Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Identification of Type I IFN in Chinese giant salamander (*Andrias davidianus*) and the response to an iridovirus infection

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ARTICLE INFO

Article history: Received 9 January 2015 Received in revised form 10 February 2015 Accepted 11 February 2015

Keywords: Chinese giant salamander Andrias davidianus Type I IFN Antiviral activity Innate immunity

ABSTRACT

The type I IFNs play a major role in the first line of defense against virus infections. In this study, the type I IFN gene designated gsIFN was identified and characterized in the Chinese giant salamander (*Andrias davidianus*). The genomic DNA of gsIFN contains 5 exons and 4 introns and has a total length of 5622 bp. The full-length cDNA sequence of gsIFN is 1113 bp and encodes a putative protein of 186 amino acids that has a 43% identity to type I IFN of *Xenopus tropicalis*. The deduced amino acid sequence has the C-terminal CAWE motif, that is mostly conserved in the higher vertebrate type I IFNs. Real-time fluorescence quantitative RT-PCR analysis revealed broad expression of gsIFN *in vivo* and the highest level expression in blood, kidney and spleen. Additionally, the expression of gsIFN at the mRNA level was significantly induced in peripheral blood leucocytes after stimulation with poly I:C and after infection with the Chinese giant salamander muscle cell line. Expression of the IFN-inducible gene Mx was up-regulated in the gsIFN-overexpressing cells after GSIV infection. The virus load and titer were significantly reduced compared with that in control cells. Additionally, a lower level of virus major capsid protein synthesis was confirmed by immunofluorescence assay compared to the control cells. These results suggest that the gsIFN gene plays an important role in the antiviral innate immune response.

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1. Introduction

Interferons (IFNs) are secreted cytokines that induce an antiviral state in cells and play a crucial role in defense against virus infection (Samuel, 2001). IFNs can induce the expression of interferon stimulated genes (ISGs), which encode antiviral proteins through JAK/STAT signaling and have a major role in initiating host innate immune responses against virus and bacterial infections (Platanias, 2005). IFNs are divided into three classes designated type I, type II, and type III based on their gene structure, receptor usage and functional properties (Pestka et al., 2004). Type I IFNs are expressed in most types of virally infected cells and trigger specific signaling pathways. However, type II IFNs are secreted by T lymphocytes and NK cells under certain conditions of activation and primarily promote cell mediated immunity (Zou and Secombes, 2011). Type III IFNs (IFN- λ s) are novel antiviral cytokines and have been

http://dx.doi.org/10.1016/j.molimm.2015.02.015 0161-5890/© 2015 Elsevier Ltd. All rights reserved. functionally classified as a new type of IFN. IFN- λ exerts antiviral activity via stimulation of host antiviral immune response (Ank et al., 2006).

In mammals, type I IFNs comprise seven major homologous subgroups including α subtypes (14 human and 11 mouse) and single β , ϵ , κ , τ , ζ , and ω subtypes (Chen et al., 2004). A characteristic of the classical type I IFNs is that they do not contain introns and are closely clustered in the same chromosome. They also bind to the same cell surface heterodimer receptor, IFNAR, containing the IFNR1 and IFNR2 subunits, triggering the JAK-STAT pathway (Kontsek et al., 2003). To date, type I IFNs have been identified in lower vertebrates such as cartilaginous and bony fish as well as amphibians. This has provided significant information on IFN gene evolution. These lower vertebrate IFNs contain multiple members but are encoded on transcripts containing 5 exons and 4 introns in contrast to reptiles, birds and mammals that have no introns (Robertsen, 2006). IFN genes identified in fish have the same exon/intron structure as the IL-10 and IFN- λ family that indicated that type I IFN genes and IL-10 like genes originated from an ancient progenitor (Lutfalla et al., 2003; Robertsen et al., 2003). Based on the protein sequence analysis, teleost type I IFNs have now been





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subdivided into two or four cysteine containing subgroups and further subdivided into four groups (IFN a to IFN d) according to their phylogeny. The four cysteine containing IFNs have a conserved CAWE motif near the C-terminus that may be the ancestral gene for the type I IFN family (Sun et al., 2009; Zou et al., 2007).

Functional studies have demonstrated that type I IFNs from zebrafish *Danio rerio* (Altmann et al., 2003), rainbow trout *Oncorhynchus mykiss* (Zou et al., 2007), Atlantic salmon *Salmo salar* (Robertsen et al., 2003), and common carp *Cyprinus carpio* (Kitao et al., 2009) have antiviral activity. This was detected by inducing expression of antiviral proteins such as Mx that subsequently exhibit increasing resistance to virus infection in fish cell lines. In the amphibian, *Xenopus laevis*, type I IFN can elicit significant increases in Mx gene expression and substantially reduce frog virus–3 (FV3) replication and infectious viral burdens both *in vitro* and *in vivo* (Grayfer et al., 2014). However, there is limited information about the amphibian immune response and the IFN antiviral defenses of the Chinese giant salamander against iridovirus infections.

