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Identification, expression profiles and antiviral activities of a type I IFN from gibel carp *Carassius auratus gibelio*

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ABSTRACT

Type I interferons, as a class of multipotent cytokines, play a key role in host antiviral immune responses. In this study, a type I IFN coding gene of gibel carp, *Carassius auratus gibelio*, *Cag*IFNa was cloned and sequenced. The full-length cDNA sequence of *Cag*IFNa consists of 724 nucleotides that encode a predicted protein of 183 amino acids. *Cag*IFNa has two highly conserved cysteine residues in the deduced protein, which is mostly conserved in the fish group I type I IFNs. *Cag*IFNa was identified as a member of the IFNa subgroup of group I type I IFNs by phylogenetic analysis. *Cag*IFNa transcripts were detected in all investigated tissues with higher levels in the liver, intestine, spleen and head kidney of gibel carp. Following injection with Cyprinid herpesvirus 2 (CyHV-2), *Cag*IFNa gene expression was significantly inhibited in the spleen but delayed and then increased in head kidneys. Similarly, while *Cag*IFNa expression was rapidly induced in gibel carp brain (GiCB) cells by poly I:C stimulation and its high induction level was delayed following CyHV-2 infection. *Cag*IFNa overexpression in GiCB cells drastically reduced virus CPE and titer. Furthermore, several genes associated with type I IFN signaling pathway including IRF3, IRF7, IRF9, STAT1, Mx1 and PKR were induced in GiCB cells overexpressing *Cag*IFNa upon CyHV-2 infection. These results show that *Cag*IFNa plays a role in antiviral immune system in gibel carp.

1. Introduction

The innate immune system plays a crucial role in early host responses against pathogens and is integrated with the adaptive immune system to provide effective immune responses [1]. Interferons (IFNs) are important secreted cytokines that have intricate functions in both innate and adaptive immunity. Activation of antiviral defense of the teleost fish relies on the host's pattern-recognition receptors (PRRs) [2,3]. PRRs recognize pathogen products and then trigger a series of downstream signal cascade, including the recruitment of IFN regulatory factor 3/7 (IRF3/7) that result in the production of type I IFNs [2,4]. Subsequently, cell surface cognate receptors recognize and bind with type I IFNs to activate an array of IFN-stimulated genes (ISGs), such as GTPase Mx and protein kinase R (PKR), to establish an host antiviral state through the JAK/STAT pathway [5]. Gibel carp IFN antiviral system possesses all the key factors involved in the JAK/STAT pathway, including the ISG factor 3 (ISGF3) complex that consists of STAT1, STAT2 and IRF9, which is the counterpart of mammalian antiviral system [6,7].

The mammalian IFNs are categorized as type I IFNs (α/β), type II IFNs (γ), and type III IFNs (λ) according to their genomic structure, biological properties as well as the receptor usage [8]. However, only IFN-I and IFN-III, known as "virus-induced IFNs", are truly specialized as innate antiviral cytokines [3]. Although gene encoding virus-induced IFNs in teleost fish share homology with mammalian type I IFN genes, phylogenetic analysis does not support a division into IFNα or IFNβ as in mammals [3,9]. Rather, fish type I IFNs are classified into two groups: group I and group II, based on the number of cysteine residues (two or four) in their mature peptide [10]. Based on phylogenetic analysis, fish type I IFNs are further subdivided into seven subgroups: IFNa, IFNd, IFNe and IFNh belong to group I type I IFNs, and IFNb, IFNc and IFNf belong to group II type I IFNs [11,12]. Interestingly, group I IFNs exist in all investigated teleost fish, but group II IFNs are only found in salmonid, cyprinid, siluriform and perciform species [13–16]. Three subgroups, IFN-a, -c and –d, exist in cyprinids such as zebrafish, common carp and grass carp [11,17]. However, to date only one group II type I IFN, IFNc, has been cloned and identified in gibel carp [18].

Although fish type I IFNs share common antiviral activities, they show distinct expression patterns and biological activities. Group I IFNs can be detected in a range of cell types and display a delayed but higher

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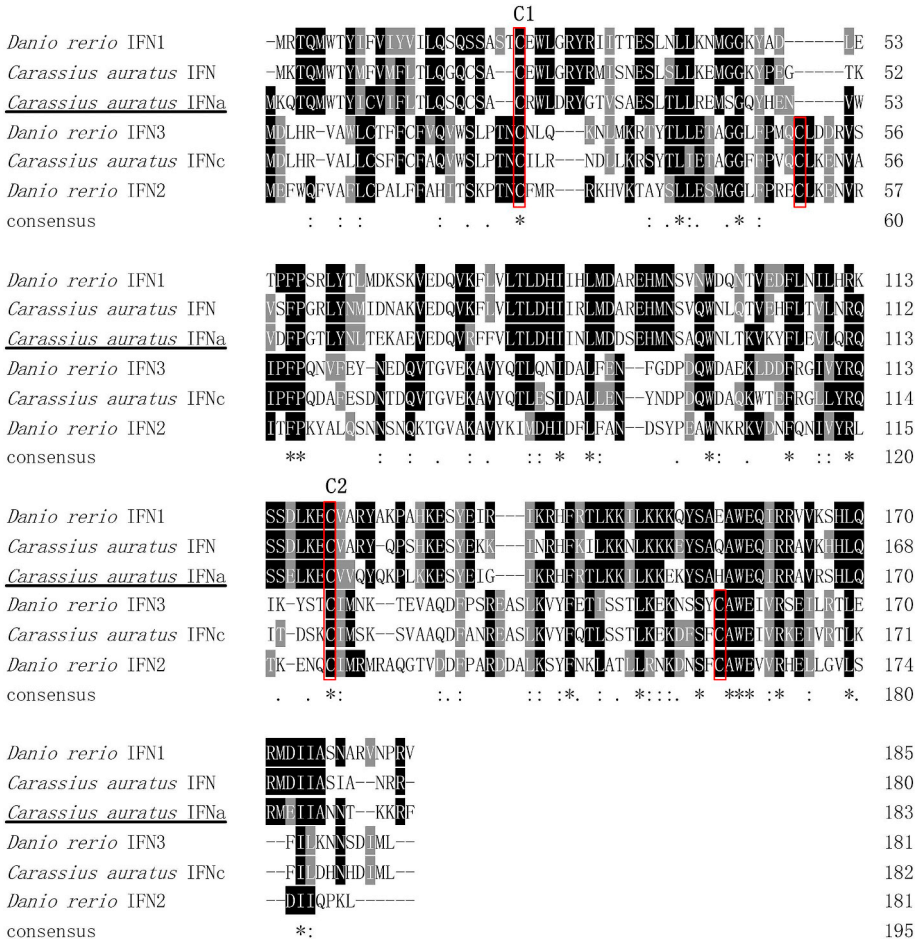

Fig. 1. Multi-alignment of *Cag*IFNa amino acid sequence with other teleost fish type I IFNs. Multiple sequence alignments among the amino acid sequences were generated with Clustal W program. The conserved residues are shaded using BoxShade (v3.21). Boxes denote the highly conserved cysteine residues. C1 and C2 indicate the sites of conserved cysteine residues of *Cag*IFNa. Identical (*) and similar (.) and (:) residues are indicated.

induction of most ISGs, whereas group II IFNs are expressed only in specific tissues and induce a rapid but transient expression of antiviral genes [11,19]. Recently, a IFNa gene from gibel carp blastulae embryonic (CAB) cells was shown to exhibit antiviral activities against the grass carp reovirus (GCRV) by inducing the expression of a series of ISGs through the STAT1 pathway [7]. The triploid crucian carp IFNa was identified as a secreted antiviral cytokine that can decrease cytopathic effect (CPE) and viral titer in IFNa-overexpressed *Epithelioma papulosum cyprini* (EPC) cell line following both the spring viremia of carp virus (SVCV) and GCRV infection [20]. In Atlantic salmon TO cells, both IFNa and IFNc exhibit significant antiviral activity and ability to induce antiviral genes following the infectious pancreatic necrosis virus (IPNV) infection, which are stronger than IFNb and IFNd [21]. These results suggest that IFNa plays a pivotal role in host antiviral defense.

