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IFNb of black carp functions importantly in host innate immune response as an antiviral cytokine

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ABSTRACT

Type I interferons (IFN-Is) play an important role in the antiviral immune response in teleost fishes. In this study, one type I interferon (bcIFNb) from black carp (*Mylopharyngodon piceus*) has been cloned and characterized. The full-length cDNA of *bcIFNb* gene consists of 806 nucleotides and the predicted bcIFNb protein contains 188 amino acids. Basing on the cysteine number and evolutionary position, bcIFNb was classified into group II type I IFN. q-PCR analysis demonstrated that bcIFNb mRNA level varied *in vivo* and *ex vivo* in response to different stimuli. bcIFNb was detected in both the whole cell lysate and the supernatant media of HEK293T cells or EPC cells transfected with bcIFNb through immunoblot assay. IFN stimulated genes (ISGs) were greatly upregulated when the host cells were treated with the bcIFNb-containing conditioned media. EPC cells showed greatly enhanced antiviral ability when the cells were transfected with bcIFNb or treated with the bcIFNb-containing conditioned media before GCRV or SVCV infection. Glycosidase digestion analysis determined that bcIFNb was modified with N-linked glycosylation, which occurred on the Asn (N) of 92 site of this cytokine. The un-glycosylated mutant bcIFNb-N92Q presented the similar antiviral ability as that of wild type bcIFNb, which demonstrated that N-linked glycosylation did not contribute directly to the antiviral property of this fish cytokine.

1. Introduction

Interferons (IFNs) are small, induce-expressed proteins after viral invasion, which are the hallmark of the innate immune response against virus replication [1,2]. IFNs play key roles in both innate and adaptive immune responses in human and mammals, which can be classified into three categories: type I IFN, type II IFN and type III IFN [3–5]. In teleost fishes, IFNs are classified into type I IFN (IFN-I) and type II IFN (IFN-II) [6]. IFNs are usually small and secreted proteins, which stimulate host cells' immune response through autocrine or paracrine [7]; however, functioning intracellular IFN system was found in rainbow trout (*Oncorhynchus mykiss*) and triploid hybrid separately, which might act as the novel defense to counteract viral infection [7,8].

In teleost fishes, IFN-Is are important antiviral cytokines and belong to two distinct groups based on their cysteine amount [1]. Group I IFN-Is contain two cysteine residues in mature peptides and group II IFN-Is contain four cysteine residues [9]. Furthermore, evolutionary studies have demonstrated that teleost type I IFNs can be classified into seven sub-groups, namely IFN-a, -b, -c, -d, -e, -f and -h, with IFN-a, IFN-d, IFN-e and IFN-h make up the group I IFN-Is and IFN-b, IFN-c and IFN-f make up the group II IFN-Is [6,10,11]. In mammals, all type I IFNs share a common receptor complex consisting of IFN-α receptor (IFNAR) 1 and IFNAR2 [2]. However, teleost fishes possess different IFN receptors. For example, it has been reported that CRFB1 and CRFB5 are receptor complexes of the group I IFN-Is (IFN 1 and IFN4) and CRFB2 and CRFB5 are receptor complexes of the group II IFN-Is (IFN2 and IFN3) in zebrafish [12].

In mammals, recent researches have established that type I IFNs have been implicated in antiviral activity, antibacterial activity, cell proliferation and the suppression of some forms of cancer [5,9,13,14]. However, type I IFNs of teleost majorly present antiviral activity during host innate immune response. For instance, IFN-a1 can protect salmonid cells against infectious pancreatic necrosis virus (IPNV) [15]. In rainbow trout (*Oncorhynchus mykiss*), recombinant group I IFNs could induce Mx expression and gain antiviral responses, whereas recombinant group II IFN was poor in antiviral activity [16]. Group I IFNs and group II IFNs could induce the expression of different antiviral genes in response to Nervous necrosis virus (NNV) and SVCV infection in zebrafish (*Danio rerio*) [17,23]. It is interesting that RIG-I specifically could activate expression of group II, but not group I, type I IFNs in

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Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Amplicon length (nt) and primer information
IFNb-C-F	ACTGACGGTACCGCCACCATGGACCTTCATCGTGTGGC	Gene cloning
IFNb-C-R	ACTGACCTCGAGGAGTTGTGGAGATTCGTAC	
5′ -RACE		
5′ GSP1	CTGGTAAACAGCCTTTTCAACT	5′ UTR 1st PCR
5′ Race Outer primer	ATGTGACGGCTTTTGGTATTG	
5′ GSP2	CCCGCAGTCTCTATAAATGT	5′ UTR 2nd PCR
5′ RACE Inner primer	AGAAACCTCACCTGGTCCTCC	
3′ -RACE		
3′ GSP1	TACCAGACACTCCAAAATATT	3′ UTR 1st PCR
UPM-Long primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM-Short primer	CTAATACGACTCACTATAGGGC	
3′ GSP2	GGAGAGCAAATGCATCATGAG	3′ UTR 2nd PCR
NUP1	AAGCAGTGGTATCAACGCAGAGT	
CMV-F	CGCAAATGGGCGGTAGGCGTG	
BGH-R	TAGAAGGCACAGTCGAGG	
IFNb-Q-F	GACCACGTTTCCATATCTTT	q-PCR
IFNb-Q-R	CATTTTTTCTTCATCCCACT	
bc-Q-actin-F	TGGGCACTGCTGCTTCCT	q-PCR
bc-Q-actin-R	TGTCCGTCAGGCAGCTCAT	
bcMX1-Q-F	TGAGCGTAGGCATTAGCAC	q-PCR (GeneBank:KX246952)
bcMX1-Q-R	CCTGGAGCAGCAGATAGCG	
bcSTAT1-Q-F	CCGTCGCAAGTAATGTATC	q-PCR
bcSTAT1-Q-R	CCTGGAAGTGCTCCTGT	
bcViperin-Q-F	CCAAAGAGCAGAAAGAGGGACC	q-PCR (GeneBank:KX384641)
bcViperin-Q-R	TCAATAGGCAAGACGAACGAGG	
bcPKR-Q-F	GAGCGGACTAAAAGGACAGG	q-PCR
bcPKR-Q-R	AAAATATATGAGACCCAGGG	
bcIFNb-N92Q-F1	GCACTGTTTGAGCAATACAGT	Gene cloning
bcIFNb-N92Q-R1	TTCTTCATCCCACTGGTCTGGAACACTGTATTGCTCAAAC	

response to NNV infection in zebrafish [17].

