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Antiviral protection mechanisms mediated by ginbuna crucian carp interferon gamma isoforms 1 and 2 through two distinct interferon gamma-receptors

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Fish genomes possess three type II interferon (IFN) genes, *ifnγ1*, *ifnγ2* and *ifnγ-related* (*ifnγrel*). The IFNγ-dependent STAT signalling pathway found in humans and mice had not been characterized in fish previously. To identify the antiviral functions and signalling pathways of the type II IFN system in fish, we purified the ifnγ1, ifnγ2 and ifnγrel proteins of ginbuna crucian carp expressed in bacteria and found them to elicit high antiviral activities against crucian carp hematopoietic necrosis virus. We also cloned two distinct *ifnγ receptor alpha chain* (*ifngr1*) isoforms, 1 and 2, and stably expressed them in HeLa cells by transfecting the cells with *ifngr1-1* or *ifngr1-2* cDNA. When receptor transfectants were treated with the ligands in a one-ligand-one-receptor manner (ifnγ1 and ifngr1-2 or ifnγ2 and ifngr1-1), the stat1 protein was phosphorylated at both serine-727 and tyrosine-701 residues. Gel shift mobility analysis and reporter assay clearly showed that the specific ligand–receptor interaction resulted in the binding of the stat1 protein to the GAS element and enhanced transcription. Therefore, the actions of ifnγ1 and ifnγ2 were found to be mediated by a specific receptor for each signalling pathway via a stat1-dependent mechanism.

Keywords: cytokines/interferon/fish immunology/receptor/signal transduction.

Abbreviations: EF1α, Elongation factor 1 alpha; GAS, IFNγ–activated site; IDO, Indoleamine dioxygenase; IFNγ, Interferon gamma; IFNγrel, Interferon gamma–related; IRF-1, Interferon regulatory factor-1; IFNGR1, Interferon gamma receptor alpha chain; JAK, Janus–activated kinase; LB, Luria–Bertani; RT, Reverse transcription; STAT, Signal transducers and activators of transcription.

Interferon gamma (IFNγ) is an antiviral and immunoregulatory cytokine that is essential for cellular defence against a variety of infectious agents in mammals (*1*, *2*). It is produced by activated T cells and natural killer cells as a dimerized soluble glycoprotein (*1*, *2*). To initiate cellular responses, IFNγ interacts with its cell-surface receptor (interferon gamma alpha chain; IFNGR1) first to form the IFNγ/IFNGR1 complex (*3*, *4*). Upon binding with the interferon gamma beta chain (IFNGR2), which was identified as an accessory factor 1 (*5*, *6*), the functional tertiary complex (IFNγ/IFNGR1/IFNGR2) activates the Janus kinase and signal transducer and activator of transcription (JAK–STAT) signalling pathway (*2*). In this case, IFNGR1 and IFNGR2 are responsible for the recruitment and subsequent activation of jak1 (*7*, *8*) jak2 (*9–11*) and stat1 (*12*, *13*). When stat1 is activated by IFNγ (*14*), the dimerized transcription factor is translocated to the nucleus and binds to a specific DNA element called the IFNγ-activated site or GAS (*14–16*). Biochemical and genetic studies have shown that stat1 plays a critical role in ifnγ-dependent signalling and that disruption of the *stat1* gene results in a loss of ifnγ-mediated antiviral activity (*17*).

The first teleost *ifnγ* gene was discovered in Japanese pufferfish through a gene synteny analysis based on the human genome (*18*). Its homologues were cloned subsequently from other teleosts including rainbow trout (*19*), Atlantic salmon (*20*), zebrafish (*21*), catfish (*22*), carp (*23*) and goldfish (*24*), but not from the ginbuna crucian carp (Table I). Injection of polyinosinic–polycytidylic acid into rainbow trout induces *ifnγ* expression in the head kidney and spleen (*19*). Additionally, recombinant rainbow trout ifnγ stimulates the gene expression of ifnγ-inducible proteins such as *γip*, *mhc class II β*-chain and *stat1*, and enhances the respiratory burst activity in macrophages (*19*). In goldfish, ifnγ primes macrophages and neutrophils for respiratory burst responses and increases the phagocytic and NO responses of macrophages (*24*). In zebrafish, ifnγ induces the expression of both *mxb* and *mxc* genes in ZF4 cells and protects zebrafish ZF4 cells from the spring viremia of the carp virus (*25*). The finding that zebrafish *stat1* can rescue the function of IFN-signalling in a *stat1*-deficient human cell line (*26*) suggests that the cytokine signalling mechanism is conserved between fish and mammals. However, it is not known whether fish ifnγ has antiviral activity or whether fish ifnγ-signalling is receptor mediated.

So far, fish are known to have at least two *ifnγ* isoforms as well as an *ifnγ-related* (*ifnγrel*) one (Table I). *ifnγ*(s) and *ifnγrel* are structurally similar to mammalian *IFNγ*; but in catfish (*22*) and goldfish (*27*), *ifnγrel*

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Table I. *Interferon gamma* (*Ifnγ*) and *Ifnγ-related* (*Ifnγrel*) genes in vertebrates.

Species	Molecule	Accession number	References
Human	*IFNG* (IFNγ)	NP_000610	(*53*)
Mouse	*ifng* (ifnγ)	NP_032363	(*54*)
Rat	*ifng* (ifnγ)	NP_620235	(*55*)
Chicken	*ifng* (ifnγ)	U27465	(*56*)
Xenopus	*ifng* (ifnγ)	XM_002938509	No report
Ginbuna	*ifng1* (ifnγ1)	AB570431	This study
crucian carp	*ifng2* (ifnγ2)	AB570432	This study
	ifngrel (ifnγrel)	AB570433	This study
Goldfish	*ifng* (ifnγ)	EU909368	(*24*)
	ifngrel (ifnγrel)	GQ149696	(*28*)
Zebrafish	*ifng* (ifnγ)	NM_001020793	(*21*)
	ifng (ifnγrel)	NM_212864	(*21*)
Carp	*ifnga* (ifnγa)	AM168523	(*23*)
	ifngb (ifnγb)	AM168524	No report
	ifngrel (ifnγrel)	AM261214	(*23*)
Catfish	*ifnga* (ifnγa)	DQ124250	(*22*)
	ifngb (ifnγb)	DQ124251	(*22*)
	ifngrel (ifnγrel)	DQ124249	(*22*)
Japanese pufferfish	*ifng* (ifnγ)	AJ616216	(*18*)
Rainbow trout	*ifng1* (ifnγ1)	NM_001160503	(*19*, *57*)
	ifng2 (ifnγ2)	NM_001160504	(*19*, *57*)
Atlantic salmon	*ifng* (ifnγ)	AY795563	(*20*)

is shorter and does not contain C-terminal cationic residues that may be required for ifnγ activity. In zebrafish and goldfish, two *ifngr1* isoforms, *ifngr1-1* and *ifngr1-2*, have been isolated (*28*). The goldfish *ifngr1-1* and *ifngr1-2* are expressed in almost all tissues, but are differentially expressed in primary cell lines (*28*). Although *in vitro* studies on goldfish cells have shown that the two receptors have different ligands (*28*), little is known about the antiviral activities of fish ifnγ ligands or the molecular mechanisms of ifnγ-signalling *in vivo*.

In this present study we generated three recombinant proteins of ginbuna crucian carp ifnγ, *i.e.* ifnγ1, ifnγ2 and ifnγrel, and determined their antiviral activities against crucian carp hematopoietic necrosis virus. We also identified and characterized two ginbuna crucian carp *ifngr1* isoforms, *ifngr1-1* and *ifngr1-2*, and expressed them in human HeLa cells to investigate their function and specificity for the ifnγ1 and ifnγ2 ligands. Finally, we examined whether the signalling was dependent on specific interactions between the ligands and the receptors. Our results clearly show that the recombinant ifnγ1, ifnγ2 and ifnγrel proteins elicited high antiviral activities and that the signalling was achieved by specific interactions between the ligands (ifnγ2 and ifnγ1) and their respective receptors (ifngr1-1 and ifngr1-2). These results suggest that ifnγ signalling in fish is similar to that in humans and mice.

Experimental procedures

Reagents

All chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Protein G Sepharose, polyvinylidine fluoride (PVDF) membrane, and ECL™ western blotting Detection kit were purchased from GE Healthcare (Piscataway, NJ, USA). Anti-actin monoclonal antibody, anti-FLAG M2 monoclonal antibody and p3XFLAG-CMV™-14 expression vector were obtained from Sigma-Aldrich (St Louis, MO, USA). Recombinant human IFNγ, rabbit anti-jak1 monoclonal antibody, rabbit phospho-stat1 (Ser-727) polyclonal antibody, rabbit phospho-stat1 (Tyr-701) polyclonal antibody and rabbit stat1 polyclonal antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Cell culture reagents, a ThermoScript RT-PCR System, and Xpress™ System Synthetic Oligonucleotides were obtained from Invitrogen (Carlsbad, CA, USA). Premix *Taq* (*Ex*Taq™ Version 2) and PrimeSTAR™ HS DNA polymerase were purchased from Takara Biomedical (Shiga, Japan).

