Genomic identification and spatial expression analysis of
*Rab-5C-like* gene identified from rock bream
*(Oplegnathus fasciatus)*

M.S. Mothishir†, Navaneethaiyer Umasuthan∗, William Shanthakumar Thulasitha∗, Ilson Whang†‡ and Jehee Lee†‡†

*Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea

**Fish Vaccine Development Center, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea

Despite its economic importance as an aquaculture species, the molecular and genetic information regarding physiologically important elements in rock bream *(Oplegnathus fasciatus)* is not completely understood. Rab proteins play a vital role in cellular mechanisms and immunity as one of the key regulators of membrane trafficking. In this investigation, a Rab gene, named as *RbRab-5C-like*, was identified from *Oplegnathus fasciatus*. *RbRab-5C-like* protein exhibited high homology with Rab proteins of other species and possessed signature characteristics of Rab proteins with four conserved cysteine residues. Phylogenetic analysis showed that *RbRab-5C-like* clustered with other fish counterparts. The *RbRab-5C-like* genomic sequence possesses six exons and five introns. Transcriptional analysis revealed that *RbRab-5C-like* was ubiquitously expressed in all examined tissues with the highest expression occurring in the liver. While the structural and homologic characteristics of *RbRab-5C-like* suggest a strong conservation of this element in different species, its mRNA distribution implies a wide range of biological significance in rock bream.

**Key words:** Rab-5C-like, Membrane trafficking, Phylogenetic analysis, Transcriptional analysis

Rock bream *(Oplegnathus fasciatus)* is an economically important species in the Korean aquaculture industry (Kim & Baek, 2010). However in recent years, with the rapid progress of rock bream industry, the infectious diseases caused by bacteria, viruses and parasites have also become more severe resulting in a great economic loss. Therefore, better understanding of the physiological and immunological elements of *Oplegnathus fasciatus* may contribute to the emerging strategies of sustainable industry.

†Corresponding author: Jehee Lee & Ilson Whang
Tel: 82-64-754-3472; Fax: 82-64-756-3493
E-mail: jehee@jejunu.ac.kr, ilswhang@daum.net

Rab proteins are highly conserved small GTPase of the Ras superfamily (ras, Rho/Rac/Cdc42, Ran, sar/Arf and RabGTPase), present in all eukaryotes, including plants, yeasts, insects and mammals (Rink, Ghigo, Kalaidzidis, & Zerial, 2005; Wennenberg, Rossman, & Der, 2005). Rab proteins play a key role in membrane trafficking transport. They are functioning as ‘molecular switches’ in the key steps of membrane trafficking process including vesicle formation, vesicle motility, membrane modeling, vesicle docking, membrane fusion and act at multiple stages of intracellular transport processes (Segev, 2001; Zerial & McBride, 2001). This proteins regulate membrane
trafficking by cycling between inactive (GDP bound) and active (GTP bound) conformation (Pan, Eathiraj, Munson, & Lambricht, 2006). It is found that Rab protein is also implicated in phagosome formation and maturation (Duclos & Desjardins, 2000; Rupper & Cardelli, 2001). Recently, Rab GTPase have been documented to be associated with immune responses (de Saint Basile & Fischer, 2001). In shrimp, it was found that Rab was up-regulated after white spot syndrome (WSSV) infection (Wu & Zhang, 2007), and a study in large yellow croaker suggested that Rab GTPase played an imperative role in fish defense against bacterial and viral infection (Han, Wang, Yao, & Wang, 2010).

However to date, the evidence of Rab-5C-like GTPase in fish is scanty. In an attempt to comprehend the role of Rab GTPase and its distribution, a Rab-5C-like gene from rock bream was characterized at in silico level in this study.

Materials and method

Rock bream cDNA library construction and isolation of RbRab-5C-like cDNA

A rock bream cDNA sequence database was constructed using the GS-FLX™ genome–sequencing technique (Droge & Hill, 2008). Animal rearing, multi-tissue collection and cDNA synthesis were performed as previously described (Saranya Revathy et al., 2012; Umasuthan et al., 2011). A cDNA GS-FLX shotgun library was created from the sequencing data obtained using the GS-FLX titanium system (DNA Link, Republic of Korea). A single putative cDNA homologous to known Ras-related Rab-5C-like was identified by homology screening using the basic local alignment sequencing tool (BLAST: http://blast.ncbi.nlm.nih.gov/Blast), and was designated as RbRab-5C-like.

