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Short communication

Characterization of the IRF2 proteins isolated from the deep-sea mussel *Bathymodiolus platifrons* and the shallow-water mussel *Modiolus modiolus*

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ABSTRACT

Interferon regulatory factors (IRFs) are transcription factors that play important roles in immune defense, stress response, hematopoietic differentiation, and cell apoptosis. IRFs of invertebrate organisms and their functions remain largely unexplored. In the present study, for the first time new IRFs (BpIRF2 and MmIRF2) were identified in the deep-sea mussel *Bathymodiolus platifrons* and the shallow-water mussel *Modiolus modiolus*. The open reading frame of *BpIRF2* and *MmIRF2* encoded putative proteins of 354 and 348 amino acids, respectively. Comparison and phylogenetic analysis revealed that both IRF2 proteins were new identified invertebrate IRF molecular. As transcriptional factors, both BpIRF2 and MmIRF2 could activate the interferon-stimulated response element-containing promoter and BpIRF2 could interact with itself. Moreover, both BpIRF2 and MmIRF2 were localized to the cytoplasm and nucleus. Collectively, these results demonstrated that IRF2 proteins might be crucial in the innate immunity of deep-sea and shallow-water mussels.

1. Introduction

Interferon regulatory factors (IRFs) are a family of important transcription factors, which play vital roles in multiple biological process, such as regulation of immune response, apoptosis, and proliferation (Tamura et al., 2008). By now, 11 members of this family have been identified in vertebrates and nine of them are present in mammals (Huang et al., 2010). IRF1, a regulator of IFN-β gene expression, was the first identified member of the IRF family (Miyamoto et al., 1988). Each mammalian IRF contains a conserved N-terminal DNA binding domain (DBD) of ~120 amino acids (Paun and Pitha, 2007; Taniguchi et al., 2001). The IRF DBD domain forms a helix-turn-helix motif that recognizes the DNA sequence of the interferon-stimulated response element (ISRE) (Darnell et al., 1994). The C-terminal region of IRFs, also known as the IRF-associated domain (IAD), is less conserved and possibly mediates interactions of specific IRFs with other family members or other transcriptional factors (Yanai et al., 2012). Based on the C-terminal region, IRFs could be classified into the following four subfamilies: IRF1 (comprising IRF1, 2, and 11), IRF3 (IRF3 and 7), IRF4 (IRF4, 8, 9, and 10) and IRF5 (IRF5 and 6) (Nehyba et al., 2002, 2009; Zhang et al., 2015a).

Although members of the IRF family have been extensively studied in mammals (Zhao et al., 2015) and in fish (Feng et al., 2015; Yuan et al., 2015; Zhan et al., 2016; Zhang et al., 2015a), there are very few reports regarding their existence and functions in invertebrates. So far, no IRF family member was identified in *Drosophila melanogaster* (Zhang et al., 2015b). However, it has been recently reported that an IRF was found in shrimp, which could activate the *Vago* gene, indicating that invertebrates possess an IFN-like system (Li et al., 2015). In mollusks, only IRF2 in *Pinctada fucata*

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(Huang et al., 2013) and *Hyriopsis cumingii* (Wang et al., 2013) was identified. The oyster genome has been also predicted to encode IRF family members (Zhang et al., 2012, 2015b). Studies of IRF homologs are therefore necessary to understand the roles IRFs play in the innate immunity and to clarify the evolution of innate immune response systems in invertebrates.

Deep-sea animals have recently begun to attract increasing research attention. Deep sea mussels belonging to the genus *Bathymodiolus* dominate cold seeps and hydrothermal vent areas (Wong et al., 2015). These mussels are known to harbor methane-oxidizing and/or thioautotrophic symbiotic bacteria in their gill epithelial cells, which probably supply the host with organic carbon and energy (Duperron et al., 2009). Whereas bivalves can identify invading microorganisms and activate their innate immune systems to maintain body homeostasis, *Bathymodiolus* mussels accept symbiotic bacteria in their gill cells. Therefore, exploration of the innate immune mechanism of deep-sea mussels would be a great interest. Analysis of the mussel transcriptome has revealed that *Bathymodiolus platifrons* genome encodes IRF family members. In the present study, the IRF genes *BpIRF2* and *MmIRF2* were identified and characterized in the deep-sea mussel *B. platifrons* and shallow-water mussel *Modiolus modiolus,* respectively. Dual-luciferase reporter assays revealed that both IRF2 proteins could activate ISRE reporter gene expression. The co-immunoprecipitation (co-IP) data demonstrated that BpIRF2 molecules can bind to each other, forming homodimers. Finally, we investigated the distinct subcellular localization of these IRF2 proteins in HeLa cells. Our findings help to explain the evolution of the IRF gene family and potential function of these IRF proteins in the immune defense of mussels. Our data are also useful for tracing the IFN-like system in invertebrates.

