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Identification and characterization of Japanese flounder, *Paralichthys olivaceus* interferon-stimulated gene 15 (Jf-ISG15)

Motoshige Yasuike, Hidehiro Kondo, Ikuo Hirono, Takashi Aoki*

Laboratory of Genome Science, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo 108-8477, Japan

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ABSTRACT

Previously, using cDNA microarray analysis, we demonstrated that an EST clone of Japanese flounder (*Paralichthys olivaceus*) with homology to mammalian interferon-stimulated gene 15 (ISG15) was strongly induced by treatment with DNA vaccine encoding the glycoprotein gene of Hirame rhabdovirus (HIRRV). In this study, we conducted molecular cloning and expression analysis of the Japanese flounder ISG15 (Jf-ISG15). Jf-ISG15 encoded two exons. The first exon was non-coding, while the second exon encoded a protein of 158 amino acids. The coded protein has two tandem ubiquitin-like domains with a carboxyl-terminus conjugation motif "LRLRGG". Phylogenetic analysis revealed an evolutionary relationship among Jf-ISG15, mammalian and fish ISG15 orthologues. The interferon-stimulated response element (ISRE) sites were conserved among DNA sequences of Jf-ISG15 and mammalian ISG15 promoter regions. An RT-PCR analysis of healthy tissues showed that Jf-ISG15 mRNA was notably strongly expressed in gills, PBLs and spleen. Expression of Jf-ISG15 was strongly induced by poly-I:C treatment in head-kidney cells, peripheral blood leukocytes (PBLs) and spleen cells, and by HIRRV infection in kidney of juvenile fish suggesting that Jf-ISG15 plays a role in fish antiviral response.

1. Introduction

The type I interferon (IFN-α/β) system plays a critical role in innate antiviral immunity in mammals [1] and fish [2–4]. In this system, direct antiviral responses are carried out by induction of hundreds of IFN-stimulated genes (ISGs) [5]. In mammals, ISG15 is one of the most strongly induced genes upon type I IFN stimulation and viral infection [6]. The molecule consists of two ubiquitin-like (UBL) domains in tandem arrangement with a carboxyl-terminus conjugation motif, "Leu-Arg-Gly-Gly (LRGG)" [7]. Through this motif, ISG15 forms an isopeptide bond with cellular targets. This conjugation, called ISGylation, is part of the primary response to type I IFN induction [8]. ISG15 exists both in the free and conjugated forms [8]. Free human ISG15 is secreted from monocytes and lymphocytes and modulates the immune system as a cytokine. After secretion of human ISG15, IFN-γ production is stimulated by $CD3^+$ cells [9], induces the proliferation of $CD56^+$ natural killer cells, and enhances non-major histocompatibility complex-restricted cytotoxicity [10]. Murine ISG15 is reported to recruit neutrophils to a local site and activate them [11]. Murine ISG15 in its conjugated forms modulate type I IFN/JAK-STAT signaling pathways [12]. In human, ISG15 conjugates with a large number of proteins; 158 proteins have been identified so far [13]. However, the role of ISGylation remains unclear.

We recently identified an EST clone of Japanese flounder (*Paralichthys olivaceus*) with homology to mammalian ISG15 that was strongly induced by polyinosinic:polycytidylic acid (poly-I:C) treatment in head-kidney and brain cells (unpublished data). This clone

* Corresponding author. Tel.: +81 3 5463 0556; fax: +81 3 5463 0690.
E-mail address: aoki@kaiyodai.ac.jp (T. Aoki).

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was also up-regulated in fish following injection with DNA vaccines encoding glycoprotein (G protein) gene of Hirame rhabdovirus (HIRRV) [14] and viral hemorrhagic septicemia virus (VHSV) [15]. Together, these results indicate that Japanese flounder ISG15 (Jf-ISG15) has a critical role in the antiviral response. Recently, fish ISG15 orthologues have been cloned from goldfish (*Carassius auratus*) [16], Atlantic salmon (*Salmo salar*) [17], Atlantic cod (*Gadus morhua*) [18], crucian carp (*Carassius auratus*) [19] and black rockfish (*Sebastes schlegeli*) [20]. However, the function of fish ISG15 is not clearly understood.

In this study, we identified a Jf-ISG15 by molecular cloning and analyzed its gene structure, phylogeny and gene expression.