The Chinese giant salamander, *Andrias davidianus*, is the largest extant amphibian species in the world. In recent years, with the rapid development of the artificial cultivation of Chinese giant salamander, a severe infectious disease caused by the Chinese giant salamander iridovirus has been spreading throughout China and has had a significant economic impact (Meng et al., 2014). A better understanding of the immune response in the Chinese giant salamander during virus infection is essential for the development of efficient disease control methods. So far, there are no members of the IFN family that have been reported and the IFN system remains unknown in the Chinese giant salamander. In this study, the full-length cDNA and genomic DNA of type I IFN were identified and characterized from the Chinese giant salamander and the antiviral effect against GSIV was examined in Chinese giant salamander muscle (GS-M) cells.

2. Materials and methods

2.1. Animals, cells, and virus

Chinese giant salamanders weighing about 180-200 g each were obtained from the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS) in Wuhan, Hubei Province. The animals were maintained in tanks for one month at 20°C and fed diced bighead carp daily. The Chinese giant salamander muscle (GS-M) cell line was generously provided by Prof. Qi-Ya Zhang (Institute of Hydrobiology, Chinese Academy of Science). GS-M cells were maintained at 20 °C in medium 199 (M199, Sigma) supplemented with 10% fetal bovine serum (FBS). The Epithelioma papilloma cyprini (EPC) cell line was obtained from the China Center for Type Culture Collection (CCTCC), Wuhan University (Fijan et al., 1983). EPC cells were incubated at 25 °C and grown in minimum essential medium (MEM, Sigma) with 10% FBS. Chinese giant salamander iridovirus (GSIV) was isolated from the Chinese giant salamander and propagated in EPC cells according to methods described previously (Meng et al., 2014).

2.2. Identification of Chinese giant salamander type I IFN

Total RNA was extracted from the kidney of naturally infected Chinese giant salamanders using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out using ImProm-IITM Reverse Transcription System (Promega). Two pairs of primers IFN-3F83/IFN-3F279 and IFN-5R315/IFN-5R151 (Table 1) ware designed based on the analysis of the transcriptome of the Chinese giant salamander (unpublished

Table 1

Primer sequences used in this study.

Primer	Sequence(5'-3')	Used for
IFN-3F83	ACAACCAGCAACACACAACAACACAG	Race-PCR
IFN-3F279	GAGCAAAAACAAGGACAAATCTGGC	
IFN-5R315	TCTCGTGTCCCAGCCAGATTTGTCCTT	Genome
		Walking
IFN-5R151	GGGGAAAGTGTCCACCCATCTGCTC	-
1-SP1	AGCACCTTGTGCGGGAACTTAATTG	Eukaryotic
		expression
1-SP2	CGCCGGGTCTGCAGATGTTTATCAA	
1-SP3	TCCACCCATCTGCTCCAGAAGTTGT	qRT-PCR
2-SP1	ATTTGCCCAGTATGTCATTTTGCAG	
2-SP2	GTCATTTTGCAGCTCCAGCCGTAGT	
2-SP3	TCCGTGAAAGATGTTGTTGAGGGTC	
3-SP1	GGTAAGCTCACTGTGATGATCCAGG	
3-SP2	TGTAAACACTAGCAACCAGAGCCAG	
3-SP3	GCGTCACAGCAACACTACTCAGAAC	
4-SP1	CGTGCACTATAACAGAAGATGGATG	
4-SP2	CAGGTAAATAGAGGTCAGCAAGACG	
gsIFN-F	CCCAAGCTTATGGCTACAACAGCTCTCCG	
gsIFN-R	CGCGGATCCCGGATCTGTAACTTTGTAA-	
	TCTTTGC	
gsIFN-rqF	ATTGGCGTGCCTTTTCGTGCTATT	
gsIFN-rqR	GGGAAAGTGTCCACCCATCTGCTC	
gsMx-rqF	GTGTTCCAGATTTCTTTGTCAGC	
gsMx-rqR	TTCCATTGAAAGTAACCAGCCT	
β-actin-rqF	TGAACCCAAAAGCCAACCGAGAAAAGAT	
β-actin-rqR	TACGACCAGAGGCATACAGGGACAGGAC	
MCP-rqF	GCGGTTCTCACACGCAGTC	
MCP-rqR	ACGGGAGTGACGCAGGTGT	
GSIV-IE-rqF	CTCCATCAGCCTCCTTGTCA	
GSIV-IE-rqR	AGTCCTGTGCCGCTACTTTTC	

Note: (1) F indicating forward primer and R reverse primer. (2) Restriction sites are underlined

data). Rapid amplification of cDNA ends (RACE) PCR was performed using the Clontech SMART cDNA synthesis kit to identify the 5' and 3' regions of the Chinese giant salamander type I IFN (gsIFN).

