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A Fas associated factor negatively regulates anti-bacterial immunity by promoting Relish degradation in *Bombyx mori*

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ABSTRACT

Negative regulation is required to keep NF-κB-dependent immune response under tight control. In previous study, we have identified a Fas associated factor (FAF) family member in *Bombyx mori*, *BmFAF*, and proposed it may act as a negative regulator in immune response. In this study, we found knock-down of *BmFAF* by RNAi led to a remarkable increase in transcriptional level of several antimicrobial peptide genes, including *BmCecropinA1* and *BmMoricin*, and higher survival rate to Gram-negative bacterial infection. We also confirmed the regulatory role of BmFAF in suppressing NF-κB-dependent transcription by employing an inducible promoter in BmE cells. Consistent with these physiological phenotypes, BmFAF suppressed the activity of the essential transcription factor, Relish, in IMD signaling pathway by promoting its proteasomal degradation through direct interaction. In addition, by constructing various truncation mutants, we further demonstrated that UBA domain in BmFAF is required for the inhibitory role, and potential ubiquitination also occurs in this domain. Taken together, our results suggest that BmFAF is a negative regulator of IMD pathway by mediating degradation of Relish.

1. Introduction

Activation of insect humoral immune response mainly depends on two signaling pathways, Toll and IMD, both of which are responsible for transducing pathogen recognition signals to NF-κB-like transcriptional factors to induce the expression of a plethora of genes encoding molecules that organize and execute immune response, such as antimicrobial peptides (De Gregorio et al., 2002; Hetru and Hoffmann, 2009; Minakhina and Steward, 2006). The essential transcriptional factors, Dorsal/Dif in Toll pathway and Relish in IMD pathway are usually sequestered in cytoplasm in the absence of immune challenges. They are released from the inhibitory complex or domain and subsequently translocated into nucleus once the pathways are turned on. After elimination of the invading pathogens, the immune homeostasis should be re-established through down-modulation and proper termination of NF-κB-mediated transcription. Otherwise, prolonged or excess NF-κB activity will result in uncontrolled tissue damage or unusual burden to individual's fecundity even viability (Ciota et al., 2011; Schneider, 2007; Zuk and Stoehr, 2002). Therefore, negative regulation of NF-κB signal pathways is essential for the fitness and survival of insect.

Through genetic screens or sequence-based analysis, many negative regulators of immune signaling pathways have been identified in *Drosophila* as well as in few other insects (Aggarwal and Silverman, 2008; Lee and Ferrandon, 2011). Several negative regulators prevent immune response from over-activation at the early steps. For instance, peptidoglycan recognition protein-LB (PGRP-LB) and PGRP-SC degrade PGN into non-stimulatory fragments through their amidase activity (Bischoff et al., 2006; Paredes et al., 2011; Zaidman-Remy et al., 2006). And Rudra disrupts the interaction between receptors and IMD, generating a potent negative feedback (Aggarwal et al., 2008). Several regulators target the essential transcriptional factors directly, often through blocking their nuclear translocation or decreasing protein stability (Chiu et al., 2005; Gordon et al., 2005; Guntermann et al., 2009; Tsuda et al., 2005). Mutation or loss of these negative regulators may result in constitutive activation of pathways and enhanced amplitude of immune response upon infection.

The *Caspar* gene, first discovered in *Drosophila*, encodes a protein homolog of the human Fas-associated factor 1 (FAF1), which negatively regulates IMD pathway possibly through suppressing Dredd-dependent cleavage of Relish, a prerequisite event for the nuclear entry of Relish (Kim et al., 2006). *Caspar* mutant flies

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showed constitutive expression of antimicrobial peptide genes and increased resistance to Gram-negative bacterial infection. Caspar homologs were later identified in *Anopheles*, phlebotomine sand fly and locust (Garver et al., 2009; He et al., 2013; Telleria et al., 2012). Sequence analysis demonstrated they all contain highly conserved ubiquitin-related protein motifs, including ubiquitin-associated (UBA) and ubiquitin regulatory X (UBX) domain. Silencing of *Caspar* by RNAi was sufficient to induce the production of certain antimicrobial peptides in the fat body, and capable of reducing parasite survival in the midgut.

By comparative genomic study, we have reported a FAF family member, *BmFAF* in silkworm genome (Wang et al., 2012). Surprisingly, it is clustered with human *FAF2* (*UBXD8*, *ETEA*) and *Drosophila FAF* instead of *FAF1* or *Caspar* on phylogenic tree. And no other FAF homology could be identified in silkworm. After bacterial infection, the transcriptional level of *BmFAF* decreased rapidly, whereas expression of antimicrobial peptide genes, such as *BmCecropinA1*, was markedly up-regulated in the fat body. Cell-based studies further revealed the potential function of BmFAF in suppressing the anti-microbial immune response, since over-expression of *BmFAF* led to a remarkable decrease of *BmCecropinA1*, *BmAttacin* and *BmMoricin*, whereas RNAi-mediated silencing of *BmFAF* resulted in increased mRNA level of these antimicrobial peptide genes (Ma et al., 2014). Although the inhibitory role of BmFAF is similar to Caspar, whether they share the same molecular mechanism is not clear.

