Fish and Shellfish Immunology 74 (2018) 509–516

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Complement component *Bf/C2b* gene mediates immune responses against *Aeromonas hydrophila* in grass carp *Ctenopharyngodon idella*

Yunfei Dang[a,b,1], Yubang Shen[a,c,d,1], Xiaoyan Xu[a], Shentong Wang[a], Xinzhan Meng[a], Meng Zhang[a], Liqun Lv[a], Rongquan Wang[e], Jiale Li[a,c,d,*]

[a] Key Laboratory of Freshwater Fishery Germplasm Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai, PR China
[b] Laboratory of Biochemistry and Molecular Biology, Ningbo University, Ningbo, PR China
[c] Shanghai Engineering Research Center of Aquaculture, Shanghai Ocean University, Shanghai, PR China
[d] National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, PR China
[e] Key Laboratory of Conventional Freshwater Fish Breeding, Health Culture Technology Germplasm Resources, Suzhou Shenhang Eco-technology Development Limited Company, Suzhou, PR China

ARTICLE INFO

Keywords:
Bf/C2b
Grass carp
Primary hepatic cell
Aeromonas hydrophila
Innate immunity

ABSTRACT

The complement system is a significant component of innate immunity. Here, we identified a *Bf/C2* homolog (*gcBf/C2b*) in grass carp. *gcBf/C2b* shares a high similarity with *Bf/C2b* counterparts in other teleosts. *gcBf/C2b* transcription was widely distributed in different tissues, induced by *Aeromonas hydrophila in vivo* and *in vitro*, and affected by lipopolysaccharide and flagellin stimulation *in vitro*. In cells over-expressing *gcBf/C2b*, transcript levels of all components except *gcC5* were significantly enhanced, and *gcBf/C2b*, *gcIL1β*, *gcTNF-α*, *gcIFN*, *gcCD59*, *gcC5aR1*, and *gcITGβ-2* were significantly upregulated after *A. hydrophila* challenge or stimulation with bacterial pathogen-associated molecular patterns (PAMPs). However, *gcBf/C2b* in interference cells down-regulated the transcript levels after *A. hydrophila* challenge, and *gcBf/C2b* induced NF-κB signaling. These findings indicate the vital role of *gcBf/C2b* in innate immunity in grass carp.

1. Introduction

The complement system is an essential humoral system and a significant component of innate immunity, forming three pathways: the classical, alternative, and lectin pathways [1]. Almost all complement components exist as inactive precursor proteins under normal physiological conditions in the serum. The components are activated when the initiators interact with the activators [2]. Although the initiator of each pathway is different, complement component 2 (C2) is a principal complement component [3,4]. In the classical and lectin pathways, the C3-convertase complex protein (C4b2b) formed by C2b and C4b produces the protein fragments C3a and C3b by cleaving C3. Then, C3b combines with C4b2b to form C5 convertase (C4b2b3b) [5]. C5 is cleaved into C5a and C5b by the C5-convertase complex protein. Finally, C5b enriches C6, C7, C8, and C9 to form the membrane attack complex (MAC), which lyses bacterial cells [6].

Many studies have been conducted on C2 in mammals [7,8]. It is associated with age-related macular degeneration [9]. Moreover, inflammation response in humans is associated with genetic variations in *C2* [10]. However, there are few studies on *Bf/C2b* in teleosts. Although we cloned and characterized *gcBf/C2a* and *gcBf/C2b*, and determined the expression patterns in juvenile and infected tissues in previous study [3], its functions in the complement system has not been illustrated.