According to the gene organization of type I IFN in *Xenopus tropicalis*, four groups of primers (1-SP, 2-SP, 3-SP and 4-SP; Table 1) were designed to obtain the sequences of gsIFN introns by using a Genome Walking Kit (TaKaRa). Primer pairs used in gsIFN intron amplification are shown in Table 1. The PCR product was examined by 1.5% agarose gel electrophoresis and the target DNA fragment was purified using the wizard[®] SV Gel and PCR Clean-up system (Promega). All purified PCR products were cloned into pMD19-T (TaKaRa) for cloning and sequencing. The positions of the introns were determined by comparing the size of the products between genomic DNA and the cDNA sequences.

2.3. Sequence alignment and phylogenetic analysis

The sequence was searched for similarity using BLAST (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://www.expasy.org). Multiple alignments of the deduced amino acid sequences were performed using Clustal program (http://www.ebi.ac.uk/Tools/clustalw/). The sig-W nal peptide region was identified using the SignalP (version3) server (http://www.cbs.dtu.dk/services/SignalP/). The secondary structure was predicted by PHD (https://www.predictprotein. org/). The positions of the exons and introns in the genomic sequences were determined by Spidey (http://www.ncbi.nlm. nih.gov/IEB/Research/Ostell/Spidey/). Exon/intron structures of IFNs were obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html) for comparison. The phylogenetic tree containing protein sequences from fish, amphibian, avian and mammals was constructed using the neighbor-joining (NJ) algorithm within MEGA version5.1.

2.4. Tissue specific gsIFN expression profiles

To examine the expression profile of the gsIFN gene *in vivo*, three healthy Chinese giant salamanders were sampled after euthanasia with tricaine methanesulfonate MS222 (100 mg/L, sigma) and tissues collected included liver, heart, skin, intestine, kidney, blood, spleen, lung and muscle that were used for RNA extraction. Total RNA was isolated using TRIzol reagent (Invitrogen) and then transcribed into cDNA as described above.

Real-time fluorescence quantitative RT-PCR (qRT-PCR) was performed using the primer IFN-rqF/IFN-rqR (Table 1). The amplification was performed by Rotor-Gene 6000 Real Time PCR system (Qiagen).

The qRT-PCR mixture consisted of 1 µl of the diluted cDNA sample, 10 µl Power 2×SYBR Real-time PCR premixture (BioTeKe), 1 µl of each primer (0.4 µM) and 7 µl H₂O. The qRT-PCR cycle profile including 1 cycle at 95 °C for 5 min, then 40 cycle at 95 °C for 15 s, 58 °C for 15 s and 72 °C for 20 s. Relative qRT-PCR gene expression analysis was performed *via* the $2^{-\Delta\Delta ct}$ method with the β-actin gene used as internal control gene for cDNA normalization (Livak and Schmittgen, 2001).

2.5. Peripheral blood leucocytes (PBLs) isolation and stimulation with poly I:C and GSIV

Chinese giant salamanders were bled from the tail vein and PBLs were isolated using Lymphocyte Separation Medium (TBD Science). Briefly, a blood sample from the tail vein were diluted with PBS at a ratio 1:1 and layered carefully onto the Histopaque 1077 for centrifugation at 400 g for 30 min at room temperature. PBLs at the interface between the plasma and the Histopaque 1077 were collected and washed twice with M199 which contained 10% FBS, 100 IU/ml penicillin and 100 μ g/ml streptomycin (Sigma). For the gsIFN gene expression study, 1 × 10⁶ PBLs were seeded into individual wells of 24-well plates and incubated for 2 h at 20 °C. After 2 h, PBLs were stimulated with 5 and 25 μ g/ml Poly1:C (Sigma) and GSIV (MOI = 0.5). The control group was treated with an equal volume of PBS. The cells were harvested at 4, 12 and 24 h post-stimulation and total RNA was extracted. The RNA transcription level of gsIFN was determined by qRT-PCR as described previously.

2.6. Construction of plasmids expressing Type I gsIFN and transfection

The ORF of the gsIFN sequence was amplified using LA TaqTM DNA polymerase (TaKaRa) by PCR with specific primers gsIFN-F/gsIFN-R (Table 1). The corresponding PCR product and the control plasmid of pEGFP-N1 (Clontech) were digested with BamHI and HindIII for 3 h. The target fragments encoding the putative mature peptide of gsIFN were purified and ligated with T4 DNA ligase, then inserted into the pEGFP-N1 vector and sequenced to verify the reading frame. The recombinant vector was designated as pEGFP-N1-IFN and the ORF of the gsIFN fragment was located between the immediate early promoter of CMV and the EGFP coding sequences. The recombinant plasmid pEGFP-N1-IFN was extracted using the Endo-free Plasmid Midi Kit (Omega). Expression construct pEGFP-N1-IFN and the empty vector were introduced into GS-M cells. Briefly, GS-M cells were seeded into 6-well plates at a density of 2×10^6 cells/ml and cultured in medium M199 that contained 10% FBS. After 24 h, the cells were transfected with a mixture containing 4 µg plasmid and 10 µl lipofectamineTM 2000 (Invitrogen) in 500 µl M199 medium per well according to the manufacturer's instructions. The medium was changed after 4 h, cells were washed with PBS and then cultured in fresh M199. At 24 h post-transfection, cells were examined under a fluorescence microscope. When GFP in the transfected cells was expressed, the cells were given 400 µg/ml of G-418 (Promega) for 3 weeks for selection. When the positive cells were approximately 50% of the culture, the cells were used for the experiments detailed below.