Cell culture

The ginbuna crucian carp (*Carassius auratus langsdorfii)* cell line GTS9, derived from thymus, was cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) at 30°C (*29*). Human epithelial carcinoma (HeLa) cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS at 37°C in a humidified incubator in 5% CO_2.

cDNA cloning of Ifnγ isoforms

Poly(A)$^+$ mRNA was extracted from ginbuna crucian carp thymus, and single-stranded cDNA was synthesized according to the method described previously (*30*). To obtain the partial nucleotide sequences of two *ifnγ* cDNAs, we performed RT–PCR using primers (sense primer 5′-GGGGA GTATG TTTGC TGACT TCAGG ATGG-3′ and antisense primer 5′-GGTGT TTTTG GCTTG TCGTC TCCT G CGC-3′) based on the cDNA sequences of the zebrafish *ifnγ* gene (*21*). PCR was carried out in 40-μl reaction mixtures containing Premix *Taq*, with reaction conditions consisting of denaturation at 96°C for 2 min and 30 cycles of denaturation at 94°C for 10 s, annealing at 60°C for 10 s, polymerization at 72°C for 30 s, and extension at 72°C for 2 min. The PCR products were subcloned into the pGEM-T Easy vector. DNA sequence analysis confirmed that the nucleotide sequences of the RT–PCR products were homologous to the sequence of the zebrafish *ifnγ* gene. Using a FirstChoice RLM-RACE Kit (Amibion, TX, USA), two cDNA fragments, coding for the full length of *ifnγ1* and *ifnγ2* were generated and cloned. A similar approach was performed to obtain the nucleotide sequence of the *ifnγrel* cDNA; *i.e.* RT–PCR was performed by using primers (sense primer 5′-TGCTA CTGTG GACTT TTGTG GATA G CA-3′ and antisense primer 5′-TGTTC TTCGT TGTAC TTGTG CTTCA-3′) based on the cDNA sequence of the zebrafish *ifnγrel* gene (*21*). The FirstChoice RLM-RACE Kit was used to generate an *ifnγrel* cDNA fragment. The PCR products were cloned into the pGEM-T Easy vector, and the cloned nucleotide sequences were determined by using a DNA sequencer (ABI 3100; Applied Biosystems, CA, USA) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequences were deposited in the DNA Databank of Japan (DDBJ) database under the accession numbers AB570431 (ginbuna crucian carp *ifnγ1*), AB570432 (ginbuna crucian carp *ifnγ2*) and AB570433 (ginbuna crucian carp *ifnγrel*).

Production and purification of recombinant protein of Ifnγ isoforms

Using the three cloned cDNA fragments encoding the three isoforms of ginbuna crucian carp *ifnγ* as templates, we used PCR to amplify the cDNA sequences coding for the *ifnγ1*, *ifnγ2* and *ifnγrel* proteins lacking the signal peptide sequence. The cDNA sequence of *ifnγ*1 was amplified by using the sense primer 5′-GGATC CACAT ACAG C GAGGC CAGCG TCCCT GAG-3′ and the antisense primer 5′-G GATC CTTAA GACTT CAGAT TTTTG GTGTT TTTGG C-3′; and each primer contained a single BamHI site. The cDNA sequence of *ifnγ2* was amplified by PCR using the sense primer 5′-CATAT G AGCG TCCCT GAGAA CCTGG ACAAG AGCAT C-3′ and the antisense primer 5′-GGATC CTCAA GATTT ATTAA GACTT TT GCT TCTTG TG-3′; and each primer contained one NdeI site and one BamHI site. The cDNA sequence of *ifnγ2* was amplified by PCR using the sense primer 5′-CATAT GTTCA GATTT CCACG GTCC A AAAGC GAC-3′ and the antisense primer 5′-GGATC CTCAT T GCAC CCTGT GATGA TGCTT TTC-3′, with each primer containing one NdeI site and one BamHI site. The amplified DNAs were subcloned into the pGEM-T Easy plasmid vector by using the TA-Cloning method (Promega, WI, USA). The cloned nucleotide

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sequence for *ifnγ1* was confirmed by sequencing and then inserted into the BamHI site of the pET-16b vector (Novagen, Madison, WI, USA) to fuse a Histag sequence at the N-terminus of the *ifnγ1* open reading frame. This construct was designated as pETIFNγ1. *ifnγ2* and *ifnγrel* constructs were also prepared in a similar way as mentioned above and designated as pETIFNγ2 and pETIFNγrel, respectively. The ifnγ proteins were expressed in *Escherichia coli* BL21(DE3)pLysS cells (Novagen) that had been transformed with the pETIFNγ1, pETIFNγ2 or pETIFNγrel. The cells were inoculated into 100 ml of Luria-Bertani (LB) broth and grown overnight at 30°C in a shaker at 200 r.p.m. The cultures were then transferred to fresh LB broth (1 l) supplemented with 100 μg/ml ampicillin in 5-l flasks, and allowed to continue to grow under the same conditions until the turbidity at 600 nm reached 0.8. Isopropyl 1-thio-β-D-galactopyranoside was added to the cultures at a final concentration of 0.2 mM, and the cultures were then incubated for an additional 4 h to induce expression of the transgene products. The bacterial cells were collected by centrifugation at 4000 *g* for 15 min. The N-terminal His-tagged *ifn* was purified from the bacterial extract by using a His Trap HP column (GE Healthcare) according to the manufacturer's protocol. The bacterial cells were re-suspended in 20 mM sodium phosphate buffer (pH 7.4) containing 1× proteinase inhibitor cocktail, 500 mM NaCl, 100 mM imidazole and 0.1% Triton X-100 and disrupted by sonication. All subsequent procedures were carried out at 4°C. The supernatant was collected by centrifuging the lysate at 10,000*g* for 30 min, and it was then dialyzed against 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl and 100 mM imidazole. The dialyzed sample was loaded onto a 1-ml His Trap HP column (GE Healthcare) that had been equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl and 100 mM imidazole (wash buffer). After sample loading, the column was washed with 150 ml of wash buffer, followed by 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl and 500 mM imidazole (elution buffer). The flow rate was 1 ml/min, and 2.5-ml fractions were collected. The His Trap HP fractions with antiviral activity were pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl (gel filtration buffer), and then loaded onto a Sephacryl S-100 column (HR *16/60*, GE Healthcare) equilibrated with the gel filtration buffer. The column was eluted at 1 ml/min with 150 ml of the gel filtration buffer. The fractions were collected into test tubes and analysed by SDS–PAGE. The antiviral activity was examined as well. Lipopolysaccharide was removed from those fractions having antiviral activity by passing it through an EndoTrap Red endotoxin removal column (Cambrex Bioscience, MD, USA); and completion of lipopolysaccharide removal was confirmed by using a Limulus ES-II Single Test (Wako, Osaka, Japan). Based on protein sequencing and western blot data, we confirmed the identity of the purified recombinant proteins.

Bioassay of recombinant Ifnγ isoform activity against crucian carp hematopoietic necrosis virus

Bioactivities of the three ifnγ recombinant proteins were examined by using a virus protection assay. Adherent GTS9 cells seeded into 96-well plates at 1×10^4 cells/well were incubated overnight at 25°C, and then the cells were either untreated or treated with various concentrations of the purified recombinant ifnγ1, ifnγ2 and/or ifnγrel recombinants proteins for 24 h. Then the cells were challenged with crucian carp hematopoietic necrosis virus at 0.5 plaque-forming units/cell (*31*) and incubated for an additional 48 h. The cells were washed twice with 0.2 ml of PBS to remove the cultured medium containing the recombinant proteins and dead cells. Surviving cells were fixed and stained with crystal violet (*31*), after which the OD_{595} was measured to determine their number. The ED_{50} of each recombinant protein was calculated by using non-linear regression analysis of GraphPad Prism 4.0 software. Graph represents the results from one of three independent experiments.

Trypan blue exclusion

The biological effects of the purified recombinant ifnγ proteins, ifnγ1, ifnγ2 and ifnγrel, on cell survival were determined by using the trypan blue exclusion method, as described elsewhere (*32*). Adherent GTS9 cells at 1×10^4 cells were plated in a single well of a 96-well plate. Cells were untreated or treated with various concentrations of ifnγ1, ifnγ2 and/or ifnγrel recombinants proteins for 24 h. Crucian carp hematopoietic necrosis virus at 0.5 plaque-forming units/cell was then added. Forty-eight hours after infection, the trypan blue assay was used to determine the cell survival. At least 200 cells were counted under a light microscope. The cells neither treated with the purified recombinant ifnγ proteins nor infected with crucian carp hematopoietic necrosis virus were used as control. The survival rate was expressed as percentage of the control. The average percentages of viable cells were determined from three independent experiments.

Western blot analysis

The proteins extracted from whole cells were resolved on a 10% SDS–polyacrylamide gel and electroblotted onto a PVDF membrane according to Yabu *et al.* (*30*, *33*, *34*). Anti-jak1 monoclonal, anti-stat1 polyclonal, anti-phospho-stat1 (Tyr-701) polyclonal, anti-phospho-stat1 (Ser-727) polyclonal, anti-actin monoclonal and anti-FLAG monoclonal antibodies were used as the primary antibodies. Following reaction with the appropriate secondary antibody, signals were detected by using an ECL™ Western blotting detection kit (GE Healthcare) according to the manufacturer's protocol.

Cloning of Ifnγ receptor alpha chain1-1 (Ifngr1-1) and Ifngr1-2

The nucleotide sequence of ginbuna crucian carp *ifngr1-1* (GenBank accession number: AB563726) was identified based on the nucleotide sequence of goldfish *ifngr1-1* (GenBank accession number: GQ149697). The nucleotide sequence of ginbuna crucian carp *ifngr1-2* (GenBank accession number: AB563727) was obtained from goldfish *ifngr1-2* (GenBank accession number: GQ149698). Total RNA from thymus was extracted by using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. For the synthesis of first-strand cDNA, 5 μg of total RNA was reverse-transcribed in a 40-μl reaction volume by using the ThermoScript RT–PCR System (Invitrogen) according to the manufacturer's protocol. The reverse-transcription products were diluted 10 times with distilled water and stored at 4°C until used. The following PCR primers for *ifngr1-1* and *ifngr1-2* were synthesized and used for amplification: for *ifngr1-1*, 5′-TATAA TGT GC ATACT GGATT GAAAC TCGCC-3′(upstream) and 5′-AACC A TTATG GTGCT TTCCT TCACT TCTAG-3′ (downstream; PCR product, 1185 bp); for *ifngr1-2*, 5′-CCTTG CGACG GGTTT CAAAA CACAT TTCTG-3′ (upstream) and 5′-ACCTA ACGCA AAGTA TCCCT CAGTA AAGTC-3′ (downstream; PCR product, 1053 bp). PCR was carried out in a total reaction volume of 50 μl containing PrimeSTAR™HS DNA polymerase. The reaction conditions were 98°C for 2 min followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 5 s, and polymerization at 72°C for 1 min, with a final extension at 72°C for 5 min. The obtained PCR products were then cloned into the pGEM-T Easy vector, and their nucleotide sequences were confirmed by DNA sequence analysis.

