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Molecular cloning, expression analysis and localization pattern of the MST family in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

The mammalian sterile 20-like (MST) family, which belongs to the serine/threonine protein kinase superfamily, has five members that can be found in mammals: STK3 (also called MST2), STK4 (MST1), STK24 (MST3), STK25 (YSK1 or SOK1), and STK26 (MST4). The MST kinases have key roles in apoptosis, immune regulation, inflammatory responses, cancer, and cell proliferation in mammals, whereas the roles and transcriptional regulatory mechanism of these kinases in teleost fish are still unclear. In this study, four *STK* genes (*CiSTK3*, *CiSTK24*, *CiSTK25*, and *CiSTK26*) were cloned and analyzed in grass carp (*Ctenopharyngodon idella*). All four *STK* genes were broadly expressed in the examined tissues, while their relative expression levels differed. In addition, after exposure to the grass carp reovirus, mRNA expression levels of the four *STK* genes were altered to different levels in the immune organs, and the levels were dramatically altered in the blood. Subcellular localization indicated that all four STK proteins were localized in the cytoplasm of transfected cells. Moreover, bimolecular fluorescence complementation analysis revealed that mouse protein-25 could interact with CiSTK3, CiSTK24, CiSTK25, and CiSTK26 independently in grass carp. Thus, our findings provide new insights for understanding the functions of the MST family in teleosts.

1. Introduction

The aquaculture industry has become a major power that promotes sustainable, rapid, and stable development of fisheries in China [1]. The grass carp (*Ctenopharyngodon idella*) is an economically important aquaculture species that accounts for 18.3% of the freshwater aquaculture production in China [2]. However, outbreaks of hemorrhagic disease caused by grass carp reovirus (GCRV) seriously restrict the development of grass carp farming [3]. GCRV, which belongs to the family *Reoviridae* and genus *Aquareovirus* (non-enveloped, double-stranded RNA), can induce apoptosis in fish cell lines, including *C. idella* kidney (CIK) cells [4,5]. Currently, the mechanism underlying hemorrhagic disease is still largely unclear. Therefore, understanding the mechanisms underlying the immune response and apoptosis induced by GCRV is important for defense against GCRV.

The Sterile 20-like (STE20) superfamily, named after the yeast sterile 20 kinase, includes several subfamilies, of which only one is named the “mammalian sterile 20-like” (MST) family in humans [6]. There are five MST kinases, STK3 (also called MST2), STK4 (MST1), STK24 (MST3), STK25 (YSK1 or SOK1), and STK26 (MST4), that could be broadly divided into two subgroups: (1) STK3 and STK4; (2) STK24, STK25, and STK26 [7]. The MST kinases have key roles in apoptosis, immune regulation, inflammatory responses, cancer, and cell proliferation in mammals [8–10].

Apoptosis is a component of innate immune mechanisms and plays a pivotal role in antiviral processes [11–13]. During apoptosis, STK3 is cleaved and undergoes irreversible autophosphorylation, which was resistant to phosphatase [14]. Caspase-mediated cleavage of STK4 results in the removal of the C-terminal inhibitory domain and subsequent translocation of the constitutively active catalytic fragment to the nucleus [15,16]. Hydrogen peroxide-induced apoptosis of trophoblast cells was suppressed by overexpression of kinase-dead STK24 or knockdown of endogenous STK24 with siRNA [17]. STK25 has been demonstrated to regulate cell death after chemical anoxia, as its downregulation by RNA interference enhances cell survival [18]. Overexpression of STK26 further enhances the transformation of Phoenix cells, as demonstrated by growth in soft agar [19].

