Developmental and Comparative Immunology 42 (2014) 323–332

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Two novel C-type lectins with a low-density lipoprotein receptor class A domain have antiviral function in the shrimp *Marsupenaeus japonicus*

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ARTICLE INFO

Article history:
Received 29 July 2013
Revised 9 October 2013
Accepted 9 October 2013
Available online 16 October 2013

Keywords:
C-type lectin
A low-density lipoprotein receptor class A domain
Innate immunity
Shrimp
Marsupenaeus japonicus

ABSTRACT

C-type lectins (CTLs) are pattern-recognition receptors (PRRs) that play important roles in immune response. In this study, two new CTLs containing a low-density lipoprotein receptor class A domain (LDLR) and a carbohydrate recognition domain (CRD) were identified in *Marsupenaeus japonicus* and designated as LdlrLec1 and LdlrLec2. The two CTLs expressed in all the tested tissues of shrimp, however, *LdlrLec1* was mainly expressed in hemocytes, heart, gill and intestines, whereas *LdlrLec2* was expressed in hepatopancreas and heart. The expression patterns of both *LdlrLec1* and *LdlrLec2* mRNA were obviously upregulated upon white spot syndrome virus (WSSV) challenge. Injection of recombinant LdlrLec1 or LdlrLec2 into shrimp inhibited WSSV replication, whereas knocking down the expression of *LdlrLec1* and *LdlrLec2* by RNA interference increased WSSV replication in vivo. The infection rates of WSSV incubated with LdlrLecs were reduced significantly compared with the control group. The LdlrLec proteins could interact with VP28, a major envelope protein of WSSV, which is necessary for the attachment and penetration of WSSV into shrimp cells. These results indicate that LdlrLec1 and LdlrLec2 may function in antiviral response by binding to WSSV and inhibiting their pervasion and replication in shrimp.

1. Introduction

Pattern recognition receptors (PRRs) can recognize pathogen-associated molecular patterns (PAMPs) found on microbial surfaces and trigger downstream reactions against infected pathogens (Christophides et al., 2004; Lee and Söderhäll, 2002). Lectins are some of the most important PRR families widely distributed in almost all organisms, including animals, plants, and microorganisms (Hardison and Brown, 2012; Lakhtin et al., 2011). In shrimp, at least seven groups of lectins have been identified, namely, C-type, L-type, P-type, M-type, fibrinogen-like domain lectins, galectins, and calnexin/calreticulin (Wang and Wang, 2013).

C-type lectins (CTLs) belong to a large superfamily of Ca^{2+}-dependent carbohydrate recognition proteins that can bind to sugar moieties with single or multiple carbohydrate recognition domains (CRDs), which are composed of nearly 130 amino acid residues and stabilized by two or three pairs of disulfide bonds (Zelensky and Gready, 2003, 2005). It has been reported 32 genes encoding CTL-like domains (CTLDs) in the *Drosophila melanogaster* genome and 278 genes in *Caenorhabditis elegans* (Dodd and Drickamer, 2001; Drickamer and Dodd, 1999; Schulenburg et al., 2008). CTLs have also been identified in crab, scallop, and especially in shrimp. Diversiform CTLs are well studied in *Penaeus monodon*, *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, and *Marsupenaeus japonicus* (Wang and Wang, 2013).