Detection of ginbuna crucian carp Ifngr1-1 and Ifngr1-2 genes by RT–PCR

The total RNA of ginbuna crucian carp GTS9 cells was extracted by using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. For the synthesis of first-strand cDNA, 5 μg of RNA in a 20-μl-reaction mixture was reverse-transcribed by utilizing the ThermoScript RT-PCR System (Invitrogen) according to the manufacturer's instructions. The resulting reverse transcription products were diluted 10 times and stored at −20°C until used. The following PCR primers for *ifngr1-1*, *ifngr1-2* and *ef1α* were synthesized and used: for *ifngr1-1*, 5′-CACGG TGTAC TTGTA TCCAG TGCTG-3′ (upstream) and 5′-GCAGT TTGGG TCCGT AACCA TCTAC-3′ (downstream; PCR product, 434 bp); for *ifngr1-2*, 5′-AGCTG AAGCT TGAAG GGGTG ATTGC-3′ (upstream) and 5′-GGATT TAGGT CTGTC ATAGT CAGAC-3′ (downstream; PCR product, 437 bp); and for *ef1α*, 5′-CGGCA GCTTC AATGC TCAGG TCATC-3′ (upstream) and 5′-GGGAA ATTCA TTTGG TCTTG GCAGC CT-3′ (downstream; PCR product, 388 bp). PCR was carried out in a 40-μl-reaction mixture (total volume) containing Premix *Taq* (*Ex Taq* Version; TAKARA). Reaction conditions were 94°C for 2 min, followed by 32 cycles (21 cycles for *ef1α*) of 15 s of denaturation at 94°C, 15 s of annealing at 55°C, 30 s of polymerization at 72°C, and 3 min of extension at 72°C. *ef1α* was chosen as an internal control. The gels were scanned and their images captured on a personal computer using Image Analysis Software (ATTO, Tokyo, Japan).

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Construction of Ifngr1-1 and Ifngr1-2

FLAG-tagged *ifngr1-1* and FLAG-tagged *ifngr1-2* constructs were created in the p3XFLAG-CMV™-14 expression vector (Sigma) by PCR using ginbuna crucian carp *ifngr1-1* and *ifngr1-2* cDNAs, or their derivatives as the templates and appropriate combinations of the forward and the reverse oligonucleotide primers. All constructs were confirmed by nucleotide sequencing.

Generation of stable HeLa transfectants

HeLa cells were cultured in DMEM medium containing 10% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin. To obtain stable transfectants, we transfected 5×10^6 HeLa cells with 4 µg of either the *ifngr1-1* or the *ifngr1-2* construct by using FuGENE 6 Transfection Reagent (Roche), according to the manufacturer's protocol; and then the cells were selected in the presence of 0.8 mg/ml geneticin (Invitrogen). The cell lines expressing the *ifngr1-1* and *ifngr1-2* stably at a high level were established; four stable clones were obtained for each cell line.

Immunoprecipitation

After ifnγ1 and/or ifnγ2 treatment, cells were washed twice with ice-cold PBS buffer, and then 1×10^6 cells were lysed for 1 h at 4°C in 1 ml of 20 mM Tris–HCl (pH. 7.5) containing 1% Nonidet P-40, 1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate and 1× proteinase inhibitor cocktail. Cellular debris was pelleted by centrifugation at 13,000*g* for 15 min at 4°C. Cell lysates were incubated with anti-FLAG antibody, followed by incubation with protein G Sepharose (GE Healthcare), sedimentation, and three washings with 20 mM Tris–HCl (pH. 7.5) containing 1% Nonidet P-40, 1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate and 1× proteinase inhibitor cocktail.

Chromatin immunoprecipition assay

HeLa cells expressing *ifngr1-1* or *ifngr1-2* seeded for 24 h in advance and grown to 80% confluence were treated with 25 ng/ml of the purified recombinant ifnγ1 and/or ifnγ2 proteins for 30 min. The cells were then washed with cold PBS and treated with 3.7% formaldehyde for 5 min at 37°C. The rest of the procedure was conducted by using an OneDay ChIP Kit from Diagenode (Philadelphia, PA, USA), as per the manufacturer's protocol. Sonication was conducted to obtain the DNA fragments of less than 500 bp. Control IgG or anti-stat1 antibody was used for immunoprecipitation. Eluted DNA fragments were utilized for PCR. The promoter region (106 bp) containing two repeats of the gamma-activated site (GAS) of the human interferon regulatory factor-1 (*Irf-1*) gene was amplified with the primers 5′-CTTCG CCGCT AGCTC TAC-3′ (−388 to −371) and 5′-G CCGC GCGGG CGCCC ATT-3′ [−283 to −321; (*35*, *36*)] whereas that (120 bp) having GAS of the human IFNγ-inducible indoleamine 2,3 dioxygenase (*Ido*) gene was amplified with the primers 5′-TAAC A CAGGT TGTGT TTCCG-3′ (−497 to −476) and 5′-AAGGC TG CAG TCCTA AACTC-3′ [−378 to −399; (*37*)]. As a control, the promoter region (66 bp) of the human *ß-Actin* gene was amplified with the primers 5′-TGCAC TGTGC GGCGA AGC-3′ (−980 to −963) and 5′-TCGAG CCATA AAAGG CAA-3′ (−915 to −932). PCR was carried out in a total reaction volume of 40 µl by using Premix *Taq* (*Ex*Taq™ Version 2) DNA polymerase. The reaction conditions were 94°C for 5 min followed by 20 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 10 s, and polymerization at 72°C for 15 s, with a final extension at 72°C for 1 min.

Gel shift mobility assay

Nuclear extracts were prepared from GTS9 cells and/or *ifngr1-1* and/or *ifngr1-2*-transfected HeLa cells that had been treated with either 25 ng/ml of ifnγ1 or 25 ng/ml of ifnγ2 recombinant protein of ginbuna crucian carp for 30 min. Nuclear fractions were extracted by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The GAS element of the human *Irf-1* gene promoter, 5′-AGCCT GATTC CCCCG AAATG ACGGC-3′, was used as a probe (*35*, *36*). A total of 5 µg of nuclear extract was incubated for 30 min with 9 pmol Cy5-conjugated double-stranded oligonucleotide, as specified above, in a 15-µl reaction mixture comprising 20 mM Hepes–NaOH (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol and 0.05% Nonidet P-40. For competition experiments, nuclear extracts were pre-incubated with a 0-, 1-, 10- and 100-fold excess of a non-Cy5-conjugated oligonucleotide before addition of the Cy5-conjugated oligoprobe. The mixture was separated on a non-denaturing 4% polyacrylamide gel. The Cy5-conjugated double-stranded oligonucleotide/stat1 complex and the free Cy5-conjugated oligoprobe were visualized by UV irradiation, and the intensity was measured by using a gel scanner attached to a fluorometer (630 nm).

Reporter assay

The plasmid pGL3 promoter, which expresses *Renilla* luciferase, was purchased from Promega (Madison, WI, USA). A nucleotide sequence containing two repeats of the GAS promoter element from the human *Irf-1* gene (*35*, *36*), 5′-AGCCT GATTC CCCCG AAAT G ACGGC-3′, was introduced into both the BglII and SmaI sites of the pGL3 promoter. A pRL-TK plasmid DNA is able to express the thymidine kinase promoter-driven *Renilla* luciferase gene constitutively was used as an internal standard. GTS9 cells and/or the HeLa cells expressing the *ifngr1-1* or *ifngr1-2* receptor were seeded at 10^4 cell/well in a 96-well plate. Then, 2 µg of luciferase-expressing plasmid DNA with the GAS promoter and 10 ng of pRL-TK plasmid DNA were used for co-transfection of the cells by using Fugene 6 (Roche). After 24 h, the cells were treated with various concentrations of the ginbuna crucian carp ifnγ1, ifnγ2 and/or ifnγrel proteins for an additional 12 h. The cell lysates were used to assay for firefly luciferase, followed by *Renilla* luciferase, by using a Dual-Glo Luciferase Assay System (Madison, WI, USA) according to the manufacturer's instructions. The relative luciferase units were calculated by dividing the firefly luciferase activity by the *Renilla* luciferase activity for each sample in three independent experiments.

Results

Cloning of cDNAs of ginbuna Ifnγ isoforms Ifnγ1, Ifnγ2 and Ifnγrel

Unlike mammals and birds, which have a single *IFNγ*, teleosts have three types of *ifnγ* (*ifnγ1, ifnγ2* and *ifnγrel*; Table I), about which little is known. To determine the antiviral activities of ifnγ ligands and to elucidate their signalling mechanisms, we cloned cDNAs encoding *ifnγ1*, *ifnγ2* and *ifnγrel* from ginbuna crucian carp. *ifnγ1* and *ifnγ2* cDNAs were identified by their similarities to zebrafish *ifnγ* (*21*). *ifnγ1* cDNA had a 531-bp open reading frame encoding a protein of 177 amino acid residues (Supplementary Fig. S1A). The deduced amino acid sequence of *ifnγ1* showed 79% and 99% identities to the sequences of zebrafish *ifnγ* (*21*) and goldfish *ifnγ* (*27*), respectively. *ifnγ2* contained a predicted open reading frame specifying a 185-amino acid protein (Supplementary Fig. S1A). The deduced amino acid sequence of *ifnγ2* showed 77% and 78% identities to the sequences of goldfish *ifnγ* (*27*) and the cloned *ifnγ1* of ginbuna crucian carp, respectively (Supplementary Fig. S1A). In a phylogenetic tree (Supplementary Fig. S1C), *ifnγ1* and *ifnγ2* were clustered with other cyprinid *ifnγ*s. By use of the SMART program (*38*), *ifnγ1* and *ifnγ2* were both predicted to have an N-terminal signal peptide, an N-linked glycosylation site, an IFNγ signature motif and a C-terminal nuclear localization signal (Supplementary Fig. S1A).

