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Genomic identification and molecular characterization of a non-mammalian *TNFAIP8L2* gene from *Oplegnathus fasciatus*

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ABSTRACT

Tumor necrosis factor alpha-induced protein 8-like 2 (TNFAIP8L2) is a newly described negative immune regulator, whose enigmatic biological functions are not clearly understood. In the present study, the *TNFAIP8L2* homolog of rock bream (*Oplegnathus fasciatus*) was identified and characterized. The genomic composition of rock bream *TNFAIP8L2* (~6.7 kb) represented a tripartite arrangement in which three exons are interrupted by two introns. The rock bream *TNFAIP8L2* transcript (1974 bp) possessed a coding sequence of 561 bp encoding a peptide of 186 amino acids. The predicted rock bream TNFAIP8L2 protein was 21.1 kDa and revealed the typical features of known TNFAIP8L2 members including the DED-like domain. Rock bream TNFAIP8L2 was composed of six α-helices and demonstrated a distinct folding pattern of the TNFAIP8L2 family. It showed a certain degree of homology and phylogenetic relationship with the corresponding tilapia counterpart. Based on an interspecies genomic organizational comparison of *TNFAIP8L2* orthologs, they could be classified into two classes, with teleost and non-teleost origin respectively. While teleost *TNFAIP8L2*s manifest a tripartite arrangement, non-teleost counterparts demonstrate a dipartite structure suggesting the loss of an intron during the post-piscine speciation. Promoter proximal region of rock bream *TNFAIP8L2* consisted of multiple immune responsive *cis*-regulatory elements. Analysis of basal transcription in eleven tissues revealed its constitutive expression in examined tissues with highest magnitude in the head kidney. The modulated temporal mRNA expression of rock bream *TNFAIP8L2* in head kidney post-challenges with stimulants (LPS and poly I:C) and pathogens (*Streptococcus iniae* and irido virus) was stimulant-specific. Additionally, a drastic down-regulation of rock bream *TNFAIP8L2* mRNA level occurred in blood cells collected from experimentally injured animals, and it was accompanied by a contemporaneous down-regulation of cytokines, *TNF*-α and *TGFβ3*. All these findings imply that rock bream *TNFAIP8L2* is potentially responsible for immune and inflammatory modulation in rock bream.

1. Introduction

The immune system often encounters numerous alien antigens and evokes a series of specific reactions typically characterized by activation and proliferation of immune cells, and expression of inflammatory genes, eventually leading to a common phenomenon known as inflammation. Restoration of the immune system to its basal state after the removal of antigens ensures protection during subsequent new antigen encounter(s). In order to maintain immune homeostasis, these immune reactions must be under tight regulation in terms of intensity and duration, since consequences of uncontrolled inflammation may lead to severe tissue damage and death. Two classes of molecules maintaining immune homeostasis are described so far: (1) inhibitory cytokines, negative modulators of immune receptors, and signal pathways and repressive transcription factors which limit the activation and proliferation of immune cells; and (2) molecules that control cell death (Van Parijs and Abbas, 1998).

Tumor necrosis factor (TNF)-α-induced protein 8 (TNFAIP8) is a recently identified family of proteins (Freundt et al., 2008; Sun et al.,

Abbreviations: TNF-α, tumor necrosis factor alpha; TNFAIP8L2/TIPE2, Tumor necrosis factor alpha-induced protein 8-like 2; DED, dead effector domain; CDS, coding sequence; aa, amino acid; CDD, Conserved Domain Database; NJ, neighbor-joining; TFBS, transcription factor binding sites; LPS, lipopolysaccharide; BHI, brain heart infusion; PBS, phosphate buffered saline; $TCID_{50}$, tissue culture infectious dose 50; qPCR, quantitative real-time PCR; SD, standard deviation; UTR, untranslated region; TSS, transcription start site.

