Cloning, characterization and expression analysis of the gene for a putative lipopolysaccharide-induced TNF-α factor of the Pacific oyster, *Crassostrea gigas*

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**Abstract**

Lipopolysaccharide-induced TNF-α factor (LITAF) is an important transcription factor that mediates the expression of inflammatory cytokines, including TNF-α, in lipopolysaccharide (LPS)-induced processes. In the present study, the Pacific oyster *Crassostrea gigas* LITAF (*Cg*-LITAF) gene was cloned and characterized. The full-length *Cg*-LITAF cDNA consists of 906 bp and encodes a polypeptide of 115 amino acids. The *Cg*-LITAF gene consists of three exons and two introns, with a length of approximately 1.8 kb. The *Cg*-LITAF protein showed 34–45% amino acid sequence identity with other known LITAF sequences. Although the *Cg*-LITAF coding sequence (115 aa) is shorter than all previously reported LITAF genes, the LITAF domain which contains two CXXC motifs is well conserved. An in vivo expression study showed that *Cg*-LITAF mRNA was expressed predominantly in gills and moderately in digestive gland and labial palps of healthy oysters. The accumulation of *Cg*-LITAF mRNA in oyster haemocytes determined by real-time PCR showed the peak 12 h after bacterial challenge. This expression pattern suggests that *Cg*-LITAF is a potent factor in the regulation of genes that are involved in innate defence mechanisms.

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Introduction

The innate immune system is the first line of inducible host defence against bacterial, fungal, and viral pathogens [1]. This defence system is essential for the survival and perpetuation of all multicellular organisms [2,3]. In particular, aquatic organisms are in constant contact with an environment that contains potentially pathogenic organisms, and they have evolved a number of constitutive and inducible innate immune responses to defend against infection [4]. These innate defence mechanisms of aquatic organisms include the production of broad-spectrum antimicrobial substances and acute phase proteins, non-classical complement activation, release of cytokines, inflammation, and phagocytosis [5]. Especially, invertebrate animals, which lack adaptive immune systems, have developed other systems of biological host defence, innate immunity, that respond to common antigens on the cell surfaces of potential pathogens [6].

To gain insight into the defence mechanisms in oysters, various approaches have been attempted at the cellular and molecular level. Gueguen et al. identified genes involved in defence mechanisms the Pacific oyster C. gigas by expressed sequence tags analysis [7]. Labreuche et al. investigated experimentally the interactions between the Pacific oyster C. gigas immune system and the pathogenic bacterium Vibrio aestuarianus strain 01/32 [8,9]. In addition, the response to a pathogen involves the activation of two major and distinct pathways, which control the transcription of both defence peptides and regulatory cytokines [10]. Among these pathways, related genes have been characterized: antimicrobial peptides (AMPs), defensins [11], pathogen-resistant protein, chitinase [12] and anti-inflammatory cytokines, TGF-β [10] in the Pacific oyster C. gigas. However, the limited research that has been undertaken to date on the exact signalling pathway, involved pathogenic bacterium infections in marine bivalve mollusc.

In recent years, the NF-κB transcription factor, which regulates the transcription of cytokines and antimicrobial effectors, has been isolated from the Pacific oyster C. gigas [13] and the gastropod abalone Haliotis diversicolor super-texta [14]. The expression of these transcription factors is changed by the stimulation with bacterial components, such as lipopolysaccharide (LPS). LPS is the major integral structural component of the outer membrane of Gram-negative bacteria and one of the most potent initiators of inflammation. LPS activates monocytes and macrophages to produce cytokines, such as TNF-α, IL-1, and IL-6, which in turn serve as endogenous inflammatory mediators [15,16]. TNF-α exhibits both beneficial and pathologic effects, thereby necessitating rigorous control of its expression [17–21]. The transcription of TNF-α is regulated by NF-κB [22]. Ets [23], NF-AT [24], AP-1[25] cAMP response element-binding protein [26], and STAT1 [27].