To date, group I IFN-Is have been cloned and characterized in variety of teleost fishes, such as zebrafish, Atlantic salmon (*Salmo salar*), catfish (*Ictalurus punctatus*) and triploid hybrid [8,13,15,18,19]. In our previous study, bcIFNa was cloned from black carp and characterized as an antiviral cytokine, which was classified into group I IFN-I [20]. In general, teleost group I IFN-Is had been widely studied, while teleost group II IFN-Is were less explored except those in salmon (*Salmo salar*), rainbow trout, zebrafish and grass carp (*Ctenopharyngodon idella*) [12,17,21–23].

In this study, one group II type I interferon of black carp, named as bcIFNb, has been cloned and characterized. Our study demonstrated that bcIFNb was an antiviral cytokine against grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV) [24], which was modified with N-linked glycosylation on the asparagine of site 92. The un-glycosylated bcIFNb mutant (N92Q) showed a similar antiviral ability with that of bcIFNb, which verified that N-linked glycan did not contribute directly to the antiviral activity of this fish cytokine.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, *epithelioma papulosum cyprini* (EPC) cells, HeLa cells, *Ctenopharyngodon idella* kidney (CIK) cells, *Mylopharyngodon piceus* kidney (MPK) cells and *Mylopharyngodon piceus* fin (MPF) cells were kept in the lab [20]. All the cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. HEK293T and HeLa were cultured at 37 °C with 5% CO_2。 EPC, CIK and MPF cells were culture at 26 °C with 5% CO_2.

pcDNA5/FRT/TO and pcDNA5/FRT/TO-HA (Invitrogen) were kept in the lab [25]. The open reading frame (ORF) of bcIFNb was amplified by PCR and cloned into pcDNA5/FRT/TO-HA between Kpn I and Xho I sites. The un-glycosylated mutant bcIFNb-N92Q was generated as previously [25], in which asparagine (N) of residue 92 was altered to glutamine (Q).

2.2. Cloning of bcIFNb cDNA

Total RNA was isolated from the liver of black carp of six months (weight of 120 g) by using TRIzol® reagent (Takara), which was injected intraperitoneally with SVCV (2.43×10^6 pfu/fish) and harvested at 33 h post infection. First-strand cDNA was synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol based on 2 μg total RNA. Rapid amplification of cDNA ends (RACE) was performed to obtain 5′ untranslated region (UTR) and 3′ UTR of bcIFNb cDNA by using 5′ Full RACE Kit and 3′ Full RACE kit separately (Takara). The full-length cDNA of bcIFNb was cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus production and titration

SVCV and GCRV were kept in the lab, which were propagated in EPC and CIK separately at 26 °C in the presence of 2% fetal bovine serum [8,19]. Virus titers were determined by plaque forming assay on EPC cells separately as previously described [20]. Briefly, the 10-fold serially diluted virus was added onto EPC cells and incubated for 2 h at 26 °C. The supernatant was removed after incubation and DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) was added. Plaques were counted at day-3 post infection.

2.4. Immunoblot assay

Immunoblot assay was performed as previously described [19]. Briefly, the whole cell lysate and supernatant media (25 μl from 2 ml for each sample) were isolated by 14% SDS-PAGE and the transferred membrane was probed with mouse monoclonal anti-HA antibody (1:3000; Sigma), then followed by the incubation with the goat-anti mouse secondary antibody (1:30000, Sigma), the proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

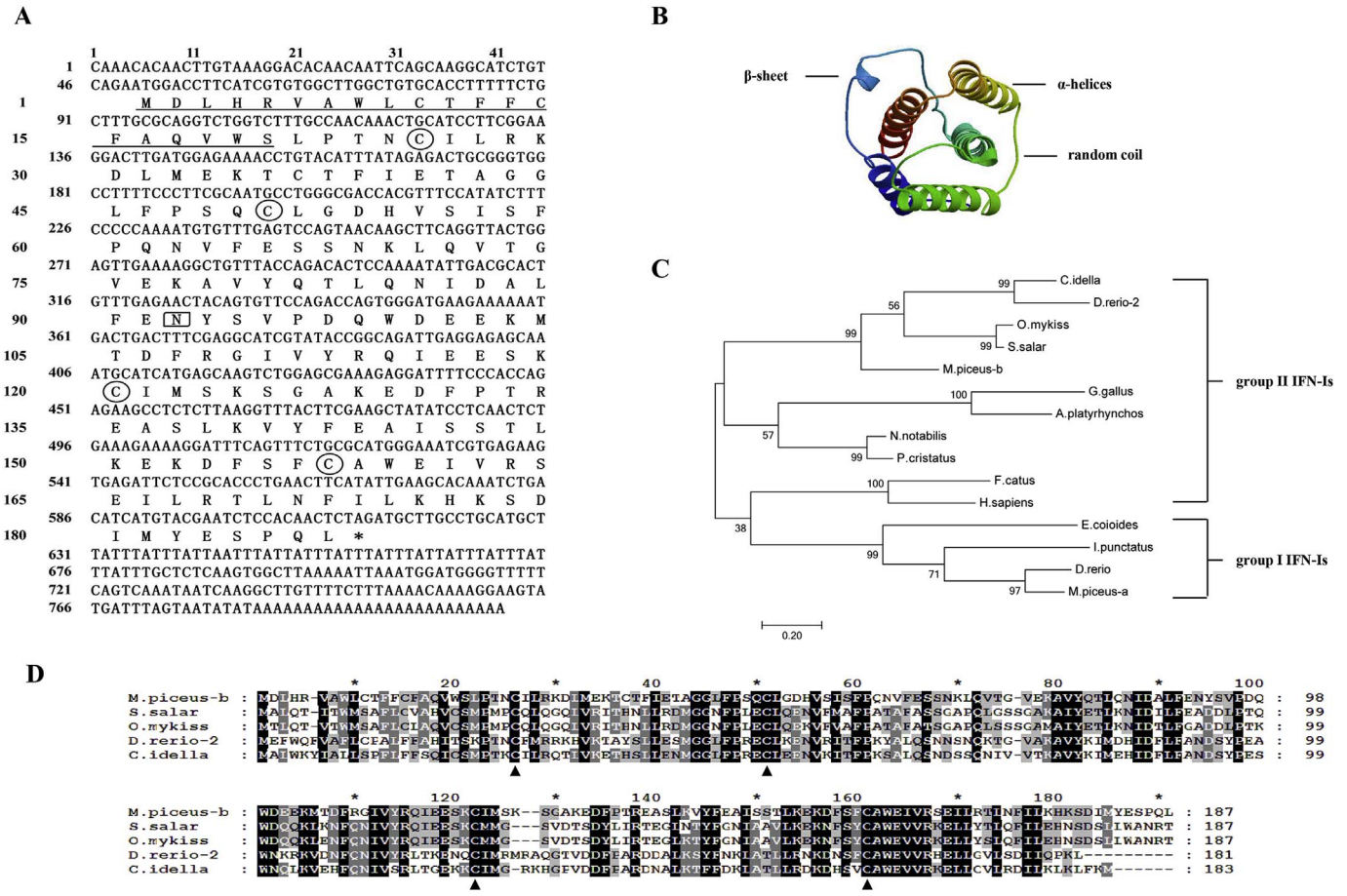

Fig. 1. Cloning of the full-length cDNA of bcIFNb.

