Short sequence report

Identification of a LPS-induced TNF-α factor (LITAF) from mollusk *Solen grandis* and its expression pattern towards PAMPs stimulation

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**Abstract**

Lipopolysaccharide-induced TNF-α factor (LITAF) is one of the most important transcription factors mediating TNF-α transcription. In the present study, a LITAF gene (designated as SgLITAF) was identified from razor clams *Solen grandis*. The full-length cDNA of SgLITAF was of 1476 bp, encoding a polypeptide of 130 amino acids showed high similarity to other known LITAFs. SgLITAF encoded a LITAF domain and the Zn\(^{2+}\)-binding motifs in the domain were well conserved. The mRNA transcripts of SgLITAF were detected in all tested tissues of healthy razor clams, including mantle, gill, gonad, hemocytes, muscle and hepatopancreas, and with the highest expression level in hepatopancreas. The expression level of SgLITAF in hemocytes was significantly up-regulated (*P* < 0.01) after razor clams were stimulated by LPS or β-1, 3-glucan, but no obvious fluctuation of SgLITAF mRNA expression was observed after PGN stimulation. All the results indicated that there might be a LITAF-regulated TNF-α signaling pathway existing in *S. grandis*, which involved in the immune response not only against gram-negative bacteria but also towards fungi.

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1. Introduction

As an important transcription factor, LPS-induced TNF-α factor (LITAF) plays a crucial role in regulation of various inflammatory cytokines, especially the TNF-α [1]. In mammals, a unique LITAF transcriptional pathway that is separated from NF-κB has been confirmed in macrophages. After LPS stimulation, LITAF initiates the signaling by transferring to nucleus and forming a functional complex with STAT6, which will subsequently result in recognizing the specific regulatory sequence of TNF-α promoter and producing several pro-inflammatory cytokines [2]. In addition, LITAF can also regulate the TNF-α-induced immune responses in vitro, for example, the recombinant chicken LITAF induces apoptosis in embryonic fibroblast cell line as well as other leukocyte tumor cell lines [3].

Although TNF-α system and its regulation mechanism has been intensively studied in mammals for almost 40 years, the research progress in invertebrate TNF-α system is still very slow. In 2002, the identification of TNF and its receptor from Drosophila, and their induction of cell death through JNK pathway, have provided more convincible evidence supporting the existence of TNF system in invertebrates [4]. Recently, several TNF system related molecules have been characterized from mollusk, such as TNF-α from abalone [5], TNFR [6], TTRAP [7], LITAF [8] from scallop, and LITAF from oyster [9], suggesting that the TNF signaling pathway also exists in marine mollusk. However, the knowledge on the regulation of TNF-α in mollusk is still limited. In present study, a LITAF was identified from razor clams *Solen grandis* (designated as SgLITAF), and the main objectives were (1) to examine its expression pattern in different tissues, (2) to investigate its temporal expression after stimulation of pathogen-associated molecular patterns (PAMPs), and (3) to provide helpful evidence to understand the regulatory mechanism of mollusk TNF-α signaling pathway.

2. Materials and methods

2.1. Razor clam

Natural captured healthy razor clams *S. grandis* with average shell length of 85 mm, from Yantai, Shandong Province, China, were
collected and maintained in the aerated seawater at 20–22 °C for a week before processing.

2.2. EST analysis and cloning of the full-length SgLITAF cDNA

A cDNA library of razor clam was constructed [10], and BLAST analysis of the EST sequences revealed that an EST of 937 bp was highly similar to the known LITAF sequences. The corresponding clone was selected and completely sequenced using primer M13-F (5'-GTAAAACGACGGCCAG-3') and M13-R (5'-CAGGAAA-CAGCTATGACC-3').

2.3. Sequence analysis

The analysis of cDNA sequence, deduced amino acid sequence, homology, protein domain and multiple sequence alignment were performed according to previous description [10].

2.4. Real-time PCR analysis of SgLITAF mRNA expression in different tissues

RNA isolation and cDNA synthesis from different tissues were consistent with our previous work [10]. Two gene-specific primers for SgLITAF, F (5'-TGCTTACTCTGCTCTTTG-3') and R (5'-GATCTCCATTCACCTGTGA-3'), were used to amplify a product of 252 bp from cDNA, and the PCR product was sequenced to verify the specificity of real-time PCR. Two β-actin primers, F (5'-TGTCACCCAAACCTGTTCTC-3') and R (5'-CATCTATGCTGTCTGATC-3'), were used to amplify a 213 bp fragment as an internal control to verify the successful transcription and to calibrate the cDNA template for corresponding samples.

Real-time PCR amplification was carried out in an ABI 7300 Real-time Thermal Cycler according to previous method [10]. The 2^−ΔΔCT method was used to analyze the expression level of LITAF [10]. All data were given in terms of relative mRNA expressed as mean ± S.E. (N = 4).

2.5. Temporal expression analysis of SgLITAF after PAMPs stimulation

Experiments of PAMPs stimulation, hemocytes harvest, RNA isolation and cDNA synthesis were performed according to our previous description [10]. Real-time PCR analysis was carried out as described above. All data were given in terms of relative mRNA expressed as mean ± S.E. (N = 4). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed t-test. Differences were considered significant at P < 0.05 and extremely significant at P < 0.01.

