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Involvement of NF-κB transcription factors in antimicrobial peptide gene induction in the red flour beetle, *Tribolium castaneum*

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ABSTRACT

We previously demonstrated that *Tribolium castaneum* antimicrobical peptide (AMP) genes can be classified to IMD-dependent group I, Toll-dependent group III and co-dependent group II genes besides non-inducible group IV. Here, we focused on NF-κB transcription factor genes, *Dif1*, *Dif2* and *Rel*, and examined their functions in AMP gene induction as well as linkages to the Toll or IMD pathway. IMD-dependent group I and Toll-dependent group III genes were revealed to be Rel- and Dif-dependent respectively through knockdown experiments, indicating that the pathway specificity of NF-κB classes found in *Drosophila* is also conserved in *T. castaneum*. The Toll–Dif and IMD–Rel pathways of *T. castaneum* were activated concomitantly by single microbe species, which may represent a distinctive feature of its immune responses. In addition, *Rel* knockdown impaired host defense against two model bacterial pathogens. Finally, potential κB motifs were searched in the regulatory regions of AMP genes, and relevance to respective NF-κB transcription factors was discussed.

1. Introduction

In response to microbial infection, insects synthesize a massive amount of antimicrobial peptides (AMPs) and secret them into the hemolymph, where they combat invading microbes. This 'systemic' response represents a hallmark of insect innate immunity, and the production of AMPs is mainly exerted by the fat body, an insect organ functionally equivalent to mammalian liver (Hultmark, 2003; Lemaitre and Hoffmann, 2007). In *Drosophila melanogaster*, robust induction of AMP genes upon microbial infection is mediated by the two distinct, NF-κB transcription factor-dependent signaling pathways, the Toll and IMD pathways (Georgel et al., 2001; Hultmark, 2003; Lemaitre, 2004; Lemaitre and Hoffmann, 2007; Valanne et al., 2011). The Toll pathway is activated mainly by Gram-positive or fungal infection, and extracellular recognition of Lys-type peptidoglycan (cell wall component of many Gram-positive bacteria), fungal β-glucan or proteases results in a proteolytic activation of the Toll receptor ligand spätzle. The intracellular TIR domain of the ligand-activated Toll receptor recruits a MyD88–Tube–Pelle heterotrimeric complex (Valanne et al., 2011). This ultimately leads to the activation and nuclear translocation of a *Drosophila* NF-κB homolog Dif or Dorsal, which regulates the expression of immune-related target genes including AMP genes (Manfruelli et al., 1999; De Gregorio et al., 2002). The IMD pathway is generally considered to provide host defense against Gram-negative infection, while this pathway has been shown to contribute to defense against several fungal species (Hedengren et al., 1999). The recognition of DAP-type peptidoglycan that composes the cell wall of most Gram-negative bacteria mainly by membrane-bound PGRP-LC triggers the IMD signaling, where a cytoplasmic death domain-containing adaptor protein IMD plays a central role. The IMD signaling eventually results in the activation by endoproteolytic cleavage and nuclear translocation of another *Drosophila* NF-κB homolog Relish, regulating a set of target genes that largely overlap those of Dif or Dorsal (Hedengren et al., 1999; De Gregorio et al., 2002).

While the two signaling pathways are well-described both mechanistically and functionally in *D. melanogaster*, the information on other insect species is relatively scarce. A series of pioneering works by Lee and his colleagues using the mealworm beetle *Tenebrio molitor* has uncovered the mechanisms for extracellular pathogen recognition and the proteolytic cascade that activates both spätzle and prophenoloxidase (Park et al., 2007; Kan et al., 2008; Kim et al., 2008; Roh et al., 2009), while the function of the IMD pathway is not clear in this species. Interestingly, the *Tenebrio* PGRP-SA can recognize both Lys-type and DAP-type peptidoglycan and induces Toll and prophenoloxidase activations

Abbreviations: AMP, antimicrobial peptide; Att1, Attacin1; Att2, Attacin2; Col1, Coleoptericin1; Def2, Defensin2; Def3, Defensin3; Cec2, Cecropin2; Cec3, Cecropin3; Att3, Attacin3; Def1, Defensin1; RNAi, RNA interference; dsRNA, double strand RNA; qRT-PCR, real-time quantitative RT-PCR; RPL32, ribosomal protein L32; malE, maltose binding protein E.

