Fish & Shellfish Immunology 44 (2015) 410–419

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Identification and expression profiles of ERK2 and ERK5 in large yellow croaker (*Larimichthys crocea*) after temperature stress and immune challenge

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ARTICLE INFO

Article history:
Received 23 January 2015
Received in revised form
3 March 2015
Accepted 4 March 2015
Available online 12 March 2015

Keywords:
ERK2
ERK5
Large yellow croaker
Subcellular localization
Temperature stress
Immune challenge

ABSTRACT

Fish is highly affected by many environmental stresses such as temperature and invasive infection. The extracellular signal-regulated kinase (ERK) pathway, part of the mitogen-activated protein kinase (MAPK) family, is found to act as crucial mediators for cell differentiation, proliferation and cell response to various stresses. In the present study, ERK2 (*Lc*ERK2) and ERK5 (*Lc*ERK2) were cloned and characterized from large yellow croaker, *Larimichthys crocea*. The full length cDNA sequence of *Lc*ERK2 was of 1910 bp, including an ORF of 1110 bp encoding a polypeptide of 369 amino acids. The full length cDNA sequence of *Lc*ERK5 was of 3720 bp, including an ORF of 3375 bp encoding a polypeptide of 1124 amino acids. Multiple alignments showed that both *Lc*ERK2 and *Lc*ERK5 contained highly conserved TEY motif and S_TKc domain in MAPK family and the unique catalytic and active structures of ERK2 and ERK5. Subcellular localization revealed that both *Lc*ERK2 and LcERK5 expressed in the cytoplasm and cell nucleus. The expression of *Lc*ERK2 and *Lc*ERK5 were detected in most tissues of large yellow croaker, with the most predominant expression of *Lc*ERK2 in brain and *Lc*ERK5 in gill, and the weakest expression of *Lc*ERK2 in liver and *Lc*ERK5 in intestine, respectively. The expression levels of *Lc*ERK2 and *Lc*ERK5 after temperature stress and poly I:C and flagellin challenge were investigated in LCK (*L. crocea* kidney) cells. After temperature stress, significant down-regulations of *Lc*ERK2 transcripts were detected after 10 °C stress ($p < 0.05$) whereas *Lc*ERK2 transcripts increased significantly after 35 °C stress ($p < 0.05$). However, significant down-regulations of *Lc*ERK5 expression were detected at most time points after both cold and heat stress ($p < 0.05$). However, significant up-regulations of *Lc*ERK2 and *Lc*ERK5 transcripts were found after immune challenge ($p < 0.05$). Our results showed that *Lc*ERK2 transcripts enhanced after heat stress and both *Lc*ERK2 and *Lc*ERK5 transcripts could be induced by immune challenge. These findings indicated that *Lc*ERK2 might be more important in fish response to high temperature stress and both *Lc*ERK2 and *Lc*ERK5 might play an important role in fish immune response.

1. Introduction

The mitogen-activated protein kinase (MAPK) superfamily contains three main protein kinase families: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 family of kinases [1,2], which plays crucial roles in the regulation of intracellular metabolism and gene expression in many biological events including immune response, apoptosis, various environmental stress, growth and development [1]. Among which, the ERK subfamily responds mainly to growth factors, mitogens, and many environmental stresses [3,4].

Till to the present study, six members have been verified in mammalian ERK subfamily, including ERK1, ERK2 (also named as MAPK1), ERK3/4, ERK5 (also named as MAPK7), ERK6 (also known as p38γ) and ERK7/8 [5,6]. ERK2 is one of the best-studied factors of the group [7] and contains highly conserved serine/threonine kinases (S_TK) domain, which is activated via phosphorylation on both threonine and tyrosine residues within the conservative TEY sequence in their activation loop by mitogen-activated protein kinase/ERK kinase (MEK) 1/2 [8]. Similar to ERK2, ERK5 also possesses a conserved TEY phosphorylation loop and S_TK catalytic (S_TKc) domain performing catalytic function, but the ERK5 encodes more amino acids than the ERK2. In addition, ERK5 is

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http://dx.doi.org/10.1016/j.fsi.2015.03.006
1050-4648/

activated by MEK5 which can not activate the ERK2 [9–11]. Although both ERK2 and ERK5 are conserved from mammals to fish, their expression patterns are varied in different species [12].