2. Materials and methods

2.1. Cloning and sequencing of Jf-ISG15 cDNA and gene

The full-length Jf-ISG15 cDNA and gene were cloned as previously described [21]. An expressed sequence tag (EST) clone (GenBank accession no. AU260555) showing putative homology to human ISG15 was used as a probe to screen a Japanese flounder kidney cDNA library. Subsequently, the open reading frame (ORF) of the Jf-ISG15 cDNA was used to screen a previously constructed Japanese flounder genomic bacterial artificial chromosome (BAC) library [22]. The sequence of the Jf-ISG15 cDNA and gene were sequenced using ThermoSequenase (Amersham Bioscience, UK) and automated sequencer LC4200 (Li-Cor, USA).

2.2. In silico analysis

The nucleotide sequence and translated amino acids were analyzed and determined using GENETYX ver. 7.0.3 (GENETYX, Japan). Amino acid identities and similarity were calculated using BLASTp (BLOSUM 62) implemented in BLAST 2 SEQUENCES [23]. The complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters [24]. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in the Mega 3.1 software package [25] employing the *P*-distance with 1000 bootstrap tests and with complete deletion of gap sites. GenBank accession numbers of ISG15 amino acid sequences used for the phylogenetic analysis are listed in Table 2. Another two-UBL domain-containing protein, FAT10, was also used for this phylogenetic analysis. Accession numbers are: chimpanzee FAT10, XP_527322; human FAT10, NP_006389; pig FAT10, NP_001153560; murine FAT10, NP_075626; rat FAT10, NP_445751.

The MEME/MAST system version 3 [26] for motif discovery and search was used to predict conserved sequence domain among Jf-ISG15 and other species ISG15 promoter regions.

2.3. RT-PCR analysis of Jf-ISG15 expression

For constitutive expression, total RNA was extracted from brain, eyes, gills, head-kidney, heart, intestine, peripheral blood leukocytes (PBLs), liver, muscle, ovary, skin, spleen, stomach and trunk kidney from three apparently healthy Japanese flounders. For the immunostimulation studies, total RNA was extracted from Japanese flounder head-kidney, spleen and PBLs cultured in RPMI and treated with final concentration of 0.5 mg/ml lipopolysaccharide (LPS) and 5.0 μg/ml poly-I:C sampled at 1, 3 and 6 h post-stimulation [27]. PBS was used as negative control. The expression of Jf-ISG15 during viral (HIRRV) and bacterial (*Edwardsiella tarda*) infection *in vivo* was also examined as previously described [28]. For the HIRRV, 15 fish samples were intramuscularly injected with the virus (3.2×10^3 $TCID_{50}$) and another 15 with PBS for the control. Kidney from 5 samples was taken at 1-day, 3 days and 7 days post-injection. For the *E. tarda* experiment, one tank containing 15 fish samples were infected with the bacteria (2.5×10^7 CFU/ml) by immersion while one untreated tank was used as a control. Kidney from 5 fish samples was sampled at 1-day, 3 days and 7 days post-infection and also from the control. For each of the sampling time points, kidney was pooled and then total RNA was extracted for cDNA synthesis and subsequent RT-PCR analysis.

RT-PCR analyses were performed as previously described [21]. Primer sets for gene amplification are shown in Table 1. The β-actin primer set was used for an internal control. The Japanese flounder interleukin-1β (IL-1β) and Mx primer sets were used for positive controls of the LPS and poly-I:C stimulations, respectively. PCR conditions were: initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and final elongation at 72 °C for 5 min. The PCR products (5 μl) were electrophoresed and visualized in a 1.0% agarose gel stained with ethidium bromide using densitometer (ATTO, Japan). The intensity of the amplification bands was semi-quantitatively measured using ImageJ software [29] and divided by the intensity of the respective β-actin signals. The data were then analyzed

Table 1
PCR primers for RT-PCR.

Gene	Accession no.	Primer	Sequence (5′→3′)	Product size (bp)
ISG15	AU260555	Jf-ISG15_F	CTCCATGTAATCTGCAGCAA	360
		Jf-ISG15_R	CAGATCTAGTGCAGGTGTGA	
IL-1β	AB070835	Jf-IL-1β_F	GCAACCGCAAAGTTCTTCTC	404
		Jf-IL-1β_R	AAGATGCTGATCCACGTTCC	
Mx	C23060	Jf-Mx_F	AACAGCCAAGGCAAAGATTG	400
		Jf-Mx_R	AATGTCCAGCTCCTCCTTCA	
β-Actin	AU090737	Jf-β-actin_F	ACTACCTCATGAAGATCCTG	510
		Jf-β-actin_R	TTGCTGATCCACATCTGCTG	