tool for genetic studies, but not biochemical studies. A lepidopteran insect system, such as *Bombyx mori* (silkworm) larvae, is a better model for biochemical studies, especially when evaluating serine proteases involved in host defense, due to its large size and greater volume of extractable hemolymph (17, 18). Previous biochemical analyses have shown that the plasma fraction of *Bombyx* hemolymph contains a serine protease known as BAEase (based on its ability to hydrolyze the synthetic substrate N<sup>α</sup>-benzoyl-L-arginine ethyl ester, BAEE) that can be activated from a zymogen form by both fungi and G+ bacteria (19), a property shared with the putative Spätzle processing enzyme suggested to be involved in *Drosophila* immunity.

BBAEase is a *Bombyx* clip-SP, and it has been shown that

cleavage between R<sup>112</sup> and I<sup>113</sup> (DR<sup>↓</sup>IFGG) (*i.e.* between the clip domain and the SP domain) is essential for inducing BAEase enzymatic activity. When the *Drosophila* homologue of *Bombyx* BAEase was evaluated, only one of the 24 clip-SPs encountered, CG16705, had an identical cleavage site (DR<sup>↓</sup>IFGG) for zymogen activation (20). This *Drosophila* homologue of *Bombyx* BAEase was subsequently named SPE (Spätzle Processing Enzyme) based on its ability to process Spätzle *in vitro* and *in vivo* (20). The SPE processes pro-Spätzle identically to *easter*, and activated-SPE rescues ventral and lateral development in embryos that lack *easter*, which demonstrates the functional homology between SPE and *easter*. Flies with reduced SPE expression show no noticeable endogenous pro-Spätzle processing and become highly susceptible to fungal and G+ bacterial infection (20). However, the essential difference between SPE and *easter* appears to be that they require distinct mechanisms for activation, which allows the Toll signaling pathway to be activated in response to different triggers and thus used in very different physiological processes. These results suggest that a single ligand/receptor-mediated signaling event can be utilized for different physiological processes by recruiting similar ligand-processing proteases with distinct activation modes during inflammation and development.

The recent development of genome-wide RNA interference (RNAi) flies has enabled screening of multiple SPs, and screening of approximately 75 distinct SPs among the 200 SPs encoded in the *Drosophila* genome resulted in the identification of four novel SPs/SPHs that are critical components of the Toll activating cascade (21). Among these SPs/SPHs, *spirit* (CG2045) is a clip-SP that is required for both fungal- and G+ bacteria-induced activation of the Toll pathway (21). The *spirit* gene appears to act as an upstream component of SPE. Transfection of the active form of *spirit* alone could induce Toll activation (*i.e.* *drosomycin* expression) (22). However, the active form of *spirit* was not able to cleave SPE in a co-transfection experiment that used *Drosophila* S2 cells, (22). Therefore, the exact relationship between *spirit* and SPE requires further investigation at the biochemical and genetic level.



**Fig. 1.** The role of clip-SPs in *Drosophila* physiology. This model is based on previous studies (5, 6, 9-16, 20-22, 31, 33, 35-39). See text for the details.

### The role of clip-SPs in PPO activation

In addition to antimicrobial peptide-producing signaling pathways such as the Toll pathway, the melanin-forming process is also believed to be an important host defense system in insects (18, 23, 24). Parasites or aberrant tissues are often melanized in insects as a result of the enzyme activity of phenoloxidase (PO). PO is normally present as pro-phenoloxidase (PPO), an inactive zymogen form of PO, however, it is converted to PO by limited proteolysis (25). Because melanin is a highly toxic and reactive compound, the activation of inactive PPO into active PO is tightly controlled by the PPO-activating cascade, which is a sequential activation of extracellular serine proteases that are homologous to the Toll-activating pathway.

PO activation is one of the first visible immune reactions in insects, with the cascade being composed of three steps: 1) the recognition of foreign microbes by pattern recognition proteins, 2) signaling amplification by sequential activation of proteases, and 3) activation of the key molecule by terminal SP (i.e. conversion of PPO to PO).

