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Molecular cloning, expression and functional characterization of tumor necrosis factor (TNF) receptor-associated factor (TRAF)-interacting protein (TRIP) in grass carp, *Ctenopharyngodon idella*

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ABSTRACT

TRIP (Tumor Necrosis Factor (TNF) Receptor-Associated Factor (TRAF)-Interacting Protein), a member of the TNF superfamily, plays a crucial role in the modulation of inflammation in vertebrates. However, no information about TRIP is available in teleosts. In this study, the full-length cDNA of TRIP, containing a 5′UTR of 112 bp, an ORF of 1359 bp, and a 3′UTR of 29 bp before the poly (A) tail, was cloned from grass carp, *Ctenopharyngodon idella*. The *TRIP* gene encoded a protein of 452 amino acids with an estimated molecular mass of 51.06 KD and a predicted theoretical isoelectric point (*p*I) of 9.11. Quantitative real-time PCR analysis revealed that *TRIP* mRNA was expressed in all the tissues examined in grass carp, with the highest expression in the kidney, followed by the intestine and thymus. However, lower levels of expression were also detected in fat, spleen, liver, gonad and heart. Subcellular localization and two-hybrid analysis revealed that TRIP was located in the nucleus and that it interacted with TRAF1 and TRAF2 in HEK293T cells. Furthermore, similar to TNF-α, IL-10 and *TRIP* mRNA expression was upregulated in the spleen of fish fed high-fat or high-carbohydrate diets, suggesting that TRIP might be associated with the response to excessive energy intake. The mRNA relative expression of TRIP was significantly reduced ($P < 0.05$) after hepatocyte of *C. idella* was treated with 2 μg/mL lipopolysaccharide (LPS) for 4 h, while the expression levels of inflammatory cytokines TNF-α and IL-10 were significantly increased ($P < 0.05$). Taken together, these results indicate that TRIP might play important roles in immune defense and has the potential to be used as a anti-inflammation target in grass carp.

1. Introduction

The tumor necrosis factor (TNF) has been recognized as a pleiotropic cytokine. The TNF receptor (TNFR) is involved in diverse biological functions, such as cell proliferation, immune regulation, inflammation, cell death and apoptosis [1,2]. TNFR-associated factor 2 (TRAF2) is a key adaptor molecule in the TNFR signaling complex that promotes downstream signaling cascades, such as the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK). TNFR-Associated Factor (TRAF)-Interacting Protein (TRIP or TRAIP) is known to be a cellular binding partner of TRAF2 and inhibits TNF-induced NF-κB activation. Upon TNF-α stimulation, TRAF2 is recruited directly to TNFR2 or via TRADD (TNF receptor 1 associated via death domain) to TNFR1, resulting in the activation of downstream NF-κB and MAPKs [3,4,5]. Recent studies demonstrated that TRIP plays multiple roles in cell signaling, proliferation, differentiation, apoptosis, and embryonic development [6]. In 293T cells, TRIP suppressed RANKL-mediated NF-κB activation [7]. The expression of TRIP is regulated by lipopolysaccharide [8]. Homozygous TRIP knockout mice die early in embryonic development (E7) due to proliferation defect and massive apoptosis [9]. Moreover, TRIP enhances TNF-induced apoptosis in 293T cells, human breast cancer cell lines, and in male rodent brain tissue [10]. In a recent study, the molecular mechanisms of TRAF2-mediated NF-κB activation via the TRAF2-TRIP interaction were identified

Abbreviations: TNF, Tumor necrosis factor; IL-10, Interleukin-10; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor; TRAF1, TNFR-associated factor 1; TRAF2, TNFR-associated factor 2; TRIP, Tumor Necrosis Factor (TNF) Receptor-Associated Factor (TRAF)-Interacting Protein; NF-κB, nuclear factor-κB; H-LIP, high lipid diet; H-CHO, high carbohydrate diet; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinases.

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NF-κB activation is crucial for the inducible expression of multiple cellular genes that encode proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and TNF-α, in addition to anti-apoptotic proteins [12,13]. Ubiquitination is involved in multiple biological functions, such as proliferation, differentiation, apoptosis and inflammation [6]. TRIP functions as a Really Interesting New Gene (RING)-type E3 ubiquitin ligase [14] and is likely involved in the immune response, cell cycle and programmed cell death (apoptosis) in mammals [15]. However, the physiological functions of TRIP have not been determined in teleosts.

Grass carp (*Ctenopharyngodon idella*) is an important economic freshwater fish in China, and grass carp production accounted for 18.10% of total freshwater fishery in 2013, one of the largest production of this fish in the world. However, the majority of cultured fishes (especially herbivorous fishes) fed with excessive carbohydrate- or lipid-containing diets accumulate extensive liver fat, resulting in lipid metabolism disorders and liver dysfunction, thus affecting the quality of the fish [16–19]. Furthermore, it has also been suggested that fish health can be affected by the fatty liver conditions [20]. Previous studies indicated that pro-inflammatory cytokines are commonly used as immune-regulatory genes in fish. However, little is known about the inflammatory response regulation. Therefore, study on the mechanism of inflammatory response in grass carp may be an important strategy against disease.

