Developmental and Comparative Immunology 49 (2015) 138–148

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

LGP2 plays extensive roles in modulating innate immune responses in *Ctenopharyngodon idella* kidney (CIK) cells

Xiaohui Chen [a], Chunrong Yang [b], Jianguo Su [a,*], Youliang Rao [a], Tianle Gu [a]

[a] College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China
[b] College of Veterinary Medicine, Northwest A&F University, Yangling 712100, China

ARTICLE INFO

Article history:
Received 28 August 2014
Revised 25 October 2014
Accepted 25 October 2014
Available online 5 November 2014

Keywords:
Grass carp (*Ctenopharyngodon idella*)
LGP2
RLR pathway
Grass carp reovirus
CIK cells

ABSTRACT

LGP2 (laboratory of genetics and physiology 2), RIG-I (retinoic acid inducible gene-I) and MDA5 (melanoma differentiation associated gene 5) constitute the RLR (RIG-I-like receptor) family. LGP2 plays a pivotal role in modulating signaling of RIG-I and MDA5 in innate immune responses. In this study, three representative overexpression vectors were constructed and transfected into *C. idella* kidney (CIK) cell line to research functional characterizations of *CiLGP2* (*C. idella LGP2*). *CiLGP2* overexpression led to the induction of *CiRIG-I* transcripts. After GCRV challenge, *CiLGP2* enhanced *CiMDA5* and *CiIPS-1* to reinforce the immune response, however, impaired the expression of *CiRIG-I*. Meanwhile, antiviral activity assays showed that overexpression of *CiLGP2* or its domains could inhibit GCRV replication and protect cells from death. Besides, *CiLGP2* lingeringly induced *CiRIG-I* mRNA expression and inhibited *CiMDA5* transcripts post poly(I:C) simulation. As a result, *CiLGP2* suppressed the RLR-mediated signaling pathway against poly(I:C). Furthermore, *CiLGP2* played active roles in RLR signaling response to bacterial PAMPs (LPS and PGN) stimulation. *CiLGP2* altered the expression pattern of *CiIPS-1* after LPS treatment, while it significantly enhanced the RLR signaling pathway against PGN stimulation. These results collectively suggested that *CiLGP2* played a strikingly broad regulation in RLR mediated innate immune responses in *C. idella*, responding to not only the dsRNA virus or synthetic dsRNA but also bacterial PAMPs, which contribute to the understanding of *C. idella LGP2* and RLR signaling pathways. In addition, these results lay a foundation for the further functional mechanism research of *LGP2* in fishes.

1. Introduction

The innate immune system serves as the first and pivotal line in creature defense against pathogens. In immune surveillance, pattern recognition receptors (PRRs) are the innate immune sensors which play significant roles in recognizing pathogen associated molecular pattern (PAMPs) derived from invading pathogens (Kumar and Bot, 2013). These sensors mainly contain TLRs (Toll-like receptors), RLRs (RIG-I-like receptors), NLRs (NOD-like receptors) and CLRs (C-type lectin receptors). Among those, cytosolic RLRs can recognize viral nucleic acids and trigger the innate immune responses (Heine, 2011).

RLRs are a family of DExD/H box RNA helicase, comprising retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Loo and Gale, 2011). RIG-I and MDA5 share structural similarities including three distinct domains: tandem caspase activation and recruitment domains (CARD) in N-terminal region, a central DExD/H box RNA helicase domain with the capacity to hydrolyze ATP and to bind/unwind RNA, and a C-terminal repressor domain (RD) for PAMP recognition and binding (Saito et al., 2007; Yoneyama et al., 2004, 2005). RNA binding causes a conformational change which promotes oligomerization and allows interaction of CARD domains with the downstream adaptor protein IFN-β promoter stimulator 1 (IPS-1, also known as MAVS, VISA and CARDIF). IPS-1 is the sole adapter in both RIG-I and MDA5 signaling that mediates effective responses against a variety of RNA viruses (Kumar et al., 2006). It initiates a signaling cascade leading to activation of the interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB), which are needed to launch transcription of interferon-β (IFN-β) (Childs et al., 2012; Goodbourn and Randall, 2009; Levy et al., 2011). RIG-I can recognize short dsRNA (<1 kb) containing a 5′ triphosphate (Pichlmair et al., 2006; Schlee et al., 2009; Zhu et al., 2014), and MDA5 appears to be activated by long dsRNA and high-order RNA structure (Kato et al., 2008; Pichlmair et al., 2009). The third member, LGP2 (namely ATP-dependent RNA helicase DHX58), lacks the N-terminal CARDs and cannot activate IPS-1 (Loo and Gale, 2011; Yoneyama et al., 2005). However, the ability of LGP2 to recognize dsRNA allows it to modulate the signaling capacities of RIG-I and MDA5 (Reikine et al.,