2.7. Antiviral activity of gsIFN in GS-M cells

The stably transfected cells were seeded into 24-well culture plates at 20° C for 24 h. Cells were then infected with GSIV at an MOI of 0.5. Total RNA was extracted at 24 and 48 h post-infection. Transcriptional levels of gsIFN and Mx genes were analyzed by qRT-PCR, as previously described. The sequence for the Mx gene was determined and submitted to GenBank (Genbank accession no: KM389533).

To examine whether gsIFN could confer antiviral effects, the GSIV major capsid protein (MCP) and encoding immediate early protein ICP-46 (IE-ICP46) genes were investigated by qRT-PCR to quantify virus yield. Supernatants in 24-well plates containing transgenic cells were harvested at 24 and 48 h after GSIV infection for subsequent viral titration assay. Virus titers were determined by the 50% tissue culture infective dose (TCID₅₀) method (Reed and Muench, 1938).

2.8. Immunofluorescence assay (IFA)

The normal GS-M cells and the stably transfected cells were seeded on glass coverslips in 6-well plates and grown at 20 °C. After 24 h, the culture medium was removed and the cells were infected with GSIV at an MOI of 0.5 in 500 µl of M199 without FBS. Non-infected cells were used as a control. Additional M199 containing 2% FBS was added 1 h later and the cells were incubated at 20°C. GSIV replication was analyzed at 24 and 48 h after infection by immunofluorescence analysis. Briefly, cells were fixed with 4% paraformaldehyde overnight and then washed three times with PBS and permeated with 0.1% TritonX-100 for 10 min. The coverslips were incubated in PBS for 1 h in blocking buffer (5% bovine serum albumin and 0.1% TritonX-100 in PBS) before being incubated for 1 h with anti-GSIV major capsid protein antibody (1:500 dilution). After washing with PBS, the coverslips were incubated with Cy3-labeled goat anti-rabbit IgG (1:1000 dilution; Beyotime) for 1 h. The cell nucleus was stained with 6-diamidino-2-phenylindole (DAPI) (Beyotime). Lastly the coverslips were washed and examined using a fluorescence microscope (Olympus).

2.9. Statistical analyses

All data were expressed as mean means \pm SD from separate experiments and then analyzed using the Student's *t*-test. A probability level of *p* < 0.05 was considered significant.

3. Results

3.1. Identification of Type I IFN gene in the Chinese giant salamander and sequence analysis

The full-length cDNA of gsIFN (Genbank accession no: KM267637) is 1113 bp long and contains a 52 bp 5'-terminal untranslated region (UTR), a 500 bp 3'-UTR and an open reading frame (ORF) of 561 bp which encodes a putative protein of 186 amino acids with a 19 aa signal peptide. Four mRNA instability motifs (ATTTA) and a conventional polyadenylation signal (AATAA) were found in the 3'-UTR of gsIFN cDNA. According to the NCBI-BLAST results, the highest amino acid identity was found between Chinese giant salamander type I IFN and *Xenopus tropicalis* IFN1 (43%) and shared approximately a 36% identity with the turtle, 33% identity with the zebrafish and 26%–29% identity with the avian type I IFN gene. Multiple alignments of the type I



Fig. 1. Comparison of the genomic structure of the Type I gene in *Xenopus tropicalis* and fish. The accession numbers for gene organization are: zebrafish *Danio rerio* IFN, NP.001104553; Atlantic salmon *Salmo salar* IFN, DQ354152; *Xenopus tropicalis* IFN1-IFN5, CAO03085-CAO03089. Boxes represent exons where coding nucleotide boxes are open, and shaded boxes represent untranslated regions (UTRs). Each exon is numbered use a roman numeral. The number of nucleotide present within the exons and introns are shown.

IFN protein sequences in fish, amphibian, avian, reptile and mammals were performed by CLUSTALW program (Supplementary Fig. S1). All encode peptides with similar lengths and a predicted signal peptide, except for two IFN genes in *Xenopus tropicalis*. The IFNs selected showed a high degree of similarity to gsIFN, including the putative IFN receptor binding sites (IFNAR1/IFNAR2) and the same pattern of cysteines in the mature peptide which can form two disulfide bridges to stabilize the structure. Higher vertebrate type I IFNs conserved motif CAWE was present in gsIFN at the C-terminal region but absent in *Xenopus tropicalis* and *Xenopus laevis*. Secondary structure analysis suggests that gsIFN possesses five alpha-helices as other type I IFNs.

