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Short communication

Mitogen-activated protein kinase kinase 6 is involved in the immune response to bacterial di-/tripeptide challenge in grass carp *Ctenopharyngodon idella*

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ABSTRACT

Mitogen-activated protein kinase kinase 6 (MKK6) is an essential component of the p38MAPK signaling pathway, which is involved in the modulation of inflammation, cell apoptosis and survival responses in mammals. However, the function of MKK6s in teleosts is still unclear. In this study, a fish MKK6 homolog (*Ci*MKK6) was first identified from the grass carp (*Ctenopharyngodon idella*), a freshwater fish. *Ci*MKK6 cDNA encodes a putative protein of 357 amino acids that contains conserved structural characteristics of the MKK6 family, including the S_TKc domain, SVAKT motif and DVD site. The deduced *Ci*MKK6 protein exhibits high sequence homology with other reported fish MKK6s and shares the closest relationship with MKK6 from *Danio rerio*. Quantitative real-time PCR (qRT-PCR) analysis revealed that *Ci*MKK6 mRNA was widely expressed in all tested tissues and stages of embryonic development. Additionally, the transcript levels of *Ci*MKK6 in the intestine were significantly up-regulated in response to bacterial muramyl dipeptide (MDP) and L-Ala-γ-D-Glu-meso-diaminopimelic acid (Tri-DAP) stimulation. Moreover, subcellular localization analysis indicated that *Ci*MKK6 was distributed in both the cytoplasm and the nucleus of HEK293T cells. Finally, overexpression of *Ci*MKK6 significantly enhanced the transcriptional activity of the AP-1 reporter gene in HEK293T cells. Overall, these findings may help better clarify the immune function of teleost MKK6s and provide new insight into the immune defense mechanisms of grass carp.

1. Introduction

Innate immunity is essential for multicellular organisms to detect and eliminate a wide range of microbial pathogens. The innate immune system provides a rapid and widespread host defense response via different cellular pattern recognition receptors (PRRs), which can specifically recognize conserved pathogen-associated molecular patterns (PAMPs) on various microorganisms [1,2]. Mitogen-activated protein kinases (MAPKs) are multifunctional signaling intermediates that can receive various immune signals triggered by upstream cellular PRRs and activate downstream nuclear transcription factors, such as nuclear factor-κB (NF-κB) and activation protein-1 (AP-1), to regulate the transcription of a subset of immune response genes to combat the invading infectious agents [3–5].

MAPKs are evolutionarily conserved intracellular serine/threonine kinases that exist widely from yeasts to mammals and are crucial for the regulation of stress responses, apoptosis, and host immune defense [6–8]. MAPKs are activated by a diverse range of stimuli, including inflammatory cytokines, growth factors, and oxidative stress, and their function is to transduce extracellular signals from cell surface receptors to the nucleus through the phosphorylation of target proteins [9,10]. MAPK activity has been shown to be regulated by upstream MAPK kinases (MKKs), and these, in turn, are phosphorylated and activated by specific MKK kinases (MKKKs) [6,11]. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38MAPK and c-Jun NH_2-terminal kinase (JNK) [9,12]. Among these, p38MAPK has been found to play important roles in immune responses, from the initiation phase of innate immunity to the activation of adaptive

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immunity [13–15].

MAPK kinase 6 (MKK6), also called MEK6 or MAP2K6, is an essential component of p38MAPK signaling pathway, that are reported to be involved in the regulation of inflammation and apoptosis during immune challenges [16–18]. Like other MKK family members, MKK6 is a dual-specificity protein that can be phosphorylated and activated by upstream MKKKs at the Ser-X-X-X-Thr motif (X is any amino acid). Activation of MKK6 results in phosphorylation of p38MAPKs through the dual phosphorylation Thr-Gly-Tyr (TGY) motif in activation loop and ultimately regulates the expression of numerous specific target genes [19]. Considering its crucial roles in immunity, MKK6 has been extensively studied in several aquatic animals up to date [20–23]. For example, shrimp *Lv*MKK6 was characterized from *Litopenaeus vannamei* and proved to be involved in regulating expression of antimicrobial peptides and host defense against *Vibrio parahaemolyticus* as well as white spot syndrome virus (WSSV) infection [21]. Wang et al. reported that a sea cucumbe MKK3/6 gene was proved to be involved in defense response to *Vibrio splendidus* infection *in vivo* [22]. In bony fish, three Atlantic salmon MKK6 orthologs were identified and shown to be upstream activators of salmon p38 which could be involved in cell response to stress stimuli [24]. Recently, several other fish MKK6 genes from *D. rerio* (NP_001299799.1), *Takifugu rubripes* (XP_003977287.2) and *Oryzias latipes* (XP_023805137.1) were reported in National Center for Biotechnology Information (NCBI) database. However, compared to studies in mammals, these reports have provided limited information of MKK6s function in aquatic animals.