LPS-induced TNF-α factor (LITAF) is one of the most important transcription factors mediating TNF-α transcription. To date, LITAF genes have been identified and characterized only in some mammalian species, ranging from humans to chickens [28–30]. LITAF exerts its effects on TNF-α expression through the formation of a complex with STAT6(B) [31]. Recently, Tang and coworkers have identified a LITAF signalling pathway in mouse macrophages, i.e., a unique LITAF signalling pathway that is separated from NF-κB and that has a role in the regulation of various inflammatory cytokines in response to LPS stimulation [32].

Although intensive studies are underway into the function of LITAF in mammals, limited information is available on LITAF in non-mammals. In the present study we describe the invertebrate LITAF gene isolated from Pacific oyster Crassostrea gigas, investigate the expression of Cg-LITAF in various tissues and compare this gene with its mammalian counterparts.

Materials and methods

Animals and bacterial challenge

Crassostrea gigas oysters (60–70 g in weight) were sampled from a farm in Tongyoung (Korea) and acclimated to laboratory conditions for 10 days before being challenged with bacteria. Oysters were challenged by filing the shell and injecting into the adductor muscle 100 μl of saline peptone water (SPW: peptone 15 g l−1 and NaCl 15 g l−1) as a control or 100 μl of a mixture of four pathogenic Vibrio strains (109 bacteria/ml) as described previously [33]. Bacteria (V. anguillarum, V. mediterranei, V. tubiashi, and V. alginae) were grown separately overnight at 24 °C in SPW, then collected by centrifugation (10,000 × g for 5 min) and resuspended in SPW. Haemolymph was collected from three oysters at 3, 6, 9 and 12 h post-injection and immediately centrifuged (1000 × g for 10 min at 4 °C). The pelleted haemocytes were used for RNA extraction. Unchallenged oysters’ digestive gland, mantle, gill, labial palps, adductor muscle and haemolymph were collected and ground immediately under liquid nitrogen for RNA preparation. Total RNA samples were extracted using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions.

Cloning and sequence analysis of Cg-LITAF

Cloning of the full-length cDNA of Cg-LITAF by 5′-RACE

Previously, we constructed a cDNA library of the oyster digestive gland and carried out expressed sequence tag (EST) analysis of 1133 clones (data not shown). One of the EST clones, oyster-hepa2-G11, shared 47% amino acid identity with the LITAF of zebrafish (accession no. NP_001002184). The oyster-hepa2-G11, which is named Cg-LITAF, contains the open reading frame (ORF) and 3′-untranslated region (UTR) of LITAF. The full-length cDNA was amplified from the first-stranded cDNA of the oyster digestive gland using 5′-RACE and primers based on the oyster-hepa2-G11 sequence (Table 1). The 5′-RACE was performed with the Cg-LITAF-5′-RACE primer and the SMART RACE cDNA Amplification Kit (Clontech), using the universal primer supplied in the kit.

Cloning of the Cg- LITAF gene and 5′-flanking region

Genomic DNA was prepared from oyster adductor muscle using 8 M TNES-urea buffer [34], and purified with phenol/chloroform. Two specific primers (Cg-LITAF-ORF-F and ORF-R) were designed based on the 5′-end and 3′-end
sequences of the Cg-LITAF ORF (Table 1). Genomic DNA (10 ng) was used as the template. PCR was performed with the following settings: denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 7 min. The 5′-upstream region was cloned using the GeneWalker kit (Clontech) with a specific primer (Cg-LITAF-5′-GSP) (Table 1), which was designed based on the nucleotide sequence of the cDNA fragment and the AP-1 primer supplied with the kit.

### Sequence analysis

The nucleotide sequences and deduced amino acid sequences were analyzed using the Genetyx-Win program ver. 4.0 (Genetyx Co., Japan). Multiple alignments of the LITAF proteins were constructed using the ClustalW program. Bootstrap values for phylogenetic tree analysis were built through ClustalW and visualized with MEGA (ver. 3). Transcription factor-binding sites were predicted using the TFSEARCH (http://www.cbrj.jp/research/db/TFSEARCH) and TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) programs.