The gibel carp (*Carassius auratus gibelio*) is an important freshwater aquaculture species in China [22]. In 2012, this species suffered a severe epizootic leading to huge economic losses in China. Cyprinid herpesvirus 2 (CyHV-2) has been identified as the causative agent of the disease named haematopoietic necrosis in gibel carp by electron microscopy, PCR assay [23], cell culture [24], etc. Given the current lack of effective methods available to control this disease, more investigation of host immune responses to CyHV-2 infection is of high relevance. In this study, a gibel carp type I IFN (*Cag*IFNa) was identified and characterized. The expression patterns of *Cag*IFNa gene both *in vivo* and *in vitro* were profiled, and the antiviral activities of *Cag*IFNa against CyHV-2 in gibel carp brain (GiCB) cells were investigated. This research may provide insights into the interaction between CyHV-2 infection and host defense in gibel carp, and lay a basis for the control of the viral disease in future.

2. Materials and methods

2.1. Fish, cell line and virus

All the animal handling and experimental procedures were approved by the Animal Care and Use Committee of Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS). Healthy gibel carps (50 ± 5 g) were obtained from the fish farm at the Yangtze River Fisheries Research Institute. Animals were kept in tanks with cycling water at 25 °C and fed with commercial foods twice a day for one week before experiment. The GiCB cells were cultured in medium 199 supplemented with 10% fetal bovine serum (FBS) at 28 °C [24]. For experimental infection, CyHV-2 was isolated from diseased gibel carp by our laboratory [23] and was propagated in GiCB cells according to methods described previously [25].

2.2. Cloning of CagIFNa cDNA

The sequences of all primers used in this study are listed in Table S1. Total RNA was extracted from gibel carp kidneys using TRIzol Reagent (Invitrogen) and reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacture's protocol. *Cag*IFNa-3F primers and *Cag*IFNa-5R primers were designed based on the result of the transcriptome analysis of gibel carp

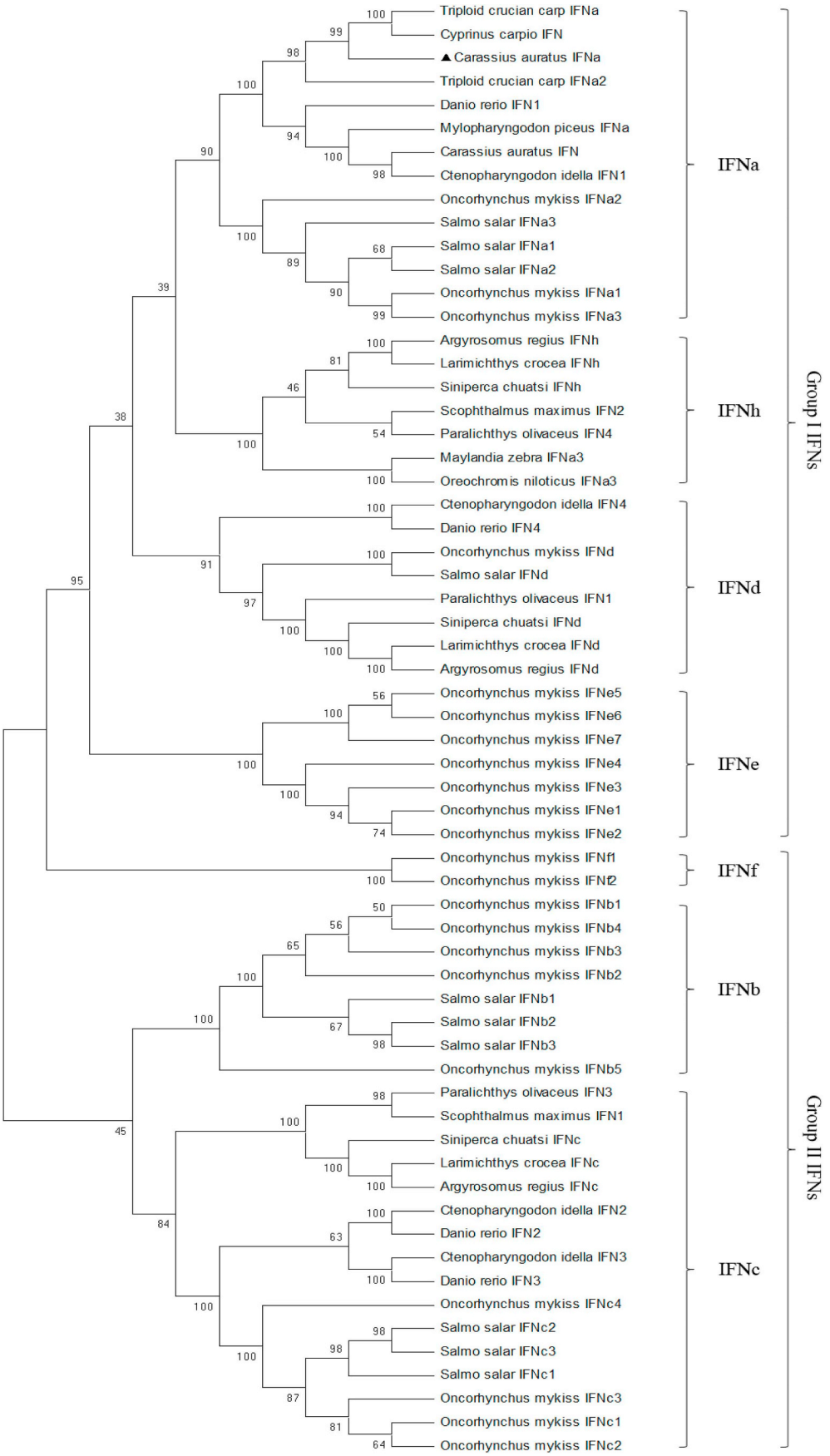

Fig. 2. Phylogenetic relationships of *Cag*IFNa and other known type I IFNs. Bootstrapping was performed 1000 times. GenBank accession numbers of the selected sequences are shown as follows: *Argyrosomus regius*, AVD96636 (IFNc), AVD96637 (IFNd), AVD96638.1 (IFNh); *Carassius auratus*, MK093763 (IFNa), AAR20886 (IFN); *Ctenopharyngodon idella*, ACZ36480 (IFN1), AMT92190 (IFN2), AMT92191 (IFN3), AMT92192 (IFN4); *Cyprinus carpio*, BAG68521 (IFN); *Danio rerio*, AAM95448 (IFN1), NP_001104552 (IFN2), NP_001104553 (IFN3), NP_001155212 (IFN4); *Larimichthys crocea*, AYP67466 (IFNc), API68651 (IFNd), API68650 (IFNh); *Maylandia zebra*, XP_014265825 (IFNa3); *Mylopharyngodon piceus*, AKM15287 (IFNa); *Oncorhynchus mykiss*, CAM28541 (IFNa1), NP_001153977 (IFNa2), CCV17397 (IFNa3), NP_001153974 (IFNb1), NP_001158515 (IFNb2), CCV17399 (IFNb3), CCV17400 (IFNb4), CCV17401 (IFNb5), CCV17402 (IFNc1), CCV17403 (IFNc2), CCV17404 (IFNc3), CCV17405 (IFNc4), CAV07949 (IFNd), CCV17406 (IFNe1), CCV17407 (IFNe2), CCV17408 (IFNe3), CCV17409 (IFNe4), CCV17410 (IFNe5), CCV17411 (IFNe6), CCV17412 (IFNe7), CCV17413 (IFNf1), CCV17414 (IFNf2); *Oreochromis niloticus*, XP_005469255 (IFNa3); *Paralichthys olivaceus*, AET71736 (IFN1), BBA46271 (IFN3), BBA46272 (IFN4); *Salmo salar*, AAP51035 (IFNa1), AAP51036 (IFNa2), ACE75687 (IFNa3), ACE75691 (IFNb1), ACE75693 (IFNb2), ACE75689 (IFNb3), ACE75692 (IFNc1), XP_014048249 (IFNc2), ACE75688 (IFNc3), DAA64377 (IFNd); *Scophthalmus maximus*, AID59461 (IFN1), AID59462 (IFN2); *Siniperca chuatsi*, AVJ47959 (IFNc), AVJ47960 (IFNd), AVJ47961 (IFNh); *Triploid crucian carp*, AMQ67073 (IFNa), AOR08324 (IFNa2).

