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Structural and functional characterization of a novel molluskan ortholog of TRAF and TNF receptor-associated protein from disk abalone (*Haliotis discus discus*)

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ABSTRACT

Immune signaling cascades have an indispensable role in the host defense of almost all the organisms. Tumor necrosis factor (TNF) signaling is considered as a prominent signaling pathway in vertebrate as well as invertebrate species. Within the signaling cascade, TNF receptor-associated factor (TRAF) and TNF receptor-associated protein (TTRAP) has been shown to have a crucial role in the modulation of immune signaling in animals. Here, we attempted to characterize a novel molluskan ortholog of TTRAP (AbTTRAP) from disk abalone (*Haliotis discus discus*) and analyzed its expression levels under pathogenic stress. The complete coding sequence of *AbTTRAP* consisted of 1071 nucleotides, coding for a 357 amino acid peptide, with a predicted molecular mass of 40 kDa. According to our *in-silico* analysis, AbTTRAP resembled the typical TTRAP domain architecture, including a 5′-tyrosyl DNA phosphodiesterase domain. Moreover, phylogenetic analysis revealed its common ancestral invertebrate origin, where AbTTRAP was clustered with molluskan counterparts. Quantitative real time PCR showed universally distributed expression of *AbTTRAP* in selected tissues of abalone, from which more prominent expression was detected in hemocytes. Upon stimulation with two pathogen-derived mitogens, lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C), transcript levels of *AbTTRAP* in hemocytes and gill tissues were differentially modulated with time. In addition, the recombinant protein of AbTTRAP exhibited prominent endonuclease activity against abalone genomic DNA, which was enhanced by the presence of Mg^{2+} in the medium. Collectively, these results reinforce the existence of the TNF signaling cascade in mollusks like disk abalone, further implicating the putative regulatory behavior of TTRAP in invertebrate host pathology.

1. Introduction

Tumor necrosis factor (TNF)-related signaling is a highly complicated and tightly regulated process, in which members of TNF ligand and receptor family proteins specifically interact with each other to initiate and propagate a wide array of cascades to regulate multiple cellular events, including cell proliferation and differentiation, immune and inflammatory responses, and apoptosis [1]. In this regard, TNF receptor-associated factors (TRAFs) and adaptor proteins play a key role in transducing the initiated signals to mount respective immune responses, by changing the signaling molecules

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within a target cell [2]. However, in order to render a vast array of cellular responses according to variety of stimuli, the activity of molecules in TNF signaling pathways are highly modulated through different regulatory factors, including TRAF family member-associated NF-kappa B activator (I-TRAF/TANK), TRAF-interacting protein (TRIP) and TRAF and TNF receptor-associated protein (TTRAP) [3–5]. Among these regulators, TTRAP plays a key role as a negative regulator of the nuclear factor κB (NF-κB) activation pathway through TNF signaling, as a result of interactions exerted with the cytoplasmic domain of CD40 and with TRAFs [4]. This further suppresses ever shorter telomere 1 (EST1) transcriptional activity and weakens synergistic transactivation by EST1 and activator protein 1 (AP1) as an E twenty-six transcription factor 1 (ETS-1)-associated protein [6]. On the other hand, overexpression of TTRAP could also trigger the activation of Janus kinase (JNK), leading to DNA fragmentation and apoptosis in human umbilical vein endothelial cells, as published in a previous report [7].

TTRAP is classified as a member of the Mg^{2+}/Mn^{2+}-dependent phosphodiesterase (MDP) superfamily, which includes sphingomyelinases, inositol-phosphatases and nucleases [8]. Among them, TTRAP is more closely related to nucleases than to sphingomyelinases or phosphatases [8]. TTRAP is known to share significant sequence and structure similarities with the human apurinic/apyrimidinic endonuclease (APE1), which is involved in both DNA repair and activation of transcription factors such as AP1 and p53 [9]. Interestingly, according to a previous report, endonuclease activity was empirically demonstrated in human TTRAP, where it was identified as a bona fide 3′- and 5′-tyrosyl DNA phosphodiesterase [10]. As suggested, this endonuclease activity was believed to be involved in DNA repair and apoptosis [11–13].

The identification and characterization of different regulatory molecules in TNF signaling pathways has been performed predominantly in vertebrates, particularly in mammals. However, reports on members of invertebrate TNF-related signaling cascades, especially mollusks are scarce, with the exception of reports on lipopolysaccharide (LPS)-induced TNF-α factor (LITAF), TNF-α and Fas ligand from *Haliotis discus discus* [14–16], and two TNF ligands, LITAF and TTRAP, exclusively from *Chlamys farreri* [17–20].