Previous studies demonstrated that both ERK2 and ERK5 could be activated by many stimuli and environmental stresses, involving in cell death, immune responses or many other physiological responses [9,13–15]. It was demonstrated that the induction of Hsp70 in response to heat stress in zebrafish (*Danio rerio*) depended on ERK activation [4]. The ERK could be activated by high temperature stress in fruit fly (*Drosophila melanogaster*) [16]. Also, in HeLa cells, the ERK2 transcripts could be induced by heat stress and the ERK5 could be activated in rat cardiomyocytes after cold stress [17,18], suggesting that both ERK2 and ERK5 might be crucial in animals' response to temperature and other environmental stresses.

In addition, ERK expression levels could be induced by immune challenge or pathogenic infection. For example, Huang and colleagues reported that ERK was essential for defense against large mouth bass virus (LMBV) infection in EPC cells [19]. The expression levels of ERK were also induced after virus injection in shrimp, *Litopenaeus vannamei* and *Fenneropeaeus chinensis* [20,21]. After challenged with bacterial LPS and flagellin, ERK1/2 increased significantly in the peripheral blood heterophils of chicken [22]. However, another investigation demonstrated that hepatitis C virus non-enveloped (HCVne) capsid-like particles were capable of inducing ERK5 pathway in a dose-dependent way [23], suggesting that ERK signaling pathway might be important in immune response and the roles of ERK2 and ERK5 might be different. However, in fish species, ERK2 and ERK5 were only reported in zebrafish. The molecular characterizations and more physiological response characterizations of ERK2 and ERK5 are poorly understood.

Large yellow croaker, *Larimichthys crocea*, is one of the economically important maricultured fish in East China Sea with the optimum sea water temperature of 18–25 °C. Recently, it has suffered from serious disease caused by viral, bacterial and many other pathogenic infections, such as iridovirus and pathogenic vibrio genus [24,25], resulting in huge economic losses, which shows a close relationship with sea water temperature. Therefore, the detailed characterizations of ERK2 and ERK5 in fish and their roles in fish response to temperature stress and pathogenic infection need to be better investigation.

In the present study, *Lc*ERK2 and *Lc*ERK5 were cloned and characterized from large yellow croaker, *L. crocea*. The subcellular localizations of them were investigated in large yellow croaker kidney cell line (LCK). Tissue expression profiles of both *Lc*ERK2 and *Lc*ERK5 were detected in non-stimulated large yellow croaker and the expression characterizations of *Lc*ERK2 and *Lc*ERK5 in LCK cells after thermal stress and immune challenge were described, which will be helpful for better understanding the roles of ERKs in large yellow croaker's physiological response.

2. Materials and methods

2.1. Fish collection and RNA extraction

Healthy juvenile large yellow croaker (total length 16 ± 1.5 cm, weighting 81 ± 25 g) were collected at the Experimental Fish Farm of Ningde Centre of Popularization of Fisheries Technology, Fujian Province, China. Before the experiments, fish was acclimated for at least 1 week in 4 m^3 tanks in salinity (28 psu), temperature (25 °C) and density conditions similar to those of the culture net cages from which the specimens were obtained. Blood was collected from eugenol anaesthetized fish by cutting the tail and the blood cells were separated in two times the volume of anticoagulant solution (0.48% citric acid, 1.32% sodium citrate and 1.47% glucose) by centrifugation at 800 g for 5 min at 4 °C and were stored in RNA fixer (Bioteke, Beijing) immediately as previously described [26,27]. Kidney, liver, spleen, head-kidney, stomach, intestine, brain, skin, gill, heart, muscle and blood were dissected out and stored in RNA fixer at −20 °C for RNA extraction. Each tissue was obtained from six independent juvenile large yellow croakers.

Total RNA was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA was incubated with RNase-free DNase I (Promega, USA) to remove any contaminating genomic DNA. First strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Fermentas, China), following the manufacturer's protocol with Oligo d (T) 18 primer.