The full-length genomic DNA sequence of gsIFN (Genbank accession no: KM288436) was determined by using Genome Walking, which consisted of 5622 bp. Comparing type I IFN genomic structures with that of the other animals, such as frog and fish, all genes were composed of five exons and four introns and all intron/exon junctions conformed to the splicing GT/AG rule. As shown in Fig. 1, the length of exon III-V of the IFNs of different species displays a high degree of similarity, while the I exon in Chinese giant salamander is smaller than that in fish and *Xenopus tropicalis*. All intron lengths are quite variable and the genomic sequence in amphibians

contains longer lengths of introns than those in fish. In addition, the *Xenopus tropicalis* type I IFNs all have four introns separated from the IFN coding region, while the first intron in the genomic DNA sequence of the gsIFN is located in the 5' untranslated region.

3.2. Phylogenetic analysis

The evolutionary relationships among vertebrate type I IFNs were analyzed by phylogenetic analysis (Fig. 2). The phylogenetic tree reveals that all the fish, amphibian and avian type I IFNs form a cluster separated from the type I IFNs in mammals and reptiles. In the amphibian, *Xenopus tropicalis*, IFN1 and IFN2 branch as a separate clade from *Xenopus tropicalis* IFN3-5 and *Xenopus laevis* IFN. The Chinese giant salamander type I IFN protein sequence appears to have a relatively closer relationship with *Xenopus tropicalis* IFN1-2 and the intron lacking type I IFNs of avians, but appears to have diverged from bony fish.

3.3. Expression pattern of gsIFN in Chinese giant salamander

In healthy animals, qRT-PCR results indicated that the transcripts of gsIFN were detected in all organs including liver, heart,



Fig. 2. Phylogenetic relationships of Type I IFN in the Chinese giant salamander and other known Type I IFNs. The tree was constructed by the Neighbour-Joining method using MEGA version 5.1. Bootstrapping was performed 1000 times. Genebank accession numbers of the selected sequences are shown as follows: fugu IFN1, CAM82750; fugu IFN2, CAM82751; salmon IFNd, AFV08801; salmon IFNa, NP.001117042; salmon IFNb, AFV08802; salmon IFNc, AFV08803; grass carp IFN1, ACZ36480; grass carp IFN2, BAD83702; medaka IFN, CAM32419; trout IFN1, CAM28538; trout IFN2, CAM28539; trout IFN3, CAM28540; zebrafish IFN1, NP.997523; zebrafish IFN1, OAM28538; trout IFN2, CAM28539; trout IFN3, CAM28540; zebrafish IFN1, NP.997523; zebrafish IFN1, NP.001104552; zebrafish IFN1, NP.001187180; catfish IFN2, AAV97699; catfish IFN3, AAV97700; goldfish IFN, BAG68521; common carp IFN1, AAR20886; *Xenopus tropicalis* IFN1-IFN5, CA003085-CA003089; *Xenopus laevis* IFN, AHN05532; anole IFNk1, XP.008101698; anole IFNb1, XP.008101700; anole IFNb3, XP.008101705; turtle IFNb, EMP34037; chicken IFNa, BAE93146; chicken IFNb, NP.001020007; duck IFN, CAA59235; turkey IFN, AAB40029; human IFNa, NP.001020007; duck IFN, CAA59235; turkey IFNa, NP.001092911; human IFNa1, AET86952; pig IFNa, NP.00102958; rat IFNa, CAA50931; bovine IFNa, NP.00117411; horse IFNb, NP.001092910; bovine IFNb, NP.776775; human IFNb, NP.001267; pig IFNb, NP.00103923; mouse IFNb, NP.034640; rat IFNb, NP.062000.

skin, intestine, kidney, blood, spleen, lung and muscle (Fig. 3). The highest expression levels of gsIFN were observed in the blood, kidney and spleen, modest levels in the liver and heart and the lowest expression levels in the skin, intestine, lung and muscle.

3.4. Expression pattern of gsIFN in peripheral blood leucocytes (PBLs)

After stimulation of PBLs with poly I:C or GSIV, the mRNA expression level of the gsIFN gene was significantly up-regulated (Fig. 4). After stimulation with 5 μ g/ml poly I:C, gsIFN mRNA expression increased by 4 h post-exposure, then reached a peak level at 12 h (2.8-fold), and returned to control expression levels by 24 h. After stimulation with 25 μ g/ml of poly I:C significant up-regulation was observed at 12 (2.5-fold) and 24 h (3.6-fold) (Fig. 4A). However, after the PBLs were exposed to GSIV the

expression of gsIFN was significantly enhanced at 12 h (13.7-fold) and declined slightly at 24 h (Fig. 4B).

3.5. Expression of Mx and gsIFN genes in transformed cells

In pEGFP-N1-IFN transfected cells, the highest IFN gene transcript level was 780-fold higher than it was in non-transfected cells at 48 h. Additionally, the expression of the IFN-inducible gene Mx was up-regulated in the gsIFN-overexpressing cells 19-fold relative to normal cells at 48 h (Fig. 5).

