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Genomic structural characterization and transcriptional expression analysis of proteasome activator PA28α and PA28β subunits from *Oplegnathus fasciatus*

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ABSTRACT

Proteasomes are multicatalytic subunit complexes involved in the degradation of cytosolic proteins and antigen presentation. In this study, we have characterized the alpha and beta subunits of proteasome activator complex from rock bream at the molecular level. RbPA28α and RbPA28β possessed the characteristic features of the subunits identified from mammals and teleosts. The RbPA28α and RbPA28β proteasome subunits contained a proline-rich motif (Region A), subunit-specific insert in the region corresponding to the KEKE motif of the known PA28α (Region B), conserved activation loop (Region C), a potential protein kinase C recognition site (Region D) and a highly homologous C-terminal region (Region E) among all three PA28 subunits. Multiple sequence alignment and pairwise alignment revealed that RbPA28α and RbPA28β proteins shared high homology with the teleosts and mammals. *RbPA28α* and *RbPA28β* genome possessed 11 exons interrupted by 10 introns. *In silico* promoter analysis of *RbPA28α* and *RbPA28β* revealed various transcription factor-binding sites displaying their regulation under various stress conditions. Tissue distribution profiling showed a higher expression in blood and gills. Transcriptional expression analysis of *RbPA28α* and *RbPA28β* showed up-regulation in the immune tissues following LPS and poly I:C challenges, providing further evidence for the immunological role of *RbPA28α* and *RbPA28β*.

1. Introduction

Proteasomes are large multi-subunit self-compartmentalizing proteolytic complexes comprising a central catalytic machine (20S proteasome) capped by two terminal regulatory sub-complexes known as 19S (PA700) or PA28. The proteasomes are made of four stacked heptameric rings formed by genetically and structurally similar two α-type and two β-type subunits in a $\alpha_7\beta_7\beta_7\alpha_7$ pattern. The subunits are axially stacked on top of each other forming a cylindrical barrel-like structure. In order to avoid unwanted damage of the cytosolic proteins, the 20S channel is usually closed by a gate formed from the N-termini of the α-subunits. The regulatory particle plays a significant role in identifying the ubiquitinated proteins and potential substrates directed for proteolysis. Upon binding of the regulatory complex (19S or PA28), the gate opens allowing the creation/formation of a conglomerate of PA28-20S-PA28 (or 19S-20S-19S) thus activating the latent central catalytic core. The catalytic sites are localized in some of the β-subunits belonging to the central core. Upon interferon-γ (IFN-γ) signaling, three of the seven constitutively expressed β-subunits in the inner ring are replaced by three inducible β-subunits forming an immunoproteasome which is responsible for the generation of peptides that can bind the major histocompatibility complex (MHC) class I molecules [1,2].

The eukaryotic 11S regulators (11S REG or PA26 or PA28) comprise three isoforms called PA28α, PA28β, and PA28γ (also

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called REGα, REGβ, and REGγ or PSME1, PSME2 and PSME3, respectively). One of the 11S REG forms a heteroheptameric ring composed of IFN-γ-inducible α and β subunits, while the other ring is composed of homo-hexameric PA28γ. The 11S REG activates the degradation of peptides in an ATP and ubiquitination-independent fashion unlike the 19S regulators that are ATP-dependent and degrade ubiquitinylated proteins. PA28α and PA28β are immunologically significant as they are involved in the activation of proteasomes and degradation of cellular proteins resulting in peptides which bind to the major histocompatibility complex MHC class I molecules facilitating antigen presentation [3–7].

Studies on antigen presentation mechanisms and activation of adaptive immunity have gained significance in fish and the molecules involved in these processes are being extensively investigated. Although PA28α, β, and γ subunits have been largely explored in human, mice and model organisms like zebrafish, few reports are available that have characterized the PA28α/β subunits and further substantiated their involvement in antigen presentation in fish [8–10]. Moreover, only a few reports are available on the genomic characterization of the proteasome activators. Rock bream are economically significant commercial species in Korea and are prone to infections by various pathogens like iridovirus, and *Edwardsiella tarda* [11–13] causing mass mortality and heavy economic loss. In order to employ novel strategies like vaccination for the prevention of such deadly diseases, it is essential to understand their antigen presentation mechanisms and adaptive immune system. In the present study, we have characterized the PA28α and PA28β subunits from rock bream at the genomic level.

2. Materials and methods

2.1. Rock bream cDNA and BAC library construction, identification of PA28α and PA28β genes

The rock bream cDNA and BAC library were custom constructed as described in our earlier reports [14]. A search for genes related to immunity in the cDNA database using BLAST led to identification of two full-length cDNA contigs that shared a high homology with PA28α and PA28β homologs available in the NCBI database. They were designated as *RbPA28α* and *RbPA28β* and considered for further studies. Two BAC clones harboring *RbPA28α* and *RbPA28β* were identified from the BAC library using the gene specific primers designed with the available cDNA as template (*RbPA28α* F1: 5′ ACTGAAGGAGAAGCTCAACACGGT 3′, R1: 5′ TCTGGGTTTGGAATGC CTCGATCT 3′; *RbPA28β* F2: 5′ ATGCTTCTGGAGAGGGTGAAACCA 3′, R2: 5′ CTGGATGGCAACACCGAAGTCATT 3′). The BAC clone search was performed according to the method specified by the manufacturer and as described in our earlier report [14].

