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Down-regulation of CD53 expression in *Epinephelus coioides* under LPS, poly (I:C), and cytokine stimulation

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ABSTRACT

Tetraspanins are a group of cell surface molecules involved in cell adhesion, motility, metastasis, signal transduction, and immune cell activation. Members of the tetraspanin family include CD9, CD37, CD63, CD53, and others. However, few tetraspanins have been investigated in teleosts. In this study, we obtained the open reading frame of CD53 cDNA from orange spotted grouper (*Epinephelus coioices*), an economically important fish. The predicted amino acid structure contains four membrane-spanning domains and a conserved CCG motif. The amino acid identity between human and grouper CD53 was only 38%; however, both CD53 proteins share the same structure. Quantitative real-time PCR revealed that mRNA is abundant in immune organs, including the head and trunk kidneys, spleen, thymus, gill, and blood. Immunochemistry and immunofluorescence analyses further revealed that CD53 was majorly expressed in the leukocytes of various organs. Finally, mRNA and protein expression for CD53 was down-regulated in fish treated with immune stimulators, including LPS, Poly (I:C), Vibrio, recombinant grouper IL-6, and CCL4. Our results indicate that the expression of CD53 may play important roles in pathogen invasion and inflammation reaction.

1. Introduction

The tetraspanin superfamily was first recognized in 1991 [1], and to date, 33 different tetraspanins have been found in mammals [2,3]. The structure of proteins belonging to the tetraspanin superfamily comprises four transmembrane domains and two, small and large, extracellular loops (1 small extracellular loop; SEL, and 1 large extracellular loop; LEL). Most tetraspanins were composed of approximately 350 amino acids [4], including highly conserved regions or residues, such as the Cys-Cys-Gly motif, as well as two other conserved cysteines in the LEL. Many studies have suggested that the LEL may be the primary region of connection between cells and other proteins or pathogens [5–7]. The SEL may also interact with viruses and assist in protein binding [8].

Tetraspanins are biosynthesized and translocated in cell membrane, binding and stabilized with other transmembrane proteins [4]. The functions of tetraspanin are diverse because the binding transmembrane proteins are involved in multiple physiological processes, such as cell motility, tissue differentiation, and signal transduction. These complexity functions may of tetraspanin was arise due to the interactions between multiple other tetraspanins, which could can induce tyrosine phosphorylation, calcium fluxes, and inositol phosphate turnover; for example, cross-linking CD81 and CD82 has been shown to modulated the proliferation of CD9, CD151, activate platelets, and human T cells [9]. In addition to physiological processes, a number of tetraspanins, such as CD63, CD81, CD9, CD151, and CD53, may be related to viral infections and tumor invasion [10,11]. Takeda et al. reports have found that the expression of tetraspanins CD9 and CD81 in macrophage was down regulated in smoking-related inflammatory response [12]. The further investigations predicted that CD9 could prevent CD14-dependent receptors from binding to the lipid raft and thereby inhibit macrophage activation [13]. Results of these and other

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studies indicate that tetraspanins play a role in the inflammation reaction.

CD53 is a 32–42 kDa glycoprotein that belongs to the tetraspanin superfamily [14]. CD53 is expressed in B cells, and CD53-mediated signaling is dependent on tyrosine kinases. CD53 is significantly associated with cytokine promotion, and therefore may be able to help decrease the level of TNF α and suppress the production of inflammatory cytokines [15–17]. Patients with a CD53 deficiency or an immunodeficiency syndrome often suffer from recurrent infections [17]. Furthermore, in elderly individuals, a connection has been suggested between the strong expression of CD53 in neutrophils and cellular apoptosis [18]. CD53 is also related to various immune responses; however, the exact function of this protein remains unknown [19]. Indeed, tetraspanin research in mammals remains in the early stages, and even less is known about tetraspanins in teleosts. Nonetheless, the CD9 genes of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) have been cloned, and mRNA expression has been quantified in various organs [20]. The CD63 gene in catfish (*Ictalurus furcatus*) [21] and zebrafish (*Danio rerio*) has been obtained and its expression has been evaluated. Those findings indicate that the biofunction of CD63 may be to assist in embryonic development [22]. Tetraspanin 3 and tetraspanin 7 (CD231) of channel catfish (*Ictalurus punctatus*) have also been obtained, and similarity with their counterpart genes in humans was found to be approximately 40% and 67%, respectively [23]. That study further compared the large extracellular loops of CD81, CD63, TSPAN3, and TSAPN7, and found low similarity between those tetraspanins with regard to amino acids.