In this study, the full length cDNA sequences of *TRIP* from *C. idella* were cloned and characterized. Expression profile in healthy fish and functionally characterized TRIP in *C. idella* hepatocyte and HEK 293T cells were also analyzed. Finally, we assessed mRNA expression after the consumption of H-LIP, H-CHO and lipopolysaccharide (LPS).

2. Materials and methods

2.1. Cell cultures

HEK293T cells were cultured at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO, USA). The *C. idella* hepatocyte line, provided by the China Center for Type Culture Collection, was derived from grass carp liver. The *C. idella* hepatocyte was cultured in the same media as HEK293T cells but were incubated at 28 °C in a humid, 5% CO_2 atmosphere.

2.2. Cloning TRIP cDNA

Total RNA was extracted from the liver of healthy grass carp (obtained from Henan Normal University, Xinxiang, China) (weight 22.35 ± 0.46 g) using Trizol reagent (Invitrogen) and reverse transcribed into first-strand cDNA using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. PCR amplification primers were designed based on the TRIP Unigene from the grass carp transcriptome database: TRIP-F: GATCAAGCTTATGAAAGCAGTGTTGTCTGAA; TRIP-R: GATCTCTAGATTATTCCAAGAAACCATCCAG. PCR amplification was performed under the following conditions: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 59.4 °C for 30 s, and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. The PCR products were gel-extracted and ligated into pMD18-T (Takara). Following transformation into competent *Escherichia coli* DH5α cells, positive clones were screened using ampicillin selection and colony PCR and then sequenced on an ABI Mode 377 automatic DNA sequencer (Invitrogen).

2.3. Sequence and phylogenetic analyses

The amino acid sequence was predicted using the translation tool, and the molecular weight, isoelectric point (*p*I), and net charge of the proteins were calculated using the ProtParam tool, both available on the ExPASy molecular biology server (http://www.expasy.org/tools). The signal peptide of the protein was identified using SignalP (version 3.0) (www.cbs.dtu.dk/services/SignalP). Multiple sequence alignments were generated using Clustal W (version 1.83) and annotated using GeneDoc software (http://www.psc.edu/biomed/genedoc). The phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 7.0 software with 1000 bootstrap replicates.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted from the gonad, heart, spleen, liver, kidney, thymus, intestine and fat of grass carp (obtained from Henan Normal University, Xinxiang, China) (weight 38.17 ± 1.61 g) and cDNA synthesis was conducted as described in Section 2.2. The specific primers for 18S (reference gene, 18S (F): TGGAATGAGCGTATCCTAAACC; 18S (R): TCTCCCGAGATCCAACTACAA) and TRIP (target gene, TRIP(F): TCGGCATCTTCATCCAACAA; TRIP(R): GAGCAGCGAGTTCCTTAACA) used in the quantitative RT-PCR (qRT-PCR) displayed a single peak in the melting curve analysis with an amplification efficiency close to the theoretical 100%. RT-PCR was carried out in 20 μL reaction volumes containing 5 μL of 1:10 diluted original cDNA, 10 μL of 2 × SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA), 2 μL of each primer (20 pmol/μL), and 3 μL of PCR grade water using a Bio-Rad CFX 96 real-time PCR machine. Triplicate fluorescence intensities of each sample, as measured by crossing-point (Ct) values, were compared and converted to fold difference using the relative quantification method [21].

2.5. Plasmid construction, cell transfection and subcellular localization

To visualize the subcellular localization of TRIP, full length *TRIP* cDNA was cloned and inserted into a pcDNA3.1-EGFP vector at the *Eco*RV and *Xho*I sites, while the ORF of grass carp TRAF1 and TRAF2 were inserted into a pcDNA3.1-Myc vector at the *Eco*RV and *Xho*I sites. The constructed recombinant-expressing plasmid was extracted using a E.Z.N.A Plasmid Mini Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol and confirmed by sequence analysis.

HEK293T cell transfection was performed using Lipofectamine 3000 (Invitrogen) reagent. Briefly, HEK293T cells were grown in a 6-well plate. Lipofectamine 3000 and the plasmids were mixed for 25 min before transfection. Then, HEK293T cells were incubated with the mixture for 4 h at 37 °C and cultured with fresh medium. The cells were then washed with PBS, fixed with 4% paraformaldehyde, and stained with DAPI (1 mg/mL). The cells were observed under a fluorescence microscope.

2.6. Immunoprecipitation and western blot analysis

Cells were distributed into 6-well plates and transfected with various plasmids using Lipofectamine transfection reagent. After 24–48 h, the cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.4), lysed with immunoprecipitation buffer containing 1.0% (vol/vol) Nonidet P40, 50 mM Tris-HCl, 50 mM EDTA, 150 mM NaCl, pH 7.4, and a protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany), and then incubated on ice for 30 min. After centrifugation for 10 min at 14,000 *g*, supernatants were collected with 1 μg monoclonal anti-