2.2. In-silico analysis of RbPA28α and RbPA28β

The *RbPA28α* and *RbPA28β* cDNAs were analyzed with DNAssist2 to obtain the open reading frame (ORF) and protein sequences. ClustalW was used to execute multiple polypeptide sequence alignments (MSA). PSORT program was used to predict nuclear localization signal (NLS). The phosphorylation sites were predicted using the NetPhos server2. The phylogenetic tree was reconstructed with MEGA 5.0 using the sequences aligned in ClustalW having bootstrap values calculated with 5000 replications to estimate the robustness of the internal branches. The amino acid identity and similarity percentages were calculated using pairwise alignment. The exon–intron structures were determined by aligning mRNA to the genomic sequences of *RbPA28α* and *RbPA28β* obtained from the BAC library using Spidey available on NCBI (http://www.ncbi.nlm.nih.gov/spidey/). The genome structures used for comparative understanding were acquired from the exon view available in Ensembl database. The transcription factor binding sites in the promoter region were predicted using TFSEARCH and TRANSFAC.

2.3. Transcriptional expression profiling of RbPA28α and RbPA28β in normal and challenged tissues

2.3.1. Tissue isolation from normal healthy fish

Healthy rock bream fish weighing an average of ~50 g were obtained from the Ocean and Fisheries Research Institute (Jeju, Republic of Korea). The animals were reconciled to the laboratory conditions (salinity 34 ± 1‰, pH 7.6 ± 0.5 at 24 ± 1 °C) in 400 L tanks. Blood samples were harvested from the caudal fin of healthy, unchallenged fish using a 22-gauge needle and centrifuged immediately for 10 min at 3000 × *g* at 4 °C, to collect the hematic cells. The tissue distribution of *RbPA28α* and *RbPA28β* was examined by harvesting the muscle, intestine, skin, kidney, head kidney, spleen, gill, heart, brain and liver tissues from healthy animals, which were immediately snap-frozen in liquid nitrogen and stored at −80 °C until RNA preparation.

2.3.2. Lipopolysaccharide (LPS) and poly I:C challenge

Healthy rock bream fish were acclimated to laboratory conditions and the mRNA expression of *RbPA28α* and *RbPA28β* determined after challenge by performing a time course experiment with immunostimulants like LPS and poly I:C. The LPS challenge was carried out by dissolving purified LPS from *Escherichia coli* (055:B5; Sigma) in PBS buffer and administering intraperitoneally at the rate of 125 μg per fish (~50 g). Similarly, the poly I:C challenge was implemented by intraperitoneally injecting the animals with a 100 μL suspension of poly I:C in PBS from an original stock of 1.5 μg/μL (Sigma), amounting to 150 μg per animal. PBS-injected animals were used as injection controls while untreated animals were used as un-injected controls. Liver, head kidney, spleen and gill tissues were isolated from the unchallenged, PBS-challenged and immunostimulant challenged fish at 3, 6, 12, 24, and 48 h postinjection/infection (p.i.). The tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further use.

2.3.3. RNA isolation and cDNA synthesis

Total RNA was obtained from isolated tissues and hematic cells pooled from three fish using the Tri Reagent™ (Sigma, USA). The concentration and purity of the RNA were determined by a UV-spectrophotometer (BioRad, USA) at 260 and 280 nm. Purified RNA samples were diluted to 1 μg/μL and pooled. Then, a PrimeScript™ first strand cDNA synthesis kit (TaKaRa) was employed to synthesize cDNA using 2.5 μg of RNA from each tissue. Concisely, RNA was incubated with 1 μL of 50 μM oligo(dT)$_{20}$ and 1 μL of 10 mM dNTPs for 5 min at 65 °C. After incubation, 4 μL of 5× PrimeScript™ buffer, 0.5 μL of RNase inhibitor (20 U) and 1 μL of PrimeScript™ RTase (200 U) were added and incubated for 1 h at 42 °C. The reaction was terminated by adjusting the temperature to 70 °C for 15 min. Finally, the synthesized cDNA was diluted 40-fold before storing at −20 °C.

2.4. Quantitative reverse transcription polymerase chain reaction (qPCR)

2.4.1. Tissue distribution

qPCR was used to examine the distribution of *RbPA28α* and *RbPA28β* mRNAs in muscle, intestine, skin, kidney, head kidney, spleen, gill, heart, brain, liver and blood tissues of healthy fish with gene specific primers (refer Section 2.1) as well as those of *β-actin* (Accession No. FJ975145; Forward primer: 5′ TCATCACCATCGGCA-