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Two novel p38 MAPKs identified from *Epinephelus coioides* and their expression pattern in response to *Cryptocaryon irritans* infection

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ABSTRACT

P38 mitogen-activated protein kinases (MAPKs) are one of the most important central regulatory proteins response to extra environmental stresses. In this study, two novel p38 MAPKs, Ec-P38γ and Ec-P38δ, were identified from *Epinephelus coioides*, an economically important cultured fish in China and Southeast Asian counties. Both of Ec-p38γ and Ec-p38δ sequences contain a serine/threonine protein kinase (S_TKc) domain and a highly conserved Thr-Gly-Tyr (TGY) motif. Analysis of phylogenetic relationships illustrated that p38 amino acid sequences were conserved between different species indicating that the functions may be similar. The four subtypes of p38 (α, β, γ, and δ) mRNA can be detected in all thirteen tissues examined, but the expression level is different in these tissues. The expression patterns of the four Ec-p38 subtypes in *E. coioides* were also detected response to *Cryptocaryon irritans* infection, one of the most important protozoan pathogens of marine fish. The expression of four p38 subtypes was up-regulated in the tissues examined, with the highest expressions of Ec-p38α (5.2 times) and Ec-p38δ (4.2 times) occurring in the skin, while Ec-p38β (24.8 times) and γ (16.6 times) occurred in the spleen. There was no significantly correlation between the expression of Ec-p38γ/Ec-p38δ and the expression of nuclear factor kappaB (NF-*k*B). The results indicated the sequences and the characters of Ec-p38γ and Ec-p38δ were conserved, the p38 subtypes showed tissue-specific expression patterns in healthy grouper, and their expressions were significantly up-regulated post *C. irritans* infection, suggesting these p38 MAPKs may play important roles in these tissues during pathogen-caused inflammation.

1. Introduction

The p38 mitogen-activated protein kinases (MAPKs) are of the MAPKs superfamily and play important roles in many processes including inflammation, wound healing, cell growth, cell differentiation, cell-cycle arrest, senescence, cytokine production, regulation of RNA splicing and apoptosis etc [1–3]. The name MAPK was originally given because an extracellular signal-regulated kinase (ERK), a MAPK family member, which was first recovered as a kinase activity in the cytosol of EGF treated cells; later, MAPKs were divided into four main categories: the extracellular signal-regulated protein kinase (ERK), Big Mitogen Activated Protein Kinase 1(BMK1/ERK5), c-Jun N-terminal kinase stress-activated protein kinases (JNK/SAPK) and p38 MAPK [4]. P38 MAPK is a family of 38 kDa kinase, and each member of the p38 family possess the same conserved domains: a serine/threonine protein kinase (S_TKc) domain and a highly conserved Thr-Gly-Tyr (TGY) motif in the activation loop [5,6]. P38 MAPK is one of the most important central regulatory proteins response to extra environmental stresses and is involved in intracellular signaling pathways that control both the production of pro-inflammatory cytokines and cellular activation [7–9], and phosphorylation of p38 activates a

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wide range of substrates that include transcription factors, protein kinases and cytosolic and nuclear proteins [10].

In mammalian, four subtypes of p38 MAPK, p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12 or ERK6), and p38δ (MAPK13 or SAPK4) have been identified, and these MAPKs share approximately 60% sequence homology of their amino acids, but differ in their expression pattern, substrate specificities, and sensitivities to chemical inhibitors [11–15]. Both p38α and p38β are ubiquitously expressed, whereas the remaining p38 subtypes are expressed in a more tissue-specific manner [11,16]. In mammals, the p38 MAPKs can activate nuclear factor kappaB (NF-κB), a nuclear transcription factor that resides in its inactive state in the cytoplasm [17,18]. In fish, several p38 subtypes have been identified, and their expression patterns in *Epinephelus coioides*, *Atlantic salmon* (*Salmo salar*), zebrafish (*Danio revio*), grass carp (*Ctenopharyngodon idella*), salmon, and rock bream (*Oplegnathus fasciatus*) was explored post virus/bacterial infections [19–24]. However, gene characters and expression patterns of p38γ and p38δ in *E. coioides* remained unknown.

Grouper *E. coioides* is an economically important cultured fish in China and Southeast Asian counties, one of the most important protozoan pathogens of marine fish [25–27]. *Cryptocaryon irritans* is capable of killing large numbers of fish in a short time and has a serious impact on aquaculture, and it is also believed to be a good model pathogen to study fish immunity [28,29]. This information provided a basis for the present study, the aim of which was to characterize the subtypes of p38γ and p38δ from *E. coioides*, study their distribution in the tissues and expression patterns response to parasitic protozoan *C. irritans* infection, and investigate the function of p38 MAPKs in the activation of NF-*k*B using luciferase reporter assays.

