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The serine protease Sp7 is expressed in blood cells and regulates the melanization reaction in *Drosophila*

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Abstract

Serine proteases play a central role in defense against pathogens by regulating processes such as blood clotting, melanization of injured surfaces, and proteolytic activation of signaling pathways involved in innate immunity. Here, we present the functional characterization of the *Drosophila* serine protease Sp7 (CG3006) by inducible RNA interference. We show that Sp7 is constitutively expressed in blood cells during embryonic and larval stages. Silencing of the gene impairs the melanization reaction upon injury. Our data demonstrate that Sp7 is required for phenoloxidase activation and its activity is restricted to a subclass of blood cells, the crystal cells. Transcriptional up-regulation of Sp7 was observed after clean, septic injury and in flies expressing an activated form of Toll; however, mutations in the Toll or the IMD pathway did not abolish expression of Sp7, indicating the existence of other regulatory pathways and/or independent basal transcription.

Keywords: *Drosophila*; RNAi; Serine protease; Melanization; Phenoloxidase; Wound; Blood cells; Innate immunity

Innate immunity in insects consists of a humoral response based on antimicrobial peptides and a cellular response mediated by blood cells that promote phagocytosis, encapsulation of pathogens, and melanization [1–3]. The immediate immune response in *Drosophila* is the melanization reaction observed at the site of injury or on the surface of pathogens and parasites invading the hemocoel. Perforation of the cuticle by injury or by parasitic infection initiates a proteolytic reaction that leads to rapid hemolymph coagulation and deposition of melanin. This reaction plays an important role in wound healing where melanized tissue forms a plug that restricts bleeding [4,5]. The melanization reaction requires the activation of phenoloxidase (PO) that catalyzes the conversion of phenols to quinones, which spontaneously polymerize to form melanin (Fig. 7). In vitro studies have shown that PO exists as an inactive precursor, prophenoloxidase (pro-PO), which is activated by a stepwise process involving serine protease cascades [6–8]. As excessive melanin is deleterious to the host, the processing of pro-PO must be strictly regulated to restrict the melanization reaction. Inactive PO is cleaved into active PO by a protease known as prophenoloxidase-activating enzyme (pro-POAE). Pro-POAEs have been identified in the tobacco hornworm, *Manduca sexta* [9,10], in the silkworm, *Bombyx mori* [7], in the beetle, *Holotrichia diomphalia* [11], and in the crayfish, *Pacifastus leniusculus* [12] but so far, no such activator gene has been studied in vivo by mutational analysis in *Drosophila*.

Negative regulators of the catalysis have been characterized and include members of the serine protease inhibitor (serpin) superfamily [13]. Serpins act as suicide substrate inhibitors of serine proteases by forming irreversible complexes. Biochemical and genetic characterization of one of them, *serpin-27A* (Spn27A) in *Drosophila*, demonstrates that the protein encoded by this gene regulates the

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melanization cascade through the specific inhibition of pro-PO processing by the terminal serine protease pro-POAE [14,15].

In addition to the regulation at the biochemical level, melanization is controlled spacially and temporally by blood cells. *Drosophila* has three blood cell types; the plasmatocytes, phagocytic cells that comprise 95% of circulating hemocytes and synthesize antimicrobial peptides; the crystal cells, named for the crystalline inclusion bodies and lamellocytes, the largest cells normally not present in healthy larvae and involved in the encapsulation response against intruders that are too large to be phagocytized. Rizki et al. [16] suggested that crystal cells are the only source of pro-PO in the fruit fly. However, lamellocytes that appeared after parasitoid wasp infection could contribute to the melanization of the capsule that isolated the parasite eggs [17,18]. The completion of the genome sequence has shown that *Drosophila melanogaster* has three pro-PO genes, Bc (DoxA1, CG5779), DoxA3 (CG2952), and CG8193. A recent proteomic analysis of larval hemolymph has shown a reduction of the pro-PO protein spots upon immune challenge [19]. The reduction is attributed to proteolytic activation, indicating that these genes code for proteins that are processed upon challenge probably by serine proteases. The protein annotation in this study corresponds to both the genes CG8193 and CG2952, and no further discrimination could be done. Based on histological location in non challenged embryos it has been inferred that pro-POs are expressed in crystal cells [20,21]. Recently it has been demonstrated that this is true for two of them, while CG2952 is exclusively expressed in lamellocytes [17]. The relative contribution of crystal cells and lamellocytes to the melanotic encapsulation of parasitoid wasp eggs is not understood. It is recognized, however, that the melanization at injury sites is mediated exclusively by crystal cells mainly because lamellocytes are not present in healthy larvae. Furthermore, three classical blood mutants: *Domino* (dom) which has fewer blood cells, *Black Cells* (Bc) which has aberrant crystal cells, and *lozenge* (Lz) which lacks crystal cells, all have severely impaired hemolymph melanization [22,23].