An *ifnγrel* cDNA homologue to zebrafish *ifnγrel* was also isolated from the ginbuna crucian carp thymus. This *ifnγrel* cDNA had a 513-bp open reading frame encoding a protein of 171 amino acid residues (Supplementary Fig. S1B). Its predicted amino acid sequence showed 76% and 55% identities to the sequences of zebrafish *ifnγrel* (*21*) and goldfish *ifnγrel* (*27*), respectively (Supplementary Fig. S1B).

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The deduced protein contained the domains well conserved among the known *ifnγrel*s (*21*), *i.e.* an N-terminal signal peptide, two N-linked glycosylation sites, an IFNγ signature-like motif in the middle region and a C-terminal nuclear localization signal (Supplementary Fig. S1B). In the phylogenetic tree, the ginbuna crucian carp *ifnγrel* was clustered with other teleost *ifnγrel*s (Supplementary Fig. S1C).

Production and characterization of recombinant Ifnγs

Amplified cDNA fragments encoding *ifnγ* isoforms without the putative signal peptide were inserted into an expression vector that added an N-terminal His-tag. Following His-tag affinity chromatography, gel filtration chromatography and endotoxin removal chromatography, the expressed recombinant proteins ifnγ1, ifnγ2 and ifnγrel were subjected to SDS–PAGE under a reducing condition. Based on the gel filtration chromatograms obtained, the purity of each recombinant protein was estimated to be more than 98.9%. They appeared as single bands on the gel with molecular weights of 22,200, 21,900 and 19,600, respectively (Fig. 1A). The molecular weights of the purified recombinant proteins based on non-denaturing Sephacryl S-100 gel filtration column chromatography were 43,500, 42,200 and 19,800, respectively (Fig. 1B). Together, these results indicate that, under native conditions, ifnγrel existed as a monomer and ifnγ1 and ifnγ2 formed homodimers, as is the case for recombinant human and mouse IFNγs (*39*, *40*).

Having the purified recombinant ifnγ1, ifnγ2 and ifnγrel proteins in hand, we then examined whether these proteins were able to elicit antiviral activities. Adherent ginbuna GTS9 cells were infected with crucian carp hematopoietic necrosis virus (CHNV) in the presence of various concentrations of ifnγs, and viable cells were detected by crystal violet staining. Each of the recombinant proteins elicited high antiviral activities; the ED_{50} values of the recombinant ifnγ1, 2 and γrel proteins were 10.10 ng/ml (Fig. 2A), 10.06 ng/ml (Fig. 2B), 9.95 ng/ml (Fig. 2C), respectively. Trypan blue exclusion analysis confirmed that the recombinant ifnγ1, ifnγ2 and ifnγrel proteins conferred resistance against CHNV infection (Table II). Thus, all three isoforms were confirmed to have strict antiviral activities, suggesting that they function as cytokines, as do human and mouse IFNγs (*39*, *40*).

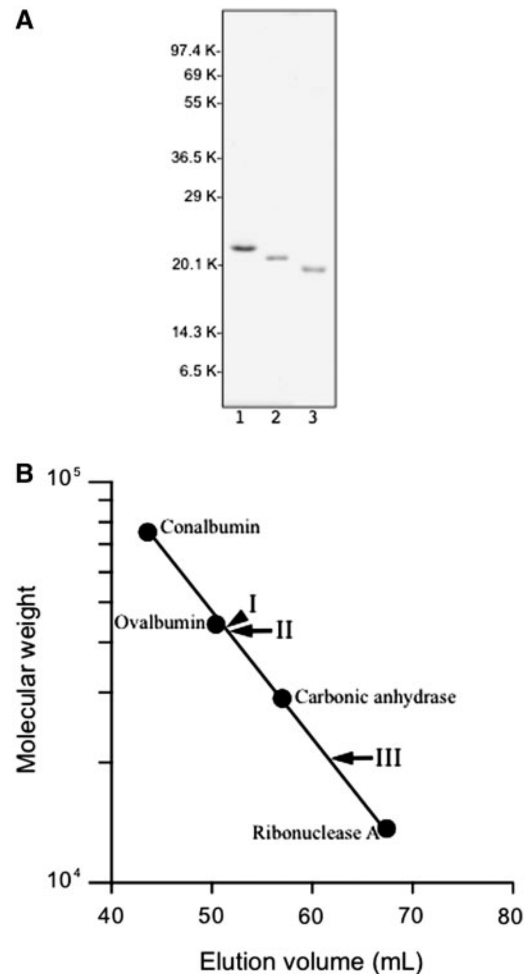

Fig. 1 Characterization of the purified recombinant ginbuna Ifnγ1, Ifnγ2 and Ifnγrel proteins. The recombinant ginbuna crucian carp ifnγ1, ifnγ2 and ifnγrel proteins were expressed in *E. coli* BL21(*DE3*)pLysS cells and purified by using affinity (His Trap HP) and gel filtration (Sephacryl S-100) column chromatographies. (A) The purified recombinant ifnγ1, ifnγ2 and ifnγrel proteins (100 ng) were loaded on a 15% SDS-polyacrylamide gel under reducing conditions and visualized by staining with Coomassie Brilliant Blue R-250. Lane 1, His-tagged ifnγ1; lane 2, His-tagged ifnγ2; lane 3, His-tagged ifnγrel. (B) Molecular weights of the purified recombinant ifnγ1, ifnγ2 and ifnγrel proteins were determined with a Sephacryl S-100 gel filtration column (*HR 16/60*) eluted with 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl; the elution rate was 1 ml/min, and the elution volume was 150 ml. The molecular weights of the standard proteins: conalbumin, 75,000; ovalbumin, 44,000; carbonic anhydrase, 29,000; ribonuclease A, 13,700. The arrowhead (I), arrow (II), and arrow (III) indicate the elution volumes for the purified recombinant ifnγ1, ifnγ2 and ifnγrel proteins, respectively.

Effects of fish Ifnγs on stat1 signalling

Human IFNγ ligand induces phosphorylation of the stat1 protein at the positions of its serine-727 and tyrosine-701 in a human cell line (*41*). Concerning the ifnγ proteins found in fish, it is still unknown whether the fish proteins are capable of inducing such phosphorylation in a human cell line. To address this question, we exposed human HeLa cells to the purified recombinant ginbuna crucian carp ifnγ1, ifnγ2 and ifnγrel proteins; human IFNγ was used as a positive control. As shown in Fig. 3, whereas the human IFNγ induced phosphorylation of the stat1 protein at serine-727 and tyrosine-701 (Fig. 3A, lanes 7–9 and B, lanes 4–6), the fish ifnγ isoforms did not (Fig. 3A, lanes 1–6 and B, lanes 1–3), revealing the phosphorylation at serine-727 and tyrosine-701 of the stat1 protein was a receptor-specific event. Thus, HeLa cells were insensitive to the ginbuna crucian carp ifnγ1, ifnγ2 and ifnγrel proteins.

Functional analysis of ginbuna crucian carp Ifngr1-1 and Ifngr1-2

Ginbuna crucian carp ifnγ's require a specific receptor for signal transduction. Following RT–PCR and cDNA cloning, we obtained two cDNA fragments

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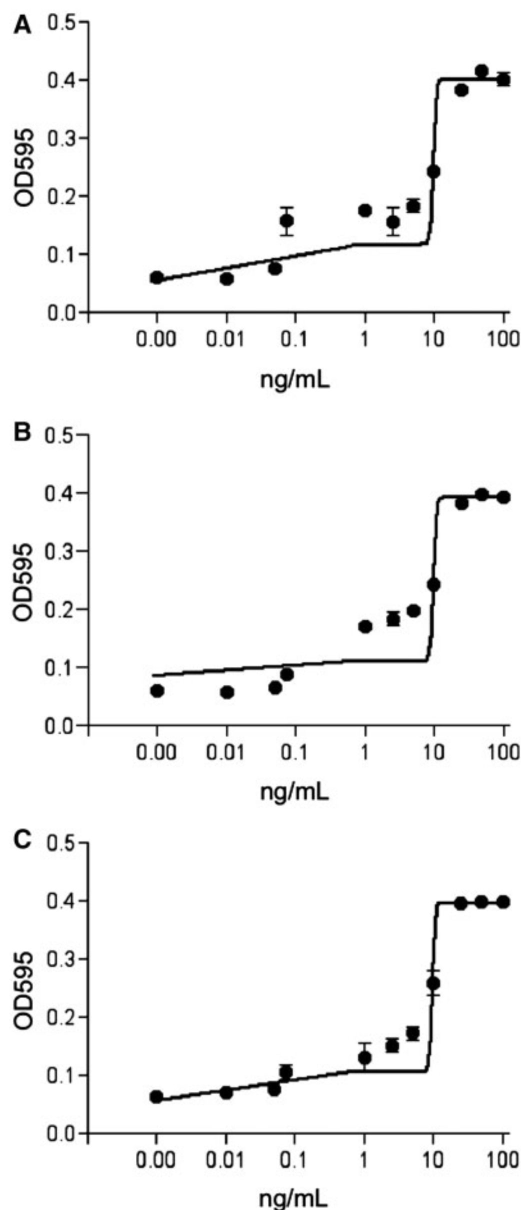

Fig. 2 Antiviral activities of the purified recombinant ginbuna Ifnγ1, Ifnγ2 and Ifnγrel proteins against crucian carp hematopoietic necrosis virus. For determination of the ED_{50}, the activity of the purified recombinant proteins at different concentrations was measured; surviving cells were stained with crystal violet after measuring the OD_{595} as described under 'Experimental Procedures' section. (A–C) Antiviral activities of the purified recombinant proteins ifnγ1, ifnγ2 and ifnγrel, respectively. Each point on the graph represents the mean of three independent experiments; error bars represent standard deviations.