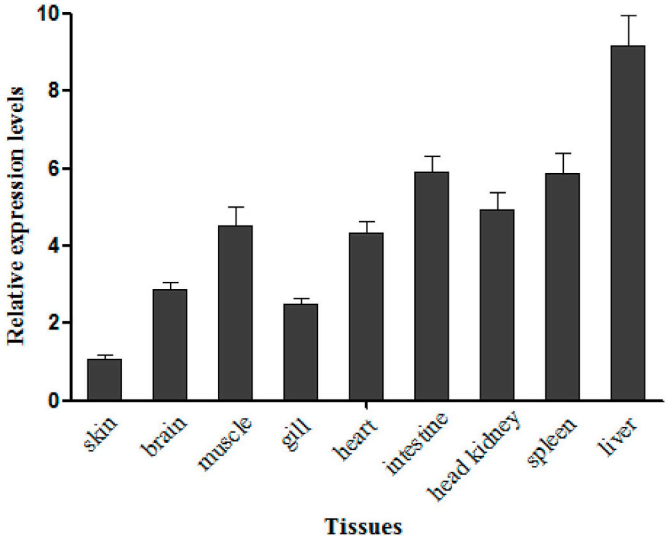

Fig. 3. Tissue expression profile of *Cag*IFNa in healthy gibel carp. Results are presented as the relative expression ratios of *Cag*IFNa in tissues versus skin, β-actin was used as an internal control. Error bars indicate the mean ± SD (n = 5).

(unpublished data). Rapid amplification of cDNA ends (RACE) PCR was performed using the SMART RACE cDNA Amplification Kit (Clontech) to identify the 5′ and 3′ untranslated region (UTR) of *Cag*IFNa gene according to the manufacturer's instructions separately. The primers *Cag*IFNa-ORF-F/R targeting 5′ and 3′ UTR regions were used to confirm the full-length coding sequence of *Cag*IFNa. All the specific PCR products were purified on 1.5% agarose gels and then cloned into pMD19-T vector (Takara) for sequencing.

2.3. Sequence analysis and alignment of CagIFNa gene encoded protein

The deduced amino acid sequence, analysis of molecular conserved structure such as cysteine residues of *Cag*IFNa were identified by the Expert Protein Analysis system (ExPASy) proteomic tool (http://www.expasy.org/tools/). The Prot-Param tool of ExPASy (http://www.expasy.org/tool/protparam/) was used to calculate both mass and PI of the putative protein. The BLAST program at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) was used to search sequences. Multiple amino acid sequence alignments were generated with Clustal W (http://www.ebi.ac.uk/Tools/clustalw/), and the conserved residues were shaded using BoxShade (v3.21). Phylogenetic tree was constructed by Mega5.1 using the neighbor-joining (NJ) algorithm [26].

2.4. Expression analysis of CagIFNa gene in vivo

For *Cag*IFNa gene tissue expression profiling, nine tissues including the liver, spleen, intestine, head kidney, heart, gill, brain, skin and muscle were collected from healthy gibel carps (n = 5). Total RNA was extracted using TRIzol Reagent (Invitrogen), treated with DNase for 6 h to eliminate contaminated genomic DNA, and then extracted by phenol/chloroform as previously described [27]. The RNA was transcribed into cDNA as described above for quantitative real-time PCR on Rotor-Gene 6000 Real-time PCR system (Qiagen). The real-time PCR amplification reaction system, cycling reaction steps and the subsequent data analysis were same as described previously [22]. The primers used for real-time PCR are listed in Table S1.

To clarify the effects of CyHV-2 infection on *Cag*IFNa in individual level, thirty-six carps were randomly divided into treated group and control group; each group contained 18 fish. In the treated group, carps were intraperitoneally injected with 300 μl of CyHV-2 (1.0×10^6 $TCID_{50}$/ml). In the control group, carps were intraperitoneally injected with equal volume of PBS. The spleen and head kidney of three individuals were collected from each group at 0, 6, 12, 24, 48 and 120 h post-injection. Subsequently, real-time PCR was performed to analyze the expression level of *Cag*IFNa. All the experiments were repeated three times.

2.5. Poly I:C stimulation and CyHV-2 infection in vitro

GiCB cells (5×10^5 cells/ml) were cultivated in 6-well plates and cultured overnight until a monolayer was formed. For poly I:C stimulation, the GiCB cells were transfected with 2 ml of medium containing 4 μg of poly I:C (Sigma) and 12 μl of Fugene HD (Promega). For CyHV-2 infection, the cells were infected with virus at an MOI of 0.5. The control group was treated with same volume of PBS. Three parallel samples in each group were collected at 0, 6, 12, 24, 48 and 120 h post stimulation and infection. Subsequently, real-time PCR was performed to analyze the expression level of *Cag*IFNa. All the experiments were repeated three times.

2.6. Construction, transfection and over-expression of CagIFNa gene

The open reading frame (ORF) of *Cag*IFNa was cloned using specific primers *Cag*IFNa-F/*Cag*IFNa-R (Table S1) including *Bgl* II and *Kpn* I sites

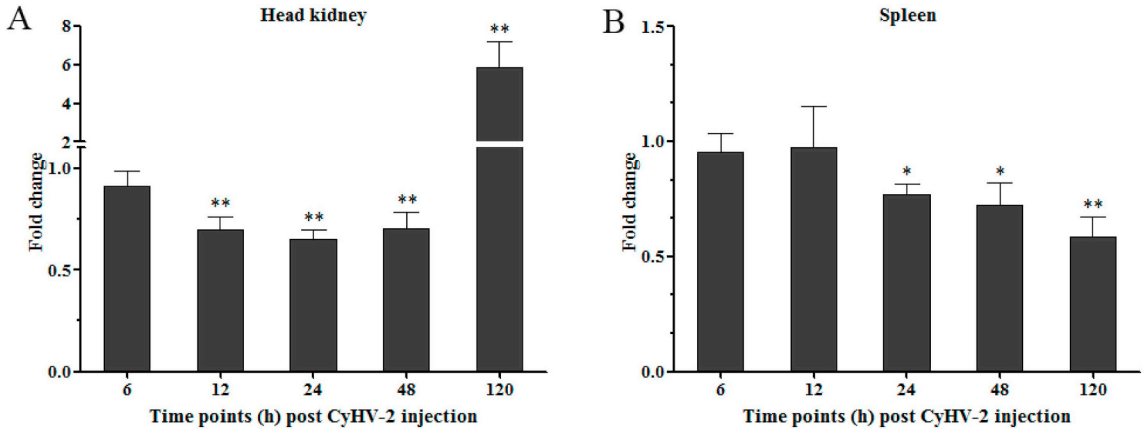

Fig. 4. The mRNA expression profiles of *Cag*IFNa in the head kidney (A) and spleen (B) at 6, 12, 24, 48 and 120 h post CyHV-2 injection. The expression level of *Cag*IFNa was determined by real-time PCR and all data were normalized to β-actin. The data are expressed as fold-induction relative to the control. Error bars indicate the mean ± SD (n = 3). The asterisks indicate significant difference ($^{**}P < 0.01$, $^{*}P < 0.05$) between treated and control (0 h) groups.