Grass carp (*Ctenopharyngodon idella*) is one of the most highly produced and economically important freshwater fish species in China, but they often encounter numerous types of pathogens, which cause tremendous economic losses to aquaculture [25,26]. Investigation into the function of *C. idella* MKK6, a conserved component of innate immune system, might contribute to a better understanding of the fish immune defense mechanism against exogenous pathogens. In this regard, we cloned the MKK6 (*Ci*MKK6) cDNA sequence from grass carp *C. idella*. The mRNA expression of *Ci*MKK6 in different tissues and developmental stages were also investigated by quantitative real-time PCR (qRT-PCR) in this study. In addition, the mRNA expression profiles of *Ci*MKK6 upon exposure to bacterial muramyl dipeptide (MDP) and L-Ala-γ-D-Glu-meso-diaminopimelic acid (tri-DAP) challenge were analyzed in intestines of grass carp. Moreover, *Ci*MKK6 was overexpressed in human embryonic kidney 293T (HEK293T) cells to determine its intracellular localization and function in signal transduction. To our knowledge, this was the first explored the potential roles of MKK6 in innate immunity of *C. idella*, and the results may lay a foundation for further researches on immune function of MKK6s in teleost.

2. Materials and methods

2.1. Experimental fish and sample collection

Healthy grass carp, averaging 30 g in weight, were purchased from the Hunan Institute of Aquatic Science. Prior to the experiment, fish were fed daily with a standard diet in a recirculating water tank system at 25 °C for 2 weeks.

After anesthetizing fish with 2-phenoxyethanol (Sigma-Aldrich), tissues, including the intestine, liver, blood, heart, kidney, gill, muscle and spleen, of healthy fish were dissected for tissue distribution analysis. For the expression analysis of different developmental stages, samples were collected from the following stages: fertilized egg, gastrula, neurula, organogenesis, hatching, 1 day post hatching (dph), 4 dph, and 7 dph. The collected samples were added to RNAiso Plus (Takara, Japan) and then stored at −80 °C until RNA isolation.

For the bacterial di-/tripeptide challenge experiments, ninety grass carp were randomly divided into 3 groups: the MDP-infected group, the Tri-DAP-infected group and the control group. Each fish in the bacterial challenge groups was injected intraperitoneally with 100 μl of MDP (10 μg/ml, Invitrogen) or Tri-DAP (10 μg/ml, Invitrogen). Each individual in the control group was intraperitoneally injected with 100 μl of PBS (phosphate-buffered saline). After injection, the grass carp were returned to the water tanks, and individuals were randomly sampled at 0, 3, 6, 12, 24 and 48 h post injection ($N = 3$). Intestines from the control and bacterial di-/tripeptide-challenged groups were collected for RNA isolation.

2.2. Cloning the cDNA sequence of CiMKK6

Upon searching the grass carp genome database, a 1339-bp nucleotide sequence was found to be homologous to the MKK6 gene of *Sinocyclocheilus anshuiensis* (XM_016480328.1). To verify and obtain the cDNA sequence of the *Ci*MKK6 gene from *C. idella*, gene-specific primers (forward primer, 5′-TCTCCCCGTTTTCGTTGCGTG-3'; reverse primer, 5′-TGCTGAGGTGGGCGTGTCTTA-3′) were designed for PCR amplification. Total RNA was isolated from the collected samples using RNAiso Plus (Takara, Japan) according to the manufacturer's protocols. RNA integrity was assessed by electrophoresis on a 1.0% agarose gel. The concentration and purity of each RNA sample were evaluated by measuring the optical density at 260/280 nm in an Eppendorf BioPhotometer. The cDNA template was then synthesized from 1 μg of total RNA with a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Japan). PCR was performed in a total volume of 50 μl containing 1 μl of cDNA template, 0.5 μl of TaKaRa LA Taq DNA Polymerase (5 U/μl), 5 μl of 10 × LA PCR Buffer II (Mg^{2+} Plus), 8 μl of dNTP mixture (2.5 mM each), 2 μl of each primer (10 μM) and 31.5 μl of ddH_2O. The following PCR program was used: 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. After PCR was finished, the products were analyzed by electrophoresis on 1.5% agarose gels in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 120 V for 20 min. The target PCR products were purified with a HiPure Gel Pure Micro Kit (Magen, China), ligated into the pMD19-T vector (TaKaRa, Japan) and transformed into competent *E. coli* DH5α cells. Positive clones were sequenced on an Applied Biosystems (ABI) DNA 3730 sequencer using the universal primers M13-47 and RV-M. The obtained cDNA sequences of *Ci*MKK6 were verified with the BLAST tool available from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Bioinformatics analysis

The amino acid sequence of *Ci*MKK6 was deduced with ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The similarity and identity of protein sequences were analyzed with MatGAT software v2.02. The compute pI/Mw tool (http://web.expasy.org/compute_pi/) was used to predict the theoretical molecular weight and isoelectric point. The protein domains were predicted with the Simple Modular Architecture Research Tool (SMART) (http://smart.cmbl-heidelberg.de/). Multiple alignments of amino acid sequences were carried out with the Clustal W method in the program MegAlign (DNASTAR software). Based on the sequence alignment results, a phylogenetic tree was constructed using the MEGA 5.05 package with the neighbor-joining (NJ) method. The GenBank accession numbers of the MKK6 sequences used for bioinformatics analysis are as follows: NP_002749.2 [*Homo sapiens*], NP_001029217.1 [*Bos taurus*], NP_036073.1 [*Mus musculus*], XP_004946297.1 [*Gallus gallus*], NP_001079947.1 [*Xenopus laevis*], NP_001299799.1 [*D. rerio*], XP_022598225.1 [*Seriola dumerili*], XP_023805137.1 [*O. latipes*], OWF39383.1 [*Mizuhopecten yessoensis*], XP_025096402.1 [*Pomacea canaliculata*], XP_012936929.1 [*Aplysia californica*], XP_011423484.1 [*Crassostrea gigas*], XP_022319505.1 [*Crassostrea virginica*], XP_013084905.1 [*Biomphalaria glabrata*].

