Fish & Shellfish Immunology 36 (2014) 467–474

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Molecular cloning and expression analysis of two lipopolysaccharide-induced TNF-α factors (LITAFs) from rock bream, *Oplegnathus fasciatus*

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ARTICLE INFO

Article history:
Received 8 October 2013
Received in revised form
17 December 2013
Accepted 19 December 2013
Available online 3 January 2014

Keywords:
LITAF
Oplegnathus fasciatus
Edwardsiella tarda
Streptococcus iniae
Red seabream iridovirus

ABSTRACT

Lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α factor (LITAF) plays an important role controlling the expression of TNF-α and the other cytokine genes in the presence of LPS. However, two LITAF homologues have not been characterized in fish. In this study, we cloned two distinct LITAF (RbLITAF1 and RbLITAF2) cDNAs from rock bream (*Oplegnathus fasciatus*) and characterized their expression profiles after infection with *Edwardsiella tarda*, *Streptococcus iniae* or red seabream iridovirus (RSIV). The coding regions of RbLITAF1 and RbLITAF2 cDNAs were 492 bp and 417 bp, encoding 153 and 138 amino acid residues, respectively. The genes consisted of a LITAF domain. RbLITAF1 was highly expressed in the spleen and heart of healthy rock bream, whereas RbLITAF2 was highly expressed in the gill, intestine and stomach. In spleen, the gene expression of RbLITAF1 and RbLITAF2 were increased until 5 days post-infection (dpi), and then decreased at 7 dpi. In kidney, *E. tarda* and RSIV infection led to induction of the RbLITAF1 gene at 1 dpi, RbLITAF2 gene was down-regulated after pathogen infection. These results suggest that RbLITAFs may be involved in the LITAF-mediated immune response and regulate systemic immune responses against pathogen infection.

1. Introduction

The innate immune response to bacterial pathogen is characterized by immediate release of pro-inflammatory cytokines, which are mediators of the immune system that enable the host to eliminate the pathogen [1]. Among the pro-inflammatory cytokines, tumor necrosis factor (TNF)-α plays an important role in a cytotoxic factor for many malignant cells and in the defense against viral, bacterial and parasitic infections, and in (auto-) immune responses. TNF-α is an important cytokine mediator of immune regulation and inflammation that can cause both beneficial and detrimental effect through its pro-inflammatory and pro-apoptotic effects [5]. TNF-α is synthesized by various cell types upon stimulation with endotoxin, inflammatory mediators, cytokines or by TNF itself [2–4]. TNF-α transcription is regulated by different transcription factors, such as nuclear factor (NF)-κB [6], Ets [7], NF-AT [8], activator protein-1 [9], cAMP response element-binding protein [10], and C/EBPβ [11,12].

Lipopolysaccharide (LPS)-induced TNF-α factor (LITAF) was initially identified and characterized in human macrophage cell line THP-1 as a novel transcription factor mediating TNF-α gene expression in the presence of LPS [1,13]. LPS is a major component of the outer membranes of Gram-negative bacteria [13] and is composed of a carbohydrate “O-antigen”, an “oligosaccharide core region” and a lipid portion termed “lipid A” that elicits a strong innate immune response in mammals and confers the endotoxic properties of LPS [14,15].

In mammals, the LPS recognition by the toll-like receptor 2 (TLR2) or TLR4 induced inflammatory response through its complex signaling cascade. TLR2 and TLR4 recognized *Porphyromonas gingivalis* LPS and *Escherichia coli* LPS, respectively [16]. TLR2 and TLR4 share the myeloid differentiation primary-response protein 88 (MyD88) adaptor protein, which is upstream of the transcription

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http://dx.doi.org/10.1016/j.fsi.2013.12.023

factors. Their signaling by MyD88 is mainly divided into two pathways based on the transcription factor: MyD88-dependent LITAF signaling pathway and MyD88-dependent NF-κB signaling pathway [17–19]. The former differs from the latter in the response to LPS. Nuclear translocation of LITAF leads to binding to the CTCCC DNA sequence of the TNF-α promoter through formation of a complex with LITAF-STAT6B and regulates TNF-α transcription [19,20]. In addition, the LITAF-STAT6B complex induces other inflammatory cytokines including GRO, interferon-γ, interleukin (IL)-1α, IL-10, regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein 2 [12].

LITAF homologues have been cloned in different vertebrate and invertebrate species including mice [19], chickens [21], grass carp (*Ctenopharyngodon idella*) [22], Pacific oyster (*Crassostrea gigas*) [23], pearl oyster (*Pinctada fucata*) [24], razor clam (*Solen grandis*) [25], zhikong scallop (*Chlamys farreri*) [26], disk abalone (*Haliotis discus discus*) [27] and whiteleg shrimp (*Litopenaeus vannamei*) [28,29]. The function of LITAF has been characterized in mice and humans. Gene expression profiles and gene structural characterization of LITAF in invertebrate species have been demonstrated in several studies. However, information on the characterization and function of LITAF in fish species is very limited, although the LITAF sequences in several fish species are available in GenBank. Two homologues of LITAF have not been characterized in fish. Therefore, molecular cloning and characterization of the fish LITAF should help elucidate the mechanism of the immune response in fish. In this study, we identified and characterized two types of LITAF in rock bream (*Oplegnathus fasciatus*) (RbLITAF). Furthermore, RbLITAF expression was analyzed by quantitative real-time polymerase chain reaction (PCR) following infection with *Edwardsiella tarda*, *Streptococcus iniae* and red seabream iridovirus (RSIV).

