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Molecular cloning and expression of black rockfish *Sebastes schlegelii* p47-*phox* (neutrophil cytosolic factor 1)

Ki-Hyuk Kim, Gun Wook Baeck, Mu Chan Kim and Chan Il Park†

Institute of Marine Industry, College of Marine Science, Gyeongsang National University, 455, Tongyeong, 650-160, Korea

The black rockfish *Sebastes schlegelii* neutrophil cytosolic factor components p47 *phox* (phagocyte oxidase) cDNA was cloned. The sequence of the cDNA showed that rockfish p47 *phox* cDNA consisted of 1,952 bp contained open reading frame encoding predicted polypeptide of 420 amino acids. Additionally analysis of the p47 *phox* amino acid sequence showed two potential SH3 domains. The functional domains are highly conserved in many animals, though the sequence of the components of the black rockfish showed low homology with that of mammals. The deduced amino acid sequence of the black rockfish p47 *phox* was similar to those of the carp (60.4%), zebrafish (59,2%), rainbow trout (68.5%), xenopus (55.2%), mouse (54.2%), rabbit (54.5%), rat (53.7%), and chicken (50.9%). The expression of the rockfish p47 *phox* molecule was induced in peripheral blood leukocytes (PBLs) from 1 to 12 h following LPS stimulation, with a peak at 6 h after the stimulation, and which increased at 1, 3, and 12 h after treated with Poly I:C compared with the control. The rockfish p47 *phox* gene was expressed in various tissues of healthy fish. The level of p47 *phox* expression was high in the PBLs, kidney and spleen.

Key words: Black rockfish, p47 *phox*, Leukocyte, Real-time PCR, RT-PCR

The aquaculture and fisheries industries in Korea and Japan consider the black rockfish to be very important due to its great demand among people.

Neutrophils play a prominent role in acute inflammatory reactions because they rapidly migrate to sites of infection and destroy invading microorganisms (Inoue *et al*., 2001). The neutrophil NADPH oxidase is a multi-protein enzyme complex that plays an essential role in host defense (DeLeo *et al*., 1995). Activation of this enzyme involves the assembly of several neutrophil proteins, some located on the plasma membrane, and others in the cytosol (Clark, 1999). Oxidative killing is mediated by oxygen metabolites generated upon activation of the neutrophil enzyme NADPH oxidase (McPhail and Harvath, 1993).

Similar to the neutrophils of mammals, the neutrophils of tilapia *Oreochromis niloticus* and eel *Anguilla japonica* and the head-kidney phagocytes of rainbow trout *Oncorhynchus mykiss* can consume oxygen and produce superoxides (Itou *et al*., 1996; Secombes and Fletcher, 1999; Shiibashi *et al*., 1999). Furthermore, the presence of flavocytochrome *b* has been indirectly or directly demonstrated in fish phagocytes using the spectrophotometric method and an anti-peptide antibody that reacts with human flavocytochrome *b* (Secombes *et al*., 1992; Itou *et al*., 1998; Shiibashi *et al*., 1999). The plasma membrane associated component directly implicated in the flow of electrons from

†Corresponding Author : Chan Il Park, Tel : +82-55-640-3103
Fax : +82-55-642-4509 , E-mail : vinus96@hanmail.net

NADPH to O_2 is a heterodimeric flavocytochrome *b*, which is composed of 91 and 22 kDa subunits (gp91 *phox* and p22 *phox*, respectively) (Jesaitis, 1995). The cytosolic NADPH oxidase proteins include p40 *phox*, p47 *phox* (neutrophil cytosolic factor 1), and p67 *phox* (Clark, 1999; Nauseef, 1999). Upon neutrophil activation, the cytosolic phagocyte oxidase (*phox*) proteins and Rac2 (Diebold and Bokoch, 2001) translocate to the membrane where they associate with flavocytochrome *b*, which results in an active O^{2-} generating complex (Clark, 1999; Inoue *et al*., 2004).

