Spätzle-Processing Enzyme-independent Activation of the Toll Pathway in *Drosophila* Innate Immunity

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ABSTRACT. **The Toll pathway regulates innate immunity in insects and vertebrates. The *Drosophila* Toll receptor is activated by a processed form of a ligand, Spätzle. Spätzle-processing enzyme (SPE) is the only enzyme identified to date that functions in converting Spätzle to an active form during the immune response. In the present study, Toll activation induced by immune challenge was almost suppressed in *spätzle* mutant larvae and adults, whereas it was present in *SPE* mutant larvae challenged with *Micrococcus luteus* and adults challenged with *Bacillus subtilis*. Our data suggest that an unidentified protease besides SPE processes Spätzle under conditions of microbial challenge.**

Key words: innate immunity, serine protease cascade, Toll pathway

Innate immunity is an evolutionarily conserved host defense system that functions in animals and plants. Toll/Toll-like receptor activation following microbial infection is one of the major innate immune responses leading to the induction of immune effector molecules, such as antimicrobial peptides or inflammatory cytokines (Lemaitre and Hoffmann, 2007; Kawai and Akira, 2010). In *Drosophila*, Toll activation in response to fungal and Gram-positive bacterial infections is triggered by the detection of microbial motifs by appropriate pattern recognition receptors (PRR) such as PGRP-SA, SD, GNBP1, and GNBP3 (PRR-dependent pathway) or the sensing of virulence factors (PRR-independent pathway) (El Chamy *et al.*, 2008). Both pathways activate proteolytic cascades that culminate in the cleavage of the cytokine Spätzle (Spz), an endogenous Toll ligand; the processed form of Spz binds to the Toll receptor (Fig. 1A, shown in black) (Ferrandon *et al.*, 2007). Numerous serine proteases required for Toll activation during infection have been identified (Kambris *et al.*, 2006; Buchon *et al.*, 2009). Among them, only Spätzle-processing enzyme (SPE) has been characterized as a protease that directly cleaves Spz in adult flies challenged with fungi and Gram-positive bacteria (Jang *et al.*, 2006). SPE is

activated by both PRR-dependent and -independent pathways, and is required for Toll activation associated with immune challenge. The role of SPE in adult flies during infection is well understood; however, whether SPE is the only Spz-processing protease with a role in innate immunity remains unclear. We recently identified a new *Drosophila* mutant, *senju*[1], in which Spz is converted to an active form in the absence of immune challenge, which leads to constitutive activation of the Toll pathway (Yamamoto-Hino *et al.*, 2015). Accordingly, Toll activation in *senju*[1] larvae was completely suppressed in the absence of Spz, whereas it was not affected by the introduction of mutant *SPE*. This result indicates that an unidentified protease may activate Spz in *senju*[1] larvae. In the present study, we investigated whether a Spz-processing protease besides SPE is active in immune-challenged larvae and adults using the *SPE* null allele *SPE^{Sk6}*, which was generated by the Cas9-mediated gene editing system (Yamamoto-Hino *et al.*, 2015).