BAC library construction, screening and identification of the RbRab-5C-like gene

A BAC library of O. fasciatus was constructed using randomly sheared genomic DNA of rock bream (Lucigen, USA). Screening of the BAC-library was carried out with a PCR-based screening method following the manufacturer’s instructions as reported earlier (Umasuthan et al., 2014). The primers used for screening were designed based on the obtained cDNA sequence (Table 1). Genomic DNA from positive clone was isolated and purified by QIAGEN Large-Construct Kit, and was subjected to sequencing by GS-FLX (Roche).

Molecular characterization and genomic structural analysis of RbRab-5C-like

The cDNA coding for Rab-5C-like was determined using a BLAST search, and analyzed using DNAAssist 2.2 to determine the open reading frame (ORF) and amino acid sequence (AA) (Patterton & Graves, 2000). The exon-intron structure was determined by aligning mRNA to the genomic sequence of RbRab-5C-like by using Spidey (http://www.ncbi.nlm.nih.gov/spi-

Table 1. Primers used in this study

| Name | Target | Purpose | Primer sequence (5’”3’)* | Amplicon size (bp) |
|------|--------|---------|--------------------------|-------------------|
| F1   | RbRab-5C-like | BAC screening and qPCR amplification | TCACAGCTTGACCCGACATGACTA | 111 |
| R1   | RbRab-5C-like | BAC screening and qPCR amplification | TGGAGCTCTTCACCCGAGTTCTTT |  |
| F3   | β-actin | qPCR internal reference | TCATCACCACGCCGAATGAGAGGT |  |
| R3   | β-actin | qPCR internal reference | TGATGCTGTTGTAGGTTGCTCGT | 108 |

F, forward; R, reverse.
Genomic identification and spatial expression analysis of Rab-5C-like gene identified from rock bream (Oplegnathus fasciatus)
Tissue isolation, RNA extraction, and cDNA synthesis

Spatial distribution of RbRab-5C-like gene

In order to investigate the physiological distribution of RbRab-5C-like in different tissues, three healthy fish were dissected on ice and tissues from muscle, intestine, skin, kidney, head kidney, spleen, gill, brain and liver were harvested. Blood was aseptically collected from the caudal fin and centrifuged at 3000×g for 10 min at 4°C. The supernatant was removed and cells were collected. All the tissue samples collected were immediately snap-frozen in liquid nitrogen and stored at -80°C until use.

RNA isolation and cDNA synthesis

Total RNA was extracted from the isolated tissue samples using TRI Reagent™. Purified RNA was diluted to 1 µg/µL, and a 2.5 µg sample was used to synthesize cDNA using PrimeScript™ first-strand cDNA synthesis kit (Takara, Japan). Finally, the syn-

Fig. 2. Multiple sequence alignment of Rab-SC homologs from different taxa. The four conserved cysteine are highlighted in red boxes. The Rab domain is flanked by black arrow.
thesized cDNA was diluted 40-fold and stored at -20°C until use.

**Tissue specific expression of RbRab-5C-like**

Quantitative real-time PCR (qPCR) was performed to determine the specific expression pattern of RbRab-5C-like in different tissues using the primers listed in Table 1. Rock bream β-actin was used as the invariant housekeeping gene. In brief, qPCR was performed in a 10 µL reaction volume containing 3 µL of diluted cDNA, 5 µL of SYBR Green mix, 0.4 µL of each primer (10 pmol/µL), and 1.2 µL of PCR-grade water under the following thermal profile: 1 cycle of 95°C for 3 min, followed by 33 amplification cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 30 s. The baseline was set automatically by the Thermal Cycler Dice™ Real Time System software (version 2, TaKaRa). The expression level of RbRab-5C-like was determined by Livak method (Livak & Schmittgen, 2001). The relative expression level calculated in each tissue was compared with the respective expression level in muscle. All the data are represented as relative Rb-Rab-5C-like mRNA expression (mean± standard deviation (SD)) and were subjected to one-way analysis of variance (ANOVA) to compare the means and followed by Duncan’s Multiple Range test. Statistically significant levels of RbRab-5C-like transcriptional expression in different tissues was determined by comparing the respective transcription levels with muscle tissue using SPSS.