Shrimp CTLs exhibit various functions. For instance, *Fc*Lec1 (*Fc*-hsL), *Fc*Lec2, *Fc*Lec3, FcLec4, and FcLec5 (Sun et al., 2008; Wang et al., 2009b,c; Xu et al., 2010; Zhang et al., 2009a) can agglutinate both Gram-negative and Gram-positive bacteria in the presence of calcium in *F. chinensis*, whereas *Mj*LecA, *Mj*LecB, and *Mj*LecC can agglutinate both Gram-negative and Gram-positive bacteria without the presence of calcium in *M. japonicus* (Song et al., 2010). *Lv*Lec (*L. vannamei*) displayed aggregation activity toward *Escherichia coli* JM109 (Zhang et al., 2009b), whereas *Pm*LT (*P. monodon*) can enhance hemocyte encapsulation (Hoi-Tung Ma et al., 2008). Meanwhile, some CTLs also have direct antiviral activity in protecting shrimp from WSSV infection. Studies have reported that *Fc*Lec3 and *Lv*CTL1 in *L. vannamei* and PMAV in *P. monodon* can respond to WSSV challenge (Costa et al., 2011; Luo et al., 2007, 2003). *Mj*LecB (*M. japonicus*) and *Mj*LecC (*M. japonicus*) can bind to VP28 of WSSV. *Mj*LecA (*M. japonicus*) exhibited similar activity and can also directly bind to VP26 (Song et al., 2010). Junkunlo et al. (2012) reported a low-density lipoprotein receptor (LDLR) class A domain CTL in *L. vannamei* and found that LvCTLD was involved in shrimp defense against yellow head virus. However, the mechanism underlying the antiviral response and the function of the LDLR domain remain unclear.

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http://dx.doi.org/10.1016/j.dci.2013.10.003

Diseases caused by bacterial and viral pathogens have brought huge losses to shrimp farming (Liu et al., 2009). Therefore, the anti-pathogenic mechanism in shrimp must be studied. In this work, we identified two new CTLs with an LDLR class A domain named LdlrLec1 and LdlrLec2 from *Marsupenaeus japonicus*. The expression patterns of *LdlrLec1* and *LdlrLec2* were studied, and the corresponding recombinant proteins and dsRNA were used to elucidate their potential functions in the antiviral response of shrimp. Our results indicated that LdlrLec1 and LdlrLec2 may play an important role in shrimp immunity against WSSV.

2. Materials and methods

2.1. Chemicals

Unizol total RNA extraction reagent was obtained from Biostar (Shanghai, China). A SMART polymerase chain reaction (PCR) cDNA synthesis kit was purchased from BD Bioscience Clontech (Mountain View, CA, USA). Genomic DNA Purification Kit was obtained from Toyobo (Japan). Taq Polymerase and DNA markers were obtained from TaKaRa Biotech (Dalian, China). An UltraSYBR Mixture was obtained from CW Biotech (Beijing, China). The LDLR domain of LdlrLec1 (LdlrLec1-LDL) peptide was synthesized by ChinaPeptides Co., Ltd. (Wuxi, China).

2.2. Immune challenge of shrimp

Shrimp weighing 8–15 g each were purchased from a market in Jinan, Shandong Province, China and cultured in water in our laboratory. For viral challenge, each shrimp was infected with 5×10^7 copies of WSSV (Wang et al., 2009a). Hemolymph was collected from the ventral sinus using a 5 ml sterile syringe preloaded with 500 μl of anticoagulant (10% sodium citrate, pH 7.0) 0, 6, 12, 24, and 48 h after injection. The collected hemolymph was then centrifuged at 800×*g* for 5 min at 4 °C to obtain hemocytes. Other tissues such as heart, hepatopancreas, gill, stomach, and intestines were also collected to extract RNA and proteins.

2.3. Total RNA isolation and cDNA synthesis

Total RNA from hemocytes, heart, hepatopancreas, gill, stomach, and intestines were extracted using Unizol reagent. cDNAs were synthesized using a SMART cDNA kit according to the manufacturer's instructions with SMART F and Oligo anchor R (Table 1).

2.4. Genomic DNA purification

Genomic DNA used for detecting viral infection was extracted from gills of WSSV challenged shrimp using the MagExtractor Genomic DNA Purification Kit (Toyobo, Shanghai, China) following the manufacturer's instructions.

2.5. Cloning of LdlrLec1 and LdlrLec2 full-length cDNA

According to the unigene sequences obtained from the transcriptome sequencing of *M. japonicus*, the specific primers LdlrLec1F and LdlrLec2F (Table 1) were designed for 3′-end amplification using rapid amplification of cDNA ends method with the 3′ anchor R primer. The PCR profile used was as follows: 95 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 90 s, and a final step of 72 °C for 10 min. The PCR fragments were sequenced and confirmed as CTL sequences by online BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis.

