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An invertebrate signal transducer and activator of transcription 5 (STAT5) ortholog from the disk abalone, *Haliotis discus discus*: Genomic structure, early developmental expression, and immune responses to bacterial and viral stresses

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ABSTRACT

Signal transducer and activator of transcription (STAT) family members are key signaling molecules that transduce cellular responses from the cell membrane to the nucleus upon Janus kinase (JAK) activation. Although seven STAT members have been reported in mammals, very limited information on *STAT* genes in molluscans is available. In this study, we identified and characterized a STAT paralog that is homologous to STAT5 from the disk abalone, *Haliotis discus discus*, and designated as AbSTAT5. Comparison of the deduced amino acid sequence for AbSTAT5 (790 amino acids) with other counterparts revealed conserved residues important for functions and typical domain regions, including the N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, and Src homology 2 (SH2) domains as mammalian counterparts. Analysis of STAT phylogeny revealed that AbSTAT5 was clustered with the molluscan subgroup in STAT5 clade with distinct evolution. According to the genomic structure of *AbSTAT5*, the coding sequence was distributed into 20 exons with 19 introns. Immunologically essential transcription factor-binding sites, such as GATA-1, HNF, SP1, C/EBP, Oct-1, AP1, c-Jun, and Sox-2, were predicted at the 5′-proximal region of *AbSTAT5*. Expression of *AbSTAT5* mRNA was detected in different stages of embryonic development and observed at considerably higher levels in the morula and late veliger stages. Tissue-specific expressional studies revealed that the highest level of *AbSTAT5* transcripts was detected in hemocytes, followed by gill tissues. Temporal expressions of *AbSTAT5* were analyzed upon live bacterial (*Vibrio parahemolyticus* and *Listeria monocytogenes*), viral (viral hemorrhagic septicemia virus), and pathogen-associated molecular pattern (lipopolysaccharides and Poly I:C) stimulations, and significant elevations indicated immune modulation. These results suggest that *AbSTAT5* may be involved in maintaining innate immune responses from developmental to adult stages in the disk abalone. Further, this study provides a basis for structural and functional exploration of STAT members in the invertebrate JAK/STAT signaling pathway.

1. Introduction

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling cascade, which is composed of three main components and includes receptors such as JAK and STAT, is important for transmitting information received from extracellular peptides to the nucleus (Aaronson and Horvath, 2002). In mammals, the JAK/STAT pathway is the principal signaling system in response to a wide array of cytokines and growth factors, and it mediates various biological events such as immune responses, proliferation, differentiation, cell migration, apoptosis, and cell survival (Harrison, 2012; Rawlings et al., 2004; Richard and

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STAT proteins are key transcription factors that transduce signals into the nucleus upon phosphorylation via JAK activation. Once the activated STAT proteins translocate into the nucleus, they can interact with the corresponding transcription factor-binding sites located in the promoters that mediate the expression of cytokine-inducible genes and activate their transcription (Darnell et al., 1994; Mitchell and John, 2005). To date, seven distinct STAT members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) have been identified and well-documented in mammals (Stark and Darnell, 2012). Structurally, these STATs resemble and share common topological domains, including the N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, Src homology 2 (SH2) domain, and transcriptional activation domain, even though they are functionally unlike (O'Shea et al., 2002).

STAT5, one of the most important members of the STAT family, is activated in response to a wide range of cytokines and hormones, such as interleukin-2 (IL-2), IL-3, IL-5, IL-7, IL-9, IL-15, erythropoietin, thrombopoietin, growth hormones, and prolactin (Takeda and Akira, 2000; Teglund et al., 1998). In mammals, two closely related isoforms, STAT5a and STAT5b, have been identified, and their specific functions were demonstrated using STAT5a and STAT5b knockout mice. STAT5a is crucial for Prl-mediated mammary gland development, whereas STAT5b is essential for sexually dimorphic growth (O'Shea et al., 2002).

Identification and functional analysis of compounds involved in the invertebrate JAK/STAT signaling cascade are still in the preliminary stage. The invertebrate JAK/STAT signaling pathway has been established in *Drosophila melanogaster* with structural and functional homologs, such as STAT members and some proteins analogous to JAKs (Hombria and Brown, 2002; Zeidler et al., 2000). The homolog of *D. melanogaster* STAT has been shown to be involved in embryonic development (Yan et al., 1996). The STAT member identified in *Anopheles gambiae*, which is mostly similar to the vertebrate STAT5 and STAT6, manifested immune responses against bacterial infections (Barillas-Mury et al., 1999). In addition, *STAT* genes have been identified in *Caenorhabditis elegans* (Liu et al., 1999) and *Dictyostelium discoideum* (Kawata et al., 1997). Studies on molluscan STAT members are scarce. In a recent study, two STAT members from a fresh water snail, *Biomphalaria glabrata*, were identified and their evolutional conservation was determined (Salinas et al., 2011).

