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Cloning, expression pattern and functional characterization of interleukin-1 receptor-associated kinase 4, an important mediator of the Toll-like receptor signaling pathway, from *Artemia sinica*

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ABSTRACT

As a crucial component of Toll-like receptor (TLR)/interleukin-1 receptor (IL-1R) signaling pathways, IL-1R–associated kinase 4 (IRAK-4) plays a central role in innate immunity and embryonic development. Herein, we have characterized the full length cDNA of IRAK4 from *Artemia sinica*. Molecular characterization revealed that the sequence includes a 2550 bp open reading frame, encoding a predicted protein of 849 amino acids. The predicted protein contains a death domain in the N-terminus and two serine/threonine/tyrosine protein kinasedomains. Bioinformatics analysis showed that it belonged to a new member of the IRAK-4 family. The expression of *As*IRAK-4 was researched in various stages during embryonic development by several molecular biology methods including real time PCR, Western blotting and immunohistochemistry. The results showed that *As*IRAK-4 was constitutively expressed at all developmental stages from embryo to adult, and it was mainly expressed in the head and thorax at the early stages and on the surface of the alimentary canal at later stages. The highest expression level was at the 0 h, 15 h and 5 d stages of *A. sinica*. While challenged by Gram-positive and Gram-negative bacteria, *AsIRAK-4* was remarkably upregulated with the rising concentration of bacteria. Our research revealed that *As*IRAK-4 plays a vital role in growth, development and innate immunity of *A. sinica*.

1. Introduction

The brine shrimp, *Artemia sinica*, belongs to Arthropoda, Crustasea, Branchiopoda, Anostacea, *Artemidae* and *Artemia* [1]. *A.sinica* is a high salt-resistant small crustacean distributed widely in saline inland lakes and coastal salterns worldwide at a wide range of temperatures [2–6]. The *A. sinica* nauplii, which contain abundant proteins and fatty acids, are not only suitable feeds for humans and animals, but also are used as a model organism in various fields, ranging from developmental biology to evolution and ecology, especially in innate immune research [7].

Interleukin-1 receptor-associated kinases (IRAKs) are important mediators in the signal transduction of toll-like receptor (TLR) family members [8,9]. To date, four members of the IRAK family have been identified including IRAK-1, 2, M and 4 [10]. It has been found that IRAK-4 is the most important member, responsible for triggering the interleukin-1 (IL-1)/TLR signaling pathway, including the MyD88-dependent IL-1/TLR signaling pathway and the MyD88-independent pathway [11,12]. In innate immunity, the only defense system in invertebrates, TLRs act as pathogen-recognition receptors (PRRs), able to differentiate between chemically diverse pathogens and activate intracellular signal transduction pathways. The highly conserved structures of intrusive antigens, which are recognized by the various TLRs, are known as pathogen-associated molecular patterns (PAMPs) [13,14].

Toll was initially recognized as an essential molecule for embryonic dorsal/ventral patterning in *Drosophila*, and was subsequently shown to be necessary for antimicrobial peptide expression in organisms resistant to fungi and Gram-positive bacteria [15–17]. In insects like *Drosophila melanogaster*, the formation of the embryonic dorsoventral axis is regulated by the Toll/Dorsal pathway, which is homologous to the vertebrate Toll/IL-1 receptor signaling pathway [15,18]. In *Drosophila*, genetic analysis of dorsal-ventral patterning of the embryo has defined a series of genes that mediate the Toll-Dorsal pathway. Binding of extracellular ligands activates the transmembrane receptor Toll, which requires the novel protein, Tube, to activate the cytoplasmic serine/threonine kinase, Pelle. Pelle activity controls the degradation of the Cactus protein, which is present in a cytoplasmic complex with the Dorsal protein. Once Cactus is degraded in response to a signal, Dorsal is free to move into the nucleus where it regulates transcription of specific target genes [15]. It has been found that the structure and

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function of Pelle from *Drosophila* are homologous to those of mammalian IRAKs [19].

In vertebrates, IRAK-4 has been identified and characterized in mammals and fish [12,20–22]. Additionally, in aquatic invertebrates, to our knowledge, IRAK-4 has been cloned from small abalone *Haliotis diversicolor*, squid *Euprymna scolopes*, sponge *Suberites domuncula* and clam *Mya arenaria* [10,23–25]. However, the structure and function of IRAK-4 in *A. sinica* has not been reported. Herein, the *AsIRAK-4* gene was isolated from *A. sinica*. The expression levels of *AsIRAK-4* at various developmental stages and in response to bacterial infection were investigated. Additionally, the protein expression of *As*IRAK-4 and its location were analyzed by Western blotting and whole mount immunohistochemistry, respectively. Our findings contribute to the functional analysis of IRAK-4 in *A. sinica*. Further studies are required to elucidate the mechanisms of IRAK-4 for regulating development and immunity of *A. sinica*.

2. Materials and methods

2.1. Animal material and sampling

Cysts of *A. sinica* were collected from the Salt Lake of Yuncheng in Shanxi Province, China, during the summer in 2010 and stored at −20 °C in the dark. The cysts were hatched in saline water under these conditions: a temperature of 28 °C, salinity of 28‰, and light intensity of 1000 lx [26]. Samples were collected at different times of development (0, 5, 10, 15, 20 and 40 h, and 3, 5, 7 and 10 d) for subsequent experiments. For the antimicrobial assay, larvae of nauplius stage *A. sinica* (20 h) were cultured in salt water and maintained in salt water with Halophilic Gram-negative bacteria *Vibrio harveyi* and Gram-positive bacteria *Micrococcus lysodeikticus* for 24 h as the control group and experimental groups, respectively. In the experimental groups, the bacteria concentrations were 10^4 cells L^{-1}, 10^5 cells L^{-1} and 10^6 cells L^{-1}. All the samples were rapidly frozen by liquid nitrogen and then stored at −80 °C.

