Developmental and Comparative Immunology 65 (2016) 91–97

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Identification and functional analysis of the toll-like receptor 20.2 gene in grass carp, *Ctenopharyngodon idella*

Wen-Ji Huang [a], Xiaomeng Yang [b], Yubang Shen [a], Xiao-Yan Xu [a], Lisen Li [a], Rongquan Wang [b], Jiale Li [a, *]

[a] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Education, Shanghai, 201306, China
[b] Key Laboratory of Conventional Freshwater Fish Breeding and Healthy Culture Technology Ministry of Agriculture, Suzhou Shenhang Eco-Technology Development Co., LTD, Suzhou, 215221, China

ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form
26 June 2016
Accepted 26 June 2016
Available online 28 June 2016

Keywords:
gctlr20.2
Aeromonas hydrophila
GCRV
Overexpression
Silencing
Inflammatory cytokines

ABSTRACT

We characterized and identified the cDNA sequence of Toll-like receptor *20.2* in *Ctenopharyngodon idella* (*gctlr20.2*); it consisted of 3197 bp, with an open reading frame of 2835 bp that encoded a 944 amino acid polypeptide. Relatively, high expression levels of *gctlr20.2* were observed in the spleen, head kidney, liver and brain tissues, with lower expression levels in the trunk kidney, intestine and heart tissues. *In vivo* and *in vitro*, after being challenged with *Aeromonas hydrophila* or grass carp reovirus (GCRV), *gctlr20.2* expression was induced in *C. idella* kidney cells stimulated with lipopolysaccharide, flagellin or polyinosinic-polycytidylic acid. Overexpression of *gctlr20.2* increased the expression of *il1β*, *il8* and *tnf-α*, but not *ifn*, and also increased the activity of the *nf-κB* signal pathway. Silencing, via siRNA-tlr20.2, inhibited *gctlr20.2* transcription by 65.7% and down-regulated the expression of inflammatory cytokine genes, but not *tnf-α*. This study increases understanding of the immune system in *C. idella*.

1. Introduction

Serious diseases occur in cultured and wild fish populations (Hedrick, 1998), raising the question of how wild fish populations protect themselves from disease? It is important to understand the relationships between hosts and their pathogens. The innate immune system is an essential and fundamental mechanism of defense for fish; it recognizes foreign substances, sending warning signals to allow defense against pathogen invasion (Magnadóttir, 2006). Many studies have shown that toll-like receptors (TLRs) are critical proteins that link innate and acquired immunity (Akira et al., 2001). TLRs are a type of germline-encoded pattern-recognition receptor (PRR), they can recognize the conserved molecular patterns of pathogens, called pathogen-associated molecular patterns (PAMPs), including mannan (a polysaccharide found in yeast cell walls), and lipopolysaccharides (LPS), lipopeptides, peptidoglycans (PGN) and teichoic acid (found in bacterial cell walls) (Kopp and Medzhitov, 2003). TLRs are type I transmembrane proteins that contain an extracellular domain composed of 20–30 leucine-rich repeats (LRRs), and are involved in the recognition of ligands. TLRs also have a transmembrane domain and an intracellular domain, the latter of which has a conservative structure, a Toll/IL-1 receptor (TIR) homologous domain, used for signal transduction (Kaisho and Akira, 2006; O'Neill and Bowie, 2007). TLRs can recognize PAMPs and might mediate the activation of pro-inflammatory pathways, mechanisms that allow hosts to protect themselves from pathogen invasions (Aderem and Ulevitch, 2000).

At least 20 types of TLRs have been identified among aquatic animals; some fish-specific TLRs identified include TLR18–20, TLR22–27, and soluble TLR5S (Wang et al., 2015). The transcription levels of *RbTLR21* and *Po-TLR21* changed by bacterial or viral stimulation (Gao et al., 2013; Priyathilaka et al., 2014). *gctlr18* expression was induced after PAMPs stimulation, *in vivo* and *in vitro*, and the transcription levels of immune-related genes also increased (Huang et al., 2015). In fish, *tlr21 and tlr22* expression is stimulated by bacterial or viral exposure (Lv et al., 2012; Wang et al., 2013a,b). These changes in fish-specific TLR levels may provide direct or indirect evidence of the regulation of particular TLRs, in the response to pathogen invasion.

