Fish & Shellfish Immunology 46 (2015) 477–485

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

IFNa of black carp is an antiviral cytokine modified with N-linked glycosylation

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ARTICLE INFO

Article history:
Received 30 May 2015
Received in revised form
17 July 2015
Accepted 21 July 2015
Available online 22 July 2015

Keywords:
Black carp
IFN
Innate immunity
SVCV

ABSTRACT

Type I interferons (IFNs) play an important role in the antiviral immune response in teleost fish. In this study, one type I interferon (bcIFNa) of black carp (*Mylopharyngodon piceus*) has been cloned and characterized. The full-length cDNA of bcIFNa gene consists of 783 nucleotides and the predicted bcIFNa protein contains 185 amino acids. Semi-quantitative RT-PCR analysis demonstrated that bcIFNa mRNA transcription level in all the selected tissues of black carp was greatly increased at 33 h post spring viremia of carp virus (SVCV) infection. The protein of bcIFNa could be detected in both the whole cell lysate and the supernatant media of HEK293T cells transfected with plasmids expressing bcIFNa through immunoblot assay. EPC cells showed greatly increased antiviral ability when the cells were treated with the bcIFNa-containing conditioned media for 24 h before SVCV infection. Mass spectrum assay and glycosidase digestion analysis determined that bcIFNa is modified with N-linked glycosylation, which occurs on the Asn (N) of 38 site of this cytokine. The un-glycosylated mutant bcIFNa-N38Q could be secreted out of the cell and showed the similar antiviral ability against SVCV as that of wild type bcIFNa, which suggested that N-linked glycosylation does not contribute directly to the antiviral property of this fish cytokine.

1. Introduction

Interferons (IFNs) are cytokines characterized with antiviral property, which are induce-expressed in host cells after viral infection. IFNs play important roles in both innate and adaptive immune responses in the host [1–3]. For innate immune system, the invade virus or viral components were detected by surface viral sensor such as toll like receptors (TLRs) or cytosolic viral sensor such as RIG-I like receptors (RLRs) [4–7]. TLRs and RLRs triggered down stream signaling after association with viral component and activated transcription factors like NF-κB and IRF3/IRF7, which transferred into nuclear and initiated the transcription of IFNs [8–10]. Secreted IFNs stimulate surrounding target cells to initiate the transcription of bulk of interferon stimulated genes (ISGs) such as Mx and veperin, which initiate host cell antiviral innate immune response [11–14]. Recent study showed that some intracellular IFN functions as antiviral cytokine through intracellular IFN receptor [15].

Telelost IFN genes have been studied among model fish and industrial species in recent years, such as zebrafish (*D. rerio*), catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), grass carp (*C. idella*), crucian carp (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), salmon (*Salmo salar* L.), fugu (*Takifugu rubripes*) and sea bass (*Dicentrarchus labrax*) [16–24]. Different types of IFNs from different fish species showed different antiviral ability *in vitro* and *in vivo*, which demonstrated that the fish IFNs possessed the diversity just as their mammalian homologues [25–29]. Post-translational modifications such as glycosylation were found in several fish IFNs although the mechanisms of these modifications were not deeply discussed [3,30].

Black carp (Mylopharyngodon piceus) is one of “four famous domestic fishes” in China and is the most highly esteemed and expensive food fish among the four domestic fishes. There are few reports about the immunity study on black carp although this industrial important species is subjected to bulk of pathogens such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV).

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http://dx.doi.org/10.1016/j.fsi.2015.07.020
1050-4648/

In our previous study, the mitochondria antiviral signaling protein of black carp (bcMAVS) was cloned and characterized. The transcription of zebrafish IFN and EPC IFN were severely activated when bcMAVS was overexpressed in tissue culture [31], which implies that bcMAVS functions as an antiviral protein like its mammalian homologue through activating IFNs of black carp. In this manuscript, one type I interferon of black carp, named as bcIFNa, has been cloned and characterized, which is modified with N-linked glycosylation on the asparagine of 38 site. EPC cells treated with conditioned media from HEK293T cells transfected with plasmid expressing bcIFNa showed much more increased antiviral ability against SVCV. The un-glycosylated bcIFNa mutant (N38Q) showed similar antiviral ability against SVCV in plaque assay, which suggested that the N-linked glycosylation of bcIFNa does not contribute directly to the antiviral property of this fish cytokine.

2. Materials and methods

2.1. Cells, plasmids and virus

HEK293T cells and EPC cells were kept in the lab and cultured at 37 °C and 25 °C accordingly. The cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

The recombinant expression plasmid of pcDNA5/FRT/TO-HA-bcIFNa was constructed by inserting the open reading frame (ORF) of bcIFNa with an HA tag at its N-terminus into pcDNA5/FRT/TO (Invitrogen); for pcDNA5/FRT/TO-bcIFNa-HA, HA tag was fused at the C-terminus accordingly. The glycosylation mutant pcDNA5/FRT/TO-bcIFNa-(N38Q)-HA was generated as previously [32], in which asparagine (N) of amino acid site 38 was mutated into glutamine (Q).

