Fish & Shellfish Immunology 43 (2015) 257–263

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

A C-type lectin (*LvCTL4*) from *Litopenaeus vannamei* is a downstream molecule of the NF-κB signaling pathway and participates in antibacterial immune response

Haoyang Li [a, 1], Yonggui Chen [b, 1], Ming Li [a, c], Sheng Wang [a], Hongliang Zuo [a], Xiaopeng Xu [a], Shaoping Weng [a], Jianguo He [a, b, **], Chaozheng Li [a, *]

[a] MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, PR China
[b] School of Marine Sciences, Sun Yat-sen University, Guangzhou, PR China
[c] Guangxi Key Laboratory of Aquatic Genetic Breeding and Healthy Aquaculture, Guangxi Institute of Fisheries, Nanning, PR China

ARTICLE INFO

Article history:
Received 2 October 2014
Received in revised form
15 December 2014
Accepted 18 December 2014
Available online 2 January 2015

Keywords:
C-type lectin
Litopenaeus vannamei
NF-κB signaling pathway
Innate immunity
Vibrio parahaemolyticus

ABSTRACT

C-type lectins (CTLs) play multiple roles in innate immune defense against invading pathogens in both vertebrates and invertebrates. In this study, a new C-type lectin gene from pacific white shrimp *Litopenaeus vannamei* (designated as *LvCTL4*) was cloned by rapid amplification of the cDNA ends (RACE) method. The full-length cDNA of *LvCTL4* was 563 bp with open reading frame (ORF) of 471 bp encoding a polypeptide of 156 amino acids, including a putative signal sequence and a single C-type lectin-like domain (CTLD). The CTLD of 137 amino acid residues contained a mutated 'EPA' (Glu^{121}-Pro^{122}-Ala^{123}) motif in the calcium-binding site 2 and three conserved disulfide bonds involved in structure maintenance. Tissue expression analysis showed *LvCTL4* was ubiquitously distributed with high levels in gill, intestine, epithelium and hepatopancreas. The expression of *LvCTL4* in gill was up-regulated in response to *Vibrio parahaemolyticus* challenge. RNAi knock-down of the *LvCTL4* gene significantly increased mortality after *V. parahaemolyticus* infection. A 103 bp 5′ flanking promoter sequence was obtained using the genome walking method and it contained a conserved NF-κB binding motif. Dual-Luciferase assay showed both *LvDorsal* and *LvRelish* could up regulate the promoter activity of *LvCTL4*. This is the first report that a shrimp C-type lectin can be regulated by both *LvDorsal* and *LvRelish*. These findings provided novel insights into the regulation of shrimp CTLs expression.

1. Introduction

The innate immune system is the dominant system of host defense in invertebrates and plays a key role in preventing pathogens invasion [1]. The innate response is usually activated when recognizing the invading pathogens by so-called pattern recognition receptors (PPRs) [2]. PPRs are germline-encoded receptors and can recognize conserved components among broad groups of pathogens termed pathogen-associated molecular patterns (PAMPs) [2]. In invertebrates and vertebrates, a multitude of PRRs have been identified, containing down syndrome cell adhesion molecules (DSCAMs), fibrinogen-related proteins (FREPs), scavenger receptors (SCRs), peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBPs), thioester-containing proteins (TEPs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) [2–7].

C-type lectins (CLTs) are a large family of proteins that bind to carbohydrates in a calcium-dependent (C-type) manner [8]. Most CTLs contain at least a C-type lectin domain (CTLD), which includes a characteristic double-loop structure stabilized by two or three conserved disulfide bonds and four Ca^{2+}-binding sites for carbohydrates binding [8]. C-type lectins are considered as PRRs because they can recognize the carbohydrates exposed on the surface of invading microbes [7,9,10]. They can recognize many diverse

* Corresponding author. School of Life Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou 510006, PR China. Tel.: +86 20 39332850; fax: +86 20 84113229.
** Corresponding author. School of Life Sciences, School of Marine Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou 510275, PR China. Tel.: +86 20 39332988; fax: +86 20 84113229.
E-mail addresses: lsshjg@mail.sysu.edu.cn (J. He), lichaozh@mail2.sysu.edu.cn (C. Li).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2014.12.024
1050-4648/

PAMPs, including mannoses, galactoses, lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acids, β-glucans, α-mannans, and so on [7,9,10]. In vertebrates, many CLRs can activate diverse signaling pathways including NF-κB signaling pathway, NLRP3 inflammasome activation pathway, Ca2+-calcineurin-NFAT pathway and MAPK signaling pathway, to tailor the immune response [7,9,11–13]. A strikingly attractive example is DC-SIGN, a C-type lectin expressed by dendritic cells (DCs) which can recognize a large array of pathogens including several viruses and microbes [12]. Upon binding to pathogens such as HIV-1, DC-SIGN activated the serine/threonine protein kinase Raf-1, which subsequently leaded to acetylation of the nuclear factor κB (NF-κB) subunit p65 at different lysines [12]. Acetylated p65 both prolonged and increased IL-10 transcription to reinforce the anti-inflammatory immune response [12]. In invertebrates, C-type lectins are also identified as PPRs or effectors to participant in many kinds of immune responses, such as agglutinating or killing microorganisms, initiating signal transduction, activation of prophenoloxidase system, stimulating phygocytosis and encapsulation, induction of respiratory burst and tailoring antiviral response [14–20]. However, there is only a few reports about signaling pathway involved in the expression of CTLs. In a very recent study, we have identified a shrimp C-type lectin (*LvCTL3*) with antibacterial and antiviral activity, which could be regulated by *LvDorsal* [21].

