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Identification and function of an evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) from *Crassostrea hongkongensis*

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ABSTRACT

Evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) is a multifunctional adaptor protein that plays a key role in the regulation of the oxidative phosphorylation (OXPHOS) system, bone morphogenetic protein (BMP) pathway and Toll-like receptor (TLR) signaling pathway in mammals. However, the function of ECSIT homologs in mollusks, the second most diverse group of animals, is not well understood. In this study, we identified an ECSIT homolog in the Hong Kong oyster *Crassostrea hongkongensis* (*Ch*ECSIT) and investigated its biological functions. The full-length cDNA of *Ch*ECSIT is 1734 bp and includes an open reading frame (ORF) of 1074 bp that encodes a polypeptide of 451 amino acids. The predicted *Ch*ECSIT protein shares similar structural characteristics with other known ECSIT family proteins. Quantitative real-time PCR analysis revealed that *Ch*ECSIT mRNA is broadly expressed in all of the examined tissues and at different stages of embryonic development; its transcript level could be significantly up-regulated by challenge with microorganisms (*Vibrio alginolyticus*, *Staphylococcus haemolyticus* and *Saccharomyces cerevisiae*). In addition, *Ch*ECSIT was found to be located primarily in the cytoplasm, and its overexpression stimulated the transcriptional activity of an NF-κB reporter gene in HEK293T cells. These findings suggest that *Ch*ECSIT might be involved in embryogenesis processes and immune responses in *C. hongkongensis*.

1. Introduction

The innate immune system is a universal and ancient form of host defense that provides a rapid and non-specific immune response against invading pathogens (Janeway Jr and Medzhitov, 2002). Among the numerous responses of the host innate immune system, the first and most crucial step is the recognition of infectious pathogens mediated by various pattern recognition receptors (PRRs) (Janeway Jr and Medzhitov, 2002; Medzhitov and Janeway, 2002). Toll-like receptors (TLRs) are the best known and most well-studied PRRs in vertebrates; these receptors recognize various types of pathogen-associated molecular patterns (PAMPs) of microorganisms, such as lipopolysaccharide (LPS), peptidoglycan (PGN) and double-stranded RNA. After PAMP recognition, TLRs can either directly or indirectly trigger downstream signaling cascades, resulting in the regulation of cytokine gene expression (Kawai and Akira, 2006; Takeda and Akira, 2004a).

In mammals, the TLR signaling cascade is activated by PAMP signals and is transduced through the homotypic interaction of TLRs (except for TLR3) and cytosolic adaptor protein myeloid differentiation factor 88 (MyD88). MyD88 then triggers the activation of a multi-step cascade of IL-1 receptor-associated kinase 4 (IRAK4), IRAK1 and tumor necrosis factor receptor-associated factor 6 (TRAF6) activation (Hopkins and Sriskandan, 2005; Takeda and Akira, 2004b). The activated IRAK1/TRAF6 complex disassociates from the receptor complex and further activates the downstream TGF-β-activated kinase-1 (TAK1) kinase complex (Besse et al., 2007; Mendoza et al., 2008). The TAK1 complex then phosphorylates and activates IκB kinase (IKK), which leads to the activation of

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nuclear factor kappa B (NF-κB) and ultimately regulates the transcription of numerous specific effector genes (Takeuchi and Akira, 2010).

Evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) was originally discovered as a TRAF6-interacting protein identified in a yeast two-hybrid screen using TRAF6 as bait (Kopp et al., 1999). ECSIT represents a novel signaling intermediate in TLR signaling pathways, and the protein can interact directly with the conserved TRAF domain of TRAF6 and provide a link to MAP kinase kinase-1 (MEKK-1), ultimately leading to the transcriptional activation of inflammatory genes (Kopp et al., 1999). A recent report demonstrated that ECSIT forms a signaling complex with TAK1 and TRAF6 through specific molecular interactions that play an essential role in regulating NF-κB-dependent gene expression and the production of proinflammatory cytokines induced by TLR4 stimulation (Wi et al., 2014). Previous studies have also shown that as an adaptor protein, ECSIT interacts with MAVS in mitochondria and positively regulates RIG-I-like receptor (RLR)-mediated signaling, one of the most important antiviral immune signaling processes in vertebrates (Kondo et al., 2012). In addition to its essential roles in the innate immune response, ECSIT is reported to play a key role in the bone morphogenetic protein (BMP) pathway, which is required for normal embryonic development and adult tissue homeostasis. Xiao et al. found that ECSIT could interact with Smad1 and Smad4 in the nucleus, thereby regulating the transcription of the Tlx2 gene, a BMP signaling pathway gene (Xiao et al., 2003). Recently, it was demonstrated that ECSIT is also involved in mitochondrial processes. Vogel et al. reported that knockdown of ECSIT expression resulted in decreased protein levels of the molecular chaperone NDUFAF1 and the mitochondrial complex (Vogel et al., 2007). Furthermore, West et al. found that ECSIT ubiquitination and enrichment at the mitochondrial periphery induced by TRAF6 may result in the augmentation of mitochondrial ROS, contributing to macrophage bactericidal activity and the generation of effector responses in mice (West et al., 2011).

