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A comparative study of three akirin genes from big belly seahorse *Hippocampus abdominalis*: Molecular, transcriptional and functional characterization

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ABSTRACT

Akirins, members of the NF-κB signaling pathway, are highly conserved nuclear proteins, which regulate gene expression in many physiological processes, including immunity, myogenesis, carcinogenesis, and embryogenesis. The akirin family in teleost fish consists of two to three genes. In the present study, three akirin genes from *Hippocampus abdominalis* were identified from a transcriptome database and designated as *HaAkirin1*, *HaAkirin2(1)*, and *HaAkirin2(2)*. The nuclear localization of *HaAkirin1* and *HaAkirin2(1)* was confirmed by subcellular localization analysis. In contrast, diffused localization of *HaAkirin2(2)* was identified in the nucleus and cytoplasm that confirmed the aberrant nature of the nuclear localization signal. Phylogenetic analysis revealed a closer relationship of HaAkirins with other known teleost akirins. All three *HaAkirin* transcripts were ubiquitously expressed in all examined tissues with higher expression in ovary tissue. Immune challenge with LPS, poly I:C, and *Streptococcus iniae* exhibited a significant increase in the expression of all three *HaAkirins* in kidney and liver tissues. NF-κB luciferase assays revealed that relative luciferase activity was significantly higher for all three *HaAkirin* genes than mock controls. These results suggest that *HaAkirin* genes might play a role in regulating NF-κB dependent immune gene expression and their expression could be induced by bacterial and viral pathogen recognition molecular patterns.

1. Introduction

Nuclear factor-kappaB (NF-κB) transcription factors regulate the expression of a wide variety of immune genes, which are involved in inflammation, lymphocyte development and maintenance, and lymphocyte activation [1]. Akirins, regulatory proteins of NF-κB dependent gene expression, are highly conserved nuclear proteins present throughout metazoa [2]. Invertebrates typically have a single akirin gene while amphibians and mammals contain two different orthologs (akirin1 and 2). Meanwhile, a single gene has been identified in reptiles and birds (akirin2), and two to three genes in teleost [akirin1(1) and akirin2(1) and/or 2(2)]. Besides, salmonid fish species have eight akirin family members (akirin1(1a), 1(1b), 1(2a), 1(2b), 2(1a), 2(1b), 2(2a), and 2(2b)) [3–5].

Akirin was first identified as a key regulator of innate immunity in drosophila. The drosophila akirin acts at the level of NF-κB in the immune deficiency (IMD) pathway and is critical for production of

antimicrobial peptides, such as attacin, eliciting an immune response against Gram-negative bacterial infection. Despite the presence of two akirin homologs in mice, only akirin2 is required for the secretion of NF-κB dependent cytokines in the regulation of toll-like receptor (TLR), tumor necrosis factor (TNF) receptor, and interleukin (IL)-1β receptor pathways [2]. Moreover, Akirin2 in macrophages and neutrophils plays a critical role in the clearance of infecting bacteria in mice [6]. Akirin2 is essential for proliferation of B cells and inhibition of apoptosis mediated by the TLR ligands and anti-CD40 stimulation [7].

Selective activation of NF-κB target genes requires chromatin remodeling factors. The interaction of akirin with chromatin remodeling complex and NF-κB has been reported in both Drosophila and mice. Studies in Drosophila revealed that expression of a set of antimicrobial peptides in the IMD pathway are akirin dependent, and their expression is involved with akirins' interaction with the Brahma-associated protein of 60 kDa (Bap60), a component of the chromatin remodeling switch/ sucrose nonfermentable (SWI/SNF) complex and the NF-κB factor,

Relish [8]. Further, mice akirin2 has been shown to interact with BAF60 proteins and inhibitor of NF-κB ζ (IκB-ζ). The BAF60–Akirin2–IκB- ζ complex links the NF-κB and SWI/SNF complexes in mammalian innate immune cell activation [6].

Seahorses (Genus *Hippocampus*, Family Syngnathidae) are one of the highly specialized teleost fish with unique biological features. They are heavily exploited for their commercial value as traditional medicine and ornamental aquaria fish [9]. Even though, the seahorse aquaculture industry has been growing, commercially intensive aquaculture demands better disease control for economic success [10]. A better understanding of immune mechanisms is crucial for disease control of fish through vaccination. Studies showed that vaccination of host animals with recombinant *Aedes albopictus* akirin could be used for control of mosquitoes and sandfly infestation [11]. Akirin might be a potential gene to be targeted for boosting immunity in seahorses against infectious agents.

Hippocampus abdominalis, introduced to Korean water, has great potential for commercial aquaculture [12]. However, there are only limited reports on the pathogens infecting seahorses in the Korean water [13]. Identification of potential pathogens and understanding fish immune responses against their infections are critical for the success of aquaculture. There are many bacterial pathogens infecting a wide range of fish species cultured in Korea, thereby causing reduction in aquaculture production [14]. *Streptococcus iniae*, which was shown to infect multiple hosts [14,15], is identified as one of the potential pathogens infecting *H. abdominalis* as well. Understanding *S. iniae*-induced immune response in seahorses is valuable for a better disease control.