2. Materials and methods

2.1. Animals and sample preparation

The deep sea mussel *B. platifrons* sampling site was a hydrothermal vent located at a depth of 996.9 m on the continental slope of the South China Sea (27° 47.442′ N, 126° 53.803′ E). *M. modiolus* specimens were collected from a farm in Dalian, Liaoning province. Sample were dissected and stored individually in TRIzol (Life Technology, USA) for subsequent RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from mussel tissues using TRIzol Reagent followed by treatment with DNase I (Promega, USA). First-strand cDNA synthesis was then carried out based on Promega M-MLV (Promega) RT Usage information using treated RNA as a template and oligo (dT) adaptor (Supplementary file 1) as a primer. The reaction mixture was incubated at 42 °C for 1 h, and the reaction was terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:50 and stored at −80 °C for subsequent gene cloning.

2.3. Gene cloning and sequence analysis

Taking advantage of the known transcriptomes of *B. platifrons* and *M. modiolus* (unpublished data), a pair of gene-specific primers, BpIRF2-F and BpIRF2-R (Supplementary Table 1) were designed to amplify the full cDNA sequence of the *BpIRF2* gene. Likewise, MmIRF2-F and MmIRF2-R were used to amplify the full cDNA sequence of the *MmIRF2* gene. The PCR products were purified and cloned into the pMD19-T vector (TaKaRa, Japan). The recombinant vector was transformed into Trans1-T1 competent cells (Transgen, China) and sequenced. The resulting sequences were subjected to cluster analysis.

We used SMART (Simple Modular Architecture Research Tool) (http://smart.embl-heidelberg.de) to predict BpIRF2 and MmIRF2 protein domains. IRF protein sequences from different species were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/guide/proteins/) and compared using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). An IRF phylogenetic tree was constructed using the MEGA program (Version 5.05) by applying the neighbor-joining algorithm. Reliability of the branching was tested using bootstrap resampling (1000 pseudo-replicates) (http://www.megasoftware.net).

2.4. Plasmid construction, cell culture, and transfection

The open reading frame (ORF) regions of *BpIRF2* and *MmIRF2* were amplified using Phusion High-Fidelity DNA polymerase (Thermo, USA) with specific primers (Supplementary Table 1). pCMV-Myc (Clotech, USA), pEGFP-N1 (Clotech) and pCMV-N-FLAG (Beyotime, China) plasmids were digested with *Eco*RI, *Xho*I, and *Eco*RI (New England Biolabs, USA) respectively, and the purified PCR product was fused with the purified digested plasmid using the Ligation-Free Cloning System (Applied Biological Materials, Inc., Canada) according to the manufacturer's instructions.

HeLa cells (ATCC, USA) were cultured in the modified Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, USA), whereas HEK293T cells (ATCC) were cultured in the Dulbecco's modified Eagle's medium-high glucose (HyClone). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1 × penicillin-streptomycin resistance solution (Solarbio, China). Cells were grown in the atmosphere of 95% air/5% CO_2 at 37 °C and subcultured every 3–4 days. Expressing plasmids were transfected into HeLa or HEK293T cells using Lipofectamine 3000 reagent (Life Technologies, USA) according to the manufacturer's instructions.

2.5. Dual-luciferase reporter assays

Dual-luciferase reporter assays to detect the effects of BpIRF2 and MmIRF2 on the ISRE promoter were performed in HEK293T cells by using pCMV-BpIRF2-Myc and pCMV-MmIRF2-Myc as IRF2-expressing vectors, respectively. Briefly, cells in each well of a 24-well plate (Corning, USA) were transfected with 0.1 μg reporter gene plasmids, pISRE-TA-luc (Beyotime, China), 0.01 μg pRL-CMV Renilla luciferase plasmid (Promega), and the correct amount of expression plasmids or empty expression vectors (as control). The pRL-CMV Renilla luciferase plasmid was used as internal control. At 24–48 h post transfection, the Dual-Luciferase Reporter Assays System (Promega) was used to measure the activity of firefly and Renilla luciferase according to the manufacturer's instructions with each experiment done in triplicate.

2.6. Co-immunoprecipitation assay

HEK293T cells were divided between two petri dishes (10 cm in diameter, Corning, USA) and cultured for 24 h pCMV-BpIRF2-Myc plasmids were co-transfected with pCMV-BpIRF2-FLAG or pCMV-N-FLAG (as control), respectively. After 24 h, cells were harvested in the cell lysis buffer (Beyotime). Input samples were prepared from the cell lysate and the remaining lysates were mixed with the anti-FLAG M2 magnetic beads (Sigma, USA) under gentle shaking on a roller shaker at 4 °C for 2 h. The beads were then washed three times with the cell lysis buffer. Input samples and Co-IP samples were incubated with 2 × protein sodium dodecyl sulfate polyacrylamide gel electrophoresis loading buffer (TaKaRa) at 100 °C for