The human (Volpp *et al*., 1989; Wientjes *et al*., 1993), bovine (Bunger *et al*., 2000) and murine (Jackson *et al*., 1994; Mizuki *et al*., 1998) p40 *phox*, p47 *phox*, and p67 *phox* genes have been fully characterized. The cloning and sequencing of rockfish p40 *phox*, p47 *phox*, and p67 *phox* genes have not yet been reported. EST analysis has been of great service in identifying immune-related genes in fish (Nam *et al*., 2000).

Our knowledge concerning the immunology of fish has increased rapidly in recent years, which is desirable because fishes comprise, in terms of species, at least one half of living vertebrates and from a phylogenetic point of view, fish serve as a good model for studying the vertebrate immune system. The aim of this study therefore is the complete sequence and physical linkage of the entire lower vertebrate p47 *phox* gene from a cDNA library. We attempted to clone immune-related genes in the EST analysis from the black rockfish leukocyte cDNA library. And examine expression of rockfish p47 *phox* since the p47 *phox* of a teleost would provide important information for the understanding of the fish immune system and neutrophil evolution.

Materials and Methods

Cloning and sequencing of back rockfish p47 *phox* cDNA

Black rockfish (200-300 g in weight) that had not been exposed to known antigenic stimulation were used as blood and tissue donors for all the in vitro experiments. The partial cDNA fragment of rockfish p47 *phox*, which was isolated in a previous expressed sequence tag (EST) study, was used for the full length of its p47 *phox* cDNA using the 5' RACE method. To obtain the complete black rockfish p47 *phox* sequence, 5' RACE was performed with the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer' s protocol, using primers (Phox-up) based on the determined partial sequences of the black rockfish p47 *phox* (Table 1).

Table 1. The oligonucleotide primers used in this study.

Primer name	Sequence of oligonucleotide primer
Phox-up	5' -CTGGTAATGACGGGTTGATTC-3'
PhoxRT-F	5'-AGCTCATTCTGGAGCGCTGCA-3'
Phox47RT-F	5' -ACCATCCGAAATCCCAAGAGC-3'
Phox47RT-R	5' -ACAACATACAGTGCGGAGAGT-3'
Beta actin-F	5' -TTTCCCTCCATTGTTGGTCG-3'
Beta actin-R	5' -GCGACTCTCAGCTCGTTGTA-3'

The sequencing of cDNA clones was performed using the ABI 3100 automatic DNA sequencer (PE Applied Biosystems, CA) and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

Multiple alignment and phylogenetic analysis of black rockfish p47 *phox*

The determined nucleotide, deduced amino acid sequences and multiple sequence alignments were analyzed with GENETYX ver. 8.0 (SDC Software Development, Japan). The deduced amino acid sequences were compared with sequences of other species in the peptide sequence databases at the National Center for Biotechnology Information (NCBI), using the BLAST network service. Amino acid sequences with complete annotations were obtained from the following species in the GenBank database: black rockfish (Accession No. AB430583), carp (Accession No. NP_001027718), zebrafish (Accession No. CAM16641), rainbow trout (Accession No. BAD60781), mouse (Accession No. BC055836), rabbit (Accession No. NP_001075571), rat (Accession No. CH473973), chicken (Accession No. NM_001030709), and xenopus (Accession No. BC055836). Nucleotide sequence comparisons were carried out using the program BLASTX (Gish and David, 1993). Phylogenies based on the p47 *phoxs* were inferred using the Mega2 software package (Kumar *et al*., 2001) and distance analysis was performed using the neighbor-joining method. The values that supported each node were derived from 2,000 re-samplings.

