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Expression and regulation of Spätzle-processing enzyme in *Drosophila*

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Abstract The *Drosophila melanogaster* Toll receptor controls embryonic dorsal-ventral axis formation and is crucial for the innate immune response. In both cases, Toll is activated by the enzymatically cleaved form of its ligand Spätzle (Spz). During axis formation, Spz is cleaved by the maternally provided serine protease Easter while the Spätzle-processing enzyme (SPE) activates Spz after infection. We confirm the role of SPE in immunity and show that it is a zygotic gene specifically expressed in immune tissues implying that the dual activation of Spz is achieved by differential spatiotemporal expression of two similar but distinct serine proteases.

Keywords: RNA interference; Serine protease; Fat body; Lymph gland; Innate immunity; Spätzle-processing enzyme; Toll; *Drosophila*

1. Introduction

Serine proteases form a large group of peptidases involved in vital processes such as digestion, blood coagulation, fertilization, immune response and embryonic development. When organized in proteolytic cascades they can mediate a rapid and localized response to physiological or foreign stimuli. Two of those cascades culminate in activation of the Toll receptor by the cytokine-like polypeptide Spz. During dorsal-ventral axis formation of the *Drosophila* embryo, local activation of Toll establishes a morphogenetic gradient of the Rel/NF-κB transcription factor Dorsal, which determines the region-specific expression of zygotic patterning genes (reviewed by [1]). The maternally contributed Easter is the terminal protease of the cascade that leads to activation of Spz. Easter is required only during the syncytial blastoderm stage as demonstrated in rescue experiments by mRNA injection and with temperature sensitive alleles [2].

In adult flies, Spz activates Toll in response to Gram-positive bacteria and fungal infections. The activation results in humoral reactions based on production of antimicrobial peptides (AMPs) [3]. The AMP *Drosomycin* (Drm) is mainly induced by this pathway, while other AMPs, such as *Diptericin* (Dpt), are strongly induced by Gram-negative bacteria and regulated by the immune deficiency (IMD) pathway (reviewed in [4]). Only recently, the serine protease that activates Spz in immunity, SPE (Sp4, CG16705) has been comprehensively characterized [5].

We have generated RNA interference (RNAi) transgenic flies targeting several proteases reported to be upregulated upon infection [6–8]. The RNAi flies were tested for susceptibility to infection. Ubiquitous drivers were used when the silencing generates viable flies (CG16705-SPE, CG3505, CG3066 and CG2045) and immune-specific drivers in the case of lethality with ubiquitous depletion (CG9733, CG6639). We observed that mutant lines targeting *SPE* had impaired resistance to the Gram-positive bacteria *Enterococcus faecalis* while the resistance to infection with the Gram-negative bacteria *Pseudomonas aeruginosa* was unaffected, indicating a role of *SPE* in the Toll signaling pathway.

We further show that *SPE* is constitutively expressed during embryogenesis in the developing fat body, the functional analog of the vertebrate liver, and in the lymph glands, the larval hematopoietic organ. In larval stages, expression is mainly confined to the fat body suggesting that SPE is secreted to the hemolymph. The expression pattern of *SPE* demonstrates that the two proteases that activate Spz, Easter and SPE, although enzymatically redundant, execute distinct physiological functions due to different spatiotemporal expression (this study) and due to distinct upstream activating proteases [5].

We address the regulation of *SPE* and show that the inducible expression of *SPE* is controlled by the Toll pathway establishing a positive feedback loop that reinforces the infection cues. We further show that depletion of *SPE* impairs the induction of Drm and this effect is exerted upstream of the receptor.

2. Materials and methods

2.1. Drosophila stocks

Fly stocks were obtained from the Bloomington Stock Center unless otherwise specified. Oregon^{R} and w^{1118} were used as wild-type controls. Mutant flies were: spätzle^{rm7}/ spätzle^{rm7} (spz); relish^{E20}/ relish^{E20} (rel); key^{1}/ key^{1} (key); tub^{1}/ tub^{2} (tube) and Tl^{10b}. The Gal4 driver line Act-Gal4/CyO was combined with the RNAi transgenic line 16705-RNAi generating ubiquitous depletion of the SPE transcript.

2.2. Transgenic RNAi

Genomic and cDNA sequences in opposite direction were cloned into pUAST to produce a dsRNA against CG16705 as described in [9]. The genomic fragment including the upstream flanking sequence and the two first introns was amplified using the primers (5′-GGGCGGCCGCCCAATGCATCGGGAGAG-3′) and

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Abbreviations: AMP, antimicrobial peptide; Dpt, Diptericin; Drm, Drosomycin; IMD, immune deficiency; RNAi, RNA interference; SPE, Spätzle-processing enzyme; Spz, Spätzle

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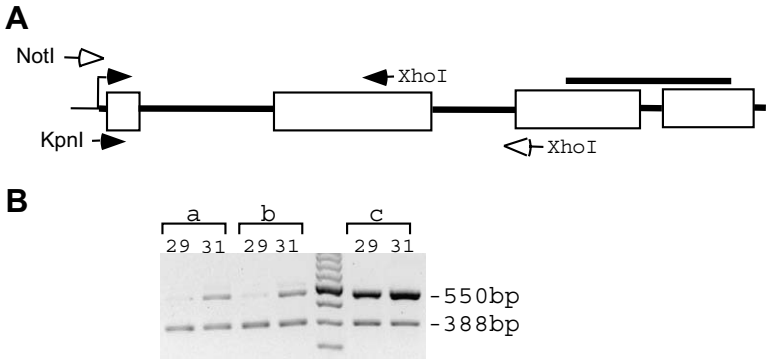

Fig. 1. RNAi strategy and depletion of *SPE* transcripts. (A) Exon-intron structure of *SPE*. Boxes represent exons. Primers and restriction sites used to construct RNAi transgenes are indicated by arrows. Hollow arrows denote primers used to amplify genomic DNA. Filled arrows denote primers used to amplify cDNAs. The line on top represents the probe used for in situ hybridization as well as the product of the RT-PCR for expression quantification. (B) Quantification of the *SPE*-depletion. RT-PCR products of *SPE* (550 bp) and of the internal control rp49 (388 bp) amplified for 29 cycles or 31 cycles. Genotypes: *Act*-Gal4/CyO;16705-RNAi/16705-RNAi (lanes a); *Act*-Gal4/CyO; 16705-RNAi/ TM3-*Sb* (lanes b); *Act*-Gal4/CyO; +/+ (lanes c).