(A): The nucleotide sequence and the predicted amino acid sequence of bcIFNb were numbered separately. The predicted signal peptide was underlined, four cysteine residues (C) are cycled and the predicted asparagine residue (N) with N-linked glycosylation was in the rectangle (predicted by the SignalP program/version4.1, Center for Biological Sequence Analysis [http://www.cbs.dtu.dk/services/SignalP/]).

(B): The amino acid sequence of bcIFNb was aligned with selected type I IFNs from different species by using MEGA 6.0 software. IFN sequences from the following organisms (GenBank accession number, unless indicated otherwise) were included: *Ictalurus punctatus* (AAP92146.1); *Salmo salar* (ACE75692.1); *Oncorhynchus mykiss* (CCV17403.1); *Homo sapiens* (AAA36123.1); *Felis catus* (AAB27160.1); *Gallus gallus* (AAA50213.1); *Danio rerio* (CAD67754.1); *Nestor notabilis* (KFQ55840.1); *Podiceps cristatus* (KFZ62568.1); *Epinephelus coioides* (AGL21770.1); *Anas platyrhynchos* (CAA59235.1); *Danio rerio*-2 (NP001104552.1); *Ctenopharyngodon idella* (AMT92190.1). The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

(C): The predicted protein structure of bcIFNb (by SWISS-MODEL).

(D): Comparisons of bcIFNb with other group II IFNs protein sequences by using MEGA 7.0 program and GeneDoc program. Four cysteine residues predicted to be engaged in disulfide bridge are indicated by a triangle (Δ).

2.5. Quantitative real-time PCR

The primers for bcIFNb, bcMX1, bcSTAT1, bcViperin, bcPKR and β-actin (as internal control) were listed in Table 1. The program of q-PCR was: 1 cycle of 50 °C/2min, 1 cycle of 95 °C/10min, 40 cycles of 95 °C/15s, 60 °C/1min, followed by dissociation curve analysis (60°C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel Sheet for subsequent data analyses where the relative expression ratios of target gene in treated group versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

2.6. Immunofluorescence microscopy

HeLa cells and EPC cells were transfected with pcDNA5/FRT/TO-bcIFNb-HA or the empty vector separately. The transfected cells were fixed with paraformaldehyde at 24 h post transfection and permeabilized with triton X-100 (0.2% in PBS). After incubated with primary and secondary antibodies, the cells were analyzed by immunofluorescence microscopy as previously described [26]. For commercial antibodies, mouse monoclonal anti-HA antibody (Sigma) was used at the dilution ratio of 1:400 and Alexa 488-conjugated secondary antibody (Invitrogen) was diluted at 1:800; DAPI was used for nucleus staining.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The bcIFNb-containing conditioned media was measured through ELISA assay as previously described [19]. Briefly, 96-well plate was coated with bcIFNb-containing conditioned media and blocked with carbonate buffer (pH 9.6) at 4 °C for overnight. After three times of wash, each well was probed with anti-HA antibody (Sigma) at different concentration and cultured at 37 °C for 2 h. After five times of wash, each well was probed with goat-anti mouse secondary antibody (1:30000, Sigma) at 37 °C for 1 h. Then, each well was added with 200 μl PNPP for 20 min and 50 μl NaOH was added to terminate the exposure. The OD value at 410 nm was measured by Synergy-2 Multi-mode reader (BioTek).

2.8. PNGase F digestion

293T cells were transfected with pcDNA5/FRT/TO-bcIFNb-HA and

transfected cells were harvested at 48 h post-transfection and lysed in lysis buffer (50 mM Tris-Hcl/pH7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.05% Tween-20) containing protease inhibitor cocktail (Roche). Whole cell lysates were divided into two aliquots, one aliquot digested with PNGase F (NEB) according to the instruction of the manufacturer; the other aliquot was treated without PNGase F. Briefly, the sample mixed with the PNGase F at 37 °C for 1 h. The PNGase F digested sample and the control were isolated by 14% SDS-PAGE and identified by immunoblot assay.

2.9. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter assay and viral titer measurement, all data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data (*$p < .05$; **$p < .01$). The data were analyzed by two-tailed Student's *t*-test.

3. Results

3.1. Molecular characterization of bcIFNb

The full-length cDNA of bcIFNb consists of 806 nucleotides including 5′ UTR, coding sequence, 3′ UTR and poly (A) tail. The open reading frame (ORF) of bcIFNb initiates at nucleotide of 50 and terminates at nucleotide of 613. The predicted bcIFNb protein contains 188 amino acids, in which the first 20 amino acids represent a putative signal peptide. The matured bcIFNb peptide contains four cysteine residues (25, 50, 120 and 157), which potentially form two disulfide bridges in this cytokine molecule and suggest that bcIFNb belongs to group II IFN-I [16]. Besides, the asparagine residue of 92 is located in the conserved N-linked glycosylation motif (N-X-S/T), which suggests that bcIFNb is a glycan protein (Fig. 1A). The protein structure of bcIFNb is predicted by SWISS-MODEL ([https://www.swissmodel.expasy.org/]), which contains α-helices, β-sheet and random coil (Fig. 1B). The calculated molecular weight of bcIFNb is 21.7 kDa and the theoretical isoelectric point of bcIFNb is 5.15 (predicted by the ProtParam tool [http://web.expasy.org/protparam/]).

To gain insight into bcIFNb evolution, the bcIFNb protein was aligned with vertebrate type I IFNs from different species and the phylogenetic tree was generated through the Clustal Omega software. The evolution study demonstrated that the selected type I IFNs could be divided into two groups, consisting of group I and group II branches, in which bcIFNb was clustered together with other salmonidae type I IFNs including salmon and trout. In the phylogenetic tree, bcIFNb was even closer with Mammals/aves group II IFN-Is (human and Kea) than teleost group I IFN-Is (Channel Catfish, zebrafish and black carp) (Fig. 1C). Comparisons of bcIFNb with other group II IFNs protein sequences demonstrated that bcIFNb was classified into group II IFN since it contains four cysteine residues similar to those of other group II IFNs (Fig. 1D).

