Clotting Factors and Eicosanoids Protect against Nematode Infections

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Key Words

Drosophila melanogaster • Eicosanoids • Entomopathogenic nematodes • *Heterorhabditis bacteriophora* • Coagulation • Transglutaminase • *Photorhabdus luminescens*

Abstract

We show that hemolymph clotting protects *Drosophila melanogaster* against infections with an entomopathogenic nematode and its symbiotic bacterium. We also provide biochemical and genetic evidence for an involvement of eicosanoids in the same infection model. Taken together, our results confirm the conserved nature of the immune function of clot formation.

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Introduction

Insect hemolymph clotting serves to seal wounds and has been proposed to contribute to preventing migration of microbes past the wound site [1], similar to other invertebrates [2]. Using proteomics and bioinformatics, we have previously identified a number of candidate clotting factors and studied their function upon wounding and in infection models [3–5]. The enzyme transglutaminase (TG) and its vertebrate homologue factor XIIIa

are amongst the best-conserved clotting factors and help to sequester bacteria at the wound site in both insects and humans. Recent evidence for an immune function of TG derives from an infection model that involves insect pathogenic nematodes and their symbiotic bacteria [6]. Infection with nematodes such as *Heterorhabditis bacteriophora* involves the release of symbiotic bacteria (*Photorhabdus luminescens*) from the gut, which helps the nematode to suppress host immunity and eventually kill the host [7]. Genetic studies in *Drosophila* show that despite the fact that antimicrobial peptides are induced in *Heterorhabditis/Photorhabdus*-infected hosts, both major pathways of immune induction (*Toll* and *imd*) appeared dispensable for the response against the nematodes and their bacteria [8]. We have previously shown that TG plays a role in the defense against *Heterorhabditis/Photorhabdus*; *Drosophila* larvae with reduced TG levels were more sensitive to nematode infections [6]. Upon contact with *Photorhabdus*, hemolymph TG targets microbial surfaces, leading to the deposition of small aggregates (microclots). Here, we screened clotting factors other than TG for their effects during a *Heterorhabditis/Photorhabdus* infection. We provide further evidence for an immune function of the clot and show that eicosanoids, which are required during blood clotting [9], also regulate the response against nematodes and their bacteria.

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Materials and Methods

Fly Strains

RNAi lines from the Vienna collection [10] were used. To account for differences in knock-down due to position effects, we used several lines for a given gene. These included lines for Tigrin (stocks 28255 and 100036), gp150 (stocks 899, 900 and 100134), hemomucin (stocks 1278, 1279 and 100286), Fbp1 (stock 37881) and CG14507 (stocks 106109 and 35713). For gp150 lines, we determined the level of knock-down by measuring the strength of the peanut agglutinin signal that detects gp150 [11], normalized to an internal Coomassie-stained control (hexamerin) in hemolymph samples from the different lines. With this method, we found stock 100134 to work best (50% knock-down; a previously published line [11] did not show efficient knock-down with the hemese driver). The line 100134 showed a significant increase in mortality after 48 h ($p = 0.0052$, as shown in figure 1; note that a second line from the Vienna collection also showed increased mortality at $p = 0.027$, data not shown). The RNAi line targeting Fondue and its level of knock-down had been characterized before [12]. The RNAi line for *NimC1* was a kind gift from I. Ando, Szeged, Hungary. Gal4-expressing driver lines with specificity for either the fat body (*pplGal4*), hemocytes (*HeGal4*) or ubiquitously expressed Gal4 (*ActinGal4*) were used. *W¹¹¹⁸* wild-type and *Bc* mutant flies were obtained from the Bloomington fly stock center (Bloomington, Ind., USA), and *imd¹* mutant flies were a kind gift from B. Lemaitre. Note that *imd¹* is a weak hypomorphic allele, a fact that might contribute to the lack of a phenotype upon nematode infection.

Identification of TG Substrates

TG substrates were identified as described before [5] using mass spectrometry.

Clot Preparation

Clots were prepared from larvae expressing a Fondue-GFP fusion protein [13] using the hanging drop method [14].

Identification of Microclots

The eicosanoid biosynthesis inhibitor dexamethasone was injected into wild-type larvae (*W¹¹¹⁸*) at a concentration of 5 $\mu\text{g}/\mu\text{l}$ in PBS. Bacteria were injected after 12 h. One hour later, hemolymph was retrieved from the larvae to perform a B-cad incorporation assay as described elsewhere [6]. As controls, larvae injected with PBS were used.