Expression analysis of black rockfish p47 *phox*

The total RNA from the normal PBLs and the PBLs stimulated with lipopolysaccharide (LPS) (500 μg/ml) or polyinosinic:polycytidylic acid (Poly I:C) (5 μg/ml) was purified as previously reported (Hirono *et al*., 2000). cDNAs were synthesized for real-time PCR from stimulated and non-stimulated leukocytes. The threshold cycle (Ct) values were automatically calculated as the cycle when the fluorescence of the sample exceeded a threshold level that corresponded to 10 standard deviations of the mean of the baseline fluorescence. The amplification was carried out as follows: 1 cycle of 94℃ for 2 min, 30 cycles of 94℃ for 30 s, 59℃ for 30 s and 72℃ for 1 min, with a final extension step of 72℃ for 5 min. Real-time PCR was conducted as previously described (Park *et al*., 2003). The primers for the real-time PCR sequences (PhoxRT-F and Phox47RT-R) are summarized in Table 1. Thermal cycling and fluorescence detection were conducted using the Gene Amp 5700 sequence detection system. All the samples were run in triplicates. The ratio between the β-actin in the standard sample and the test samples were defined as the normalization factor. All the calculations were made using in MS Excel 2000 (Microsoft Corporation). Statistical comparisons were performed using ANOVA (Fisher's protected least squares difference). Values were considered to be significant at $P < 0.05$.

The total RNA (50 ng) from the brain, intestine, kidney, liver and spleen were reverse-transcribed into cDNA using an AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Invitrogen, CA). PCR was performed on the resulting cDNA using the Phox47RT-F and Phox47RT-R specific primer sets (Table 1). β-actin was amplified as a control using the Beta actin-F and Beta actin-R primers (Table 1). The PCR mixtures were denatured at 94℃ for 2 min and then subjected to 25 cycles of 94℃ for 30 s, 54℃ for 30 s and 72℃ for 1 min. The products were visualized by separation on a 1.5% agarose gel.

Results

Cloning and Sequencing of black rockfish p47 *phox* cDNA

The partial cDNA fragment of the black rockfish p47 *phox* was isolated via a previous expressed sequence tag (EST) analysis. Using poly I:C stimulated black rockfish peripheral blood leukocyte (PBL) cDNAs as templates, 5' RACE was performed with the specific primer (Table 1) and the CDS primer to isolate full-length fragments of the black rockfish p47 *phox* cDNA.

The full-length cDNA obtained from the p47 *phox* was 1,952 nucleotides long and contained an ORF of 1,260 nucleotides that encoded 420 amino acids (Fig. 1). The 3' UTR contained a polyadeny-