The disk abalone, *Haliotis discus discus*, is one of the most important marine gastropods commercially cultured in the Republic of Korea. During the past few decades, frequent disease outbreaks were observed because of habitat contamination by chemicals and microbial pathogens, leading to demolition of the disk abalone industry (Cai et al., 2008; Chang et al., 2005; Hooper et al., 2007). Similar to other invertebrates, the disk abalone has to rely primarily on the innate immune system, since it does not have an adaptive immune system (Loker et al., 2004). Investigation of signaling cascades like JAK/STAT has not yet been fully established in mollusks, but it may be essential for improvement of health management and disease control in disk abalone culture. In the present study, a member of the STAT family, homologous to STAT5, was identified in the disk abalone and characterized at cDNA and genomic levels. To understand the molecular evolution, comprehensive sequence analysis was conducted in terms of phylogeny and genomic organization by using other STAT family members. For the functional aspects, spatial expression in early stages of embryonic development and several adult tissues and temporal expression in adult gills and hemocytes upon pathogenic and PAMP stress were investigated.

2. Materials and methods

2.1. Identification and characterization of disk abalone STAT5 gene

A cDNA contig, homologous to *STAT5*, was identified in the disk abalone transcriptome database established by the Roche 454 Genome Sequencer FLX system (GS-FLX™), a next-generation sequencing platform (Droege and Hill, 2008) and termed *AbSTAT5*. The sequence was confirmed on the basis of BLASTp results in the National Center for Biotechnology Information (NCBI). The genomic sequence of *AbSTAT5* was obtained from a disk abalone bacterial artificial chromosome library (BAC) constructed by Lucigen® Co. (Middleton, WI, USA) by using randomly sheared genomic DNA from gill tissue (Umasuthan et al., 2013). Screening of the BAC library was performed using a polymerase chain reaction (PCR)-based technique with gene-specific primers (*AbSTAT5-BAC-F*: 5′-ACCAATCTAGTTCAGGGCAGCTCA-3′ and *AbSTAT5-BAC-R*: 5′-AAACACAAGCTCATTGCCTCCGAC-3′), according to the manufacturer's instructions, and a positive BAC clone was sequenced using the 454 GS-FLX™ pyrosequencing approach.

Primary sequence characterization of AbSTAT5 was conducted using various bioinformatic tools available in the Expert Protein Analysis System (http://www.expasy.org/). Characteristic domain regions were predicted on the basis of the mouse STAT5a crystal structure and previous studies (Hwa et al., 2011; Neculai et al., 2005). ClustalW was used to align multiple amino acid sequences, and the BioEdit (v.7.2) sequence alignment editor was used to generate a graphic view of the alignment. A phylogenetic tree was constructed using MEGA 6.0 software (Tamura et al., 2013) by using the neighbor-joining (NJ) method based on the ClustalW alignments. About 5000 iterations of bootstrapping were used to test the topology of the phylogenetic tree. Tertiary structure of AbSTAT5 was modeled using SWISS-MODEL, a fully automated protein structure homology-modeling server (Schwede et al., 2003), and the mouse STAT5a crystal structure (PDB: 1Y1U, 3.21 Å resolution), which shared 57.01% identity with AbSTAT5, as a template. PyMOL molecular graphic software (DeLano, 2002) was used to visualize the tertiary structure of AbSTAT5. Spidey program of NCBI (Wheelan et al., 2001) was used to determine the exon-intron boundaries in the genomic sequence of *AbSTAT5*. Possible transcription factor-binding sites in the 5′-flanking region of AbSTAT5 were predicted using AliBaba 2.1 with default parameters (http://www.gene-regulation.com/pub/programs/alibaba2/index.html).

2.2. Fertilization and collection of different stages of embryonic development

Eggs and sperm of disk abalones (*H. discus discus*) were collected from the Ocean and Fisheries Research Institute, Jeju Island, Republic of Korea, and allowed to fertilize in filtered sea water at 18 °C. Different stages of early embryonic development, namely, egg, 16-cell stage (3 h post fertilization [pf]), morula (4 h and 30 min pf), gastrula (6 h pf), trochophore (16 h pf), and three time points of the veliger stage (early: 24 h pf, middle: 36 h pf, and late: 48 h pf) were collected. Before the collection of samples, developmental synchronies of each stage were examined under the light microscope to confirm that more than 75% of the sample was at the same stage. Subsequently, the collected samples were washed with cold phosphate-buffered saline and stored at −80 °C until RNA extraction.

2.3. Animal rearing and immune challenges

Adult disk abalones (~50 g) were purchased from a commercial