Infection Experiments

Nematode infection of larvae was performed as described previously [6], using *H. bacteriophora* (strain H222) naturally carrying *P. luminescens* ssp. *kayaii*. All experiments were run in triplicate using 48 larvae per group at different temperatures and nematode doses (22°C and 100 nematodes/larva for inhibitor injections, 25°C and 100 nematodes/larva for the experiments shown in figure 3C or 29°C and 25 nematodes/larva for the experiments shown in figure 1 and 3B).

Eicosanoid Inhibitor Injections

The eicosanoid biosynthesis inhibitors esculetin (6,7-dihydroxycoumarin), dexamethasone [(11 β ,16 α)-9-fluoro-11-17,21-trihydroxy-16-methylpregna-1,4-diene-3, 20-dione] and indo-

Fig. 1. Identification of clot components that contribute to the defense against *Heterorhabditis/Photorhabdus*. RNAi lines for individual clotting factors were crossed either with a hemocyte-specific (A) or a fat body-specific driver (B) and infected with *Heterorhabditis/Photorhabdus*, and mortality was scored after 24 and 48 h (see Materials and Methods for further details on infection and efficiency of the knock-down). Data represent means $\pm$ SD. ** $p < 0.01$.

methacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid], all from Sigma-Aldrich (St. Louis, Mo., USA), were used. Seventy-two-hour-old larvae of *D. melanogaster* were injected with 0.05 μl of inhibitors dissolved in PBS (5 $\mu\text{g}/\mu\text{l}$) using the automatic nanoliter injector Nanoject II (Drummond Scientific, Pa., USA). As controls, untreated larvae and larvae injected with the same volume of PBS were used. The larvae were exposed to nematode infection 12 h after injection. Data were analyzed using Student's t test and comparison with the PBS-injected control.

Fig. 2. Fondue contributes to both the clot matrix and microclots on *Photorhabdus*. **A, B** Hemolymph was incubated with a biotin-containing substrate (biotin-cadaverine) which labels TG substrates. The sample was split into two parts, which were run in parallel and processed either by staining with Coomassie (**A**) or blotting and probing with biotin-specific streptavidin (**B**). The arrows indicate the position of Fondue, which is among the preferred TG substrates. The pH range was between 3 and 10. **C, D** Fondue is part of the clot matrix and microclots. A clot was prepared as described from larvae expressing a Fondue-GFP fusion protein [13] and visualized using fluorescence microscopy. In **C**, a part of the clot matrix, which has partially folded back onto

Results

To test whether clotting factors other than TG are required for the response against nematodes and their bacteria we infected knock-down (RNAi) lines for previously identified clotting factors including hemomucin [15], gp150 (or Eig71Ee, CG7604 [11]), Fbp1, Tiggrin and Fondue [4, 5]. To minimize side effects due to ubiquitous knock-down, we relied on known information on the expression of these factors using driver lines with specificity for the fat body, hemocytes or both. Significant increases in susceptibility were observed when gp150 expression was reduced in hemocytes (fig. 1A). Similarly, knock-down of Fondue in the fat body (fig. 1B) increased mortality after nematode infection. In contrast, knock-down of the phagocytic receptor Nimrod C1 in hemocytes had no effect, adding further support to the idea that phagocytosis does not play a major role (fig. 1A) [6]. Nor did knock-down of any other clotting factor show any effects (fig. 1). Although due to the partial nature of knock-downs an influence of genes that did not show an effect on mortality cannot be excluded, these results further support a role for clotting factors in the response against nematodes and their bacteria.

Fondue is one of the preferred hemolymph substrates for TG (fig. 2A, B) [5]. To get further insight into its function, we decided to trace Fondue using a fusion construct with GFP [13]. In hemolymph clots, the protein is part of both the fibrous matrix that forms the clot itself and local aggregates that resemble microclots (fig. 2C, D). When *Photorhabdus* was injected into larvae expressing the fusion protein, microclots were also found on the surface of the bacteria (fig. 2E, F). Taken together, these results confirm the importance of clot formation as part of the response against entomopathogenic nematodes.

itself (left part), is shown, while in **D** the matrix has folded up more extensively into longer strands, part of which is shown. **E, F** Microclots bind to bacteria. *Photorhabdus* were injected into Fondue-GFP-expressing larvae and retrieved by bleeding followed by immunohistology with a GFP-specific and a TRITC-conjugated secondary antibody. Note the punctate structures on the bacteria (microclots), which had been observed before using TG-specific reagents [6] (fluorescence and phase contrast exposure of the same field are shown). Scale bars = 10 μ m (**C, D**, same magnification) and 1 μ m (**E**).