### Quantitative real-time RT-PCR

The levels of Cg-LITAF expression in digestive gland, mantle, gill, labial palps, adductor muscle, and haemolymph tissues using TRIzol reagent (Invitrogen). Subsequently, first-strand cDNA synthesis was carried out using the Advantage RT-for-PCR Kit (BD Biosciences). The gene encoding the 28S ribosomal DNA was used as internal control. For 28S ribosomal DNA, the forward and reverse primers were 28SrDNA-RF and 28SrDNA-RR, respectively (Table 1). The real-time RT-PCR assay was carried out with the LightCycler FastStart DNA Master SYBR Green I (Roche). The thermal profile for dissociation consisted of: denaturation steps at 95°C for 10 min, followed by 35 amplification cycles of 10 s denaturation at 95°C, 5 s annealing at 55°C and 15 s extension at 72°C. For further expression level analysis was determined for each transcript using the LightCycler software. The copy ratio of each analyzed cDNA was determined as the mean of triplicates. The levels of mRNA were quantified by expressed relative to the 28SrDNA transcript level. The amplified PCR products were analyzed on 1.5% agarose gel containing ethidium bromide (100 ng/ml).

### Table 1

<table>
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<th>Primers used in this study</th>
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<tr>
<td>Cg-LITAF-5′-RACE</td>
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<tr>
<td>Cg-LITAF-ORF-F</td>
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<tr>
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<td>Cg-LITAF-5′-GSP</td>
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<td>Cg-LITAF-RT-F</td>
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<tr>
<td>Cg-LITAF-RT-R</td>
</tr>
<tr>
<td>28SrDNA-RT-F</td>
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<td>28SrDNA-RT-R</td>
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</table>

Cg-LITAF gene. The start (ATG) codon is underlined and the stop (TAA) codon is indicated by an asterisk (*). The polyadenylation signal is shown in bold and underlined. The three ATTTA sequence motifs in the 3′-UTR region are shown in bold. The exon/intron junction site (GT-AG) is in italic and the polyadenylation signal is shown in bold and underlined.
Analysis of the Cg-LITAF sequence

The nucleotide and deduced amino acid sequences of Cg-LITAF (GenBank accession no. EF157939) spans 906 nt and comprises a 5'UTR of 129 nt, a 3'UTR of 429 nt and an ORF of 348 nt that encodes a polypeptide of 115 amino acids (Fig. 1). The deduced polypeptide has a molecular weight of 12.53 kDa and an expected isoelectric point of 6.5. The 3'UTR of the Cg-LITAF transcript contains three mRNA instability motifs (ATTTA) and a polyadenylation signal (AATAAA) 26 nucleotides upstream of the poly(A) tail. The Cg-LITAF gene consists of three exons and two introns, with GT/AG junctions (Fig. 1). The 5'-flanking region of the Cg-LITAF gene has the putative binding sites for several transcription factors, such as STATx (754 to 762 nt, −1017 to −1023 nt), HSF (−977 to −981 nt), IRF-2 (−957 to −968 nt), AP-1 (−921 to −931 nt), C/EBPβ (−668 to −679 nt), Ntx-2.5 (−523 to −529 nt), C/EBPα (−402 to −411 nt), and NF-kB (−259 to −268 nt) (Fig. 1).

Alignments and phylogenetic tree construction

Comparison of the amino acid sequences of Cg-LITAF and the LITAF proteins of vertebrates, including mammals and teleosts, revealed a higher level of homology at the carboxyl-terminus than at the amino-terminus (Fig. 2). The Cg-LITAF protein showed overall identity of 35–56% with the other LITAF proteins (Table 2). A phylogenetic tree was generated using MEGA. Cg-LITAF showed the closest relationship with scallop LITAF, and these proteins formed a cluster with the LITAF proteins of mammals, maintaining a high bootstrap value (Fig. 3).

Expression of the Cg-LITAF transcript in different tissues

Quantitative real-time RT-PCR analysis revealed that Cg-LITAF was expressed at the highest level in gills, with

<table>
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<th>Table 2</th>
<th>Amino acid sequence identities of Cg-LITAF</th>
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<td>Species</td>
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<td>Scallop</td>
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Cloning, characterization and expression analysis of the gene for a putative LITAF

intermediate levels in digestive gland and labial palps, and the lowest levels in adductor muscle, mantle, and haemolymph (Fig. 4A).