2. Materials and methods

2.1. Molecular cloning of RbLITAF1 and RbLITAF2

The full-length sequences of RbLITAF1 and RbLITAF2 cDNAs were obtained by analyzing expressed sequence tags (ESTs) in the cold shock-stimulated rock bream erythrocyte library (unpublished data). This analysis revealed that the sequences were homologous to other known LITAFs, and were therefore denoted as RbLITAF1 and RbLITAF2. Primer-walking methods were conducted using an ABI 3730 automatic DNA Sequencer (Life Technologies, Carlsbad, CA, USA) and an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit to identify the full-length LITAF cDNAs (Life Technologies).

2.2. Sequence analysis of RbLITAF1 and RbLITAF2

The functionally important domains of RbLITAF1 and RbLITAF2 were determined using the SMART program. The deduced amino acid sequence of the LITAF genes was compared to those of known LITAF genes in the GenBank database using the Clustal W program. A phylogenetic tree analysis based on the entire LITAF amino acid sequence in rock bream and other species was constructed by the neighbor-joining algorithm in MEGA software ver. 4.0. Bootstrap sampling was performed with 1000 replicates.

2.3. Gene expression of RbLITAF1 and RbLITAF2 in healthy fish

Peripheral blood leukocytes (PBLs) were isolated using Percoll density gradients. Head kidney, trunk kidney, spleen, liver, intestine, gill and muscle were isolated from healthy rock bream. Total RNA was extracted using TRIzol reagent (Life Technologies). cDNA was synthesized from the RNA template using a First-strand cDNA Synthesis Kit (Takara, Kyoto, Japan). Real-time PCR was performed with SYBR Green Master Mix (Takara), following the manufacturer's protocol. Real-time PCR was carried out with cDNA templates of each organ and specific primer sets of RbLITAF1 and RbLITAF2 (Table 1). Relative expression levels of RbLITAFs mRNAs were determined using the rock bream EF-1α gene as an internal reference by the comparative Ct ($2^{-\Delta\Delta Ct}$) method, according to the Thermal Cycler DICE Real-Time System (Takara). The level of gene expression was normalized to expression level of EF-1α gene and was expressed as fold change relative to the value of the lowest expression among the tested tissues. Significant differences in gene expression among tissues were determined by analysis of variance (ANOVA) compared to values for muscle (RbLITAF1) and PBLs (RbLITAF2).

2.4. Effects of S. iniae, E. tarda and RSIV infection on RbLITAF1 and RbLITAF2 gene expression

Healthy rock bream (body length: 11–13 cm) were challenged with an intraperitoneal injection of pathogenic *S. iniae*, *E. tarda* and RSIV, which were adjusted to 1.5×10^5, 1.5×10^5 cells/fish and 1.1×10^4 copies/fish in phosphate-buffered saline (PBS), respectively. Control fish were injected with PBS alone. Bacteria- and virus-infected fish were maintained in seawater at 20 °C. At 1, 3, 5 and 7 days post-infection (dpi), kidney and spleen were collected from three fish in each group. The RNA extraction, cDNA synthesis and real-time PCR were performed as described above. Relative expression levels of RbLITAF1 and RbLITAF2 genes were normalized to the expression of EF-1α gene, and were expressed as fold changes relative to the control value. The significance differences between gene expression levels of the infected and control groups were evaluated by ANOVA.

3. Results

3.1. Characterization of RbLITAF1 and RbLITAF2 cDNAs

The full-length cDNA sequences of RbLITAF1 and RbLITAF2 were 913 bp and 923 bp, respectively. The RbLITAF1 cDNA contained an open reading frame (ORF) of 492 bp encoding 153 amino acid (aa) residues. The 5′-untranslated region (UTR) of RbLITAF1 cDNA was 153 bp in length, and the 3′-UTR was 268 bp with a poly-A tail (Fig. 1A, GenBank accession no. AB747088). The RbLITAF2 is constituted by an ORF of 417 bp encoding 138 aa, a 5′-UTR of 39 bp and a 3′-UTR of 532 bp with a poly-A tail (Fig. 1B, GenBank accession no. 747089).

3.2. Multiple alignment and phylogenetic analysis of RbLITAF1 and RbLITAF2

The C-terminal region of the RbLITAFs shows much higher homology than that of other regions in multiple alignment

Table 1
PCR primers used in this study.

Primer	Primer sequence (5′—3′)
LITAF1	
LITAF1 F	TGATGTCCCAGGACAAACTG
LITAF1 R	TCAATGCAGAACGGGATACA
LITAF2	
LITAF2 F	GATGCTCACCTGGCTGATCT
LITAF2 R	GTTACAGGAGGGGCAGGAAT
EF-1α	
EF-1α F	CCCCTGCAGGACGTCTACAA
EF-1α R	AACACGACCGACGGGTACA