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Anti-viral effects of interferon administration on sevenband grouper, *Epinephelus septemfasciatus*

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ABSTRACT

Interferon (IFN) plays crucial roles in innate immune responses against viral infections. In the present study, we report cloning and characterization of the IFN gene from the sevenband grouper (*Epinephelus septemfasciatus*), and the anti-viral effects of its recombinant IFN protein *in vivo*. The isolated cDNA from sevenband grouper IFN encoded a protein consisting of 178 amino acids, and its first 22 amino acids represented a putative signal peptide. We named the identified sevenband grouper IFN gene as SgIFNa1 based on the result from phylogenetic analysis that categorized the deduced protein sequence into fish IFNa family. The expression of SgIFNa1 mRNA in the head kidney cells was induced by synthetic Poly(I:C), which is known as an inducer of IFN. It has also been confirmed that injection of recombinant SgIFNa1 protein (rSgIFNa1) upregulates expression of the Mx gene, which is known as an IFN-responsive gene, in head kidney cells. Moreover, we observed that preliminarily injection of rSgIFNa1 provided significant protection against a lethal challenge of nervous necrosis virus (NNV), which is a serious disease of sevenband grouper. These results demonstrate that SgIFNa1 has anti-viral activity and the administration of rSgIFNa1 to sevenband grouper is effective in preventing severe symptom development after NNV infection.

1. Introduction

Interferons (IFNs) are a large number of secretary cytokines that are involved in immune responses against viral pathogens and they have a major role in the defense against viral infection in vertebrates [1]. Three families of IFNs, type I (α/β), II (γ) and III (λ), have been identified, and it has been shown that type I and III IFN are induced by viral infection whereas type II IFN is induced by mitogenic or antigen stimulation [1,2]. Anti-viral effects of type I IFN are mediated via a specific transmembrane receptor which is present in all nucleated cells [3]. The IFNα/β-receptor binding triggers the JAK-STAT signal transduction pathway and leads to induction of transcription of several hundred genes. Some of these induced genes, such as Mx genes, dsRNA-dependent protein kinase R, 2′ 5′ oligoadenylate synthetase and RNaseL, encode anti-viral proteins that inhibit viral replication in mammals [1].

Recently, several studies have reported that the type I IFN-mediated defense mechanism in teleost fish is similar to mammals. IFN genes have been identified from several different fish species, such as zebrafish (*Danio rerio*) [4], spotted green puffer fish (*Tetraodon nigroviridis*) [5], Atlantic salmon (*Salmo salar*) [6], channel catfish (*Ictalurus punctatus*) [7], European sea bass (*Dicentrarchus labrax L.*) [8], and common carp (*Cyprinus carpio* L.) [9]. Furthermore, in a few fish species, the presence of multiple IFN genes have been confirmed, which suggests that the IFN system in fish has a control mechanism as complex as that in mammals [10–12]. It has been shown that all fish IFN genes contain five exons and four introns, and this gene structure is similar to mammalian IFNγ [8,9,13]. However, the fish IFN protein sequence and structure are more similar to mammalian IFNα/β [8].

In mammals, recombinant IFNα/β protein is widely utilized as a drug for viral diseases and cancer [14–19]. Administration of recombinant IFN protein (rIFN) is very effective for prevention of viral infection and progressive organ damage. In the case of fish, several groups have reported the effects of rIFN on anti-viral reactions *in vitro* [6,7,9,20–22]. Administration of rIFN provided effective protection against virus infection *in vivo* [23,24].

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Sevenband grouper (*Epinephelus septemfasciatus*) is one of the most commercially valuable fish species for marine aquaculture. However, this species is especially vulnerable to infections by viral nervous necrosis (VNN) disease, which is caused by a piscine nodaviruses belonging to the family *Nodaviridae* (genus *Betanodavirus*) [25]. VNN infection is characterized by neurological abnormality in the central nervous tissues, and VNN-infected fish showed abnormal swimming behavior [26,27]. Furthermore, this disease often occurs at early developmental stage, and it can lead to fish mortality [26,28]. Therefore, it is very important to understand the innate immune responses of sevenband grouper against virus infection in order to establish an effective treatment for defense against NNV. In the present study, we describe the isolation and characterization of a gene encoding fish IFNa homolog, SgIFNa1, from sevenband grouper. By using recombinant SgIFNa1 protein, we demonstrated an induction of anti-viral defense-related gene expression *in vitro* and *in vivo*, and anti-viral effects of the recombinant protein in injected sevenband grouper.

2. Materials and methods

2.1. Experimental fish

Sevenband grouper (38.3 g average body weight) were maintained at 23 ± 2 °C in 60-L tanks with a flow-through system using seawater.

2.2. Virus

A red-spotted grouper nervous necrosis virus (RGNNV) strain, SGEhi00, originally isolated from infected sevenband grouper in Japan [29], was used for virus challenge tests. E-11 cells that are a clone of the snakehead fish cell line, SSN-1 [30], were grown at 20 °C in Leibovitz's L-15 medium (L-15; Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). Monolayer cultures in 25-cm^2 tissue culture flasks were washed twice with Hanks' balanced salt solution (HBSS) and then inoculated with SGEhi00 at a dose of 10^5 $TCID_{50}$/ml. After incubation with 5 ml of L-15 containing 2% FBS at 25 °C for 3 days when the monolayer was mostly disintegrated, the culture fluid was collected by centrifugation at 4 °C at 2500 g for 10 min and stored at −80 °C until use. A portion of the virus stock was titrated using 96-well plates seeded with E-11 cells and the virus infective titer was quantified according to the method of Reed and Muench (1938) [31].