3.2. bcIFNb expression in vivo and ex vivo

To investigate the transcription of *bcIFNb* gene *in vivo*, total RNA was extracted separately from kidney, liver, spleen, heart, intestine, muscle, gill and skin of the black carp injected with GCRV, SVCV or PBS. bcIFNb mRNA level in different tissues was examined by q-PCR, in which the PCR of β-actin was used as the internal control. The result demonstrated that *bcIFNb* gene was constitutively transcribed in all the selected tissues of black carp under healthy condition, in which the mRNA level of bcIFNb in spleen was the highest and bcIFNb mRNA level in kidney and liver was much lower (Fig. 2A). For GCRV infected group, the bcIFNb mRNA level in intestine, gill, liver and skin increased post GCRV injection, especially that in liver and skin. However, bcIFNb mRNA level in spleen was reduced at 33 h post GCRV injection. It was interesting that the mRNA level of bcIFNb in liver obviously increased but decreased in spleen, intestine and gill post SVCV injection (Fig. 2A). The variation of bcIFNb mRNA level in different tissues after RNA virus infection suggested that bcIFNb was involved in the black carp innate immune response, especially after GCRV infection.

To learn the profile of bcIFNb mRNA expression in host cells during the innate immune response, MPF cells were subjected to different stimuli and applied to q-PCR analysis. For LPS stimulation, bcIFNb mRNA expression increased right after treatment and the highest bcIFNb level emerged at 2 h point for all doses. The data showed that the highest relative bcIFNb mRNA level (0.5 μg/ml; 2 h point) was up to 28.7 folds (Fig. 2B). For poly(I:C) stimulation, bcIFNb mRNA in MPF cells increased right after treatment; the highest bcIFNb level merged at 2 h point for 5 μg dose and at 24 h point for both 5 μg and 50 μg dose. The data showed that the highest relative bcIFNb mRNA level (50 μg/ml; 24 h point) was up to 605.0 folds (Fig. 2C). These data suggested that bcIFNb might be an important component of host defense mechanism responsible for both virus infection and bacteria invasion.

3.3. bcIFNb is a secreted cytokine

To study the protein expression of bcIFNb, both HEK293T cells and EPC cells were transfected with pcDNA5/FRT/TO-bcIFNb-HA or the empty vector separately. The whole cell lysate and the media supernatant of the transfected cells were harvested for immunoblot (IB) assay at 48 h post transfection separately. For the IB of the whole cell lysate, there were two bands detected by anti-HA antibody in the lane bcIFNb-HA, one was around 22 kDa and the other was around 19 kDa (Fig. 3A&B, lift panel). For the IB of media supernatant, two bands were detected in the lane of bcIFNb-HA, one was around 22 kDa and the other was around 19 kDa (Fig. 3A&B, right panel). The data clearly demonstrated that bcIFNb was well expressed in and secreted out from HEK293T cells and EPC cells. The calculated molecular weight of bcIFNb is 21.7 kDa and the predicted molecular weight of matured bcIFNb is 19.2 kDa (Fig. 1A). It was clear that the band of 19 kDa was thicker than the band of 22 kDa in the IB of both HEK293T and EPC cells. The IB data suggested that the larger band of bcIFNb might be a modified form; most likely, this fish cytokine was modified with glycosylation.

To determine the intracellular distribution of bcIFNb before its secretion, both EPC cells and HeLa cells were transfected with pcDNA5/FRT/TO-HA-bcIFNb or the empty vector separately and analyzed by immunofluorescence (IF) staining. The IF data showed bcIFNb was a cytosolic protein before its secretion since the bcIFNb-expressing region (green) surrounding the nucleus (blue) (Fig. 3C&D).

3.4. bcIFNb induces the expression of downstream gene and ISGs

To test whether bcIFNb induced antiviral signaling or not, both MPF cells and MPK cells were treated with bcIFNb-containing conditioned media and the expression of downstream gene and ISGs was examined by q-PCR whose counterparts of mammals functioned importantly in host antiviral innate immunity. After bcIFNb stimulation, the mRNA level of all the selected genes increased in both MPK cells and MPF cells, including bcSTAT1, bcMX1, bcViperin and bcPKR (Fig. 4A–H). Interestingly, the mRNA level of these genes was up regulated to the highest level at 4 h after bcIFNb stimulation in both MPK cells and MPF cells (Fig. 4).

3.5. bcIFNb is an antiviral cytokine

In our previous study, bcIFNa, a group I IFN-I of black carp, has been identified as an antiviral cytokine during host innate immune response [20], which triggered us to test whether bcIFNb owned a