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CTGACTCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTC 60
GGCACGAGGCGGTGTATCTCAGCATGGAGGAGACCTACGTCAGGCATGTGGAGCTGTTGG 120
                      M  E  E  T  Y  V  R  H  V  E  L  L
GCTTTGAAAAGCGTTCGTACCCGAGTCAGCACTTCGTTTACATGCTGATGGTGAAATGGA 180
G  F  E  K  R  S  Y  P  S  Q  H  F  V  Y  M  L  M  V  K  W
GCGACCTCTCCGAGAAACTCATCTACAGGACGTATCCTGAGATGTACGTCTTCCACAAAT 240
S  D  L  S  E  K  L  I  Y  R  T  Y  P  E  M  Y  V  F  H  K
CGCTGAAGGAGATGTTTCCCATCGAGGCGGGTCAAATAGAAAAGAAGGACAGAATCATCC 300
S  L  K  E  M  F  P  I  E  A  G  Q  I  E  K  K  D  R  I  I
CGTCATTACCAGCGCCGCGGTGGCTGTACAGCGAGAAGTCACCAGAAACCAGGCAGACCA 360
P  S  L  P  A  P  R  W  L  Y  S  E  K  S  P  E  T  R  Q  T
CCTTGACGGAGTACTGCCACTCGCTCATCAACCTGCCGCCTCACATCTCCCGCAGCAAAC 420
T  L  T  E  Y  C  H  S  L  I  N  L  P  P  H  I  S  R  S  K
ACCTCTCCAACTTCCTCAGGGTTCGACCCGAAGACGAGAACCCACCTACCGCAAACCCAA 480
H  L  S  N  F  L  R  V  R  P  E  D  E  N  P  P  T  A  N  P
TGAAAAGAAACGAGACGTTTGTGGTGTCCAGGGAGTTGGCCAGAGGCAATGTATTCGAGA 540
M  K  R  N  E  T  F  V  V  S  R  E  L  A  R  G  N  V  F  E
TTTCCAGCCCCATCATATTGGACACCTACAGCGTGATCGCAGACTTCACCAAGACGTCCA 600
I  S  S  P  I  I  L  D  T  Y  S  V  I  A  D  F  T  K  T  S
AACACGAGATCAATCTGCACGCCGGAGACCTGGTGGAAATTGTGGAGAAAAATCAGAACG 660
K  H  E  I  N  L  H  A  G  D  L  V  E  I  V  E  K  N  Q  N
GTTGGTGGTTCTGCCAGTGTGAGTCTAAACGAGGCTGGATTCCTGCGTCCTACTTGGAGC 720
G  W  W  F  C  Q  C  E  S  K  R  G  W  I  P  A  S  Y  L  E
CCCTGGATGGACCGGAGGAGGCCGAGGACGCTGACCCAGACTACGAAGGAGAGCTGTACG 780
P  L  D  G  P  E  E  A  E  D  A  D  P  D  Y  E  G  E  L  Y
TCACCACCACAGCCTACAAGGCGGAGCAGGAGGACGAGATGTCTCTGGAGGTCGGTGAAA 840
V  T  T  T  A  Y  K  A  E  Q  E  D  E  M  S  L  E  V  G  E
CCGTCGCCGTCATTCACAAGCTGCTGGACGGCTGGTGGGTCATCAGGAAAGGAGATGAGA 900
T  V  A  V  I  H  K  L  L  D  G  W  W  V  I  R  K  G  D  E
CCGGTCATTTCCCCTCCATGTACCTGCACAAGGCCGGCAAGAGAGAGTTATATGGAGCCG 960
