Developmental and Comparative Immunology 46 (2014) 208–221

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Gene structure, cDNA characterization and RNAi-based functional analysis of a myeloid differentiation factor 88 homolog in *Tenebrio molitor* larvae exposed to *Staphylococcus aureus* infection

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ARTICLE INFO

Article history:
Received 7 January 2014
Revised 13 April 2014
Accepted 14 April 2014
Available online 19 April 2014

Keywords:
Tenebrio molitor
MyD88
Toll/IL-1 domain
Immune challenge
RNA interference
Staphylococcus aureus

ABSTRACT

Myeloid differentiation factor 88 (MyD88), an intracellular adaptor protein involved in Toll/Toll-like receptor (TLR) signal processing, triggers activation of nuclear factor-kappaB (NF-κB) transcription factors. In the present study, we analyzed the gene structure and biological function of MyD88 in a coleopteran insect, *Tenebrio molitor* (TmMyD88). The TmMyD88 gene was 1380 bp in length and consisted of five exons and four introns. The 5′-flanking sequence revealed several putative transcription factor binding sites, such as STAT-4, AP-1, cJun, cfos, NF-1 and many heat shock factor binding elements. The cDNA contained a typical death domain, a conservative Toll-like interleukin-1 receptor (TIR) domain, and a C-terminal extension (CTE). The TmMyD88 TIR domain showed three significantly conserved motifs for interacting with the TIR domain of TLRs. TmMyD88 was grouped within the invertebrate cluster of the phylogenetic tree and shared 75% sequence identity with the TIR domain of *Tribolium castaneum* MyD88. Homology modeling of the TmMyD88 TIR domain revealed five parallel β-strands surrounded by five α-helices that adopted loop conformations to function as an adaptor. TmMyD88 expression was upregulated 7.3- and 4.79-fold after 12 and 6 h, respectively, of challenge with *Staphylococcus aureus* and fungal β-1,3 glucan. Silencing of the TmMyD88 transcript by RNA interference led to reduced resistance of the host to infection by *S. aureus*. These results indicate that TmMyD88 is required for survival against *Staphylococcus* infection.

1. Introduction

The innate immune system serves as the first line of defense in multicellular organisms and responds to pathogens in a nonspecific manner. This system is triggered upon recognition of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000; Akira et al., 2006). PAMPs conserved in microbes include lipopolysaccharides (LPS) in Gram-negative bacteria, peptidoglycan (PGN) and lipoteichoic acids (LTAs) in Gram-positive bacteria, β-glucan in fungi, and unmethylated bacterial CpG motifs in viruses (Janeway and Medzhitov, 2002; Valanne et al., 2011). In vertebrates, the immune system also undergoes the adaptive responses that consist of several other immune-related molecules, such as T and B lymphocytes, which express specific antigen receptors. Previous studies have suggested that adaptive immune responses need to be primed by innate immunity signals (Janeway, 2001).

Insect immunity mainly relies on the innate immune response, which consists of two components: the humoral and cellular arm. Secretion of antimicrobial peptides (AMPs) and phenoloxidase-mediated melanin synthesis are humoral immune responses, while phagocytosis and encapsulation are cellular immune responses (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). In *Drosophila*, the induction of AMPs upon microbial infection is regulated by two distinct pathways: the Toll and immune deficiency (Imd) pathways (Cherry and Silverman, 2006; Lemaitre and Hoffmann, 2007). The Toll pathway is activated upon recognition of Lys-type PGN from Gram-positive bacteria and β-glucan in fungi, resulting in cleavage of the Toll ligand spätzle (Jang et al., 2006). The intracellular events require activation of the myeloid differentiation factor

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http://dx.doi.org/10.1016/j.dci.2014.04.009
0145-305X/

(MyD88) and the nuclear factor-kappa B (NF-κB) to regulate the expression of AMP genes.

The role of MyD88 as a central adaptor molecule in the Toll signaling pathway has been extensively studied after it was identified in 1990 (Lord et al., 1990). The role of MyD88 in innate immunity was attributed to amino acid homology with the cytoplasmic domains of Toll and mammalian IL-1 receptors (Muzio et al., 1997; Wesche et al., 1997). MyD88 genes have been characterized from different species of vertebrates and invertebrates (Bonnert et al., 1997; Hardiman et al., 1997; Prothmann et al., 2000; Tausig-Delamasure et al., 2002; Takano et al., 2006; Qiu et al., 2007; Wheaton et al., 2007; Rebl et al., 2009; Skjaeveland et al., 2009; Yao et al., 2009; Zhang et al., 2012; Wen et al., 2013). The carboxy-terminal Toll-like/interleukin-1 receptor (TIR) domain of MyD88 is known to bind directly to the TIR domain of activated Toll-like receptors (TLRs). TIR domains are involved in numerous interactions, including the oligomerization of receptor TIR domains (R-interface), oligomerization of adaptor TIR domains (A interface), and the receptor adaptor association (S-interface) (Xu et al., 2000). The N-terminal death domain superfamily plays crucial roles in death signal transduction, the regulation of apoptosis, and inflammatory responses (Motshwene et al., 2009; Lin et al., 2010; Cataisson et al., 2012; Ochi et al., 2012).