First, we analyzed whether the Toll pathway is activated in the absence of SPE in larvae challenged with the Gram-positive bacterium *Micrococcus luteus* (*M. luteus*), an activator of Toll signaling. Larval bacterial challenge was performed by microinjection of *M. luteus* via a glass capillary. After 12 h, larvae were collected and the expression of the antimicrobial peptide gene *Drosomycin* (*Drs*), a target of the Toll pathway, was assessed using real-time PCR. The *y*, *cho*, *v* strain was used as control because it was used to generate *SPE* mutants. Homozygous *SPE^{Sk6}* died at the late larval stage likely because of a secondary mutation. Therefore, a heterozygote harboring both a *SPE^{Sk6}* allele and a
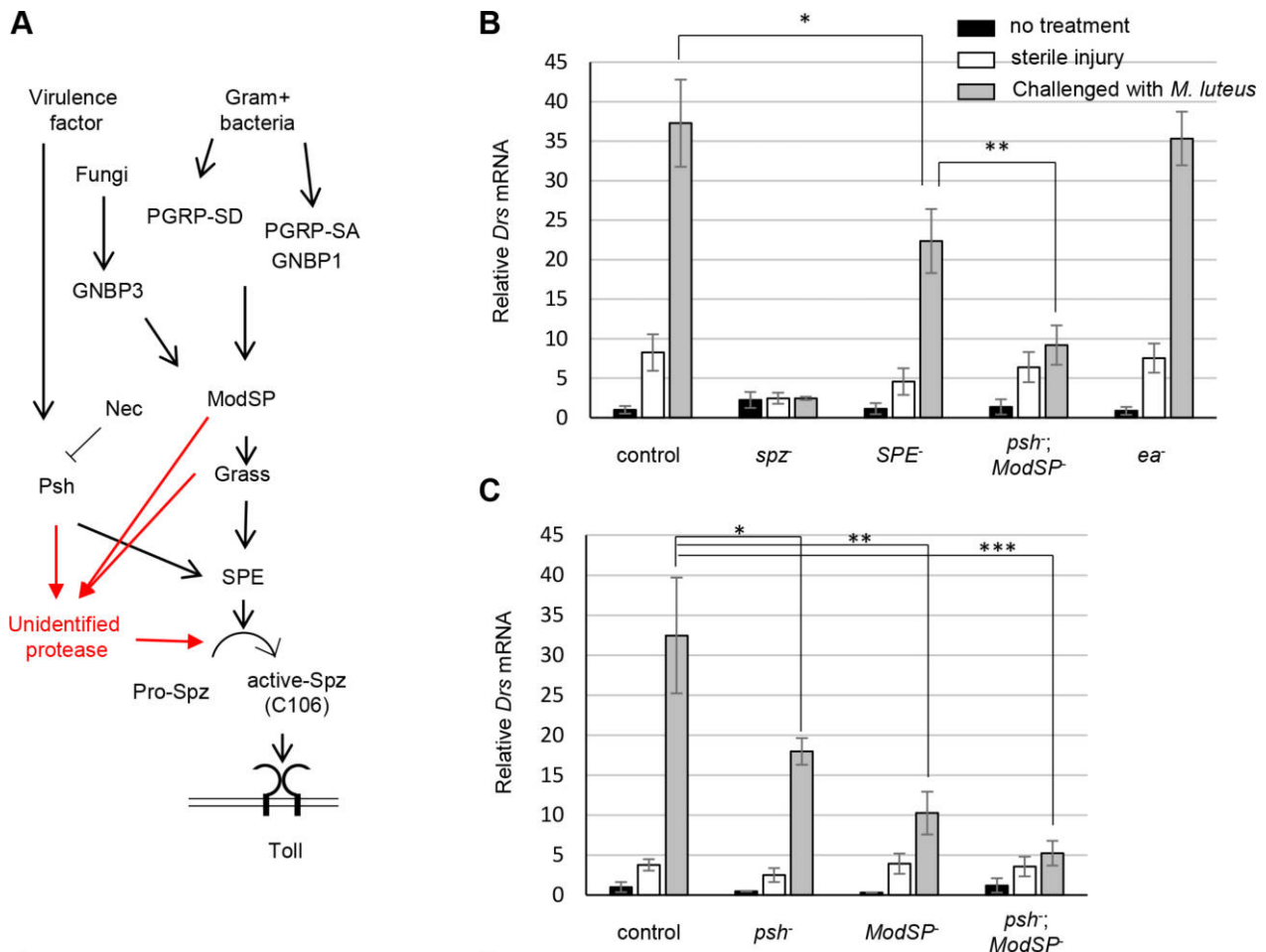

