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Identification and primary immune characteristics of an amphioxus *akirin* homolog

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ABSTRACT

Akirin is a recently described nuclear protein that is thought to be required for the NF-κB signaling pathway in insects and vertebrates. Here, functional investigations of akirin are described in the basal chordate amphioxus *Branchiostoma belcheri tsingtauense* in an attempt to link this gene between insect and vertebrate lineages. Phylogenetic analysis indicated that amphioxus *akirin* represented a true ortholog of the two characterized vertebrate *akirin* paralogs. Amphioxus *akirin*, coding 219 amino acids with two nuclear localization signal (NLS) sequences and one 14-3-3 binding motif, was widely expressed in various tissues and up-regulated in response to *Escherichia coli* (Gram-negative bacterium) and *Staphylococcus aureus* (Gram-positive bacterium) challenges. Furthermore, amphioxus akirin was strictly localized to the nucleus of HEK293T cells in a confocal analysis. Our work identified and characterized for the first time an amphioxus *akirin* homolog and will promote a better understanding of the evolution and transcriptional network of the *akirin* gene family.

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1. Introduction

The first line of host defense against infectious agents depends on pathogen recognition and immunity activation [1,2]. The basic underlying mechanisms of these processes are conserved throughout much of the animal kingdom, including one of the hallmarks, activation of NF-κB family transcription factors [3]. The sophisticated transcriptional regulation of NF-κB requires the help of proteins that localize exclusively in the nucleus [4]. An excellent example from the recent studies is the highly conserved *akirin* gene family [5].

Akirin is a recently identified nuclear protein that was first discovered as a protective antigen in the tick *Ixodes scapularis* (Clone ID: 4D8) [6–8]. Homologs were subsequently identified in nematodes, insects, mammals and fish [5–15]. In invertebrates, typically only one *akirin* family member is retained, while mammals have two (*akirin1* and *2*), and teleost genetic models have 2–8 [12,16,17].

The expression patterns of *akirins* in tissues differ both within and among species. For example, mouse *akirin1* (also known as *Mighty1*) is widely distrusted in several tissues [18], whereas rat *akirin2* (also known as *FBI1*) is predominantly expressed in the testes, cerebrum and cerebellum, and at lower levels in the liver, heart, spleen and muscle [14]. In Atlantic salmon *Salmo salar*, eight distinct *akirin* family members are known; they are ubiquitously expressed in 10 tissues, although at different levels [16]. In addition, *akirin* is observed during all developmental stages of the sea lice *Caligus rogercresseyi* (where it is also known as *my32*) [19], nematode *Caenorhabditis elegans* [9] and tick *I. scapularis* [7]. The up-regulation of *akirin* expression has been observed in the salivary glands of the tick *Dermacentor variabilis* after infection with the parasite *Anaplasma marginale* (where it is also known as *subolesin*) [20], and throughout the liver, spleen and kidney of turbot *Scophthalmus maximus* after infection with *Vibrio anguillarum* or lymphocystis disease virus [13], implying its role in immune defense against various pathogens.

There has been pronounced interest in akirin functions, which appear to be many and varied. Drosophila akirin acts in parallel with Relish transcription factor (a fly homolog of vertebrate NF-κB) downstream of the immune deficiency (Imd) pathway, which responds to Gram-negative bacterial infection [5]. The difference in gene expression between akirin and NF-κB knockdown in ticks implied that akirin may be involved in NF-κB-dependent and -independent gene expression [10]. In mice, akirin2 acts with or downstream of NF-κB in the regulation of toll-like receptor (TLR)- and interleukin-1β (IL-1β)-inducible gene expression [5]. In addition, rat akirin2 can promote carcinogenesis via the interaction

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with 14-3-3β [14]. Moreover, the systemic silencing of akirin by RNA interference (RNAi) reduced tick survival, weight and oviposition, and caused degeneration of gut, salivary gland and reproductive tissues [21–24]. Mouse *akirin2* knockouts are embryonic lethal, indicating that *akirin2* is essential for normal embryonic development in mice [5].

