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Research paper

Expression profiles of the p38 MAPK signaling pathway from Chinese shrimp *Fenneropenaeus chinensis* in response to viral and bacterial infections

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ABSTRACT

Mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in cellular response to inflammatory cytokines, environmental stress and pathogenic infection, However, compared with mammals and insects, the potential function of p38 MAPK from Chinese shrimp (*Fenneropenaeus chinensis*) in response to viruses and bacterial infection is limited. In the study, the immune responses of four genes MKK3, MKK4, p38 and ATF-2 from *F. chinensis* were investigated in defending against white spot syndrome virus (WSSV), *Vibrio anguillarum* (*V. anguillarum*) and *Staphylococcus aureus* (*S. aureus*) infection. Results demonstrated that the expression levels of these four genes were apparently modulated in hemocytes and gills when shrimp were stimulated by WSSV or bacteria, particularly at 3–24 h after infection. MKK3, p38 and ATF-2 were most sensitive to *V. anguillarum* (Gram-negative bacteria), followed by WSSV and *S. aureus* (Gram-positive bacteria), while MKK4 gene was most sensitive to *S. aureus*, followed by WSSV and *V. anguillarum*. Knockdown of *Fcp38* by RNA interference (RNAi) resulted in a reduction in the expression of *FcMKK3* and *FcATF-2*. The results indicate that p38MAPK signaling pathway plays a role in defending against viral and bacterial infections in *F. chinensis*.

1. Introduction

Mitogen-activated protein kinase (MAPK) signaling pathways are highly conserved from mammals to yeast and play important roles in cellular responses to extracellular stimuli (Roux and Blenis, 2004; Cargnello and Roux, 2011). In mammalian cells, three subfamilies of MAPK have been characterized: extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNKs), and p38 MAPK (Garrington and Johnson, 1999; Kyriakis and Avruch, 2001). Of these, p38 MAPK can be activated by various extracellular stimuli, including inflammatory cytokines, environmental stress, UV irradiation and pathogen infection (Han et al., 1994; Raingeaud et al., 1995; Pietersma et al., 1997; Neuder et al., 2009; Regan et al., 2009; Huang et al., 2011). Similar to other MAPKs, p38 MAPK contains a serine/threonine protein kinase domain and a highly conserved dual phosphorylation motif of Thr-Gly-Tyr in the activation loop (Hanks and Hunter, 1995).

Innate immunity is essential for multicellular organisms to defend themselves against microbial infections (Chen et al., 2010). To date, several signaling pathways such as the Toll (Yang et al., 2007; Huang et al., 2010), immune deficiency (Imd) (Huang et al., 2009; Wang et al., 2009), and JAK-STAT (Chen et al., 2010; Cheng et al., 2010) pathways have been proven to participate in the immune response of shrimp to invading pathogens. In mammals, the p38 pathway is known to play a vital role in inflammatory responses, and further evidence has shown that p38 MAPK played crucial roles during viral replication *in vitro* (Johnson et al., 2000; Banerjee et al., 2002; Hirasawa et al., 2003; Khatri and Sharma, 2006). In lower vertebrates, such as *Epinephelus coioides*, p38 MAPK plays a crucial role in regulating virus replication during iridovirus infection (Cai et al., 2011). In invertebrates, recent research in *Litopenaeus vannamei* indicated that p38 MAPK plays an important role in defending against bacterial infections (Yan et al.,

Abbreviations: F. chinensis, *Fenneropenaeus chinensis*; MAPK, mitogen-activated protein kinase; MKK3, mitogen-activated protein kinase kinase 3; MKK4, mitogen-activated protein kinase kinase 4; MKK6, mitogen-activated protein kinase kinase 6; ATF-2, activating transcription factor 2; WSSV, white spot syndrome virus; *V. anguillarum*, *Vibrio anguillarum*; *S. aureus*, *Staphylococcus aureus*; RNAi, RNA interference; ERKs, extracellular signal-regulated kinases; SAPK/JNKs, stress-activated protein kinases/c-Jun N-terminal kinases; RT-PCR, reverse transcription PCR; dsp38, *Fcp38* dsRNA; ANOVA, One-way analysis of variance

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2013). These results indicate that p38 MAPK pathways play a crucial role in the defense against bacterial or viral infections in mammals and lower vertebrates, as well as in invertebrates.

