Fish & Shellfish Immunology 31 (2011) 1005–1012

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

The Rab1 GTPase of *Sciaenops ocellatus* modulates intracellular bacterial infection

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ARTICLE INFO

Article history:
Received 4 July 2011
Received in revised form
21 August 2011
Accepted 22 August 2011
Available online 26 August 2011

Keywords:
Rab
GTPase
Sciaenops ocellatus
Bacterial infection

ABSTRACT

The Rab family proteins belong to the Ras-like GTPase superfamily and play important roles in intracellular membrane trafficking. To date no studies on fish Rab have been documented, though *rab*-like sequences have been found in a number of teleosts. In this study, we identified and analyzed a Rab homologue, SoRab1, from red drum, *Sciaenops ocellatus*. The cDNA of *SoRab1* contains a 5′- untranslated region (UTR) of 358 bp, an open reading frame (ORF) of 612 bp, and a 3′-UTR of 265 bp. The ORF encodes a putative protein of 203 residues, which shares 92–99% overall sequence identities with the Rab1 from fish, human, and mouse. SoRab1 possesses a typical Rab1 GTPase domain with the conserved G box motifs and the switch I and switch II regions. Recombinant SoRab1 purified from *Escherichia coli* exhibits apparent GTPase activity. Quantitative real time RT-PCR analysis showed that *SoRab1* expression was detected in a number of tissues, with the lowest expression found in blood and highest expression found in muscle. Bacterial and lipopolysaccharide challenges significantly upregulated *SoRab1* expression in liver, kidney, and spleen in time-dependent manners. Transient overexpression of SoRab1 in primary hepatocytes reduced intracellular bacterial infection, whereas interference with SoRab1 expression by RNAi enhanced intracellular bacterial invasion. These results provide the first indication that a fish Rab1 GTPase, SoRab1, regulates intracellular bacterial infection and thus is likely to play a role in bacteria-induced host immune defense.

1. Introduction

The Ras superfamily of small GTPase is composed of several families that regulate diverse cellular functions [1]. Rab GTPases form the largest family within the Ras superfamily and function in the regulation of intracellular vesicle trafficking and protein transport between different organelles such as endoplasmic reticulum (ER), Golgi complex, endosomes, and various secretory vesicles [2,3]. Like other GTPases, Rab switches between two different forms, one is that bound with GDP, which is inactive and cannot interact with effector proteins, and the other is the GTP-bound form, which is the active form that recruits target effectors [4]. Switch between these two forms is regulated by factors named guanine nucleotide exchange factors (GEFs), which catalyze GDP release and GTP binding, and GTPase activating proteins (GAPs), which catalyze the hydrolysis of GTP to GDP [5]. Structurally, Rab shares with other Ras-like GTPases a common GTPase fold that contains five sequence motifs called ‘G boxes’, which are required for guanine nucleotide interactions, and two ‘switch regions’ called switch I and switch II, which are two surface loops that differ in conformation between the GTP- and GDP-bound forms and are essential for interactions with regulatory partners [6–10]. The five G boxes are characterized by the sequences of GXXXXGKT/S, T, DXXG, N/TKXD, and C/SAK/L/T, respectively [1,11]. In addition to the GTPase fold, most Rab GTPases also possess a C-terminal cysteine motif consisting of CC, CXC, or CCX. This motif serves as a recognition site for geranylgeranyl transferase II, which catalyzes the addition of geranylgeranyl groups [1,12].

Rab GTPases play key roles in membrane traffic and are modulated at activity by a number of regulatory factors. As a newly synthesized protein, Rab is presented by Rab escort protein (REP) to the geranylgeranyl transferase for prenylation at the cysteine motif. The modified Rab is then delivered by REP to the target membrane, where Rab is anchored to the cytosolic face of the membrane via the hydrophobic prenyl groups [13,14]. Membrane targeting of Rab is accompanied with the GEF-catalyzed conversion of the GDP-bound form to GTP-bound form and the dissociation of REP [12,13]. In the GTP-bound state, Rab interacts with various effector proteins that function in different membrane trafficking processes, such as cargo selection, vesicle formation, motor protein-connection for vesicle transport, and membrane tethering and fusion [15–18]. Thus, through interactions with multiple effector molecules, Rab GTPases carry out various functions. Rab is inactivated by the hydrolysis of the bound GTP to GDP under the catalysis

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doi:10.1016/j.fsi.2011.08.019

of GAP. GDP-bound Rab is released from the membrane by GDP dissociation inhibitor (GDI), which inhibits the exchange of GDP for GTP and facilitates the recycling of Rab between membranes [12].

Consistent with their central roles in membrane traffic, Rab GTPases have been found to be involved in the process of pathogen infection. It is known that in mammals, Rab5 and Rab7 levels affect the entry, transport, and intracellular replication of some viruses, such as West Nile viruses and hepatitis C virus, and intracellular bacterial pathogens, such as *Mycobacterium tuberculosis* and *Salmonella enterica* serovar Typhimurium [19,20]. In aquatic animals, it has recently been reported that shrimp Rab is implicated in white spot syndrome virus infection and required for optimal hemocytic phagocytosis [21,22].

