Fish & Shellfish Immunology 46 (2015) 243–251

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Identification and characterization of tumor necrosis factor receptor (TNFR)-associated factor 3 from humphead snapper, *Lutjanus sanguineus*

Jia Cai [a, b, c, 1], Hongli Xia [a, b, c, 1], Yucong Huang [a, b, c], Jufen Tang [a, b, c], Jichang Jian [a, b, c], Zaohe Wu [b, c, d], Yishan Lu [a, b, c, *]

[a] College of Fishery, Guangdong Ocean University, Zhanjiang 524088, China
[b] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang 524088, China
[c] Guangdong Key Laboratory of Control for Diseases of Aquatic Economic Animals, Zhanjiang 524088, China
[d] Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China

ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form
15 June 2015
Accepted 18 June 2015
Available online 21 June 2015

Keywords:
Humphead snapper
TRAF3
Immune response
Over-expression

ABSTRACT

Tumor necrosis factor receptor (TNFR)-associated factor 3(TRAF3) is a key regulator in TNFR and Toll-like receptor (TLRs)/RIG-I-like receptors (RLRs) signal pathway. Here, a TRAF3 gene (Ls-TRAF3, GenBank Accession No: KJ789921) is cloned from humphead snapper (*Lutjanus sanguineus*). The Ls-TRAF3 cDNA contains an open reading frame of 1788 bp, which encodes a polypeptide of 595 amino acids. The deduced amino acid of Ls-TRAF3 possesses a RING finger, two TRAF-type zinc fingers, a coiled-coil and a MATH domain. Ls-TRAF3 protein shares high identities with other known TRAF3 proteins. In healthy fish, Ls-TRAF3 transcripts were broadly expressed in all examined tissues with highest expression levels in spleen, liver and head kidney. Quantitative real-time PCR (qRT-PCR) analysis revealed that Ls-TRAF3 could be induced by bacteria or viral PAMP poly I:C stimulation *in vivo*. Here, we also showed Ls-TRAF3 that, positively regulated IRF3 and Mx upon poly I:C stimuli, whereas prevented production of proinflammatory cytokine IL-6 after LPS injection. Moreover, over-expression of wide type (WT) Ls-TRAF3 and truncated forms, including ΔZinc finger 1, ΔZinc finger 2 and Δcoiled-coil suppressed NF-κB activity significantly, whereas the inhibitory effect of NF-κB was partially impaired when the RING finger or MATH domain deletion, suggesting the latter was more important for downstream signal transduction. Taken together, these results implicated that Ls-TRAF3 might play regulatory roles in immune response to pathogen invasion.

1. Introduction

Tumor necrosis factor receptor-associated factors family members (TRAF1-7) are kinds of crucial signal transduction docking proteins that regulate various immune signal pathways [1–14]. Most TRAF proteins share a well-conserved TRAF-C domain (MATH domain) in C-terminus, which mediates interactions of the receptors, downstream signaling molecules, and the formation of TRAF oligomerization [15–17]. Except for TRAF1, all members of this family also contain several zinc fingers followed by a RING finger that mediated the activation of downstream effectors via polyubiquitin [15,16,18,19].

TRAF3, a distinct member of TRAF family, is initially isolated by virtue of its binding to the cytoplasmic domain of CD40 and LMP-1 [3,4]. TRAF3 can act as adaptor protein and E3 ubiquitin ligase, which are essential for the activation of receptor-mediated signaling transduction [20–23]. On the other hand, TRAF3 is a positive regulator of type I interferon production through recruiting TBK1 and IKK- ε and can lead to direct phosphorylation of IRF-3 [21,24]. Except positive regulatory roles, TRAF3 also function as a negative regulator in the alternative NF-κB pathway by inhibiting NIK-mediated induction of p100 process [25–28]. Additionally, TRAF3 negatively regulates MAPK activation and inflammatory

* Corresponding author. Fisheries College, Guangdong Ocean University, Fisheries College, Guangdong Ocean University, No. 1 of Haida Road, Mazhang District, Zhanjiang, Guangdong Province 524088, China.
E-mail address: fishdis@163.com (Y. Lu).
[1] These authors contributed to the work equally and should be regarded as co-first authors.

http://dx.doi.org/10.1016/j.fsi.2015.06.021
1050-4648/

cytokines such as TNF, IL-6 and IL-12 productions as well [22,29]. Therefore TRAF3 is thought to be a "traffic cop" because of the multiple roles in different signal pathway [30]. However, piscine TRAF3 has only been recorded in common carp so far [31], the regulatory roles of TRAF3 in fish immune response need to be elucidated.

Humphead snapper (*Lutjanus sanguineus*) is one of the most important marine fish in the south coastal regions of China. However, outbreaks of disease caused by *Vibrio* spp. threaten snapper culture seriously. While the immune defense of humphead snapper against pathogen invasion remains largely unknown. In the present study, a TRAF3 (Ls-TRAF3) homolog is isolated form humphead snapper (*L. sanguineus*). The tissue distribution and expression profiles of Ls-TRAF3 upon LPS and poly I:C stimulation is investigated *in vivo*. Moreover, the association of Ls-TRAF3 and IL-6, IgM, IRF3 as well as Mx after LPS or poly I:C inductions are analyzed *in vitro*. Moreover, effects of Ls-TRAF3 (wild type (WT) or mutants) over-expression on signal transduction function are also detected. These data will expand our knowledge about the role of TRAF3 in immune response of fish during pathogen invasion.