---

Fig. 3. The neighbor joining phylogenetic tree of Rab homologues and relative position of RbRab-5C-like. Accession numbers of orthologs used are following: house mouse (NP_077776.2), brown rat (NP_001099310.2), cow (NP_001029915.1), human (NP_004574.2), African clawed frog (XP_001025499.1), turkey (XP_003213173.1), chicken (NP_989856.1), rainbow smelt (ACO_09401.1), puffer fish (XP_003964847.1), Nile tilapia (XP_003442080.1), fairy cichild (XP_006798303.1), zebra mbuna (XP_003964847.1), southern platyfish (XP_005796754.1), Japanese rice fish (XP_004071340.1), Atlantic salmon (ACM_09114.1), zebra fish (XP_005163861.1). The tree was constructed based upon an alignment corresponding to full-length amino acid sequences, using MEGA (5.0). The topological stability of the trees was determined by 5000 bootstrap replications and the bootstrap values are shown on the corresponding branches of the tree.
(11.0) program. A $p$-value of less than 0.05 was considered to be significant. The relative mRNA expression has been provided in terms of mean ± standard deviation (SD).

**Results and discussion**

It is well established that the Rab GTPases play fundamental roles in a number of intracellular processes including signal transduction, cell proliferation, cytoskeletal organization, transduction and membrane trafficking. To date, more than 60 Rab GTPases have been identified in mammals (Zerial & McBride, 2001). In this study a Rab-5C-like GTPase was isolated from rock bream and characterized. The cDNA of Rab-5C-like (GenBank accession. No. KP190140; 2191 bp) was identified from the transcriptomic library using BLAST tool in NCBI and was consisted of a 5′ un-translated region (UTR) of 179 bp, an open reading frame (ORF) of 663 bp encoding a 220 amino acid protein and a 3′-UTR of 1349 bp (Fig. 1). The calculated molecular mass of the RbRab-5C-like protein was 23.55 kDa with a theoretical isoelectric point of 8.64. RbRab-5C-like contained a Rab small GTPase-like domain that belongs to the Ras-superfamily. The RbRab-5C-like was aligned with Rab-5C-like GTPase counterparts of other taxa and re-

![Genomic structural comparison of RbRab-5C-like with other Rab homologs. The size of the exon and intron is indicated above the exon boxes and below the intron lines, respectively. While shaded boxes show ORFs, UTRs are indicated with blank boxes.](image-url)
Genomic identification and spatial expression analysis of Rab-5C-like gene identified from rock bream (Oplegnathus fasciatus) revealed the conservation of four conserved cysteine residues (Fig. 2). This multiple sequence alignment further showed a tight conservation of Rab-5C-like in all the examined vertebrates (Fig. 2). The pairwise alignment showed that RbRab-5C-like demonstrates the higher identity of 99.1% with Osmerus mordax and 98.2% with Oreochromis niloticus, Neolamprologus brichardi, and Xiphophorus maculatus (Table 2). A phylogenetic tree was constructed with the Rab-5C-like homologues available in GenBank. RbRab-5C-like was placed closer to other fish homologues; whereas, the other homologs form the separate cluster of tetrapod taxa (Fig. 3), suggesting that RbRab-5C-like has the origin from a common ancestor.

The genomic structure of RbRab-5C-like consisted of six exons and five introns similar to other vertebrates (Fig. 4). All the introns showed the splice signals consistent with the GT/AG rule. Although RbRab-5C-like possessed six exons, the first, half of the second and sixth exons comprised of noncoding nucleotides (UTRs). The coding sequence was distributed in the second, third, fourth, fifth and sixth exons. Based on the inter-lineage gene structural comparison of vertebrate Rab-5C-like, the ORF seems to be distributed within five exons. Intriguingly, the size of each exon was almost similar, and except the first and last exons in ORF, others were identical in all the vertebrate species accounted in the comparison. The variations observed in the genomic size of the Rab-5C-like homologues, in spite of the high conservation of the coding exons, were due to the presence of additional exon in a few species and varying