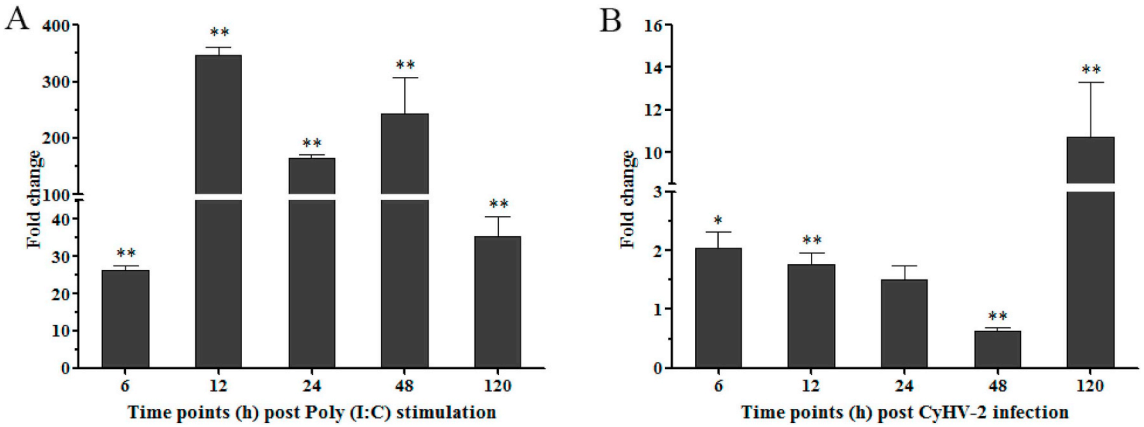

Fig. 5. The mRNA expression profiles of *Cag*IFNa in GiCB cells at 6, 12, 24, 48, and 120 h post poly I:C stimulation (A) and CyHV-2 infection (B). The expression level of *Cag*IFNa was determined by real-time PCR and all data were normalized to β-actin. The data are expressed as fold-induction relative to the control. Error bars indicate the mean ± SD (n = 3). The asterisks indicate significant difference ($^{**}P < 0.01$, $^{*}P < 0.05$) between treated and control (0 h) groups.

for orientation into the expression vector under a CMV promoter (pCMV-HA-*Cag*IFNa). GiCB cells (2×10^5 cells/ml) were cultivated in 6-well plates for 24 h until the cells should be approximately 70%–80% confluency. Then three wells in each plate were transiently transfected with 2 μg/ml pCMV-HA-*Cag*IFNa using 16 μl/well Fugene HD. And the remaining three wells were transiently transfected with 2 μg/ml pCMV-HA-N empty vector using 16 μl/well Fugene HD as control. Cell lysates and the supernatant media were respectively harvested from three parallel samples in each group at 24 h post-transfection. The protein concentration in supernatant media was compressed using ProteoMiner Protein Enrichment Kit (Bio-Rad) according to the manufacturer's instructions. Then Western blot analysis was used to examine whether the transfected plasmid produce and secrete HA-*Cag*IFNa to extracellular. And the cell lysates of transfected cells were harvested from three parallel samples in each group at 30, 36, 48, 72 and 144 h post-transfection to clarify the continued over-expression of HA-*Cag*IFNa by Western blot analysis, respectively. Cell lysates and the enriched supernatant media were normalized to 0.5 mg/ml using the BCA Protein Assay Kit (Beyotime) and separated by 10% SDS-PAGE at the quantity of 15 μg in 30 μl per lane. The method of Western blot analysis was performed as described previously [28].

2.7. Antiviral activities of CagIFNa in GiCB cells

GiCB cells (2×10^5 cells/ml) were cultivated in 6-well plates for 24 h until the cells should be approximately 70%–80% confluency. Then three wells in each plate were transiently transfected with 2 μg/ml pCMV-HA-*Cag*IFNa or pCMV-HA-N (as control) using 16 μl/well Fugene HD. After 24 h, the transfected GiCB cells were infected with CyHV-2 at an MOI of 0.5. CPE in cells was observed by microscopy at 24, 48, and 120 h post CyHV-2 infection. Meanwhile, the cells containing supernatants were collected from three parallel samples in each group and the virus titrated by standard 50% tissue culture infective dose ($TCID_{50}$).

GiCB cells (2×10^5 cells/ml) were cultivated in 6-well plates for 24 h until the cells should be approximately 70%–80% confluency. Then three wells in each plate were transiently transfected with 2 μg/ml pCMV-HA-*Cag*IFNa or pCMV-HA-N (as control) using 16 μl/well Fugene HD. After 24 h, the transfected GiCB cells were infected with CyHV-2 at an MOI of 0.5. The transfected GiCB cells were respectively harvested from three parallel samples in each group at 6, 12, 24, 48, and 120 h post CyHV-2 infection. Real-time PCR was performed to quantify the expression levels of IRF3, IRF7, IRF9, STAT1, Mx1 and PKR genes at each time point. To further analyze the antiviral effect of *Cag*IFNa, the expression levels of CyHV-2 genes encoding for capsid triplex protein (CTP), major capsid protein (MCP) and thymidine kinase (TK) were also examined by real-time PCR to quantify the virus yield at 24, 48 and 120 h post CyHV-2 infection. All the experiments were repeated three times. The primers used for real-time PCR are listed in Table S1.

2.8. Statistical analysis

The GraphPad Prism 5.0 software was used for statistical analysis. All data was expressed as the mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) to reveal the statistical significance between samples. $P < 0.05$ value was considered to be statistically significant difference and $P < 0.01$ value as extreme difference.

3. Results

3.1. Identification and molecular characterization of CagIFNa

The full-length cDNA of *Cag*IFNa (GenBank accession no: MK093763) is 724 bp and contains a 57 bp 5′ UTR, a 552 bp ORF and a 115 bp 3′ UTR with a poly (A) tail. The ORF encodes a predicted *Cag*IFNa protein of 183 amino acids with a calculated molecular mass of 21.943 kDa and a theoretical isoelectric point of 9.23. The *Cag*IFNa deduced amino acid sequence also includes two cysteine residues ($C1{:}C^{24}$, $C2{:}C^{120}$), which are highly conserved in group I type I IFNs (Fig. 1).

Phylogenetic analysis (Fig. 2) further supports an evolutionary relationship of *Cag*IFNa within the fish type I IFNs. In the reconstructed phylogenetic tree, *Cag*IFNa clusters with all the other piscine IFNa subgroup genes of group I type I IFNs. And, *Cag*IFNa has higher identity supported by high bootstrap value with selected *Cyprinidae* IFNa subgroup genes, such as triploid crucian carp IFNa (AMQ67073) (85.3% identity) and common carp IFN (BAG68521) (86.3% identity).

3.2. Expression profiles of CagIFNa in vivo

*Cag*IFNa gene expression was detected in nine tissues of healthy gibel carp (Fig. 3), with the highest level in the liver, intermediate levels in the intestine, spleen, head kidney, muscle, and heart and low levels in the brain, gill and skin. Following CyHV-2 infection, *Cag*IFNa expression level significantly decreased in the head kidney at 12, 24, and 48 h but dramatically increased up to 6-fold higher at 120 h post-injection compared to sham-infected controls (Fig. 4A). In the spleen, *Cag*IFNa expression level significantly decreased at 24, 48 and 120 h post CyHV-2 injection (Fig. 4B).