Spring viremia of carp virus (SVCV) was propagated in EPC cells at 25 °C in the presence of 2% FBS and virus titer was determined by plaque assay in EPC cells as previously [33].

2.2. Cloning of bcIFNa cDNA

Degenerate Primers (Table 1) were designed to amplify the cDNA of bcIFNa basing on the conserved domains of type I IFNs of *C. auratus* (AY452069), *Danio rerio* (AJ544821), *Ctenopharyngodon idella* (DQ357216) and *C. carpio* (GQ168341). Total RNA was isolated from the liver of black carp by using TRIzol® reagent (Takara), which was injected intraperitoneally with SVCV (2.43×10^6 pfu/fish) and sacrificed at 33 h post injection. First-strand cDNA was synthesized by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. Rapid amplification of cDNA ends (RACE) was performed to obtain 5′UTR (untranslated region) and 3′ UTR of bcIFNa cDNA by using 5′ Full RACE Kit and 3′ Full RACE kite separately (TaKaRa). The full-length cDNA of bcIFNa was cloned into pMD18-T vector and sequenced by Invitrogen (Shanghai).

2.3. Semi-quantitative RT-PCR of bcIFNa

Black carps of six months (weight of 20 g) were injected with SVCV (2.43×10^6 pfu/fish) or sterile PBS (used as control) separately and cultured at 25 °C. The injected black carps were sacrificed at 33 h post injection and total RNA was isolated from tissues of gill, kidney, heart, intestine, liver, muscle, skin and spleen independently. The primers of bc-320-FULL-HA-C1-F1 and bc-320-FULL-HA-C1-R1 (Table 1) were used for the detection of bcIFNa mRNA transcription in the above tissues. The semi-quantitative RT-PCR program was: 94 °C for 5 min, then 30 cycles of 94 °C/30 s, 58 °C/30 s and 72 °C/30 s, finally extension at 72 °C for 5 min.

2.4. Immunoblotting

HEK293T cells in six-well plate were transfected with FRT/TO-HA-bcIFNa, FRT/TO-bcIFNa-HA or the empty vector separately and the transfected cells were harvested at 48 h post-transfection. The whole cell lysate and the supernatant media (20 ul from 2 ml for each sample) were applied to immunoblot (IB) separately as previously reported [34]. Briefly, the proteins were isolated by 12% SDS-PAGE and the transferred membrane was probed with anti-HA antibody (1:2000; Sigma), then followed by incubation with the goat-anti mouse secondary antibody (1:5000, Sigma), bcIFNa was visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.5. Glycosidase digestion

HEK293T cells were transfected with FRT/TO-bcIFNa-HA and the transfected cells were harvested at 48 h post-transfection. The whole cell lysate (WCL) was divided into two aliquots; one was

Table 1
Primers used in the study.

Primer name		Sequence (5′→3′)	Amplification length (nt) and primer information
F2		GTGGAGGA (C/T)CAGGTGA	ORF(558 bp)
R2		GCGATGATGTCCATCCTC	
5′GSP1		GACTCCTTATGTGATGGCTTGTG	5′UTR 1st PCR
5′Race Outer primer		CATGGCTACATGCTGACAGCCTA	
5′GSP2		CAAGAAACTTCACCTGGTCCTC	5′UTR 2nd PCR
5′RACE Inner primer		CGCGGATCCACAGCCTACTGATGATCAGTCGATG	
3′GSP1		CCACAAGCCATCACATAAGG	3′UTR 1st PCR
UPM	Long primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
	Short primer	CTAATACGACTCACTATAGGGC	
3′GSP2		ATCACCTTCAGAGGATGGAC	3′UTR 2nd PCR
NUP1		AAGCAGTGGTATCAACGCAGAGT	
M13-F		GGAAACAGCTATGACCATGATTAC	
M13-R		CGACGTTGTAAAACGACGGCCAGT	
bc-320-FULL-HA-C1-F1		ACTGACGGTACCGCCACCATGAATACAAAGATGAAAACTC	Expression construct
bc-320-FULL-HA-C1-R1		ACTGACCTCGAGTCGTCTGTTGGCAATGCTTGCAATGAT	RT-PCR
CMV-F		CGCAAATGGGCGGTAGGCGTG	
BGH-R		TAGAAGGCACAGTCGAGG	
Fish-actin-F		CATGTTCGAGACCTT	RT-PCR
Fish-actin-R		AGGCAGCTCATAGCT	