Fig. 1. Spätzle-processing enzyme (SPE)-independent Toll activation in *Drosophila* larvae challenged with Gram-positive bacteria. (A) Schematic depiction of the extracellular Toll pathway. The currently accepted pathway is represented in black, and a new pathway proposed in this study is represented in red. (B) qPCR analysis of *Drs* expression in various mutant larvae exposed to sterile injury and infection with *M. luteus*. *$p<0.05$ and **$p<0.01$ (Student's *t*-test). The genotypes of larvae used in the study were as follows: control (*y*, *cho*, *v*), *spz*⁻ (*spz*rm7/*spz*rm7), *SPE*⁻ (*SPE*sk6/*Df(3R)BSC491*), *psh*⁻;*ModSP*⁻ (*psh*1/*psh*1;;*ModSP*1/*MpdSP*1), and *ea*⁻ (*ea*1/*ea*1). (C) qPCR analysis of *Drs* expression in various mutant larvae exposed to sterile injury and infection with *M. luteus*. *$p<0.05$, **$p<0.01$ and *** $p<0.005$ (Student's *t*-test). The genotypes of larvae used in the study were as follows: control (*y*, *cho*, *v*), *psh*⁻ (*psh*1/*psh*1), *ModSP*⁻ (*ModSP*1/*MpdSP*1), and *psh*⁻;*ModSP*⁻ (*psh*1/*psh*1;;*ModSP*1/*MpdSP*1).

SPE deficiency allele (*Df(3R)BSC491*) was used for analysis since it survived until the adult stage. The expression of *Drs* was strongly induced in the control larvae challenged with *M. luteus* (Fig. 1B), whereas it was downregulated in immune-challenged *SPE* mutants, although a high level of expression was still observed (60–70% of that observed in the control larvae challenged with *M. luteus*). This result indicated that Toll activity after infection with *M. luteus* is partly dependent on SPE and partly mediated by an unidentified larval molecule. This molecule may act upstream of Spz and directly process Spz instead of SPE because the *spz* mutation completely suppressed the induction of *Drs* mRNA after infection with *M. luteus* (Fig. 1B).

Toll signaling is activated by PRR-dependent and -independent pathways (Fig. 1A). The ModSP and Grass serine proteases are required for the PRR-dependent pathway, whereas Psh, a serine protease that senses virulence factors from microbes, triggers the PRR-independent pathway. Both pathways regulate the Toll activation in control larvae challenged with *M. luteus* because *psh* and *ModSP* mutation reduced expression of *Drs* mRNA to 55% and 31% of that observed in the control larvae, respectively (Fig. 1C). We next investigated whether the unidentified protease acts independently from these proteases. Larvae harboring a *ModSP* and *Psh* double mutation and challenged with *M. luteus* showed a reduction of *Drs* expression to the basal level (Fig. 1B), suggesting that the unidentified protease is activated by Psh and/or ModSP, and functions in parallel with SPE (Fig. 1A, shown in red).

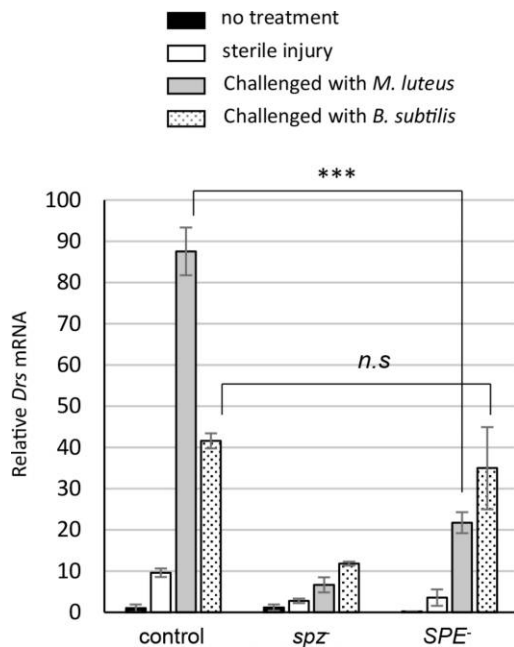The Toll pathway was originally identified as a series of

Legend:
- no treatment (filled black)
- sterile injury (white)
- Challenged with *M. luteus* (gray)
- Challenged with *B. subtilis* (dotted)

Fig. 2. SPE is involved in Toll pathway activation in response to infection with *M. luteus* but not *B. subtilis* in adult flies. qPCR analysis of *Drs* expression in various mutant adult flies exposed to sterile injury and infection with *M. luteus* or *B. subtilis*. ***$p < 0.005$, n.s. indicates not significant (Student's *t*-test).

maternal effect genes that control dorsal-ventral patterning of the *Drosophila* embryo (Anderson and Nusslein-Volhard, 1984). During axis formation, the Easter (Ea) protease processes Spz to generate the Toll ligand (DeLotto and DeLotto, 1998). We investigated whether Ea is the unidentified protease responsible for Toll activation in immune-challenged larvae. *Drs* expression was comparable between the *ea* null mutant and the control after infection with *M. luteus* (Fig. 1B), suggesting that *ea* is not required for Toll pathway activation in response to microbial infection in larvae, as is the case in adult flies (Lemaitre *et al.*, 1996).

