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Three members in JAK/STAT signal pathway from the sea cucumber *Apostichopus japonicus*: Molecular cloning, characterization and function analysis

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ABSTRACT

The JAK/STAT signal transduction pathway plays a critical role in host defense against bacterial infections. In the present study, we firstly cloned the full-length cDNAs of three molecules in JAK/STAT cascade, *STAT5*, *FOXP* and *SOCS2*, from sea cucumber *Apostichopus japonicus* (denoted as *AjSTAT5*, *AjFOXP*, *AjSOCS2*, respectively) and investigated their immune functions towards *Vibrio splendidus* infection and LPS exposure. The *AjSTAT5* cDNA was composed of 2643 bp consisting of 787 amino acid residues which included protein interaction domain, STAT-α domain, DNA binding domain and SH2 domain. The putative AjFOXP contained a ZnF_C2H2 domain, the leucine zipper-like domain and FH domain, all of which were thought to be the representative characteristics of FOXP subfamily. The deduced amino acids sequence of AjSOCS2 included an SH2 domain and SOCS box domain similar to vertebrate SOCS counterparts. Phylogenetic trees further supported that all these three identified proteins belonged to novel members of JAK/STAT signal pathway in sea cucumber. Tissue specific expression analysis showed that three genes were ubiquitously expressed in all examined tissues. *AjSTAT5* and *AjFOXP* were both dominantly expressed in intestine, tentacle and respiratory tree, and weak in muscle. In contrary, the peak expression of *AjSOCS2* was observed in muscle and lowest in respiratory tree. The *V. splendidus* challenge and LPS exposure could both significantly up-regulate the mRNA expression of three genes, in which *AjSOCS2* showed opposite expression trends to those of *AjSTAT5* and *AjFOXP*. Silencing the *AjSTAT5* by siRNA depressed the *AjFOXP* expression, but induced the expression level of *AjSOCS2*, revealing that *AjSTAT5* might directly modulate *AjFOXP*, and *AjSOCS2* function primarily by acting as a potent inhibitor involve in JAK/STAT pathway. The present study would expand our understanding on JAK/STAT signaling transduction pathway in modulating the innate immune responses of sea cucumber.

1. Introduction

Cytokines activate cell surface receptor complexes and regulate a vast array of biological processes including survival, proliferation, differentiation and immune response [1,2]. The Janus family of protein tyrosine kinases (JAKs) and the signal transducers and activators of transcription proteins (STATs) both play important roles in this cytokine-driven regulation of innate and acquired immunity [3,4]. As one of the key members of JAK/STAT signaling pathway, STATs family is activated to regulate gene expression in response to a large number of extracellular signals [5]. When cytokines, interferons, or other stimulation activate JAK kinases, tyrosine residues in STATs are phosphorylated by activating JAKs, which activate STAT dimers or tetramers and then translocate signals to the nucleus where they bind to specific DNA sequences and induce target genes transcription [3,6,7]. At present, a total of seven different STAT proteins (STAT1, 2, 3, 4, 5A, 5B and 6) have been described in mammals. These proteins demonstrate structural homology with an N-terminal domain, a coiled coil domain, a DNA binding domain, a linker domain, a Src homology 2 (SH2) domain and C-terminal transcription activation domain [8,9]. In marine invertebrates, *STAT* genes have been identified from black tiger shrimp *Penaeus monodon* and kuruma shrimp *Marsupenaeus japonicus*, and displayed induced expression profiles after WSSV or peptidoglycan exposure [10,11].

Triggering phosphorylation of *STAT5* and its translocation to the

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nucleus where it can activate the transcription factor *FOXP3*, which acting as one of JAK/STAT signal pathway downstream molecules and stimulating the differentiation of regulatory T (Treg) cells in vertebrate [12]. The FOXP subfamily is a member of the Forkhead box (FOX) gene family of transcription factors that comprises four members with diverse functions [13]. *FOXP1/2/4* are abundantly expressed throughout the development, whereas *FOXP3* is predominantly expressed in the immune system [14,15]. *FOXP3* is a T cell-specific transcription factor and plays a key role in the development of Treg cells and in the immune regulatory process during inflammation. The FOXP subfamily has been well documented in vertebrate, however, their invertebrate counterparts still remain unclear.

When activation of innate immunity results in the induction of a pro-inflammatory release which is intended to defend against the invading pathogen. However, overshooting immune reaction is harmful to host cells and requires down regulation genes to mediate. Suppressor of cytokine signaling (SOCS) proteins act as negative regulators are crucially involved in the control of excessive cytokine effects and inflammatory responses through their impact on various signaling pathways including the JAK/STAT pathway [1,16]. The discovery of the SOCS proteins seemed to explain how the cytokine-JAK-STAT pathway was negatively regulated. So far, eight SOCS proteins have been investigated, including cytokine-inducible SH2 containing protein (CISH) and SOCS1 to SOCS7, each of which shares a central SH2 domain and a carboxy-terminal SOCS-box domain [17]. The SH2 domain in SOCS family compete with STATs-SH2 domain or hinder the STAT binding sites of receptors, inhibiting STATs activation, especially in the case of STAT5 [18]. Previous studies had shown that microorganisms can induce different SOCS proteins [19,20], but only few reports have been characterized the relationship between *JAK/STAT* and *SOCS* in invertebrate after immunostimulation [11,21]. In vertebrate, SOCS proteins have other functions. For example, *SOCS1* and *SOCS3* mainly regulate T cells as well as antigen-presenting cells, such as macrophages and dendritic cells to promote host development and against pathogenic infections [1]. *SOCS1* plays important roles in T cell integrity and function by maintaining *FOXP3* expression and by suppressing IFN-γ and IL-17 production driven by *STAT1* and *STAT3*, respectively [22]. To date, Knosp et al. [23] have been demonstrated that *SOCS2* is also required for stable expression of *FOXP3* in periphery for inducible T cells (iTregs) in *vitro* and *vivo*. It is well established that lower vertebrates possess T cells and T cell-mediated immune responses like mammals [24,25]. Whereas, no evidences demonstrate T cells or *FOXP3* gene exists in invertebrate.