Very recently, mouse akirin1 was found to be a novel promyogenic factor to regulate muscle regeneration and cell chemotaxis [18,25]. Fly akirin (also known as Bhringi) can interact with Brahma SWI/SNF class chromatin remodeling complexes, which induce changes in the chromatin environment leading to the optimal expression of some Twist-regulated genes [26,27].

Overall, research in the last decade has shown that the broad functions of invertebrate akirins in immune responses, survival/reproduction/embryonic development, and myogenesis are strongly conserved in vertebrates, although akirin1 and akirin2 have diverged in function [17,12]. Clearly, vertebrate *akirins* were generated by a gene duplication event during chordate evolution [17]. Nevertheless, to date, no research has been carried out on this gene family in early chordates.

The basal chordate amphioxus is becoming a new model organism for studying the origin of the vertebrate immune system [28]. Here, for the first time, we successfully cloned an *akirin* homolog from cephalochordate *Branchiostoma belcheri tsingtauense* and evaluated its expression and primary immune function. Our study will provide insights into the evolution of the *akirin* gene family.

2. Material and methods

2.1. Amphioxus care and maintenance

Adult amphioxus *Branchiostoma japonicum* (formally known as *B. belchri tsingtauense*) were collected from sandy sea floor at Shazikou near Qingdao, China, and cultured in the containers with daily changes of filtered seawater at ambient temperature (20–25 °C) and dark. Prior to the experiments, they were starved to clear the gut for three days in sterilized seawater.

2.2. Cloning of amphioxus akirin cDNA

Total RNA was extracted from adult amphioxus using Trizol (Takara), according to the manufacturer's instructions. The first cDNAs were synthesized by reverse transcription system using oligo-d (T) primers (Takara). Specific primers (*AmphiAkirin*-PCR-F 5′ CAAAATGG CGTGTGCTACTCTG 3′, *AmphiAkirin*-PCR-R 5′ CTCTGTTGTTCCCTCTA CTGGTG 3′) were designed based on a hypothetical protein jgi|Brafl1|115111 and used for RT-PCR. The PCR parameters were as follows: 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and 1 cycle at 72 °C for 10 min. Then PCR products were cloned into pEASY-T3 (Transgene) vector for sequencing.

2.3. Sequence analysis and phylogenetic tree construction

Akirin homolog sequence of *B. floridae* was obtained using BLASTP at the Joint Genome institute website (JGI, http://www.jgi.doe.gov) or GenBank (NCBI, http://www.ncbi.nlm.nih.gov) with amino acid sequences of fruit fly akirin (NP_648113), mouse akirin1 (NP_075912) and mouse akirin2 (NP_001007590). Akirin homologs in other organisms were extracted as follows: amphioxus akirin amino acids were used as queries for BLASTP searches against the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez) for Mammals (human *Homo sapiens*), Aves (chicken *Gallus gallus*), Reptile (green anole *Anolis carolinensis*), Amphibian (clawed frog *Xenopus tropicalis*), Teleost (zebrafish *Danio rerio*), Echinodermata