2.4. Quantitative real-time PCR analysis of CiMKK6 expression levels

The mRNA expression levels of *Ci*MKK6 were determined by

quantitative real-time PCR (qRT-PCR) using a pair of gene-specific primers (forward primer, 5′- TGTCCCTCTCGGCAAAACTGA-3'; reverse primer, 5′-TGCTGAGGTGGGCGTGTCTTA-3′). The grass carp β-actin gene was used as an internal standard to normalize the amount of total RNA added to the reverse transcription reactions. The primers for β-actin (forward primer, 5′-CTTGACTTCGAGCAGGAG-3'; reverse primer, 5′-GGCATACAGGTCTTTACGG-3′) were designed for qRT-PCR analyses. Reverse transcription (RT) was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan) with 1 μg of total RNA as a template according to the manufacturer's instructions. The qRT-PCR reactions were carried out with the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher, USA) in a volume of 20 μl containing 10 μl of 2 × SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Japan), 1 μl of cDNA template, 0.8 μl of each primer (10 μM) and 7.4 μl of ddH_2O. The qRT-PCR cycling procedure was as follows: an initial denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, followed by dissociation curve analysis. The relative expression level of *Ci*MKK6 was calculated according to the $2^{-\Delta\Delta Ct}$ method. The amplification efficiencies of the target and reference genes were verified and found to be approximately equal. All expression data obtained from three independent biological replicates were analyzed with SPSS 16.0 software. One-way analysis of variance (ANOVA) was used to determine differences among the groups. Differences were considered statistically significant at $P < 0.05$ and extremely significant at $P < 0.01$.

2.5. Construction of eukaryotic expression plasmids

Eukaryotic expression plasmids, including pCMV-N-Flag-*Ci*MKK6 (*Ci*MKK6-Flag) and pEGFP-N1-*Ci*MKK6 (*Ci*MKK6-GFP), were constructed and used for mammalian cell transfections. The primers used for the construction of the *Ci*MKK6-Flag (forward primer, 5′-GATAAGAGCCCG GGCGGATCCATGGAAGGAGGGAGC-3'; reverse primer, 5′-ATCGAATTC CTGCAGAAGCTTTCAGTCCCCAAGGAT-3′) and *Ci*MKK6-GFP (forward primer, 5′-CTACCGGACTCAGATCTCGAGATGGAAGGAGGGAGC-3'; reverse primer, 5′-ATGGTGGCGACCGGTGGATCCCGGTCCCCAAGGA TGC-3′) vectors were designed based on the cloned *Ci*MKK6 cDNA sequence. The complete ORF region of *Ci*MKK6 was amplified using PrimeSTAR® Max DNA Polymerase (TaKaRa, Japan) with a 50 μl reaction volume containing 25 μl of PrimeSTAR Max Premix (2 ×), 2 μl of each primer, 1 μl of template and 20 μl of ddH_2O. The PCR program was as follows: 98 °C for 2 min, followed by 32 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s, followed by a final extension at 72 °C for 8 min. A ClonExpress® II One Step Cloning Kit (Vazyme, China) was used to construct the *Ci*MKK6-Flag and *Ci*MKK6-GFP vectors according to the manufacturer's instructions. Briefly, the empty pCMV-N-Flag and pEGFP-N1 plasmids were double digested by the restriction enzymes *BamH*I/*Hind*III and *Xho*I/*BamH*I, respectively. Then, the *Ci*MKK6 ORF containing a 15 bp sequence of empty plasmid cloning site and the linearized vector were mixed in the presence of Exnase® for 30 min at 37 °C and immediately placed on ice for 5 min. Finally, the recombination products were added to 100 μl of *E. coli* DH5α fresh competent cells for transformation. Positive colonies were verified by direct colony PCR and sequenced by Biosune Company (China).

2.6. Cell culture, subcellular localization and luciferase reporter assays

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% FBS (fetal bovine serum, Gibco BRL) and antibiotic (100 mg/L streptomycin and 10^5 U/L penicillin, Gibco) in a humidified incubator with 5% CO_2 at 37 °C. Prior to transfection, the cells were seeded overnight and grown until they were 70% confluent by the time of transfection. The plasmids were transfected into the cells in serum-free culture medium using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Six hours after transfection, the medium was replaced with complete medium containing 10% FBS. All of the plasmids used for transfection were prepared from overnight bacterial cultures using a HiPure Plasmid EF Micro Kit (Magen, China) according to the manufacturer's protocol.