In shrimp, there are many kinds of lectins identified, including C-type, L-type, M-type, P-type, fibrinogen-like domain lectins, galectins, and calnexin/calreticulin [10]. Among these lectins, C-type lectins are the most well studied family [10]. To data, a number of CTLs have been reported in *Litopenaeus vannamei*, *Litopenaeus schmitti*, *Litopenaeus setiferus*, *Fenneropenaeus chinensis*, *Fenneropenaeus merguiensis*, *Penaeus monodon*, *Marsupenaeus japonicus*, *Macrobrachium rosenbergii* [10]. According to previous study, on the basis of their molecular structure, shrimp CTLs can be classified to three groups: those containing only one CTLD, two CTLDs, and those with one CTLD and an additional other domain [10]. In this study, we reported a new C-type lectin from *L. vannamei* named *LvCTL4* with a single CTLD and a putative signal peptide. *LvCTL4* was responsive to *Vibrio parahaemolyticus* challenge and its expression could be regulated through the NF-κB signaling pathway. Furthermore, RNA silencing assay indicated *LvCTL4* was involved in antibacterial immune response. Taken together, we speculated that *LvCTL4* could function as a downstream molecule of the NF-κB signaling pathway to participate in antibacterial immune response.

2. Materials and methods

2.1. Cloning of LvCTL4 full-length cDNA and its 5′ flanking regulatory region

Based on the *L. vannamei* transcriptome data in our laboratory [22], a partial cDNA sequence was obtained to amplify the full length cDNA sequence using the rapid amplification of cDNA ends (RACE) method as previously described [21]. Two primers 3RACE1 and 5RACE1 were used to perform the first-round 3′-RACE and 5′-RACE amplification, respectively. The second-round PCR was performed with 3RACE2 and 5RACE2 primers, subsequently the products were cloned into pMD-20T vector (TaKaRa, Japan) and 12 positive clones were selected for sequencing. Transcription starting site (TSS) of *LvCTL4* was determined according to the 5′-RACE PCR amplification.

The 5′ flanking regulatory region of *LvCTL4* was isolated using GenomeWalker™ Universal Kit (Clontech, Japan) according to previously described [21]. Two pairs of primer AP1/5′GW-LvCTL4-1 and AP2/5′GW-LvCTL4-2 were used to perform the first and second rounds of genome walking PCR amplification, respectively. The PCR products were cloned to pMD-20T vector (TaKaRa, Japan) and sequenced. Primers were listed in Table 1.

2.2. Bioinformatics analysis

Protein domains and signal peptide were analyzed using the SMART program (http://smart.embl-heidelberg.de/). The potential transcription factor binding sites in the 5′ flanking regulatory region were predicted using the TRANSFAC® 6.0 program [23] using high quality matrices and 0.85 as matrix and core similarity cut-off.

2.3. Plasmid constructions

The primer pair of pGL3-LvCTL4-F/pGL3-LvCTL4-R was used to amplify the 5′ flanking regulatory region of *LvCTL4* and subsequently cloned into pGL3-Basic (Promega) vector at KpnI/XhoI sites (named as pGL3-κB). The vector pGL3-κBm, containing promoters of LvCTL4 with deletion of the NF-κB binding motif (TGGGAAAATC), was obtained by the overlap extension polymerase chain reaction method using the two pairs of primers pGL3-LvCTL4-F/pGL3-LvCTL4-κBmutant-R and pGL3-LvCTL4-κBmutant-F/pGL4-LvCTL4-R, similar to previous described [21]. The *LvDorsal* and *LvRelish* expression vectors were obtained from previous study [24,25].

2.4. Dual-luciferase reporter assays

Because shrimp has no usable and stable cell lines, *Drosophila* S2 cells can be the candidate to study the function of *LvCTL4*. For DNA transfection, *Drosophila* S2 Cells were plated into a 96-well plate (TPP, Switzerland) and transfections were performed on the next day, and plasmids were transfected using the Effectene Transfection Reagent (Qiagen) according to the user manual. For dual-luciferase reporter assays, S2 cells in each well of a 96-well plate were transfected with 0.05 μg reporter gene plasmids (pGL3-κB or pGL3-κBm), 0.005 μg pRL-TK renilla luciferase plasmid (as an internal control), and 0.05 μg (0.02 μg or 0.03 μg) expression plasmids (*LvDrosal* or *LvRelish*) or empty pAc5.1/V5-His A plasmid (as control). At 48 h post transfection, the activities of the firefly and renilla luciferases were measured according to user i**nstruction**. Each experiment was done at least three times.

2.5. Tissue expression and immune challenge analysis by real-time RT-PCR

Healthy shrimps *L. vannamei* (average 5 g each) were obtained from a shrimp farm of Zhuhai (Guangdong province, China). For tissue expression distribution, shrimp tissues of eyestalks, epitheliums, gills, hepatopancreases, hemocytes, intestines, muscles, nerves, pyloric cecas, scapes and stomachs were sampled and pooled from 15 shrimps. For immune stimulations, shrimps were cultured in air-pumped salt-water tanks (27 °C). The treated groups were injected with 50 μl of *V. parahaemolyticus* suspension (1×10^6 colony-forming units, CFU) or white spot syndrome virus (WSSV) (1×10^6 particles, newly extracted) at the second abdominal segment of each shrimp, and the control group was injected with PBS solution. Gills of challenged shrimps were sampled at 0, 4, 8, 12, 24, 36, 48, 72 h post injection (hpi), and each time point samples were collected and pooled from 15 shrimps. Total RNA extraction and real-time RT-PCR assays were performed according to former described [21]. Expression levels of *LvCTL4* were calculated using the Livak ($2^{-\Delta\Delta CT}$) method after normalization to *L. vannamei EF-1α* (GU136229). Primer sequences were listed in Table 1.