2.3. Cloning of IFN cDNA

Head kidneys were obtained from juvenile sevenband groupers. Total RNA was extracted with Sepasol (R) RNA I (Nacalai tesque, Japan) according to the manufacturer's instruction. Poly(A)$^+$ mRNA was purified from total RNA using the Oligotex™ -dT30 mRNA Purification Kit (Takara, Japan). The cDNA was synthesized from Oligo(dT)-primed mRNA using Superscript III reverse transcriptase (Invitrogen, USA) for 1 h at 50 °C. The cDNA fragments of IFN gene were obtained by RT-PCR using first strand cDNA of sevenband grouper head kidney as template. Two primers were designed based on conserved amino acid sequences of known fish IFN genes in the GenBank database. The forward primer was 5′-TCC TCG CTG AGC TGC AGA TGG-3′; the reverse primer was 5′-TCC TTC CTG ATC AGC TCC CA-3′. The PCR amplification was performed in a 25 μl volume using KOD FX DNA polymerase (Toyobo, Japan). The cycling protocol was 1 cycle of 94 °C for 2 min, 40 cycles of 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 1 min. After electrophoresis with 1.5% agarose gel, a band was extracted from agarose gel using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Sweden). The purified PCR product was ligated into the pTA2 vector (Toyobo), which was then used to transform JM109 *Escherichia coli* cells. Plasmid DNA was sequenced by the Big-Dye termination method using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

Subsequently, both 5′ and 3′ RACE were performed using a SMART™ RACE cDNA amplification kit (Clontech Laboratories, USA). To confirm the nucleotide sequence of IFN cDNA, cDNA fragments that included the open reading frame of IFN were sequenced. The fragments were obtained by RT-PCR using KOD FX DNA polymerase (Toyobo) with proofreading ability. All PCR products were subcloned into pTA2 vector (Toyobo). Plasmid DNA from at least six independent clones were sequenced by the Dye-termination method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

2.4. Structural and phylogenetic tree analyses

Signal peptide and potential N- and O-linked glycosylation sites were predicted from the deduced amino acid sequences of the IFN cDNA using the internet portal of the Center for Biological Sequence Analysis (www.cbs.dtu.dk). Multiple amino acid sequence alignments were constructed using ClustalW in the DDBJ website (www.ddbj.nig.ac.jp) [32]. The Neighbor-joining method in ClustalW was used to construct a phylogenetic tree [33]. The ClustalW analysis was performed using default settings except for gaps. Gaps were treated as missing characters. Relative branch support was evaluated by bootstrap analysis. NJplot software was used to generate a graphical representation of the phylogenetic tree [34].

2.5. Production and purification of rSgIFNa1

To evaluate biological activity of SgIFNa1, we produced and purified the recombinant protein (rSgIFNa1) through an *E. coli* protein expression system. The pCold IV vector (Takara) was used to construct the expression vector. The cDNA fragments corresponding to the putative mature peptide of SgIFN were amplified by RT-PCR using primers rSgIFN-FW (5′- TATACATATGTGCAGATGGGTGGATCA-3′) and rSgIFN-RV (5′- GGGATCCTAGTGATGGTGATGGTGATGGTGATGGTTA-GATAAAAGCAGCTGGT-3′). The PCR products were digested with *Nde*I and *Bam*HI restriction enzymes and inserted into the plasmid pCold IV to generate the pCold IV-SgIFNa1 expression vector. To allow expression of soluble protein, the pCold IV-SgIFNa1 plasmid were transformed into *E. coli* Rosetta-gami B (DE3) strain (Novagen, USA). A single colony of Rosetta-gami B (DE3)/pCold IV-SgIFNa1 was used to inoculate a LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol, and grown at 37 °C. A starter overnight culture was used to inoculate fresh medium at 1/20 dilution. It was then cultured at 37 °C until OD 600 of 0.5. After incubation at 15 °C for 30 min, isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM to induce expression and it was then cultured at 15 °C for 24 h. The cells were harvested by centrifugation, resuspended in lysis buffer (phosphate buffered saline, 1% Triton X-100, 20 mM imidazole, one tablet of protease inhibitor cocktail (Roche)), and sonicated 4 times for 30 sec with 30-sec pauses. The lysate was centrifuged at 20,400 g at 4 °C for 30 min. The supernatant was filtered through a 0.22 μm filter (Millipore, USA) to remove undissolved particles. The supernatant containing the soluble rSgIFNa1 was loaded to a His Trap HP column (1 ml: GE Healthcare) pre-equilibrated with PBS at 4 °C, and nonspecific bound proteins were removed using ten column volumes of wash buffer (PBS, 100 mM imidazole). Elution of the bound rSgIFNa1 was carried out using ten column volumes of elution buffer (PBS, 250 mM imidazole). The eluted fractions containing the rSgIFNa1 were pooled and then dialyzed for 2 days with 5 × 1L of PBS. The solution containing rSgIFNa1 was centrifuged at 20,400 g at 4 °C for 30 min, and the supernatant was stored at −80 °C. Protein