T  G  H  F  P  S  M  Y  L  H  K  A  G  K  R  E  L  Y  G  A
AGAGGACGCAGCTGCAGGGAAAGAAACCACCACCTCGCAGGTCCACCATCCGAAATCCCA 1020
E  R  T  Q  L  Q  G  K  K  P  P  P  R  R  S  T  I  R  N  P
AGAGCATCCACAAAAAGGCTCGCCAGCAGCTCAGCCAGGACGCCTACCGCAGGAACAGCC 1080
K  S  I  H  K  K  A  R  Q  Q  L  S  Q  D  A  Y  R  R  N  S
GCCGGTACCTCCAGCAGAAAGGTGTTCCGCAAAAGAACTCCAAGAACCCTGCAAAGTCAC 1140
R  R  Y  L  Q  Q  K  G  V  P  Q  K  N  S  K  N  P  A  K  S
CGCTAAGGGAGATGAAGAACCAGGACAACATTCCTGAGGTGTCCAGCTCCAGTTCGGAGA 1200
P  L  R  E  M  K  N  Q  D  N  I  P  E  V  S  S  S  S  S  E
GCGAGTCGAAGAAGGAGGCTCCGATCATCCCCCCTCGGCCCAGTCCGGAGCTCATTCTGG 1260
S  E  S  K  K  E  A  P  I  I  P  P  R  P  S  P  E  L  I  L
AGCGCTGCAGCGACAACACCCGCAAGAAAGTCAGCATCCACGGGTCACGCACCAGTTCCA 1320
E  R  C  S  D  N  T  R  K  K  V  S  I  H  G  S  R  T  S  S
GCTCCAGCACCAGCTCCACGTCCTAGCACCGGGACTCTCCGCACTGTATGTTGTAAAAGG 1380
S  S  S  T  S  S  T  S  *
CGCAACGAAATACTTGAAGAGCTTTTTATTGTTTCTATTGTTTGAGTAAAGATTTTGGGG 1460
AAATAAGTTATGTTGCATTACATTACAATACATTATATGTTGTTTGGAAAAATTGTGACT 1520
TTAAATAATAACTGATAACTTATTTTATATTTTTCTTATAGTCAAAAAATCCTTTGAAAA 1580
GACCAAAACCAATAATGTGTTAGTTCAAGTTTTTTTTTCGTACATTTATTATTTTATTAC 1640
CTCAGAGAATATTTAAGTTTTTTGCTCATAAATGTGTTTTTTTTTTTCGTACATTTTTAC 1700
TTCCCAATGTCAGAAAATATTTAAGTTTTTTTTTCATAGTTTATGAGGTTTTCTCATATA 1760
TATTACTTTATAATGTCAGAAAATATTCAAGTTTTTTTCTCATAATTTGCTGAGTTTTTT 1820
TCAAAAATATATTGCTTTATAATCTCAGAGAATATTAAAGTTTTTTCTTATTAATTTGT 1880
AAATTTTTTTCTTCATACATTTATTACTGTATAATGTCAGAAAATATTAAAGATTTGTTC 1940
TCATAAAAAAAAAAAAAAAAAAAAAAAAAAAA                             1952
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Fig. 1. Nucleotide and deduced amino acid sequences of the black rockfish p47 *phox*. The arrows indicate the oligonucleotide primers used in this study. The putative signal peptide are underlined. The polyadenylation signal ATTAAA is shown in boldface.