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(Yu et al., 2010), suggesting coleopteran systems may be somewhat different from the *Drosophila* system.

In a previous paper, we characterized nine AMP genes of a model beetle *Tribolium castaneum* in terms of the induction profiles in pupae by three microbial species, Gram-negative *Escherichia coli*, Gram-positive *Micrococcus luteus* and the budding yeast *Saccharomyces cerevisiae*, and categorized them into four groups (Yokoi et al., 2012). Group I genes *Attacin1* (*Att1*), *Attacin2* (*Att2*), *Coleoptericin1* (*Col1*) and *Defensin2* (*Def2*) showed acute and robust responses against infection of both *E. coli* and *M. luteus* while responses to *S. cerevisiae* were weaker. Group II gene *Defensin3* (*Def3*) also showed acute and strong responses to the two bacterial species whereas the degree of induction was weaker than the cases of group I genes. In contrast, group III genes *Cecropin2* (*Cec2*) and *Cecropin3* (*Cec3*) exhibited slower and more sustained responses to microbes, and the degrees of induction were modest and did not vary greatly for the three microbes tested. Group IV genes *Attacin3* (*Att3*) and *Defensin1* (*Def1*) were not or very weakly induced by the three microbes as well as by two additional bacterial species *Enterobacter cloacae* and *Bacillus subtilis*. We subsequently examined these AMP genes in terms of dependence on the Toll and IMD pathways by utilizing RNA interference (RNAi) of two intracellular adaptor protein genes *MyD88* and *IMD*, which are representative of the two pathways respectively (Yokoi et al., 2012). We estimated: the induction of group I genes by *E. coli* and *M. luteus* was mainly mediated by the IMD pathway while that of group III genes was largely dependent on the Toll pathway; the both pathways were responsible for the induction of group II gene by *E. coli* and *M. luteus*; the induction of group I genes by *S. cerevisiae* was likely to be mediated by the both pathways while the Toll pathway predominantly regulated the activation of group II and group III genes by *S. cerevisiae*. These results indicate that in *T. castaneum* a single microbe species can activate both the Toll and IMD pathways and that respective AMP genes are likely to be regulated based on a balance of their dependence on the two signaling pathways rather than by elicitor classes, suggesting more promiscuous activation and usage of the two signaling pathways occur in *T. castaneum* than in *D. melanogaster* (Yokoi et al., 2012).

Questions arise here as to whether the epistatic relationships of signaling components to distinct terminal NF-κB transcription factors in the two pathways, such as MyD88 to Dif/Dorsal and IMD to Relish found in *D. melanogaster* are also conserved in *T. castaneum* and whether the dependence of respective AMP genes on the two signaling pathways is accounted for by their preferences to respective NF-κB transcription factors. To address these questions, here we focused on NF-κB transcription factors that presumably act in the terminal steps of the two signaling pathways of *T. castaneum*. According to the annotation of immunity-related genes by Zou et al. (2007), the *T. castaneum* genome encodes two transcription factors homologous to *D. melanogaster* Dif/Dorsal, namely Dif1 and Dif2, as well as one homolog of *D. melanogaster* Relish, Rel. We conducted knockdown of these *T. castaneum* NF-κB transcription factor genes followed by expression analyses of AMP genes and survival assays using two model bacterial pathogens.