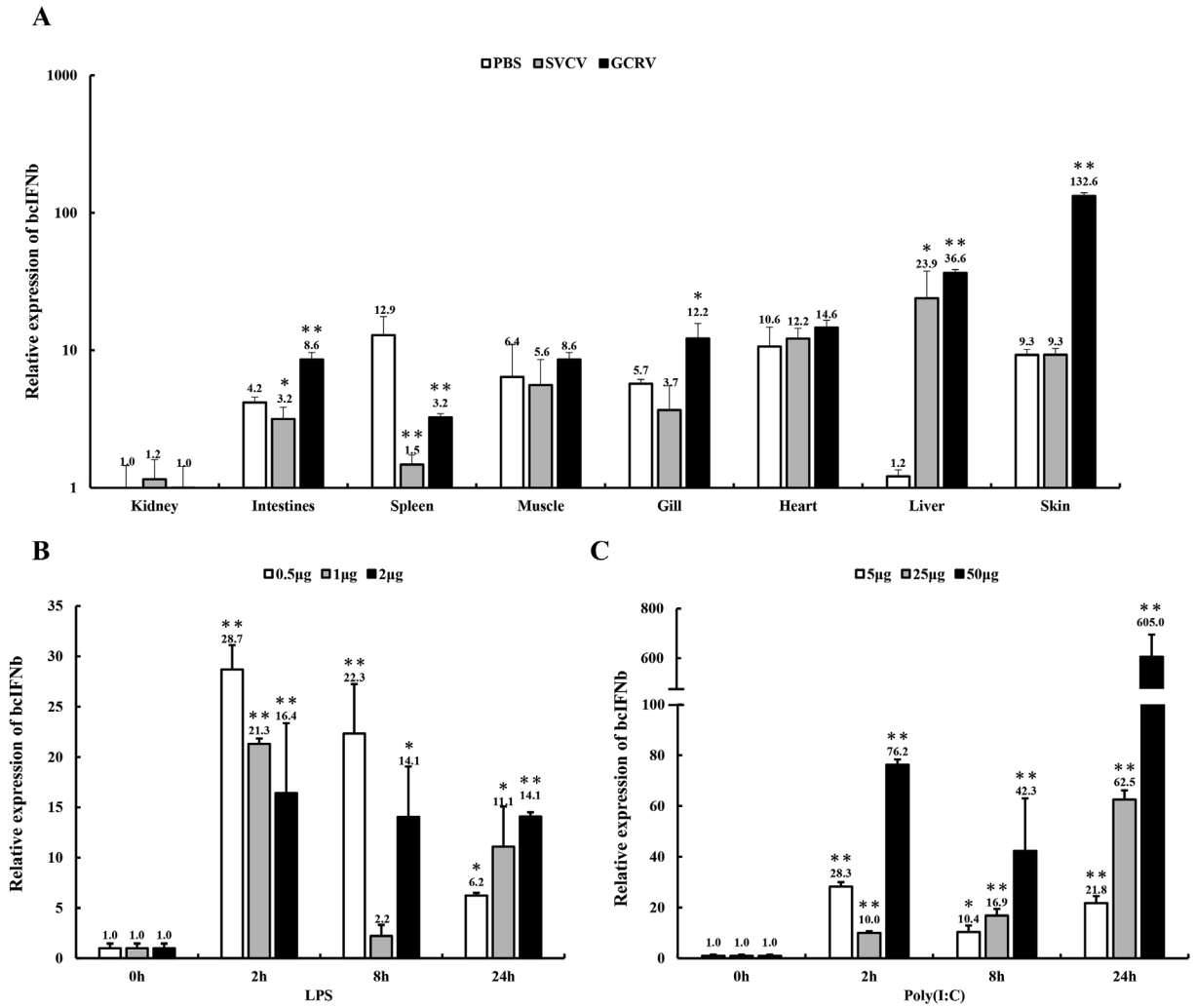

Fig. 2. bcIFNb expression *in vivo* and *ex vivo*.

(A) Black carp of six months (weight of 120 g) were injected intraperitoneally with GCRV (2.52×10^6 pfu/fish), SVCV (2.43×10^6 pfu/fish) or sterile PBS separately and cultured at 25 °C. Three fish were collected for each injected group at 33 h post injection and total RNA was isolated from the indicated tissues independently. The transcription of bcIFNb after viral challenge or in healthy condition (PBS injection) was examined by q-PCR separately. The relative bcIFNb mRNA level in healthy kidney was defined as 1 and the number above the error bar stands for the average bcIFNb mRNA level. The significant difference analysis was conducted between the values in healthy condition and viral infected condition in each tissue. (B and C): MPF cells in 6-well plate (2×10^6 cells/well) were treated with LPS or poly (I:C) at the indicated concentration separately and harvested at 2, 8 or 24 h post stimulation separately. The relative bcIFNb mRNA level was examined by q-PCR. The bcIFNb mRNA level without stimulation (0 h) was set up as 1 (as control) and the number above the error bar stands for the average bcIFNb mRNA level. The significant difference analysis was conducted between the values of the control and the stimulated groups.

similar function or not. For the EPC cells infected with GCRV at different MOIs (0.01, 0.1, 1 and 2.5), both the CPE rate and the viral titer in the supernatant media of the EPC cells over-expressing bcIFNb were much lower than those of the controls, which were determined by crystal violet staining and classic plaque assay separately (Fig. 5A and B). For the EPC cells infected with SVCV at different MOIs (0.01, 0.1, 1 and 5), both the CPE rate and the viral titer in the supernatant media of the EPC cells over-expressing bcIFNb were much lower than those of the controls, which were similar to those of GCRV infected EPC cells (Fig. 5C and D).

To further test the antiviral activity of bcIFNb, EPC cells in 24-well plate (4×10^5 cells/well) were treated with bcIFNb-containing conditioned media for 24 h before being infected with GCRV or SVCV. Both the CPE rate and the viral titer in the supernatant media of the EPC cells pre-treated with the conditioned media were much lower than those of the controls, which were determined by the classic plaque assay (Fig. 5E and G) and the crystal violet staining (Fig. 5F and H) separately. These data demonstrated clearly that bcIFNb was an antiviral cytokine against both GCRV and SVCV and it had a similar function as group I IFN-Is.

3.6. bcIFNb is modified with N-linked glycosylation

Two bands were detected in the IB of both HEK293T and EPC cells expressing bcIFNb (Fig. 3A). Considering that asparagine residue of 92 of bcIFNb was within the conserved motif (N-X-S/T) of N-linked glycosylation (Fig. 6A), it was speculated that the larger band of bcIFNb might be its glycosylated form. To explore if bcIFNb was modified with N-linked glycosylation or not, the whole cell lysate of HEK293T expressing bcIFNb was digested with PNGase F, which is a glycosidase specific for digesting the N-linked glycan chains. After digestion with PNGase F, there was only one band of bcIFNb left, which migrated identically to the small band of bcIFNb (Fig. 6B). The data demonstrated that bcIFNb possessed N-linked glycosylation, in which the smaller band stood for the un-glycosylated form and the larger one was the glycosylated form.

To see if the N-linked glycosylation of bcIFNb impact on its antiviral function or not, the un-glycosylated mutant bcIFNb-N92Q was constructed, in which the asparagine (N) of 92 has been replace with glutamine (Q). There was only one band of N92Q detected in IB of the whole cell lysate of HKE293T cells transfected with pcDNA5/FRT/TO-