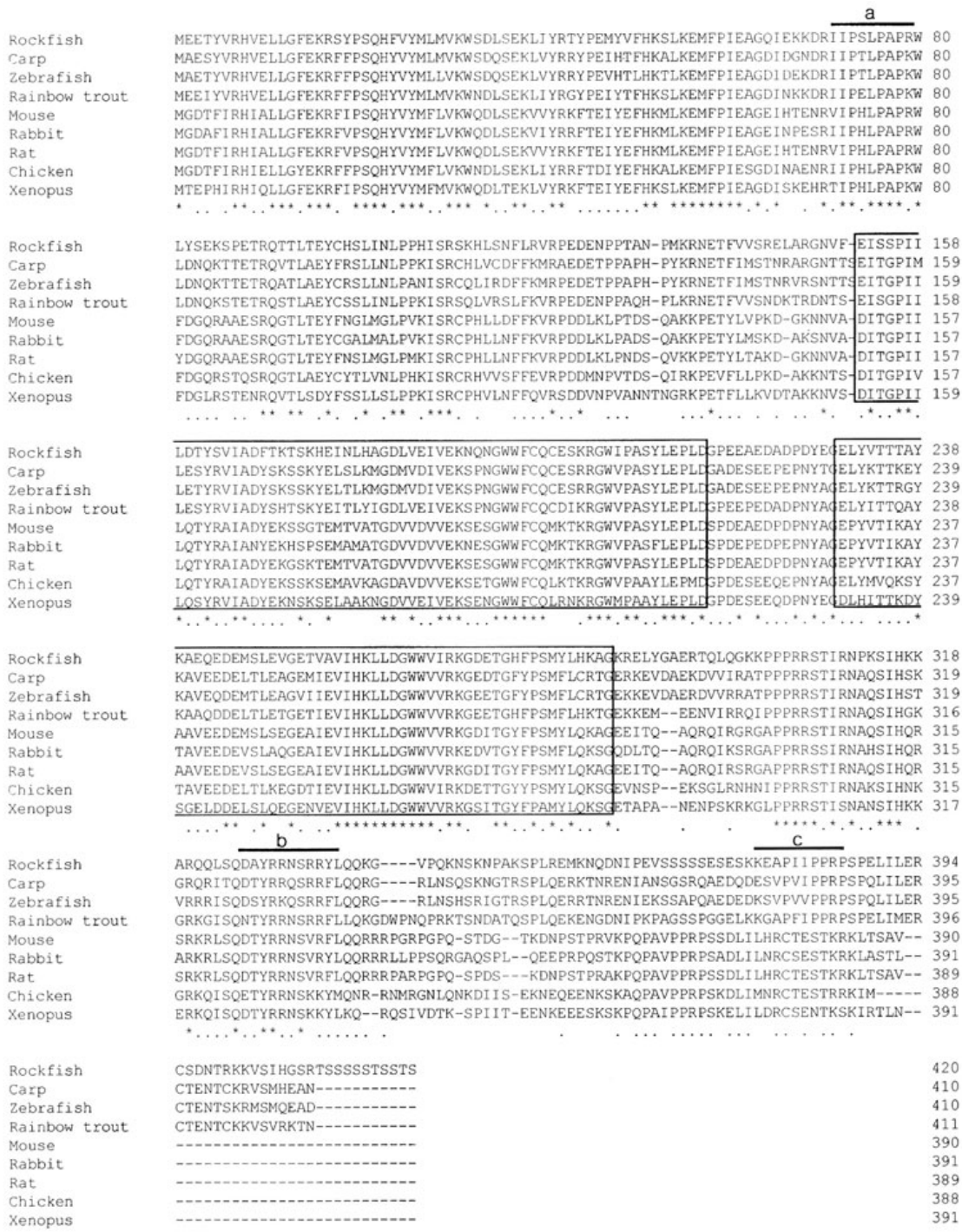

Fig. 2. Comparison of the black rockfish p47 *phox* amino acid sequence with other known p47 *phox* sequences. The amino acids identical to the rock sequence are indicated by an asterisk (*), and the amino acids absent are indicated by a dash (-). Both SH3 domains are shown in boxes, and the consensus sites of the phosphorylation homologs are also indicated in boldface. The solid lines indicate (a) the putative N-terminal, proline-rich SH3 domain-binding domain; (b) the p67 *phox*/flavocytochrome *b*-binding region; and (c) the C-terminal prolinerich, p67 *phox* SH3 domain-binding site.

lation signal (AUUAAA) and a polyadenylation site (Fig. 1).

The gene encoded a putative amino acid protein of 48.4 kDa that contained a 20 amino acid signal sequence and conserved two potential SH3 domains as well as the potential amino-terminal proline-rich SH3 domain binding site, the potential putative p67 *phox*/favocytochrome *b* binding site and the carboxy-terminal proline-rich p67 *phox* SH3 domain binding site (Fig. 2), which are characteristics of mammalian and non-mammalian p47 *phox* proteins.

Phylogenetic analysis of black rockfish p47 *phox*

In the phylogenetic analysis, the black rockfish p47 *phox* was grouped with other teleosts p47 *phox* peptides (Fig. 3). This grouping was well-supported by bootstrapping. This result is possibly due to the differences between mammalian and non-mammalian species. The phylogenetic analysis indicated that the known rainbow trout and rockfish are more closely related than the other species.

Expression analysis of black rockfish p47 phox

As shown in Fig. 4, the black rockfish p47 *phox* levels seemed to have reached a peak in expression at 6 h after LPS post-stimulation, with a decrease at