Here, we present the functional characterization of the first serine protease in any arthropod species that is necessary for activation of the PO pathway and melanization upon injury. Sp7 is constitutively expressed in blood cells and up-regulated after injury, infection, and in immune deficient mutations. Silencing of transcription in crystal cells using the early transcription factor Lozenge is sufficient to impair hemolymph melanization.

Materials and methods

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: $spaetzle^{rm7}/spaetzle^{rm7}$ (spz); $relish^{E20}/relish^{E20}$ (rel); key^1/key^1 (key); tub^1/tub^2 (tube); the constitutive active form of Toll, Tl^{10b}; *domino* (dom); *Black cells* (Bc) and the Gal4 driver lines *Act-Gal4/CyO*, *Tub-Gal4/TM3, hem-Gal4-GFP*, and *Lz-Gal4-GFP*. RNAi transgenic lines were generated by P element-mediated transformation using w^{1118} as recipient stock. Flies were maintained on standard medium at 19, 25, or 29 °C, as necessary.

Transgenic RNA interference. A pUAST-vector containing genomic and cDNA sequences in opposite direction was constructed to produce dsRNA. All the described transcripts of the gene CG3066 were targeted. The genomic fragment encompassing 729 bp and including the last three introns was amplified with the primers: (5′- GGGCGGCCGCAACGA CACTGCTATTGACG-3′) and (5′-GGCTCGAGGTGCTCTTGCGGG CTATAGA-3′). The corresponding cDNA encompasses 425 bp and was amplified with the primers: (5′- GGGGTACCACTGCTTGAGTACG TGGATA-3′) and (5′-GGCTCGAGTTCGGCCCCACCCGCTGACCA-3′) (Fig. 1B). Subcloning and transformation were done as described in [24]. The induced dsRNA is expected to form a 425-bp hairpin linked by a 148 nucleotide loop. Ten independent transgenic lines were recovered and the efficiency of the silencing was examined by RT-PCR in flies induced with the ubiquitous drivers *Act-GAl4* or *Tub-Gal4* (Fig. 1B). The endogenous transcript was reduced between 60% and 98% depending on the line, the number of transgenic copies, and rearing temperature. On average, the increase of temperature from 25 to 29 °C improved the silencing by at least 25%. Two insertions with strong silencing effect, one in chromosome II (line i3066-42a) and one in chromosome III (line i3066-17a), were selected for further characterization.

Semi-quantitative RT-PCR. One microgram of total RNA extracted from adults flies was reverse transcribed using oligo-(dT) and 1/20 of the reaction was amplified for 29 or 32 cycles with the primers (5′-GA GCACCATAAAGCAGCGA-3′) and (5′-CTCAGGGACGAATGGTC TCCA-3′) targeting the 3′ end of the gene excluded in the RNAi construct and the primers (5′-GACCATCCGCCCAGCATACAGG C-3′) and (5′-GAGAACGCAGGCGACCGTTGG-3′) targeting the housekeeping gene rp-49 as internal control. Primer concentrations were 200 nM for CG3066 and 40 nM for rp-49. Quantification of the bands was done by densitometry using the 1D program (Kodak).

In situ hybridization. Single stranded DNA probes were generated by asymmetric PCR. 200 ng of the 746 bp band amplified with the forward primer: (5′-ACGACTACCAGTTCAAGTTCA-3′) and the reverse primer: (5′-TCAAAACCCCTTCGCATCAGC-3′) corresponding to the 3′ of the CG3066 transcripts was labeled using a digoxigenin-labeling kit (Roche Applied Science). The reverse primer was used to generate the antisense probe and the forward primer to produce the sense probe and used as a negative control. In situ hybridizations were carried out according to Tautz and Pfeifle [25].

Melanization and phenol oxidase activity. The paper assay of phenoloxidase activity was done by dropping the hemolymph of a single dissected larva on a filter paper soaked with 10 mM phosphate buffer (pH 6.5) containing 10 mM L-DOPA [26]. Photographs of the spots were taken at timed intervals. Phenoloxidase activity in the total protein extract was quantified 4 h after injury or infection with a mixture of Gram-negative (*Escherichia coli*) and Gram-positive (*Micrococcus luteus*) bacteria. Five L3 instar larvae were homogenized in 100 µl cold 10 mM phosphate buffer. After centrifugation at 4 °C, 50 µl of clear lysate was collected avoiding the floating fat tissue. The protein concentration was determined using the Coomassie Plus Protein Assay kit (Pierce), using BSA as a standard. Ten micrograms of protein was assayed as in [14] measuring the absorbance at 490 nm after 3 min. Larva cuticle melanization was monitored by dissection and exposure to air for 1 h [27].

Results

The gene CG3066 encodes a clip-domain serine protease with four splicing forms

Searches of amino acid sequence databases showed that Sp7 has the highest similarity to arthropod proteins containing a carboxyl-terminal serine protease domain