Mitogen activated protein kinase kinase 3 (MKK3) is a member of the p38 MAPK signaling pathway, which plays an important role in inflammatory processes (Pettus and Wurz, 2008). Various studies, both *in vitro* and *in vivo*, have demonstrated that MKK3 plays a pivotal role in the development of non-allergic inflammatory processes (Wang et al., 2002; Inoue et al., 2005, 2006). MKK4 belongs to a network of MAPK pathways, and is involved in various physiological and pathophysiological processes (Ganiatsas et al., 1998). In some cases MKK4 is also the upstream activator of p38 (Kang et al., 2006). Activating transcription factor 2 (ATF-2) is a member of the ATF/cAMP response element binding family of transcription factors, and its transcriptional activation requires phosphorylation, which was mediated by p38, JNK and ERK to enhance its trans-activating capacity in response to various stresses including inflammation, reactive oxygen species and hypoxia (Bhoumik et al., 2007; Vlahopoulos et al., 2008).

Fenneropenaeus chinensis (*F. chinensis*), is one of the most commercially important farmed aquatic species in China. However, frequent outbreaks of viral and bacterial diseases have posed a significant challenge to the shrimp culture industry, resulting in drastically decreased production and catastrophic economic losses (Hikima et al., 2003; Liu et al., 2005).Compared with mammals and insects, information on the molecular mechanisms involved in the p38 MAPK signaling pathway in response to viral and bacterial infections in *F. chinensis* is limited. This study explored the potential role of the p38 MAPK signaling pathway in *F. chinensis* immunity by investigating the expression of these four genes in the hemocytes and gills of shrimp after infection with white spot syndrome virus (WSSV), *Vibrio anguillarum* (*V. anguillarum*) and *Staphylococcus aureus* (*S. aureus*). The results may provide important information on *FcMKK3*, *FcMKK4*, *Fcp38* and *FcATF-2* defense against viral and bacterial infections and will improve our understanding of the role of p38 MAPK signaling pathway in the innate immunity response of *F. chinensis*.

2. Materials and methods

2.1. Culture of F. chinensis

Healthy *F. chinensis* with an average weight of 1.33 ± 0.22 g and an average length of 40.17 ± 3.59 mm were collected from a commercial farm in Changyi city, China. They were cultured in oxygenated seawater at a salinity of 31, a pH of 7.6–8.2 and a temperature of 24 ± 1.0 °C for 2 weeks to acclimatize to the laboratory conditions. Thirty prawns were placed in tank (200 L). They were fed twice a day with commercial feed at 10% body weight, and two-thirds of the seawater was exchanged daily throughout the experimental period.

2.2. Experimental design

Experiments were divided into the WSSV challenged group, the *V. anguillarum* challenged group, the *S. aureus* group and the control group. Each group was subjected to three parallel tests, and thirty shrimp were included in each test. WSSV crude extract was obtained from 10 g of gill tissue from WSSV-infected *F. chinensis*. WSSV-infected shrimps were provided by the Mariculture Disease Control and Pathogenic Molecular Biology Laboratory, Yellow Sea Fisheries Research Institute (Qingdao, China), and extraction was performed according to the method of Xie et al. (2005). The final concentration of crude extract was about 2×10^8 copies/mL by means of absolute quantification. *V. anguillarum* and *S. aureus* strains were obtained from the Germplasm Resources and Genetic Breeding Laboratory, Yellow Sea Fisheries Research Institute (Qingdao, China), and they were activated in Luria broth medium overnight at 37 °C. Then, the bacterial cells were pelleted at 5000 × *g* for 10 min, washed and resuspended in 1 × phosphate-buffered saline (PBS: containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.3). Bacterial suspensions were quantified by counting the microbial colony-forming units (CFU) per milliliter on Luria broth agar plates. The *V. anguillarum* suspension contained about 6.5×10^7 CFU/mL and the *S. aureus* suspension contained about 5.0×10^7 CFU/mL.