The identification, subcellular localization and expression pattern after infection of *tlr20* has been studied in several fish,

* Corresponding author.
E-mail address: jlli2009@126.com (J. Li).

http://dx.doi.org/10.1016/j.dci.2016.06.019
0145-305X/

including *Carassius auratus* (GenBank: KT284363) (Zhang et al., 2015), *Cyprinus carpio* (GenBank: KF482527.1) (Pietretti et al., 2014), *Ictalurus punctatus* (GenBank: HQ677723.1) (Quiniou et al., 2013), and *Danio rerio* (Gene ID: 403140) (Pietretti et al., 2014). Lee et al. also found that there were four copies of *tlr20* in the *Salmo salar* genome (Lee et al., 2014). However, its functions have not been clearly elucidated. Grass carp (*Ctenopharyngodon idella*), the most important aquaculture species in China, is highly impaired with grass carp reovirus (GCRV; caused by *Aquareovirus* spp.) (Yan et al., 2015) and *Aeromonas hydrophila* (Liu et al., 2015). Understanding how grass carp defends itself against these diseases is very important. In the present study, the *tlr20* gene was cloned and characterized from grass carp, its gene expression patterns in various tissues were analyzed. In addition, we investigated the part of signal pathway of *tlr20* using overexpression and RNAi interference.

2. Materials and methods

2.1. In vivo exposure of Ctenopharyngodon idella

Three-year-old and two-year-old *C. idella* were obtained from Nanhui Fish Farm, Shanghai, China. The fish specimens weighed 2500 g and 200 g, on average, respectively. They were kept healthy and maintained at 26 °C until the experiment started. Total RNA from the blood, gill, muscle, liver, spleen, trunk kidney, intestine, head kidney, heart and brain of the healthy three-year-old grass carp (n = 3), were extracted by RNAiso Plus (Takara, Japan).

The two-year-old fish were divided into three groups; one group (n = 12) received an intraperitoneal inoculation of GCRV-JX01 (1.0×10^7 $TCID_{50}$ in 100 μL PBS); fish in another group (n = 12) were injected with *A. hydrophila* S2 (5.0×10^7 cells in 100 μL PBS); and the control (n = 12) fish received a corresponding volume of PBS. GCRV-JX01 and *A. hydrophila* were obtained from the Aquatic Pathogen Collection Center of the Ministry of Agriculture, China. After the fish had been infected for 4 h, 1 day, 3 days and 7 days, grass carp specimens were sacrificed from each treatment and their gill, liver, spleen and kidney tissues were collected. All samples were stored at −80 °C until total RNA extraction. The integrity of the RNA extracted was measured using a NanoDrop 2000C (Thermo Fisher Scientific, MA, USA) and the $OD_{260/280}$ values were 1.8–2.0.

2.2. Culture of Ctenopharyngodon idella kidney cells

C. idella kidney (CIK) cells, provided by the China Center for Type Culture Collection, were cultured in M199 medium (Gibco, China) supplemented with 10% heat inactivated Fetal Bovine Serum (Gibco, China). Cells were grown in a 5% CO_2 incubator maintained at 28 °C.

