Fish & Shellfish Immunology 44 (2015) 88–99

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Isolation and expression of grass carp toll-like receptor 5a (CiTLR5a) and 5b (CiTLR5b) gene involved in the response to flagellin stimulation and grass carp reovirus infection

Yao Jiang [a, c, 1], Libo He [a, 1], Changsong Ju [a, b], Yongyan Pei [a, c], Myonghuan Ji [b], Yongming Li [a], Lanjie Liao [a], Songhun Jang [a, b], Zuoyan Zhu [a], Yaping Wang [a, *]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
[b] Department of Zoology, College of Life Sciences, Kim Il Song University, Pyongyang, Democratic People's Republic of Korea
[c] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form
25 January 2015
Accepted 25 January 2015
Available online 7 February 2015

Keywords:
Grass carp
Toll-like receptor 5
Flagellin
GCRV

ABSTRACT

Toll-like receptor 5 (TLR5), a member of Toll-like receptors (TLRs) family and is responsible for the bacterial flagellin recognition in vertebrates, play an important role in innate immunity. In the study, two TLR5 genes of grass carp (*Ctenopharyngodon idellus*), named CiTLR5a and CiTLR5b, were cloned and analyzed. Both CiTLR5a and CiTLR5b are typical TLR proteins, including LRR motif, transmembrane region and TIR domain. The full-length cDNA of CiTLR5a is 3054 bp long, with a 2646 bp open reading frame (ORF), 78 bp 5′ untranslated regions (UTR), and 330 bp 3′ UTR. The full-length cDNA of CiTLR5b is 3326 bp, with a 2627 bp ORF, 95 bp 5′ UTR, and 594 bp 3′ UTR. Phylogenetic analysis showed that CiTLR5a and CiTLR5b were closed to the TLR5 of *cirrhinus mrigala*, *cyprinus_carpio*, and *danio rerio*. Subcellular localization indicated that CiTLR5a and CiTLR5b shared similar localization pattern and may locate in the plasma membrane of transfected cells. Real-time quantitative PCR revealed CiTLR5a and CiTLR5b were constitutively expressed in all examined tissues, whereas the highest expressed tissue differed. Following exposure to flagellin and GCRV, CiTLR5a and CiTLR5b were up-regulated significantly. Moreover, the downstream genes of TLR5 signal pathway such as MyD88, NF-κB, IRF7, IL-1β, and TNF-α also up-regulated significantly, whereas the IκB gene was down-regulated, suggesting that CiTLR5a and CiTLR5b involved in response to flagellin stimulation and GCRV infection. The results obtained in the study would provide a new insight for further understand the function of TLR5 in teleost fish.

1. Introduction

The grass carp (*Ctenopharyngodon idellus*) has been an important aquaculture species in China for over 60 years, accounting for more than 18% of total freshwater aquaculture production. The production of grass carp reached 478.2 million tons in 2012, making it the most highly consumed freshwater fish worldwide [1]. However, disease outbreaks are frequently and resulted in huge economic losses to the aquaculture of grass carp. The grass carp haemorrhagic disease, caused by the grass carp reovirus (GCRV), is one of the most serious diseases [2]. In the GCRV infected fish, gills and intestines are the organs that show haemorrhagic symptoms [3]. The liver, spleen, kidney, intestine, and muscle are susceptible to GCRV and had a higher number of viral RNA copies [4]. Nevertheless, no effective drugs or vaccines against GCRV were developed until now. Therefore, identification of grass carp genes that involved in innate immunity is important for antiviral drug development and fish breeding programs.