In the present study, by employing an inducible promoter we characterized previously (Hua et al., 2015) and various biochemical assays, we investigated whether BmFAF is involved in modulation of Relish activity. We found BmFAF suppresses Relish activity by promoting its proteasomal degradation through direct interaction. In addition, by constructing various truncation mutants, we further demonstrated that its UBA domain is required for the regulatory function of BmFAF, and potential ubiquitination also occurs in this domain. Taken together, our results suggest that BmFAF is a negative regulator of IMD pathway by mediating degradation of Relish.

2. Materials and methods

2.1. Insects, antibodies and reagents

Silkworm DaZao P50 strain was originally obtained from Silkworm Genetic Resource Supply in Southwest University. Larvae were reared following standard procedure. The following antibodies have been used: anti-Flag (Sigma, USA), anti-Flag conjugated with HRP (Sigma), anti-Myc (Life Technologies, USA), anti-GAPDH (Beyotime, China), anti-Tubulin (Beyotime) and anti-GST (Beyotime). LPS (Sigma) and MG132 (Millipore, USA) was purchased from the indicated manufacturers.

2.2. Plasmid construction and dsRNA synthesis

BmFAF (SILKDB: BGIBMGA008874; GI: 512929299) and *BmDredd* (SILKDB: BGIBMGA006726; GI: 168823406) was cloned from silkworm larvae cDNA. Expression vector of N-terminal Flag tagged full-length BmFAF (FAF-FL) or truncated forms and HA tagged-BmDredd (HA-Dredd) was constructed following the same procedures of construction of Myc tagged-BmRelish (Myc-Relish) expression vector (Hua et al., 2015). Interfering dsRNA against *BmFAF* (dsFAF) or *EGFP* (dsEGFP) was generated using T7 *in vitro* Transcription Kit (Promega, USA) following the manufacturer's instruction. Primers used in this study were listed in Supplementary Table 1.

2.3. Cell transfection

BmE cells were maintained at 27 °C in Grace medium (Gibco, UK) supplemented with 10% FBS and penicillin and streptomycin (Hyclone, USA). Plasmids or dsRNA were transfected into cells using X-treme GENE transfection reagent (Roche, Switzerland) following the manufacturer's instruction.

2.4. Infection experiments

dsRNA was injected into silkworm larvae (20 μg/larva) at second day of fifth instar through the second last stoma in abdomen with a fine needle. 24 h later, overnight-cultured *Serratia marcescens* or *Bacillus bombyseptieus* (1×10^3/larva) were injected at the same site. Survival experiments were performed with at least 30 larvaes for the same treatment. Surviving larvae counts were taken every day until larvaes reached to wandering stage. The average of three replicate experiments is presented as a graph, and the standard deviation (S.D.) is indicated as an error bar.

2.5. Luciferase reporter assay

BmE cells were transfected with plasmids encoding full length or truncated Flag-FAF together with Myc-Relish, HA-Dredd, 0.2 μg firefly luciferase reporter and 0.02 μg Renilla luciferase plasmid. 24 h after transfection, cells were treated with 5 μg LPS (10 μg mL^{-1}) for 6 h. Luciferase activity was measured using Dual-Glo luciferase Reporter Assay System (Promega) following the manufacturer's instruction. Firefly luciferase readings were divided by the Renilla luciferase readings to calculate the relative fold change.

2.6. Quantitative RT-PCR analysis

Total RNA was extracted from silkworm larvae or BmE cells using Total RNA Kit (Omega, USA) and reverse transcribed by GoScript™ Reverse Transcription System (Promega). qRT-PCR was performed using SYBR Premix Ex Taq II (Takara, Japan) on a 7500 fast Real-Time PCR System (Applied Biosystems, USA) with a program consisting of an initial denaturing step of 30 s at 95 °C and 40 amplification cycles consisting of 3 s at 95 °C followed by 30 s at 60 °C. The expression level of *BmFAF*, *BmCecropinA1*, *BmMoricin* and *BmAttacin* was normalized to the control (SilkDB Probe number: sw22934). Primers used in qRT-PCR were listed in Supplementary Table 1. The experiment was repeated three times independently, and at least three silkworm larvae were sampled for each treatment every time.

2.7. Immunoprecipitation and immunoblotting

Cells were lysed in NP40 lysis buffer (Beyotime). Nuclear- and cytosolic-enriched fractions were prepared as described previously (Liu et al., 2014). 1 mg of total protein was incubated with 2.5 μg of corresponding antibodies for 3 h at 4 °C followed by incubation with 30 μL Protein A/G PLUS-Agarose (Santa Cruz Biotech, USA) for 4 h at 4 °C. The immunoprecipitates were washed for five times and subjected to immunoblotting. For immunoblotting analysis, whole-cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membrane (GE Healthcare, USA). The membranes were incubated with the primary antibodies, then with anti-mouse or anti-rabbit IgG conjugated with HRP (Beyotime) followed by visualization with ECL detection system (Thermo Fisher Scientific, USA).