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Original article

Functional analysis of immune response genes in *Drosophila* identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells

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Abstract

The templates of innate immunity have ancient origins. Thus, such model animals as the fruit fly, *Drosophila melanogaster*, can be used to identify gene products that also play a key role in the innate immunity in mammals. We have used oligonucleotide microarrays to identify genes that are responsive to Gram-negative bacteria in *Drosophila* macrophage-like S2 cells. In total, 53 genes were induced by greater than threefold in response to *Escherichia coli*. The induction of all these genes was peptidoglycan recognition protein LC (PGRP-LC) dependent. Twenty-two genes including 10 of the most strongly induced genes are also known to be up-regulated by septic injury in vivo. Importantly, we identified 31 genes that are not known to respond to bacterial challenge. We carried out targeted dsRNA treatments to assess the functional importance of these gene products for microbial recognition, phagocytosis and antimicrobial peptide release in *Drosophila* S2 cells in vitro. RNAi targeting three of these genes, *CG7097*, *CG15678* and *β-Tubulin 60D*, caused altered antimicrobial peptide release in vitro. Our results indicate that the JNK pathway is essential for normal antimicrobial peptide release in *Drosophila* in vitro.

Keywords: *Drosophila melanogaster*; Innate immunity; JNK signaling

1. Introduction

Recent progress in understanding the basic templates of innate immunity has revealed striking conservation in the first-line host defense from insects to human [1,2]. This similarity is clearly illustrated by the role of Toll-like receptors and related intracellular pathways in acute humoral response to microbial infection [3,4]. Consequently, genetically tractable model organisms like *Drosophila*, in particular, have been widely used to dissect genes and pathways that are of importance in innate immune response of the host.

Upon microbial challenge, both cellular and humoral arms of the *Drosophila* innate host defense are required for optimal immune competence in vivo [5]. Invading microbes are phagocytosed by circulating macrophages and the antimicrobial peptides are synthesized by both hemocytes and the fat body, a functional equivalent of the mammalian liver. In addition, proteolytic cascade that leads to activation of phenoloxidase, which catalyses the conversion of dopamine to microbicidal melanine, is activated. *Drosophila* humoral immunity distinguishes between different classes of pathogens through the immune defiency (Imd) and the Toll pathways. These two pathways, which are very similar to mammalian Toll/IL and

Abbreviations: aop, *anterior open*; dSR-CI, *Drosophila* scavenger receptor CI; GNBP, Gram-negative bacteria-binding protein; *hep*, *hemipterous*; Imd, immune defiency; *kay*, *kayak*; MAPK, mitogen-activated protein kinase; *msn*, *misshapen*; PAMP, pathogen associated molecular pattern; PGRP, peptidoglycan recognition protein; PRR, pattern recognition receptor; TNF, tumor necrosis factor.

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tumor necrosis factor (TNF)-receptor pathways [2], are the major regulators of the immune response in *Drosophila* in vivo [6]. Remarkable conservation between human and *Drosophila* pathways points to an evolutionary link from insects to man.

Earlier we have shown that *Drosophila* S2 cells are macrophage-like [7,8], which makes them a valid in vitro system to study hemocyte-mediated response to microbial challenge. In these cells, exposure to Gram-negative bacteria leads to rapid transcriptional induction of several well-characterized antimicrobial peptide genes including *Attacin*, *Cecropin* and *Metchnikowin* via the Imd pathway [8]. In addition, S2 cells recognize and phagocytose efficiently both Gram-negative and Gram-positive bacteria. In contrast, the activation of the Toll pathway, which is initiated by Gram-positive bacteria through a circulating peptidoglycan recognition protein (PGRP) SA in vivo [9], requires introduction of the active form of Spätzle by transfection in these cells [10]. Therefore, S2 cells provide an opportunity to study the Imd pathway mediated response to Gram-negative bacteria.

2. Materials and methods

2.1. Cell cultures

S2 cells were maintained as described earlier [11].