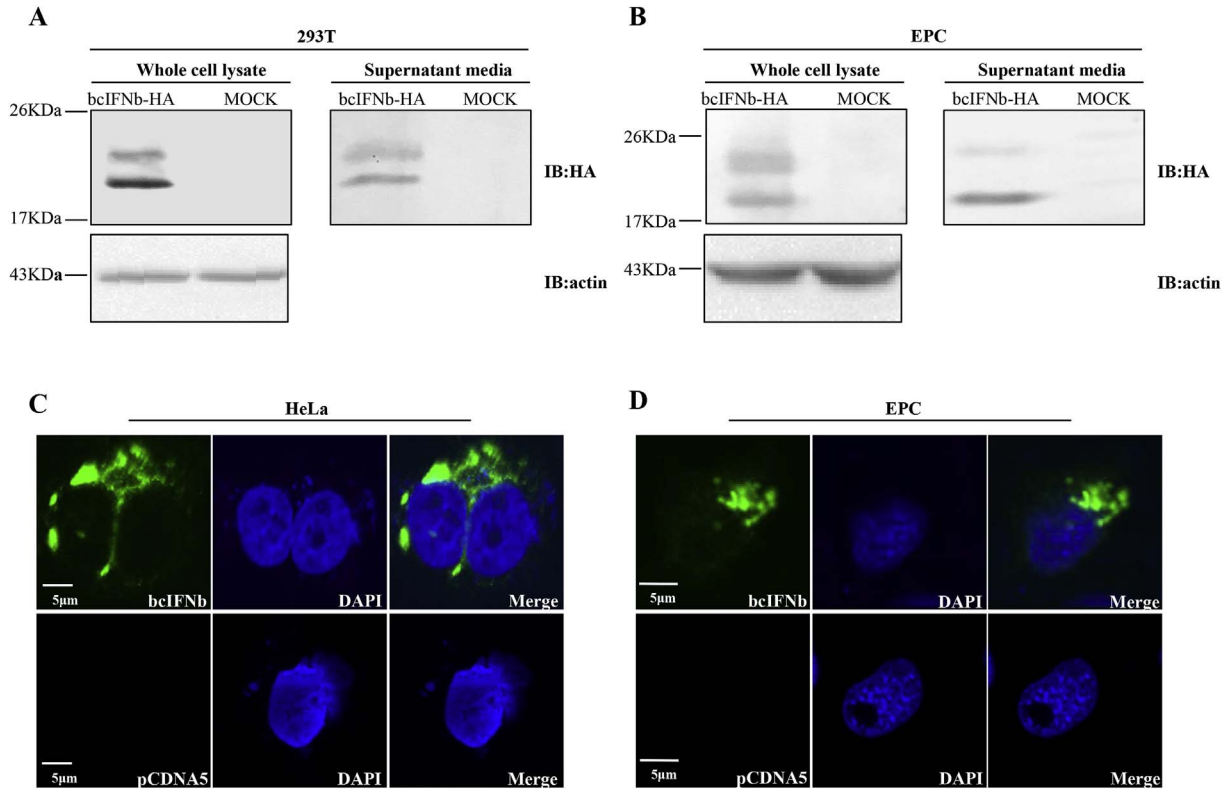

Fig. 3. Protein expression of bcIFNb.

(A and B): HEK293T cells (A) and EPC cells (B) were transfected with indicated plasmids; and the whole cell lysate and supernatant media of the transfected cells were used for immunoblot (IB) as described in methods separately. (C and D): Both EPC cells and HeLa cells were transfected with bcIFNb or the empty vector separately and used for immunofluorescence staining according to the methods. bcIFNb (green) indicated intracellular expression of bcIFNb, DAPI (blue) indicated nucleus of EPC or HeLa cells; the bar stands for the scale of 5 μm bcIFNb-HA: pcDNA5/FRT/TO-bcIFNb-HA; MOCK: HEK293T cells without transfection; pcDNA5: pcDNA5/FRT/TO-HA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

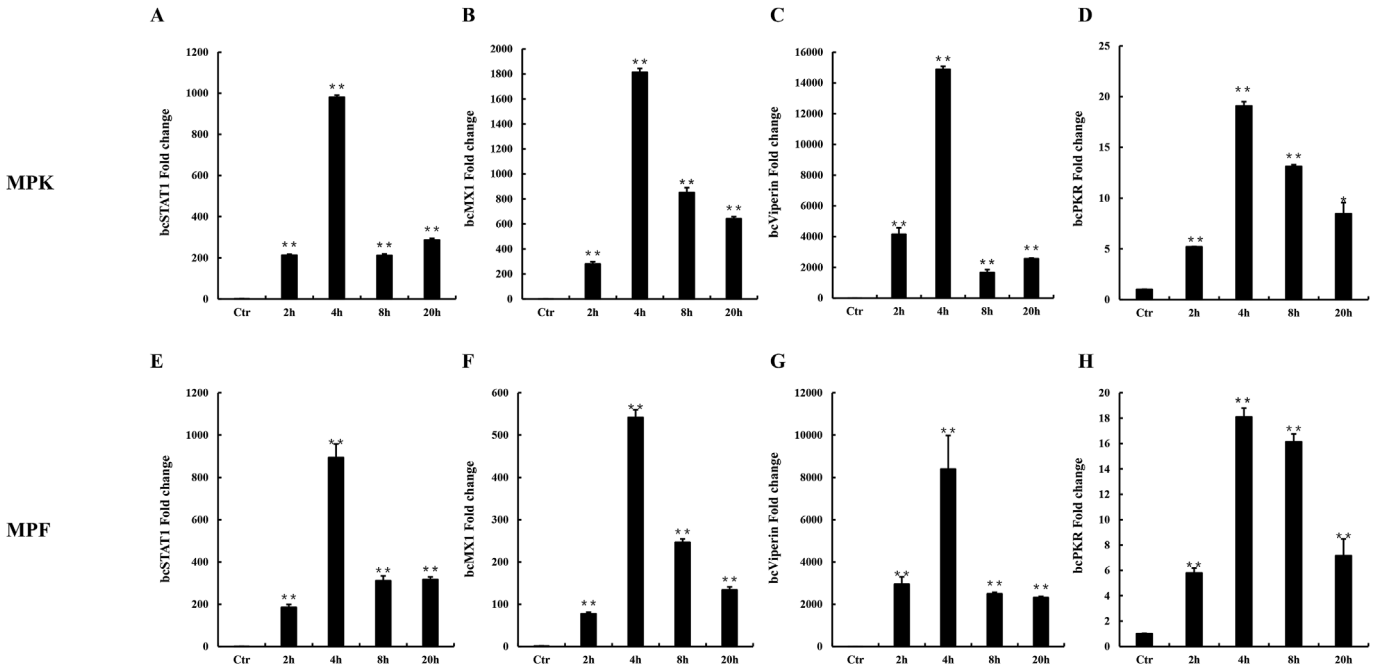

Fig. 4. bcIFNb induces the expression of downstream gene and ISGs.

MPK cells (A–D) or MPF cells (E–H) in 6-well plate (2×10^6 cells/well) were treated with the bcIFNb-containing conditioned media (1.7 ng/μl). The cells were harvested at 0, 2, 4, 8 or 20 h post stimulation separately and used for RNA isolation. The relative downstream gene and ISGs mRNA level was examined by q-PCR. The concentration of bcIFNb in the media was 1.7 ng/μl, which was determined by ELISA. The cells without treatment were used as control and the relative mRNA level of the indicated gene in the control was defined as 1.