For subcellular localization analysis of *Ci*MKK6, cells were seeded onto sterile glass microscope coverslips and placed in a 12-well cell culture plate prior to transfection. After cell adhesion for 24 h, the cells were transiently transfected with the recombinant endo-free plasmid *Ci*MKK6-GFP in serum-free culture medium. The transfections were performed using Lipofectamine 2000 with a 2:1 ratio of transfection reagent to DNA. After transfection for 48 h, the transfected cells were washed with PBS (pH 7.4), fixed with 4% paraformaldehyde for 10 min, and then stained with 6-diamidino-2-phenyl-indole (DAPI) (1 μg/ml) for 5 min. Finally, the cells transfected with *Ci*MKK6-GFP vectors were washed with PBS and directly observed by fluorescence microscopy.

To assess the ability of *Ci*MKK6 to activate AP-1 transcriptional activity, *Ci*MKK6-Flag was cotransfected with pRL-TK and pAP-1-Luc (Catalog #219073) reporter plasmids into HEK293T cells grown in a 48-well plate. After 48 h of transfection, the luciferase activity of total cell lysates was measured using a Dual-Luciferase® Reporter Assay System (Promega, USA). In brief, the transfected cells were washed with PBS and lysed in 100 μl of 1 × passive lysis buffer at room temperature for 15 min. For each assay, 20 μl of cell lysate was added to 100 μl of Luciferase Assay Reagent II (LAR II) in a reaction tube to measure the firefly luciferase activity. Next, Stop & Glo® reagent (100 μl/well) was added to each tube to quench firefly luciferase and initiate the Renilla luciferase reaction. The values were calculated as the mean relative stimulations for a representative experiment from three separate experiments, with each experiment performed in duplicate. Significant differences among the different groups were analyzed using SPSS v.16.0 software by one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Cloning and characterization of the CiMKK6 sequence

The cDNA sequence of grass carp MKK6 (*Ci*MKK6) was cloned based on the sequence retrieved from the genome database and was submitted to GenBank under accession number MH491995. To the best of our knowledge, this is the first report to describe the molecular characterization of *Ci*MKK6 in *C. idella*. The nucleotide and deduced amino acid sequences of *Ci*MKK6 are shown in Fig. S1. The *Ci*MKK6 cDNA consisted of 1393 bp containing a 5′ untranslated region (UTR) of 155 bp, a 3′-UTR of 110 bp and an open reading frame (ORF) of 1074 bp encoding a polypeptide of 357 amino acids (aa) with a calculated molecular weight of 39.96 kDa and a theoretical pI of 6.67. Upon comparing *Ci*MKK6 with other reported MKK6 genes, we found that the length of the *Ci*MKK6 protein sequence was similar to that of homologs from other fish but was longer than that of high vertebrates and shorter than that of most invertebrates. Sequence analysis of *Ci*MKK6 revealed that it contained the main features characteristic of the MKK6 family [21,22], including a serine/threonine protein kinase (S_TKc) domain (262 aa, positions 76-337), a conserved dual phosphorylation site (230Ser and 234Thr) in the SVAKT motif (5 aa, positions 230-234) and one domain of versatile docking (DVD) site (24 aa, positions 334-357).

Earlier studies have indicated that MKKs are a group of highly conserved serine/threonine-specific kinases and that their function relies on the conserved S_TKc domain, which is critical for kinase phosphorylation and ATP binding. Additionally, the dual phosphorylation site of Ser and Thr in the S-X_3-T motif and the docking site (DVD) are essential for MKK activation by upstream MKKKs during physiological and pathological processes [19,27]. These conserved motifs and structures were observed in *Ci*MKK6, suggesting that *Ci*MKK6 may, similarly to its reported homologs, play important roles in the MAPK signal transduction pathway. Multiple sequence alignment showed that the

deduced *Ci*MKK6 protein exhibited high sequence homology with other reported MKK6s. As shown in Fig. S2A, the S_TKc domain and SVAKT motif of MKK6s were highly conserved among different kinds of species. A BLAST analysis showed that *Ci*MKK6 shared 34.5–44.9% identity and 54.1–66.3% similarity with MKK6 sequences from other species, including *H. sapiens* (81.5% identity, 86.6% similarity); *B. taurus* (81.2% identity, 86.3% similarity), *M. musculus* (80.7% identity, 86.3% similarity), *G. gallus* (82.4% identity, 88.8% similarity), *X. laevis* (80.8% identity, 87.1% similarity), *D. rerio* (95.0% identity, 96.1% similarity), *S. dumerili* (90.8% identity, 94.4% similarity), *O. latipes* (89.9% identity, 93.3% similarity), *M. yessoensis* (59.2% identity, 72.6% similarity), *P. canaliculata* (55.5% identity, 68.9% similarity), *A. californica* (53.9% identity, 71.5% similarity), *C. gigas* (55.8% identity, 72.8% similarity), *C. virginica* (56.2% identity, 70.2% similarity) and *B. glabrata* (81.5% identity, 86.6% similarity) (Fig. S2B). Among the sequences, the deduced amino acid sequence of *Ci*MKK6 was closest to that of *D. rerio,* followed by that of *S. dumerili* and *O. latipes*. A neighbor-joining (NJ) phylogenetic tree was constructed using the amino acid sequences of *Ci*MKK6 and other known MKK6s. Overall, the relationships displayed in the cladogram generally agreed with those of traditional taxonomy. Phylogenetic analysis revealed that the MKK6 homolog proteins could be divided into two groups: vertebrates and invertebrates. *Ci*MKK6 was located in the vertebrate cluster and exhibited the closest evolutionary relationship with *D. rerio* MKK6 (Fig. S3), which was consistent with the sequence alignment results. These findings suggest that *Ci*MKK6 is a novel member of the MKK6 family in bony fishes.

