Fish & Shellfish Immunology 70 (2017) 140–148

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Molecular characterization and expression analysis of interferon-gamma in black seabream *Acanthopagrus schlegelii*

Yangxi Xiang [1], Wei Liu [1], Peng Jia, Yunlong Li, Yilin Jin, Limin Chen, Jing Zhang, Kuntong Jia**, Meisheng Yi*

Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering, Zhuhai Key Laboratory of Marine Bioresources and Environment, School of Marine Sciences, Sun Yat-sen University, Guangdong, China

ARTICLE INFO

Article history:
Received 8 June 2017
Received in revised form
23 August 2017
Accepted 25 August 2017

Keywords:
Black seabream
IFN-gamma
Red spotted grouper nervous necrosis virus
JAK-STAT
ISG

ABSTRACT

Interferon gamma (IFN-γ) is a major component in immunological signaling and plays a key role in resisting viral infection. In this study, we identified and characterized an IFN-γ gene (*As*IFN-γ) in the marine fish black seabream (*Acanthopagrus schlegelii*). We cloned *As*IFN-γ genomic sequence, which comprises four exons, three introns and an upstream promoter including several conserved regulatory elements. The complete cDNA of *As*IFN-γ was 816 bp in length and encoded a putative 194 amino acids (aa) protein with a 22 aa signal peptide, six α-helices and one nuclear localization signal (NLS). Multiple alignment showed that *As*IFN-γ protein shared 31–60% identity with IFN-γ of other fish but low identity with fish IFN-γrel and IFN-γ of other vertebrates. *As*IFN-γ was constitutively expressed in all examined tissues with the highest expression level in immune organs, such as spleen, gill and kidney. In black seabream infected by red spotted nervous necrosis virus (RGNNV), the expression of *As*IFN-γ was significantly up-regulated in most tissues, and RGNNV infection *in vitro* also induced significant up-regulation of *As*IFN-γ, indicating that *As*IFN-γ was involved in immune response to RGNNV infection. Overexpression of *As*IFN-γ in cultured *Acanthopagrus schlegelii* brain (AsB) cells rapidly and transiently stimulated the expression of JAK-STAT signaling pathway related genes including STAT1, STAT2 and IRF9, as well as the downstream antiviral genes MX1 and ISG15. Furthermore, overexpression of *As*IFN-γ was able to significantly inhibit RGNNV replication and virus production in AsB cells. In summary, we identified a conserved IFN-γ gene of black seabream, and demonstrated the rapid and strong antiviral activities of *As*IFN-γ against RGNNV in black seabream.

1. Introduction

Interferon (IFN) response, the first line of defense in innate immunity, protects the host from uninvited pathogens. The IFN family has been classified into three major types according to their genetic loci, protein structures, receptors, and the cell types in which they are distributed [1]. Type I IFN primarily consists of IFN-α/β and some subtypes such as IFN-ε, IFN-ω, and IFN-κ. Type III IFN, also named IFN-λ, has three family members IFN-λ1/2/3. Type II IFN has only one member IFN-γ in most vertebrates [2]. Type I and III

Abbreviations: AP-1, activator protein 1; bp, base pair; CaMKII, calcium/calmodulin kinase II; GAS, gamma IFN-activated sequences; ORF, open reading frame; GATA3, GATA-binding protein 3; hpi, hour post infection; IFN, interferon; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; ISRE, interferon-stimulated response element; ISGF3, IFN-stimulated gene factor 3; JAK-STAT, Janus kinase-signal transducers and activators of transcription; MAP kinase, mitogen activated protein kinase; MX, myxovirus resistance protein; NF-κB, nuclear factor kB; NLS, nuclear localization signal; OCT1, octamer transcription factor-1; PBS, phosphate buffer solution; PI3-K, phosphoinositide 3-kinase (PI3-K)/Akt; Poly I:C, polyinosinic-polycytidylic acid; RDRP, RNA-dependent RNA polymerase; RGNNV, red spotted grouper nervous necrosis virus; qRT-PCR, quantitative real time polymerase chain reaction; $TCID_{50}$, 50% tissue culture infective dose; UTR, untranslated region.

* Corresponding author.
** Corresponding author.
E-mail addresses: xiangyx6@mail2.sysu.edu.cn (Y. Xiang), liuw68@mail.sysu.edu.cn (W. Liu), 825143529@qq.com (P. Jia), 932920971@qq.com (Y. Li), jinyilin915@126.com (Y. Jin), 2562492896@qq.com (L. Chen), jingyuansunshine@sina.com (J. Zhang), jiakt3@mail.sysu.edu.cn (K. Jia), yimsh@mail.sysu.edu.cn (M. Yi).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2017.08.046
1050-4648/

IFNs are ubiquitously expressed in different cell types. IFN-γ is mainly produced by activated immune cells, such as natural killer (NK) cells, macrophages, NKT cells and dendritic cells during innate immune response, and $CD4^+$ T helper 1, $CD8^+$ cytotoxic T lymphocyte effector T cells in adaptive immunity [3,4].