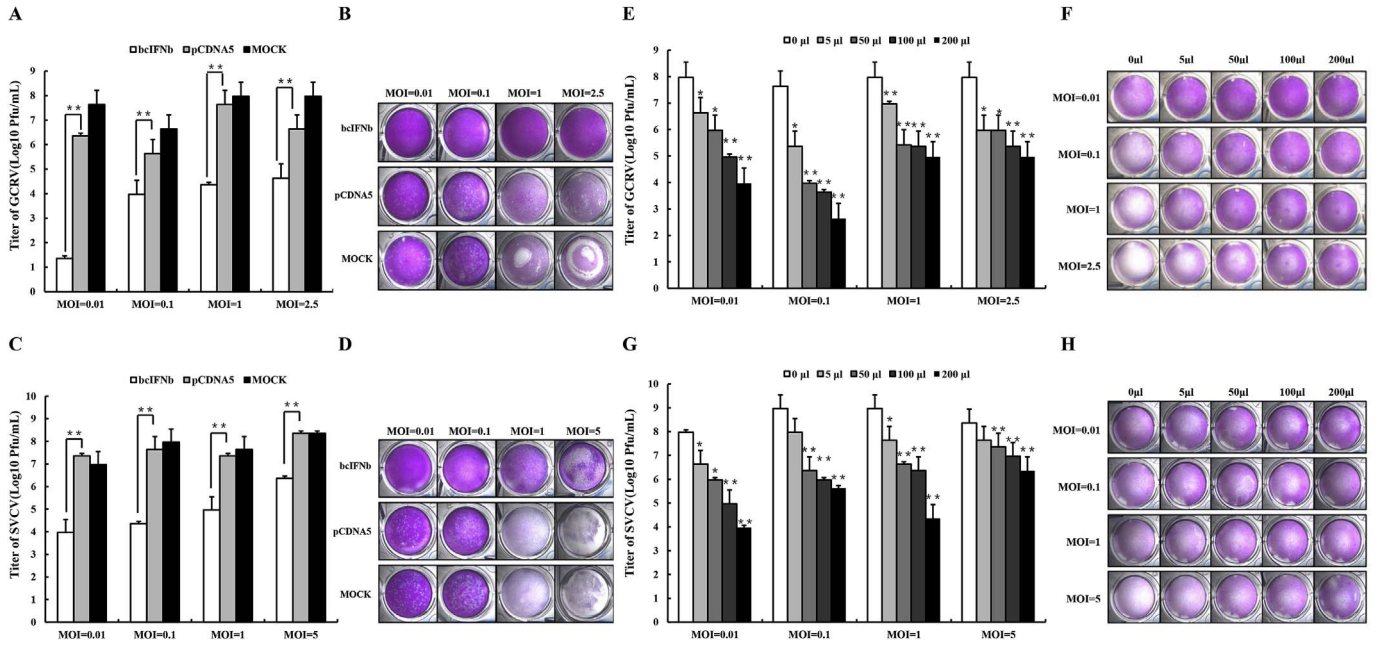

Fig. 5. Antiviral activity of bcIFNb.

(A–D):EPC cells in 24-wells plate were transfected with 300 ng of bcIFNb or the empty vector separately and infected with GCRV (A and B) and SVCV (C and D) at indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet (B and D) and virus titers in the supernatant media were examined by plaque assay at 24 h post-infection (A and C). MOCK: EPC cells without transfection; bcIFNb: pcDNA5/FRT/TO-bcIFNb-HA; pcDNA5: pcDNA5/FRT/TO-HA.

(E–H): EPC cells in 24-well plate were treated with indicated amount of the bcIFNb-containing conditioned media for 24 h before GCRV (E and F) or SVCV (G and H) infection separately. The concentration of bcIFNb in the media was 1.7 ng/μl, which was determined by ELISA. Virus titers in the supernatant media of EPC were determined by plaque assay (E and G) and the cell monolayers were stained with crystal violet (F and H). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bcIFNb-N92Q-HA (Fig. 6C), which demonstrated that bcIFNb was a glycoprotein with a single N-linked glycan chain on Asn of 92 site. Both the cytopathic effect (CPE) and the viral titer of the supernatant media of the EPC cells transfected with bcIFNb or bcIFNb-N92Q were obviously decreased compared with those of the controls (Fig. 6D and E). These data demonstrated that bcIFNb-N92Q possessed the similar antiviral ability against both SVCV and GCRV to that of wild type bcIFNb, which demonstrated that N-linked glycosylation was not crucial for the antiviral property of this fish cytokine.

4. Discussion

Teleost fishes possessed both innate and adaptive immune systems, however, they depended more on their innate immunity to defend themselves against the invasion of pathogenic microbes [27–29]. IFNs of teleost fishes functioned as the key components in both innate immunity and adaptive immunity liking their mammalian counterparts, which are the small natural proteins induce-expressed by many stimuli, such as virus and bacteria [27]. These cytokines are able to induce the expression of a variety of antiviral genes, including myxovirus resistance (MX), protein kinase R (PKR), viperin, and IFN-stimulated gene 15 (ISG15), thus leading to an enhanced antiviral state of host cells [30–32].

In this paper, the expression, subcellular location, antiviral activity and post-translational modification of bcIFNb had been characterized, which was classified into group II IFN-Is because of its four cysteine-containing sequences in the mature peptide. Type I IFNs of teleost fishes presented differential expression patterns [1]. In our previous study, the transcription of *bcIFNa* gene could not be detected through semi-quantitative RT-PCR in most selected tissues of black carp under healthy condition although the obviously increased bcIFNa mRNA level was detected after viral infection [20]; in this paper, the transcription of *bcIFNb* gene was detected through q-PCR in most selected tissues of black carp without virus challenge (Fig. 2A). However, this might be explained by the difference of the sensitivity of the two methods. bcIFNb showed strong antiviral activity against both GCRV and SVCV in EPC cells transfected with bcIFNb, however, it was interesting that the mRNA level of bcIFNb decreased in spleen, intestine and gill of black carp post SVCV injection. And the mechanism behind the high basal level of bcIFNb mRNA in these tissues under healthy condition was deserved to be further explored (Fig. 2A).