2.3. In vitro exposure of CIK cells to PAMPs, Aeromonas hydrophila S2 and GCRV

For the *in vitro* studies, the CIK cells (1×10^6 cells/mL final concentration) were incubated in six-well dishes (Thermo Fisher Scientific, China) for 24 h prior to *A. hydrophila* S2 or GCRV infection; doses of 1×10^2 cells and 1 MOI (multiplicity of infection, 1/cell), respectively, were added to the CIK cells, which were then collected at 6 h and 24 h. The cells collected were stimulated with purified flagellin from *Salmonella typhimurium* (FLA-ST), LPS (extracted from *Escherichia coli* and purified by phenol extraction) or polyinosinic-polycytidylic acid (poly(I:C)), all purchased from Sigma-Aldrich, at final concentrations of 10 ng/mL, 10 μg/mL and 5 μg/mL, respectively. In order to analyze the time-dependent expression of genes, the cells from three wells (per treatment) were harvested at 0, 6, 12, 24, and 48 h and treated with 1 mL RNAiso Plus (Takara, Japan) for 5 min. All treatments were analyzed in triplicate.

2.4. Cloning of full-length gctlr20.2 cDNA

The cDNA from a healthy *C. idella* spleen was used as the template for amplification of the *gctlr20.2* (grass carp TLR20) partial sequence, using the gene specific primers PSF1/R1 (Table 1), which were designed based on the conserved regions of TLR20a from *D. rerio* (Gene ID: BC163786.1) using Primer Premier 5. To obtain the full-length of *gctlr20.2* cDNA, a rapid amplification of cDNA ends (RACE) procedure was followed; the 5′ RACE and 3′ RACE were based on the manufacturer's specifications, with gene specific primers (F31 or F51) and adaptor primers (Table 1), and the SMART RACE cDNA Amplification Kit (Clontech, USA) was used.

2.5. Creation of overexpression plasmid and siRNA

To comprehensively analyze the functional characteristics of *gctlr20.2*, overexpression plasmids PTLR20.2 was constructed using the primers 20F-Xhoi and 20R-Bamhi inserted with full-length open reading frame (ORF) of *gctlr20.2* and linked to the PEGFP-C3 (Clontech, Japan) plasmid using T4 ligase (New England BioLabs, USA). They were identified using DNA sequencing by Mapbioo (Shanghai, China).

siRNA was designed and synthesized by Bioneer (Shanghai, China) to analyze interference of *gctlr20.2*. The siRNA sequence, named siRNA-tlr20.2, spanned from 1645 to 1664 bp of the ORF and was as follows: the sense (5′–3′) was CAC UCU UGA CAU CUG UAG U (dtdt) and the antisense (5′–3′) was A CUA CAG AUG UCA AGA GUG (dtdt). Corresponding volume of PBS was added into the control group.

2.6. gctlr20.2 overexpression and interference analysis

The function of *gctlr20.2 in vitro* was explored using overexpression: the recombinant plasmid PTLR20 (1.2 μg) was transfected using 4.5 μL Attractene Transfection Reagent (QIAGEN, Germany) into the CIK cells that were cultivated in six-well plates 24 h before transfection. 48 h post transfection, the CIK cells were detected and collected for RNA extraction; PEGFP-C3 was used as a control.

To analyze the function of *gctlr20.2* using interference, siRNA-tlr20.2 (75 pmol) was transfected into the prepared CIK cells in six-well dishes, using 3.5 μL HiPerFect Transfection Reagent (QIAGEN, Germany). After 24 h, the CIK cells were collected for RNA extraction, this allowed an analysis of the interference efficiency of siRNA-tlr20.2. The expression of the associated immune-related genes (*il-1β*, *il-8*, *tnf-α* and *ifn*), which are downstream effectors of the TLR signal pathway, was analyzed in the overexpression and interference experiments.

2.7. Luciferase reporter assays

CIK cells (2×10^5) were transfected with 100 ng NF-κB luciferase reporter plasmid (Promega, USA), 10 ng pRL-TK vector (Promega, USA) and 100 ng of either PTLR20 or PEGFP-C3. Cells were washed with PBS and lysed with lysis buffer (Promega, USA) 24 h after transfection. Firefly and Renilla luciferase activities were measured. The firefly luciferase activity was normalized to that of Renilla. The results were calculated from three independent replicates.