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Genomic organization, promoter characterization and expression analysis of the leukocyte cell-derived chemotaxin-2 gene in *Epinephelus akaraa*

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ABSTRACT

Leucocyte cell-derived chemotaxin 2 (LECT2) was first identified as a chemotactic factor and has been subsequently proven to be a multifunctional protein that mediates the regulation of liver regeneration, carcinogenesis and Natural killer T (NKT) cell homeostasis in mammals. In fish, it has been recently found to be critical for the inflammatory response to stimuli. However, the *in vivo* function of LECT2 in fish remains obscure. Base on the full-length cDNA of the *Epinephelus akaraa* LECT2 (EaLECT2) gene we previously isolated, we sought to analyze its genomic structure and context. The genomic DNA of the EaLECT2 gene spans 2866 bp from the transcription start site to the termination codon. As in most LECT2 genes in other vertebrates, the EaLECT2 genomic DNA contains four exons and three introns. An analysis of the promoter region revealed the presence of a TATA box and several putative transcription factor-binding sites. And transcriptional activity analysis suggested that most basal DNA regulatory elements required for EaLECT2 transcriptional activity might be contained within the 581 bp region upstream of the transcription start codon. A real-time PCR analysis showed that the EaLECT2 expression levels were slightly increased in the head kidney, liver, gill and brain by bacterial challenge with *Vibrio harveyi*. Furthermore, the transcriptional level of the EaLECT2 gene in the liver was significantly up-regulated within 1 h and reached its peak level at 12 h post-stimulation. Higher levels of LECT2 expression were also observed in head kidney in challenged individuals.The expression pattern demonstrates the role of EaLECT2 in the immune response and its functions under other conditions. Additionally, we found that the recombinant EaLECT2 could be expressed as a soluble protein using a prokaryotic expression system with the expression vector pET32a(+) and the soluble protein was further proved to be the recombinant EaLECT2 with the rat antiserum against EaLECT2 we obtained. This work provides a unique basis for substantial work in future projects.

1. Introduction

Leucocyte cell-derived chemotaxin 2 (LECT2), originally discovered in 1996, was identified as a chemotactic factor for neutrophils [1] despite the absence of a cysteine motif (Cys-X-Cys, Cys-X3-Cys and Cys-Cys) [2] in the molecule. LECT2 exhibited neutrophilic chemotaxis *in vitro* as a secreted protein initially isolated from the cultured fluid of phytohemagglutinin-activated human T-cell leukemia SKW-3 cells. However, its wide distribution in human tissues [3] suggested that it may have additional functions. LECT2 was subsequently demonstrated to have a multifunctional role in such disparate processes as the regulation of liver regeneration [4], carcinogenesis [5] and Natural killer T (NKT) cell homeostasis [6]. In addition, LECT2 was shown to be identical to chondromodulin II [7], a cartilage-derived growth promoting factor found to be expressed when cartilage cell growth or repair was initiated after cell damage [3]. In conjunction with the functions described above, recent studies have described a prominent role of LECT2 in the regulation of neuritic development through a unique mechanism that differs from that of other cytokines [8].

LECT2 is known to be present in mammals, and several LECT2 homologs have recently been identified in fishes, including *Salmo salar* (BT046607.2), *Oncorhynchus mykiss* (BT073541.1), *Oncorhynchus masou formosanus* (EU325849.1), *Esox lucius* (BT079031.1), *Plecoglossus altivelis* (FM253748.1), *Lates calcarifer* (EU136177.1), *Larimichthys crocea* (AM709638.1), *Aristichthys nobilis* (FJ502111.1),

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and *Epinephelus coioides* (GU988725.1). LECT2 has attracted more and more attention because of its potentially important roles in the inflammatory responses of fish following pathogenic infection and in the development of possible mitigation strategies for disease resistance. However, progress toward related research has been slow. LECT2 has been recognized as a candidate anti-infection and anti-inflammatory agent with a fairly high sensitivity to bacterial infections in fish [9]. A functional clue about LECT2 has been recently revealed by the discovery of two proteins, a transferring protein and a C-type lectin receptor, which strongly interact with LECT2. It has been therefore accepted that LECT2 likely influences the immune system via the activation of a C-type lectin receptor and participates in the regulation to prevent bacteria from stripping the iron from host materials [10,11]. All these evidences imply that LECT2 acts as an important factor in the fish immune system, but a better understanding of LECT2 function is obviously required.

In the present study, *Epinephelus akaraa*, an important cultured marine species of grouper in Asia, was investigated. *E. akaraa* is a popular seafood species with a high market value. The importance to better understand this species is highlighted by the recent disease outbreaks caused by viral and bacterial pathogens, especially the nervous necrosis virus (VNN) [12] and Vibrio species [13]. Based on the full-length cDNA of the *E. akaraa* LECT2 gene (EaLECT2) we have previously cloned, in this paper, we performed a genomic structure analysis of the EaLECT2 gene. As a major first step toward identifying the elusive mechanisms regulating LECT2 expression, EaLECT2 5′-flanking region was cloned for the first time in our study, and the transcriptional activity of this region was subsequently analyzed. To determine the participation of LECT2 in innate immune responsiveness and to identify the key mechanistic steps for improving immunity, we also examined the real-time quantitative expression of LECT2 in various tissues in response to infection with pathogenic bacteria. After constructing recombinant expression plasmids, we surprisingly found that mature LECT2 could be expressed in *E. coli* as a recombinant fusion protein; this expression is the first time that LECT2 has been obtained in soluble form, to the best of our knowledge. And the antiserum against EcLECT2 was further obtained. It allows us to prepare for the further investigation of LECT2. All of the discoveries presented here will allow us to gain more insight into the genesis and evolution of the LECT2 gene and its immunological function in lower vertebrates, including fish.

