



Cloning of IRAK1 and its upregulation in symptomatic mandarin fish infected with ISKNV

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ABSTRACT

Interleukin-1 receptor activated kinases (IRAKs) play crucial roles in the Toll-like receptor (TLR) mediated signal transduction pathways that control host innate immune responses. Here we report the cloning of an IRAK1 cDNA (named ScIRAK1) from the mandarin fish. The predicted ScIRAK1 peptide contains a death domain and a serine/threonine-specific kinase domain. Quantitative RT-PCR showed that ScIRAK1 mRNA was primarily expressed in blood cells and posterior kidney. Seven days following infection with infectious spleen and kidney necrosis virus (ISKNV), the ScIRAK1 mRNA level was significantly higher in the blood cells of clinically symptomatic fish than in the blood cells of asymptomatic fish or control fish injected with phosphate-buffered saline. Additional experiments showed that overexpression of ScIRAK1 in the 293T cells could induce NF- κ B activation. These results suggest that ScIRAK1 may play a role in the pathology of ISKNV infection in the mandarin fish.

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Introduction

The Toll-like receptors (TLRs) can detect specific features of evolutionarily distant pathogens, and the Interleukin-1 receptor (IL-1R) family proteins enable cells to initiate early host defense responses [1]. IL-1 receptor activated kinases (IRAKs) play crucial roles as mediators in the TLR/IL-1R signal transduction pathways [2]. Moreover, IRAK family member IRAK1 has been identified as a key component of the IL-1R signaling pathway in mammals [3]. Upon ligand binding to TLR/IL-1R, IRAK1 is recruited to the receptor complex to initiate the NF- κ B signaling pathway, leading to induction of inflammatory target genes such as TNF- α , IL-6, IL-1 β , and IL-12 [4]. Furthermore, IRAK1 is involved in activation and nuclear translocation of STAT3 (signal transducer and activator of transcription 3), STAT1 and IRF7 (interferon regulation factor 7), as well as in downstream gene expression [2,5,6]. Phosphorylated IRAK1 also undergoes ubiquitin-mediated degradation or sumoylation resulting in nuclear translocation and transcriptional activation of inflammatory target genes [7]. However, little is known about IRAK family member functions in fish, with the exception of zebrafish [8,9].

The mandarin fish *Siniperca chuatsi* is a predominantly farmed fish species in China, with an annual output of 210,000 tons with a value of 1.5 billion US dollars in 2007. In the last decade, the spread of infectious spleen and kidney necrosis virus (ISKNV) has resulted in significant economic loss for many fish farms [10,11]. ISKNV is classified in the *Megalocystivirus* genus of the *Iridoviridae* family. Thirteen cultured fish species and 39 wild fish species have been confirmed to be hosts of ISKNV-like viruses [12], including the mandarin fish. Infected fish develop serious systemic diseases [13], and ISKNV infection is capable of causing severe epizootics resulting in mass mortalities. Therefore, it is important to study the pathology and immunity of the mandarin fish in order to search for effective ways to protect the fish from diseases. In the present study, we cloned an IRAK1 cDNA from the mandarin fish (named ScIRAK1), and showed that after ISKNV infection, the ScIRAK1 mRNA level was significantly higher in fish with typical disease symptoms than in fish without symptoms or control fish injected with phosphate-buffered saline (PBS). Furthermore, overexpression of ScIRAK1 in the 293T cells could induce NF- κ B activation. These results suggest that ScIRAK1 may play a role in the pathology of ISKNV infection in the mandarin fish.

Materials and methods

Experimental animals. Healthy mandarin fish (average weight = 250 g) were obtained from Nanhai Fish Culture Farm

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(Guangdong, China) and kept in aquaria at 28 °C. Fish were fed with healthy specimens at an average feeding rate of 10 g grass carp fry/kg/day. Before the experiments, the fish were maintained for at least 2 weeks and were determined to be free of ISKNV infections by PCR [14].

Cloning of ScIRAK1 cDNA by RT-PCR and rapid amplification of cDNA ends (RACE). Total RNA was extracted from the mandarin fish spleen using the RNeasy Mini Kit (Qiagen, Germany) as per the manufacturer's instructions and then used to synthesize the 3'- and 5'-RACE cDNA templates with the BD SMART RACE cDNA amplification kit (Clontech, Japan) according to the user's manual. Two degenerate oligonucleotide primers (F1 and R1, Table 1) were designed based on the conserved cDNA sequences of IRAK1 from human, mouse, dog, frog and zebrafish. Touchdown PCR reactions were performed using primers F1 and R1, with 1 cycle of denaturation at 94 °C for 3 min, 10 cycles of 94 °C for 30 s, 58–1 °C/cycle for 40 s, 72 °C for 40 s, and 28 cycles of 94 °C for 35 s, 48 °C for 40 s and 72 °C for 40 s, followed by a 5 min extension at 72 °C. PCR products were cloned into the pGEM-T Easy vector (Promega, USA) and sequenced. Based on the partial sequence obtained, specific primers (5'RACE and 3'RACE, Table 1) were designed to obtain the 3'- and 5'-end cDNA sequences of ScIRAK1 by RACE.

Expression profiles of ScIRAK1 in healthy and ISKNV-challenged mandarin fish. To determine expression of ScIRAK1 mRNA in healthy mandarin fish, the gills, anterior kidney, posterior kidney, spleen, liver, intestine, heart, brain, skin, muscle and stomach were dissected from three healthy mandarin fish and used for isolation of total RNA using the RNeasy Mini Kit (Qiagen, Germany). Total RNA from blood cells was also extracted from three healthy mandarin fish using TRIzol reagent (Invitrogen, USA). Individual total RNA samples from the same tissues were pooled and treated with RNase-free DNase I for quantitative RT-PCR.