3.6. Antiviral effect of gsIFN in vitro

In the pEGFP-N1-IFN transfected cells, the transcription levels of the MCP and IE-ICP46 genes were reduced 4 and 7-fold, respectively, at 24 h relative to non-transfected cells. At 48 h, MCP and IE-ICP46 transcripts decreased about 8 and 12-fold, respectively,



Fig. 3. The expression pattern of gsIFN in healthy Chinese giant salamanders. The expression of gsIFN was examined in 9 different tissues by qRT-PCR relative to a reference gene, β -actin (Genbank accession no: HQ822274). The values of relative expression level were normalized against with the expression level of gsIFN in the skin. Vertical bars represent the mean \pm S.D. from three individual samples.

compared with the normal cells (Fig. 6A and B). Similarly, the GSIV viral titer in the transfected cells was reduced 23 and 56-fold at 24 and 48 h relative to the non-transfected cells, respectively (Fig. 6C). The cells transfected with pEGFP-N1-IFN exhibited significant antiviral activity.

Cytopathic effect (CPE) in GS-M cells that were overexpressing gsIFN appeared delayed and more subtle compared to the CPE induced in non-transfected cells and cells that were transfected with the empty vector (Fig. 7). To confirm the antiviral effect of gsIFN on the synthesis of viral protein, we analyzed GSIV infected cells by IFA using an antibody against the viral major capsid protein (MCP). As shown in Fig. 8, the viral MCP was detected 24 h after infection. Compared to the pEGFP-N1-IFN transfected cells, a higher level of MCP was detected in the non-transfected cells and cells transfected with empty vector that were infected with GSIV. No expression of viral protein was detected in uninfected cells. At 48 h after GSIV infection, the MCP was synthesized only in sites where the CPE appeared severe, however only a low concentration of MCP was detected in the pEGFP-N1-IFN transfected cells. Additionally, in pEGFP-N1-IFN transfected cells, gsIFN appeared predominantly uniform and localized in the cytoplasm and intercellular space, which was determined by GFP green fluorescence. Additionally, in the GSIV infected cells, strong green fluorescence signals were observed in the MCP synthesis region (Fig. 8, right column).

4. Discussion

Type I IFNs are critical mediators of host defense mechanisms and appear to play a key role in the vertebrate innate immune response against a viral infection. In the study reported herein, the type I IFN gene was identified in the Chinese giant salamander, Andrias davidianus. The genomic sequence of gsIFN has a common structure with fish and other amphibians that includes five exons and four introns in contrast with higher vertebrates that have a single exon. Introns are also found in the IFN genes of cartilaginous fish, suggesting that the type I IFN genes evolved from an intron containing ancestral gene (Zou and Secombes, 2011). The Chinese giant salamander, which originated about 160 million years ago, is considered as an ancestral organism (Gao and Shubin, 2003). A shift may have occurred in the immune system genes during the evolutionary transition from fish to tetrapods. It now generally believed that intronless type I IFN genes found in higher vertebrates originated from a retroposition event that led to the intron disappearing (Zou et al., 2007). Type I IFNs family consists of multiple members which are clustered on chromosome 9 in human. There are also multiple type I IFN genes that were found in bony fish like zebrafish and Atlantic salmon (Aggad et al., 2009; Sun et al., 2009). Similarly, five multiple type I IFN genes are present in the genome in Xenopus (Qi et al., 2010). We suggest that the Chinese giant salamander might have other type I IFN subtypes that could be induced by viral infection and show antiviral activity. Interestingly, the first intron of gsIFN was located in the 5' untranslated region unlike the introns separating the IFN coding region among fish (Fig. 1). Several reports have suggested that there may be more introns in the 5' untranslated region in salmonids and cyprinids (Long et al., 2004; Purcell et al., 2009; Robertsen et al., 2003). Similar to other reported type I IFN genes, the 3'-UTR of the gsIFN gene contains four ATTTA instability motifs which is a characteristic of inflammatory mediator



Fig. 4. The mRNA expression patterns of gsIFN gene after stimulation with different concentrations of poly I:C (A) and GSIV (B). Peripheral blood leucocytes (PBLs) were isolated and stimulated with 5 or 25 μ g/ml Poly I:C and GSIV or PBS as a control. The transcript level of gsIFN was examined at 4, 12 and 48 h post-stimulation. The expression of gsIFN was normalized to the expression of β-actin and relative to the expression level of the PBS group at same time points. Vertical bars represent the mean ± S.D. from PBLs isolated from three individual samples. Asterisks (*) mark the significant difference between experimental and control groups (P < 0.05).



Fig. 5. Analysis of the levels of the Chinese giant salamander type I gene (A) and Mx gene (B) expression after Chinese giant salamander iridovirus (GSIV) infection in transfected Chinese giant salamander muscle cells (GS-M) and normal cells. The GS-M cell line was non-transfected (normal cells) or transfected separately with either pEGFP-N1 (empty vector) or PEGFP-N1-IFN. After G418 selection, cells were infected with 0.5 MOI GSIV and the gene expression level was examined at 24 and 48 h post-infection. Vertical bars represent the mean \pm S.D. from three experiments. Asterisks (*) mark the significant difference between non-transfected and transfected groups (P < 0.05).