To date, more than 60 different types of Rab GTPases have been identified in humans [23,24]. In teleosts, genes encoding Rab-like proteins have been found to exist in a number of species; however, the potential functions of piscine Rab GTPases remain uninvestigated. In this study, we identified and analyzed a Rab1 homologue from red drum (*Sciaenops ocellatus*), an important cultured fish species in China. We found that red drum Rab1 is a functional GTPase that is required for deterring intracellular infection of bacterial pathogen.

2. Materials and methods

2.1. Fish

Red drum (*S. ocellatus*) were purchased from a commercial fish farm in Fujian Province, China and maintained at 22 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were anaesthetized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving tissue collection.

2.2. Bacterial strains and culture condition

Edwardsiella tarda TX1 is a fish pathogen that has been reported previously [25]. *Escherichia coli* BL21(DE3) was purchased from Tiangen (Beijing, China). All strains were cultured in Luria-Bertani broth medium at 28 °C (for *E. tarda*) or 37 °C (for *E. coli*) as described previously [25].

2.3. Cloning of SoRab1

A full-length cDNA library was constructed from the mRNA of red drum head kidney, spleen, and liver [26]. Plasmid was isolated from ~1000 clones of the library and subjected to sequence analysis. One plasmid was found to contain the open reading frame (ORF) of a *Rab1* homologue (named *SoRab1* for "*S. ocellatus* Rab1") with 5′- and 3′- untranslated regions (UTRs). The nucleotide sequence of *SoRab1* has been deposited in GenBank database under the accession number HQ185378.

2.4. Sequence analysis

The cDNA and amino acid sequences of SoRab1 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the conserved domain search program of NCBI. The calculated molecular mass and theoretical isoelectric point (pI) were predicated by EditSeq of the DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was created with the ClustalX program.

2.5. Plasmid construction

To construct pEtSoRab1, the ORF of *SoRab1* was amplified by PCR with primers F1 (5′- GATATCGCCACCATGAATCCCGAATACGACT -3′; underlined sequence, EcoRV site) and R1 (5′- CGCGATATCGCAGCATCCTCCTGAT -3′; underlined sequence, EcoRV site); the PCR products were ligated with the T—A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid pTASoRab1 was digested with EcoRV to retrieve the 0.6 kb fragment, which was inserted into pET259 [27] at the SwaI site. To construct pISoRab1, pTASoRab1 was digested with EcoRV, and the 0.6 kb fragment was inserted into pID3 [28] at the EcoRV site. pID3 is a derivative of the mammalian expression plasmid pIRES2-EGFP (Clontech, Mountain View, CA, USA), which is designed to express the gene of interest (in this case, *SoRab1*), under the human cytomegalovirus (CMV) immediate early promoter. For DNA vector-based siRNA technology, the siRNA expression vector pRNAT-CMV3.1 (GenScript, Piscataway, NJ, USA) was used, in which CMV promoter drives the expression of siRNA that can be inserted into the vector between BamH I and Afl II sites. In our study, the *SoRab1*-specific siRNA (5′-CCAGTGCCAAGAACGCCAC-3′) and the *SoRab1*-nonspecific siRNA (5′-CCACAACTTGCTCAACCAC-3′) were inserted into pRNAT-CMV3.1, resulting in plasmids pRiSoRab1 and pRiNC1 respectively.

2.6. Purification of recombinant protein

Recombinant SoRab1 expressed in *E. coli* (rSoRab1) was purified as follows. *E. coli* strain BL21(DE3) was transformed with the plasmid pEtSoRab1. The transformant was cultured in LB medium at 37 °C to mid-logarithmic phase (OD_{600} 0.8), and expression of SoRab1 was induced by adding into the culture isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM. The growth was continued at 30 °C for an additional 4 h, and recombinant protein was purified under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) columns (GE Healthcare, USA) as recommended by the manufacturer and as described previously [29]. The protein was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250.

2.7. rSoRab1 activity analysis

The GTPase activity of rSoRab1 was determined using the EnzChek Phosphate Assay Kit (Molecular Probes, Leiden, the Netherlands) according to manufacturer's instructions. Briefly, undenatured rSoRab1 or heat-denatured (by boiling at 100 °C for 20 min) rSoRab1 in different concentrations were incubated with 0.5 mM GTP in 50 mM Tris/Hepes (1:1) (pH 8.0) buffer at 37 °C. The reactions were stopped by transferring the samples on ice. For quantitation of inorganic phosphate (Pi), the 2-amino-6-mercapto-7-methylpurine riboside/purine nucleoside phosphorylase system was added to each sample, and the mixture was incubated at 22 °C for 30 min. Free Pi was determined by measuring absorbance at 360 nm.

2.8. Quantitative real time reverse transcriptase-PCR (qRT-PCR) analysis of SoRab1 expression in fish tissues

Brain, heart, gill, kidney, spleen, liver, muscle, and blood were taken aseptically from red drum and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR