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Molecular cloning, characterization and function of a germinal center kinase MST4 gene from *Litopenaeus vannamei* in response to *Vibrio alginolyticus* challenge in TLR-TRAF6 signaling pathway

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ABSTRACT

The serine/threonine protein kinase MST4 plays multiple roles in the regulation of signaling pathways that govern cellular processes including mitosis, migration, homeostasis, polarity, proliferation, differentiation and apoptosis. Here we report the identification and characterization of the full-length sequence of *Lv*MST4 from the shrimp *L. vannamei*, and investigations into its role in the shrimp's immune response to infection by the pathogenic bacterium *Vibrio alginolyticus*. Subcellular localization assays demonstrated the enzyme's presence in the shrimp's cytoplasm, and tissue-specific expression analysis revealed that it is expressed ubiquitously but at different levels in different tissues. Infection with *V. alginolyticus* increased *Lv*MST4 expression and induced a rapid response via the TLR-TRAF6 signaling pathway, causing a decline in the total hemocyte count (THC) and an increase in respiratory burst (RB) activity. In non-infected shrimp, RNAi silencing of *Lv*MST4 with dsRNA had no significant effect on THC but seemed to activate the TRAF6-MKK6-p38 pathway and reduced RB activity. In shrimp challenged with *V. alginolyticus*, *Lv*MST4 silencing reduced bacterial clearance and increased the initial upregulation of *Lv*TRAF6 while reducing the expression of *Lv*MKK6 and *Lv*p38. *Lv*MST4 silencing also slightly reduced the THC but caused pronounced increases in RB activity and cumulative mortality. These findings suggest that *Lv*MST4 contributes to antimicrobial responses via the TLR-TRAF6 signal pathway, and helps maintain immunological homeostasis in *L. vannamei*.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, is a luxuriant penaeid shrimp and one of the most economically important shrimp species in the world. Like other invertebrates, penaeid shrimps lack true adaptive immune response systems (Hoffmann et al., 1999; Li and Chen, 2008; Li and Xiang, 2013; Shi et al., 2014) and are therefore prone to infection by pathogenic bacteria. *L. vannamei* is subject to infectious diseases including viral infections and vibriosis, especially caused by the gram negative marine bacterium *Vibrio alginolyticus* (PC et al., 1996). *V. alginolyticus* infections can cause severe economic losses in shrimp aquaculture (Li et al., 2008). Adverse environmental conditions associated with intense cultivation, together with potentially high levels of the infectious agent in the Vibrio population of aquaculture waters, often result in disease outbreaks (Lavilla-Pitogo et al., 1998). The immunity status and health of white shrimp populations with respect to *V. alginolyticus* infection can be measured using a range of indicators including the total hemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB, release of superoxide anion) activity, superoxide dismutase (SOD) activity, phagocytic activity, and bacterial clearance efficiency (Le Moullac and Haffner, 2000; Li and Chen, 2008; Rodríguez and Le Moullac, 2000). Because its effects can be studied in such diverse ways, *V. alginolyticus* is an important model organism for studying the adaptability of shrimp immune responses and their susceptibility to environmental stressors.

MST4, a novel member of the germinal center kinase (GCKIII) subfamily of mammalian Sterile 20 serine/threonine kinases,

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regulates cell growth and apoptosis, and also appears to influence cell polarization (Fidalgo et al., 2010; Klooster et al., 2009; Nogueira et al., 2008). It has a highly conserved kinase domain in its the N-terminal region and a C-terminal regulatory domain that mediates its dimerization and regulates its kinase activity. The gene encoding MST4 was localized to a disease-rich region of chromosome Xq26 by fluorescence *in situ* hybridization, and was first cloned by exploiting the interaction between c-Raf and a LexA yeast two-hybrid system (Fidalgo et al., 2010). Northern blot analysis revealed that it is distributed across a wide range of tissues, but is particularly strongly expressed in the placenta, thymus, lymphocytes and peripheral blood leukocytes (Lin et al., 2001; Qian et al., 2001).

Sterile 20 (Ste20) is a serine threonine kinase first identified in yeast, which acts upstream of MAPK kinase (Leberer et al., 1992; Ling et al., 2008). MST4 combines directly with and phosphorylates tumor necrosis factor receptor-associated factor 6 (TRAF6), which plays an important signaling role during inflammatory responses and is a vital signal transducer in the Toll-like receptor (TLR) signaling pathway. The phosphorylation of TRAF6 by MST4 restricts the former protein's auto-ubiquitination and thus limits the production of downstream pro-inflammatory cytokines and signaling activity. After a septic shock, mice in which MST4 was knocked down showed exacerbated inflammation and reduced survival. These adverse effects were alleviated by heterozygous deletion of *Traf6* (*Traf6*$^{+/-}$) (Jiao et al., 2015; Kim and Ausubel, 2005; Leulier and Lemaitre, 2008; Wang et al., 2011). TLR-TRAF6 signaling plays a critical role in responses to bacterial infection and tissue injury in many species, including shrimp. In addition, bacterial infections activate the TLR-dependent TRAF6-MKK3/6-p38 MAPK signaling pathway, inducing a synergistic inflammatory response (Jono et al., 2012).