![Fig. 5. Tissue-specific differential expression analysis of RbRab-5C-like mRNA by SYBR green qPCR. The relative RbRab-5C-like transcript level of each tissue was calculated by the 2^ΔΔCT method using rock bream beta-actin as reference gene, and results were compared to that of muscle tissue. Tissues: peripheral blood cells (blood), gill, liver, spleen, head kidney, spleen, kidney, skin, muscle, heart, and brain, intestine. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test using SPSS (11.0) program. Data with different letters are significantly different at p<0.05.](image)
Table 2. Percentage similarity and identity of the rock bream Rab-5C-like (RbRab-5C-like) to other homologues at amino acid level

| Organism                        | Common name        | Similarity | Identity | Accession No. |
|---------------------------------|--------------------|------------|----------|---------------|
| Oreochromis niloticus           | Nile tilapia       | 99.10%     | 98.20%   | XP 003442080.1|
| Neolamprologus brichardi       | Fairy Cichlid      | 99.10%     | 98.20%   | XP 006795830.1|
| Xiphophorus maculatus           | Southern Paltyfish | 99.10%     | 98.20%   | XP 0057966754.1|
| Osmerus mordax                  | Rainbow smelt      | 99.10%     | 99.10%   | ACQ 09401.1   |
| Astyanax mexicanus              | Mexican tetra      | 98.60%     | 98.60%   | XP 007256330.1|
| Maylandia zebra                 | Zebra Mbuna        | 98.20%     | 98.20%   | XP 003964847.1|
| Takifugu rubripes               | Puffer fish        | 97.30%     | 97.30%   | XP 003964847.1|
| Oryzias latipes                 | Japanese rice fish | 96.80%     | 96.40%   | XP 004071340.1|
| Danio rerio                     | Zebra fish         | 97.70%     | 96.40%   | XP 005163861.1|
| Ictalurus punctatus             | Channel catfish    | 96.40%     | 95.50%   | AHI 29950.1   |
| Lepisosteus oculatus            | Spotted gar        | 93.20%     | 91.80%   | XP 004633830.1|
| Salmo salar                     | Atlantic Salmon    | 94.10%     | 93.70%   | ACM 09114.1   |
| Condylura cristata              | Star-nosed mole    | 92.30%     | 90.50%   | XP 004684231.1|
| Mus musculus                    | House mouse        | 91.80%     | 90.50%   | NP 077776.2   |
| Microtus ochrogaster            | Prairie vole       | 92.30%     | 90.50%   | NP 001099310.2|
| Rattus norvegicus               | Brown rat          | 91.80%     | 90.50%   | XP 005369081.1|
| Octodon degus                   | Degu               | 92.30%     | 89.50%   | XP 004633830.1|
| Sorex araneus                   | Common shrew       | 91.80%     | 90.50%   | XP 004608856.1|
| Canis lupus familiaris          | Dog                | 91.40%     | 90.00%   | NP 001003261.1|
| Jaculus jaculus                 | Jerboa             | 91.40%     | 90.50%   | XP 004055430.1|
| Echinops telfair                | Hedgehog           | 91.80%     | 89.50%   | XP 004707230.1|
| Loxodonta africana              | African bush elephant | 91.40%     | 90.00%   | Xp 003414284.1|
| Bos taurus                      | Cow                | 91.40%     | 90.00%   | NP 001029915.1|
| Ailuropoda Africana             | Giant panda        | 90.90%     | 90.00%   | XP 002922175.1|
| Homo sapiens                    | Human              | 90.90%     | 90.00%   | NP 004574.2   |

Intron sizes (Fig. 4).

qPCR results revealed that the RbRab-5C-like was broadly expressed in all the tissues examined. Predominant level of expression was detected in liver (Fig. 5). Significantly higher transcription was detected in liver, heart, and blood cells as compared to that in muscle (p<0.05). This ubiquitous expressional pattern in tissues was consistent with the expression profile of Rab8 in zebra fish and Rab6 in shrimp (Wu & Zhang, 2007). Recently the Rho family and Ran family were found to be relevant to phagocytosis (Han et al., 2010; Niedergang & Chavrier, 2005) implicating that the small GTPases may have an imperative role in innate immunity. In this context, our work might provide a hint to explicate the association of RbRab-5C-like in different biological roles such as signal transduction and innate immunity in rock bream. To understand the molecular mechanism underlying the tissue-specific response on different immune stimuli will be necessary in future studies.

In conclusion, Rab-5C-like belonging to the conserved Rab GTPases family was isolated from rock bream Oplegnathus fasciatus and characterized. RabRab-5C-like was broadly expressed in most of the tissues in rock bream with the highest expression in liver. The results presented in this study would be useful for further understanding of the expression, regulation and mechanism of Rabbs in bony fishes and their functions in immune response.
Acknowledgements

This research was a part of the project titled ‘Fish Vaccine Research Center’, funded by the Ministry of Oceans and Fisheries, Korea.