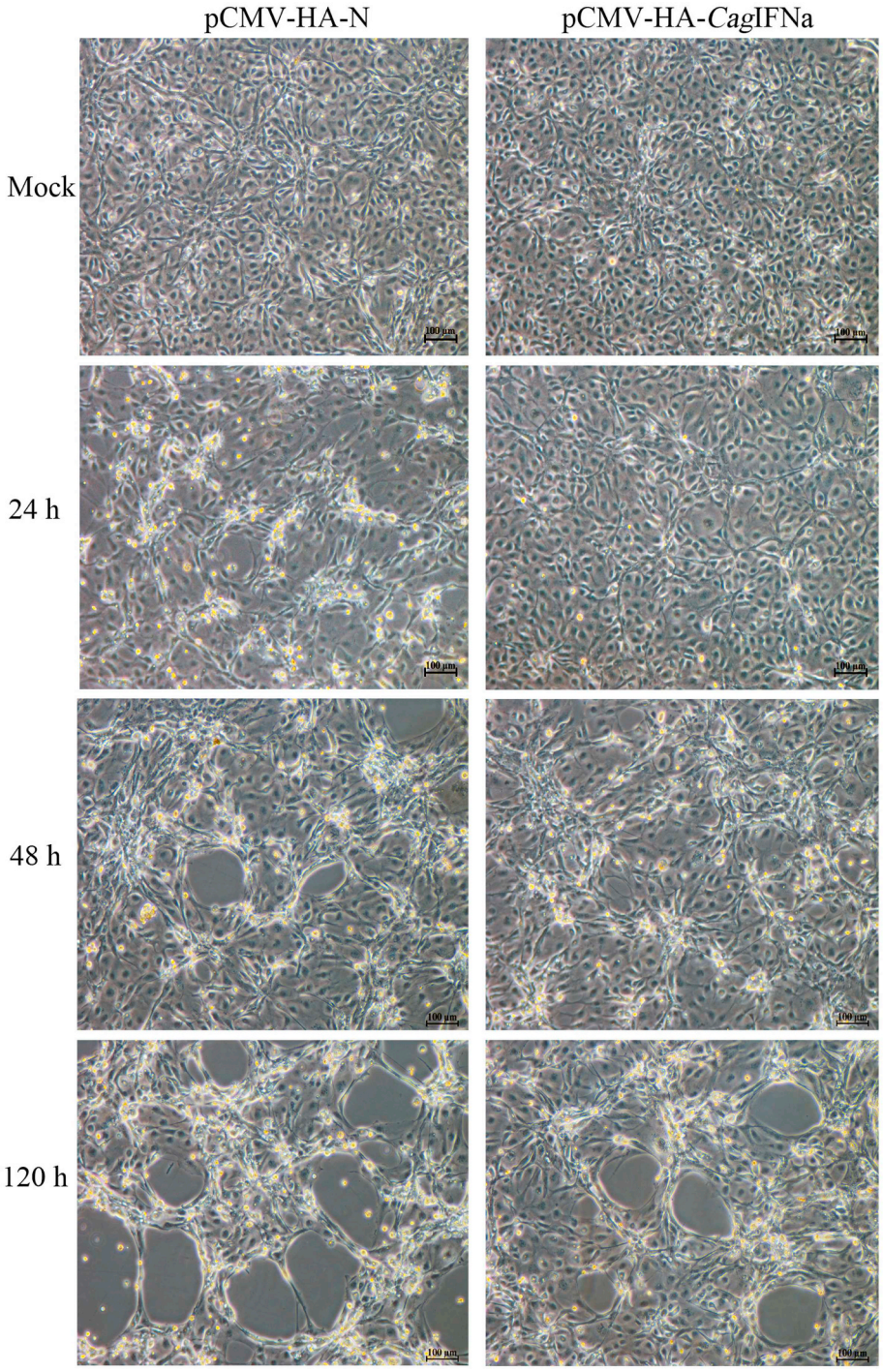

Fig. 6. Cytopathic effect (CPE) in transfected GiCB cells. GiCB cells were transfected with pCMV-HA-*Cag*IFNa or pCMV-HA-N plasmid (as control) for 24 h and then infected with CyHV-2 at an MOI of 0.5. Mock: GiCB cells without infection. The CPE in transfected GiCB cells was observed by microscopy at 24, 48 and 120 h post CyHV-2 infection. Scale bar, 100 μm.

3.3. Expression profiles of CagIFNa following poly I:C stimulation and CyHV-2 infection in vitro

After stimulation of GiCB cells with poly I:C, the expression level of *Cag*IFNa was rapidly up-regulated at 6 h, peaked at 12 h with a 347-fold induction and remained high even at 120 h compared with control group (Fig. 5A). Interestingly, after CyHV-2 infection, the expression level of *Cag*IFNa significantly increased at 6 and 12 h, decreased at 48 h then sharply increased again at 120 h (10.7-fold) compared with control group (Fig. 5B).

3.4. Antiviral activities of CagIFNa

The production and secretion of recombinant HA-*Cag*IFNa protein with the expected molecular weight by GiCB cells transfected with pCMV-HA-*Cag*IFNa plasmid were confirmed by Western blot (Fig. S1). After infection with CyHV-2, CPE in pCMV-HA-*Cag*IFNa transfected GiCB cells was significantly delayed compared to cells transfected with pCMV-HA-N plasmid (Fig. 6). In addition, CyHV-2 viral titers were considerably reduced compared with control group transfected with pCMV-HA-N plasmid (Fig. 7A). At 24, 48 and 120 h post CyHV-2

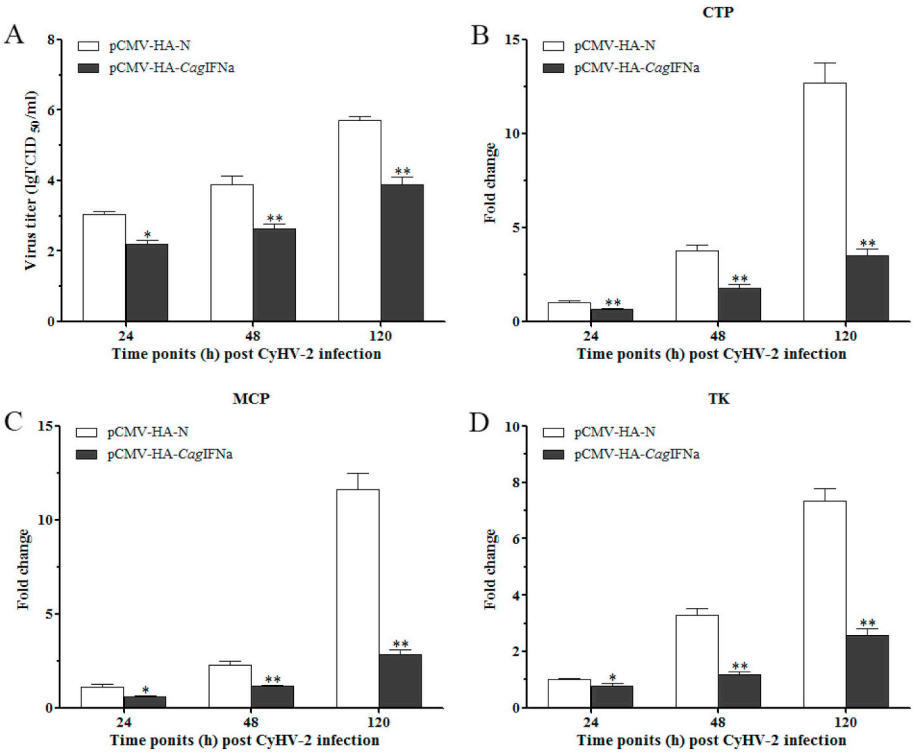

Fig. 7. Antiviral activities of *Cag*IFNa on the virus titer (A) and the expression of the viral capsid triplex protein (CTP) gene (B), major capsid protein (MCP) gene (C) and thymidine kinase (TK) gene (D). (A) GiCB cells were transfected with pCMV-HA-*Cag*IFNa or pCMV-HA-N plasmid (as control). After 24 h, the transfected GiCB cells were infected with CyHV-2 at an MOI of 0.5. Then the cells in 6-well plates containing supernatants were harvested at 24, 48 and 120 h post CyHV-2 infection for virus titration assay by $TCID_{50}$. (B–D) GiCB cells were transfected with pCMV-HA-*Cag*IFNa or pCMV-HA-N plasmid (as control). After 24 h, the transfected GiCB cells were infected with CyHV-2 at an MOI of 0.5. Then the cells were sampled at 24, 48 and 120 h post CyHV-2 infection and used for real-time PCR analysis of target genes. The relative expression levels of target genes were normalized to β-actin. The data are expressed as fold-induction relative to the 24 h control group. Error bars indicate the mean ± SD (n = 3). The asterisks indicate significant difference ($^{**}P < 0.01$, $^{*}P < 0.05$) between pCMV-HA-*Cag*IFNa transfected (black columns) and pCMV-HA-N transfected (white columns) groups at the same time point.