Orange spotted grouper (*Epinephelus coioides*) is a fish species of economic importance in Taiwan and Southeast Asia. However, intensive culturing has led to increases in viral and bacterial infections, which can be hard to resolve, thereby decreasing the survival ratio of larvae. One solution is to improve the culture environment and promote grouper immunity. However, this approach relies on a comprehensive understanding of fish immunology. In our previous study, a grouper CD53 gene was identified in the subtractive cDNA library (prepared from *E. coioides* peripheral blood leukocytes [PBL] and stimulated by lipopolysaccharide [LPS] and phosphate buffered saline [PBS]). Our results suggest that CD53 might be involved in the immune response of grouper. However the functions of CD53 are not clear now. In the current study, the full length of CD53 encoding region was obtained; recombinant proteins of the large extracellular loop region of CD53 were produced using an *Escherichia coli* expression system; and mice anti grouper CD53 antibodies were prepared. The tissue distribution of CD53 was evaluated using quantitative real-time PCR and immunohistochemistry analyses. Finally, mRNA expression in various grouper organs stimulated with LPS, poly (I:C), or recombinant grouper cytokines was evaluated. In addition to those stimulators, bacterial pathogens, *Vibrio anguillarum* serotype O1, was also used to stimulated grouper and analysis CD53 expression. Our aim was to expand our understanding of the role that teleost CD53 plays in the innate immune responses of fish.

2. Materials and methods

2.1. Experiment animals

The orange-spotted groupers (*E. coioides*) used in this research were acquired from the Laboratory of Marine Biotechnology, National Cheng Kung University. Fish were kept in a 60 l aquarium with aerated, sand-filtered, and UV-treated seawater. Water quality was maintained using a filter, and 30% of the seawater in the tank was exchanged daily. Fish were fed dry pellets (Uni-President, Tainan, Taiwan) twice a day. Juvenile groupers used in experiments measured approximately 2 inches in length and weighed approximately 5 g. Fish rearing and sample preparation were both conducted in accordance with methods described in our previous report [24].

To investigate CD53 cDNA expression and identify proteins in various organs, different immunestimulator (LPS and poly (I:C), Sigma–Aldrich, St Louis, MI, USA, 50 μg per gram of fish body weight), recombinant grouper cyctokines (rCCL4, rIL-6, expression in our lab, 50 μg per gram of fish body weight), and pathogen for grouper (*V. anguillarum* serotype O1, isolated from ill grouper, 10^6 cfu per gram of fish body weight) was inoculated in different group of fish, five randomly sampled juvenile groupers were scarified by administering an overdose of anesthetic (600 ppm 2-phenoxyethanol; Sigma–Aldrich) at 0, 2, 8, 24 and 48 h post-induction (hpi). Samples were obtained from the head and trunk kidneys, spleen, thymus, gill, blood, brain, heart muscle, stomach, intestine, liver, and pancreas in order to conduct RNA extraction, cDNA synthesis, and quantitative real-time PCR. Samples of head and trunk kidneys, liver, intestine, blood and muscle were also fixed to enable immunohistochemistry assays.

2.2. Cloning and sequencing grouper CD53

Partial grouper CD53 cDNA was identified from the subtractive cDNA library which had been previously prepared from grouper PBLs stimulated using lipopolysaccaride (LPS) from *E. coli* O55:B5 (Sigma–Aldrich) and phosphate buffered saline (PBS) (0.15 M NaCl, pH 7.2). The full length cDNA sequence of open reading frame in grouper CD53 was obtained through Rapid Amplification of cDNA Ends (RACE) (GeneRacer™ Kit, Invitrogen, Carlsbad, CA, USA) using primer specific to the partial sequence of interest, and cloned into the plasmid pGEM-T easy (Promega, Madison, WI, USA). The process was performed in accordance with the protocol of the manufacture.

MEGA 4.0 Software was used to alignment of grouper CD53 amino acid sequence, and other tetraspanins CD63, CD81 from other species was used to be an outgroup. A phylogenetic tree was constructed by the neighbor-joining method and 1000 bootstrap values to calculate.

2.3. Extraction of mRNA and cDNA synthesis of tissue samples

RNA was extracted from tissue samples using TRIzol®reagent (Invitrogen, Waltham, MA, USA). Specifically, samples were transferred into a 1.5 ml Eppendorf and homogenized in 1 ml of TRIzol reagent before being gently mixed with 200 μl of chloroform and allowed to stand at room temperature for 10 min. The mixture was then centrifuged at 12000 g for 20 min, whereupon the supernatant was transferred into a new Eppendorf and mixed with an equal volume of isopropanol before being centrifuged at 12000 g for 15 min. We then collected the precipitate and added 1 ml of 75% ethanol to wash the pellets prior to centrifugation at 12,000 g for 10 min. The supernatant was discarded, and the RNA pellet was air-dried and resolved by adding diethylpyrocarbonate treated water (DEPC water; ddH_2O contained 0.1% DEPC). Finally, the extracted total RNA was stored at −70 °C.

To synthesize cDNA, RNA was incubated using 2.5 mM dNTP, 0.1 M DTT, 3 μl of primer (25 μM), cDNA synthesis buffer (100 mMTris–HCl, pH 8.4, 250 mM KCl), and 1 μl superScriptll reverse transcriptase (200 U/μl) (Invitrogen). The RNA was then incubated at 42 °C for 60 min and then at 65 °C for 10 min to deactivate the enzyme. Finally, cDNA was stored at −70 °C.