Fish & Shellfish Immunology 42 (2015) 108–113

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Nuclear factor of activated T cells (NFAT) in pearl oyster *Pinctada fucata*: Molecular cloning and functional characterization

Xian-De Huang, Guo-jian Wei, Hua Zhang, Mao-Xian He*

Key Laboratory of Tropical Marine Bio-Resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China

ARTICLE INFO

Article history:
Received 23 September 2014
Received in revised form
23 October 2014
Accepted 28 October 2014
Available online 6 November 2014

Keywords:
Pearl oyster
PfNFAT
Gene cloning
Gene expression

ABSTRACT

Nuclear factor of activated T cells (NFAT) plays an important role in nonimmune cells and also in T cells and many other cells of the immune system, by regulating the expression of a variety of genes involved in the immune response, organ development, developmental apoptosis and angiogenesis. In the present study, the NFAT homology gene, PfNFAT, from the pearl oyster *Pinctada fucata* was cloned and its genomic structure and promoter were analyzed. PfNFAT encodes a putative protein of 1226 amino acids, and contains a highly conserved Rel homology region (RHR) with DNA-binding specificity, and a regulatory domain (NFAT homology region, NHR) containing a potent transactivation domain (TAD). The PfNFAT gene consists of 12 exons and 11 introns, and its promoter contains potential binding sites for transcription factors such as NF-κB (Nuclear factor κB), STATx (signal transducer and activator of transcription), AP-1 (activator protein-1) and Sox-5/9 (SRY type HMG box-5/9), MyoD (Myogenic Differentiation Antigen) and IRF (Interferon regulatory factor). Comparison and phylogenetic analysis revealed that PfNFAT shows high identity with other invertebrate NFAT, and clusters with the NFAT5 subgroup. Furthermore, gene expression analysis revealed that PfNFAT is involved in the immune response to lipopolysaccharide (LPS) and Polyinosinic-polycytidylic acid (poly I:C) stimulation and in the nucleus inserting operation. The study of PfNFAT may increase understanding of molluscan innate immunity.

1. Introduction

Nuclear factor of activated T cells (NFAT) was initially identified as a transcription factor that could bind the antigen receptor response element (ARRE-2) of the human interleukin-2 (IL-2) promoter in activated T cells [1]. In mammals, five NFAT genes (NFAT1–5) have been identified, encoding NFAT1 (NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3) and NFAT5 (TonE-BP or NFATL1). These proteins are found to play an important role in nonimmune cells and also in T cells and many other cells of the immune system, by regulating the expression of a variety of genes involved in the immune response, organ development, developmental apoptosis and angiogenesis [2–4].

Similar to the NF-κB (Nuclear factor κB) transcription factors, NFAT proteins have a well-conserved Rel homology region (RHR) with DNA-binding specificity, and a moderately conserved regulatory domain (NFAT homology region, NHR) containing a potent transactivation domain (TAD). The NHR contains multiple serine-rich regions (SRRs) for phosphorylation and docking sites for calcineurin and the NFAT kinases. As transactivation sites, the N- and C-terminal amino acid sequences of NFAT have less sequence similarity and their molecular functions remain poorly understood. The NFAT proteins have weak DNA-binding capacity and they must cooperate with other nuclear resident transcription factors, which forces NFAT proteins to integrate their signal pathways with other signaling pathways [3,5,6].

Most NFATs (NFAT1–4) are dephosphorylated in response to activated calcium flux and tightly controlled by calcineurin, a serine/threonine phosphatase [2,7]. They translocate to the nucleus and bind to the promoter regions of target genes in cooperation with other NFATs and additional co-activators. Unlike the other NFAT proteins, NFAT5 is regulated in a calcineurin-independent manner, and is activated by osmotic stress [8,9].

Unlike the situation in vertebrates, especially mammals, little is known about NFATs in invertebrates. A NFAT5-like gene emerged in *Nematostella vectensis* [10]. In *Drosophila melanogaster*, the NFAT5-like protein (DmNFAT5) was found to be expressed by almost all cells. It is activated in response to osmotic stress, and regulates presynaptic development and activity-dependent plasticity [11,12]. In

* Corresponding author. Tel./fax: +86 20 89023144.
E-mail address: hmx@scsio.ac.cn (M.-X. He).

http://dx.doi.org/10.1016/j.fsi.2014.10.029
1050-4648/

Branchiostoma belcheri, a NFAT gene was identified that is involved in innate immunity [13], which is the only experimental report that invertebrate NFAT involved in the immune response. To provide insight into the role of NFATs in the innate immunity of invertebrate animals, it is necessary to isolate and characterize NFAT homologs from these species.

In the present study, we cloned and characterized the PfNFAT homolog, and analyzed its genomic structure, from the pearl oyster *Pinctada fucata*, one of the most important bivalve molluscs for seawater pearl production. In recent years, disease outbreaks, especially those caused by bacteria and viruses, have become severe. Combined with environmental pollution and the injury arising from the nucleus inserting operation, this has resulted in mass mortality of pearl oysters and brought heavy economic losses [14,15]. It is necessary to improve understanding of the innate immune defense mechanism of the pearl oyster. To investigate the possible biological roles of PfNFAT, its patterns of expression were analyzed after immune stimulation and the nucleus inserting operation.