2. Materials and methods

2.1. Experimental fish

E. akaraa, with a body weight ranging from 200 to 300 g, were purchased from a commercial fish farm in Fujian Province, China, and maintained at 25 °C in aerated seawater. Before the experimental manipulation, the fish were acclimatized in the laboratory for one week. Throughout the experiment, the seawater was changed daily. The fish were not fed during the infection experiment.

2.2. LECT2 genomic DNA sequence amplification and gene organization analysis

The *E. akaraa* genomic DNA was extracted from muscle with phenol-chloroform method. The specific primers genomeF and genomeR (Table 1) were designed to amplify the LECT2 genomic DNA sequence based on the full-length LECT2 cDNA isolated from *E. akaraa* (GenBank: GU722597). The amplification regime was 5 min at 95 °C, followed by 35 cycles consisting of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 3 min. The genomic DNA sequence obtained was then aligned with the cDNA to identify the exons and introns, and the LECT2 genomic DNA sequences from other species were also consulted.

Table 1
Primer sequences and their application.

Primer name	Sequence (5′–3′)	Application
GenomeF	ATGAGACGTGTCACCGTTTT	Genomic DNA amplification
GenomeR	TTAATTAAAGTACTGCGTGGG	
LECT2_G_F1195	TGTTTCAGGACGACCAGGCTAC	Copy number determination
LECT2_G_R1276	GACAATGACCTTTCCGTTTAGAGT	
ENC_F510	GGTGGACCAGAAAGCCAAAGAGAT	
ENC_R653	CATTCCTCGTGGACGGTGTCATAGA	
walking_R97	TTTGTCACCTTTGTCCGTGCTCTAC	Genomic walking
walking_R85	TCCGTGCTCTACAGTTCACAGGTC	
walking_R15	ATTCATCATCTTACCCAGCAAAACG	
Lect2F	AACAAGAGCCGAGATTTGAAGAT	Real-time PCR
Lect2R108	TCCGTAGTGTCCCTGTCCCCAT	
Exp_MatF	GGCTATTCCATATGGTAAGGTTC GGACAGCTCTGC	Recombinant expression
Exp_R	CCGGAATTCTTAATTAAAGTACTGCG TGGGG	
GST_MF	CCGGAATTCGTAAGGTTCGGACAGC TCTGC	
GST_R	ACGCGTCGACTTAATTAAAGTACTG CGTGGGG	
ActinF	AAGCCAACAGGGAGAAGATGAC	Real-time PCR (control)
ActinR	TGTGGTGGTGAAGGAGTAGCC	
PF1191	CGACGCGTCAGTTCACAGGTCGAGA GAAAGGAT	Plasmid constructs
PF537	CGACGCGTCTGACAAGAGGCTGAAT GAGACG	
PF811	CGACGCGTCATCCATGAACATGATT TCACT	
PR1	CCGCTCGAGCTTCAAATCTCGGCTCT TGTTCCAG	
PR2	CCGCTCGAGGTCTCATTCAGCCTCTT GTCAGC	

2.3. Cloning the E. akaraa LECT2 gene 5′-flanking region

The LECT2 gene 5′-flanking region was obtained with a genome walking kit (TaKaRa, Japan). Following the manufacturer's instructions, the three primers, walking_R97, walking_R85 and walking_R15 (Table 1), were designed and used in PCR in combination with the forward primer AP1. The PCR product was purified, ligated into the pMD19-T vector and sequenced. The 5′-flanking region was analyzed for potential transcriptional factor (TF) binding sites with the following online software: TFSAERCH (http://www.cbrc.jp/research/db/TFSEARCH.html), TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess), TFBIND (http://tfbind.hgc.jp/) and CpG (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). NNPP version 2.2 (http://fruitfly.org:9005/seq_tools/promoter.html) was used to search for the promoter sequence.

2.4. Transcriptional activity analysis of EaLECT2 promoter

Different genomic fragments upstream of the transcription start codon of LECT2 gene were amplified from *E. akaraa* genomic DNA by PCR using three forward primers (PF1191, PF811 and PF537) that contained MluI site and reverse primer (PR1) contained XhoI site. The forward primers were designed at bp −1191, −811 and −537 relative to the transcriptional start site of the LECT2 gene. And the reverse primer was complementary to bp +18 to +43 in the LECT2 gene. A 3′-nested deletion mutant was also generated by PCR with primers PF1191 and PR2. Primers used were shown in Table 1. The target PCR products were digested and subcloned into the MluI and XhoI sites of pGL3-basic vector (Promega, USA).