Table II. Ifnγ1-, Ifnγ2- and Ifnγrel-induced survival of GTS9 cells.

	Ligand concentration (ng/ml)			
	0	1	10	50
Recombinant protein	Percentage of cell survival[a]			
ifnγ1	0	12.2 ± 3.4	35.9 ± 5.4	101.2 ± 10.9
ifnγ2	0	8.4 ± 1.5	30.3 ± 9.2	109.5 ± 9.5
Ifnγrel	0	8.6 ± 2.4	25.2 ± 6.7	98.9 ± 10.6

[a]Mean ± S.D. ($n = 3$)

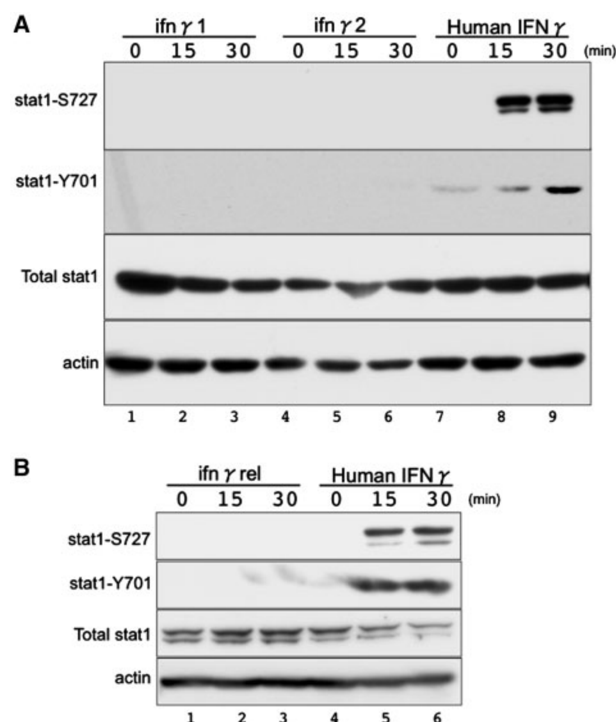

Fig. 3 Detection of stat1 phosphorylation in response to human IFNγ, and the purified recombinant ginbuna crucian carp Ifnγ1 and Ifnγ2 in human HeLa cells. (A) HeLa cells were treated with 25 ng/ml of the purified recombinant ifnγ1 protein (lanes 1–3), with 25 ng/ml of the purified recombinant ifnγ2 protein (lanes 4–6) or with 1 ng/ml of the recombinant human IFNγ protein (lanes 7–9). (B) HeLa cells were treated with 25 ng/ml of the purified recombinant ifnγrel protein (lanes 1—3) or with 1 ng/ml of the recombinant human IFNγ as a positive control (lanes 4–6). (A and B) The cellular proteins were extracted at the times indicated after treatment with the designated recombinant ifnγ ligands. The cell lysates (16 µg protein) were loaded on an SDS-polyacrylamide gel under a reducing condition. The phosphorylated and non-phosphorylated stat1 proteins as well as actin in the cell lysate were separated on a 10% SDS-polyacrylamide gel, blotted onto a PVDF membrane and detected with anti-stat1-S727/anti-stat1-Y701, anti-stat1 and anti-actin antibodies, respectively, as described under 'Experimental Procedures' section.

encoding fish *ifnγ* receptors. Their deduced amino acid sequences were similar to *IFNGR1* sequences of other animals and contained five putative N-linked glycosylation sites and a putative stat1-binding site, and were thus designated *ifngr1-1* and *ifngr1-2*; *ifngr1-1* encoded a protein of 377 amino acid residues with a predicted molecular weight of 42,900 (Supplementary Fig. S2A) and *ifngr1-2* encoded a protein of 344-amino acid residues with a predicted molecular weight of 38,600 (Supplementary Fig. S2B). In a phylogenic tree, these two receptors were clustered with other teleost *ifngr1*'s (Supplementary Fig. S2C).

To prove that the isolated cDNAs indeed coded for functional receptors, we conducted RT–PCR and found the expression of mRNAs coding for *ifngr1-1* and *ifngr1-2* in ginbuna crucian carp GTS9 cells (Supplementary Fig. S3A and B). In addition, we transfected HeLa cells, which did not show a response to ginbuna crucian carp ifnγ1, ifnγ2 and ifnγrel ligands, as shown in Fig. 3, with the created constructs of the FLAG-tagged *ifngr1-1* and *ifngr1-2*. Western blot

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analysis showed that the anti-FLAG antibody specifically reacted with a protein having a molecular weight of ~72,000 or 78,000 in the cells expressing the ifngr1-1 or ifngr1-2 proteins, respectively (Fig. 4A and B); whereas no signal was detected in the non-transfected and the mock-transfected cells.

To investigate whether the ginbuna crucian carp ifngr1-1 and/or ifngr1-2 receptors interacted with their potential ligands, *i.e.* ifnγ1, ifnγ2 and/or ifnγrel, we exposed FLAG-tagged *ifngr1-1*-transfectants or FLAG-tagged *ifngr1-2*-transfectants to each of the three purified ligand proteins, and then examined the phosphorylation at serine-727 and tyrosine-701 of the stat1 protein. As shown in Fig. 4C and D, a rapid increase in stat1 phosphorylation at serine-727 and tyrosine-701 residues was observed to occur in a time-dependent manner when the *ifngr1-1*-transfected cells were exposed to the ifnγ2 protein or when the *ifngr1-2*-transfected cells were treated with the ifnγ1 protein. Thus the *ifngr1-1*-transfected cells responded to ifnγ2 protein, but not to the ifnγ1 ligand. On the other hand, the *ifngr1-2*-transfected cells did not respond to the ifnγ2 but did to the ifnγ1 ligand. Upon treating the *ifngr1-1* (Supplementary Fig. S4A) or the *Ifngr1-2* (Supplementary Fig. S4B) transfectant with the ifnγrel protein, no induction of stat1 phosphorylation was observed.

Jak1 and stat1 associate with two ginbuna crucian carp Ifnγ receptors, Ifngr1-1 and Ifngr1-2

To examine whether the jak1 and stat1 proteins associated with ifngr1-1 and/or ifngr1-2 receptors, we incubated the FLAG-tagged *ifngr1-1*-transfectant and the FLAG-tagged *ifngr1-2*-transfectant with the ifnγ2 and the ifnγ1 ligands, respectively, solubilized them in Nonidet P-40 and Triton X-100, and carried out an immunoprecipitation using anti-FLAG antibody. As shown in Fig. 5, both jak1 and stat1 proteins were

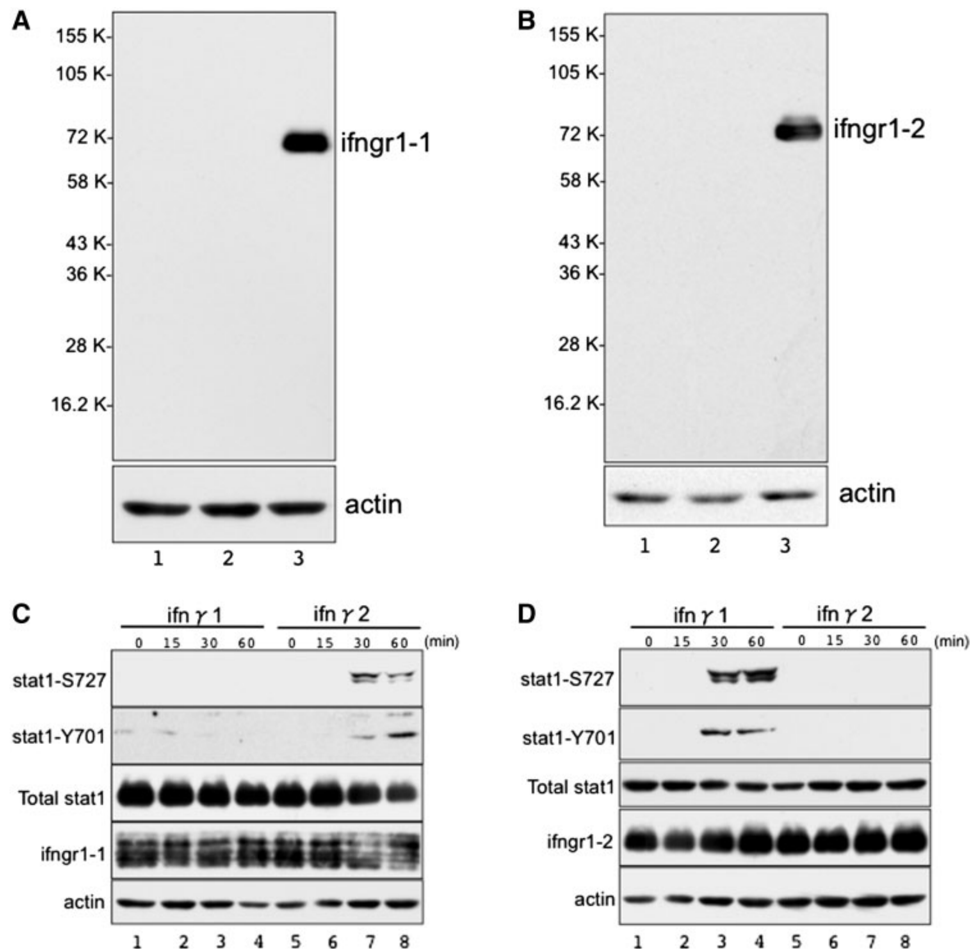

Fig. 4 Activation of transcriptional factor stat1 in response to the specific interaction between the ginbuna crucian carp Ifnγ ligands and their designated Ifngr1 receptors. (A) Lysates of HeLa cells transfected with a FLAG-tagged *ifngr1-1* construct were examined for expression of the construct. Lane 1, HeLa cells; lane 2, mock; lane 3, FLAG-tagged ifngr1-1. (B) Lysates of HeLa cells transfected with a FLAG-tagged *ifngr1-2* construct were examined. Lane 1, HeLa cells; lane 2, mock; lane 3, FLAG-tagged ifngr1-2. (C) A cell line stably expressing ifngr1-1 was treated with 25 ng/ml of either the purified recombinant ifnγ1 protein (lanes 1–4) or the purified recombinant ifnγ2 protein (lanes 5–8). (D) The cell line expressing ifngr1-2 stably was treated with 25 ng/ml of either the purified recombinant ifnγ1 protein (lanes 1–4), or the purified recombinant ifnγ2 protein (lanes 5–8). (C and D) the cellular proteins were extracted at the indicated times after exposing the cells to the Ifnγ's. The cell lysates (16 μg protein) were loaded on an SDS-polyacrylamide gel under a reducing condition. The expressed receptor proteins, the phosphorylated Stat1 protein, Stat1 protein, and actin in the cell lysates were separated on a 10% SDS-polyacrylamide gel, blotted onto a PVDF membrane and detected with anti-FLAG, anti-stat1-S727/anti-stat1-Y701, anti-stat1 and anti-actin antibodies, respectively, as described under 'Experimental Procedures' section.