2. Materials and methods

2.1. Experimental fish and sampling

Healthy grouper *E. coioides* (weighing 12.3 ± 3.3 g) from the Marine Fisheries Development Center of Guangdong Province, China were maintained at 25 °C in a flow-through water system and fed daily with commercial grouper feed for two weeks before experimentation.

A total of 80 grouper were exposed to *C. irritans* at a dose of 4800 theronts per fish in 150 L of continuously circulated seawater as previously described [30,31], and *C. irritans* was maintained by serial passage on *Traohinotinae ovatns* in our laboratory. At day 4 the fish were transferred into a new tank to avoid re-infection. Fish of the control group were treated the same as the infected group except no *C. irritans* were added. Five fish were randomly selected at 0, 6, 12 h and 1, 2, 3, 5, and 7 d after challenge, and the gill, skill, head kidney, and spleen from each fish were collected and immediately frozen in liquid nitrogen for further analysis.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the tissues using TRIzol Reagent (Invitrogen, Canada), and digested with DNase I (Promega, USA). The concentration was measured spectrophotometrically, and RNA integrity was conformed by 1% agarose gel electrophoresis. The first-strand cDNA was synthesized using ReverTra Ace-α reverse transcriptase following the manufacturer's instruction (Toyobo, Japan) and was used as a template in subsequent polymerase chain reaction (PCR) (TakaRa, Japan) and real-time PCR (TakaRa).

2.3. Identification of grouper P38γ and P38δ MAPK sequences

The transcriptome data of grouper skin, gill, spleen and head kidney post *C. irritans* infection was obtained in our lab (data unpublished). According to the transcriptome data and the tBLAST information, the complete open reading frames (ORF) of p38γ and partial p38δ genes were obtained. Various primers were designed to amplify the 5′ and 3′ unknown regions of p38 δ using the SMARTer™RACE cDNA Amplification Kit (Clontech, USA). The names and sequences of the primers were listed in Table 1. In brief, the first-round PCR was performed with the primers UPM/p38δ-GSP1 or UPM/p38δ-GSP2, and the second-round PCR with the primers NUP/p38δ-NGSP1 or NUP/p38δ-NGSP2, respectively. Full length grouper p38δ gene sequence was obtained by splicing together the three sequences isolated above. The primers, p38δ-F/p38δ-R and p38γ-F/p38γ-R were then designed to amplify the ORFs of grouper p38δ and p38γ genes, respectively. The PCR cycling conditions were as follows: 35 cycles of 98 °C for 10s, 60 °C for 15s, and 72 °C for 2 min, followed by 72 °C for 10 min. All PCR products were cloned into the pEASY®-Blunt Simple Cloning vector (TransGen Biotech, China), and sequenced by the Life Technologies Corporation, Guangdong, China.

2.4. Bioinformatic analysis

The ORFs were identified using the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A multiple-sequence alignment was constructed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The protein structure was analyzed with the SMART program (http://smart.embl-heidelberg.de/). The isoelectric point (pI) and molecular weight (Mw) were computed with an online program (http://web.expasy.org/compute_pi/). The *N*-glycosylation sites were predicted with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). A phylogenic tree was constructed with the MEGA 7.0 program.

2.5. Expression analysis

Real-time PCR was used to determine the tissue specific expression profiles of grouper p38 (α, β, γ, and δ) subtypes in both healthy and *C. irritans* infected fish. The primers, qp38α-F/qp38α-R, qp38β-F/qp38β-R, qp38γ-F/qp38γ-R, qp38δ-F/qp38δ-R, β-actin-F/

Table 1
Primers used in this study.

Primer	Sequence (5 ′ to 3')
p38δ-GSP1	CCAGTTCAGAATGACCTCAGGCGC
p38δ-NGSP1	AGGCAGGCGAAAACACATCAAGAAG
p38δ-GSP2	ACTGACGCTGAGATGACAGGCTACG
p38δ-NGSP2	CGCCTGAGGTCATTCTGAACTGGAT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGG TATCAACGCAGAGT
NUP	AAGCAGTGGTATCAACGCAGAGT
p38 γ-F	ACCACAGCCTGTAGTCGGTAGGG
p38 γ-R	ATCATATTTTTGGGGTGCTGGTGTC
p38δ-F	CAGGAGGAGGGAGTAGAACTTTTTA
p38δ-R	GTAGGGCGGGGTGTCAGTAAT
qp38α-F	CCTCAACAACATCGTCAAGTG
qp38α-R	GGCTTCAAGTCTCTGTGGAT
qp38β-F	TCTCTCCTCCCCCATCCTCCG
qp38β-R	GCCACATACCCTGTCATCTCGTCAT
qp38γ-F	GCACTCTGTCTTCTCCAA
qp38γ-R	CTCGGCATCTCTGAACTC
qp38δ-F	CTTCTTGATGTGTTTTCGCCTGC
qp38δ-R	CCTTGTGAATATACCTGAGTCCGC
β-actin-F	TGCTGTCCCTGTATGCCTCT
β-actin-R	CCTTGATGTCACGCACGAT