In recent years, the function of invertebrate ECSITs has attracted much attention due to the crucial roles of their mammalian homologs in innate immunity. For example, Kopp et al. identified *Drosophila* ECSIT (dECSIT) and demonstrated that dECSIT specifically binds to *Drosophila* TRAF6 (dTRAF6) and induces the transcription of host defense genes in insect cells (Kopp et al., 1999). Additionally, MjECSIT1, a crustacean homolog of ECSIT, was cloned and reported to be essential for the antibacterial immunity of *Marsupenaeus japonicas* (Ding et al., 2014). The first mollusk ECSIT was recently reported in Pacific oyster and was shown to be involved in the immune defense response to bacterial challenge (Zhang et al., 2012). However, compared to studies in vertebrates, these reports have provided limited information of ECSITs in invertebrates.

Hong Kong oysters (*Crassostrea hongkongensis*) are one of the most economically important shellfish along the southern coastline of China, but they often encounter numerous types of pathogens, including bacteria, fungi and viruses, which cause serious economic losses to oyster aquaculture. Similar to other bivalves, *C. hongkongensis* lacks adaptive immunity, and their defense mechanisms rely on an innate immune system to fight foreign microbes. By acting as pattern recognition receptors, TLRs play an essential role in the activation of the innate immune response in mollusks (Zhang et al., 2013). Investigation into the TLR pathway of *C. hongkongensis* might contribute to the development of strategies for the prevention and treatment of diseases associated with pathogen-induced inflammation. In this regard, ECSIT, a conserved adaptor protein in the TLR signaling pathway, was identified from *C. hongkongensis*, and its mRNA expression in different tissues and developmental stages were investigated by quantitative real-time PCR (qRT-PCR). In addition, the mRNA expression profiles of *Ch*ECSIT upon exposure to immune challenge were also examined in hemocytes. Finally, *Ch*ECSIT was overexpressed in human embryonic kidney 293T (HEK293T) cells to determine its intracellular localization and function in signal transduction.

2. Materials and methods

2.1. Cloning and sequencing of ChECSIT cDNA

A search of the transcriptome data of *C. hongkongensis* hemocytes (unpublished) revealed an EST contig homologous to the ECSIT gene of *Crassostrea gigas* (AEN71568.1). Based on the identified EST sequence, gene-specific primers (Table 1) were designed to amplify the full-length *Ch*ECSIT cDNA using the rapid amplification of cDNA ends (RACE) approach. To obtain the 3'-end of *Ch*ECSIT, primer pairs Takara3P/*Ch*ECSIT-F1 and Takara3NP/*Ch*ECSIT-F2 (Table 1) were employed for primary PCR and nested PCR, respectively. Similarly, the 5'-end of the *Ch*ECSIT gene was obtained by nested PCR using the primer pairs Takara5P/*Ch*ECSIT-R1 and Takara5NP/*Ch*ECSIT-R2 (Table 1). All of the specific PCR products were cloned into the pMD19-T vector (TaKaRa, Japan) and sequenced with universal primers M13-47 and RV-M using an Applied Biosystems (ABI) 3730 DNA Sequencer (PerkinElmer, Wellesley, MA, USA). Full-length cDNA sequences were obtained by combining 3'- and 5'-end sequences.

2.2. Sequence and phylogenetic analyses

The cDNA sequences and deduced amino acid sequences of *Ch*ECSIT were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The deduced amino acid sequence was analyzed using ORF Finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi), and identity and similarity analyses were performed with MatGAT2.02 (Campanella et al., 2003). The SMART program (http://smart.embl-heidelberg.de/) was used to predict functional sites and domains in the deduced amino acid sequence. The isoelectric point (pI) and molecular weight (MW) of the deduced *Ch*ECSIT protein were calculated by the compute pI/Mw tool (http://web.expasy.org/compute_pi/). Multiple protein sequences were aligned using the MegAlign program via the Clustal W method in the DNASTAR software package. A phylogenetic tree was constructed using MEGA 5.05 (http://www.megasoftware.net/) based on the neighbor-joining method with 1000 bootstrap repetitions (Tamura et al., 2011). The GenBank accession numbers corresponding to the ECSIT protein sequences are listed in Table 2.

2.3. Oysters, immune challenge and sample collection

Healthy *C. hongkongensis* (two years of age and with an average shell length of 10.0 cm) were collected from a local farm in Zhanjiang, Guangdong Province, China, and cultured in aerated seawater (salinity, 20‰) at 25 °C for a week before processing.

For *Ch*ECSIT expression analysis in different tissues, hemocytes, hearts, mantles, adductor muscles, digestive glands, gonads and gills were collected from five Hong Kong oysters. For analysis of the developmental expression patterns of *Ch*ECSIT, samples were collected from the following stages: fertilized egg, 2-cell, 4-cell, blastula, gastrula, trochophore and D-larva. The collected samples were immersed in TRIzol, quickly frozen in liquid nitrogen, and then stored at −80 °C until RNA isolation.

An *in vivo* infection experiment was performed according to our previous work (Qu et al., 2015). Briefly, healthy oysters were