infection, the virus titers were $10^{3.04}$, $10^{3.89}$ and $10^{5.72}$ $TCID_{50}$/ml in control group. However, in treated group, the virus titers were $10^{2.21}$, $10^{2.62}$ and $10^{3.88}$ $TCID_{50}$/ml, showing a 6.8, 18.6 and 69.2-fold inhibition, respectively.

Meanwhile, the expression levels of viral genes CTP, MCP and TK were examined in pCMV-HA-*Cag*IFNa and pCMV-HA-N transfected cells at 24, 48 and 120 h post CyHV-2 infection by real-time PCR. Notably, compared to control group, the expression levels of these viral genes were markedly decreased in cells expressing recombinant HA-*Cag*IFNa protein at all time points post CyHV-2 infection (Fig. 7B–D). These data demonstrate that expression of recombinant *Cag*IFNa effectively prevents CyHV-2 infection.

3.5. Activation of type I IFN signaling pathway associated genes by CagIFNa

To determine whether the ectopic expression of the recombinant *Cag*IFNa in transfected GiCB cells induced the activation of the type I IFN signaling pathway, change in relative expression of IRF3, IRF7, IRF9, STAT1, Mx1 and PKR genes was monitored during CyHV-2 infection compared to GiCB cells transfected pCMV-HA-N empty control plasmid (Fig. 8). The results showed a marked induction of all these genes from early time post-infection (6 h) and up to 120 h post-infection in cells transfected *Cag*IFNa compared to control plasmid.

4. Discussion

IFN-mediated innate immune response is a pivotal component of innate immunity that provides a broad-spectrum antiviral effect. Great progress has been made in the characterization of the fish interferon system in a variety of piscine species [11,13]. However, there is still little known about piscine interferon response against *alloherpesviruses*. Here, we have identified a type I IFN (*Cag*IFNa) in the gibel carp and characterized its antiviral function against the *alloherpesvirus* CyHV-2 *in vitro* and *in vivo*.

The putative *Cag*IFNa protein exhibits the key features of piscine group I type I IFNs including two cysteine residues at conserved positions that establish disulfide bridge to stabilize the structure of the mature protein potentially [10]. Furthermore, the phylogenetic analysis reveals that *Cag*IFNa is closely related to IFNa genes of teleost fish including cyprinid fish. Taken together, the cloned gibel carp IFN is identified as IFNa subgroup gene of group I type I IFNs.

In the present study, the constitutive gene expression of *Cag*IFNa was detected in all examined tissues of healthy gibel carp, which is consistent with the IFNa expression profiles reported for other cyprinid fish, including triploid crucian carp [20] and black carp [29]. However, the expression level of *Cag*IFNa in the liver, intestine, spleen and head kidney was higher than that in the muscle, heart, brain, gill and skin. In teleost fish, the liver, spleen and head kidney are major immune tissues and their immune responses are easily elicited, and the intestine is a vital immune barrier [30]. Thus, *Cag*IFNa may mainly perform functions in immune-related organs.

In fish, the head kidney is the functional equivalent of mammalian bone marrow, whereas in absence of lymph node, the spleen is both a primary and secondary lymphoid organ that is important for antiviral immune responses [30,31]. Interestingly, *Cag*IFNa gene in the head kidney and spleen had a rather low expression level during infection with CyHV-2 until 120 h post-injection. Similarly, injection with the infectious spleen and kidney necrosis virus (ISKNV) also causes a delayed IFN gene expression response in the head kidney and spleen of mandarin fish [32]. In black carp, the expression of type I IFN gene is inhibited in the spleen and is not significantly changed in the kidney following infection with SVCV and GCRV [33]. In addition, infection of salmon cells *in vitro* with the infectious salmon anemia virus (ISAV) delays induction of type I IFNs [34,35]. This inhibitory effect may be due to virus gene products that could disable the interferon antiviral system by modulating the expression of multiple host factors for their invasion and survival [36,37]. A contribution from the host autoimmune regulation to prevent harmful excessive activation of type I IFNs is also possible [38]. As such, mechanisms behind the inhibition of *Cag*IFNa gene expression in the spleen and head kidney during CyHV-2 infection deserved to be further explored. It is noteworthy that unlike the spleen, the expression of *Cag*IFNa in the head kidney was drastically

