Fish & Shellfish Immunology 30 (2011) 1031–1041

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

# A new Akirin1 gene in turbot (*Scophthalmus maximus*): Molecular cloning, characterization and expression analysis in response to bacterial and viral immunological challenge

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ARTICLE INFO

*Article history:*
Received 14 October 2010
Received in revised form
29 January 2011
Accepted 30 January 2011
Available online 15 February 2011

*Keywords:*
Akirin
Turbot
NF-κB
NLS
Immunity
*Scophthalmus maximus*

ABSTRACT

*SmAkirin1*, a member of the NF-κB signaling pathway, was isolated from turbot by RACE. Its cDNA was 564 bp and encoded a putative protein of 187 amino acids with a predicted molecular mass of 21 kDa and an isoelectric point (pI) of 9.05. Amino acid sequence alignments showed that *SmAkirin1* was 91% identical to the *Salvelinus alpinus* Akirin1 protein ACV49694. Transient expression of *SmAkirin1*-GFP in the turbot kidney cell line SMKC revealed a nuclear localization of the protein, and a typical NLS signal was found at the N-terminal region of the *SmAkirin1* protein. Trans-activation assay in yeast demonstrated that *SmAkirin1* has no transcriptional activation. Transcriptional analysis showed that *SmAkirin1* was expressed in all of the tissues examined, with the highest expression in the spleen and brain. Real-time quantitative reverse-transcriptase polymerase chain reaction analysis showed that the *SmAkirin1* transcript was induced by bacterial and viral infection.



## 1. Introduction

Despite more than ten years of research since the initial discovery of NF-κB and its signaling pathway, the pathway is still not fully understood, although additional participants in the pathway continue to be identified. The highly conserved protein Akirin is one such example.

Recently, a new protein named Akirin [1] was discovered and shown to have an essential function in the *Drosophila melanogaster* immune deficiency (Imd) pathway, which responds to gram-negative bacterial infection. Various Akirin genes have been identified from eukaryotes. Akirin can be separated into five groups based on function, generally in terms of regulating gene expression. For example, Akirin was characterized as a growth factor named "subolesin" in arthropods [2,3], and in Drosophila as a myogenetic factor [4]. In vertebrates, Akirin1 was named "mighty" in mice and was shown to regulate myogenesis [5], whereas akirin2 was named "FBI1" in rats and shown to promote carcinogenesis [6], acting as a transcriptional repressor when bound to a 14-3-3 protein [7–10], or to function downstream of NF-κB to induce the transcription of several immune-response genes, including the anti-inflammatory cytokine, interleukin-6 (IL-6) [1].

In fish, the character and function of the Akirin genes at present are only poorly described, and in fact are restricted to the analysis of a few Akirin genes in zebrafish (*Danio rerio*), four salmonid fishes. Macqueen and Johnston [7] analyzed the intron–exon structure of Akirin genes in fish and showed that the duplication of Akirin which occurred in the teleost lineage was followed by lineage-specific patterns of paralog loss. Macqueen *et al.* [11] demonstrated an expanded repertoire of eight family members in the genome of four types of salmonid fishes, and determined the transcript levels for the Akirin family members and 26 other genes using quantitative real-time PCR (qPCR). The results showed that one class of Akirins in these fishes was co-expressed with catabolic genes coding the NF-κB p65 subunit, E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases, and IGF-I receptors, and another class of Akirins was unregulated with subsequent feeding, and was co-expressed with 14-3-3 protein genes. However, as yet there is no reported evidence for a relationship between the Akirins and the immune pathway in fish.

Turbot (*Scophthalmus maximus*) is a widely cultured marine fish of considerable economic importance in Europe and China.

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doi:10.1016/j.fsi.2011.01.028

However, outbreaks of diseases caused by parasites, bacteria and viruses such as *Philasterides dicentrarchi*, *Vibrio anguillarum* and nodavirus have seriously threatened the turbot aquaculture. Recently, several immune-related EST libraries have been constructed from turbot [12,13] in order to explore the molecular mechanisms for disease resistance and host–pathogen interactions in this species. In addition, numerous immune-related genes, including hepcidin, Nramp (natural resistance associated macrophage protein), MHCIIA and B (Major Histocompatibility Complex Class II) and CXC chemokines have also been investigated [14–16]. To date, there has been no report in turbot of Akirin, a highly conserved nuclear protein required for NF-κB-dependent genes.