(5′-GGCTCGAGGTTTCAGAAAAAACTGAAAGG-3′), and the cDNA fragment using the primers (5′-GGGGTACCCATAGCGACGAGATGGCT-3′) and (5′-GGCTCGAGTCCATGATATTGCCCCG-3′), (Fig. 1A). Subcloning and transformation was done as described in [10]. Eight independent transgenic lines were recovered.

2.3. Semi-quantitative RT-PCR

Quantitative estimation of the knockdown effect was performed by RT-PCR. One μg of total RNA extracted from adult flies was reverse transcribed using oligo-(dT) and 1/20 of the reaction was amplified for 29 or 32 cycles with the primers (5′-AACTCGCGCTACGTCCTGACC-3′) and (5′-ATAGGCACCATAAGGGGACCG-3′) targeting the 3′ end of the gene and the primers (5′-GACCATCCGCCCAGCATACAGGC-3′) and (5′-GAGAACGCAGGCGACCGTTGG-3′) targeting the ribosomal protein-encoding gene *rp-49* as control. In order to compensate the distinct abundance of transcripts, primers for *SPE* were used at 200 nM and 40 nM for rp-49.

Induction of AMPs was tested by multiplex PCR of random primed reverse transcription. In addition to the internal primers for rp-49 (40 nM), the reaction included primers for Drm (100 nM) (5′-CGTGAGAACCTTTTCCAATATGATG-3′) and (5′-GAATATGTGTAAGTAGTGGAGAG-3′) and Dpt (100 nM) (5′-ACTTTGCTGCGCAATCGCTTCTAC-3′) (5′-CCATATGGTCCTCCCAAGTGC-3′).

2.4. Immune challenge

Septic injury was performed by pricking the thorax of adult flies (aged 2–4 days) with a needle dipped into a concentrate (O.D. 200) of overnight bacterial culture. For survival experiments, 20 flies of each sex per vial were infected and incubated at 29 °C. Vials were positioned upside down to avoid the sticking of injured flies to the food and to facilitate the counting of dead flies.

2.5. In situ hybridization

Single stranded DNA probes were generated by asymmetric PCR. 200 ng of the 550 bp band corresponding to the 3′ end of the *SPE* transcript was labeled using a digoxigenin-labeling kit (Roche Applied Science). The reverse primer was used to generate the anti-sense probe. The forward primer was used to produce the sense probe for negative control experiments. In situ hybridizations were carried out according to Tautz and Pfeifle [11].

3. Results and discussion

3.1. Inherited RNAi efficiently knocks down SPE transcript levels

In order to quantify the reduction of *SPE* transcription in silenced lines, we performed RT-PCR on RNA from adult flies induced with the ubiquitous driver *Act*-GAL4 (Fig. 1B). One copy of the RNAi transgene reduced transcript levels by 94% while two copies reduced the expression by 97%. The driver line, as well as the parental line w^{1118}, showed constitutive expression of *SPE* in the absence of infection (Fig. 1B, lanes 29c and 31c and data not shown). Silencing of *SPE* did not cause lethality and a stock in which *SPE* was ubiquitously depleted could be maintained at RT. At 29 °C, however, this stock produced few viable progeny. This temperature sensitivity could explain the contradictory results on survival reported in two recent studies targeting *SPE* [5,12]. The rate of lethality after infection with the Gram-positive bacteria *E. faecalis* was increased in *SPE*-depleted flies, reaching 80% lethality 10 h post-infection, while the lethal rate was only 20% for wild-type flies.

3.2. SPE is expressed in immunological tissues

The spatial and temporal expression of *SPE* was examined by *in situ* hybridization to whole mount embryos and in dissected tissues. A maternal contribution of *SPE* was not detected. During embryogenesis, *SPE* is expressed in the lymph glands and in the fat body. Both organs are involved in the immune response. The lymph glands are the larval hematopoietic tissue and the fat body is the main source of circulating immune-related components. The fat body arises from segmentally repeated primary and secondary cell clusters located in the lateral and ventral mesoderm of parasegments (PSs) 3–12, respectively [13]. An additional dorsal cell cluster is located in the dorsal mesoderm of PS 13. The GATA transcription factor Serpent (spr) is the earliest fat body-cell marker but *spr* is also expressed in several other tissues [14]. Expression of *SPE* is first detected at early stage 11 at the posterior tip of the germ-band (Fig. 2A). During germband retraction, expression appears dorsally in PS 13 (arrowheads) and ventrally in PS 3–12 (stars) (Fig. 2C and E). The dorsal view at stage 12 (Fig. 2D) reveals expression at the tip of the retracting germband and in PS 13 (arrowhead). Tissue movements during embryogenesis suggest that cells at the tip of the germ-band may contribute to the posterior-most portion of the lateral fat body. Our results suggest that cells cluster in PS 13 make up dorsal fat body projections whereas the ventral