Fig. 6. Antivirus effects of gsIFN on the transcription of the major capsid protein (MCP) gene (A), immediate early protein ICP-46 (IE-ICP46) gene (B) and the virus titer (C) of the Chinese giant salamander iridovirus (GSIV) in non-transfected (normal cells) or transfected cells. GS-M cell line was non-transfected or transfected with pEGFP-N1 (empty vector) or PEGFP-N1-IFN separately. After G418 selected, cells were infected with 0.5 MOI of GSIV. The gene expression levels were examined 24 and 48 h post infection. Vertical bars represent the mean ± S.D. from three experiments. Asterisks (*) mark the significant difference between non-transfected and transfected groups (*P* < 0.05).



Fig. 7. Cytopathic effect (CPE) in GS-M cells transfected with pEGFP-N1 (empty vector) or pEGFP-N1-IFN after GSIV infection. Non-transfected cells (normal cells) served as the control. Areas of severe CPE are indicated by black arrows. Scale bar, 100 μ m.

coding genes that reflect the highly inducible nature of these antiviral cytokines (Sachs, 1993). In teleost fish, type I IFN genes can be divided into two groups based on the cysteine patterns with group I possessing two cysteines (2C) and group II having extra pair of cysteines (4C) (Zou et al., 2007). The conservative cysteine residues that form the disulfide bridge are critical for protein activity. The inhibition of the formation of the disulfide bridge or just the displacement of its location by only one position causes loss of activity (Viscomi, 1997). In this study, a CAWE motif was found in the gsIFN amino acid sequence with the presence of C 4. Interestingly, in spite of the presence of C 4 in *Xenopus* IFNs, they lack the CAWE motif (Supplementary Fig. S1). Fourth cysteine patterns containing IFNs which have a conserved C-terminal CAWE motif are likely to be the ancestral gene for the Type I IFNs family. (Wan et al., 2012; Zou et al., 2007).

The gsIFN gene was found to be highly and constitutively expressed in blood, kidney and spleen in the giant salamander (Fig. 3). In agreement with observations in fish and amphibians (De Jesus Andino et al., 2012; Parhi et al., 2014), these tissues are associated with the immune system and an immune response is easily elicited. Moreover, the gsIFN expression level was significantly increased in freshly isolated peripheral blood leucocytes after poly I:C and GSIV stimulation, suggesting the importance of this cytokine in the immune response of the Chinese giant salamander. Compared with the gsIFN mRNA expression pattern after poly I:C stimulation, the response was more rapid and intense after



Fig. 8. Effect of gsIFN on the viral major capsid protein (MCP) expression in cells infected with GSIV. Normal cell and cells transfected with pEGFP-N1 (empty vector) or pEGFP-N1-IFN were infected with GSIV at an MOI of 0.5. At 24 and 48 h the cells were analyzed by IFA. Nuclei were stained with DAPI and appear blue. The MCP and gsIFN were detected in red and green fluorescence, respectively. Scale bar, 50 μm.

exposure to GSIV (Fig. 4). The response of gsIFN appeared more sensitive to viral infection suggesting that the two stimuli differ in their abilities to activate signaling pathways. The innate responses constitute the first line of defense and can establish host susceptibility to pathogens. In mammals, IFN- α is primarily produced by virally infected leukocytes and IFN- β is synthesized by most cell types but especially in fibroblasts (Derynck et al., 1980; Goeddel et al., 1981). In addition, innate immune cells such as macrophages play an instrumental role in both innate and adaptive immune responses to viral infections (Ellermann-Eriksen, 2005). In Atlantic salmon, the expression of type I IFNs was examined in leucocytes in vitro and IFNa1/a2 genes were strongly up-regulated by poly I:C (Sun et al., 2009). In amphibians, the occurrence, composition, activation status and permissiveness of peritoneal leukocytes (PLs) to frog virus - 3 (FV3) was investigated. The results indicated that PLs may play a dual role in anti-FV3 immune responses and permissively harbor asymptomatic FV3 aspect. (Morales et al., 2010).

