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Molecular characterization and immune responses of Rab5 in large yellow croaker (*Larimichthys crocea*)☆

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ABSTRACT

Rab5 regulates key steps in membrane traffic transport and endocytic pathway of host immune responses. In the present study, a full-length cDNA of Rab5 (LcRab5) was cloned from large yellow croaker, *Larimichthys crocea* and contained an open reading frame (ORF) of 648 bp encoding a 215 amino-acid protein. The amino acid sequence identities between LcRab5 and the homologues in mammals and other fish are from 87% to 97%. Quantitative real-time PCR (qRT-PCR) analysis revealed that LcRab5 mRNA was predominantly expressed in blood and gill. The expression levels of LcRab5 were investigated in liver, spleen, and head-kidney after fish were challenged with LPS, polyinosinic polycytidynic acid and *Vibrio parahaemolyticus*. LcRab5 transcripts were significantly induced in the spleen after immune challenge and were 24.1 times more abundant compared with the control 24 h after injection of *V. parahaemolyticus* ($P < 0.05$). Recombinant LcRab5 protein was expressed and purified from *Escherichia coli*. Immunoelectron microscopy analysis showed that LcRab5 protein was localized in the mitochondria of liver and fibrous tissue of spleen. Additionally, overexpression of LcRab5 in *L. crocea* kidney (LCK) cells significantly enhanced tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) transcripts ($P < 0.05$). Our results indicate that LcRab5 is involved in the immune response of the large yellow croaker and improves the inflammatory response through activation of TNF-α and IL-6 expression.

1. Introduction

Large yellow croaker (*Larimichthys crocea*) is one of the most economically important marine fish in China. However, increasing environmental pollution has severely affected *L. crocea* immunity, which resulted in considerable economic losses (Martinez-Urtaza et al., 2004; Zheng, Liu, Ao, & Chen, 2006). In particular, *L. crocea* aquaculture has been affected by various pathogenic diseases caused by parasitic, bacterial, and viral infection (Han, Xiao, Zhang, & Wang, 2015; Zhu et al., 2016), leading to the outbreak of epidemics, especially for fish seedlings and leading to an infection rate and mortality of up to 100% (Niu et al., 2013). Several important immune factors of large yellow croaker have been investigated, such as TLRs, IL6, complement component C, scd1 and some small G proteins (Liu et al., 2016; Han, Wang, Yao, & Wang, 2010; Han, Wang, Huang, Zheng, & Wang, 2011; Han, Wang, Yang, Cai, & Wang, 2011; Han, Wang & Wang., 2013; Han, Xiao, Zhang, & Wang, 2015; Mu et al., 2014; Xu, Zhang, Lv, Luo, & Wang, 2015; Zheng et al., 2006; Zhu et al., 2016), with the objective of understanding its immune response to pathogens and to develop strategies for disease management and long-term sustainability of aquaculture.

RabGTPase, a member of the Ras superfamily which comprises Ras, Rho/Rac/Cdc42, Ran, Sar/Arf, and Rab, are found in all eukaryotes, including yeast, plants, insects, and mammals (Rink, Ghigo, Kalaidzidis, & Zerial, 2005; Segev, 2001; Zerial & McBride, 2001). In the process of exocytic and endocytic membrane trafficking, Rab proteins regulate plasma membrane delivery, organelle biogenesis, and degradative pathways (Kroemer and Jäättelä, 2005; Posiri, Panyim, & Ongvarrasopone, 2016). These proteins regulate membrane trafficking by cycling between inactive (GDP-bound) and active (GTP-bound) conformations (Pan, Eathiraj, Munson, & Lambright, 2006).

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Rab5, located in the plasma membrane, controls the fusion of the early body and the endocytic vesicle (Letunic et al., 2006; Rothman & Wieland, 1996), and therefore, Rab5 is a major rate-limiting component of the early endocytic pathway (Barbieri et al., 2000; Naj & Linder, 2015). Not only does Rab5 participate in intracellular protein transport and sorting, but Rab5 is also involved in signal transduction (Bucci & Chiariello, 2006; Diao, Frost, Morohashi, & Lowe, 2008). Mutations of Rab5A lead to cell dysfunction, which in turn, causes various diseases (Niu et al., 2013). In addition, Rab5A regulates the function of $CD8^+$ T cells through the integrin LFA-1 or the chemokine receptor CXCR4, which induces PKCε-dependent phosphorylation of Rab5a at Thr-7, suggesting that Rab5A is crucial for cytoskeleton remodeling and cell migration (Ong et al., 2014). Mutation of Rab5 leads to cell dysfunction and subsequently various diseases in human (Nikoshkov et al., 2015), implying that Rab5 might have crucial functions in the fish immune response. However, the roles of Rab5 in the immune response of large yellow croaker are still poorly understood.

In the present study, the full-length cDNA of Rab5 from *L. crocea* (LcRab5) was cloned. The tissue expression profiles and temporal expression levels of LcRab5 in the spleen, head-kidney, and liver after bacterial lipopolysaccharides (LPS), viral mimic polyinosinic polycytidynic acid (poly I:C), and *Vibrio parahaemolyticus* challenge were detected by real-time PCR. Furthermore, the expression of two key immune factors, TNF-α and IL-6, were examined in *L. crocea* kidney (LCK) cells after LcRab5 over-expression in order to better understand the roles of Rab5 in the fish immune response.