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2008), playing multiple regulatory roles related to apoptosis, cancer, inflammation and immunity (Lou and Liu, 2011). To date, four different sub-classes of the TNFAIP8 family, namely TNFAIP8, TNFAIP8L1, TNFAIP8L2 and TNFAIP8L3 (TNFAIP8-like 1, 2 and 3) have been identified. The TNFAIP8 family members share a high degree of sequence homology and are crucial for maintaining immune homeostasis (Sun et al., 2008). Notably, except in a dead effector domain (DED)-like region, TNFAIP8 family members share no significant homology with other known protein families; and hence, they are classified as a new class of DED-like region containing proteins (Lou and Liu, 2011). Differential mRNA distribution of these members in different rat organs and their association with diabetic nephropathy has been determined (S. Zhang et al., 2010). Regardless of the structural relatedness of TNFAIP8 members, experimental evidence was provided, that they were responsible for distinct biological function(s) (Lou and Liu, 2011).

Following the identification of TNFAIP8, TNFAIP8L2 (TIPE2) was originally discovered from a mouse model for autoimmune inflammation with a characteristic abnormal expression profile (Carmody et al., 2002). Although TNFAIP8L2 reveals appreciable homology with other TNFAIP8 proteins, and consists of a putative DED-like region, the crystal structure of TNFAIP8L2 revealed a previously uncharacterized fold with a central cavity for potential cofactor binding, varying from the typical 3D fold of DED (Zhang et al., 2008). It has been shown that, while TNF-α induces the transcription of *TNFAIP8L2*, the protein expression of TGFβ3 is governed by *TNFAIP8L2* (Luan et al., 2011; Sun et al., 2008). Several experimental findings support the strong notion that TNFAIP8L2 is an essential negative regulator of inflammation and immune homeostasis (Sun et al., 2008), which has also been implicated with several immuno-pathophysiological conditions (Li et al., 2009; Xi et al., 2011; S. Zhang et al., 2010). However, mechanism of action and other roles of TNFAIP8L2 remain incompletely characterized.

Regardless of many studies focusing on mammalian *TNFAIP8L2* (Li et al., 2010; G. Zhang et al., 2010, 2011), no lower order vertebrate homologs have been studied at molecular and genomic levels so far. The present study describes a piscine *TNFAIP8L2* from the rock bream (*Oplegnathus fasciatus*), a popular delicacy in Southeastern Asia. We determined the genomic structure of rock bream *TNFAIP8L2*, and predicted its protein structure and possible *cis*-regulatory elements of transcriptional expression. In addition, mRNA expression was examined under normal state, injury condition and established pathological conditions using different immune stimulants to demonstrate the possible involvement of *TNFAIP8L2* in the innate immunity of rock bream.

2. Materials and methods

2.1. Experimental fish and rearing conditions

Healthy rock breams (~50 g) were obtained from Jeju Special Self-Governing Province Ocean and Fisheries Research Institute (Jeju, Republic of Korea) and maintained in 400 L tanks with aerated sand-filtered seawater (salinity 34 ± 1 psu, pH 7.6 ± 0.5 at 24 ± 1 °C). Animals were acclimatized to laboratory conditions for one week prior to any experimentation.

2.2. Identification of cDNA- and genomic-sequences of rock bream TNFAIP8L2

We have built a normalized multi-tissue shotgun transcriptome library for rock bream, based upon sequence data using the Roche 454, GS-FLX™ titanium system (Umasuthan et al., 2011). A cDNA sequence orthologous to the known *TNFAIP8L2* counterparts was identified from the transcriptome library by BLAST search on NCBI and designated as rock bream *TNFAIP8L2*. The coding sequence (CDS) was cloned into T-Vector pMD20 (TaKaRa), using the gene specific primers flanking the CDS (F1, 5′-GTTCTGCTCGTGATGAAGATGAAGGGAT-3′ and R1, 5′-CTCC TCTCCAGAATACATGACATCAGTAGTTCTG-3′) and sequenced (Macrogen) in order to confirm the sequence of rock bream *TNFAIP8L2*.