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tcgcccttcaaaatggcgtgtgctactctgaaacggacttacgagttggaccctttgcac
              M  A  C  A  T  L  K  R  T  Y  E  L  D  P  L  H
agccgcccgtccaagagacccaggcggtgtatgcctatggcgggacacaaccctaacgtt
 S  R  P  S  K  R  P  R  R  C  M  P  M  A  G  H  N  P  N  V
tcccctcacagctccggtctggccacaccacctcggcagaagccctcatcctttcccgaa
 S  P  H  S  S  G  L  A  T  P  P  R  Q  K  P  S  S  F  P  E
gtcgagcccaaactgacctcagaacaaatttcccagtttatccgggatgagtaccgccgc
 V  E  P  K  L  T  S  E  Q  I  S  Q  F  I  R  D  E  Y  R  R
atgcaccgtaggcgccgcctgtgggtagcaacagactcaaacgtgcaacagcccgccggc
 M  H  R  R  R  R  L  W  V  A  T  D  S  N  V  Q  Q  P  A  G
acctccctgtccccaagccatcacggctccctctcacccatgcacgaaagctcccacatg
 T  S  L  S  P  S  H  H  G  S  L  S  P  M  H  E  S  S  H  M
gccggcggctcctccccaatgcattatggggcgtcgccgatgcattatggtacactttcg
 A  G  G  S  S  P  M  H  Y  G  A  S  P  M  H  Y  G  T  L  S
ccgacccacgggagttcgtcgccgatccccggggacgcagtcgccgatcaacaaggacaag
 P  T  H  G  S  S  S  P  I  P  G  T  Q  S  P  I  N  K  D  K
ccactgttctcgatgcggcaggtgagcatgatctgcgagcggatgatcaaggaacgcgac
 P  L  F  S  M  R  Q  V  S  M  I  C  E  R  M  I  K  E  R  D
acccaggtgcgagaggagtacgacaaggtgctgtcctgcaagctagcagagcaatatgat
 T  Q  V  R  E  E  Y  D  K  V  L  S  C  K  L  A  E  Q  Y  D
gccttcatcaggttcaatcaagactatttgcagaggcgatttggcgagacagccgccagc
 A  F  I  R  F  N  Q  D  Y  L  Q  R  R  F  G  E  T  A  A  S
tatgtatcataagcccagcagcacccatacaacctgtcaaccaaccctgtagcagtcaca
 Y  V  S  *
accagccagctatttctccagtttaacatcaacaacaaccctgtaccaccacagcagcta
acttacaccagtagagggaacaacagag
```

Fig. 1. The cDNA and deduced amino acid sequences of amphioxus akirin. Two putative nuclear localization signals are underlined. The putative 14-3-3 binding site with the highest scoring site (position 53; black arrowhead) is boxed. The stop codon is labeled with *.

(purple sea urchin *Strongylocentrotus purpuratus*), Arthropoda (fruit fly *Drosophila melanogaster*) and Choanoflagellida (choanoflagellate *M. brevicollis MX1*); the EST database (http://www.ncbi.nlm.nih.gov/nucest) for Teleost (blue catfish *Ictalurus furcatus*, channel catfish *Ictalurus punctatus*, common roach *Rutilus rutilus*, altantic salmon *S. salar*), Cyclostomata (lamprey *Petromyzon marinus*) [17], Urochordate (ascidians *Halocynthia roretzi*), Mollusca (eastern oyster *Crassostrea virginica*), Annelida (*capitella teleta*), Nematoda (pork worm *Trichinella spiralis*), Rotifera (*Brachionus plicatilis*). Data of Platyhelminthes blood fluke *Schistosoma japonicum*, Amoebozoa (slime mold *Dictyostelium discoideum AX4*) and Chromalveolata (cryptomonad alga *Guillardia theta*) were retrieved from article by Macqueen and Johnston, (2009) [17].

Multiple protein sequence alignments were performed using Clustal-X. Phylogenetic analysis by Neighbor-Joining (NJ) algorithm was constructed by MEGA (4.0) package, and in each case, branch confidence values were obtained by bootstrapping with 1000 iterations. Data were analyzed using P-distance, and gaps were removed by Pairwise Deletion. Deduced protein forecasting was conducted using *ExPASy* (http://www.expasy.ch). NLS (nuclear localization signal), DNA binding motifs and ribosomal protein motifs were predicted by *PSORT II* (http://psort.hgc.jp/cgi-bin/runpsort.pl). Other potential domains and functional sites were determined using *SMART* (http://smart.embl-heidelberg.de) and *ScanSite* (http://scansite.mit.edu), respectively.

2.4. In situ hybridization histochemistry

The Digoxigenin-labeled sense and antisense RNA probes of amphioxus *akirin* were synthesized *in vitro* from the linearized plasmid according to the DIG-UTP supplier's instructions (Roche). The preparation of paraffin section and the hybridization procedure were performed as described by Holland [29].

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