Reference

De Saint Basile, G., & Fischer, A. (2001). The role of cytotoxicity in lymphocyte homeostasis. Current opinion in immunology, 13(5), 549-554.

Droege, M., & Hill, B. (2008). The Genome Sequencer FLX™ System—Longer reads, more applications, straight forward bioinformatics and more complete data sets. Journal of biotechnology, 136(1), 3-10.

Duclos, S., & Desjardins, M. (2000). Subversion of a young phagosome: the survival strategies of intracellular pathogens. Cellular microbiology, 2(5), 365-377.

Han, F., Wang, X.-Q., Yao, C.-L, & Wang, Z.-y. (2010). Molecular characterization of Rangene up-regulated in large yellow croaker (Pseudosciaena crocea) immunity. Fish & shellfish immunology, 29(2), 327-333.

Kim, J. W., & Baeck, G. W. (2010). Preliminary EST analysis of immune-relevant genes from the liver of LPS-stimulated rock bream Oplegnathus fasciatus. J. Fish Pathol, 23(2), 229-238.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(Delta Delta CT) Method. Methods, 25(4), 402-408.

Niedergang, F., & Chavrier, P. (2005). Regulation of phagocytosis by Rho GTPases Bacterial Virulence Factors and Rho GTPases (pp. 43-60): Springer.

Pan, X., Eathiraj, S., Munson, M., & Lambright, D. G. (2006). TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism. Nature, 442(7100), 303-306.

Patterson, H.-G., & Graves, S. (2000). DNAssist: the integrated editing and analysis of molecular biology sequences in Windows. Bioinformatics, 16(7), 652-653.

Rink, J., Ghigo, E., Kalaidzidis, Y., & Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell, 122(5), 735-749.

Rupper, A., & Cardelli, J. (2001). Regulation of phagocytosis and endo-phagosomal trafficking pathways in Dictyostelium discoideum. Biochimica et Biophysica Acta (BBA)-General Subjects, 1525(3), 205-216.

Saranya Revathy, K., Umasuthan, N., Whang, I., Lee, Y., Lee, S., Oh, M.-J., Jung, S., Choi, S. Y., Park, C.-Y., Park, H.-C. (2012). A novel acute phase reactant, serum amyloid A-like 1, from Oplegnathus fasciatus: Genomic and molecular characterization and transcriptional expression analysis. Developmental & Comparative Immunology, 37(2), 294-305.

Segev, N. (2001). Ypt and Rab GTPases: insight into functions through novel interactions. Current opinion in cell biology, 13(4), 500-511.

Umasuthan, N., Bathige, S., Revathy, K. S., Wickramaarachchi, W. D. N., Wan, Q., Whang, I., Kim, E., Park, M.-A., Park, H.-C., Lee, J. (2014). A C1 inhibitor ortholog from rock bream (Oplegnathus fasciatus): Molecular perspectives of a central regulator in terms of its genomic arrangement, transcriptional profiles and anti-protease activities of recombinant peptide. Developmental & Comparative Immunology, 42(2), 197-210.

Umasuthan, N., Whang, I., Kim, J.-O., Oh, M.-J., Jung, S.-J., Choi, C. Y., Yeo, S.-Y., Lee, J.-H., Noh, J. K., Lee, J. (2011). Rock bream (Oplegnathus fasciatus) serpin, protease nexin-1: Transcriptional analysis and characterization of its antiprotease and anti-coagulant activities. Developmental & Comparative Immunology, 35(7), 785-798.

Wennenberg, K., Rossman, K. L., & Der, C. J. (2005). The Ras superfamily at a glance. Journal of cell science, 118(5), 843-846.

Wu, W., & Zhang, X. (2007). Characterization of a Rab GTPase up-regulated in the shrimp Penaeus japonicus by virus infection. Fish & shellfish immunology, 23(2), 438-445.

Zerial, M., & McBride, H. (2001). Rab proteins as membrane organizers. Nature reviews Molecular cell biology, 2(2), 107-117.

Manuscript Received : Nov 26, 2014
Revised : Jul 7, 2015
Accepted : Jul 23, 2015