The expression of Cg-LITAF in haemocytes after injection of Vibrio sp. was also monitored. In the initial stage of infection, the levels of Cg-LITAF increased slightly. However, Cg-LITAF expression was clearly up-regulated after infection, especially at 12 h post-infection, at which time-point Cg-LITAF expression was at the highest level, as compared to the control (Fig. 4B).

Discussion

In the present study, we cloned, sequenced, and characterized the LPS-induced TNF-α factor (LITAF) from the oyster moderate homology to other known LITAF genes. The Cg-LITAF polypeptide (115 aa) is shorter than all other reported mammalian LITAF.

In multiple alignment analysis, the C-terminal region of Cg-LITAF showed high homology to all other known LITAF proteins, which is consistent with previous reports [28–30]. All the other known LITAF coding sequences showed the highest levels of homology in their C-terminal regions, which contain the LITAF domain. The LITAF domain is a potential membrane-associated motif that contains the CXXC and (H)XCXXC motifs, which are highly characteristic of Zn²⁺-binding domains. This suggests that the LITAF domain, by virtue of the two CXXC motifs, forms a compact Zn²⁺-binding structure instead of inserting itself into the membrane [35].

A phylogenetic tree derived from sequence of vertebrate to non-vertebrate LITAF showed that the Cg-LITAF sequence was split into a distinct group. The scarcity of additional LITAF sequences for other vertebrates, such as fish, birds, and mammals, prevents a more detailed analysis of the position of the Cg-LITAF sequence in the phylogenetic tree. However, it is clear that the LITAF proteins of mammals diverged after the divergence of lower vertebrates from invertebrates.

The LITAF mRNAs in the human, mouse, and chicken are primarily expressed in the lymphoid tissues that constitute the immune system [28–30]. Cg-LITAF mRNAs were presented in all examined tissue and predominantly expressed in gill and moderately in digestive gland and labial palps (Fig. 4A). This result is consistent with expression pattern of TGF-β in oyster [10]; it can be suggested that macrophage-like cells are located in major tissues such as gill, digestive gland and labial palps. In oyster, the fact that Cg-LITAF is ubiquitously expressed also suggests that it might have a pleiotropic function and is involved in a wide variety of biological processes.

Since gills represent the main interface between aquatic organisms and the external environment, mollusc gills are the first line of defence against bacterial infection [36]. Recently, the LITAF gene homologue (CfLITAF) was cloned and characterized first from an invertebrate, Zhikong scallop Chlamys farreri in the course of our studies. CfLITAF mRNA was also expressed primarily in gonad and with intermediate levels in gills [37]. When oyster was challenged with a mixture of pathogenic Vibrio species, the levels of Cg-LITAF mRNA expression were up-regulated 3 h post-infection. These expression patterns are in accordance with those of Yu and Song [37] who observed that CfLITAF mRNA expression was enhanced in cultured haemocytes activated with LPS up to 9 h. However, the expression of CfLITAF transcript was not tested after 9 h stimulation. The Cg-LITAF mRNA expression was up-regulated four-fold after 3 h post-infection, and then decreased gradually after 6 h and 9 h.
post infection. However, nine-fold up-regulation of Cg-LITAF mRNA was observed after 12 h post-infection. It could be speculated that Cg-LITAF mediates not only TNF-α transcription but also other cytokines, which are induced to produce by TNF-α, such as IL1β and RANTES. Recently, there was a report showing that the LITAF directly interacted with STAT6 and forms a LITAF–STAT6 complex, and significantly up-regulated several cytokines [31]. Interestingly, the analysis of these cytokine promoters showed that most of them contain a CTCCC sequence upstream of their TATA signal, a specific DNA-binding site that is specific to LITAF on TNF-α promoter [38]. Even though it is difficult to demonstrate a direct relationship between Cg-LITAF and the expression of cytokines described above, due to the lack of information about the cytokines of molluscs, it can be speculated that Cg-LITAF plays the role of transcription factor for several cytokines, not only TNF-α.

Further studies will focus on identifying the inflammatory cytokines that are linked to this regulatory pathway, with the goal of determining their exact functions and interactions.

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References


