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Identification and characterization of Rab7 from orange-spotted grouper, *Epinephelus coioides*

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ABSTRACT

Rab7 is a small GTPase that regulates vesicular traffic from early to late endosomal stages of the endocytic pathway. During the virus–host co-evolution, host Rab7 was also exploited by virus to complete their life cycle. To date, however, the roles of fish Rab7 in virus infection remained largely unknown. Here, we cloned and characterized a Rab7 gene from grouper, *Epinephelus coioides* (Ec-Rab7). The full-length Ec-Rab7 cDNA was composed of 1182 bp and encoded a polypeptide of 207 amino acids which shared 99% identity with that from *Anoplopoma fimbria* or *Oreochromis niloticus*. Ec-Rab7 contained five conserved domains of Rab GTPase family including GTP-binding or GTPase regions as well as an effector site. RT-PCR analysis revealed that Ec-Rab7 ubiquitously expressed in all detected tissues and its transcript in spleen was up-regulated after challenge with Singapore grouper iridovirus (SGIV). Subcellular localization analysis revealed that Ec-Rab7 was distributed in the cytoplasm as spots and mostly colocalized with lysosomes. Notably, the ectopic expressed Ec-Rab7 partly aggregated into the viral factories in cells infected by SGIV. Furthermore, overexpression of Ec-Rab7 accelerated the occurrence of cytopathic effect (CPE) induced by SGIV infection and promoted viral gene transcription. In addition, far western blotting assay revealed that Ec-Rab7 might interact with viral proteins, including SGIV VP69 and VP101. Taken together, our data suggested that Ec-Rab7 might be potentially involved in SGIV replication.

1. Introduction

Ras-associated binding (Rab) proteins are small GTPases of the Ras superfamily and are central regulators of endocytic pathway including vesicle formation, motility, tethering or docking to their target membranes. To date, more than 60 Rab genes have been identified in human genome [1]. Different Rab GTPases were localized to specific membrane-enclosed compartments of exocytic and endocytic pathways, where they acted as regulators of distinct steps in membrane traffic pathways [2,3]. Among them, Rab7 was ubiquitously expressed in most tissues and regulated the traffic from early to late endosomes and from late endosomes to lysosomes. In addition, Rab7 was also a key regulatory protein for the biogenesis and maintenance of the lysosomal compartment [4,5].

Endocytosis not only played critical roles in the physiological process by regulating many signaling pathways during development, but also was utilized for viruses infection by mediating virus internalization or trafficking the virus particles to the site of replication [6,7]. As an important regulator in endocytosis, Rab7 has been demonstrated to exert important roles in the process of virus infection [8–11]. For example, Semliki Forest Virus (SFV) particles partly colocalized with Rab7 after 30 min of infection [9]. Influenza A virus could not infect the cells expressing the constitutively inactive Rab7 mutant protein [8]. In addition, Rab7 was also required for virus release of HIV-1 at the late stage of the viral replication cycle [12]. Besides the mammalian viruses, shrimp Rab7 was also found to interact with invertebrate virus-white spot syndrome virus (WSSV) envelop protein VP28 during infection [13]. Although several fish Rab7s were cloned and identified [14], the information on the roles of Rab7 in fish virus infection remained scarce.

Orange-spotted grouper, *Epinephelus coioides*, is an important marine fish species cultured in China and Southeast Asian counties.

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In recent years, prevalent outbreaks of viral diseases have caused heavy economic losses in grouper aquaculture industry. Singapore grouper iridovirus (SGIV), a novel iridovirus belonged to genus Ranavirus, is one of the major pathogens isolated from diseased grouper [15]. Our previous studies revealed that SGIV induced non-apoptosis in grouper cells and activated mitogen-activated protein kinases (MAPKs) signaling pathway [16,17]. Moreover, SGIV entered into cells via endocytosis like other iridovirus isolates [18,19]. However, little information was explored on the roles of endocytosis pathway regulators-Rab GTPases during virus infection. Here, Ec-Rab7 was cloned and characterized from grouper, *E. coioides*, and its effect on SGIV infection was investigated. Our results might contribute greatly to understanding the molecular mechanisms of iridovirus pathogenesis.

2. Materials and methods

2.1. Fish, cell line and virus

Juvenile orange-spotted grouper, *E. coioides* (50–60 g) were purchased from a fish farm in Huizhou, Guangdong province, China. Fish were maintained in a laboratory recirculating seawater system at 25–30 °C and fed twice daily for further study.

Grouper spleen (GS) cells used in this study were grown in Leibovitz's L15 medium containing 10% fetal bovine serum (Invitrogen, USA) at 25 °C [20]. Singapore grouper iridovirus (SGIV) were kept in our lab and propagated in GS cells. Viral stocks were stored at −70 °C until use.

2.2. Rapid amplification of cDNA ends (RACE) of Ec-Rab7 cDNA

According to the partial EST sequence of Ec-Rab7 from our published transcriptome library [21], the 5′ and 3′ ends of Ec-Rab7 cDNA were obtained using a SMART RACE cDNA amplification kit (Clontech, USA) following the manufacturer's protocol. All the primers used are listed in Table 1. In detail, the primers 5′EcR7-R1, 3′ EcR7-F1 and UPM Mix (supplied by the kit) were used for first-round PCR, and the corresponding product was diluted for the nested PCR using primers 5′EcR7-R2, 3′ EcR7-F2 (Table 1) and NUP (supplied by the kit). Finally, the PCR products of 3′ and 5′ RACE were separated on 1.0% agarose gel and purified for sequencing by ABI3730, respectively.