2. Materials and methods

2.1. Fish, challenge, and sampling

Healthy humphead snapper (average weight 500–600 g) were obtained from local commercial market (Zhanjiang, China). The fish were maintained in aerated sea water tank at 28 °Cand fed for one week prior to experimental manipulation. A series of samples including head kidney, gill, heart, intestine, thymus, muscle, liver, skin, spleen, stomach, kidney, brain were collected from three fish and immediately frozen by liquid nitrogen until used for analysis of tissue expression patterns.

The synthetic dsRNA analog polyinosinicpolycytidylic acid sodium salt (poly I:C, product number: P9582) and Lipopolysaccharides (LPS, product number: L2630) was purchased from Sigma Corporation (USA) and dissolved in PBS. Each fish was injected intraperitoneally (i.p.) with 200 μl LPS or poly I:C (1 μg/μl). The control group was injected with 200 μl PBS. Each sample contained 3 independent individuals respectively to eliminate the individual differences. At 0, 3, 12, 18, 24, 48, 60, 72 and 84 h post injection, head kidneys samples were collected immediately from each group at each time point for further analysis.

2.2. Preparation of head kidney leukocytes (HKLs) and sample collection

The head kidney leukocytes were prepared according to the protocol in Ref. [32] with minor modification. Briefly, the head kidney was placed on a stainless steel mesh filter (100 μm), and pressed through with 5 ml of Leibovitz's L15 medium (Invitrogen, USA) to create cell suspensions. Released cells were collected through a centrifugation (400 g for 5 min). After discarding the supernatant, 2 ml of distilled water was added to the cell pellet to lyse erythrocytes and gently mixed several times with a pipette. Subsequently, remaining head kidney leukocytes were washed twice by centrifugations with L15 medium. Finally the cells were dispersed in L15 medium containing 20% fetal bovine serum. Cell concentration and viability were determined by trypan blue dye exclusion with a hemocytometer. Viability of cells was approximately 90%.

Primary HKLs were randomly divided into LPS-treated or Poly I:C-treated group (1×10^6 cells/well) and untreated group. The treated cells were incubated with LPS or Poly I:C at a dose of 10 μl, whereas the control cells were not treated. Samples were collected at 0 h, 6 h, 12 h, 18 h from all groups. Three wells were sampled from each group at each time point.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the sampled tissues of humphead snapper using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The quality of total RNA was detected by electrophoresis on 1% agarose gel. The first-strand cDNA synthesis was carried out according to protocols of M-MLV Reverse Transcriptase (Promega, USA) after DNaseI treatment (Promega, USA). The RNA isolated from spleen was used for templates synthesis of RACE PCR through SMARTer RACE cDNA Amplification Kit (Clontech, USA) after DNaseI treatment (Promega, USA).

2.4. Cloning of cDNA sequence

To clone the partial sequence of snapper TRAF3(Ls-TRAF3), degenerate primers were designed based on a multiple sequence alignment of the fish TRAF3 genes reported in GenBank, including those of *Oncorhynchus mykiss*, *Oreochromis niloticus and Danio rerio.* Using primers LsTRAF3-DS/DA (Table 1), a 190 bp cDNA sequence was obtained. The PCR products were purified, ligated into the pMD18-T vector (TaKaRa, Japan) and cloned. Then the positive clones were sequenced by SANGON BIOTECH (Shanghai, China).

To amplify the full-length sequence of Ls-TRAF3, the 5′ and 3′ ends of the Ls-TRAF3 cDNA were amplified following the manufacturer's protocol of SMARTer RACE cDNA amplification kit (Clontech, USA). The sequences of the primers used were listed in Table 1. The RACE PCR condition and assembly of Ls-TRAF3 cDNA were performed as mentioned in Ref. [33].

2.5. Bioinformatics

The similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide and predicted amino acid sequences of Ls-TRAF3 were analyzed using Genetyx 7.0 software. The protein motif features were predicted by Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Multiple-sequence alignment of the reported TRAF3 amino acid sequences was performed using ClustalX2.0 and a phylogenetic tree was constructed using the MEGA 5.0 software.

2.6. Real-time PCR

After total RNA was extracted as described above, cDNA was prepared using TransScript First-Strand cDNA Synthesis SuperMix (Trans Gen, beijing). Then the expression of Ls-TRAF3 was detected in different tissues. qRT-PCR was operated on IQ5 Real-time PCR System (Bio-Rad laboratories) with SYBR Green Master mix (TOYOBO). Gene-specific primers were designed based on the sequences from transcriptome library of *L. sanguineus* spleen constructed in our laboratory (unpublish data Table 1). Reactions were performed in a final volume of 20 μl, comprising of 1 μl cDNA sample, 10 μl SYBR Green Master mix, and 0.8 μl each primer. The PCR conditions were as follows: 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Each sample was assayed in triplicate. The specificity of the PCR products was confirmed by melting curve analysis and sequencing. The qRT-PCR data was analyzed by $2^{-\triangle\triangle Ct}$ method [34]. The results were expressed as mean ± SD and statistical analysis was performed using SPSS software.