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detected upon either treating the FLAG-tagged *ifngr1-1*-transfectant with the ifnγ2 ligand or exposing the FLAG-tagged *ifngr1-2*-tansfectant to the ifnγ1 ligand. Neither the control cells nor the non-treated cells gave a signal. The immunoprecipitation experiment demonstrated that the jak1 and stat1 proteins associated with the ifnγ2/ifngr1-1 and/or the ifnγ1/ifngr1-2 complex.

Dual Ifnγ ligands induce binding of stat1 protein to promoters of interferon regulatory factor-1 gene and indoleamine dioxygenase gene

A chromatin immunoprecipitation assay was performed to find out whether the ginbuna crucian carp ifnγ1 and ifnγ2 ligands were able to cause the stat1 protein to bind to the IFNγ-activated site (GAS) element in the promoter regions of the human interferon regulatory factor-1 (*Irf-1*) gene (*35*, *36*) and human indoleamine dioxygenase (*Ido*) gene (*37*). The *ifngr1-1*-transfectant and the *ifngr1-2*-transfectant exposed to the ginbuna crucian carp ifnγ2 and ifnγ1 ligands, respectively, for 30 min were used for the experiment. The PCR product selected for amplification extends from nucleotide −388 to −283 of the promoter region of the *Irf-1* gene and from nucleotide −497 to −378 of that of the *Ido* gene. As a control, the PCR product chosen for the promoter region of the *ß-Actin* (nucleotide −980 to −915) was used. As shown in Fig. 6, the expected PCR products derived from the promoter regions of *Irf-1* and *Ido* genes were observed on an agarose gel when the *ifngr1-1*-transfected cells were incubated with the ifnγ2 ligand and when the *ifngr1-2*-transfected cells were treated with the ifnγ1 ligand; in

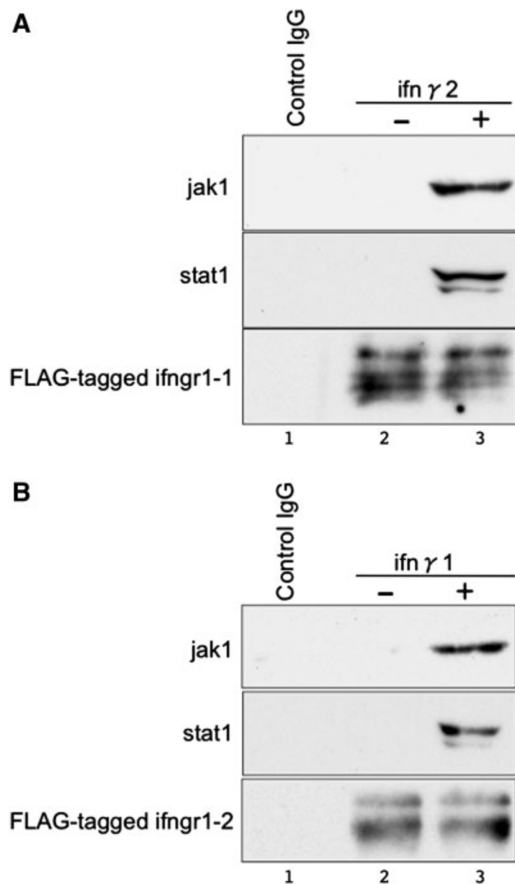

Fig. 5 Association of jak1 and stat1 with Ifngr1-1 and/or Ifngr1-2 receptors. (A) HeLa cells transfected with *ifngr1-1* were incubated with or without 25 ng/ml of ifnγ2 for 30 min. Lane 1, Control IgG; lane 2, ifnγ2-untreated cells; lane 3, ifnγ2-treated cells. (B) HeLa cells transfected with *ifngr1-2* were incubated with or without 25 ng/ml of ifnγ1 for 30 min. Lane 1, Control IgG; lane 2, ifnγ1-untreated cells; lane 3, ifnγ1-treated cells. (A and B) After treatment, the cell lysates (500 μg protein) were incubated with an anti-FLAG antibody first and then with protein G Sepharose. The resulting immunoprecipitates were visualized on western blots as described under 'Experimental Procedures' section.

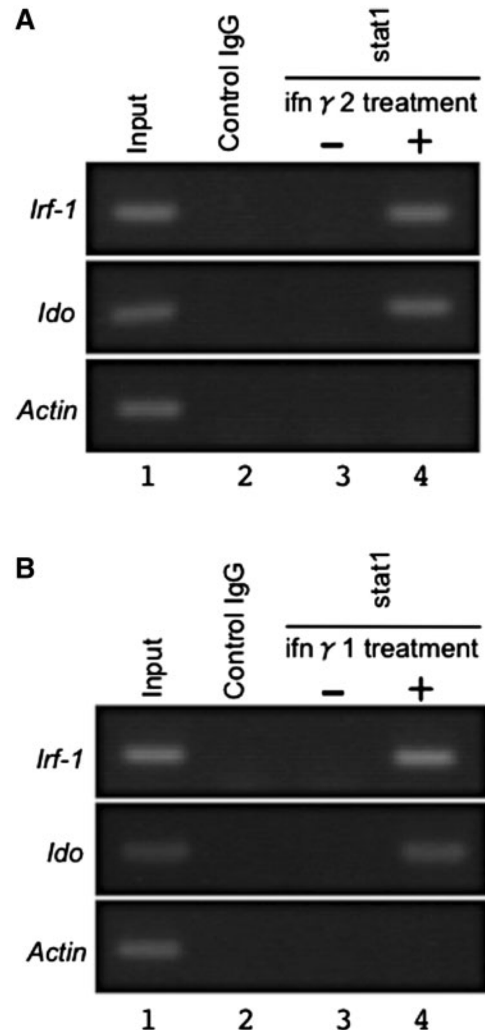

Fig. 6 Binding of transcriptional factor stat1 to the GAS element in promoters of interferon regulatory factor-1 and indoleamine dioxygenase genes. (A) HeLa cells transfected with *ifngr1-1* were incubated with or without of 25 ng/ml of the purified recombinant ifnγ2 for 30 min. Lane 1, input; lane 2, control IgG; lane 3, ifnγ2-untreated cells; lane 4, ifnγ2-treated cells. (B) HeLa cells transfected with *ifngr1-2* were incubated with or without 25 ng/ml of the purified recombinant ifnγ1 for 30 min. Lane 1, input; lane 2, control IgG; lane 3, ifnγ1-untreated cells; lane 4, ifnγ1-treated cells. (A and B) chromatin from cross-linked cells was sheared by sonication and reacted with the specific stat1 antibody overnight, and then incubated with protein G Sepharose for the chromatin immunoprecipitation assay as described under 'Experimental Procedures' section. The immunoprecipitated promoter DNAs coding for *Irf-1*, *Ido* and *Actin* were amplified by PCR using the promoter-specific primers. The obtained PCR products were resolved on a 2.5% agarose gel and stained with ethidium bromide.

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contrast, the control antibody and the transfectant without ifnγ treatment gave no PCR product. In both cases, no amplified product of the promoter region of *ß-Actin* was observed. These findings unambiguously show that the stat1 bound to the promoter regions of the *Irf-1* and *Ido* only when the ifnγ2 and ifnγ1 ligands interacted with their designated receptors.

Binding of fish Ifnγ-activated stat1 protein to the GAS element

stat1 is known to be translocated to the nucleus where it binds to the GAS promoter upon activation by IFNγ in mammals (*15*, *16*) and zebrafish (*26*). By using a gel shift mobility assay, we then sought to confirm that the binding of the stat1 protein at the GAS element occurred in our experimental system. When *ifngr1-1*- or *ifngr1-2*-transfected cells were treated with the ifnγ2 or ifnγ1 ligands, respectively, a shifted band corresponding to the stat1 complex was detected (Fig. 7A and B). Cy5-conjugated double-stranded oligonucleotide derived from the GAS element of the *Irf-1* gene promoter was used as a probe. The intensity of the detected band was decreased in a dose-dependent manner as the amount of Cy5-unlabeled double-stranded oligonucleotide was increased. In the case of GTS9 cells, a similar result was obtained (Fig. 7C). Namely, the shifted band of the stat complex was also detected when the cells were incubated with either ligand. These findings prove that the stat1 protein bound to the GAS promoter