* Corresponding author. College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China. Tel.: +86 29 87092139; fax: +86 29 87092164.
E-mail address: su.jianguo@gmail.com (J. Su).

http://dx.doi.org/10.1016/j.dci.2014.10.012
0145-305X/

2014). And evidences are accumulating that LGP2 can impact a wide range of cellular responses (Rodriguez et al., 2014).

Currently, the role of LGP2 as a negative or positive regulator of RIG-I or MDA5 signaling has been clarified on many species. Early study indicated that overexpression of LGP2 blocked IFN induction in response to NDV (Newcastle disease virus) and poly(I:C) (polyinosine–polycytidylic acid), while knockdown of LGP2 enhanced gene activation induced by NDV infection (Yoneyama et al., 2005). In addition, paramyxovirus V proteins interacted with LGP2 and cooperatively inhibited RIG-I-dependent interferon induction (Childs et al., 2012). Additionally, LGP2 downregulated IFN production during infection by seasonal influenza A viruses (Malur et al., 2012). On the contrary, mouse LGP2 has been found to be essential and positive for producing effective antiviral responses against many viruses that are recognized by RIG-I and MDA5, such as VSV (vesicular stomatitis virus), SeV (Sendai virus), JEV (Japanese encephalitis virus) and EMCV (encephalomyocarditis virus) (Satoh et al., 2010). Furthermore, some researches on antiviral function of LGP2 have been reported in teleost. For example, Japanese flounder LGP2 played an important role in the recognition of both viral ssRNA and dsRNA to induce the antiviral activity by the production of IFN stimulated proteins (Ohtani et al., 2010). And functional characterization of LGP2 in rainbow trout also suggested that LGP2 likely acted as a positive regulator for IFN production (Chang et al., 2011). In contrast, crucian carp LGP2 functioned as a negative regulator of both RIG-I and MDA5 in cytosolic dsRNA-induced IFN signaling (Sun et al., 2011). These results infer that fish LGP2 acts as distinct roles in different species.

As one of the important freshwater fish in China, grass carp (*Ctenopharyngodon idella*) seriously suffers from hemorrhagic disease caused by grass carp reovirus (GCRV). Roles that *C. idella* RLRs play in antiviral immune response to GCRV are worthy of study. Fortunately, *C. idella* RLRs have been identified and preliminarily examined in expression profiling to GCRV challenge *in vivo* or *in vitro* (Huang et al., 2010; Su et al., 2010; Yang et al., 2011). Meanwhile, three SNP loci of *CiLGP2* were significantly associated with the resistance/susceptibility to grass carp reovirus (GCRV) (Wan et al., 2013). CpG methylation in the 5′ flanking region of *LGP2* gene was no striking relevance on methylation with resistance against GCRV (Shang et al., 2014).

In this study, a series of overexpression vectors were constructed to research functional characterizations of *LGP2* in *C. idella*, including full-length *CiLGP2* and two domain containers. Then they were transfected into *C. idella* kidney (CIK) cell line to obtain steadily expressing recombinant proteins. The mRNA expressions of relevant genes (*CiRIG-I, CiMDA5* and *CiIPS-1*) were examined post GCRV and poly(I:C) challenges. *CiLGP2* transcripts were detected upon lipopolysaccharides (LPS) and peptidoglycan (PGN) stimulation in CIK cells transfected with overexpression vectors. In addition, the expression profiles of *CiRIG-I, CiMDA5* and *CiIPS-1* were tested in cells transfected with full-length *CiLGP2* vector after LPS and PGN treatment. The results set the foundation for the further functional mechanism exploration of *LGP2* in teleost.