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′–3′)
cDNA synthesis	
SMART F	TACGGCTGCGAGAAGACGACAGAAGGG
Oligo anchor R	GACCACGCGTATCGATGTCGACT16(A/C/G)
cDNA cloning	
LdlrLec1F	CCAGCCTCAAATCCCAATA
LdlrLec2F	TTGACGACACTCCCATGCCT
3′anchor R	GACCACGCGTATCGATGTCGAC
RT-PCR and qRT-PCR	
LdlrLec1rtF	CCGTCACCTTCCGCCACTACT
LdlrLec1rtR	TGAGGCTGGGCAGGGATAATAA
LdlrLec2rtF	AAGCGACTTCTGGATTGGAGG
LdlrLec2rtR	GGCTAGACTCCCTCAGGTTCAT
β-actin F	GCATCATTCTCCATGTCGTCCCAGT
β-actin R	TACGGCTGCGAGAAGACGACAGAA
Recombinant expression	
LdlrLec1exF	TACTCAGGATCCGGCTCCTGTGAGACCGGATAC
LdlrLec1exR	TACTCAGTCGACTTCACACAAAGGGCTCTTGA
LdlrLec1CRDexF	TACTCAGAATTCTGCCCCAAGCTCTATACCAG
LdlrLec2exF	TCCGAATTCGAGCTCGCGTCCTGCAGTTCGAACCA
LdlrLec2exR	GTGGTGGTGCTCGAGCTAGACTCCCTCAGGTTCAT
RNAi	
dsLdlrLec1F	GCGTAATACGACTCACTATAGGTTGCAATTATCTGGGACCA
dsLdlrLec1R	GCGTAATACGACTCACTATAGGCCTCAACTTTGCCTATATG
dsLdlrLec2F	TAATACGACTCACTATAGGCTCAACGCTCAGTCAGAAGTAC
dsLdlrLec2R	TAATACGACTCACTATAGGTTGCTTGGGTGCTTGAATAT
dsGFP F	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
dsGFP R	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC

2.6. Semi-quantitative RT-PCR and quantitative real-time PCR (qRT-PCR) analysis

The transcription levels of *LdlrLec1* and *LdlrLec2* in different tissues were evaluated by semi-quantitative RT-PCR using two pairs of primers (LdlrLec1rtF and LdlrLec1rtR, as well as LdlrLec2rtF and LdlrLec2rtR in Table 1). The following PCR profile was used: 95 °C for 3 min, 27 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, and a final step of 72 °C for 10 min. *β-actin* was amplified using specific primers *β*-actin F and *β*-actin R (Table 1) as an internal control. The PCR products were run on 2% agarose gels. The transcription levels of *LdlrLec1* and *LdlrLec2* in the same tissue at different time points after immune challenge were evaluated by qRT-PCR. The following PCR profile used was: 95 °C for 10 min, 40 cycles of 94 °C for 15 s, 60 °C for 60 s, and read at 78 °C for 2 s. mRNA expression level was analyzed using the comparative C_T method. The discrepancies among the C_T values of *LdlrLec1*, *LdlrLec2*, and *β-actin* (ΔC_T) were calculated to normalize the variation in the amount of cDNA in each reaction. The mRNA expression level was calculated by the $2^{-\Delta\Delta CT}$ method. The normalized data were subjected to statistical analysis followed by an unpaired sample *t*-test. Significant difference was accepted at $P < 0.05$.

2.7. Recombinant expression and purification of LdlrLec1 and LdlrLec2

Based on the full-length cDNA sequence, primers (Table 1) were designed for the amplification of *LdlrLec1* (LdlrLec1exF and LdlrLec1exR), *LdlrLec1-CRD* (LdlrLec1CRDexF and LdlrLec1exR), and *LdlrLec2* (LdlrLec2exF and LdlrLec2exR) (Table 1). After digestion with restriction enzymes, the fragments were subcloned into the pET30a(+) plasmid, which were also digested with the same enzymes. The recombinant pGEX4T-1/*LdlrLec1-CRD* was also constructed. The recombinant plasmids were then transformed into competent *E. coli* Rosetta cells for recombinant expression. All recombinant proteins including mature LdlrLec1 and LdlrLec2 proteins, as well as the CRD domain of LdlrLec1, were expressed as