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The *Drosophila* Thioester containing Protein-4 participates in the induction of the cellular immune response to the pathogen *Photorhabdus*

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ABSTRACT

The function of thioester-containing proteins (TEPs) in the immune defense of the fruit fly *Drosophila melanogaster* is yet mostly unexplored. Recently, we showed the involvement of TEP4 in the activation of humoral and phenoloxidase immune responses of *Drosophila* against the pathogenic bacteria *Photorhabdus luminescens* and *Photorhabdus asymbiotica*. Here, we investigated the participation of *Tep4* in the cellular defense of *Drosophila* against the two pathogens. We report significantly lower numbers of live and dead plasmatocytes in the *tep4* mutants compared to control flies in response to *Photorhabdus* infection. We also find fewer crystal cells in the control flies than in *tep4* mutants upon infection with *Photorhabdus*. Our results further suggest that *Drosophila* hemocytes constitute a major source for the transcript levels of *Tep4* in flies infected by *Photorhabdus*. Finally, we show that *Tep4* participates in the phagocytic function in *Drosophila* adult flies. Collectively our data support the protective role for TEP4 in the cellular immune response of *Drosophila* against the entomopathogen *Photorhabdus*.

1. Introduction

Thioester containing proteins (TEPs) serve a major role in the host innate immune response of organisms by recognizing and promoting the elimination of the invading microbes (Christophides et al., 2004). TEPs can be categorized into two protein subfamilies, complement factors and α2-macroglobulins (α2M). A characteristic feature of complement proteins is the presence of a thioester (TE) motif (GCGEQ) followed by a catalytic histidine-residue. The TE motif binds covalently to the microbes as opsonin to promote phagocytosis and cell lysis with the contribution of membrane attacking complex (Merle et al., 2015). Additionally, α2M are protease inhibitors that contain TE motifs but lack accompanying histidine residues, a bait region and a receptor-binding domain. These protease inhibitors neutralize pathogenic proteases resulting in reduced virulence along with pathogen clearance (Armstrong, 2010; McTaggart et al., 2009). In invertebrates, TEPs have been studied in the mosquito *Anopheles gambiae* and shrimp *Paneaus mondon* (Christophides et al., 2004; Wang and Wang, 2013). Few studies have also examined the participation of TEPs in the immune defense of the fruit fly, *Drosophila melanogaster* (Bou Aoun et al., 2011; Igboin et al., 2011; Lageaux et al., 2000; Shokal and Eleftherianos, 2016; Stroschein-Stevenson et al., 2006).

The *D. melanogaster* genome includes 6 *Tep* genes (*Tep1-Tep6*), of which *Tep5* is a pseudogene and *Tep6* contains a mutated TE motif (Bou Aoun et al., 2011). Hemocytes, specifically plasmatocytes, constitutively express *Tep1-Tep4* genes. Other tissues that express *Tep* genes include the adult head, the abdominal epidermis and the spermatozoon (Aradska et al., 2015; Bou Aoun et al., 2011; Wasbrough et al., 2010). *Tep1*, *Tep2* and *Tep4* are upregulated at both larval and adult stages of *D. melanogaster* upon bacterial challenge (Lageaux et al., 2000). Genome wide transcriptome analyses have shown induction of *Tep2* and *Tep4* in response to certain bacteria, fungi and parasitoids (Bou Aoun et al., 2011; Wertheim et al., 2005). Moreover, *Tep3* and *Tep4* are induced against parasitic nematode infection (Arefin et al., 2014). Recently, we have shown the importance of *Tep4* in regulating the humoral immunity and phenoloxidase response of *D. melanogaster* adult flies against the Gram-negative pathogenic bacteria *Photorhabdus* (Shokal and Eleftherianos, 2016). We reported stronger induction of Toll and Imd pathways and higher levels of phenoloxidase activity in *tep4*

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mutants infected by *Photorhabdus* compared to control flies, effects that promote the survival of *tep4* mutants following infection with the pathogens.

Photorhabdus spp (family: *Enterobacteriaceae*) are bioluminescent bacteria that exist in a unique mutualistic symbiosis with nematodes from the genus *Heterorhabditis* (Waterfield et al., 2009). *Photorhabdus* bacteria are pathogenic to a wide range of insect species because they produce a variety of toxins and virulence factors that cause apoptosis or ADP-ribosylation of actin in certain tissues including the midgut epithelium and hemocytes (Aktories et al., 2011; Rodou et al., 2010; Simon et al., 2014). In addition, *Photorhabdus* interacts dynamically with the insect immune system by altering the antimicrobial peptide immune response, blocking hemocyte phagocytosis and other cellular reactions, and suppressing the phenoloxidase/melanization activity (Eleftherianos et al., 2010). These features have established *Photorhabdus* as an excellent model for studying the molecular basis of microbial pathogenesis and identifying virulence factors that interact with host molecules that regulate innate defense mechanisms (Nielesen-Leroux et al., 2012).

In this study we investigated the function of *Tep4* in the cellular immune defense of *Drosophila* against two different species of *Photorhabdus*, the insect pathogen *P. luminescens* and the emerging human pathogen *P. asymbiotica*. We have demonstrated that *Tep4* plays an important role in the regulation of hemocyte activity and function in the *Drosophila* antibacterial immunity.