2.3. Bioinformatic analysis of Ec-Rab7

The nucleotide and predicted amino acid sequences of Ec-Rab7 were analyzed using Genetyx7.0 software. The similarity of Ec-Rab7 with other Rab7s was analyzed using the BLASTX search program at the NCBI (www.ncbi.nlm.nih.gov/blast). Multiple-sequence alignment of the reported Rab7 amino acid sequences was performed using ClustalX2.0 and phylogenetic tree was constructed using the MEGA 4.0 software.

Table 1
Sequences of primers used in this study.

Primers	Sequence (5′–3′)
5′EcR7-R1	ACTGGTTCATCAACGAGGTCTTCCCAA
5′EcR7-R2	TTCATCAACGAGGTCTTCCCAACTCC
3′EcR7-F1	ACAGGTAACAACCAAGCGAGCACAGGC
3′EcR7-F2	ACAGGTAACAACCAAGCGAGCACAGG
RT-actin-F	TACGAGCTGCCTGACGGACA
RT-actin-R	GGCTGTGATCTCCTTCTGCA
RT-Ec-Rab7-F	AGAGGTTCCAGTCCTTAGGTGTAGCGTT
RT-Ec-Rab7-R	GGTTGTTACCTGTCTGTTCTCCAAGTCG
pEGFP-Rab7-F	GAAGATCT ATGACTTCAAGGAAGAAAGTACTAC
pEGFP-Rab7-R	GCGTCGAC TCAGCAGCTGCAGGTCTCTGCTGATG
pGEX-Ec-Rab7-F	CGGAATTCATGACTTCAAGGAAGAAAGTACTAC
pGEX-Ec-Rab7-R	GCGTCGAC TCAGCAGCTGCAGGTCTCTGCTG
RT-ORF072-F	GCACGCTTCTCTCACCTTCA

2.4. Tissue distribution of Ec-Rab7

Total RNA was extracted from healthy orange-spotted grouper head kidney, heart, liver, spleen, intestine, muscle, brain, skin, gill, stomach and kidney, respectively, using TRIzol Reagent (Invitrogen, USA) according to manufacturer's instructions. Expression of Ec-Rab7 in different tissues was determined by RT-PCR using primers shown in Table 1. The PCR conditions were applied as follow: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Primers RT-actin-F and RT-actin-R were used to amplify β-actin as an internal control (Table 1).

2.5. Expression profiles of Ec-Rab7 after challenge with SGIV

To determine the expression profiles of Ec-Rab7 in response to SGIV infection, groupers were injected intraperitoneally (i.p.) with 200 μl PBS (control group) or SGIV of 10^5 $TCID_{50}$/ml. At indicated time points (0, 6, 12, 24, 48, 72, 96 h p.i.), the spleen of control or infected group ($n = 3$) were collected for RNA extraction. Expression levels of Ec-Rab7 were examined by qRT-PCR using the following cycling condition: 94 °C for 5 min, followed by 40 cycles of 5 s at 94 °C, 10 s at 60 °C and 15 s at 72 °C. The results were expressed as relative fold of one sample in each experiment as mean ± SD.

2.6. Expression and purification of recombinant Ec-Rab7

Primers pGEX-Ec-Rab7-F and pGEX-Ec-Rab7-R (Table 1) were used to amplify the open reading frame (ORF) sequence of Ec-Rab7. The recombinant expression plasmid pGEX-Ec-Rab7 was transformed into *Escherichia coli* BL21 (DE3) cells. After induction with the isopropyl-1-thio-b-D-galactopyranoside (IPTG), the fusion protein was purified using the GST-Bind purification kit (Novagen) for the following far western blotting assay.

2.7. Subcellular localization of Ec-Rab7 and its roles on viral replication

To elucidate subcellular localization of Ec-Rab7 in fish cells, the recombinant pEGFP-Rab7 or pEGFP-C1 plasmid was transfected using Lipofectamine-2000 Reagent (Invitrogen, USA) as described previously [22]. After transfection, cells with or without SGIV infection were washed with PBS and fixed with 4% paraformaldehyde for 1 h. After staining with DAPI (1 mg/ml) for 5 min, cells were observed using fluorescence microscopy (Leica). To label the lysosome with the lysosome marker, cells were stained with 100 nM Lyso-Tracker Red for 30 min, and then examined under fluorescence microscopy.

To detect the roles of Ec-Rab7 on SGIV replication, plasmids pEGFP-Ec-Rab7 or pEGFP-C1 (vector control) were transfected and GS cells, respectively. After incubation with SGIV for 36 h, the transcription of viral gene MCP was detected using qRT-PCR as described above.

2.8. Far western blotting

Protein samples were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore), then denatured and renatured gradually at 4 °C in AC buffer (100 mM NaCl, 20 mM Tris pH 7.6, 0.5 mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skim milk powder and 1 mM