In this study, we report the successful cloning and characterization of an Akirin1 family gene from *S. maximus* (*SmAkirin1*). NLS analysis and transcriptional activity assay were used to determine the functions and characteristics of *SmAkirin1*. Moreover, the quantitative real-time RT-PCR results provide evidence that *SmAkirin1* mRNA is responsive to bacterial and viral infection in the antigen presenting processes of the immune system.

## 2. Materials and methods

### 2.1. Experimental animals and cell line

Turbot obtained from Haiyang Fisheries Company in Yantai were raised in a breeding tank with seawater (16 °C), with an average weight of 100 g. For the cloning and tissue expression analysis of *Akirin1* cDNA, 11 tissues consisting of the brain, gill, skin, muscle, fin, heart, liver, spleen, kidney, head kidney, and intestine were collected from three individuals for RNA extraction. To investigate bacterial and viral challenge, 3 tissues consisting of the liver, spleen, and kidney were collected from three individuals for RNA extraction.

The SMKC (*S. maximus* kidney cell line) cell line developed in our lab [17] was used in the present study. The cells were cultured as described [18] in modified eagle's medium containing 10% fetal calf serum (JIBC) and antibiotics. For infection, SMKC cells were grown on a 6-well culture plate ($8 \times 10^5$ cell/well) with serum-free medium.

### 2.2. Bacterial and viral challenges

The bacterium *V. anguillarum*, which has been shown to be pathogenic to turbot, was cultured at 28 °C to mid-logarithmic growth on medium 2216E, centrifuged to collect the bacteria and suspended in 0.9% saline [13,19]. The number of bacteria in the suspension was measured by cell counter. *V. anguillarum* at the final concentration of $7 \times 10^6$ cfu was used for each fish injection, and 0.9% saline was used as the negative control. At 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post-injection, three individuals from each time point were sacrificed and tissues were used for RNA extraction. For the negative control, tissues were taken 12 h after the saline injection.

For viral infection, LCDV (Lymphocystis disease virus) was used for virus susceptibility analysis. LCDV is an important pathogen in many fish species [20] which can lead to lymphocystis disease. The virus was isolated and used to detect the viral susceptibility in a previous study [21]. The culture and titration determinations were performed as described in a previous study [18,21]. Turbot were injected abdominally with LCDV ($1 \times 10^8$ TCID50/ml, 100 μl per fish), and the negative control was injected with medium MEM. At 6 h, 12 h, 24 h, 48 h, 72 h, 96 h post-injection, tissues were isolated for RNA extraction. For SMKC infection, after 2 days of culture, cells were treated for 1 h with 1ul LCDV ($1 \times 10^8$ TCID50/ml) or Poly I:C (500 μg/ml, Sigma). The treated cells were then supplemented with medium, and immediately frozen at −70 °C until RNA extraction.

### 2.3. Cloning of Akirin1 cDNA

For the cloning of Akirin1, turbot liver cDNA was used as the template. The primers AKI-O-S1 and AKI-O-A1 were designed based on the high homology with the Akirin1 of human, mouse and zebrafish. Then the 5′ and 3′ fragments were amplified according to the BD smart™ RACE cDNA amplification kit, using the designed primers AKI-F-S1, AKI-R-S1, AKI-P-A1 and AKI-P-A2.