Teleost group II IFN-Is possessed strong antiviral activity during host innate immune response initiated by virus invasion. In zebrafish, IFNø2 and IFNø 3, which belong to group II IFN, induced the rapid and transient expression of antiviral genes and displayed antiviral activity in the early stages viral infections against SVCV [23]. In salmon, the pretreatment of TO cells with rIFNb or rIFNc led to strong inhibition of the replication of IPNV and salmon α virus (SAV) [21,30,33]. In this paper, bcIFNb induced the expression of ISGs and showed the similar antiviral activity against GCRV and SVCV like those in zebrafish and salmon (Figs. 4 and 5). IFN3 of rainbow trout was classified into group II IFN and its transcription was up-regulated after stimulation with poly (I:C) in host cells, however, this group II rtIFN was poor in antiviral responses [16]. In this study, bcIFNb mRNA level was up-regulated by the treatment of LPS, which implied this fish cytokine was recruited into the innate immune response against the invasion of pathogenic bacteria (Fig. 2B).

Glycosylation is one of the post-translational modifications of eukaryotic cells, which serves various functions such as to help protein folding correctly [34]. N-linked glycosylation is the most common glycosylation type, in which the glycan chain is added to the asparagine in the conserved motif of N-X-S/T. The asparagine of 92 site of bcIFNb is in the conserved domain of N-linked glycosylation and two bands (2–3 kDa different in molecular weight) have been detected through IB, which suggests that bcIFNb is a glycoprotein (Fig. 3A). The glycosidase digestion verified that bcIFNb is modified with N-linked glycosylation and the glycan chain is attached to the asparagine of 92, which was determined by the analysis of mutant N92Q (Fig. 5B and C). Several studies showed that N-linked glycosylation did not affect the antiviral property of fish IFNs, such as IFNs from Atlantic salmon, black carp and

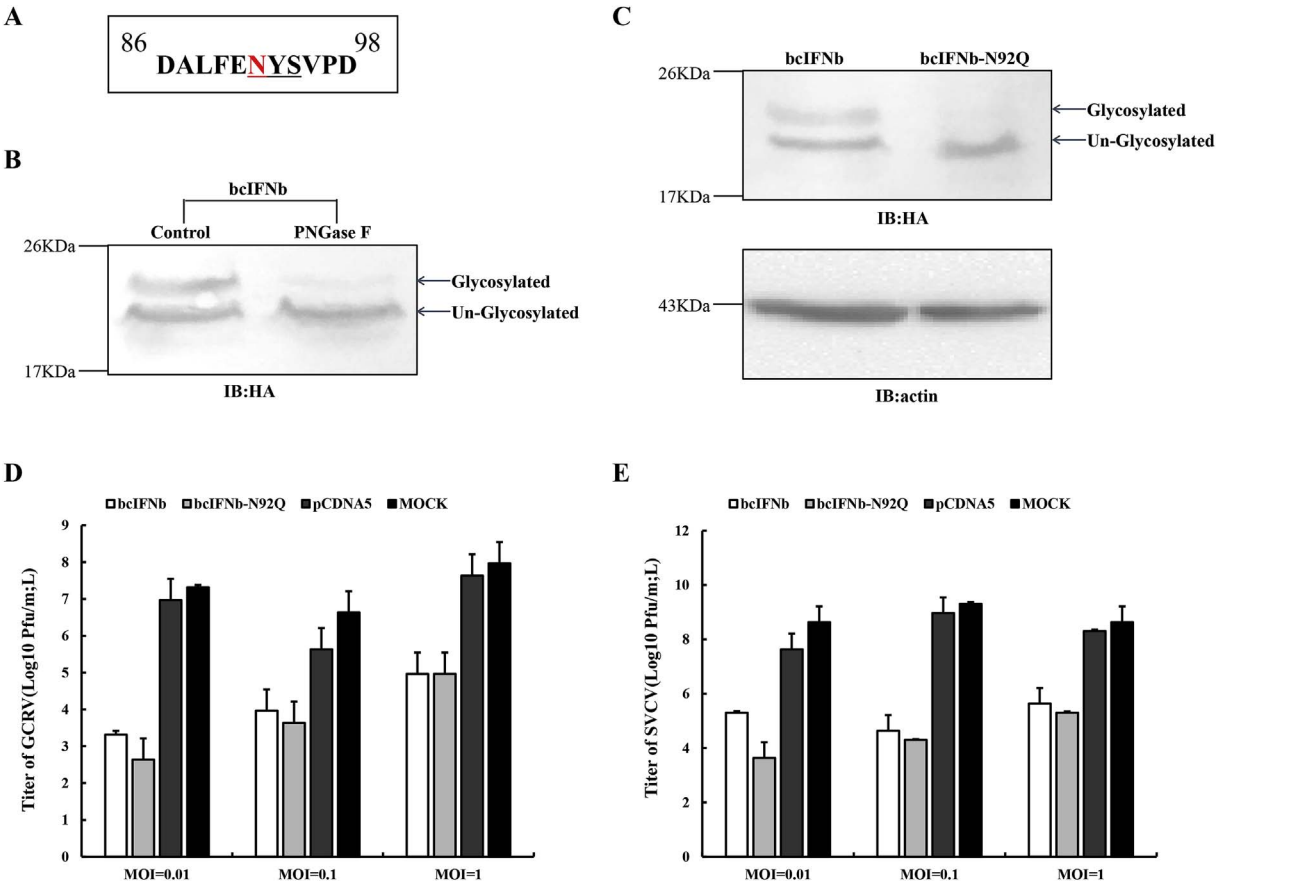

Fig. 6. bcIFNb possesses N-linked glycosylation.

(A): N-X-S/T consensus sequence (underlined) for N-linked glycosylation in bcIFNb. (B): Glycosidase digestion of bcIFNb: HEK293T cells were transfected with bcIFNb and the whole cell lysate was digested with PGNase F or the enzyme buffer (as control).

(C):HEK293T cells were transfected with bcIFNb, bcIFNb-N92Q or the empty vector separately and the whole cell lysate was applied to immunoblot assay. IB: immunoblot.

(D and E): EPC cells in 24-wells plate were transfected with bcIFNb, bcIFNb-N92Q or the empty vector separately and infected with GCRV (D) or SVCV (E) at indicated MOIs at 24 h post transfection. The virus titers in the supernatant media were determined by plaque assay at 24 h post-infection.

MOCK: cells without transfection; pcDNA5:pcDNA5/FRT/TO-HA; bcIFNb: pcDNA5/FRT/TO-bcIFNb-HA; bcIFNb-N92Q: pcDNA5/FRT/TO-bcIFNb-N92Q-HA.

triploid fish [8,15,20]. The data of viral titer showed that wild type bcIFNb and un-glycosylated mutant bcIFNb-N92Q possessed similar ability against SVCV and GCRV (Fig. 6), which demonstrated that N-linked glycosylation of bcIFNb did not contribute directly to its antiviral property. However, the functional mechanism of glycosylation of this fish antiviral cytokine needs further exploration.

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