Recently, we constructed a custom BAC library using rock bream genomic DNA by random shearing approach (Lucigen®, USA; Umasuthan et al., 2012). The positive BAC clone bearing the rock bream *TNFAIP8L2* was localized by a two-step PCR-based screening of BAC library with gene-specific primers (F2, 5′-GATTGCTGTGAAGATTGGCGTGCT-3′ and R2, 5′-AGCTCCTCCATCACTGCATTGTCA-3′) following the manufacturer's instructions. Then, the positive clone was sequenced by GS-FLX™ system (Macrogen) and the complete genomic sequence of rock bream *TNFAIP8L2* was obtained.

2.3. Computer assisted sequence analysis

The searches for orthologous nucleotide and deduced amino acid (aa) sequences were carried out at the NCBI using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) and/or Ensembl database. The deduced polypeptide sequence of rock bream TNFAIP8L2 was analyzed using the ExPASy tools (http://www.Expasy.Org/), and the protein domain profiles were examined at Conserved Domain Database (CDD) of NCBI (http://www.ncbi.nlm.nih.gov/cdd). All the sequence alignments were performed in pairwise or multiple modes using ClustalW2. The genome organization and exon–intron junctions were inferred by aligning the cDNA with the respective genomic sequence of rock bream *TNFAIP8L2* and/or orthologous genes using the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/). To construct the 3D structure, aa sequence of rock bream TNFAIP8L2 was submitted to the automated platform of Swiss-Model (http://swissmodel.expasy.org/), and a crystal structure of human TNFAIP8L2 with a resolution of 1.7 Å (PDB, 3F4M) which shared a 78.5% similarity with rock bream TNFAIP8L2 was chosen as the template. Generated models were visualized using PyMOL system (DeLano, 2002). A neighbor-joining (NJ) phylogenic tree was reconstructed using MEGA 5.0 platform based on an alignment of 40 orthologous TNFAIP8 aa sequences. Bootstrap values of 5000 generations were calculated to estimate the reliability of the tree. A 2.1 kb of 5′-flanking promoter proximal region was subjected to predict the potential *cis*-acting elements and immune responsive transcription factor binding sites (TFBS) with the following online tools: TFSAERCH (http://www.cbrc.jp/research/db/TFSEARCH.html), TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and AliBaba (http://www.gene-regulation.com/pub/programs/alibaba2/index.html).

2.4. Tissue sampling, RNA extraction and cDNA synthesis

In order to investigate the mRNA distribution profile of rock bream *TNFAIP8L2*, different tissues including the gill, liver, heart, spleen, intestine, head kidney, kidney, skin, muscle and brain were examined. Tissues were aseptically sampled from three healthy fish (n = 3) following the withdrawal of blood from each fish (~1 mL fish^{-1}) using sterile syringes. Blood cells were harvested by centrifugation (3000 ×*g* at 4 °C for 10 min). All the tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C. A 50 mg sample of each tissue from three individuals was mixed and the tissue pool of 150 mg was then subjected to the total RNA extraction using TriReagent™ (Sigma). A 2.5 μg of RNA sample was reverse transcribed into cDNA using PrimeScript RTase derived from M-MLV (PrimeScript™ first-strand cDNA synthesis kit, TaKaRa) following the vendor's protocol.

2.5. Immune and injury challenges and tissue collection

To examine the immune response of rock bream *TNFAIP8L2*, we devised five immune challenge experiments using lipopolysaccharide (LPS), poly I:C, *Streptococcus iniae* and rock bream iridovirus (RBIV) as described in our previous report (Umasuthan et al., 2012).

Bacterial strain was cultured in brain heart infusion (BHI) broth supplemented with 1% sodium chloride at 30 °C for 12 h. Cells were harvested and resuspended in sterile phosphate buffered saline (PBS) prior to dilution. RBIV infected kidney tissue was obtained from moribund