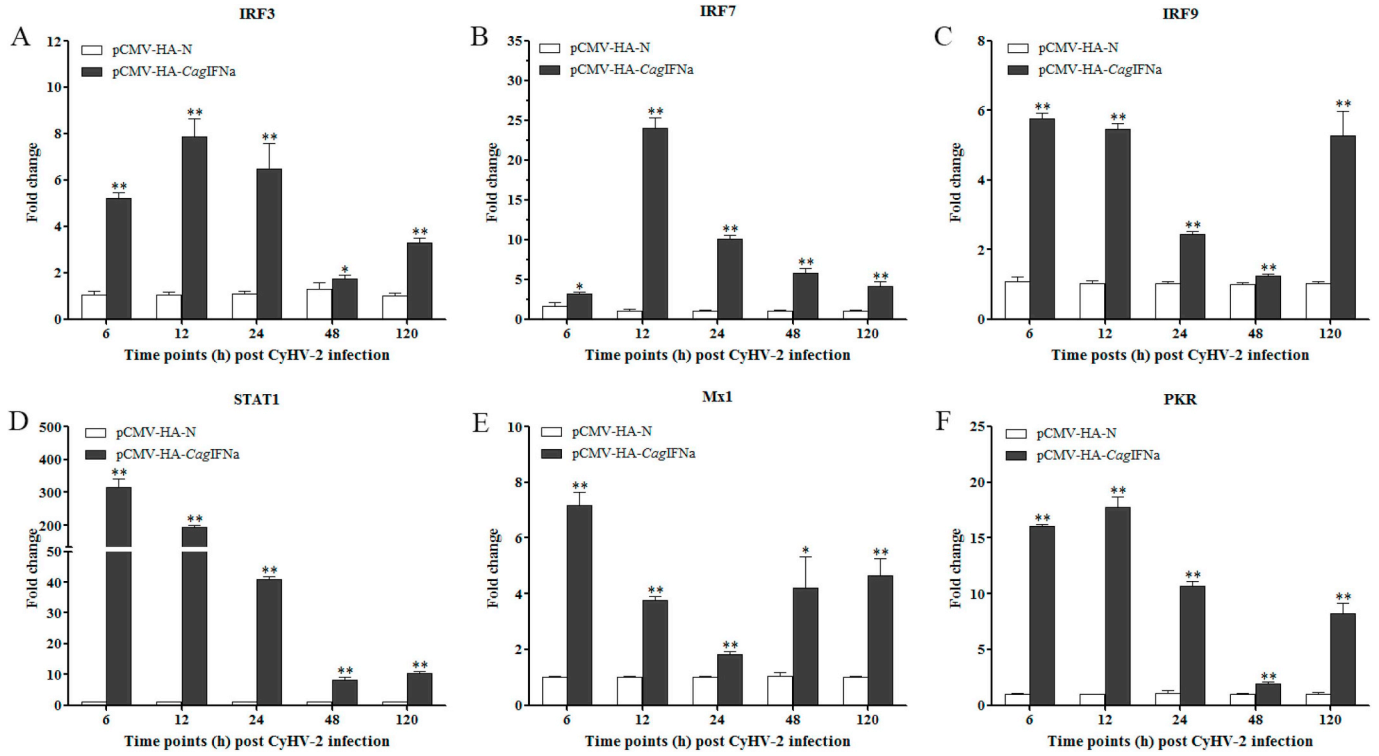

Fig. 8. Analysis of mRNA expression levels of IRF3 (A), IRF7 (B), IRF9 (C), STAT1 (D), Mx1 (E) and PKR (F) genes in transfected GiCB cells. GiCB cells were transfected with pCMV-HA-*Cag*IFNa or pCMV-HA-N plasmid (as control). After 24 h, the transfected GiCB cells were infected with CyHV-2 at an MOI of 0.5. Then the cells were sampled at 6, 12, 24, 48 and 120 h post CyHV-2 infection and used for real-time PCR analysis of target genes. The relative expression levels of target genes were normalized to β-actin, and fold-induction was calculated by comparing the relative expression levels of these genes in pCMV-HA-*Cag*IFNa transfected groups with those in pCMV-HA-N transfected groups at the same time point. Error bars indicate the mean ± SD (n = 3). The asterisks indicate significant difference ($^{**}P < 0.01$, $^{*}P < 0.05$) between pCMV-HA-*Cag*IFNa transfected (black columns) and pCMV-HA-N transfected (white columns) groups at the same time point.

increased at 120 h post-injection.

In previous studies, the induced expression of teleost type I IFNs in different cells has been obtained using different immune stimulants, including poly I:C, LPS, R848 and so on. Among those, poly I:C, which mimicks viral dsRNA, is often used and is a well-established inducer of type I IFNs. *Cag*IFNa gene expression was rapidly (6 h) induced and sustained (up to 120 h) in GiCB cells stimulated with poly I:C, which is similar to the response of Atlantic salmon IFN-α1 in TO cells [39], although *Cag*IFNa exhibited more persistent activation. CAB cells transfected with poly I:C (intracellular poly I:C) can establish a clear type I IFN antiviral response to against GCRV infection and was more efficient than the extracellular poly I:C [7].

In contrast to poly I:C stimulation, the expression of *Cag*IFNa in GiCB cells was not significantly induced at 24 h and even inhibited at 48 h following CyHV-2 infection. Consistent with these results, salmon IFNa1/a2 genes have been reported to strongly up-regulated by poly I:C but only weakly by the imidazoquinoline S-27609 [40]. Moreover, in common carp brain (CCB) cells, the type I IFN response induced by poly I:C, SVCV and the Cyprinid herpesvirus 3 (CyHV-3) are fundamentally different [41]. This may be due to the induction of a variety of other molecules such as PRRs during the process of virus-infecting cells. The different induction of these molecules may depend on the type of viruses, which affect the expression of target gene(s) to some extent [40,42]. Intriguingly, the *Cag*IFNa gene expression kinetics *in vivo* and *in vitro* were different following CyHV-2 infection. At the early stage of CyHV-2 infection, the expression of *Cag*IFNa was inhibited in the spleen and head kidney but significantly up-regulated in GiCB cells, although the increase was relatively modest compared to later time points. The different expression profiles of *Cag*IFNa might due to the expression of diverse transcriptional regulators in different fish tissues or cells.

The delay of virus-mediated CPE, the significant inhibition of virus titer, and the low expression levels of the viral CTP, MCP and TK genes in GiCB cells overexpressing *Cag*IFNa upon CyHV-2 infection collectively indicated an inhibition of CyHV-2 replication. It is well known that the activation of type I IFNs stimulate the expression of ISGs through JAK-STAT pathway, which establish the antiviral state in host [5]. Overexpression of *Cag*IFNa in GiCB cells induced the expression of the two ISGF3 members (STAT1 and IRF9), IRF3, IRF7 and the two ISGs (Mx1 and PKR) in response against CyHV-2 infection. The activation of these genes associated with the type I IFN system confers a cellular environment non-permissive to viral infection [2]. In CAB cells, either transfection of IFN plasmid or treatment with rIFN protein significantly activated the strong expression of a set of type I IFN related genes such as Mx and PKR, which were identified as orthologues to mammalian IFN stimulated genes (ISGs) [7]. The activation of type I IFN in CyHV-3 infected CCB cells can promote the expression of genes associated with type I IFN system such as IRF3/7, Mx and PKR, which limit the expression of viral genes and significantly reduced the virus titer [41].

In summary, the gibel carp *Cag*IFNa full length cDNA was identified as a new member of the fish IFNa subgroup of group I type I IFN. The expression of *Cag*IFNa was up-regulated upon CyHV-2 infection *in vi*tro. The over-expression of *Cag*IFNa could activate expression of genes associated with the type I IFN signaling pathway and exhibited effective antiviral activities. This study provides new insights into the type I interferon response against CyHV-2 pathogens in gibel carp and will serve as a foundation for the control of gibel carp CyHV-2 infection in future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2019.04.063.

