Fish & Shellfish Immunology 35 (2013) 1829–1837

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Genomic structure and immunological response of an STAT4 family member from rock bream (*Oplegnathus fasciatus*)

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ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form
4 September 2013
Accepted 5 September 2013
Available online 18 September 2013

Keywords:
Rock bream
STAT4
Genomic analysis
Immune challenge
Gene expression analysis

ABSTRACT

The Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a critical role in host defense against viral and bacterial infections. STAT proteins are a group of transcription factors that translocate into the nucleus and are critical for the induction of many genes crucial for the allergic cascade and immune defense. In the present study, a member of the STAT4 family was identified from rock bream (*RbSTAT4*) at the genomic level, and its transcriptional regulation in response to different pathological stimuli under *in vivo* conditions was investigated. The genomic sequence of *RbSTAT4* is approximately 15.6 kb in length, including a putative core promoter region and 24 exons interrupted by 23 introns. Bioinformatics analysis of RbSTAT4 identified the presence of typical and conserved features of the STAT4 family, including the STAT_int domain, STAT alpha domain, STAT bind domain, linker domain, SH2 domain, and transcriptional activation domain. According to the phylogenetic analysis, RbSTAT4 exhibited the closest evolutionary proximity with the STAT4 member from mandarin fish (*Siniperca chuatsi*). The *RbSTAT4* transcript in healthy rock breams was detected to have ubiquitous expression in 11 different tissues examined, where liver and spleen tissues showed moderate expressions compared with the highest expression level detected in gill tissue. The time-course *in vivo* immune stimulation of rock bream with lipopolysaccharide, poly I:C, live *Edwardsiella tarda*, and rock bream iridovirus caused significant transcriptional regulation of the *RbSTAT4* expression in gill, head kidney, and spleen tissues, suggesting that *RbSTAT4* is involved in immune regulation mechanisms and/or signaling cascades, orchestrating against both bacterial and viral pathogens.

1. Introduction

Mariculture is a rapidly developing industry worldwide, providing valuable sources of essential fatty acids and essential amino acids along with enriched sources of other nutrients. Many edible fish species are produced either through culturing or capture fisheries. Rock bream (*Oplegnathus fasciatus*) is one of the most economically important and highly consumed fish species, especially in eastern Asia. However, production losses have also increased in recent years with the growing and intensified mariculture industry owing to the occurrence of many infectious diseases. In particular, Edwardsiellosis caused by *Edwardsiella tarda* [1] and iridoviral disease caused by rock bream iridovirus (RBIV) [2] occur frequently, causing a significant mortality of farmed rock breams. In this regard, disease control plays an important and critical role to minimizing production losses, either by pathogen control with chemotherapeutics or by host control with vaccines and immunostimulants [3]. Studies on fish immunogenetics will provide more precise approaches to develop new strategies for efficient disease control.

The immune system of an organism plays a critical and indispensable role, where the innate immune system of fish relies on both cellular and humoral responses that are mediated via the activation of several signaling pathways that have already been identified in mammals [4]. The Janus tyrosine kinase (JAK)/signal

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http://dx.doi.org/10.1016/j.fsi.2013.09.011

transducer and activator of transcription (STAT) signaling pathway plays a critical role in host defense against viral and bacterial infections. The JAK/STAT signaling cascade is activated as a response to the various chemical signals induced by interferons (IFNs), interleukins (ILs), growth factors, or other chemical messengers. STAT proteins are a group of transcription factors that transmit signals to the nucleus and are critical for the induction of many genes crucial for the development of allergic inflammations and immune defense [5]. The members of the STAT family have been found to be involved in cell proliferation, apoptosis, survival, immune functions, and certain aspects of tissue differentiation [6,7]. In mammals, STAT proteins are grouped into 7 families (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), where each STAT member has been found to be bound to different DNA sequences in the promoter region of the target gene.

STAT4 is an important element in mediating IL-12 responses, and it has been most extensively investigated in both human and murine T lymphocytes [8–10]. Many studies have reported that Th1 cell differentiation requires STAT4 activation through the IL-12 signaling cascade [11,12]. Moreover, STAT4 was reported to bind directly with genes involved in Tfh cell differentiation, including *Bcl6* and *IL-21* [13]. STAT4 was also reported to function in conjunction with STAT1 in order to produce IFN-γ and to enhance the expression of T-box transcription factor (T-bet) in Th1 cell differentiation through the IL-12 signaling cascade [14]. Although STAT4 is activated by both IL-12 and type I IFN-α/β in humans, it is activated only by IL-12 in mice [15–17], and it was also reported that STAT4 is activated by IFN but not by IL-12 in human vascular endothelial cells [18]. In addition to T lymphocytes, STAT4 was also reported to be expressed by different immune cells, including B lymphocytes [19], natural killer (NK) cells [20], dendritic cells, monocytes, and macrophages [21,22]. However, the role of STAT4 in teleosts is not well understood, and no STAT or IL genes from rock bream have been identified yet. In this study, the genomic organization of the STAT4 gene from rock bream was identified and its temporal mRNA expression was investigated in animals challenged with different immune stimuli derived from both bacterial (*E. tarda* and lipopolysaccharide (LPS)) and viral origins (rock bream iridovirus and poly I:C).