Abalones (*Haliotis* species) are marine gastropods that are cultivated as aqua-crops, contributing to an eminent proportion of the yield in commercial aquaculture industry in East and Southeast Asia. However, environmental stresses in their natural habitats, mostly from a wide range of pathogenic infections, have a negative impact on their survival and growth. Abalones are known to be infected by bacteria [21,22], viruses [23] and some parasites [24]. Nevertheless, the innate immune mechanisms functioning in abalones can combat these pathogenic threats to a certain extent. Therefore, revealing the mechanisms that exist in abalones is a constructive way to further develop appropriate strategies for their disease management.

In this study, an invertebrate ortholog of TTRAP from disk abalone (*H. discus discus*) (*AbTTRAP*) was identified and molecularly characterized, further analyzing its transcriptional modulation upon pathogen-derived mitogen stimulation. Furthermore, we demonstrated the endonuclease activity of AbTTRAP, using its purified recombinant protein, which is a typical functional property of TTRAPs, previously reported from invertebrates as well as vertebrates.

2. Materials and methods

2.1. Identification and sequence characterization

A cDNA library of disk abalone was constructed as described in our previous report [25]. A single putative clone, the sequence of which showed higher similarity to known TTRAP homologs, was identified using the Basic Local Alignment Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the DNA clone (containing TTRAP cDNA of disk abalone) was isolated using *AccuPrep*® Plasmid Mini Extraction kit (Bioneer, Korea). The *AbTTRAP* internal sequencing primer, TTRAP-I1 (Table 1), was designed based on the 3′-end of the known sequence, and then sequencing was carried out by ABI3700 sequencer (Macrogen, Korea). Finally, the full-length cDNA sequence of *AbTTRAP* in disk abalone was identified. The coding sequence and respective amino acid sequence of AbTTRAP were derived using DNAssist 2.2 software (www.DNAssist.org).

Characteristic domains and sequences existing in TTRAP molecules were predicted in AbTTRAP protein sequence using the NCBI-CDD server (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [26] and SMART online server (http://smart.embl-heidelberg.de). In order to compare the derived AbTTRAP protein sequence with its orthologs, pairwise sequence alignments and multiple sequence alignment were performed using emboss needle (http://www.Ebi.ac.uk/Tools/emboss/align) and ClustalW2 (http://www.Ebi.ac.uk/Tools/clustalw2) online programs, respectively. In addition, some of the physicochemical properties of AbTTRAP were determined through DNAssist 2.2 software. The phylogenetic relationship of AbTTRAP with its orthologous sequences was determined using the Neighbor-joining method under bootstrapping values taken from 1000 replicates by Molecular Evolutionary Genetics Analysis (MEGA) software version 5 [27].

2.2. Prediction of protein function

In order to improve the accuracy of our *in-silico* predictions, domain analysis, biological processes and molecular functions associated with AbTTRAP were further annotated using gene ontology (GO) hierarchical prediction by using the I-TASSER online server and COFACTER algorithm [28,29], which simulates the derived AbTTRAP protein sequence.

2.3. Generation of a recombinant AbTTRAP plasmid construct

The coding sequence of *AbTTRAP* was PCR-amplified and cloned into the pMAL-c2X vector, as described in the protocol of pMAL™ Protein Fusion and Purification System (New England Biolabs, UK), resulting a recombinant plasmid construct. Briefly, the open reading frame (ORF) of AbTTRAP gene was amplified using the sequence-specific primers, AbTTRAP-EF and AbTTRAP-ER, consisted with the restriction enzyme sites for *Eco*RI and *Hin*dIII, respectively (Table 1). PCR was performed in a TaKaRa thermal

Table 1
Primers used in the study. F and R refer to forward and reverse oligomers, respectively.

Name	Target	Primer sequence (5′–3′)
TTRAP-I1	Internal sequencing	CAGAATGTATCATTGCCTCCAGGAT
AbTTRAP-F	Real time PCR amplification	AAATGTCGTGTGAAGGGCATCAGC
AbTTRAP-R	Real time PCR amplification	AGTGTCGCCACCGAATATGACAG
AbTTRAP-EF	ORF cloning for expression	gagagaGAATTCTCTGACAGTGAGGCTGACAGTG
AbTTRAP-ER	ORF cloning for expression	gagagaCTGCAGCTAGACTGACGCACAGGTTGG
AbRibosomal-F	Real time PCR amplification	GGGAAGTGTGGCGTGTCAAATACA
AbRibosomal-R	Real time PCR amplification	TCCCTTCTTGGCGTTCTTCCTCTT