To examine the role of SPE in the adult, 30 adult flies were pricked in the thorax with a thin needle dipped in a concentrated pellet of microbial culture. Insects were collected 24 h after infection, and the expression of *Drs* was assessed. *M. luteus*-challenged control flies showed approximately 90-fold higher *Drs* mRNA levels than unchallenged control flies (Fig. 2). *Drs* mRNA expression in response to *M. luteus* was markedly downregulated in *SPE*-deficient flies. This was consistent with previous reports (Jang *et al.*, 2006; El Chamy *et al.*, 2008) and suggests that Toll activation by Lys-type Gram-positive bacteria in adult flies is mostly mediated by SPE, which differs from larval immunity.

The Toll pathway is preferentially activated by infection with Gram-positive bacteria containing Lys-type peptido-

glycans; however, it can also be directly activated by virulence factors such as fungal or bacterial proteases via PRR-independent pathways (Gottar *et al.*, 2006). *B. subtilis*, which is a Gram-positive bacterium with diaminopimelic (DAP)-type peptidoglycans, moderately induced *Drs* expression (Fig. 2). Unlike flies challenged with *M. luteus*, *SPE*-deficient flies challenged with *B. subtilis* showed the same or slightly lower levels of *Drs* mRNA as control flies challenged with *B. subtilis*. The upregulation of *Drs* mRNA expression in response to infection with *B. subtilis* was abolished in the *spz* mutant, suggesting that Toll activation by *B. subtilis* in adult flies was also mediated by an unidentified protease functioning upstream of Spz.

SPE and Ea belong to the CLIP-domain serine protease (clip-SP) family, which is characterized by the presence of one or two regulatory amino-terminal clip domains and a chymotrypsin-like serine protease domain at the C-terminus (Jiang and Kanost, 2000). Clip-SPs are essential for the extracellular serine protease cascade, in which proteins are secreted as inactive zymogens and activated by an upstream protease (Krem and Di Cera, 2002). SPE and Ea are terminal proteases, which function at the end of the cascade and cleave the terminal substrate. Twenty-two clip-SPs were identified in *Drosophila*, and a phylogenetic analysis of the catalytic domains of *Drosophila* clip-SPs showed that CG1102 is closely related to SPE and Ea (Ross *et al.*, 2003). CG1102/MP1 is the protease responsible for the melanization cascade that activates phenoloxidase in response to bacterial and fungal infection (Tang *et al.*, 2006). To test whether CG1102/MP1 can cleave Spz, we generated an activated form of MP1 consisting of the catalytic domain, and coexpressed it with Flag-tagged Spz in S2 cells. Immunoblot analysis with the anti-Flag antibody M2 revealed the production of the processed form of Spz, which has the same molecular size of that generated by activated SPE (Fig. 3A). These data indicated that CG1102/MP1 can process Spz to generate the Toll ligand *in vitro*. Therefore, we investigated whether CG1102/MP1 is involved in Toll activation in response to infection with *M. luteus* in larvae. *MP1*-knockdown larvae generated by crossing the 1102R2 strain carrying the CG1102/MP1-RNAi construct and the ubiquitously expressed driver, *da-Gal4*, showed the same level of *Drs* mRNA as that of control larvae (Fig. 3B), suggesting that Toll activation in larvae infected with *M. luteus* is not mediated by CG1102/MP1.