2. Materials and methods

2.1. cDNA sequence identification and bioinformatics analysis

The complete cDNA sequence of rock bream STAT4 (*RbSTAT4*) was identified from the rock bream multi-tissue normalized cDNA GS-FLX database, as described in our previous report [23]. The complete cDNA sequence was used to analyze the open reading frame (ORF) sequence by using the DNAssist version 2.2 software. Both the nucleotide and amino acid sequences were subjected to a homology search using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/), and functional domains were identified in the protein sequence by analyzing with the Conserved Domain Database (CDD) at the NCBI (http://www.ncbi.nlm.nih.gov/cdd). Identity and similarity percentages were calculated by comparing with other known STAT4 members from different species identified from a BLAST search by using EMBOSS Needle—Pairwise sequence alignment at the amino acid level. Multiple sequence alignment was carried out with STAT4 orthologous sequences by using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed by applying the neighbor-joining method available in the MEGA version 5.0 program, which represents all STAT family members in different taxonomic classes with bootstrap values from 1000 replicates.

2.2. Genomic sequence comparison and promoter sequence analysis

The genomic sequence of *RbSTAT4* was identified from a rock bream bacterial artificial chromosome (BAC) library by screening with the polymerase chain reaction (PCR) and a sequencing-based method, as described in our previous report [24]. Genomic DNA (gDNA) and cDNA sequences were analyzed using the Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) in order to identify the intron–exon structure of *RbSTAT4*. The genomic structures of other species obtained from the Ensembl Genome Browser (http://asia.ensembl.org/index.html) were mapped using the GeneMapper version 2.5 software and compared with the *RbSTAT4* genomic structure. A putative core promoter sequence of around 940 bp from the transcription start site towards the upstream of the gene was analyzed with the TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and Alibaba 2.1 Transcription Factor Binding Prediction (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) programs to locate the potential transcription factor binding sites.

2.3. Experimental animals and tissue isolation

Rock breams (mean weight 50 g), provided by the National Fisheries Research and Development Institute, Republic of Korea, were maintained in our laboratory in 400-L tanks under controlled conditions at 24 °C with sand-filtered aerated seawater. The fish were acclimatized for a period of 1 week under laboratory conditions and fed daily with standard commercial feed. A total of 11 different tissue samples (muscle, blood, brain, intestine, kidney, head kidney, heart, liver, spleen, gill, and skin) were collected for total RNA extraction. Blood samples (approximately 1 mL/fish) were collected from the caudal vein using a 22 G syringe, and samples were immediately centrifuged at 3000 × *g* at 4 °C for 10 min to separate the blood cells. All the tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until used for the RNA extraction.

2.4. Total RNA extraction and cDNA synthesis

Pooled tissue samples from 3 fish (50 mg/fish) were used for the total RNA isolation using TRIzol reagent (Sigma–Aldrich), according to the manufacturer's protocol. Purified RNA samples were diluted to 1 μg/μL, and 2.5 μg of RNA from each tissue was used for cDNA synthesis using the PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Japan) following the manufacturer's protocol. The resultant cDNA was then diluted 40-fold (total 800 μL) before storage at −20 °C.

2.5. Tissue-specific gene expression analysis by qPCR

To analyze the tissue-specific *RbSTAT4* gene expression, 11 types of tissues were collected from 3 healthy rock breams, and the respective cDNAs were synthesized as described in Sections 2.3 and 2.4. Gene expression analysis was carried out by the quantitative real-time PCR (qPCR) technique using the following gene-specific primers: sense primer 5′-TTGTGAGTAAAGAGATGGAGCG-3′ and anti-sense primer 5′-AACTTCACCTCCCCATTGTC-3′. Briefly, the reaction was carried out in a 15 μL reaction volume containing 4 μL of cDNA from each tissue, 7.5 μL of 2 × TaKaRa Ex Taq™ SYBR premix, 0.6 μL of each gene-specific primer (10 pmol/μL), and 2.3 μL of PCR-grade H_2O. The qPCR cycle program consisted of 1 cycle of 95 °C for 10 s, followed by 45 cycles of 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 20 s, and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The baseline was set automatically to maintain consistency,