3.2. *CiMKK6 was broadly expressed in various tissues and embryonic stages*

Previous studies have reported that MKK6 is a protein kinase that is widely expressed in various tissues and organs of vertebrates and invertebrates [20,23]. For example, another carp MKK6 gene was found to be broadly transcribed in tested fish tissues [23]. A tissue distribution analysis from sea cucumber demonstrated that the expression of *Aj*MKK3/6 could be detected in all the tested tissues of *Apostichopus japonicus* [22]. In shrimp, MKK6 was also shown to be broadly expressed in the stomach, intestine, hemocytes, heart, eyestalk, nerve, gill, epithelium, scape, pyloric caecum, muscle and hepatopancreas [21]. To determine the tissue distribution of grass carp MKK6, the mRNA expression levels of *Ci*MKK6 were detected by qRT-PCR in different adult tissues. The qRT-PCR results showed that *Ci*MKK6 transcript was widely expressed in all the examined tissues, including blood, kidney, intestine, spleen, liver, heart, muscle and gill (Fig. 1), suggesting that *Ci*MKK6 may be involved in various biological processes in grass carp. Additionally, the highest expression level of *Ci*MKK6 was observed in blood, an important tissue for both innate and acquired immunity in vertebrates [28–30]. In fact, in addition to *Ci*MKK6, other immune-related genes involved in the host response to pathogen infection have also been found to be highly expressed in fish blood [29–31]. For example, Xu et al. reported that the mRNA of matrix metalloproteinase-9 (MMP-9) was expressed at high levels in the blood of healthy grass carp [31]. In bivalve mollusks, the Yesso scallop MKK6 was found to be predominately expressed in hemocytes, which is similar to fish blood and is thought to be a key immune tissue for the recognition and elimination of cellular pathogens in shellfish [20]. It is generally accepted that fish gills are in direct contact with the aquatic environment and serve as the first line of defense against a variety of aquatic pathogens. Previous studies have demonstrated that many immune-related signaling genes, including tumor necrosis factor receptor 1 (TNFR1) [32], toll-like receptor (TLR4) [33] and nucleotide-binding oligomerization domain protein 1/2 (NOD1/2) [30], are abundantly expressed in the gills of grass carp. In our study, a relatively high transcript level of *Ci*MKK6 was also observed in the gills, suggesting that *Ci*MKK6 may play roles in maintaining gill homeostasis in grass carp. These earlier results and recent progress suggest that *Ci*MKK6 may play a broad functional role in grass carp and should be studied further.

A large number of studies have shown that p38MAPKs play an important regulatory role in embryonic development in amphibians [34], fishes [35] and mollusks [15]. Although MKK6 is a key upstream kinase of p38MAPK signaling pathways, whether MKK6 is involved in embryogenesis remains unclear. The expression patterns of *Ci*MKK6 during different embryonic and larval stages were detected in this study to determine the possible development-related functions of *Ci*MKK6. The qRT-PCR results revealed that the expression of *Ci*MKK6 was widely distributed in all of the tested developmental stages, including the fertilized egg, gastrula, neurula, organogenesis, hatching, 1 day post hatching (dph), 4 dph, and 7 dph stages (Fig. 2). The broad expression patterns of *Ci*MKK6 are largely similar to those found in studies on *Patinopecten yessoensis,* in which PyMKK3/6 mRNA was also found to be expressed in all ten developmental stages of the scallop [20]. In our study, we also found that *Ci*MKK6 transcript levels varied throughout the process of embryonic development. Certainly, the level of *Ci*MKK6 mRNA significantly increased and peaked at the gastrula stage (at which stage it was 2.5-fold higher than that in the fertilized egg stage, $P < 0.05$); decreased significantly at the neurula stage; increased gradually from the neurula stage to hatching; and finally declined and displayed low levels from 1 dph to 7 dph. In particular, the highest level of *Ci*MKK6 was observed in the gastrula stage, an important stage in the embryonic development of fish during which other grass carp MKK family members (MKK4 and MKK7) also showed elevated mRNA expression (data not published). These findings suggest that *Ci*MKK6 may participate in major events in the gastrula stage of grass carp. Further

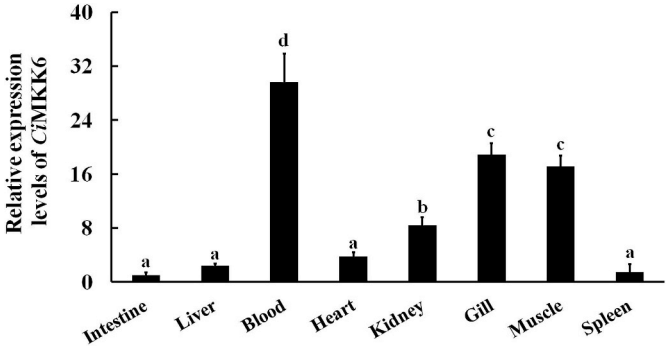


Fig. 1. The transcript levels of *Ci*MKK6 in eight tissues of adult grass carp were analyzed by qRT-PCR. The mRNA expression levels were normalized to β-actin expression, and the error bars indicate the standard deviation. The expression levels of *Ci*MKK6 in the intestine were used as a control and were set as 1.0. Data without shared letters are significantly different ($P < 0.05$).

