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Expression and biological activity of two types of interferon genes in medaka (*Oryzias latipes*)

Shun Maekawa [a], Yi-An Chiang [a], Jun-ichi Hikima [b], Masahiro Sakai [b], Chu-Fang Lo [c], Han-Ching Wang [a, *], Takashi Aoki [a, **]

[a] Institute of Biotechnology, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan
[b] Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[c] Institute of Bioinformatics and Biosignal Transduction, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan

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ABSTRACT

Type I interferon (IFN) is one of most important cytokines for antiviral responses in fish innate immunity, after the induction pathway following pattern recognition. In this study, 2 types of type I IFN mRNA from a medaka (Japanese rice fish; *Oryzias latipes*) were identified and classified (phylogenetic analysis) into subgroup-a and -d by (designated *ol*IFNa and *ol*IFNd, respectively). Both *ol*IFNa and *ol*IFNd (encoding 197 and 187 amino acid residues, respectively) contained 2 cysteines. Gene expression pattern of *ol*IFNa, *ol*IFNd and IFN-stimulated genes (ISGs) was assessed (quantitative real-time reverse transcriptase PCR, qRT-PCR) in various organs (*i.e.*, whole kidney, liver and spleen) of medaka stimulated by polyI:C or infected with nervous necrosis virus (NNV). Expression of *ol*IFNa, *ol*IFNd and ISGs, especially the ISG15 gene, were significantly upregulated after NNV-infection. Furthermore, *ol*IFNa, *ol*IFNd and ISGs mRNAs were sufficiently induced in DIT cells (*i.e.*, medaka hepatoma cell line) transfected with polyI:C or infected with NNV. In addition, *in vitro* biological activities of recombinant *ol*IFNa and *ol*IFNd (r*ol*IFNa and r*ol*IFNd) produced by mammalian cell line HEK293T were also characterized. Expression of GIG1a and ISG15 genes in kidney cells of adult medaka were induced by r*ol*IFNa or r*ol*IFNd. The *ol*IFNs-over-expressing DIT cells had reduced viral titers following NNV infection. Therefore, we inferred that 2 type I IFNs were involved in innate immunity (antiviral response) in medaka fish.

1. Introduction

Type I interferon (IFN) is among the most important cytokines for antiviral responses in innate immunity. Mammalian type I IFNs constitute a multigene family grouped into IFN-α, IFN-β, IFN-δ IFN-τ, and IFN-ω [1]. Mammalian type I IFN genes do not contain introns and are closely clustered within the same chromosome. Expression of type I IFN is induced by pattern recognition receptors (PRRs), e.g., toll-like receptor (TLR)s, RIG-I-like receptors (RLRs) and cytosolic DNA sensors (CDSs). There are 3 RLRs have 3 members, namely RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2); they are localized in intracellular vesicles and recognize double strand RNA (dsRNA) of virus genome and polyI:C (dsRNA analog). Activated RLRs induces transcription of type I IFN, by activating signal cascades of mediator of IRF3 activation (MITA), TANK-binding kinase 1 (TBK1) and IFN regulatory factors (IRFs) 3 and 7 [2,3]. The secreted type I IFN binds with IFN-receptor (IFNR)-1 and IFNR-2, which delivers a signal through the JAK/STAT pathway. As a consequence, STAT associates with IRF9 to form a heterotrimeric complex (IFN-stimulated gene factor 3, ISGF3), which binds to the upstream IFN-stimulated response elements (ISRE) and activates transcription of various IFN-induced genes to promote antiviral activity, including production of inflammasome proteins [4].

Teleost type I IFN genes have been identified in various fish species. Teleost type I IFN genes retain the gene organization of 5 exons and 4 introns, unlike in mammals [5,6]. The number of type I IFN genes varies among teleost fish species. There are 4 type I IFN genes in zebrafish, *Danio rerio* [7–9], 5 in rainbow trout (*Oncorhynchus mykiss*) [7,10,11] and 11 in Atlantic salmon (*Salmo salar*)

* Corresponding author.
** Corresponding author.
E-mail addresses: wanghc@mail.ncku.edu.tw (H.-C. Wang), takashi.aoki65@gmail.com (T. Aoki).

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[6,12,13]. Meanwhile, Japanese pufferfish (*Takifugu rubripes*) and medaka (*Oryzias latipes*) each have only a single type I IFN gene [7]. Based on the cysteine patterns in the mature peptide, teleost type I IFNs could be divided into 2 major groups, with either 2 or 4 cysteine residues (2C or 4C groups, respectively). Phylogenetically, teleost type I IFNs are also classified into 4 subgroups, denoted IFNa, -b, -c, and -d, with IFNa and -d constituting the 2C type I IFNs and IFNb and -c the 4C type I IFNs [6,11]. In zebrafish, type I IFNs of the 2-cysteine group (IFNφ1 and φ4) bound to the cytokine receptor family B (CRFB)1-CRFB5 complex, whereas the CRFB5 chain associated with CRFB2 to form the receptor for 4-cysteine IFNφ2 and φ3 [9]. Although IFNφ1-3 had an anti-viral effect, IFNφ4 (subgroup d type I IFN) did not have [9]. In a salmon study, IFNa and -c had antiviral activities and induced antiviral genes, although IFNb was less active, and IFNd had no activity [13]. Antiviral activity of IFNd has apparently not been demonstrated in fish. However, green spotted puffer (*Tetraodon nigroviridis*) IFN, an IFNd homolog, induced strong expression of the Mx gene in primary head kidney cells [14]. Orange-spotted grouper (*Epinephelus coioides*) and rock bream (*Oplegnathus fasciatus*) IFNs, which belong to subgroup-d, also have important biological functions, including stimulation of Mx expression [15,16]. It was suggested that the IFNd subgroup is the only type I IFN in the superorder Acanthopterygii, including Japanese pufferfish, medaka, stickleback and rock bream [5,7,16]. However, 4C group and 2C group IFN genes were recently identified in turbot (*Scophthalmus maximus*) [17]. The 4C group of type I IFN in turbot had clear antiviral activity, whereas the 2C group did not. Consequently, it was suggested that there was another subtype of type I IFN, in addition to IFNd, that has been identified in the superorder Acanthopterygii.