2. Materials and methods

2.1. Fly stocks and bacterial strains

The *D. melanogaster* fly strain *yw* as well as a *tep4* P-element insertion mutant (15936, Bloomington) were used for all experimental infections. The flies were maintained and amplified with instant *Drosophila* media (Carolina Biological Supply) made in deionized water. All stocks were maintained at 25 °C and a 12:12-h light:dark photoperiod. Adult flies 7–10 days old were used in infection assays with bacteria.

Bacterial strains *Photorhabdus luminescens* subsp. laumondii (strain TT01), *P. asymbiotica* subsp. *asymbiotica* (strain ATCC 43949) and *Escherichia coli* (strain K12) were used for all fly infections. Bacterial cultures were prepared in sterile Luria–Bertani (LB) broth and grown for approximately 18–22 h at 30 °C on a rotary shaker at 220 rpm. Bacterial cultures were pelleted down and then washed and re-suspended in 1x sterile phosphate-buffered saline (PBS, Sigma Aldrich). Bacterial concentrations were adjusted to Optical Density (OD, 600 nm) of 0.1 for *P. luminescens*, 0.25 for *P. asymbiotica* and 0.015 for *E. coli* using a spectrophotometer (NanoDropTM 2000c – Thermo Fisher Scientific).

2.2. Hemolymph collection

Female flies (n = 4) were injected with *P. luminescens*, *P. asymbiotica*, *E. coli* or 1XPBS. After 18 h, the hemolymph was collected using a modified version of a previously published protocol (Castillo et al., 2006). Briefly, flies were anesthetized using CO_2 and then injected into the thorax with 2–3 μL of incubation solution [60% Grace's Medium (GM) supplemented with 10% of Fetal Bovine Serum (FBS) and 20% of Anticoagulant Buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, pH 4.5)] using a blunt end needle (16 gauge) fitted with a tubing connected to a 20 mL glass syringe. After 20 min of incubation on ice, flies were kept on a petri dish and an incision was made between the 2nd and 3rd abdominal segments. Flies were again injected into the thorax with 5 μL of collection solution (90% of GM supplemented with 10% of FBS). Hemolymph was then collected in a 1.5 mL tube and used for further assays.

2.3. Total hemocyte counts, cell type counts and cell viability

Hemolymph samples (10 μL) were loaded on a hemocytometer and total numbers of cells as well as the different hemocyte types were estimated using 40X magnification of a compound microscope (Olympus CX21). For cell viability, Trypan blue exclusion assay was performed. All experiments were repeated at least three times. The following equations were used for estimation of percent hemocyte sub-types and cell viability.

Percentage of crystal cells or plasmatocytes = (Total number of plasmatocytes/total number of cells) * 100

Percentage of viable cells or plasmatocytes or crystal cells = (total number of viable cells or plasmatocytes or crystal cells / total number of cells)*100

2.4. Inactivation of hemocyte phagocytosis and survival experiments

To inactivate hemocyte phagocytosis in *D. melanogaster*, flies were anesthetized with CO_2 and then injected with 69 nL of Latex beads (0.3 μm diameter, Molecular Probes, Invitrogen) into the thorax using a Nanoject II apparatus (Drummond Scientific) equipped with glass capillaries prepared with a micropipette puller (Sutter Instruments). Latex beads were washed with sterile 1XPBS and used 4X concentrated in PBS (corresponding to 5–10% solids). 1XPBS served as control for the first round. After 18 h, the flies were injected again, with 18.4 nL (100–300 CFU) of each bacterial suspension (*P. luminescens*, *P. asymbiotica* or *E. coli*) or PBS (negative control). Following injection, the flies were transferred to fresh vials with instant media at 25 °C and survival was scored at 6-h intervals and up to 48 h. Fifteen flies were used for each treatment and each assay was replicated three times.

2.5. Gene transcription

Three-four adult flies from each strain were injected as described above with *P. luminescens*, *P. asymbiotica, E. coli* or PBS, and they were frozen at 6 and 18 h after infection. Total RNA was extracted using the PrepEase RNA spin kit (Affymetrix USB). Complementary DNA (cDNA) synthesis, quantitative RT-PCR (qRT-PCR), and analysis of the data were performed as previously described (Castillo et al., 2013). All primers used for the PCR assays are listed in Table 1. Data are presented as the ratio between pre-injected flies with either beads or 1XPBS (baseline controls) versus flies injected in the second round. The experiments were performed three times.

2.6. Phagocytosis assay

Seven flies from each strain were injected with 50.4 nL of 1 mg/

Table 1
List of primers used in the study.

Gene	FlyBase ID	Primer	Primer Sequence (5′ to 3′)	Tm (°C)
RpL32	FBgn0002626	Forward	GATGACCATCCGCCCAGCA	61
		Reverse	CGGACCGACAGCTGCTTGGC	
Tep4	FBgn0041180	Forward	GCTGCAGAACCAGATCGAAATC	61
		Reverse	ATGACTTTGGCGACGTCTTGAT	
Eater	FBgn0243514	Forward	ATAACGATCCATCTAACCGATGTGT	57
		Reverse	GATTGGCAGGTTCCTCGACTAC	