3. Results and discussion

One EST from S. grandis cDNA library was found homologous to the previously identified LITAF genes. The clone corresponding to the EST was re-sequenced, and yielded a cDNA sequence of 1476 bp. The complete sequence of SgLITAF (GenBank accession JN642122) cDNA consisted of a 5’ terminal untranslated region (UTR) of 15 bp, a long 3’ UTR of 1068 bp with a poly (A) tail, and an open reading frame (ORF) of 393 bp by encoding a polypeptide of 130 amino acids with a theoretical isoelectric point of 8.05 and predicted molecular weight of 14.4 kDa. Be similar to LITAFs from other mollusk, such as Zhikong scallop [8] and pacific oyster [9], there was no typical signal peptide in the N-terminus of SgLITAF by the SignalP software, suggesting SgLITAF was also not a secretory protein. SMART analysis showed that the amino acids 60 (P) ~ 129 (R) constituted a LITAF domain, which was usually found in LITAF superfamily members [5,7,8,11]. BLAST analysis revealed that SgLITAF shared significant sequence similarities with other known LITAFs such as 83% identity to Meretrix meretrix LITAF (ADA77533) and 67% identity to Crassostrea gigas LITAF (AB070331). Based on the multiple sequences alignment between SgLITAF and other six LITAFs from scallop, human, clam, oyster, chicken and mouse, ten conserved cysteine residues were identified. Furthermore, the Zn^2+—binding motifs CXXC and XXCC, which were potential membrane-associated motifs, were also identified in SgLITAF, suggesting SgLITAF forms a compact Zn^2+—binding structure instead of inserting itself into the membrane like this molecule in other animals [9,12].

Investigating the mRNA distribution of SgLITAF in different tissues would benefit understanding its potential function and regulatory mechanism towards TNF-α signaling pathway. The mRNA transcript of SgLITAF was found to be constitutively expressed in a wide range of tissues with different levels in the healthy razor clams, including hepatopancreas, gill, hemocyte, gonad, mantle and muscle (Fig. 1). Considering razor clams completely expose themselves to the aquatic environment with large number of pathogens, the wide distribution of SgLITAF also suggested its important roles in immune defense against invaders. Furthermore, SgLITAF mRNA was expressed high in hepatopancreas and gill, which was 1610.7 and 691.4-fold compared with that in muscle, while the expression level in hemocyte, gonad and mantle were relatively lower, and was lowest in muscle (Fig. 1). Hepatopancreas is an important organ in mollusk synthesizing proteins involved in mollusk immune defense, while gill is the organ that acquired oxygen and algae from water containing various pathogenic microorganisms and is the first line in immune defense against bacterial infection [8]. The highest expression level in hepatopancreas and gill also particularly suggested its potential crucial role in immune defense of razor clams.

To preliminarily unravel the regulation mechanism of SgLITAF in innate immunity, the temporal expression of its mRNA in hemocytes was examined by real-time PCR after razor clams were stimulated by three typical PAMPs. In LPS stimulation group, significant up-regulation of SgLITAF mRNA expression level was
observed at most time points, and the high level lasted to the end of the experiment. The expression level was 4.99 ($P < 0.01$), 5.00 ($P < 0.05$) and 5.29-fold ($P < 0.01$) compared with blank group at 6, 12 and 24 h, respectively (Fig. 2). When *S. grandis* was stimulated by PGN, no significant changes were detected in the expression level of SgLITAF mRNA during the whole examine period (Fig. 2), suggesting LITAF might not be associated with the gram-positive bacteria infection. Similar result was also observed in scallop LITAF [8]. After β-1, 3-glucan stimulation, transcript level of SgLITAF mRNA reached the maximal level at 12 h post-stimulation, which was 38.0-fold ($P < 0.01$) compared with the blank group. As time progressed, the expression of SgLITAF transcript decreased gradually and reached to the original level at 48 h post-stimulation (Fig. 2).

LPS, the major component of gram-negative bacteria outer membrane that was usually used as a potent stimulator of immune-related cells, could induce the secretion of TNF-α and other pro-inflammatory cytokines [13]. The LITAFs from other mollusks, for example, scallop [8] and pearl oyster [11], were also proved to significantly respond to LPS stimulation, providing a clue for the existence of TNF-α or TNF-α-like molecules in mollusk. Except for LPS, live gram-negative bacteria challenge could also up-regulate the same gene in mollusk [9]. To date, there was little evidence illustrating the involvement of molluscan LITAFs in the immune response against fungi. In present study, the transcript level of SgLITAF exhibited a significant up-regulation after stimulation of β-1, 3-glucan from fungi, suggesting not only LPS but also β-1, 3-glucan might induce the activation of LITAF-dependent cytokine signaling pathway in *S. grandis*. However, further study was still needed to clarify the potential mechanism of such expression pattern. Our results which revealed the expression patterns of LITAF towards PAMPs stimulation in *S. grandis*, indicated SgLITAF might take part in the regulation of immune response against gram-negative bacteria and fungi, and would also provide helpful evidence to understand the regulation mechanism of the TNF-α signaling pathway in marine mollusk.

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**References**