Previous studies have shown that STK proteins are participants of

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both adaptive and innate immune regulation [10]. STK3/4 may control the activation of Rho GTPase and egress of mature T cells from the thymus by activating Dock8 [20]. In STK3 and STK4 double-knockout mice, mature T cells could not efficiently migrate from the thymus to the circulatory and secondary lymphoid organs. Animal studies have indicated that STK4 plays a critical role in lymphocyte chemotaxis and thymocyte emigration [21]. STK4 is involved in the regulation of immune cell polarity and adhesion through the Rap1–RapL signaling pathway via its direct interaction with RapL or RIAM [22,23]. Furthermore, STK4 controls lymphocyte trafficking and interstitial migration, which are important for efficient immunosurveillance and effective immune responses [24]. Previous studies have shown STK24/25/26 are linked to TNF-induced NF-κB signaling [25]. STK26 has been identified as a regulator that limits TLR-related inflammatory responses through TRAF6 and dynamically responds to LPS treatment [26].

In our study, four *STK* genes from the grass carp, named *CiSTK3*, *CiSTK24*, *CiSTK25*, and *CiSTK26*, were cloned and analyzed. The expression profiles of the *CiSTK* genes in different tissues and their response to GCRV infection were examined *in vivo*. Additionally, the subcellular location patterns of CiSTK3, CiSTK24, CiSTK25, and CiSTK26 were investigated. Moreover, mouse protein-25 (MO25) was detected to interact with CiSTK3, CiSTK24, CiSTK25, and CiSTK26 independently. This study may provide new insights for understanding the functions of MST family in teleosts.

2. Materials and methods

2.1. Fish, GCRV challenge, and sample collection

A total of 50 four-month-old healthy grass carps (weight, about 10 g; average length, 7 cm) were obtained from the GuanQiao Experimental Station, Institute of Hydrobiology, Chinese Academic of Sciences, and acclimatized in aerated freshwater at 28 °C for one week before the infection experiment. The fish were fed twice a day with a commercial feed. After no abnormal symptoms were observed, the grass carps were used for further experiments. GCRV used in the experiment was obtained as follows. Dead fish with typical symptoms of hemorrhagic diseases were collected and homogenized together with an equal volume of 0.75% saline. The mixture was centrifuged, and then, the supernatant was filtered through a 0.22-lm Millex filter (Millipore, USA). For the viral challenge experiment, the grass carps were intraperitoneally injected with 200 μl of GCRV. All the injected fish were fed with the commercial feed twice a day and monitored carefully every day. The temperature was maintained at 28 °C throughout the experiment.

Three uninfected fish were selected, and samples of the blood, gill, spleen, liver, intestine, middle kidney, head kidney, heart, muscle, skin, and brain were obtained. The RNA was obtained from these tissues for full-length cDNA cloning. In addition, blood, intestine, kidney, head kidney, and liver were sampled from three infected fish at different days after GCRV infection (1, 2, 3, 4, 5, and 6 days). The RNA was obtained from these tissues to analyze the response of *CiSTK3*, *CiSTK24*, *CiSTK25*, *CiSTK26*, and apoptosis-related genes after GCRV infection. All the samples were homogenized in TRIzol reagent (Invitrogen, USA) and stored at −80 °C before RNA extraction.

2.2. Cloning the full-length cDNAs of CiSTK3, CiSTK24, CiSTK25, and CiSTK26

The total RNA was extracted from the samples of healthy grass carps by using TRIzol reagent, according to the manufacturer's instructions. Then, first-strand cDNA synthesis was performed using DNase I (Promega, USA) with total RNA as the template and random nonamer primers as the control for reverse transcriptase (Toyobo, Japan). Incomplete but specific fragments of *CiSTK3*, *CiSTK24*, *CiSTK25*, and *CiSTK26* were obtained by blasting the sequences of the zebrafish (*Danio rerio*) *STK* genes with draft genome of grass carp with [27]. The 5′ Full RACE Kit and 3′ Full RACE Kit (TaKaRa, Japan) were used to obtain the 5′ and 3′ untranslated regions (UTRs) of the *CiSTK* genes. The primers for RACE were listed in Table 1. The full-length cDNA sequences were amplified using PCR with primers (Table 1) within the 5′- and 3′-UTRs. The PCR products were purified, ligated into pMD18-T vectors (TaKaRa, Japan), and transformed into competent *Escherichia coli* DH5α cells (TransGen, China). Ten positive colonies were random selected and sequenced by a commercial company (TsingKe, China).