The grass carp (*Ctenopharyngodon idella*) is an economic freshwater food resource in Asia, because of its excellent growth performance, delicious meat, and strong stress resistance [11]. However, its production is greatly reduced by bacterial septicemia caused mainly by *Aeromonas hydrophila*, leading to a high mortality. Thus, a better understanding of the innate immune response in grass carp could accelerate disease-resistant breeding [12]. In this study, we used the sequence of *gcBf/C2b* which was obtained from transcriptome data to analyze its amino acid sequence. The distribution profile of *gcBf/C2b* in the tissues of grass carp was determined. In addition, the time-dependent expression pattern of *gcBf/C2b* post bacterial challenge was analyzed both *in vitro* and *in vivo*. Besides, mRNA abundance of the lectin pathway components was evaluated in *gcBf/C2b* over-expressed primary hepatic cells. Moreover, expression levels of immune-related genes were examined after *A. hydrophila* challenge or stimulation with bacterial pathogen-associated molecular patterns (PAMPs) in *gcBf/C2b*

* Corresponding author. College of Fisheries and Life Science, Shanghai Ocean University, 201306, Shanghai, PR China.
E-mail address: jlli2009@126.com (J. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2018.01.030
Received 25 November 2017; Received in revised form 13 January 2018; Accepted 16 January 2018

over-expressed and interferential primary hepatic cells. The interaction between *gcBf/C2b* and the NF-κB signal pathway was studied. Our results provide insight into a crucial molecule of the complement system during innate immune response in grass carp.

2. Materials and methods

2.1. Ethics statement

In this study, all the experiments with fish were conducted in accordance with the guidelines on the care and use of animals for scientific purposes, set up by the Institutional Animal Care and Use Committee (IACUS) of Shanghai Ocean University, Shanghai, China. IACUS approved this study within the project "Breeding of Grass Carp" (approval number, SHOU-09-007).

2.2. Sequence analysis

The cDNA sequence of *gcBf/C2b* was obtained from our previously published data deposited in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/Traces/sra/; accession number, SRP060308). Primer Premier 5.0 software was used to predict the theoretical protein sequence. ExPASy (http://web.expasy.org/protparam/) was used to analyze the isoelectric point of the amino acid sequence. The open reading frame (ORF) was searched using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The homology of *gcBf/C2b* cDNA and theoretical protein sequences was analyzed using BLASTp and BLASTn (http://www.ncbi.nlm.nih.gov/) of NCBI. To predict the signal peptide, we used SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The domain architecture was analyzed using the SMART program (http://smart.eC2heidelberg.de/). MEGA 5 program was used to construct a phylogenetic tree; the reliability analysis was conducted with 1000 bootstrap replicates on the basis of the deduced amino acid sequences. cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) was used to predict the nuclear localization signal.

2.3. Fish infection and sample collection

The grass carps (24 ± 2.2 g, n = 180) were obtained from Wujiang National Farm of Chinese Four Family Carps, Jiangsu Province, China. The fish were maintained in a sterilized circulating water system (28 ± 0.1 °C) that was inflated several days before use. The grass carps were fed three times (8:00 a.m., 12:00 p.m., and 5:00 p.m.) per day with 3% of the total biomass. After two weeks, the fish were randomly separated into four tanks (45 individuals per tank); two of the tanks were set as the control group, and the others as the experimental group.

Six fish (three from each control tank) were selected for random sampling. We used tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) to euthanize the fish, which were maintained on ice before tissue collection. Blood and 11 tissues (gill, liver, spleen, intestine, trunk kidney, head kidney, heart, skin, muscle, brain, and fin) were sampled for the *gcBf/C2b* distribution profile analysis. Furthermore, before the experiment, a pre-experiment was performed to evaluate the medial lethal concentration (LC_{50}) of *A. hydrophila* (AH10; Aquatic Pathogen Collection Center of Ministry of Agriculture, China). The fish were administered an intra-peritoneal (100 μL) injection of 1 × phosphate-buffered saline (PBS) with or without *A. hydrophila* at a dose of 1.2×10^7 CFU/mL. For the time-dependent expression profile analysis of *gcBf/C2b*, immune-related tissues (from the gill, liver, spleen, intestine, trunk kidney, and head kidney) were collected from six randomly selected fish (three from each experimental group) at 0, 4, 8, 12, 24, 48, and 72 h after bacterial infection. All the samples were immediately frozen in liquid nitrogen and then stored at −80 °C until total RNA extraction.