2. Materials and methods

2.1. Cells, GCRV and PAMPs treatment, sampling

The CIK cell line, provided by China Center for Type Culture Collection, was derived from grass carp kidney (Zuo et al., 1986). CIK cell line was cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Biosource, USA), 100 IU/ml of penicillin (Sigma, USA) and 100 μg/ml of streptomycin sulfate (Sigma). The cells were incubated at 28 °C in 5% CO_2 humid atmosphere.

Table 1
Primers for the construction of vectors and qRT-PCR analyses.

Primer name	Sequence (5′→3′)	Application
LF990	TCAG*ggtacc*ACACAGACCTCGACGCTA	pLGP2/pDExD/H
LR991	TCAG*gggccc*TGTGTCAGTCGTCTAAGTCCA	pLGP2/pRD
LR1067	TCAG*gggccc***TCA**CACCGAGTAGACGCTGT	pDExD/H
LF1068	TCAG*ggtacc***ATG**CGGATTGAAGCCGAGAGA	pRD
LF215	CGTCTACTCGGTGGTGGCT	*CiLGP2*
LR216	AAACTCCCTGGGACTCATACTCT	qRT-PCR
EF125	CGCCAGTGTTGCCTTCGT	*EF1α*
ER126	CGCTCAATCTTCCATCCCTT	qRT-PCR
RF230	ACTACACTGAACACCTGCGGAA	*CiRIG-I*
RR231	GCATCTTTAGTGCGGGCG	qRT-PCR
MF150	CAGGAGCGACTCTTGGACTATG	*CiMDA5*
MR151	AAAGACGGTTTATTTGAATGGAAG	qRT-PCR
IF217	GACCGTAAGAAGTCAGCCTCC	*CiIPS-1*
IR218	CCTGAATAACTCTTGATAGCCCTC	qRT-PCR
VF146	CGAAAACCTACCAGTGGATAATG	*VP4*
VR147	CCAGCTAATACGCCAACGAC	qRT-PCR

Note: The nucleotides in bold mark the initiation codon or the termination codon. The nucleotides in italicized lowercase represent the site of restricted enzyme.

For viral infection, cells were infected with GCRV (097 strain, 3.63×10^7 $TCID_{50}$/ml) at a multiplicity of infection (MOI) of 1. For PAMP stimulation, 5 μg/ml of poly(I:C) (Sigma), 10 μg/ml of LPS (Invitrogen) and PGN (Invitrogen) were performed, respectively. Preparation of these solutions and the detailed instructions were provided by previous study (Yang et al., 2013). The control was treated with phosphate buffer solution (PBS).

Cells were collected at different time points post-treatment by centrifuging at 1000 rpm for 8 min. The samples were homogenized in TRIZOL reagent (Invitrogen) and total RNAs were isolated according to the manufacturer's instruction. Total RNAs were incubated with RNase-free DNase I (Roche, USA) to eliminate contaminated genomic DNA before being reversely transcribed into cDNA using random hexamer primers and M-MLV Reverse Transcriptase (Promega, USA).

2.2. Overexpression vectors construction

The full-length cDNA sequence and structural domains of *CiLGP2* were reported previously (Huang et al., 2010). Structural domains of CiLGP2 protein contain DExD/H helicase domain and RD. The primers for the full-length cDNA, RD and DExD/H helicase domain constructs were designed (Table 1). Each fragment was amplified using LA Taq™ DNA polymerase (TaKaRa, Japan). The corresponding PCR products were purified and then digested with the enzymes of *Apa*I and *Kpn*I (Fermentas, Canada), meanwhile, the plasmid of pCMV-EGFP-CMV-SV40 (pCMV) (Fig. S1) was digested. After purification, *CiLGP2* fragments were ligated with pCMV by T4 DNA ligase (Fermentas). Target fragments were sequenced to validate the insert sequences without mutations. The plasmids with correct insert were extracted by TIANpure Midi Plasmid Kit (TIANGEN, China). Three overexpression plasmids were respectively named as pLGP2, pRD and pDExD/H (Table 2).

Table 2
The abbreviation of constructed vectors.

Plasmid name	Abbreviation	Domain
pCMV-EGFP-CMV-SV40	pCMV	Empty vector
pCMV-EGFP-CMV-CiLGP2-SV40	pLGP2	Full length
pCMV-EGFP-CMV-CiLGP2-DExD/H-SV40	pDExD/H	DExD/H helicase domain remained
pCMV-EGFP-CMV- CiLGP2-RD-SV40	pRD	RD reserved