Table 1
Primers for full-length cDNA cloning.

Primers	Sequences (5'—3')	Purpose
3 CiSTK3-F	AGGACTCAAACAAAGCACAA	CiSTK3 3' RACE
5 CiSTK3-R	CTTCCTCGGTGAGCGTTTTG	CiSTK3 5' RACE
3 CiSTK24-F	GAAGAGGAGCCCAATAAGAG	CiSTK24 3' RACE
5 CiSTK24-R	CCGGGCTCCAGCAAATCTAG	CiSTK24 5' RACE
3 CiSTK25-F	GGAGAATGCCTTCAACCTGG	CiSTK25 3' RACE
5 CiSTK25-R	CTGAGCACCGTGATCTCCTG	CiSTK25 5' RACE
3 CiSTK26-F	GTCAGCCAGTTTAACCACCG	CiSTK26 3' RACE
5 CiSTK26-R	GGATGTCTTCGATTTCGTCT	CiSTK26 5' RACE
CiSTK3-F	ATGGAGCATTCGGTGCCC	CiSTK3 cDNA cloning
CiSTK3-R	TTAGAAGTTCTGCTGCCG	
CiSTK24-F	ATGGCTCATTCTCCAGTGCAG	CiSTK24 cDNA cloning
CiSTK24-R	TCACTGGTGCGATGAGGATG	
CiSTK25-F	ATGGCGCACCTCCGCGAC	CiSTK25 cDNA cloning
CiSTK25-R	TCACCGTGTTGATGGCGTG	
CiSTK26-F	ATGGCTCAGTCGCCGGTAGC	CiSTK26 cDNA cloning
CiSTK26-R	CTAACTCACAGAGAACCTCTG	

2.3. Sequence analysis

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for the gene sequences in other species. The amino acid sequences of CiSTK3, CiSTK24, CiSTK25, and CiSTK26 were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), and multiple sequence alignments were performed using ClustalW 2.1 (http://www.ebi.ac.uk/tools/clustalw2.1). SMART (http://smart.embl-heidelberg.de/) was used to predict the protein domain features. Neighbor-joining (NJ) phylogenic trees were constructed on the basis of the amino acid sequences by using MEGA 7.0 software (http://www.megasoftware.net/index.html), and Evolview (http://www.evolgenius.info/evolview/) was used to mark the trees.

2.4. Expression levels of CiSTK3, CiSTK24, CiSTK25, and CiSTK26 in different tissues

Eleven tissues (blood, gill, spleen, liver, intestine, middle kidney, head kidney, heart, muscle, skin, and brain) were isolated from three healthy grass carps, and the total RNA was extracted and then reverse-transcribed to obtain cDNA. The relative mRNA expression levels of *CiSTK3*, *CiSTK24*, *CiSTK25*, and *CiSTK26* in the different tissues were examined using qRT-PCR and the CFX96^{TM} real-time PCR detection system (Bio-Rad, USA). The housekeeping gene *β-actin* was used as the reference gene. Relative expression levels were calculated as the ratio of gene expression in each tissue to that in the skin. The specific qRT-PCR primers for *β-actin*, *CiSTK3*, *CiSTK24*, *CiSTK25*, and *CiSTK26* were listed in Table 2. The relative expression levels of *CiSTK3*, *CiSTK24*, *CiSTK25*, and *CiSTK26* were calculated using the $2^{-\triangle\triangle Ct}$ method [28]. All data were expressed as mean ± standard deviation (SD) values of three replicates.

2.5. Expression analysis of CiSTK3, CiSTK24, CiSTK25, CiSTK26 and apoptosis-related genes after GCRV infection

Total RNA was extracted from five tissues (blood, intestine, kidney,