The invertebrate sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea) as a commercially important species in Chinese marine culture, lacks an acquired immune system and is therefore depending on its innate immune system to detect infectious pathogens [26]. Identification of novel immune-related genes involved in specific signaling pathways will give new insights into the immune defense mechanisms and control the disease outbreaks in this specie, particularly the JAK/STAT signal transduction pathway, which plays a critical role in host defense against bacterial infections [11,27]. In current study, we will firstly give insights into the structural evolution and functional characteristics of *STAT*, *FOXP*, and *SOCS* genes in *A. japonicus*. The main objectives of the present study are: (1) to clone the full-length cDNA of *STAT5*, *FOXP*, and *SOCS2* from *A. japonicus* (designated as *AjSTAT5, AjFOXP, AjSOCS2*); (2) to investigate tissue-specific expression of three members and their response to *Vibrio splendidus* and LPS challenge *in vivo* or *in vitro*; and (3) to elucidate the connection among *AjSTAT5, AjFOXP* and *AjSOCS2* after *AjSTAT5* silencing, in order to provide more information to understand the immune defense roles in invertebrate.

2. Materials and methods

2.1. Experimental animals and challenge studies

Sea cucumber *A. japonicus* (weight: 125 ± 15 g) were collected from Bowang Aquaculture Company (Ningbo, China) and acclimatized in 30 L aerated natural seawater (salinity 28 psu, temperature 16 °C) for three days. For immune challenged experiment, one tank served as the control, and the other five tanks contained live *V. splendidus* at a high density, with a final concentration of 10^7 CFU mL^{-1}. The infection dose and sampling points were determined by immune gene expression analysis. The coelomocytes from 5 individuals for the control and the challenged groups were collected at 0, 6, 24, 48, 72 and 96 h, respectively. Sea cucumber were dissected by sterilized scissors on ice and the coelomic fluids were filtered through a 300 Mesh CellCribble to remove large tissues debris. The coelomocytes were harvested by syringe and then centrifuging at 800 × g for 5 min for time-course expression analysis. For spatial expression analysis, coelomocytes and other four tissues including muscle, tentacle, respiratory trees, intestine were collected from control individuals using sterilized scissors and tweezers. These tissues (*approx.* 100 mg wet weight) were ground into powder in liquid nitrogen using a mortar and a pestle. We performed 5 replicates in experimental group as well as the control group and all samples were stored at −80 °C for RNA extraction.

2.2. Rapid application of cDNA ends (RACE)

Partial sequences of *STAT5*, *FOXP*, and *SOCS2* genes were generated by screening *A. japonicus* transcriptome database [28]. Gene-specific primers for *AjSTAT5*, *AjFOXP* and *AjSOCS2* (Table 1) were designed based on the acquired unigenes and the full-length cDNA sequences were subsequently cloned using the 3′,5′-Full RACE Kit (TaKaRa) following the manufacturer's instructions. The desired PCR products were purified and then cloned into the pMD18-T simple vector (TaKaRa). Three positive clones for each product were sequenced at Sangon (Shanghai, China).

2.3. Sequence analysis

Sequences homology were obtained using BLAST program at

Table 1
Primers used for cloning and quantitative real-time PCR.

Primer name	Primer sequence (5′-3′)	Used for
AjSTAT5 3-1	GGTAGGAGTCTGTCCAATGATAA	3′ RACE
AjSTAT5 3-2	CTTACAAGGAAGCACTTTAGAGG	
AjSTAT5 5-1	TGTTCAAGTCTTTGGCAGTATCC	5′ RACE
AjSTAT5 5-2	TTGAATACCGCATCTGGAACTCT	
AjFOXP 3-1	TCTCAGGAAATCCACCACAATGC	3′ RACE
AjFOXP 3-2	GAAATGCTGCCACCTGGAAGAAT	
AjFOXP 5-1	GCGGAGCCAGTAAATAGCCAGGA	5′ RACE
AjFOXP 5-2	AGGTGAGACATCCCTGGTGGCAT	
AjSOCS2 3-1	GGGCGAAGCAGCCAAGAAGACAT	3′ RACE
AjSOCS2 3-2	TAAAACGCCGAGAGGGACGACGA	
AjSOCS2 5-1	GTTCGTGTCTATCTCTGCCTTCG	5′ RACE
AjSOCS2 5-2	CGATTCTAACACTCGTCGTCCCT	
AjSTAT5 F	TACAAGGAAGCACTTTAGAGGGC	Real-time PCR
AjSTAT5 R	CCTCTCACCTCTGTCATTTTCGC	
AjFOXP F	TCTCAGGAAATCCACCACAATGC	Real-time PCR
AjFOXP R	ATTCTTCCAGGTGGCAGCATTTC	
AjSOCS2 F	GCGAAGCAGCCAAGAAGACAT	Real-time PCR
AjSOCS2 R	TAACACTCGTCGTCCCTCTCG	
Ajβ-actin F	CCATTCAACCCTAAAGCCAACA	Real-time PCR
Ajβ-actin R	ACACACCGTCTCCTGAGTCCAT	