Fig. 3. Eicosanoids and the *imd* pathway contribute to immunity against *Heterorhabditis/Photorhabdus*. **A** Infection experiments were performed after previous injection of eicosanoid biosynthesis inhibitors [dexamethasone (DEX), indomethacine (IND) and esculetin (ESC)]. Control larvae were injected with the inhibitors alone without subsequent infection with nematodes, and survival was scored after 48 h. The inset shows bacteria introduced into dexamethasone- and PBS-injected larvae, which were retrieved after 1 h and treated as described elsewhere [6] to detect micro-

clots. Scale bar = 1 μ m. Mortality was scored after the indicated times. **B** Mortality after infection of knock-down (k/d; using the actin driver line) of the lines for secreted PLA₂ (CG14507), a control cross (w1118, crossed with the actin driver line) and the responder line alone. **C** Infection of control larvae, *imd*¹ and *Bc* mutants alone and *imd*¹/*Bc* double mutants reveals an influence of the *imd* pathway in the absence of melanization. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All inhibitors showed the same significance level at 48 h in **A**.

Since eicosanoids are well-established regulators of blood clotting [9] and have been implicated in insect immunity [16, 17], we speculated that they might also play a role during *Heterorhabditis/Photorhabdus* infection. To test this, we injected wild-type larvae with known in-

hibitors of eicosanoid synthesis prior to infection with nematodes (fig. 3A). All three inhibitors led to an increase in mortality 48 h after infection, which suggests that eicosanoids or related lipids do play a role and agrees with the observation that *Photorhabdus* induces immu-

nosuppression in its host by inhibiting phospholipase A2 (PLA₂), the most upstream enzyme in the eicosanoid biosynthesis pathway [18]. In addition, the formation of microclots was inhibited in the presence of dexamethasone (fig. 3A, inset). To obtain independent genetic evidence for a role of eicosanoids in this infection model, we performed similarity searches for *Drosophila* homologues of PLA₂. Amongst the phospholipases identified, we focused on the secretory PLA₂ CG14507, since secretory phospholipases had been implicated in the response against entomopathogenic bacteria [19] and CG14507 had previously been shown to be induced upon wounding by laser ablation [20]. CG14507 RNAi larvae were indeed more susceptible to nematodes and their bacteria (fig. 3B), providing additional genetic evidence for a function of eicosanoids in insect immunity. Conversely, knock-down of two genes which had been electronically annotated as potentially involved in eicosanoid synthesis (CG10602 and CG1742, Flybase) had no effect (not shown). Since eicosanoid synthesis had previously been shown to act in parallel to the *imd* pathway, and recent evidence suggests a complex role for the *imd* pathway in the response against *Photorhabdus* [21, 22], we also decided to reevaluate the role of IMD in the response against *Heterorhabditis/Photorhabdus* (fig. 3C). To facilitate detection of *imd*-dependent effects, we performed the infection in *Bc* mutants, which lack phenoloxidase activity due to a defect in hemocyte development [23], and in *Bc/imd* double mutants. The double mutants were indeed more sensitive, and since *Bc* mutants alone showed no increased susceptibility, this result further supports the idea that eicosanoid metabolism is at least partially connected to *imd* signaling [21] and is in line with previous results which show synergy between *Bc* and *imd*¹ [24].

Discussion

Here we confirm and expand our previous findings and show that hemolymph clotting is an integral part of immunity. We also observe that both steroidal and non-steroidal inhibitors of eicosanoid biosynthesis interfere with nematode infections, findings which are in line with previous work in other insect hosts and provide, to our knowledge, the first direct genetic evidence for a role of eicosanoids in insect immunity. This supports earlier biochemical data [16, 18, 25] and opens up the interesting perspective that regulation of clotting through eicosanoids has been phylogenetically conserved. Future work will reveal the exact nature of the lipids produced in this pathway in insects. It should be noted that similar to other invertebrates that produce eicosanoids, *Drosophila* lacks clearly identifiable homologues of many key enzymes in their synthesis [9]. These include human pathogens such as *Leishmania* and trypanosomes where synthesis relies on distant members of the vertebrate enzymes or on proteins that have acquired similar activities independently [26]. The work presented here shows that genetic screens may open up new pathways to identify invertebrate enzymes that produce lipid regulators of innate immunity.

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