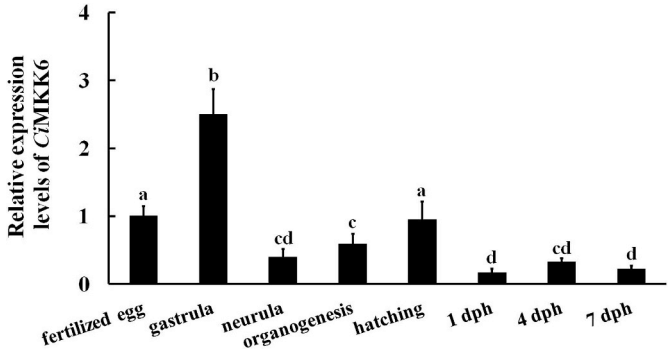


Fig. 2. The expression levels of *Ci*MKK6 in different embryonic stages (fertilized egg, gastrula, neurula, organogenesis, hatching, 1 day post-hatching (dph), 4 dph, and 7 dph) were determined by qRT-PCR. The mRNA expression was normalized to that of the β-actin gene. Each vertical bar represents the mean ± SEM ($N = 3$). The transcript levels of *Ci*MKK6 at the fertilized egg stage were used as a control and were set as 1.0. Data without shared letters are significantly different ($P < 0.05$).

studies are needed to clarify this speculation.

3.3. The expression of CiMKK6 was significantly regulated by bacterial di-/tripeptide stimulation

The intestine is an important digestive and immune organ of the body that plays central roles in nutrient digestion/uptake and innate immunity in teleosts. It is well known that fish intestines are often exposed to a large and diverse assortment of harmful bacterial infections that can cause significant intestinal inflammation and challenge the health status of the fish [36,37]. The intestinal immune response plays a pivotal role in maintaining intestinal health in fish [38,39]. Previous studies have revealed that MDP and Tri-DAP are bioactive motifs present in the cell wall of both gram-positive and gram-negative bacteria that can significantly induce inflammatory responses in animal intestines. As typical bacterial peptides, MDP and Tri-DAP can be transported by the oligopeptide transporter PepT1 from the intestinal lumen into epithelial cells. Upon the accumulation of di-/tripeptides in intestinal epithelial cells via PepT1-mediated transport, the intracellular pattern recognition receptor NOD1/2 recognizes and interacts with these bacterial di-/tripeptides and then activates receptor-interacting serine/threonine-protein kinase 2 (RIP2) to trigger downstream activation of MAPK and nuclear factor kappa (NF-κB) pathways leading to the production of innate immune effector molecules [40]. In recent years, NOD1/2-RIP2 pathways have been identified in several fish species and have been proven to play important roles in bacterial di-/tripeptide-induced immune responses [41–43]. For instance, Maharana et al. reported that zebrafish NOD2 could bind bacterial MDP and interact with the downstream adaptor protein RIP2, providing direct evidence for the important immune function of the NOD2-RIP2 pathway during MDP challenge [44]. However, compared to that in higher vertebrates, understanding of the intestinal defense mechanisms of fish in response to bacterial di-/tripeptide stimulation remains limited, and the mechanisms need further exploration. In this study, the mRNA profile of *Ci*MKK6, a key upstream kinase of MAPK in the NOD1/2-RIP2 pathway, was analyzed in the intestines of grass carp after MDP and Tri-DAP challenge.

Our results revealed that *Ci*MKK6 transcript levels in the intestine were significantly increased in a time-dependent manner upon MDP and Tri-DAP challenge. As shown in Fig. 3A, the expression level of *Ci*MKK6 in MDP-challenged fish was increased significantly compared to that in the PBS group fish at 6 h post challenge (3.4-fold elevation, $P < 0.05$), peaked at 12 h post challenge (18.5-fold elevation, $P < 0.05$), and then decreased from 24 h (7.5-fold elevation, $P < 0.05$) to 48 h post challenge (2.1-fold elevation, $P > 0.05$). For the Tri-DAP challenge experiment, the *Ci*MKK6 transcripts were significantly upregulated at 3 h post challenge (2.9-fold elevation, $P < 0.05$), reached the highest levels at 6 h post challenge (7.4-fold elevation, $P < 0.05$), decreased at 12 h (5.8-fold elevation, $P < 0.05$), increased again from 24 h post challenge (5.7-fold, $P < 0.05$), and finally returned to normal levels at 48 h post challenge (Fig. 3B). Overall, *Ci*MKK6 displayed a broad and strong response to MDP and Tri-DAP stimulation, suggesting that *Ci*MKK6 may play important roles in the host immune response against bacterial di-/tripeptide challenge. Previously, a bacterial challenge experiment in *L. vannamei* revealed that shrimp MKK6 was induced significantly in response to *V. parahaemolyticus* infection [21]. Recently, a scallop MKK6 was shown to be involved in innate immune defense responses to *Micrococcus luteus* (gram-positive) and *Vibrio anguillarum* (gram-negative) bacterial infections [20]. These findings suggest that the immune function of the MKK6 response to bacterial stimulation may be conserved in vertebrates and invertebrates.