The role of MyD88 as a "central linker" to activate downstream signaling in the Toll pathway of invertebrates has been established based on evidence of MyD88 upregulation induced by immune elicitors and microbial pathogens (He et al., 2013; Li et al., 2013; Toubiana et al., 2013). In *Drosophila*, MyD88-mutant flies were susceptible to Gram-positive bacterial and fungal infections (Tausig-Delamasure et al., 2002). In penaeid shrimp (*Fenneropenaeus chinensis*) and Pacific white shrimp (*Litopenaeus vannamei*), antibacterial and antiviral responses were mediated by the MyD88-dependent pathway (Wen et al., 2013). MyD88 gene silencing revealed that the Toll pathway controls the response to Dengue virus (RNA virus) infection in *Aedes aegypti* (Xi et al., 2008) and *Drosophila* antiviral response (Zambon et al., 2005). An evolutionary 'loss' of the MyD88 signaling pathway has been reported in *Caenorhabditis elegans* based on the absence of a MyD88 ortholog and identification of a Toll homolog (Kim and Ausubel, 2005). This suggests that regulation of the MyD88 gene in the Toll signaling pathway is critical for innate defense in insects. Therefore, increasing our understanding of MyD88 gene in other invertebrates and on the Toll signaling pathway is important from an evolutionary perspective.

The yellow mealworm *Tenebrio molitor* is a freeze-susceptible, stored-product pest. It is also considered a useful model for studying insect biochemistry, immunology and physiology. The extracellular pattern recognition and the proteolytic cascades in *T. molitor* has been examined using elegant biochemical studies, with a focus on Toll signaling activation (Kim et al., 2008; Roh et al., 2009; Jiang et al., 2011). However, the downstream role of MyD88 in Toll interactions and activation of NF-κB factors in the beetle remains unclear. Previously, we screened immune-related genes from *T. molitor* using expressed sequence tag (EST) (Jeong et al., 2013) and RNAseq based strategies. Using these approaches, we identified a MyD88 homolog from *T. molitor* (denoted as TmMyD88). The purposes of this study were to clone and characterize full-length cDNA of TmMyD88, and examine specific sequence and structural features using molecular informatics tools; characterize the TmMyD88 gene structure and promoter; examine the stage and tissue-specific mRNA expression patterns of TmMyD88; clarify the time-course expression profiles of TmMyD88 under *Staphylococcus aureus* and fungal β-1,3 glucan challenge; and study the effects of TmMyD88 gene silencing on the susceptibility of *T. molitor* larvae against *S. aureus* infections.

2. Materials and methods

2.1. Insect collection and maintenance

T. molitor larvae were procured from the College of Pharmacy, Pusan National University, Busan, South Korea, and maintained at 26 ± 1 °C, 60% ± 5% relative humidity, and a 16:8 h light: dark cycle. Only last instar, uniformly sized, and healthy larvae were used for experiments unless otherwise stated.

2.2. Microorganisms

Escherichia coli ATCC 25922 and *S. aureus* strain RN4220 were cultured overnight in Luria–Bertani (LB) broth at 37 °C. Cells were harvested, washed twice in phosphate-buffered saline (PBS), and centrifuged at 5000 rpm for 5 min. The resuspension culture was serially diluted in 0.9% saline to an OD_{600} of 1.0. In these cases, the cell densities reached a concentration of 10^9 cells/ml. The OD_{600} values were confirmed by aseptically spread-plating the serially diluted samples of *E. coli* and *S. aureus* on LB agar plates. The plates were incubated at 37 °C for 16 h prior to colony counting. β-1,3 glucan was obtained from the College of Pharmacy, Pusan National University, Korea.

2.3. Identification of the genomic sequence of TmMyD88

Genomic DNA was isolated from last-instar *T. molitor* larvae (gut was removed) using the standard phenol–chloroform method (Sambrook and Russell, 2001). The quality of total DNA was assessed using agarose gel electrophoresis, and DNA was stored at 4 °C for future use. For fosmid library construction, DNA was sheared into approximately 35–40 kb fragments using HydraShear (GeneMachines Inc., San Carlos, CA). The sheared DNA (20 μg) was end-repaired in an 80 μl reaction volume (8 μl 10× end-repair buffer, 8 μl 2.5 mM dNTP mix, 8 μl 10 mM ATP, and 4 μl end-repair enzyme mix) at room temperature for 45 min to generate blunt-ended 5′-phosphorylated DNA. The DNA was subsequently ligated into the *Eco*72I blunt-end site of the CopyControl pCC1FOS vector (Epicentre Biotechnologies, Madison, WI) in a 10:1 M ratio. The ligated DNA was packaged using MaxPlax™ lambda packaging extracts (>10^9 pfu/μg DNA) and plated on TransforMax™ EPI300™ *E. coli* (Epicentre Biotechnologies, Madison, WI). The titer of the phage library was determined by serial dilutions and clones that were eight times larger than each of the selected clones were randomly picked for plasmid preparation and sequencing with pCC1/pEpiFOSTM forward and reverse primers (Table 1). Sequencing was performed on an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) using the cycle sequencing method. Lane tracking and trace extraction was performed using the Applied Biosystems sequence software and the data was transferred to UNIX workstations for further processing.

For screening the fosmid library, gene-specific primers (Table 1) were designed based on the cDNA sequence of TmMyD88 and semiquantitative polymerase chain reaction (PCR) was conducted with the following reaction conditions: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 36 °C for 35 s, annealing at 54 °C for 35 s, and extension at 72 °C for 2 min. After three rounds of PCR screening, the fosmid clones were end-sequenced to confirm the identity using the cycle sequencing method (see above).

2.4. Sequence analysis

The fosmid clone corresponding to TmMyD88 was analyzed using the FGENESH 2.6 program at Softberry (http://linux1.