2.2. Microbial challenge, RNA isolation and oligonucleotide microarray analysis

3.0×10^6 S2 cells were incubated for 6 h with 3.0×10^7 heat-killed *Escherichia coli* and thereafter total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Gene expression analysis was performed using the Affymetrix (Santa Clara) *Drosophila* Genechips according to the standard Affymetrix Genechip protocol as outlined in the GeneChip Expression Analysis Technical Manual by Affymetrix (2001). Gene expression levels of four unchallenged, control dsRNA treated S2 cells were compared pair-wisely to four *E. coli* exposed samples (altogether 16 comparisons) and to three *E. coli* exposed, PGRP-LC dsRNA treated samples (12 comparisons).

2.3. Data analysis

We identified the genes whose RNA levels are affected by an exposure to *E. coli* using the following criteria. First, the majority of the 16 comparisons had to be called increased (or decreased) and none of the remaining ones was called decreased (or increased). Second, there must be at least a threefold increase (or decrease) in the relative expression level. Finally, the *t*-test was used to measure statistical significance. A *P*-value of < 0.05 was considered to be significant. Those genes that fulfilled all of these criteria are shown in Table 1.

2.4. dsRNA treatments

Total RNA was isolated with TRIZOL® Reagent (GIBCO BRL, Gaithersburg, MD) and first-strand cDNA was synthesized from 1.0 μg of total RNA. The templates for dsRNA synthesis were generated from cDNA by a two-step PCR reaction. The first primers consisted of 15 base gene-specific sequences designed for each gene of interest. A second PCR reaction was performed using primers containing a T7 promoter sequence (GAATTAATACGACTCACTATAGGGAGA) attached to the 5′-end of the gene-specific sequences. Both sense and antisense RNAs were synthesized simultaneously from a single PCR product using the T7 MegaScript RNA polymerase (Ambion, Austin, TX) as recommended by the manufacturer. dsRNA was precipitated with LiCl and treated with DNAase. Quality of dsRNA was analyzed by agarose gel electrophoresis and the concentration was measured by spectrophotometer. The concentration of dsRNA was 10 μg per 10^6 S2 cells in each experiment (if not stated otherwise). Cells were incubated with dsRNA for 72 h.

2.5. FACS analysis to quantify phagocytosis and microbial binding

Flow cytometry was used to analyze the ability of the dsRNA treated cells to bind and phagocytose heat-killed, FITC-labeled *E. coli* and *Staphylococcus aureus* (Molecular probes, Leiden, the Netherlands) compared to GFP dsRNA treated control cells. A total of 5.0×10^5 dsRNA treated S2 cells were plated onto 48-well plates and incubated for 1 h at room temperature. Thereafter the cells were cooled down to 4 °C for 30 min, and heat-killed, FITC-labeled *E. coli* or *S. aureus* was added. Plates were centrifuged for 1 min at $100 \times g$ (at 4 °C) and incubated for 1 h at 4 °C to let S2 cells bind bacteria. To allow phagocytosis, the plates were incubated at 26 °C (16 min for *E. coli* and 30 min for *S. aureus*). Thereafter plates were placed on ice, medium was replaced with ice-cold PBS and cells transformed into flow cytometry tubes. Samples were analyzed with FACS using EXPO32 program (Beckman Coulter, Ordior). The fluorescence of extracellular particles was quenched by adding 0.2% Trypan blue in 1× PBS (pH 4.85) shortly before the actual measurement. The amount of phagocytosis was quantified as the percentage of fluorescence-positive cells multiplied by the mean fluorescence of these cells. 5000–10,000 cells were counted from each sample. The ability of dsRNA treated cells to bind bacteria was measured accordingly except plates were kept at +4 °C at all times and no Trypan blue was added prior to measurements.

2.6. Luciferase reporter assay

A luciferase reporter assay was used to analyze the effect of dsRNA treatments on antimicrobial peptide release by both the Imd and the Toll pathway. The S2 cells were transfected either with 0.5 μg of *Attacin*-reporter plasmid (the Imd path-