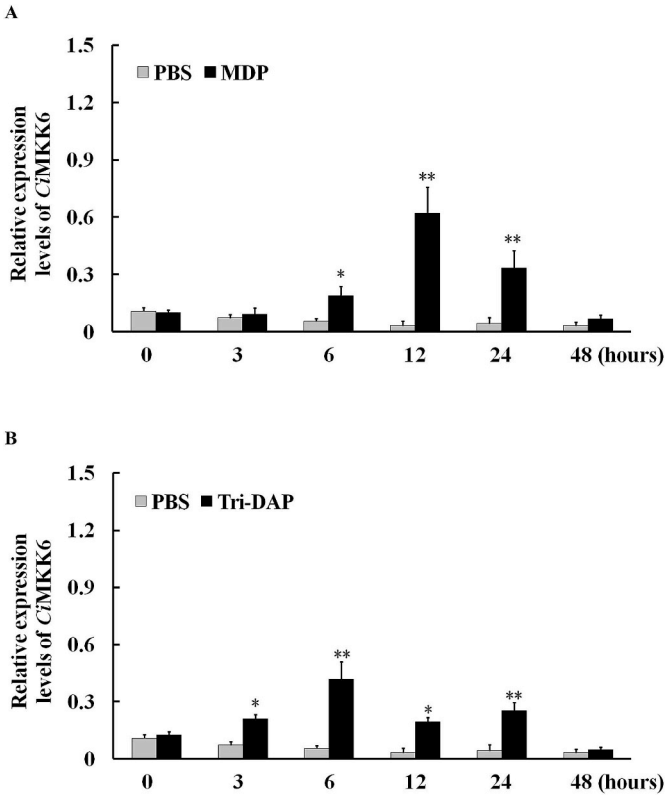


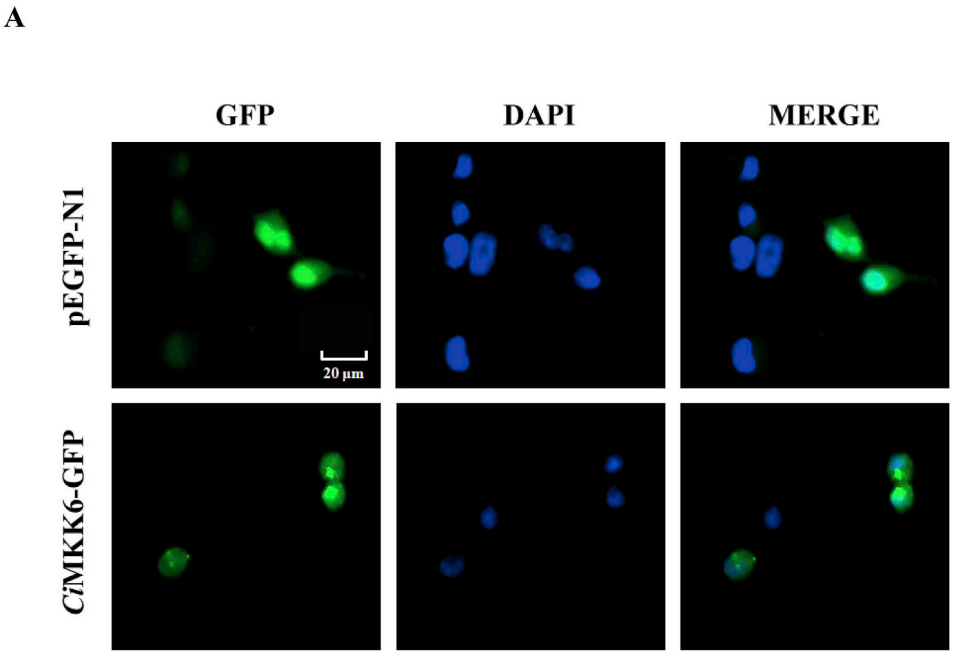
Fig. 3. Quantitative RT-PCR analysis of *Ci*MKK6 mRNA levels in the intestine upon MDP (A) and Tri-DAP challenge. Grass carp β-actin expression was used as an internal control. Each bar represents the mean of the normalized expression levels of the replicates ($N = 3$). Significant differences are indicated by asterisks (* and ** represent $P < 0.05$ and $P < 0.01$, respectively).