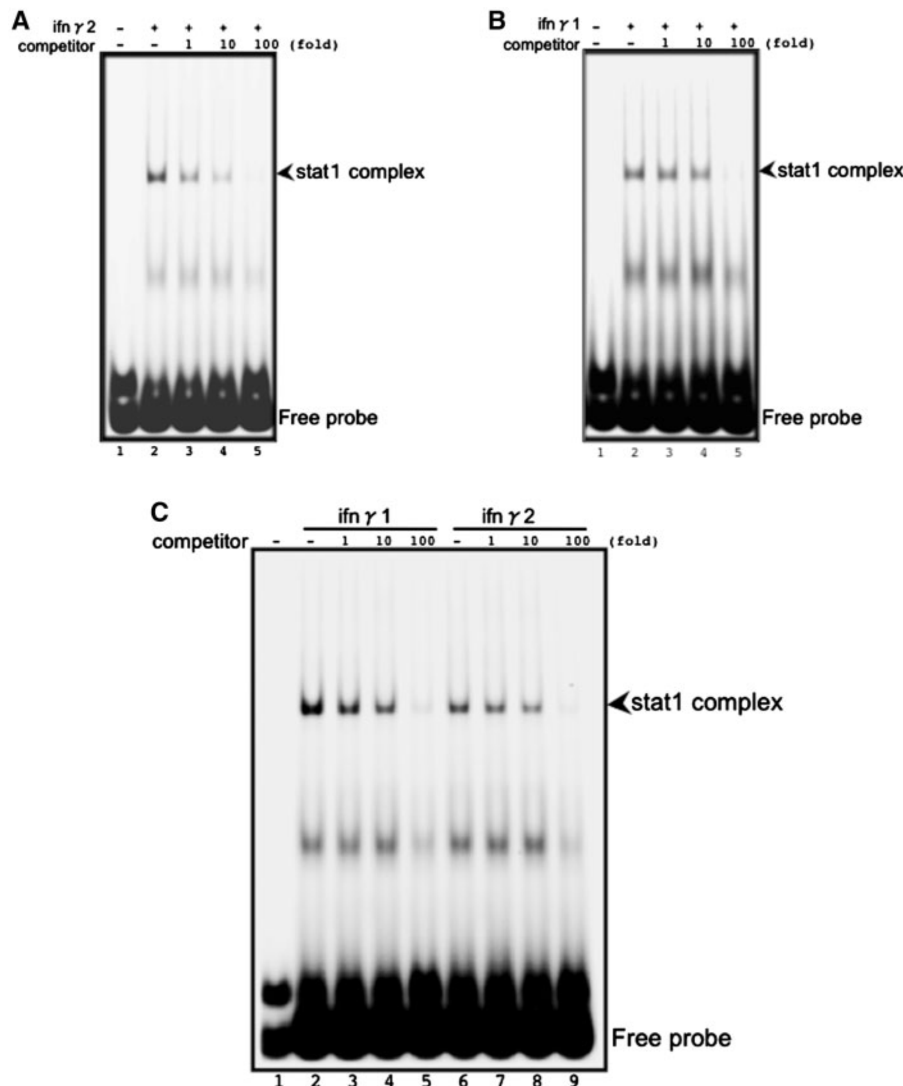

Fig. 7 Ifnγ-induced binding of a stat1 complex to the GAS element of interferon regulatory factor-1 promoter. Gel mobility shift assays were conducted to analyse the binding of the stat1 complex to the GAS element of the human *Irf-1* promoter. (A and B) Nuclear extracts were prepared from *ifngr1-1*- (A) or *ifngr1-2*- (B) transfected HeLa cells incubated without (Lane 1) or with 25 ng/ml of the purified recombinant ifnγ2 (A, Lanes 2–5) or purified recombinant ifnγ1 (B, Lanes 2–5) for 30 min. A competition experiment was conducted with various amounts of double-stranded oligonucleotide in excess over the probe concentration. Lane 2, no excess; lane 3, 1-fold excess; lane 4, 10-fold excess, lane 5, 100-fold excess. (C) Nuclear extracts were prepared from GTS9 cells incubated without (Lane 1) or with either 25 ng/ml of the purified recombinant ifnγ1 (Lanes 2–5) or 25 ng/ml of the purified recombinant ifnγ2 (Lane 6–9) for 30 min. A competition experiment was conducted with various amounts of double-stranded oligonucleotide in excess over the probe concentration. Lanes 2 and 6, no excess; lanes 3 and 7, 1-fold excess; lanes 4 and 8, 10-fold excess; lanes 5 and 9, 100-fold excess. Each gel mobility shift assay was performed by using a 4% polyacrylamide gel as described under 'Experimental Procedures' section. The stat1 complex is indicated by the arrowhead.

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upon activation by the ginbuna crucian carp ifnγ1 or ifnγ2.

Ifnγ-activated stat1 protein was able to induce Ifngr1-1- and Ifngr1-2-dependent transcription in GAS promoter

To confirm that the ginbuna crucian carp ifnγ-activated stat1 protein was able to induce the transcriptional activation in the GAS promoter, we fused it to a luciferase expression vector and conducted a luciferase reporter assay. Two reporter plasmids, the pGL3 promoter vector with or without the GAS element and the pRL-TK vector, were used to co-transfect *ifngr1-1* transfectant (Fig. 8A), *ifngr1-2* transfectant (Fig. 8B) and GTS9 cells (Supplementary Figs. S5 and S8C). Twelve hours after co-transfection, their luciferase activities were measured. The luciferase activities were induced in a ligand dose-dependent manner, whereas the control vector without the GAS promoter resulted in no elevation of luciferase activities. When GTS9 cells were treated with either ligand, luciferase activity increased in a ligand dose-dependent manner as well (Fig. 8C). Upon treating the GTS9 cells with the ifnγrel protein, no induction of luciferase activities was observed (Supplementary Fig. S5). Thus, the luciferase reporter assay showed that the specific receptor–ligand interaction of each ifnγ receptor with its ligand enhanced transcription by promoting the binding of active phosphorylated stat1 protein to the GAS element.

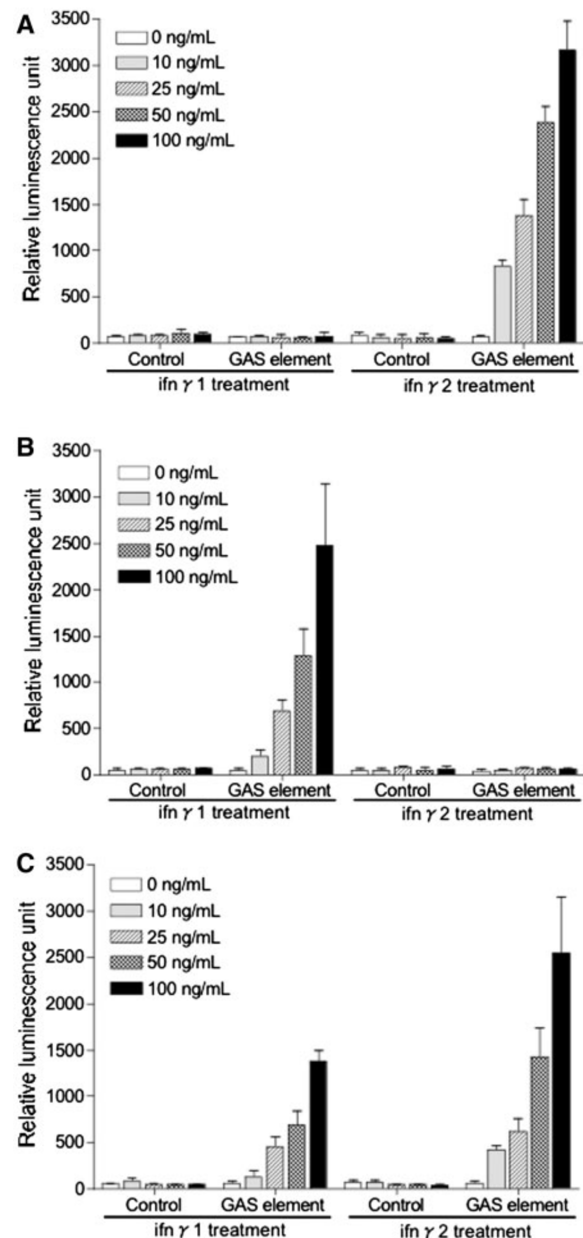

Fig. 8 Transcriptional activation at GAS promoter in response to specific interaction between the ginbuna crucian carp Ifnγ ligands and their designated ifngr1 receptors. (A–C) HeLa cells expressing FLAG-tagged *ifngr1-1* (A) or FLAG-tagged *ifngr1-2* (B) or ginbuna crucian carp GTS9 cells (C) were transfected with a construct containing either no promoter element (*Control*) or the GAS element fused to the luciferase gene (*GAS element*). Constitutively expressed *Renilla* luciferase vector was included in all of the experiments. The transfected cells were treated with 0, 10, 25, 50 or 100 ng/ml of either the purified recombinant ifnγ1 or the purified recombinant ifnγ2 protein for 12 h. Preparation of the cell extracts and measurement of the luciferase activity were carried out as described under 'Experimental Procedures' section. Each value represents the mean of three independent experiments; and error bars represent standard deviations.

Discussion

IFNγ is an antiviral cytokine and an important signalling molecule in mammals. Mammalian genomes possess a single copy of the *IFNγ* gene, whereas teleost genomes have at least two copies (Table I). However, the antiviral activities of fish ifnγ ligands and molecular mechanisms of ifnγ-signalling *in vivo* remained uncharacterized until now. As an approach to clarify these matters, we first cloned the ginbuna *ifnγ1*, *ifnγ2* and *ifnγrel* cDNAs and expressed their proteins in *E. coli*. Using the purified recombinant ifnγ proteins, we then examined their subunit structures and antiviral activities. Our results show that the recombinant ifnγ1 and ifnγ2 were homodimeric proteins, as in the case of human and mouse IFNγ recombinant proteins (*39*, *40*); whereas the recombinant ifnγrel was a monomeric one. Our finding that all three ginbuna recombinant proteins elicited high antiviral activities against crucian carp hematopoietic necrosis virus (Fig. 2) suggest that these fish proteins function as cytokines similarly as do human and mouse IFNs (*39*, *40*).