In medaka, a well characterized experimental model fish, it has been reported that adult fish and some cell lines are susceptible to red-spotted grouper and striped jack nervous necrosis viruses (NNV) [18,19]. However, anti-virus activity though IFN systems in medaka have apparently not been reported. In this study, we report cDNA cloning 2 kinds of type I IFN cDNAs from medaka, designated *ol*IFNa and *ol*IFNd. Furthermore, *in vitro* biological activity of medaka IFNs were also investigated using recombinant proteins.

2. Materials and methods

2.1. Fish

Cab strain medaka were maintained in several transparent plastic tanks with a water circulating system (28 °C) and under a 14-h light and 10-h dark cycle. In all experiments, fish that were 3–4 month old and weighing 200–300 mg were used. PolyI:C was injected in adult medaka (10 μg/g BW injected intraperitoneal, IP), Control group fish were injected with PBS. For NNV challenge, adult medaka were injected IP with 10 μL orange-spotted grouper NNV suspension (10^8 $TCID_{50}$/mL).

2.2. Cloning olIFNa and olIFNd cDNA

Two putative sequences of type I IFN genes were identified in the medaka genome database at the Ensembl Genome Database Project (http://asia.ensembl.org/Oryzias_latipes/). Based on the 2 putative sequences, primers were designed to clone the cording regions of *ol*IFNa and *ol*IFNd cDNAs. Total RNA was extracted from various organs using REzol™ C&T reagent (Protech Technology, Taipei, Taiwan) according to manufacturer's instructions. Total RNAs were subjected to cDNA synthesis using SuperScript™II Reverse transcriptase (Life technologies, Grand Island, NY, USA) and Anchor dTv primer or random primer. The PCR products were cloned into RBC T&A cloning vector (RBC Bioscience) and sequenced. Multiple alignments of IFN amino acid sequences were performed using the ClustalW program (Version 2.1, DDBJ). The putative cleavage site of the signal peptide was predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/).

2.3. Quantitative real-time reverse transcriptional PCR analysis

Quantitative reverse transcription PCR (qRT-PCR) was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA). Gene expression levels were normalized to that of elongation factor 1α (EF1a). The mean threshold cycle was used to determine relative expression levels. Primers used for qRT-PCR were designed using Primer3 software [20]. Sequences of primers used for the qRT-PCR study are listed in Table S1.

2.4. Expression of recombinant proteins in HEK293T cells

A modified vector from pFUSE-mIgG2a-Fc (Invitrogen, Carlsbad, CA, USA) was used. A fragment containing a 10-histidine-tag (His-tag) and stop codon was generated by PCR. Sequence encoding mature *ol*IFNa, *ol*IFNd and EGFP peptides were amplified using Ex Taq and cloned into pFUSE-His vector. Sequences of primers used for the plasmid construction are listed in Table S1. Then, HEK293T cells were seeded in a 10-cm culture dish in 10 mL Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (PAA Laboratory, Pasching, Austria). When cultures reached ~80% confluence, the culture medium was changed to Opti-MEM (Gibco). Then, cells were transfected with 10 μg plasmid vector by adding 15 μL of FuGENE HD Transfection Reagent (Roche Diagnostics, Basel, Switzerland) to each dish containing 500 μL Opti-MEM (Gibco). After 72 h, the culture supernatant containing recombinant His-tag *ol*INFs and EGFP protein were purified using 2 column chromatography steps, nickel-chelating Sepharose Fast Flow (GE Healthcare BioSciences Co., Piscataway, NJ, USA) and NAP-5 columns (GE Healthcare BioSciences Co.). Each fraction sample was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting (the latter was performed using anti-histidine polyclonal antibody). Signals were detected using a Western Lightning Plus-ECL chemiluminescence detection kit (Perkin–Elmer, Waltham, MA, USA).

2.5. In vitro culture of DIT cell line

The DIT cell line, derived from medaka hepatoma [21], was obtained from the Riken cell bank and maintained in Leibovitz's L-15 (Gibco) medium containing 20% FBS (Sigma–Aldrich, St. Louis, MO, USA) and 10 mM HEPES (pH 7.6) at 33 °C. For transfection, DIT cells were cultured in 24-well plates at a concentration of 1×10^5 cells/well. Before transfection, cells were washed with PBS, and given fresh culture medium. For each well, 50 ng of pFUSE-His vectors containing mature peptides region of *ol*IFNs described above were mixed with 0.75 μL of FuGENE HD Transfection Reagent (Roche Diagnostics). The DNA mixtures were transfected to DIT cells in 25 μL of Opti-MEM (Gibco). To determine effects of intracellular polyI:C stimulation, 500 ng of polyI:C was transfected as described above.

2.6. In vitro culture of renal hematopoietic cells

Kidney marrow cells from adult medaka (1×10^5 cells/well) were cultured in a mixture containing L-15 medium (Gibco), 5% fetal bovine serum (Sigma–Aldrich), and 100 ng/mL of recombinant proteins or 50 μg/mL of polyI:C. Cells were incubated at 28 °C.