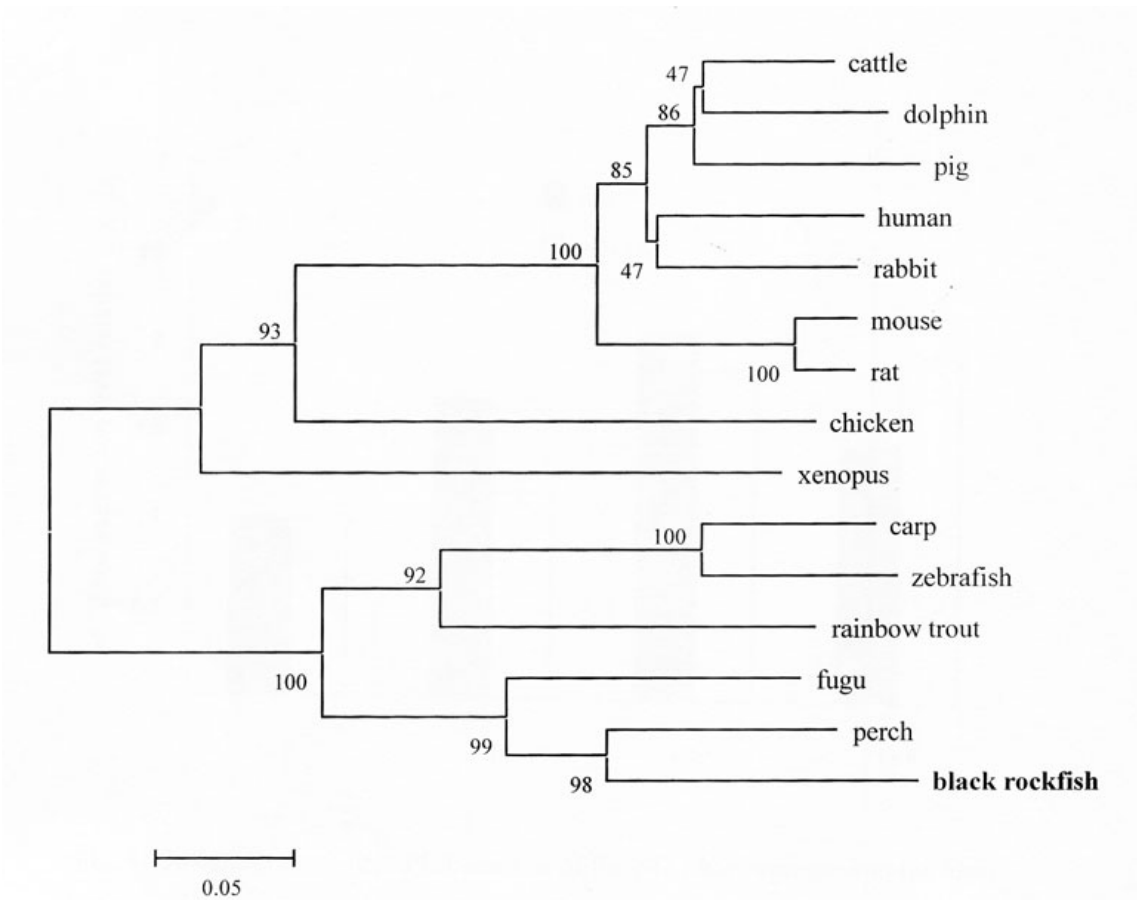

Fig. 3. The phylogenetic tree of p47 *phox* proteins, including of the black rockfish p47 *phox*, from 15 representative species. The positions of the black rockfish p47 *phox* were estimated using the neighbor-joining method of clustering in the Mega2 package program. The sequences were obtained from the DDBJ/EMBL/GenBank.

12 h. The black rockfish p47 *phox* expression was induced from 1 to 12 h following the poly I:C stimulation and peaked at 12 h.

The expression of the p47 *phox* gene in the tissues of the black rockfish was detected with RT-PCR. The black rockfish p47 *phox* transcript was predominantly expressed in the PBLs, kidney, spleen and gills, but not in the liver, brain, intestine and muscles (Fig. 5).

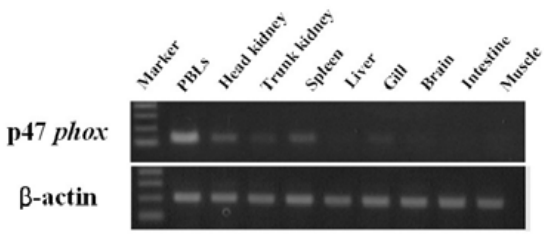

Fig. 5. Detection of the black rockfish p47 *phox* mRNA levels and β actin from various tissues of healthy black rockfish by RT-PCR. The marker is a 100-bp ladder marker.

Discussion

In this study, we report the cloning and sequencing of black rockfish NADPH oxidase cytosolic protein, p47 *phox*. The comparison of the black rockfish p47 *phox* amino acid sequence with the other known p47 *phox* sequences demonstrated considerable similarity with the homologues of the carp (60.4%), zebrafish (59,2%), rainbow trout (68.5%), xenopus (55.2%), mouse (54.2%), rabbit (54.5%), rat (53.7%), and chicken (50.9%).