Toll-like receptors (TLRs) family is an important class of pattern recognition receptors (PRRs), which specifically recognize a series of highly conserved pathogenic microorganism structures, termed pathogen-associated molecular patterns (PAMPs) [5]. TLRs family is the best understand receptors and play an important role in the innate immunity of vertebrate and invertebrate [6]. TLRs are typical type I transmembrane proteins that included three major domains: tandem repeat leucine-rich repeat (LRR) motifs that identifies PAMPs, a transmembrane region, and an intracellular Toll/IL-1

* Corresponding author. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. Tel.: +86 27 68780081.
E-mail address: wangyp@ihb.ac.cn (Y. Wang).
[1] These authors contributed equally to the study.

http://dx.doi.org/10.1016/j.fsi.2015.01.024
1050-4648/

receptor domain (TIR) domain that responsible for signal transmission [7]. When the corresponding ligands were recognized by TLRs, the subsequent signaling pathway was activated [8]. The signaling pathway of TLRs was divided into two types: myeloid differentiation primary response protein 88 (MyD88)-dependent and MyD88-independent signaling pathway [9]. The MyD88-dependent signaling pathway induced inflammatory-cytokine production via NF-κB activation, whereas the MyD88-independent signaling pathway leaded the production of interferon inducible gene via interferon regulatory factor 3 (IRF3) [9].

Toll-like receptor 5 (TLR5), a member of TLRs family and is responsible for the bacterial flagellin recognition in vertebrates, played an important role in the host defense against bacterial pathogens [10]. After the flagellin was recognized by TLR5, NF-κB was activated through the MyD88-dependent signaling pathway, and some inflammatory-cytokines such as IL-1 and TNF-α were induced [11]. In mammalians, TLR5 was expressed in the surface of many kinds of cells, such as intestinal epithelial cells, dendritic cells, monocytes cells, and splenic macrophages and so on [12,13]. In teleost fish, two types of TLR5 were existed: the membrane form of TLR5 (TLR5M) and the soluble form of TLR5 (TLR5S). Until now, TLR5S was cloned from rainbow trout (*Onchorhynchus mikiss*) [14], atlantic salmon (*Salmo salar*) [15], catfish (*Ictalurus punctatus*) [16], Japanese flounder (*Paralichthys olivaceus*) [17], and gilthead seabream (*Sparus aurata*) [18]. However, in zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*), and Indian major carp (*Cirrhinus mrigala*), only TLR5M was found [19,20]. The results obtained in the above studies showed that TLR5 of teleost fish could also recognize flagellin of bacterial and activate the immunity response. However, it is remain unknown that whether other ligands could be recognized by TLR5 and the particular role of TLR5 in teleost fish was still unclear.

In the study, two TLR5 genes of grass carp (*C. idellus*), named CiTLR5a and CiTLR5b, were cloned and analyzed. The gene structure, tissue distribution, localization pattern, and the response to flagellin stimulation and GCRV infection were investigated. Moreover, the responses of the TLR5 downstream genes to flagellin stimulation and GCRV infection were also analyzed. Our study would provide a new insight for further understand the function of TLR5 in teleost fish.

2. Materials and methods

2.1. Ethical procedures

Animal experimental procedures were conducted under the institutional guidelines of Hubei Province. The protocol was approved by the committee of institute of hydrobiology, Chinese Academy of Sciences (CAS). All surgery was performed under eugenol anesthesia, and all efforts were made to minimize suffering.

2.2. Experiment fish, sample collection, and virus exposure

Healthy grass carp at three months old were used in the study. The grass carp, weighing about 2–4 g with an average length of 5 cm, were obtained from the Guanqiao Experimental Station, institute of hydrobiology, CAS and acclimatized in aerated freshwater at 28 °C for one week before processing. The fish were fed with commercial feed twice a day, and the water was exchanged daily. After no abnormal symptom was observed, the grass carp was subjected to further study.

The virus exposure was conducted by feeding as follows. Dead fish with apparent symptoms of GCRV infection were collected and pestled together with an equal volume of 0.75% saline water, and then mixed with an equal amount of commercial feed. The resulting feed mixture was used as the source virus. The experimental group of fish was fed with the feed mixture on the first day, then with commercial feed on the other days. The temperature was maintained at 26–28 °C throughout the experiment.

Five uninfected fish were selected and samples from the gill, spleen, liver, intestine, kidney, head kidney, heart, muscle, skin and brain were prepared. RNA from these tissues was prepared to analyze the tissue distribution of CiTLR5a and CiTLR5b. In addition, kidney, gill, head, spleen and intestine samples were isolated from five infected fish at 0–6 days post infection. RNA from these tissues was prepared to analyze the response of CiTLR5a and CiTLR5b and their downstream genes to GCRV infection.