3.4. CiMKK6 was distributed in the cytoplasm and nucleus of HEK293T cells

In mammals, previous studies have shown that MKK6 is a key cytosolic adaptor protein of the p38MAPK signaling pathway [19,23]. When cells are exposed to various extracellular stimuli, MKK6 can receive stimulus signals from upstream MKKKs and then activate downstream p38MAPKs via dual phosphorylation of the TGY motif at threonine and tyrosine residues. Upon phosphorylation by MKK6, p38MAPKs can trigger the activation of downstream transcription factors and then induce the expression of various specific target genes [19]. It is generally believed that the cytoplasmic localization of MKK6 is critical for signal transduction in the p38MAPK pathway. In our study, the subcellular localization of *Ci*MKK6 was preliminarily analyzed by fluorescence microscopy to reveal whether *Ci*MKK6 is a cytoplasm-localized signaling adaptor molecule. The fluorescence microscopy observations revealed that the green fluorescence of *Ci*MKK6 was distributed in the cytoplasm and nucleus of HEK293T cells (Fig. 4A), suggesting that *Ci*MKK6 may not only work in the cytoplasm but also be involved in some physiological processes in the nucleus. Similar results were reported in shrimp, in which *Lv*MKK6 showed a uniform presence of green fluorescence in both the cytoplasm and the nucleus of Drosophila S2 cells [21]. These data may suggest that MKK6 is a cytoplasm- and nucleus-localized signaling molecule in bony fishes and shrimp.

3.5. Conserved role of CiMKK6 in activating AP-1 signaling pathway

Activator protein 1 (AP-1) is often considered a key immune-related transcription factor that plays essential roles in regulating the production of numerous specific effector genes, such as interleukin-6 (IL-6), interleukin-8 (IL-8) and matrix metalloproteinase-1 (MMP-1), in



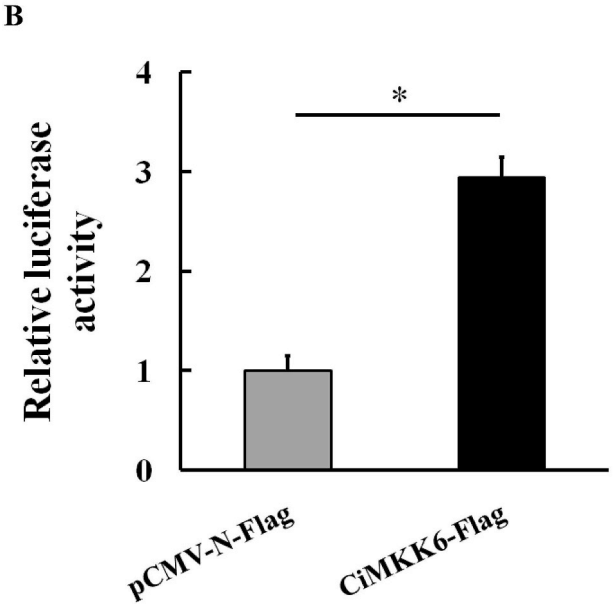


Fig. 4. Overexpression of *Ci*MKK6 in HEK293T cells. (A) Subcellular localization analysis of *Ci*MKK6. The *Ci*MKK6-GFP plasmid was transfected into HEK293T cells. At 48 h post transfection, the cells were observed using a fluorescence microscope. (B) Effects of *Ci*MKK6 overexpression on the activity of an AP-1 reporter gene. Plasmids, including *Ci*MKK6-Flag, pAP-1-Luc and pRL-TK, were cotransfected into HEK293T cells. After transfection for 48 h, the luciferase activity of the cells was analyzed with the Dual-Luciferase® Reporter Assay System. The luciferase activity of cells transfected with pCMV-N-Flag alone was used as a control. Significant differences are indicated by different letters ($P < 0.05$).

response to a variety of immune stimuli [45]. Earlier evidence showed that mammalian MAPKs are involved in the activation of AP-1 signaling pathways [5,45,46]. Our previous studies also demonstrated that oyster MAPKs can significantly enhance AP-1 transcriptional activity in bivalve mollusks [47]. Although MKK6s are key upstream regulator of MAPKs, whether fish MKK6s could activate the AP-1 signaling pathway remained unclear. To this end, the *Ci*MKK6-Flag expression vector and AP-1-Luc reporter gene were cotransfected into HEK293T cells. Forty-eight hours after transfection, the enhancing effects of *Ci*MKK6 on AP-1 transcriptional activation were observed in *Ci*MKK6-Flag-transfected cells. Overexpression of *Ci*MKK6 significantly increased AP-1-Luc activity by 2.94-fold compared to transfection with pCMV-N-Flag alone ($P < 0.05$) (Fig. 4B). These data indicated that *Ci*MKK6 is an efficient activator of the AP-1 signaling pathway.

4. Conclusion

In summary, a novel fish MKK6 homolog (*Ci*MKK6) was cloned and identified for the first time from *C. idella*. The deduced *Ci*MKK6 sequence shared high structural similarity and a close evolutionary relationship with its homologs from other fish. Quantitative RT-PCR analysis showed that *Ci*MKK6 mRNA was ubiquitously expressed in all selected tissues and in different developmental stages of grass carp. Additionally, *Ci*MKK6 mRNA transcripts were significantly upregulated by bacterial MDP and Tri-DAP in a time-dependent manner. Moreover, fluorescence microscopy observations revealed that *Ci*MKK6 was distributed in the cytoplasm and nucleus, and its overexpression could increase the transcriptional activity of an AP-1 reporter gene in HEK293T cells. These findings provide new insight into the immune

defense mechanisms of *C. idella* and lay a foundation for further functional studies on MKK6s in teleosts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.073.

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