The phagocyte respiratory burst oxidase (NADPH oxidase) remains inactive until the phagocyte is stimulated by engagement of receptors for chemoattractants, the receptors mediating phagocytosis, or responses to various cytokines. Active NADPH oxidase appears to be a multi-component enzyme system, consisting of at least five proteins: the heterodimeric flavocytochrome *b* and the cytosolic proteins, a second low molecular weight GTP-binding protein, Rac2, p40 *phox*, p47 *phox* and p67 *phox* (DeLeo and Quinn, 1996; Nauseef, 1993).

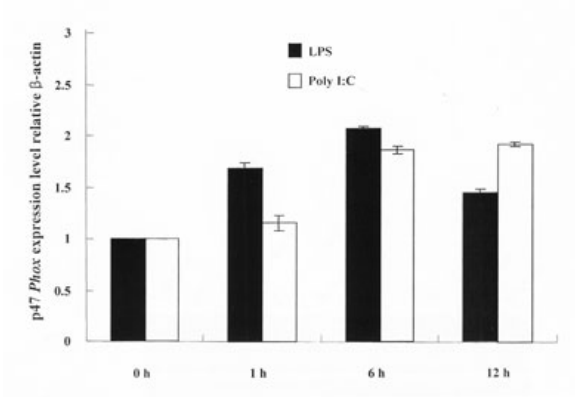

Fig. 4. Quantitative real-time PCR analysis of the p47 *phox* expression in the black rockfish leukocytes stimulated with LPS (500 μg/ml) or Poly I:C (5 μg/ml)at 1, 3, 6, and 12 h.

The rockfish p47 *phox* contained SH3 motif domains (NH2-terminus: Glu152-Asp215; COOH-terminus: Glu230-Gly285) similar to those seen in mammalian and non-mammalian p47 *phox*. The phosphorylation site included residues such as serines 307, 314, 324, 346 and 352. The phosphorylation site of the human p47 *phox* has been shown to be involved in the activation of the oxidase (Huang and Kleinberg, 1999). The serines at residues 307 in the black rockfish p47 *phox* are regarded as part of the protein kinase C substrate consensus sequences (K/R)X(S/T) or (S/T)X(K/R), which suggests that these kination sites in the black rockfish p47 *phox* play the same role in phosphorylation that they play in human (Vignais, 2002). Furthermore, a proline-rich region that conformed to a consensus SH3 domain-binding site was found. All the amino acids in the proline-rich region were conserved in the black rockfish p47 *phox*, as in the other species. Inoue *et al*. reported that stimulation of fugu granulocytes with LPS for 6 h resulted in a significant increase in p47 *phox* transcript expression (Itou *et*

al., 1998). In addition, the human p47 *phox* mRNA level in the macrophages incubated with LPS for 24 h increased (Cassatella *et al*., 1991). Both the LPS and the poly I:C up-regulated the expression of the black rockfish p47 *phox*, but in different manners. This might have been due to the existence of distinct signal pathways activated by these two stimulants. All together, the findings clearly show that both LPS and poly I:C induced rockfish p47 *phox* expression in vitro. Phagocytosis of the phagocytes could have been an intermediate phase of this response.

Relatively large quantities of the black rockfish p47 *phox* transcript were expressed in the PBLs, kidney, spleen and gills could be attributed to the presence of phagocytic cells in these tissues, thus suggesting that this gene plays important roles in the immune system. In fish, phagocytic cells are abundant in the ellipsoids of the spleen, intertublar haemopoietic tissues of the kidney and blood (Ellis and Munroe, 1976).

In conclusion, the black rockfish NADPH oxidase component gene was found to be not highly homologous with those of mammals. The basic structures relevant to the function in particular, the sites and motifs essential for the production of oxygen radicals were well conserved. These structural features together with the patterns of expression in the tissues strongly suggest that the gene encoding NADPH oxidase component in the black rockfish has functions similar to those of the mammalian homologs.

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