IFN-γ is believed to be capable of modulating immune system in pathogen infection [5]. The signaling pathways of IFN-γ in immune response have been widely studied in mammals. Upon pathogen infection, the activated immune cells produce IFN-γ, which binds to its cell surface receptor IFN-γR, to recruit various kinases and subsequently activate a series of signaling cascades and initiate downstream gene transcriptions in a gene- and cell type-specific manner to protect the host from pathogen infection. It has been reported that IFN-γ can activate different signaling pathways including JAK-STAT, MAP kinase, PI3-K, CaMKII and NF-κB in mammals, among which the best characterized is JAK-STAT signaling pathway [6]. In the conventional JAK-STAT signaling pathway, IFN-γ binds to IFN-γR1 and IFN-γR2, resulting in oligomerization of the receptors, which brings the pre-associated JAK kinases JAK1 and JAK2 into close proximity and allows them to *trans*-phosphorylate each other and cytoplasmic domains of IFN-γR. The phosphorylated IFN-γR provides a docking site for SH2 domain of STAT1, which is subsequently phosphorylated by JAK kinase. The phosphorylated STAT1 forms a homodimer, which enters into the nucleus and binds with IRF9 to form an IFN-stimulated gene factor 3 (ISGF3) complex. The ISGF3 complex binds to gamma activated sequence (GAS) or interferon-stimulated response element (ISRE) in the promoter region of downstream interferon-stimulated genes (ISGs) such as viperin, myxovirus resistance protein (MX) and ISG15, and activates their transcription [7].

IFN-γ has been widely studied in mammals, birds, amphibians and reptiles [8–11]. In teleost fish, an IFN-γ homolog has also been cloned and identified in some species such as fugu, rainbow trout and Atlantic cod [12–14]. Unlike in mammals, an additional IFN-γ related (IFN-γrel) homolog was identified in some teleost fish including channel catfish and some cyprinids [15–19]. Although IFN-γrel lacked a classical nuclear localization signal (NLS), it shared many common characteristics with IFN-γ in protein structure, cell distribution and immune response [5]. Both fish IFN-γ and IFN-γrel were capable of protecting host from bacterial infection by stimulating the expression of pro-inflammatory genes including tumor necrosis factor α and interleukins, and enhancing the activation of phagocytic cells by increasing the production of inducible nitric oxide synthase and reactive oxygen intermediates [14,15,20]. Moreover, fish IFN-γ and IFN-γrel played important roles in repressing viral infection, such as spring viremia of carp virus, grass carp reovirus and viral hemorrhagic septicemia virus [15,17,21,22].

Nervous necrosis virus (NNV) is the causative agent of viral nervous necrosis (VNN) disease, which causes massive mortalities of the larvae of many cultured marine fish species [23]. Previous studies provided insights into the involvement of host immune system, especial for IFN response, defense against NNV [23–26]. However, the role of IFN-γ in NNV infection was remained unclear.

Black seabream (*Acanthopagrus schlegelii*), a relatively new aquaculture species with high economic potential, is mainly distributed in Northern Europe and Asia. One of the major threats in the culture of black seabream is VNN disease outbreak [27]. However, little is known about the role of IFN response against NNV infection in black seabream. In this study, we cloned and identified an IFN-γ homolog (*As*IFN-γ) in black seabream, characterized its expression patterns, and investigated its antiviral role in red spotted grouper nervous necrosis virus (RGNNV) infection.

2. Material and methods

2.1. Fish, cell and virus

Black seabream larva (approximately 200 mg/fish) and adult (approximately 200 g/fish) were supplied by Haomiao aquaculture station in Zhuhai, China. Fish were acclimated for one week before experiment as previously described [28]. All procedures with black seabream were approved by the Ethics Committee of Sun Yat-Sen University.

Two cell lines *Acanthopagrus schlegelii* brain (AsB) and fin (AsF) cells have been cultured over 30 passages in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco) at 28 °C (unpublished).

RGNNV was previously isolated and kept in laboratory [29].

2.2. RGNNV challenge in vivo and tissue collection

Each black seabream larva and adult individual was infected by intraperitoneal injection with 30 μl and 300 μl of RGNNV (3×10^7 $TCID_{50}$/ml) respectively, and fish injected with equal amount of PBS were used as controls. Eight tissues including heart, liver, spleen, kidney, brain, gut, eyes and gills were collected. The larval tissues were pooled from 10 individuals at 48 h post RGNNV infection, and the adult tissues were collected individually at 3 days post infection.

2.3. Cloning of AsIFN-γ cDNA

The SMART cDNA library of black seabream spleen was constructed by SMART™ RACE cDNA Library Kit (Takara). Based on multiple alignment of other fish IFN-γ, degenerate primers were designed for cloning the conserved fragment of IFN-γ in black seabream. According to the obtained *As*IFN-γ sequence, gene-specific primers were designed for 3′- and 5′-RACE PCR for the full length of *As*IFN-γ cDNA as previously described [30]. The primer sequences were listed in Table S2.

2.4. Cloning of the AsIFN-γ promoter

A genome walking library of black seabream was constructed by Universal Genome Walker Kit (Takara). The *As*IFN-γ promoter was determined by two rounds nested PCR from the genome walking library. The PCR fragments of *As*IFN-γ were cloned into pMD-18T vector and sequenced. The *As*IFN-γ genomic sequence had been submitted to Genbank (Genbank accession number: KY921614). The primer sequences were listed in Table S2.

2.5. Bioinformatic analysis of AsIFN-γ

The signal peptide was predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The global identities of amino acid were calculated by DNAMAN 7.0. The exon-intron structure of the identified genomic sequences was performed by alignment of the full-length cDNA to the genomic sequence by online BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment of amino acid sequences of vertebrate IFN-γ and fish IFN-γrel were performed by the Bioedit program [31]. The phylogenetic tree based on amino acid sequences of vertebrates IFN-γ and fish IFN-γrel homologs was constructed by MAGE 6.0 using neighbor-joining methods with 1000 replicates [32]. The transcription factor binding sites were predicted using TFBIND (http://tfbind.hgc.jp/).