2.2. Cloning of AsIRAK-4

Total RNA was extracted from *A. sinica* (10 d) with RNAiso Plus (Takara, Dalian, China) according to the user manual and then was reverse transcribed into cDNA. The Primer Premier 5.0 was used to design the specific primers (IRAK-4 F, IRAK-4 R; Table 1), which were based on the transcriptomic database of *A. sinica*. All primers used in this study were synthesized by Genewiz (Suzhou, China). The amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (56 °C, 30 s), extension (72 °C, 1 min) and a final extension (72 °C, 10 min) step. The PCR products were detected using electrophoresis with 1.0% agarose/TAE gels, purified from the gel using a DNA gel extraction kit (TransGen Biotech), and then sequenced by TSINGKE.

Table 1
Primer used in this study.

Primer name	Sequences (5′–3′)
IRAK4F	ATTCCTGAGTTTATGCCTG
	CAAGGTGAAAGTATGAAC
IRAK4R	GCTTTCTTCCACCTTGATT
	ACAAGAGGTAACAACAAC
IRAK4-RT-F	TGGTTAGAGGGTGTTGCTGTAT
IRAK4-RT-R	TTAGGTGGGCGAAAAACGAGT
3′ IRAK-4	GATGTTGACCCTAAGGCAGGAGATTG
5′ IRAK-4	CAAGTTCGTCTTCGGTTTCAGGCGG
β-actinF	GTGTGACGATGATGTTGCGG
β-actinR	GCTGTCCTTTTGACCCATTCC
exIRAK4-F	CCGGAATTCACCATCAGCGAAGCAA
exIRAK4-R	CCGCTCGAGTTACTTTCTTCCACCTTGATTC

The full-length sequence was obtained by 5′–3′ rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA Amplification Kit (Clontech, Dalian, China), following the manufacturers' instructions. The primers used in 5′–3′ RACE (3′ IRAK-4, 5′ IRAK-4; Table 1) were shown in Table 1. The target RACE products were purified and sequenced.

2.3. Bioinformatic and phylogenetic analysis

The sequence analysis of *AsIRAK-4* was performed using BLAST program at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). The open reading frame and deduced amino acid sequences were identified by the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The ProtParamtool of ExPASy (http://web.expasy.org/protparam) analyzed the molecular weight and theoretical isoelectric point of the protein. The SMART (http://smart.embl-heidelberg.de) software and the prositetools of ExPASy (http://prosite.expasy.org/prosite.html) were used to predict the *As*IRAK-4 functional domains and protein structure. SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP) and TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were employed to predict the signal peptide of *As*IRAK-4 amino acid sequences and transmembrane region. ClustalX2.0 program and DNAman were used to carry out protein multiple sequence alignments among 15 species including 10 species of vertebrate and 5 species of invertebrate. Multiple protein sequence alignment was constructed by ClustalX2.0 and MEGA6.0. Phylogenies of protein sequences were estimated using neighbor-joining (NJ) method and MEGA6.0 software. The bootstrap values were replicated 1000 times to obtain the confidence value for the analysis.

2.4. Quantitative analysis of AsIRAK-4 transcript levels

2.4.1. Expression of AsIRAK-4 during different developmental stages

RNA samples of *A. sinica* were extracted from different growth time periods (0, 5, 10, 15, 20 and 40 h; 3, 5, 7 and 10 d) and cDNA templates were prepared using the method detailed in Materials and methods Section 2.2. The gene-specific primers of qPCR were designed in The Primer Premier 5.0 (Table 1). Real-time qPCR was performed in triplicate for every sample in a parallel design using the LightCycler 96 System (Roche). Each PCR was run in 10 mL reaction volume containing 5 mL 2 × SYBR Premix Ex Taq (Takara, Dalian, China), 1 mL diluted cDNA, 0.4 mL each primer (10 mM), and 3.2 mL sterile distilled H_2O. The qPCR program was 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s, melting curve was performed at the end of qPCR reaction followed by 95 °C for 10 s and 58 °C for 30 s. β-actin primers (β-actinF, β-actinR, Table 1) were used as a normalization control for target genes expression [27]. The $^{2-\Delta\Delta}$Ct method was used to calculate the relative quantification (comparative method) [28]. Data obtained from qPCR analysis were analyzed using one-way ANOVA in SPSS 16.0 software. The significance threshold of the means values between the experimental and control groups was set at $P < 0.05$.

2.4.2. Expression of AsIRAK-4 in response of bacterial challenge

V. harveyi, halophilic Gram-negative bacteria and Gram-positive bacteria *M. lysodeikticus* were inoculated into 1 mL liquid medium, respectively, and cultivated with shaking for 12 h. The nauplius larva of *A. sinica* (20 h) was challenged with two kinds of bacteria respectively, which were diluted to 10^4 cells L^{-1},10^5 cells L^{-1} and 10^6 cells L^{-1}, for 24 h. Total mRNA was extracted from *A. sinica* treated with each dilution and reverse transcribed into cDNA. Real-time qPCR was performed on the samples using the primers and reaction conditions detailed in Section 2.4.1.