Previously, it was shown that type I IFNs induce expression of a wide range of IFN stimulated genes (ISGs), including PKR, OAS, Mx and ISG15, that possibly contribute to enhancing the antiviral state of a host (Robertsen, 2006; Samuel, 2001). In crucian carp Carassius auratus L, IFN induces a set of ISGs to inhibit virus replication through the Stat1 pathway (Yu et al., 2010). Mx proteins are dynamin-like GTPases which appear to target viral nucleocapsids and inhibit RNA synthesis (Haller and Kochs, 2002). In vitro, cells transfected with IFN expression plasmids can elevate the expression of Mx. The antiviral state coincides with a strong expression of Mx mRNA and Mx protein in a salmon cell line infected with salmonid alphavirus - 3 (Xu et al., 2010). Recombinant type I IFN proteins were potent inducers of Mx expression and exhibited antiviral activities in zebrafish, Atlantic salmon and rainbow trout (Altmann et al., 2003a; Robertsen et al., 2003; Zou et al., 2007). The immune functions of type I IFNs have also been studied in vivo. Xenopus laevis type I IFN could induce Mx gene expression in tadpoles and conferred antiviral effects (Grayfer et al., 2014). In the present study, gsIFN could induce the Mx protein in the permissive GS-M cell line quickly (Fig. 5), suggesting that gsIFN might use a similar signaling network as that in other vertebrates. Since the Mx gene is up-regulated by type I IFNs, the Mx gene could be used as a marker gene to assess the biological activity of gsIFN both in vitro and in vivo.

In present study, GSIV replicated slowly as indicated by the reduction in virus titer and the low transcript levels of the MCP and IE-ICP46 genes in the gsIFN- overexpressing GS-M cell line (Fig. 6). This result indicated that the gsIFN has an inhibitory effect on the gene transcription kinetics of GSIV and showed a significant antiviral effect on GSIV replication. Furthermore, a reduction in the MCP synthesis was observed by immunofluorescence assay (Fig. 8). These results indicated that GSIV replication was inhibited at both the mRNA and protein level. In other vertebrate species, the antiviral effects of type I IFNs have also been confirmed. In Atlantic salmon, cells transfected with the SasaIFN- α 1 gene produced high titers of acid-stable antiviral activity that induced the Mx protein. The induced Mx protein protected the salmonid embryo cell line, CHSE-214, against infectious pancreatic necrosis virus (IPNV) (Robertsen et al., 2003). Infectious salmon anemia virus replication is transiently inhibited by Atlantic salmon type I interferon in cell culture (Svingerud et al., 2013). Additionally, zebrafish embryo fibroblast cells transfected with a recombinant IFN gene exhibited increased resistance against snakehead rhabdovirus infection (Altmann et al., 2003). In grouper, type I IFN induced Mx gene expression that enhanced the host immune response against nodavirus infection (Chen et al., 2014). In an amphibian, pretreatment with a recombinant X. laevis IFN substantially reduced FV3 virus replication and infectious viral burdens in frog kidney cells (Grayfer et al., 2014). Modulation of type I

IFNs is complicated and IFN signaling provides a broad-spectrum antiviral function. Type I IFNs can be activated by many different molecules, such as viral dsRNA, and then mediate the activation by several effector pathways to control viral replication (Sadler and Williams, 2008; Zhang and Gui, 2012). However, there have only been a limited number of functional studies in amphibians using type I IFNs.

The causes of the global decline of amphibians are complex and infectious diseases such as viral infections by Ranaviruses (Iridoviridae family) are now considered important (Chen and Robert, 2011). Members of the genus Ranavirus have been recognized as major pathogens of economically and ecologically important cold-blooded vertebrates, including fish, amphibians and reptiles (Jancovich et al., 2010; Williams et al., 2005). Chinese giant salamander iridovirus (GSIV) has been confirmed as an emerging severe viral disease in farmed Chinese giant salamanders in China. The complete genome sequence of GSIV has been determined and phylogentic analysis showed that this virus belongs to the common midwife toad ranavirus (CMTV) subset in the amphibian-like ranavirus (ALRV) group of the genus Ranavirus (Chen et al., 2013; Mavian et al., 2012). In our previous studies, the viral replication cycle and ultrastructural alterations that occur were examined in an infected fish cell line (Ma et al., 2014). Additionally, a killed GSIV vaccine induced protection against iridovirus induced hemorrhagic disease in Chinese giant salamanders (Liu et al., 2014). The relatively recent and rapid increase in the prevalence of GSIV infection, suggests that there is an urgent need to understand more about the hosts immune system in the etiology of viral diseases. Characteristics of molecules associated with innate and adaptive immunity such as MHC, IFN-inducible protein 6 (IFI6) have been investigated in the Chinese giant salamander (Zhu et al., 2014a, b). In this study, the molecular and *in vitro* anti-viral functions of gsIFN were demonstrated. These results highlights that there is a powerful immune system that exists in this primitive amphibian and makes this species to be a suitable candidate to understand the immune system of lower vertebrates. More research into the involvement of the amphibian immune responses in virus infections will unravel the antiviral mechanisms in amphibians and the strategies employed by viruses to avert those immune responses.

In summary, a novel type I IFN gene was successfully identified in Chinese giant salamander, which contains four cysteines as well as exists in every order of vertebrates. The gsIFN mainly exerts its function in the host innate immune response. The results of the study reported herein provide a fundamental base for further studies of gsIFN in the innate response and signalling pathways, which may be of significance for the control of viral diseases.

Acknowledgments

The work was supported by Special Fund for Agro-scientific Research in the Public Interest (201203086).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm. 2015.02.015.

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