Recently, Kellenberger *et al.* analyzed the crystal structure of Grass Clip Serine Protease and proposed a structure-based classification of clip-SPs (Kellenberger *et al.*, 2011). These authors divided *Drosophila* clip-SPs into three groups according to the number of α-helices in the clip domain and two groups according to the presence of the 75-loop region, which prevents spontaneous activation of the catalytic domain. Terminal proteases including *Drosophila* (Dm)-Easter, Dm-SPE, *Manduca sexta* (Ms)-SPE, Ms-HP8,
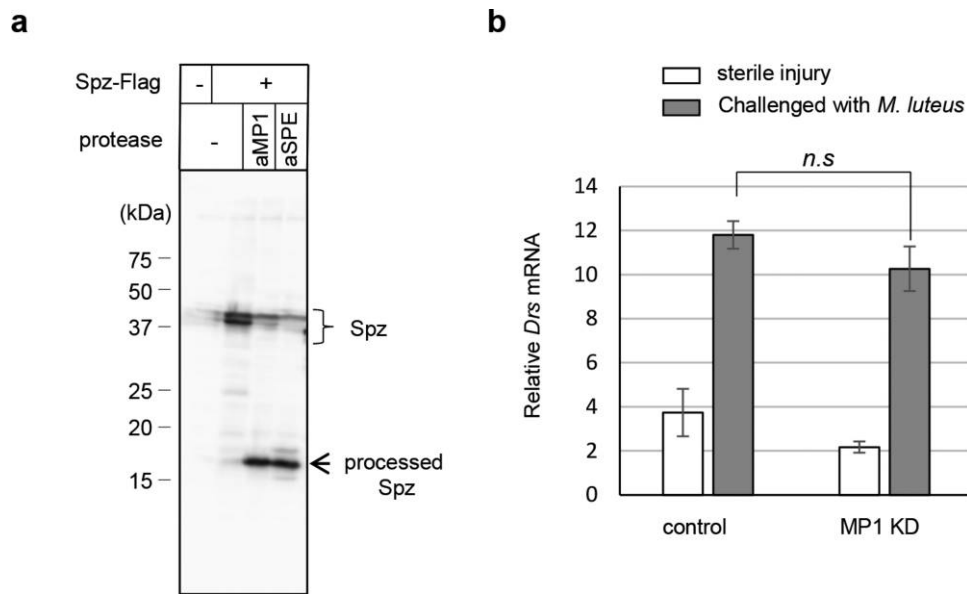
Fig. 3. CG1102/MP1 processes Spz *in vitro*, but does not regulate Toll activity in larvae after infection with Gram-positive bacteria. (A) Immunoblot analysis of Flag-tagged Spz (Spz-Flag) expression in S2 cells. Spz-Flag was coexpressed with activated SPE (aSPE), and activated MP1 (aMP1). Immunoblot analysis using anti-Flag antibody showed that aMP1 produced the processed form of Spz. (B) qPCR analysis of *Drs* expression in CG1102/MP1-knockdown larvae after infection with *M. luteus*. The genotypes of larvae used in the study were as follows: control (*da-Gal4>UAS-lacZ RNAi*) and MP1 KD (*da-Gal4>1102R-2*).

and *Tenebrio molitor*-SPE have a 75-loop in the catalytic domain and two α-helices in their clip domain. The unidentified protease was expected to have these structural features characteristic of terminal proteases. The sequences of *Drosophila* clip-SPs harboring a 75-loop region in the catalytic domain were aligned using the MUSCLE program, and a phylogenetic tree was built using the phyML program (http://www.phylogeny.fr/) (Dereeper *et al.*, 2008). The results showed a close relation between SPE and Ea, and a weak relation with CG16710 (Fig. 4), suggesting that CG16710 is a candidate protease mediating Toll activation in response to microbial infection in larvae. However, CG16710 is expressed at low levels throughout the *Drosophila* life cycle (http://flybase.org/reports/FBgn0039101.html). Moreover, the transcription of immune-related genes is often regulated by immune challenge, and this phenomenon was not observed for CG16710 (De Gregorio *et al.*, 2001; Irving *et al.*, 2001; De Gregorio *et al.*, 2002). Further genetic and biochemical analyses are needed to identify the protease involved in the conversion of Spz to its processed form in immune-challenged larvae.