In the present study, we have characterized three akirin genes *HaAkirin1*, *HaAkirin2(1)*, and *HaAkirin2(2)* from big belly seahorse *H. abdominalis*. Spatial expression was determined in different tissues, and temporal expression was determined upon exposure to lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (poly I:C), and *S. iniae* in kidney and liver tissues. Moreover, the nuclear localization and effect of seahorse akirins in NF-κB pathway were analyzed. Results from this study will broaden the understanding of akirins' role in seahorse immune defense.

2. Materials and methods

2.1. Identification and sequence analysis

The cDNA sequences of seahorse akirins were identified using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) from a previously established *H. abdominalis* transcriptome library [16]. Open Reading Frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the ORF and amino acid sequence of the *HaAkirins*. The molecular weight and isoelectric point of HaAkirins were estimated using ExPASy ProtParam tool [17]. Nuclear Localization Signal (NLS) was predicted by PSORT II (https://psort.hgc.jp/form2.html) [18]. Multiple sequence alignments of HaAkirins with orthologs of other vertebrate species were performed by ClustalW from Bioedit [19]. Phylogenetic analysis of HaAkirins with ortholog sequences from different invertebrates and vertebrates were performed using MEGA 7.0 with neighbor-joining (NJ) algorithm and 5000 bootstrap resampling iterations [20].

2.2. Acclimatization of fish and tissue sampling

Seahorses with an average weight of 8 g were obtained from the Korea Marine Ornamental Fish Breeding Center, Jeju, and acclimatized to the laboratory aquarium tanks (300 L) with average water temperature (18 ± 2 °C) and average water salinity (34 ± 0.6‰) for a week. To analyze the tissue specific expression of *HaAkirins* in the seahorse, skin, gill, intestine, liver, muscle, heart, kidney, brain, pouch, testis, and ovary tissues were dissected from six seahorses (3 males and 3 females). Blood was obtained by cutting the tails, and blood cells were

isolated by centrifugation at 3000*g* for 10 min at 4 °C. The collected tissue samples were snap-frozen in liquid nitrogen and kept at −80 °C.

2.3. Challenge experiment

For immune challenge experiments, seahorses were divided into four groups, and challenged with LPS, poly I:C, *Streptococcus iniae*. Phosphate-buffered saline (PBS) was used as a control. While five unchallenged individuals were sampled at 0 h post-injection, and a total of thirty individuals were challenged in each group. Each fish in the respective group was injected intraperitoneally with 100 μL suspension of LPS (1.25 μg/μL), poly I:C (1.5 μg/μL), and *S. iniae* (10^5 CFU/μL) prepared with PBS. Fish in the control group were injected with 100 μL of PBS. At time intervals of 3, 6, 12, 24, 48, and 72 h post-injection, five individuals from each challenge group were sacrificed to obtain liver and kidney tissue samples.

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted from a pool of tissues from six healthy unchallenged fishes and five challenged fishes corresponding to each time point by using RNAiso plus (TaKaRa, Japan), and purified by using an RNeasy spin column (Qiagen, USA). RNA concentrations were measured at 260 nm in a μDrop Plate reader (Thermo Scientific,USA), and RNA purity was examined by 1.5% agarose gel electrophoresis. The first strand cDNA was synthesized using purified RNA (2.5 μg) by PrimeScript™ II 1st strand cDNA synthesis kit (TaKaRa, Japan). The cDNA samples were diluted 40-fold and stored at −80 °C until further use.

2.5. Quantitative real-time PCR (qPCR) analysis

To study the tissue distribution and temporal expression of *HaAkirin* mRNAs in *H. abdominalis* upon immune challenge, qPCR reactions were conducted using the TaKaRa TP950 Thermal Cycler Dice™ in a 12-μL reaction volume containing 3 μL of diluted cDNA template, 6 μL of TOPrealTM qPCR 2x PreMIX (SYBR Green, Enzynomics, Korea), 1 μL of each gene-specific primer (10 pmol/μL) (Supplementary Tables 1) and 1 μL dH₂O. PCR conditions were as follows: 1 cycle of 95 °C for 10 min; 45 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 20 s; 1 cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The qPCR reactions for all the samples were conducted in triplicate. The relative mRNA expression of *HaAkirins* was calculated using the $2^{-\Delta\Delta Ct}$ method [21] using *H. abdominalis* 40S ribosomal protein S7 (Accession number: KP780177) as the internal control. For the tissue distribution analysis, mRNA expression levels within different tissues were normalized with the lowest expression values. The relative temporal *HaAkirins* expression for the challenge experiments was normalized to the corresponding mRNA expression of the PBS injected control.

2.6. Construction of the recombinant vector

For the subcellular localization study and NF-κB luciferase assay, specific primers were designed to incorporate the corresponding restriction site (Supplementary Table 2). The coding sequences of *HaAkirin1*, *HaAkirin2(1)*, and *HaAkirin2(2)* were amplified using specific primers with cDNA synthesized from ovary tissue. The PCR reaction was performed in a total volume of 50 μL containing 4 μL template, 5 μL 10 × ExTaq buffer, 4 μL of 2.5 mM dNTP, 2 μL of each 10 pmol forward and reverse primers, and 0.2 μL of 5 U of ExTaq polymerase (Takara, Japan). The PCR condition was as follows: 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; final extension of 72 °C for 5 min. PCR products of *HaAkirins* and plasmids (pCDNA3.1(+) and pEGFP-N1) were restriction-digested with corresponding enzymes and gel purified using Accuprep™ purification kit (Bioneer Co., Korea). Ligations were performed using Mighty Mix