### 2.4. Sequence analysis

Similarity analysis was performed with the Blast program at the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/blast). The protein motif features and functional domains, such as the signal peptide and nuclear localization signals, were predicted using the PSORT II server (http://psort.ims.u-tokyo.ac.jp). The alignment of Akirins from different species was performed using the ClustalW alignment program, and the phylogenetic tree was constructed on the basis of the proportion of the amino acid differences (p-distances) determined by the Neighbor-joining (NJ) method [22] using MEGA 3 software [23]. The following proteins were used in the alignment: *SmAkirin1* [*S. maxima*] ADK27484.1, akirin, isoform B [*D. melanogaster*] AAN12062.1, akirin, isoform A [*D. melanogaster*] AAF50569.1, akirin [*Marsupenaeus japonicus*] BAI49701.1, akirin [*Gallus gallus*] ADK26453.1, akirin-PA [synthetic construct] ACL89590.1, akirin1 [*Salmo salar*] NP_001161992.1, akirin2(2b) [*Oncorhynchus mykiss*] ACV49724.1, akirin2(2a) [*O. mykiss*] ACV49723.1, akirin2(1b) [*O. mykiss*] ACV49722.1, akirin2(1a) [*O. mykiss*] ACV49721.1, akirin1(2b) [*O. mykiss*] ACV49720.1, akirin1 (2a) [*O. mykiss*] ACV49719.1, akirin1(1b) [*O. mykiss*] ACV49718.1, akirin1(1a) [*O. mykiss*] ACV49717.1, akirin2(2b) [*Salmo trutta*] ACV49716.1, akirin2(2a) [*S. trutta*] ACV49715.1, akirin2(1b) [*S. trutta*] ACV49714.1, akirin2(1a) [*S. trutta*] ACV49713.1, akirin1(2b) [*S. trutta*] ACV49712.1, akirin1(2a) [*S. trutta*] ACV49711.1, akirin1 (1b) [*S. trutta*] ACV49710.1, akirin1(1a) [*S. trutta*] ACV49709.1, akirin2 (2b) [*S. salar*] ACV49708.1, akirin2(2a) [*S. salar*] ACV49707.1, akirin2 (1b) [*S. salar*] ACV49706.1, akirin2(1a) [*S. salar*] ACV49705.1, akirin1 (2b) [*S. salar*] ACV49704.1, akirin 1(2a) [*S. salar*] ACV49703.1, akirin1 (1b) [*S. salar*] ACV49702.1, akirin1(1a) [*S. salar*] ACV49701.1, akirin2 (2b) [*Salvelinus alpinus*] ACV49700.1, akirin2(2a) [*S. alpinus*] ACV49699.1, akirin2(1b) [*S. alpinus*] ACV49698.1, akirin2(1a) [*S. alpinus*] ACV49697.1, akirin1(2b) [*S. alpinus*] ACV49696.1, akirin1 (2a) [*S. alpinus*] ACV49695.1, akirin1(1b) [*S. alpinus*] ACV49694.1, akirin1(1a) [*S. alpinus*] ACV49693.1, akirin2 [*Caligus rogercresseyi*] ADK39312.1, akirin1 [*Xenopus laevis*] NP_001089245.1, akirin1 isoform 1 [*D. rerio*] NP_001107272.1, akirin1 isoform 2 [*D. rerio*] NP_001007187.1, akirin2 [*Xenopus (Silurana) tropicalis*] NP_988914.1, akirin2 [*X. laevis*] NP_001085484.1, akirin1 [*Rattus norvegicus*] NP_001025225.1, akirin1 [*Bos taurus*] DAA31047.1, akirin2 [*B. taurus*] DAA26175.1, akirin1 [*Oryctolagus cuniculus*] XP_002715780.1, akirin2 [*O. cuniculus*] XP_002714617.1, akirin1 isoform 2 [*O. cuniculus*] XP_002708555.1, akirin1 isoform 1 [*O. cuniculus*] XP_002708554.1, akirin2(2a)-like [*Saccoglossus kowalevskii*] XP_002736520.1, akirin2 [*D. rerio*] AAH97074.1, akirin1 [*Mus musculus*] AAH03291.1, akirin2 [*X. (Silurana) tropicalis*] AAH61612.1, akirin2 [synthetic construct] dbj BAI46847.1, akirin2 [*Taeniopygia guttata*] XP_002195619.1, akirin2 [*M. musculus*] CAM16479.1, akirin1 [*Homo sapiens*] AAI19746.1, akirin2 [*H. sapiens*] AAH05051.1, akirin2 [*B. taurus*] NP_001103557.1, akirin1 [*X. (Silurana) tropicalis*] NP_001016080.1, akirin2 [*R. norvegicus*] NP_001035003.1, akirin1 [*M. musculus*] NP_075912.2, akirin1 isoform 1 [*H. sapiens*] NP_078871.1, hypothetical protein [*Bactrocera oleae*] ADQ64468.1, CF166 protein [*S. salar*] NP_001158769.1, akirin2 [*G. gallus*] NP_001180524.1, akirin1 [*B. taurus*] NP_001094706.1, akirin2 isoform 1 [*D. rerio*] NP_998707.1, akirin2 isoform 2 [*D. rerio*] NP_998459.1, akirin1 isoform 2 [*H. sapiens*] NP_001129747.1.