In conclusion, our analysis strongly suggests the existence of a protease besides SPE that processes Spz to an active form during the immune response of *Drosophila* larvae. The contribution of the unidentified protease to Toll pathway activation in larvae was greater than that in adult flies challenged with Lys-type Gram-positive bacteria, suggesting that different proteases are active under different
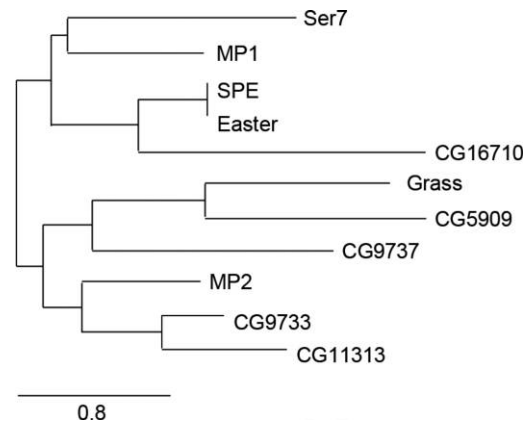

Fig. 4. Phylogenetic analysis of the complete sequences of *Drosophila* clip-SPs harboring a 75-loop in the catalytic domain. The phylogenic tree was constructed using the phyML program based on the multiple sequence alignment by the MUSCLE program. The branch length indicates the evolutionary distance between each member. The scale at the bottom represents evolutionary distance.

conditions. Serine protease cascades control the immune system of flies and mammals, immune responses in invertebrates, and blood clotting and the complement system in mammals. The identification of novel serine proteases and the analysis of their activation/suppression mechanisms will improve our understanding of the regulation of Toll

activity by serine protease cascades during the immune response and facilitate research on these cascades in invertebrate and vertebrate species.

Materials and Methods

Fly stocks

ea[1] flies was obtained from Bloomington Stock Center. 1102R2 was provided by the National Institute of Genetics. Other strains were described previously (Yamamoto-Hino *et al.*, 2015).

Infection experiments

M. luteus or *B. subtilis* strains were used for the infection experiments. Thirty adult flies were pricked in the thorax with a thin needle dipped in a concentrated pellet of microbial culture. Larval challenge was performed by microinjection of a bacterial pellet via a glass capillary. Larvae (12 h after infection) and adult flies (24 h after infection) were collected and used for RT-qPCR.

RT-qPCR

Ten larvae or ten flies were frozen at –80°C and then ground up. Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's protocol. Superscript reverse transcriptase (Invitrogen) and oligo(dT) primers were used for the reverse transcription reaction. Real-time PCR was performed using a 7500 HT Fast Real-Time PCR system (Applied Biosystems) and Power SYBR Green (Applied Biosystems). The amount of amplified transcript was normalized against an internal control (*ribosomal protein 32* (*rpl32*)). The primers were as follows: *rpl32* sense, 5'-GCAAGCCCAAGGGTATCGA-3'; *rpl32* anti-sense, 5'-CGA-TGTTGGGGCATCAGATACTG-3'; and *Drs* sense, 5'-TTGTTC-GCCCTCTTCGCTGTCCT-3'; *Drs* anti-sense, 5'-GCATCCTTC-GCACCAGCACTTCA-3'.

Plasmid, Expression and Western blotting

The expression plasmid Spz-Flag, activated SPE (aSPE), and activated MP1 (aMP1) were generated. To express Flag-tagged Spz, a DNA fragment encoding the 3× Flag sequence was ligated to the 3' end of the *spz* coding region and inserted into the pRmHa vector. A plasmid expressing aSPE was generated according to ref. (Jang *et al.*, 2006). For aMP1, the aMP1 DNA fragment corresponding to the Easter signal peptide was fused directly to the MP1 catalytic domain (amino acids 128–390) and inserted into the pRmHa vector. The plasmid Spz-Flag was coexpressed with the plasmid aSPE or aMP1 in S2 cells using calcium phosphate method. Cells lysed with RIPA buffer were subjected to SDS-PAGE. The proteins were transferred to PVDF membranes (Millipore), which were then blocked with PBS containing 0.05% Tween 20 and 5% skim milk, followed by an overnight incubation with the anti-Flag antibody M2. The primary antibody was detected with HRP-conjugated anti-mouse antibody (Jackson Immunoresearch), and the signals were visualized using the Supersignal West Pico Chemiluminescent Substrate (Thermo).

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