2.2. Cloning and sequencing of LcERK2 and LcERK5 cDNA

Based on the partial cDNA sequences of ERK2 and ERK5 from RNAseq (RNA sequence) database of large yellow croaker, the 3′ and 5′ end were obtained by rapid amplification of cDNA ends (RACE) approaches with brain cDNA as template (Table 1). The 3′end PCR of *Lc*ERK2 gene was performed using the gene specific primers ERK2-3′-F1/F2 and adapter primers AOLP/AP (Table 1) by nest PCR at annealing temperature of 52 °C and 54 °C, respectively. To clone the 5′ ends of *Lc*ERK2 gene, the spleen mRNA were transcribed by M-MLV reverse transcriptase using gene specific primer ERK2-5′-R2 (Table 1), followed by purifying the cDNA with a DNA purification kit (Omega, Bio-Tek) and tailed with poly(C) according to the manufacture's protocol. The first round PCR was performed at annealing temperature of 54 °C with primer ERK2-5′-R1 and adapter primer AAP using the tailed spleen cDNA as template, and then nested PCR with a specific primer ERK2-5′-R2 and adapter primer AP were used with annealing temperature at 53 °C. The ORF sequence of *Lc*ERK2 was verified using primer ERK2-ORF-F1/F2 and ERK2-ORF-R1/R2 at annealing temperature of 50 °C. PCR products were purified with Gel Extraction Kit (Omega, Bio-Tek). The purified PCR product was inserted into PMD19-T vector and transformed into the competent *Escherichia coli* TOP10 cells. Positive clones containing the expected-size inserts were screened by colony PCR and then sequenced by Sangon Corp (Shanghai, China).

Table 1
Primers used for *Lc*ERK2 and *Lc*ERK5 gene cloning and expression analysis.

Primers	Sequence (5′–3′)	Tm (°C)
ERK2-3′-F1	GAGGATTGAGGTGGAGG	54.6
ERK2-3′-F2	GGATGACCTACCCAAAGAG	56.1
ERK2-5′-R1	CTGGTGCTCAAATGGACTG	56.1
ERK2-5′-R2	CGATGTAGGAGAGGTTGC	55.4
ERK2-ORF-F1	ACAGCGACGACGGATAT	52.2
ERK2-ORF-F2	ATGTCAACTAAGGAGAGTG	46.9
ERK2-ORF-R1	AGTGGCAAACGCAGAAGC	55.4
ERK2-ORF-R2	TCAGGCATCAGGGGGAATG	47.2
ERK5-3′-F1	GTTCTCAGTCACCCCTAAAG	56.8
ERK5-3′-F2	CTGATTGGAGCGAGGTTC	55.4
ERK5-5′-R1	TGAGGAAGGTTGGGCTGT	55.4
ERK5-5′-R2	GGCATTTGTCACCACTTC	53.2
ERK5-ORF-F1	ACACAACTACGGATGTCG	53.2
ERK5-ORF-F2	ATGTCAACTAAGGAGAGTG	46.9
ERK5-ORF-R1	ACTTGAGGTTTCCATGCT	51.0
ERK5-ORF-R2	TCAGGCATCAGGGGGAATG	47.6
ERK2-L-F	CCGCTCGAGGCCACCATGTTAAGACCT	68.9
ERK2-L-R2	CGGAATTCATAATGGCGACAGCTGC	64.1
ERK5-L-F	CCCAGCGCTGCCACCTATGTCAACTAAGG	70.4
ERK5-L-R	CGGGATCCGAGGCATCAGGGGGAATGG	72.0
ERK2-RT-F	GAAGGTGCCTACGGGATGG	60.4
ERK2-RT-R	GGTCAATGGTTGGTGTGCGG	60.8
ERK5-RT-F	CCGTGACCTCAAACCCTCC	60.4
ERK5-RT-R	TGCACCCCACAGACCACAAG	60.8
β-actin-RT-F	TTATGAAGGCTATGCCCTGCC	63.0
β-actin-RT-R	TGAAGGAGTAGCCACGCTCTGT	63.0