2.4. Primary hepatic cell culture

Liver tissues were sampled from three one-year-old grass carps by using sterile scissors and forceps at a laminar flow cabinet. Then, the tissues in the sterile culture dish were rinsed three times with DPBS (Gibco) and 3% Antibiotic-Antimycotic 100 × (Gibco) to eliminate impurities. The samples were cut into pieces (about 1 mm^3) under sterile conditions and suspended in DPBS. Then, the suspension was centrifuged for 20 min at 24 °C and 60 g to remove the blood cells and fat tissues. The tissue fragments were digested with trypsin (0.25% with EDTA; Sigma, USA) for 25 min at 27 °C in a tissue culture incubator, and 10% heat-inactivated fetal bovine serum (HI FBS; Thermo Fisher, US) was used to stop the digestion. Then, the solution was centrifuged at 800 rpm for 5 min, and the supernatant was removed. Subsequently, the tissue fragments and digested cells were re-suspended and transferred to six-well plates supplemented with Medium 199 (M199; Gibco), 20% HI FBS, and 3% Antibiotic-Antimycotic 100 × . The plates were stored at 28 °C in a 5% CO_2 humidified incubator. After five days, the cells were washed three times with DPBS and digested with trypsin for about 1 min at 37 °C. After centrifugation (24 °C and 60 g), the cells were re-suspended in M199 and 20% HI FBS and seeded into six-well plates at a final concentration of 2×10^6 cells/mL.

2.5. Expression profiles of gcBf/C2b after A. hydrophila challenge and flagellin and lipopolysaccharide stimulation in the hepatic cells

For the time-dependent expression profile of *gcBf/C2b in vitro*, the cells in the six-well plates were washed three times with DPBS, and 500 μL of M199 and 20% HI FBS were added. The cells were then infected with *A. hydrophila*, FLA-ST (Sigma-Aldrich; flagellin purified from *Salmonella*), or lipopolysaccharide (LPS; Sigma-Aldrich; purified from *Salmonella*) at final concentrations of 1×10^4 CFU/mL, 15 ng/mL, and 15 μg/mL, respectively. Meanwhile, two plates were treated with PBS or sterile water as the control and blank groups, respectively. All the groups from the three wells were collected in parallel. The groups challenged with *A. hydrophila* were sampled at 4, 8, 12, and 16 h; the FLA-ST and LPS groups were sampled at 12, 24, 36, and 48 h and treated with 500 μL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for 5 min.

2.6. Over-expressed plasmid construction and RNAi efficiency evaluation

To construct the over-expressed-vector of *gcBf/C2b*, the ORF was amplified using primers *gcBf/C2bF-Hind*III/*gcBf/C2bR-Sac*II (Table S1). Then, the ORF was inserted into pEGFP-N1 (Clontech) between the *Hind*III and *Sac*II sites to obtain pEGFP-N1-gcBf/C2b (pBf/C2b). The over-expressed recombined plasmid was validated by sequencing.

The *gcBf/C2b*-specific siRNA oligonucleotide was synthesized by Bioneer Corporation (Daedeok-gu Daejeon, Korea), and the sequences are listed in Table S2. The primary cells in the six-well plates were transfected with 100 pmol of siRNA and HiPerFect Transfection Reagent (Qiagen, Germany), according to the manufacturer's instructions. The control group was treated with the same volume of negative control siRNA. After 48 h of cultivation, three parallel cells were washed three times with PBS and then treated with TRIzol reagent for 5 min. The efficiency of RNAi was checked using quantitative real-time (qRT)-PCR (Fig. S1).

2.7. Expression trends of genes in the over-expressed/RNAi cells and after A. hydrophila infection and flagellin and LPS stimulation

The hepatic cells in the six-well culture plates (2×10^6 cells/well) were maintained in a constant temperature incubator, and then, the cells were washed three times with PBS at approximately 80% confluence. Two sets of cells were transfected with 2.4 μg of pBf/C2b or the control pEGFP-N1, and the other two sets of cells, with 100 pmol of