References

[1] D. Ori, M. Murase, T. Kawai, Cytosolic nucleic acid sensors and innate immune regulation, Int. Rev. Immunol. 36 (2) (2017) 74–88.
[2] Y.B. Zhang, J.F. Gui, Molecular regulation of interferon antiviral response in fish, Dev. Comp. Immunol. 38 (2) (2012) 193–202.
[3] C. Langevin, E. Aleksejeva, G. Passoni, N. Palha, J.P. Levraud, P. Boudinot, The antiviral innate immune response in fish: evolution and conservation of the IFN system, J. Mol. Biol. 425 (24) (2013) 4904–4920.
[4] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (6) (2010) 805–820.
[5] W.M. Schneider, M.D. Chevillotte, C.M. Rice, Interferon-stimulated genes: a complex web of host defenses, Annu. Rev. Immunol. 32 (1) (2014) 513–545.
[6] J. Shi, Y.B. Zhang, T.K. Liu, F. Sun, J.F. Gui, Subcellular localization and functional characterization of a fish IRF9 from crucian carp *Carassius auratus*, Fish. Shellfish Immunol. 33 (2) (2012) 258–266.
[7] F.F. Yu, Y.B. Zhang, T.K. Liu, Y. Liu, F. Sun, J. Jiang, J.F. Gui, Fish virus-induced interferon exerts antiviral function through Stat1 pathway, Mol. Immunol. 47 (14) (2010) 2330–2341.
[8] A.J. Sadler, B.R.G. Williams, Interferon-inducible antiviral effectors, Nat. Rev. Immunol. 8 (7) (2008) 559–568.
[9] B. Robertsen, The interferon system of teleost fish, Fish. Shellfish Immunol 20 (2) (2006) 172–191.
[10] J. Zou, C. Tafalla, J. Truckle, C.J. Secombes, Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates, J. Immunol. 179 (6) (2007) 3859–3871.
[11] J. Zou, B. Gorgoglione, N.G. Taylor, T. Summathed, P.T. Lee, A. Panigrahi, C. Genet, Y.M. Chen, T.Y. Chen, M. Ul Hassan, S.M. Mughal, P. Boudinot, C.J. Secombes, Salmonids have an extraordinary complex type I IFN system: characterization of the IFN locus in rainbow trout *oncorhynchus mykiss* reveals two novel IFN subgroups, J. Immunol. 193 (5) (2014) 2273–2286.
[12] Y. Ding, J. Ao, X. Huang, X. Chen, Identification of two subgroups of type I IFNs in perciforme fish large yellow croaker *Larimichthys crocea* provides novel insights into function and regulation of fish type I IFNs, Front. Immunol. 7 (2016) 343.
[13] J. Zou, C.J. Secombes, Teleost fish interferons and their role in immunity, Dev. Comp. Immunol. 35 (12) (2011) 1376–1387.
[14] Z.A. Laghari, S.N. Chen, L. Li, B. Huang, Z. Gan, Y. Zhou, H.J. Huo, J. Hou, P. Nie, Functional, signalling and transcriptional differences of three distinct type I IFNs in a perciform fish, the Mandarin fish *Siniperca chuatsi*, Dev. Comp. Immunol. 84 (2018) 94–108.
[15] D.J. Milne, C. Campoverde, K.B. Andree, X. Chen, J. Zou, C.J. Secombes, The discovery and comparative expression analysis of three distinct type I interferons in the perciform fish, meagre (*Argyrosomus regius*), Dev. Comp. Immunol. 84 (2018) 123–132.
[16] Y. Ding, Y. Guan, X. Huang, J. Ao, X. Chen, Characterization and function of a group II type I interferon in the perciform fish, large yellow croaker (*Larimichthys crocea*), Fish. Shellfish Immunol 86 (2019) 152–159.
[17] Z. Liao, Q. Wan, J. Su, Bioinformatics analysis of organizational and expressional characterizations of the IFNs, IRFs and CRFBs in grass carp *Ctenopharyngodon idella*, Dev. Comp. Immunol. 61 (2016) 97–106.
[18] S. Xia, H. Wang, X. Hong, J. Lu, D. Xu, Y. Jiang, L. Lu, Identification and characterization of a type I interferon induced by cyprinid herpesvirus 2 infection in crucian carp *Carassius auratus gibelio*, Fish. Shellfish Immunol 76 (2018) 35–40.
[19] A. Lopez-Munoz, F.J. Roca, J. Meseguer, V. Mulero, New insights into the evolution of IFNs: zebrafish group II IFNs induce a rapid and transient expression of IFN-dependent genes and display powerful antiviral activities, J. Immunol. 182 (6) (2009) 3440–3449.
[20] J. Yan, L. Peng, Y. Li, H. Fan, Y. Tian, S. Liu, H. Feng, IFNa of triploid hybrid of gold fish and allotetraploid is an antiviral cytokine against SVCV and GCRV, Fish. Shellfish Immunol 54 (2016) 529–536.
[21] T. Svingerud, T. Solstad, B. Sun, M.L. Nyrud, O. Kileng, L. Greiner-Tollersrud, B. Robertsen, Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc-producing cells in fish lymphoid tissues, J. Immunol. 189 (12) (2012) 5912–5923.
[22] Y. Fan, Y. Zhou, L. Zeng, N. Jiang, W. Liu, J. Zhao, Q. Zhong, Identification, structural characterization, and expression analysis of toll-like receptors 2 and 3 from gibel carp (*Carassius auratus gibelio*), Fish. Shellfish Immunol. 72 (2018) 629–638.
[23] J. Xu, L. Zeng, H. Zhang, Y. Zhou, J. Ma, Y. Fan, Cyprinid herpesvirus 2 infection emerged in cultured gibel carp, *Carassius auratus gibelio* in China, Vet. Microbiol. 166 (1–2) (2013) 138–144.
[24] J. Ma, N. Jiang, S.E. LaPatra, L. Jin, J. Xu, Y. Fan, Y. Zhou, L. Zeng, Establishment of a novel and highly permissive cell line for the efficient replication of cyprinid herpesvirus 2 (CyHV-2), Vet. Microbiol. 177 (3–4) (2015) 315–325.
[25] L. Zhang, J. Ma, Y. Fan, Y. Zhou, J. Xu, W. Liu, Z. Gu, L. Zeng, Immune response and protection in gibel carp, *Carassius gibelio*, after vaccination with beta-propiolactone inactivated cyprinid herpesvirus 2, Fish. Shellfish Immunol 49 (2016) 344–350.
[26] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol. 28 (10) (2011) 2731–2739.
[27] N. Jiang, X. Jin, J. He, Z. Yin, The roles of follistatin 1 in regulation of zebrafish fecundity and sexual differentiation, Biol. Reprod. 87 (3) (2012) 54.
[28] J. Ma, Y. Fan, Y. Zhou, W. Liu, N. Jiang, J. Zhang, L. Zeng, Efficient resistance to grass carp reovirus infection in JAM-A knockout cells using CRISPR/Cas9, Fish. Shellfish Immunol 76 (2018) 206–215.
[29] Z. Huang, S. Chen, J. Liu, J. Xiao, J. Yan, H. Feng, IFNa of black carp is an antiviral cytokine modified with N-linked glycosylation, Fish. Shellfish Immunol. 46 (2) (2015) 477–485.
[30] P.R. Rauta, B. Nayak, S. Das, Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms, Immunol. Lett. 148 (1) (2012) 23–33.
[31] S.T. Solem, J. Stenvik, Antibody repertoire development in teleosts—a review with emphasis on salmonids and *Gadus morhua L*, Dev. Comp. Immunol. 30 (1) (2006) 57–76.
[32] Z.A. Laghari, S.N. Chen, L. Li, B. Huang, Z. Gan, Y. Zhou, H.J. Huo, J. Hou, P. Nie, Functional, signalling and transcriptional differences of three distinct type I IFNs in a perciform fish, the Mandarin fish *Siniperca chuatsi*, Dev. Comp. Immunol. 84 (2018) 94–108.
[33] H. Wu, L. Liu, S. Wu, C. Wang, C. Feng, J. Xiao, H. Feng, IFNb of black carp functions importantly in host innate immune response as an antiviral cytokine, Fish. Shellfish Immunol 74 (2018) 1–9.
[34] V. Bergan, S. Steinsvik, H. Xu, O. Kileng, B. Robertsen, Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions, FEBS J. 273 (17) (2006) 3893–3906.
[35] O. Kileng, M.I. Brundtland, B. Robertsen, Infectious salmon anemia virus is a powerful inducer of key genes of the type I interferon system of Atlantic salmon, but is not inhibited by interferon, Fish. Shellfish Immunol 23 (2) (2007) 378–389.
[36] F. Ke, Q.Y. Zhang, Aquatic animal viruses mediated immune evasion in their host, Fish. Shellfish Immunol. 86 (2018) 1096–1105.
[37] C.J. Guo, J. He, J.G. He, The immune evasion strategies of fish viruses, Fish. Shellfish Immunol 86 (2019) 772–784.
[38] L.B. Ivashkiv, L.T. Donlin, Regulation of type I interferon responses, Nat. Rev. Immunol. 14 (1) (2014) 36–49.
[39] O. Kileng, V. Bergan, S.T. Workenhe, B. Robertsen, Structural and functional studies of an IRF-7-like gene from Atlantic salmon, Dev. Comp. Immunol. 33 (1) (2009) 18–27.
[40] O. Kileng, A. Albuquerque, B. Robertsen, Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for Toll-like receptor 7, Fish. Shellfish Immunol 24 (5) (2008) 514–522.
[41] M. Adamek, K.Ł. Rakus, J. Chyb, G. Brogden, A. Huebner, I. Irnazarow, D. Steinhagen, Interferon type I responses to virus infections in carp cells: in vitro studies on Cyprinid herpesvirus 3 and Rhabdovirus carpio infections, Fish. Shellfish Immunol 33 (3) (2012) 482–493.
[42] S. Biacchesi, M. LeBerre, A. Lamoureux, Y. Louise, E. Lauret, P. Boudinot, M. Bremont, Mitochondrial antiviral signaling protein plays a major role in induction of the fish innate immune response against RNA and DNA viruses, J. Virol. 83 (16) (2009) 7815–7827.