In the challenge experiment, healthy *F. chinensis* were intramuscularly injected individually at the fourth abdominal segment with 20 μL of live WSSV crude extract, 20 μL of live *V. anguillarum* solution, or 20 μL of live *S. aureus*. These concentrations could induce shrimp disease, but most of the shrimp survived and tissues were collected for study. Healthy *F. chinensis*, as the control group, were treated with 20 μL of PBS individually. Then, both the challenged and control groups were returned to the tanks containing oxygenated seawater and feed at 24 ± 1.0 °C as described previously. The waste seawater was disposed of in a way that protected against contamination of the environment.

Viruses or bacteria invade shrimp *via* the gill, and then infect the entire organism through the blood circulation. At 0, 3, 6, 12, 24, 48 and 72 h post-injection, the hemocytes and gills of six shrimps from each group were randomly sampled. Hemocytes were collected with a syringe which contained an equal volume of anticoagulant solution, and centrifuged at 800 × *g* for 10 min at 4 °C (Yang and Pan, 2013). Shrimp gills were excised and immediately ground in liquid nitrogen. There were three replicates for each sampling point from each group. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA samples were analyzed by 2.0% agarose gel electrophoresis and quantitated at 260 nm, all OD_{260}/OD_{280} readings were between 1.9 and 2.1. Then RNA (5 μg) was reverse transcribed into cDNA using the PrimeScript™ real time PCR kit (TaKaRa, Tokyo, Japan) for real-time quantitative RT-PCR analysis.

2.3. Expression of FcMKK3, FcMKK4, Fcp38 and FcATF-2 after WSSV, V. anguillarum and S. aureus challenge

Real-time quantitative RT-PCR assays were performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) to investigate the expression profiles of *FcMKK3*, *FcMKK4*, *Fcp38* and *FcATF-2*. To analyze the mRNA expression level of *FcMKK3*, *FcMKK4*, *Fcp38* and *FcATF-2*, specific primers were designed according to full-length cDNA sequences obtained from our previous experiments (Yao et al., 2015, 2016a, 2016b) for RT-PCR (Table 1). The β-actin gene of *F. chinensis* was used as the internal reference in RT-PCR, and two primers β-actin-F and β-actin-R (Table 1) were used to amplify a product of 240 bp to verify successful reverse transcription and to calibrate the cDNA template(Liu et al., 2009; Song et al., 2010; Qiao et al., 2015).

RT-PCR was carried out in a total volume of 10 μL, containing 5 μL

Table 1
Primer sequences used in this study.

Primer name	Sequence (5′-3′)	product size of RT-PCR (bp)	GenBank No.
MKK3-F	ACCGTGATGTGAAACCATCC	209	KF994775
MKK3-R	CGATATGCCAAGTGACCACA		
MKK4-F	ACCGTCCACAGAACAACCTT	220	KJ023198
MKK4-R	AAACCTCCTCTTCCAATCTCC		
p38-F	AGGCTCGCAACTACATACGC	214	KF991368
P38-R	CTGGTCATAGGGCTCACTGTC		
ATF-2-F	GGCACCACTACGGAAATCTG	236	KF991367
ATF-2-R	GGCAGTCGCAATAAATGAGC		
β-actin-F	AGTAGCCGCCCTGGTTGTAGA	240	DQ20546.1
β-actin-R	TTCTCCATGTGGTCCCAGT		
dsRNA-F	GGUACCACACGAUAGAGUUTT		
dsRNA-R	AACUCUAUCGUGUGGUACCTT		