2. Materials and methods

2.1. Fish collection and immune challenge

Healthy large yellow croakers (100 g ± 20 g) were obtained from the Fishery Extension Station of Ningde (Fujian, China), kept in net cages at a salinity of 25–26 and temperature 25 °C, and fed with a commercial feed. Samples of tissues including blood cells, head-kidney, intestine, spleen, liver, gill, skin, heart, and dorsal muscle were dissected from three fish, and then frozen in liquid nitrogen immediately. These samples were stored at −80 °C for RNA extraction. For challenge experiments, the fish were cultured at least two weeks and then injected intraperitoneally with 0.5 mL PBS (control group), 0.5 mL polyinosinic polycytidynic acid (poly I:C) (27472901, GE Healthcare, England; experimental group 1) at a titer of 0.8 mg/mL, 0.5 mL formalin-inactive Gram-negative bacterium *V. parahaemolyticus* (isolated from diseased fish; experimental group 2) at a titer of 10^8 cfu/mL, and 0.5 ml LPS (L2880, Sigma, USA; experimental group 3) at a titer of 0.5 mg/ml, respectively. Finally, head-kidney, spleen, and liver from six control fish and six experimental fish were harvested at 0, 3, 6, 12, 24, 48, and 72 h after injection (fish number: $4 \times 6 \times 7 = 168$), frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Each group was set up in triplicate.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Afterwards, the total RNA was incubated with RNase-free DNase I (Promega, USA) to remove any contaminating genomic DNA. The First strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega, USA), according to the manufacturer's protocol with an Oligo (dT) primer.

2.3. Cloning and sequence analysis of LcRab5

The full-length cDNA sequence of LcRab5 was obtained from previous transcriptomic sequences of various tissues from large yellow croaker in our laboratory (unpublished data). Two specific primers (Rab5-cF1 and Rab5-cR1, Table 1) for RT-RCR were designed based on the sequence of LcRab5. PCR was performed under the following conditions: denaturation at 94 °C for 3min, 30 cycles of 94 °C for 30 s, 55 °C annealing for 30 s, and 72 °C for 1min, followed by a 7 min extension at 72 °C. PCR products were cloned into pMD-18T vector (TaKaRa, Dalian, China) and sequenced.

2.4. Real-time PCR analysis of LcRab5 transcript expression

Total RNA from various tissues at different time points following the immune challenge was extracted as described above. 2 μg total RNA treated with RNase-free DNase I (Fermentas Life Science, Germany) was used for synthesizing first-strand cDNAs using Superscript reverse transcriptase (Fermentas Life Science, Germany) and oligo (dT) primers in a 20 μL reaction volume according to the manufacturer's instructions. Quantitative real-time PCR was performed using iQ™ SYBR Green Supermix (Bio-Rad, Singapore) on an ABI 7500 Real-time Detection System (Applied Biosystems, USA). PCR amplifications were carried out under the following conditions: 3 min at 95 °C, followed by 40 cycles of 15 s at 94 °C, 20 s at 58 °C and 30 s at 72 °C. All reactions were performed in triplicate in a 96 well plate, and the mean value recorded. The relative expression of target genes was normalized to the expression of β-actin and analyzed using the comparative Ct method ($2^{-\Delta\Delta C_T}$) (Livak & Schmittgen, 2001). Statistical analysis was conducted using one-way ANOVA by SPASS 16.0 and a probability level of $P < 0.05$ was considered as statistically significant. All primers used for quantitative real-time PCR are listed in Table 1.

2.5. Recombinant expression of LcRab5 in Escherichia coli

The cDNA fragment, encoding the deduced mature peptide of LcRab5, was amplified with two gene-specific primers Rab5-F and Rab5-R (Table 1), containing the *Bam*HI and *Xho*I restriction sites, respectively. The plasmid pMD19-T-LcRab5 was used as the template and the amplified product was purified using a DNA Purification System (OMEGA, Shanghai), and then digested with *Bam*HI and *Xho*I. The digested products were subcloned into the expression vector pET32a(+) (EMD Biosciences, Novagen), which was digested by the same enzymes. The cDNA sequence was verified by sequencing as indicated above. The recombinant and sequencing-confirmed plasmid pET32a(+)-LcRab5 was introduced into *E. coli* BL21 codon plus (DE3). A single bacterial colony containing pET32a(+)-LcRab5 was transferred into 5 mL of LB medium containing ampicillin (50 μg/mL) and cultured at 37 °C with 200 rpm shaking. The overnight cultures were transferred into fresh LB medium at a 1:100 dilution, and incubated at 37 °C until the logarithmic phase (at OD_{600} of 0.5–0.6), then isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The cultures were incubated at 37 °C to induce the expression of the recombinant LcRab5 fusion protein. After 6 h incubation, cells were harvested by centrifugation at 6,000 g for 5 min at 4 °C, and the cell pellets were re-suspended in lysis buffer (50 mM KH2PO4, 300 mM KCl, 5 mM imidazole, pH 8.0) and sonicated at 65 W for 20 min (each treatment lasted 3 s with a 4 s interval) in an ice-water bath. The bacterial lysate was centrifuged at 12,500 g for 20 min at 4 °C and the supernatant was purified using a Protein Purification System (BIO-RAD, USA) to obtain the recombinant protein. Finally, the purified protein was stored in 20% glycerol at −80 °C until use.

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