Concerning the molecular mechanism of ginbuna ifnγ receptor-mediated signalling, when the *ifngr1-1* transfected cells were treated with the ifnγ2 ligand or when the *ifngr1-2* transfected cells were treated with the ifnγ1 ligand, stat1 was phosphorylated at both serine-727 and tyrosine-701 residues in a time-dependent manner. The immunoprecipitation experiments demonstrated that jak1 and stat1 proteins became associated with the ifnγ2/ifngr1-1 or ifnγ1/ifngr1-2 complex. In addition, chromatin immunoprecipitation showed that stat1 protein bound to the promoter regions of the human interferon regulatory factor-1 (*Irf-1*) gene and the human *Ido* gene only when the ifnγ2 and ifnγ1 ligands interacted with their designated receptors. Furthermore, the gel shift assay indicated that the

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stat1 protein bound to the GAS element of human *Irf-1* as a consequence of activation of the receptor signalling mechanism by the ifnγ1 or ifnγ2 ligand. Finally, the luciferase reporter assay clearly showed that the luciferase activities were induced in a ligand dose-dependent manner and that the control vector without the GAS promoter gave no enzyme activity. As summarized in Fig. 9, the two isolated cloned ligands, ifnγ1 and ifnγ2, of ginbuna crucian carp stimulated stat1-dependent signal transduction mechanisms regulated by the dual ifnγ receptors, ifngr1-1 and ifngr1-2.

The IFNγ signalling pathway in mammals requires jak1/stat1 phosphorylation. When the switch is on, the phosphorylated stat1 protein is translocated into the nucleus and binds there to the GAS consensus sequence, TTC$N_{2\text{-}4}$GAA, within the promoter region of *IFNγ*-responsive genes (*35*, *36*). The *mx* gene promoter in transfected teleost cell lines responds less to Ifnγ and predominantly to the other type I IFN (*42–44*). Recently, the transfected promoter of the antigenic peptide 2 transporter gene was shown to be activated by trout ifnγ (*45*) even though the GAS sequence was absent. Our present study shows that ginbuna crucian carp ifnγ-activated stat1 protein initiated the transcriptional activation by the GAS promoter, as occurs in the case of the human and mouse proteins. Further studies are required to resolve the above discrepancy and to elucidate the molecular mechanism(s) of antiviral protection acquired by the transcription activation of ifnγ-activated stat1 protein in response to the type II IFN in teleosts and other higher vertebrates.

Using the recombinant ginbuna crucian carp ifnγ and ifnγrel proteins, we showed dual ifnγ signalling pathways operating through two distinct receptors, ifngr1-1 and ifngr1-2, in strict specific responses to ifnγ2 and ifnγ1. Based on the amino acid sequence identity, we speculate that zebrafish *ifnγ* (*21*) and goldfish *ifnγ1* (*24*, *27*) correspond to ginbuna crucian carp *ifnγ1*, which interacted with the *ifngr1-2* receptor, but not the *ifngr1-1* receptor. Indeed, *in vitro* cross-linking experiments showed that the recombinant ligand proteins ifnγ1 and ifnγrel from goldfish bind to the recombinant receptor proteins ifngr1-2 and ifngr1-1, respectively, and that the receptor proteins lack putative transmembrane and cytoplasmic domains (*28*). *In vivo* knockdown experiments showed that zebrafish *ifnγ1* associates with the cytokine receptor family 17 (crfb17) receptor, a homologue of *ifngr1-2*, whereas zebrafish ifnγrel interacts with both crfb17 receptor and cytokine receptor family 13 (crfb13) receptor, a homologue of *ifngr1-1* (*46*). The interaction between the ginbuna crucian carp ifnγ1 and ifngr1-2 that we found in the transfection experiments is consistent with these results (*46*). However, in the case of the ifngr1-1 receptors, we showed that ifnγ2 rather than ifnγrel is the interacting ligand. So far, the ifnγ2 ligand protein has not been found in other fish; and the *ifngr1-1* has not been isolated or characterized in goldfish or zebrafish. Further kinetics analyses and crystal structural analyses of the ligand–receptor complex are

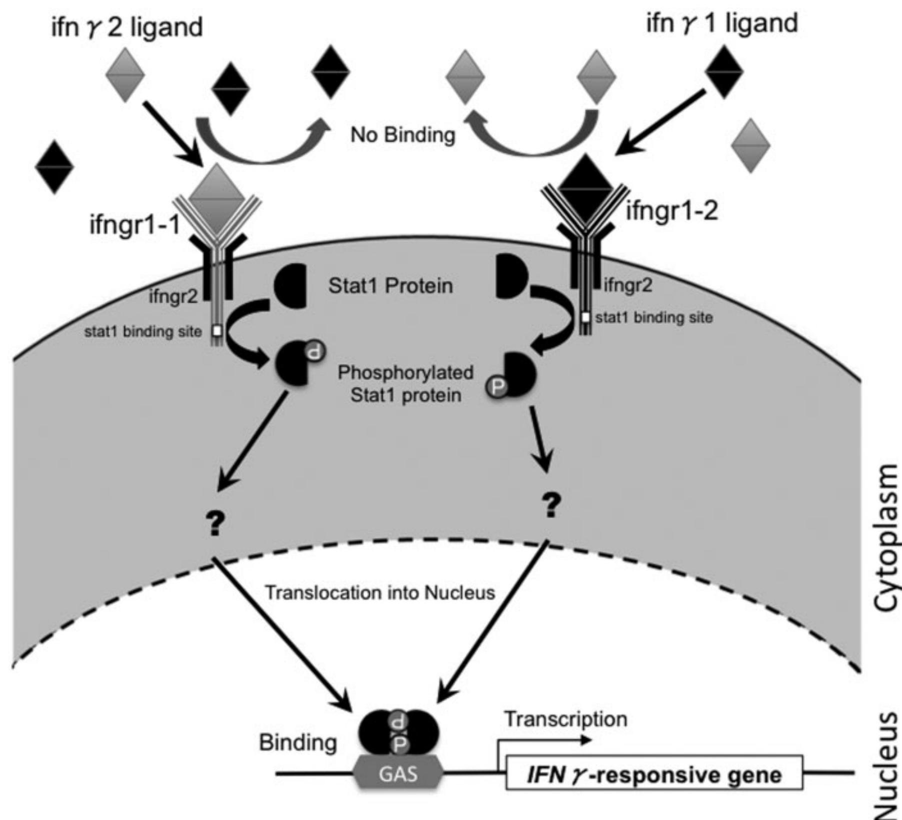

Fig. 9 Diagram of the dual ginbuna crucian carp ifnγ signalling pathways.

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needed to elucidate the interaction between ifnγ ligands and their receptors in teleosts. Since the gene expressions of *ifnγ1* and *ifnγ2* are tissue-specific and responsive to mitogenic stimulation (Araki *et al. in preparation*), teleosts may possess dual antiviral protection mechanisms for their innate and adaptive immune responses. The biological significance of these dual *ifnγ* signalling pathways remains to be elucidated.

Our finding that the recombinant ifnγrel protein induced neither stat1 phosphorylation in the *ifngr1-1* and *ifngr1-2* transfectants (Supplementary Fig. S4) nor transcriptional activation at GAS promoter in the GTS9 cells (Supplementary Fig. S5) indicates that stat1 was not used in the ifnγrel-mediated signalling pathway. Thus, we speculated that other pathway(s), presumably a previously unrecognized one, which may be specific to fish, must be utilized instead.

The IFN proteins initiate signalling by binding to their cognate receptors. The multiple human type I IFN ligands, including the subspecies IFNα, IFNβ, IFNε, IFNκ and IFNω, act through the shared type I receptor complex composed of two chains, IFNα receptor 1 (IFNαR1; *IFNAR1*) and IFNαR2c (*IFNAR2*) (*47*, *48*). Also, there is an IFNλ protein, a type III IFN, that possesses many of the functional properties of type I IFN: IFNλ acts through a receptor complex composed of the IFNλR1 (*IFNLR1*) and cytokine receptor family 2-4 (*IL10R2*) chains (*49*, *50*). Concerning the signalling mediated by type I (*51*) and III IFNs (*52*), jak1 and tyk2 are activated, leading to the activation of stat1 and stat2, respectively, following the binding of IFN ligands to their cognate cell-surface receptors. The dimerized stat proteins associate with interferon regulatory factor-9 (Irf-9) to form the trimeric IFN-stimulated regulatory factor 3 (ISGF3) transcription complex (*51*). The ISGF3 is then translocated to the nucleus, where it binds to the IFN-stimulated response element (ISRE) to activate the transcription of the IFN-stimulated genes. In zebrafish, ifnγrel signalling seems to be mediated through a distinctly different receptor complex consisting of crfb17 and crfb13 chains (*46*). These results, together with ours, lead us to speculate that the ginbuna IFNγrel induces antiviral activity by activating a mechanism similar to the type III IFN system of mammals, although a fish type III IFN system has not yet been discovered.

Ginbuna and zebrafish *ifnγrel*s (*21*) are thought to be cytokines different from goldfish (*28*) and carp (*23*) ones. Although goldfish, ginbuna, carp and zebrafish *ifnγrel*s have similar primary structures and biological activities, the ginbuna and zebrafish *ifnγrel*s contain a putative nuclear localization signal in their C-terminal region. Further studies are needed to understand the signalling pathway(s) mediated by *ifnγrel*. We are presently conducting a functional analysis of the ligand and receptor proteins (Shibasaki *et al. in preparation*).

The type II IFN system can be used to investigate other aspects of the immune system such as the regulation of host defence by viruses, bacteria, and parasites. Also, it can be applied to the research studies on the effects of fish diseases on ifn biology.

In conclusion, we showed that ginbuna ifnγ1, ifnγ2 and ifnγrel elicited high antiviral activity and that the molecular mechanism of *ifnγ1* or *ifnγ2* signalling was similar to that of the human and mouse types. Further studies are needed to address the role of the ifnγ1 and ifnγ2 ligands in virus-induced activation of antiviral genes *in vivo* and to identify the mechanisms of *ifnγrel*-induced antiviral activity in host-defence. Characterization of fish *ifnγs* at the molecular level will certainly contribute to our understanding of signalling pathways that mediate antiviral activity.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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