MST4 could potentially be useful as a “molecular brake” for preventing overactive inflammatory responses during the treatment of diseases such as cancer. Consequently, there have been several studies on the mechanism by which it controls innate immune responses in humans. However, little is known about the functions of MST4 in *L. vannamei*. In this study, we first cloned and characterized the full-length cDNA of *Lv*MST4. Then, to clarify the protein's role in the stress responses of *L. vannamei*, shrimp were subjected to RNAi silencing of *Lv*MST4 and *V. alginolyticus* challenge, and the resulting effects on the total hemocyte count, respiratory burst activity, bacterial numbers, cumulative mortality, and the expression of TLR-TRAF6 signaling-related genes were investigated.

2. Materials and methods

2.1. Animals

Pacific white shrimp, *L. vannamei*, averaging 4–5 g in weight and 8–9 cm in length, were collected from a shrimp farm in Panyu, Guangdong Province of China. The shrimp were kept for 3 days prior to the experiments in 20 L fiberglass tanks containing filtered circulating seawater at 15‰ salinity, pH 7.4–7.9, and 24–25 °C. The animals were fed commercial feed at 5% of body weight twice daily.

2.2. V. alginolyticus infection experiment

Healthy L. *vannamei* were randomly divided into two groups. *V. alginolyticus* stored in the laboratory was activated in LB liquid culture medium and suspended in 1 × PBS (phosphate-buffered saline, pH 7.2) to a concentration of 1 × 10^7 CFU/mL. One group of *L. vannamei* individuals were injected with 10 μL of this *V. alginolyticus* suspension using a 20 μL microsyringe. The other group was injected with 1 × PBS as a negative control. At 0, 1.5, 3, 6, 12 and 24 h post-injection, shrimp were selected randomly from each group for collection. Their hemolymph from six shrimps was sampled using a 2.5 mL sterilized syringe containing an equal volume of Alsever's anticoagulant solution (AS: 8 g/L sodium citrate, 20.5 g/L glucose, 4.2 g/L sodium chloride, pH 7.5) and was stored on ice. Meanwhile, the hepatopancreas, heart, muscle, pleopod, intestine, stomach, eyestalk and gill tissues were collected from four individuals at each timepoint and sharp-frozen separately in liquid nitrogen for RNA extraction.

2.3. Total RNA extraction and cDNA template synthesis

Total RNA was isolated from the hepatopancreas of harvested shrimp using Trizol (Invitrogen, USA) following the manufacturer's instructions, then dissolved in DEPC (diethylpyrocarbonate)-treated water and stored at −80 °C. A single-strand cDNA template was prepared from 1 μg of total RNA by reverse transcription using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) following the manufacturer's protocol.

2.4. Gene cloning and full-length sequence analysis

The template for cloning *Lv*MST4 was synthesized using the BD SMART RACE (rapid amplification of cDNA ends) cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions. Primers (Table 1) for amplifying the *Lv*MST4 fragment were designed using conserved sequence data for other species drawn from the NCBI database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). These primers were then used in conjunction with UPM (Universal Primer Mix A) and NUP (Nested Universal Primer A) primers (Table 1) to amplify 3′ and 5’-fragments in two-round PCR reactions.

The sequences obtained in this way were analyzed using NCBI BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The program ClustalW2 was used to perform multiple sequence alignment. Another search was performed to explore conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) in the sequence. A phylogenetic tree was constructed with MEGA 4.0 using a neighbor-joining algorithm based on the deduced amino acid sequences of MST4 for some other species, with 1000 bootstrap replicates.

2.5. Subcellular localization assay

The plasmid pAc5.1V5-HisB was reconstructed with an inserted sequence encoding GFP (green fluorescent protein). The *Lv*MST4 ORF (open reading frame) sequence was then cloned into this new plasmid using the primers listed in Table 1, yielding the new plasmid pAc5.1V5-HisB-GFP. Before transfection, S2 cells were cultured in a 24-well plate. The lipofectamine 2000 reagent (Invitrogen, USA) was then mixed with the recombinant plasmid and serum-free medium according to the manufacturer's instructions. After 15 min' standing at room temperature, this mixture was added to the cells and the resulting suspension was mixed evenly before being left to continue incubating for 24 h at 25 °C. Redundant medium was then discarded and cells were washed twice with PBS, fastened with 4% paraformaldehyde for 15 min, washed three times for 10 min each with PBS, then treated with PBS containing 0.1% Triton X-100 for 15 min. Finally, the cells were again washed with PBS, stained with 500 μL of DAPI (1 mg/mL) for 12 min, and visualized with a laser scanning confocal microscope (Olympus, Japan).