2. Materials and methods

2.1. Insect rearing

T. castaneum was reared as in a previous paper (Yokoi et al., 2012).

2.2. Microorganisms

E. coli DH5α and *M. luteus* ATCC4698 were cultured overnight in LB broth at 37 °C and 30 °C, respectively. *S. cerevisiae* S288C was cultured overnight in YPD medium at 30 °C. Cells were collected, washed in D-PBS (−) (WAKO Chemicals), and A_{600} values adjusted to 0.5. In these cases, the cell densities of *E. coli*, *M. luteus* and *S. cerevisiae* suspensions were equivalent to 2.9×10^8, 2.9×10^7 and 6.3×10^6 cells/ml, respectively. These cell suspensions were incubated for 10 min at 75 °C, aliquoted from single pools and stored at −80 °C as heat-killed microbial suspensions until use. Suspensions of live *E. cloacae* and *B. subtilis* were prepared as follows. They were cultured overnight in LB broth at 30 °C. Then, the culture was diluted 100 times with fresh LB broth, and the cells of logarithmic growth phase were obtained by culturing them for additional 3–4 h at 30 °C. The cells were harvested and washed, and the suspensions of live *E. cloacae* and *B. subtilis* in D-PBS prepared by adjusting A_{600} values to 0.1 and 4.0, respectively. Fifty nanoliters of microbe suspensions, either heat-killed or live, were injected into naïve day 3 pupae or day 3 pupae pretreated with double strand RNA (dsRNA) using a Nanoject II (Drummond Scientific Company). *M. luteus* was provided by the RIKEN Bioresource Center in Japan. *S. cerevisiae* was from Dr. T. Ushimaru of Shizuoka University, Japan. *E. cloacae* and *B. subtilis* were the generous gifts of Dr. Y. Yagi at Nagoya University, Japan.

2.3. Sequences of immune-related genes and primers used for real-time quantitative RT-PCR (qRT-PCR)

The immune-related genes of *T. castaneum* dealt in this study were as follows: *Dif1* (GLEAN_07697), *Dif2* (GLEAN_0896), *Rel* (GLEAN_11191), *Att1* (GLEAN_07737), *Att2* (GLEAN_07738), *Att3* (GLEAN_07739), *Cec2* (GLEAN_00499), *Cec3* (GLEAN_00500), *Col1* (GLEAN_05093), *Def1* (GLEAN_06250), *Def2* (GLEAN_10517), *Def3* (GLEAN_12469), and the normalizer of qRT-PCR *ribosomal protein L32* (*RPL32*) (GLEAN_06106). The sequences were retrieved from the Beetlebase (http://www.beetelebase.org), and primer pairs of respective target genes designed for qRT-PCR. The primer sequences for *Dif1*, *Dif2* and *Rel* are listed in Table 1. The other primer sequences appear in Yokoi et al. (2012).

2.4. RNA extraction and qRT-PCR

Total RNA was extracted from the whole body of *T. castaneum* and subjected to qRT-PCR analyses as described in a previous paper (Yokoi et al., 2012).

2.5. RNAi

RNAi was performed basically as described in Yokoi et al. (2012). Briefly, dsRNAs were synthesized using cDNA templates possessing T7 RNA polymerase promoter sequences on both ends. One hundred nanograms of dsRNA was injected into day 0 pupae with Nanoject II. dsRNA possessing a partial *maltose binding protein E* (*malE*) sequence was used as a negative control. For double or triple knockdown experiments, 100 ng each of respective dsRNAs were combined in a volume less than 100 nl, and injected into the pupae as above. The pupae were kept at 30 °C for 72 h, then subjected to qRT-PCR to confirm knockdown of targeted genes at the mRNA level, or challenged with microbes for further analyses. Sequences of primer pairs used for preparing T7 RNA polymerase promoter sequence-tagged cDNA fragments of *Dif1*, *Dif2*, *Rel* and *malE* are presented in Table 2.

2.6. Survival assay

Day 0 pupae were treated with dsRNA of *Dif1*, *Dif2, Rel*, *Dif1* plus *Dif2* mixture or *malE*. Seventy-two hours later, 50 nl of live *E. cloacae* or *B. subtilis* suspension was injected into the pupae (day 3), and the numbers of surviving pupae counted every 24 h.