2.3. Cloning the full-length cDNA of CiTLR5a and CiTLR5b

Total RNA was extracted from the tissues of healthy grass carp using Trizol reagent (Invitrogen, USA). The first-strand cDNA synthesis was carried out using DNase I (Promega, USA)-treated total RNA as a template and oligo (dT)-adaptor primer as the control for the reverse transcriptase (TOYOBO, Japan). Specific primers (Table 1) were designed according to the cDNA sequences of zebrafish TLR5a and TLR5b and the deduced cDNA sequences of grass carp TLR5a and TLR5b. PCR amplification was conducted using the above cDNA as a template and the following program: 94 °C for 2 min, 31 cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were gel purified, cloned into pMD18-T vector (Takara, Japan), transformed into DH5α competent cells. The positive clones were picked and sequenced in both directions. The coding sequences of CiTLR5a and CiTLR5b were obtained by sequence assembly and the 5′- and 3′-untranslated region (UTR) sequences were then obtained using a SMARTer™ RACE cDNA Amplification Kit (Invitrogen, USA) and a 5′ RACE System for Rapid Amplification of cDNA Ends kit (Clontech, USA).

Table 1
Primers used in the study (enzyme cleavage site is underlined).

Primers	Sequences (5′ to 3′)	Usage
T5a-F	TACCCCAACTGACTCTGAGAAG	Tlr5a cDNA cloning
T5a-R	TCTACAGGGATCTCACAATGGC	
T5aI-F	CTTGCTTTGCTCCCAGGTTCT	Tlr5a 3′-race
T5aI-R	GTCCAGGAATATGAGTACGTGG	Tlr5a 5′-race
T5b-F	GGCTGAAGGATTATTGAAGCGT	Tlr5b cDNA cloning
T5b-R	CAGCCCTATTGCTACATTTCC	
T5bI-F	CCCGCTGCCAGTGGATAAAGAC	Tlr5b 3′-race
T5bI-R	CGAGACCGTGGACTACTTCTG	Tlr5b 5′-race
pTLR5a-F	GGAGAATTCAATGGCAACAATACAC	pEGFP-5a
pTLR5a-R	TTAGGATCCCACTGCAGTGTCTG	
pTLR5b-F	TTCGAATTCAATGGGATTTACATTTAT	pEGFP-5b
pTLR5b-R	TCAGGATCCTACTGATGTGTTTGCA	
Qt5a-F	TGACGCAGCAAATGTTCAAGC	qPCR of Tlr5a
Qt5a-R	GAGAACCTGGGAGCAAAGCAA	
Qt5b-F	CATTATCTCATGTTTCCTATG	qPCR of Tlr5b
Qt5b-R	GTTGAAGATGTTAAGATTTTGC	
Qβ-actin-F	AGCCATCCTTCTTGGGTATG	qPCR of β-actin
Qβ-actin-R	GGTGGGGCGATGATCTTGAT	
QMyD-F	TGGAGGACTGTCGCCGAAATG	qPCR of MyD88
QMyD-R	TGTGGCCTCTGGACGAGTTTC	
QIκB-F	GGCAGATGTAAACGCAAAG	qPCR of IκB
QIκB-R	GCCGAAGGTCAGGTGGTA	
QNF-κBF	GGCAGATGTAAACGCAAAG	qPCR of NF-κB
QNF-κBR	GCCGAAGGTCAGGTGGTA	
QIRF7-F	GGTGGAAAGTGGGCGGTAT	qPCR of IRF7
QIRF7-R	TCGTTAGGGTGCTCGTTGA	
QTNF-α-F	CATCCATTTAACAGGTGCATAC	qPCR of TNF-α
QTNF-α-R	GCAGCAGATGTGGAAAGAGAC	
QIL-1β-F	GATTCGAAAGTTCGATTCAATCT	qPCR of IL-1β
QIL-1β-R	TTCAGTGACCTCCTTCAAGAC	