ISKNV filtrates were prepared as described previously [11]. One milliliter of the filtrates was used for infection by intramuscular injection to each fish. Fifty fish were injected with ISKNV filtrates. For controls, 15 fish were injected with PBS (pH 7.2) (1 mL/fish). All the mandarin fish were maintained in 30 L aquaria (3 fish/aquarium) at 30 °C for 15 days. At 0, 1, and 4 days after ISKNV or PBS injection, RNA samples were isolated from the blood cells of three randomly selected mandarin fish from each group. On the 7th day after infection, three fish exhibiting lethargy, unresponsiveness to disturbances, pale body pigmentation, cessation of eating, and gill

pallor were selected, and RNA was extracted from blood cells. Additionally, three fish without clinical symptoms and three fish from the PBS-injected control group were selected, and RNA was extracted from blood cells. On the 15th day after ISKNV infection, some fish recovered from the infection and showed no more clinical signs. Three of these recovered fish were selected, and RNA was extracted from blood cells. Three fish were also selected from the PBS-injected group 15 days post-injection, and RNA was extracted from blood cells.

RNA from the three fish in the same group was pooled and reverse transcribed with ReverTra Ace MMLV reverse transcriptase (TOYOBO, Japan). The resultant cDNAs were analyzed by quantitative RT-PCR.

Quantitative RT-PCR analysis. Primers Real-5F and Real-3R (Table 1) were used to amplify the ScIRAK1 cDNA fragment. Since 18S rRNA is one of the most reliable reference genes for quantitative RT-PCR of total RNA [15], primers 18S-F and 18S-R (Table 1) were used for amplification of 18S rRNA cDNA as an internal control. All amplifications and detections were carried out using the cDNAs described above as templates in the LightCycler 480 instrument (Roche, Sweden) with SYBR Green Realtime PCR Master Mix (QPK201) (TOYOBO, Shanghai, China) by using the following program: 95 °C for 30 s, 1 cycle; 95 °C for 5 s, 55 °C for 10 s, 72 °C for 15 s, 40 cycles, followed by a 30 min dissociation curve that was used to identify primer dimers. Statistical analyses were performed using LightCycler 480 Basic Software Version 1.2. All quantitative RT-PCR assays were repeated three times.

Construction of expression plasmids. The pCMV-Flag vector was prepared from the pEGFP-C3 vector (Clontech, Japan). pEGFP-C3 was digested with NheI and XhoI to remove the GFP sequence. Two primers (5'-gctagcgcccgccatgattacaagatgacgacgataaggccctcg ag-3'; 5'-ctcgaggcccttatctgctgcatcttgaatccatggcggcgctagc-3') were then synthesized and annealed to produce an adaptor. The adaptor was digested with NheI and XhoI and ligated into the NheI/XhoI digested pEGFP-C3 to prepare the pCMV-Flag vector. The entire open reading frame of ScIRAK1 was amplified using primers F2 and R2 (Table 1); the resultant PCR product was digested with EcoRI and BamHI and inserted into linearized pCMV-Flag to produce pCMV-IRAK1. Plasmid DNAs were prepared using Endo-free Plasmid Extraction Kit (Omega, China), and the sequences were confirmed by sequencing. HEK293T cells were transfected with pCMV-Flag or pCMV-IRAK1 plasmid using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions.

Cell transfection and NF- κ B activity assay. HEK293T cells were maintained at 37 °C in Opti-MEM I medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA). For DNA transfection, cells were seeded in 96-well plates. When the cells were 70–90% confluent, they were co-transfected with 150 ng pCMV-Flag or pCMV-IRAK1, 50 ng pNF- κ B-Luc (Promega, USA) and 1 ng pRL-TK Renilla luciferase plasmid (Promega, USA) as an internal control. At 24 or 48 h after transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. Significance was analyzed by one-way ANOVA followed by Bonferroni's post hoc adjustment. All statistics were performed using the SPSS program.

Results

Nucleotide and deduced amino acid sequences of ScIRAK1

By PCR amplification with degenerate primers and RACE, a full-length cDNA of the mandarin fish IRAK1 (ScIRAK1) was obtained (GenBank Accession No. FJ436359). The ScIRAK1 cDNA is 2676 bp with an open reading frame of 2265 bp, which encodes a putative

Table 1
Summary of primers used in this study.

Primers	Sequence (5'–3') ^a
<i>For fragment amplification</i>	
F1	tgggmdmkkgaggrrtyggagtkg
R1	accacdcraagctgyagahrctc
<i>For RACE</i>	
5'RACE	gaacactggcgctacgtccgtgat
3'RACE	tcctccagcagagtcctccag
Universal Primer a Mix (UPM)	ctaatacgactcactataggcgaagcagtggtatcaacgcagagt
Nest Universal Primer (NUP)	ctaatacgactcactataggc
3'-RACE cds primer A	aagcagtggtatcaacgcagagtagt(t) ₃₀ vn
<i>For real-time PCR</i>	
Real-5F	ccacggagacatcaagagttca
Real-3R	tttaccacacgatgcccgtc
18S-F	atggtagctttaggcgcctac
18S-R	tatacgtctattggagctgg
<i>For plasmid construction</i>	
F2	ccggaattcagatgtcggcaggagaccggagg
R2	cgggtagcctatcagtcgtgttcagcgggaagataa

^a D = not C; H = not G; M = A or C; N = A, C, G or T; R = A or G; V = not T; Y = C or T.

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