2. Materials and methods

2.1. Animals and sample collection

Pearl oysters (shell length 4.5–5.5 cm) were collected from two places: Daya Bay in Shenzhen and Xuwen in Zhanjiang, Guangdong Province, China. The immune stimulation experiment were performed in Daya Bay. Pearl oysters were acclimated in indoor cement ponds at ambient seawater temperature for one week before experimentation. The nucleus inserting operation experiments were performed in Xuwen, and pearl oysters were directly collected from the sea. For analyses of gene expression in different tissues, the digestive gland, gills, adduct muscle, hemocytes, heart, mantle, foot, intestine and gonad were collected from pearl oysters. Among these tissues, the hemocytes were used for quantitative PCR (qPCR) expression analyses. To harvest hemocytes, the haemolymph was collected from the pericardial cavity through the adductor muscle using a syringe, and immediately centrifuged at 5000 × rpm for 2 min. Nine individuals were randomly selected at each time point. Each group of nine individuals was randomly divided into three replicates, then the appropriate tissues were obtained from each replicate and pooled as one sample. All samples were stored in Sample Protector (TaKaRa, Japan) until used.

2.2. Obtaining full-length cDNA and genomic sequence of PfNFAT

Gigabase-scale transcriptome sequencing, assembly and functional annotation of the pearl oyster *P. fucata* have been performed by our laboratory [16]. By BLAST and contig analysis of the all annotation sequences (ESTs) from our lab and predicted transcripts from the *Pinctada fucata* genome Ver 1.00 (http://marinegenomics.oist.jp/), an approximately 4000 bp fragment of the NFAT homolog sequence was obtained. To obtain full-length cDNA of the PfNFAT homolog, rapid amplification of cDNA ends (RACE) was performed. Total RNA was extracted from the digestive gland and gills of pearl oysters using a Mollusc RNA Kit (Omega, USA), following the manufacturer's instructions. Sequence analysis showed that the PfNFAT cDNA had a 5′-terminal but lacked 3′-terminal. Then 3′-RACE was performed using a SMART RACE cDNA Amplification Kit (Clontech, Japan), following the manufacturer's instructions. The 3′-RACE was performed using specific primers (3RPNFATseq3771 and 3RPNFATseq3958) and Universal Primer A Mix/Nest Universal Primer (Table S1). Nested or semi-nested PCR was employed. The PCR products were purified with a Gel Extraction Kit (Omega, USA), following the manufacturer's instructions, and sequenced. Meanwhile primers (RT5NFATseq197 and RT3NFATseq4082) were used to verify the full-length cDNA by performing RT-PCR (Table S1).

To obtain the genomic DNA sequence of PfNFAT, the BlastN program using the full-length PfNFAT cDNA was used on the *Pinctada fucata* genome Ver 1.00. It showed that scaffold3719.1 covered the full-length of PfNFAT cDNA, except for three gaps. For the complete PfNFAT genomic structure, genomic DNA was isolated from one *P. fucata* adduct muscle using a Mollusc DNA Kit (OMEGA, USA), following the instruction manual. Primers (Table S1) were designed to overlap the gaps in the genomic sequence of the fragments, and the PCR products were treated as mentioned above.

2.3. Sequence analysis and amino acid alignment

Sequence similarities were analyzed using the BLAST algorithm at the NCBI web site (http://www.ncbi.nlm.nih.gov/blast). SMART (Simple modular architecture research tool, http://smart.embl-heidelberg.de) and the NCBI CDS program (Conserved Domain Search, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to analyze the deduced amino acid sequences. The *Pinctada fucata* genome Ver 1.00 was searched by the BlastN program using the full-length PfNFAT cDNA to obtain the genomic DNA sequence of PfNFAT. The exon/intron structure was analyzed using Spidey, an mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/spidey/). The sequence of the 5′-promoter region of PfNFAT was analyzed using TRANSFAC software for potential transcriptional factor binding sites (http://www.gene-regulation.com/pub/programs.html). Based on amino acid sequences, the comparison and phylogenetic analysis were performed with Clustal omega – Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA software 6 using the neighbor-joining algorithm and performing 10,000 bootstrap replications.

2.4. Immune stimulation and nucleus inserting operation experiments

The immune stimulation experiment comprised three groups: the lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (poly I:C) and phosphate-buffered saline (PBS) stimulation groups. Each pearl oyster was injected in the adductor muscle with 100 μl of LPS (Sigma, Germany) dissolved in sterile PBS (LPS 1 μg/μl), 100 μl poly I:C (Invivogene, USA) dissolved in sterile PBS (polyI:C 1 μg/μl), or 100 μl sterile PBS, respectively. Nine untreated pearl oysters were used as the blank group (0 h). A total of five time points, 6, 12, 24, 48 and 72 h after injection respectively, were selected. The nucleus inserting operation can trigger a series of physiological responses including the immune response, and bring the injury and even death to the pearl oyster. For the nucleus inserting operation experiment, a total of six 6 points, 1, 3, 5, 7, 15 and 30 days after the nucleus inserting operation, respectively, were selected, and day 0 was recorded as the blank group.

2.5. RT-PCR and quantitative PCR (qPCR) analysis

After the total RNA had been extracted as described above, cDNA was prepared using the PrimeScript™ reagent kit with gDNA Eraser (TaKaRa, Japan), following the manufacturer's instructions, and mRNA expression of PfNFAT and the internal control, 18S (GI:62549260), were analyzed. For analyses of the expression in different tissues, RT-PCR was performed. The PCR reactions used the following conditions: denaturation at 94 °C for 3 min, cycles of 94 °C for 35 s, 59 °C for 40 s and 72 °C for 30 s, followed by elongation at 72 °C for 5 min. The number of PCR cycles was 34 for both PfNFAT and 18S. For analysis of gene expression in the