Most studies conducted to evaluate the PPO-activating cascade have been performed using lepidopterans and coleopterans, which is the model of choice for biochemical analyses (26-29). Some of the studies conducted have shown that the PPO system is an important defense system against microbial or parasitic infections (30-32), however, others have shown that PO is not required for the survival of the host after bacterial infection (33, 34). Therefore, the *in vivo* relevance of the

PO system in innate immunity is poorly known, primarily due to the lack of genetic analysis at the organism level.

Surprisingly, very few genetic studies regarding the PPO-activating cascade have been conducted in *Drosophila*. However, flies carrying a mutation on a serpin, called *spn27A*, have been identified and genetically analyzed. These *spn27A* mutant flies show a spontaneous melanization phenotype (35), which indicates that the target SP of the *spn27A* is involved in the PPO-activating cascade. Furthermore, the PPO-activating cascade requires Toll pathway activation and depends on the removal of the *spn27A* gene via Toll-dependent transcription of an acute immune component (36). Therefore, PPO and Toll signaling are intertwined because 1) both signaling pathways require similar activation modes in terms of pattern recognition and protease cascade, and 2) Toll signaling activation is then required for the PO-dependent melanization process to occur.

The clip-SPs involved in the *Drosophila* PPO-activating cascade, CG1102 and CG3066 (also called M1 and M2, respectively), were identified during screening for suppressors of the melanization phenotype of the *spn27A* mutant (31). Independently, CG3066 has also been identified as a PPO-activating enzyme (PAE1) (33). The loss of function (or RNAi) of these clip-SPs can suppress the phenotype induced by *spn27a* mutation, and it has been shown that both clip-SPs are dispensable for host resistance against bacterial infection (31, 33). Interestingly, however, *MP2-RNAi* flies, but not *MP1-RNAi* flies, were less viable after natural infection with the fungus, *B. bassiana* (31). In contrast to this report, *PAE1* mutant flies survived as wild-type flies after various microbial infections, including infection with *B. bassiana* (33), however, we can not currently explain this discrepancy, therefore further studies evaluating various mutants affecting the PO cascade at different levels should be conducted.

Initially, *spn27A* was presumed to inhibit PPO-activating enzyme (PPAE, a terminal enzyme that converts PPO to PO), because *spn27A* is also maternally required for embryonic dorso-ventral patterning (37, 38) where its target is easter, the terminal protease in a cascade leading to activation of Toll signaling. However, the active forms of MP1 or MP2 cannot directly cleave PPO in a biochemical assay (Jang and Lee, unpublished observation). Taken together, these results suggest that MP1/MP2 act as upstream components of a putative PPAE for PPO activation. However, the terminal clip-SPs that act as the PPAE are still unknown.

### Conclusion and future perspective

Among the 24 clip-SPs evaluated, 7 were subjected to genetic analyses by generating null mutant or RNAi flies. Five clip-SPs were found to be deeply involved in the Toll-activating pathway: snake and easter, which are involved in the Toll activation pathway during development, and *psh*, *spirit* and *SPE*, which are involved in the Toll pathway during immune

response. In the case of the PO activation pathway, CG1102 and CG3066 are known to be members of the PO-activating cascade.

However, our understanding of both cascades is still incomplete. Specifically, we do not yet know 1) how the recognition event can transmit the signals necessary for activation of clip-SPs to occur, 2) whether the pattern recognition molecules are shared between the Toll- and PPO-activating cascades, 3) whether any cross talk between the PPO cascade and Toll cascade exists, 4) how many clip-SPs are involved in both cascades, 5) the molecular identity of putative PPAE, and 6) the exact *in vivo* role of the PPO-activating cascade during immune response at the organism level. Further genetic analyses to evaluate the remaining clip-SPs will certainly enhance our understanding of these complex and important host defense systems.

### Acknowledgements

This work was supported by the Korea Research Foundation Grant (KRF-2005-C00316) and Ewha Womans University Grant.

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