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Short communication

First molecular cloning and gene expression analysis of teleost CD42 (glycoprotein Ib beta chain) GPIb-IX-V subunit from rock bream, *Oplegnathus fasciatus*

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ABSTRACT

CD42 is a platelet membrane glycoprotein Ib that plays a key role in haemostasis and thrombin-induced platelet activation. Here, we report the molecular cloning and sequence analysis of the CD42c gene from rock bream (*Oplegnathus fasciatus*). Rock bream CD42 (RbCD42c) gene expression profiles were determined after infection with *Streptococcus iniae, Edwardsiella tarda* and red seabream iridovirus (RSIV). The full-length RbCD42c cDNA contained an open reading frame of 624 bp encoding 207 amino acids. The deduced amino acid sequences of the leucine-rich repeat (LRR)-N terminal and LRR-C terminal were conserved between fish and mammals. RbCD42c was highly expressed in red blood cells, spleen, gill, liver and kidney of healthy rock bream. The RbCD42c gene was not significantly up- or downregulated after *E. tarda* exposure. However, RbCD42c gene expression was upregulated in kidney, spleen and gill after *S. iniae* infection. RbCD42c was upregulated in spleen, liver and gill, but downregulated in kidney 24 and 48 h after RSIV infection. These results suggest that RbCD42c has different expression patterns after infection with bacterial or viral pathogens. This gene may be directly involved in haemostasis.

1. Introduction

The initial haemostatic response to blood vessel injury is platelet plug formation, and one of the critical events in this reaction is adhesion of platelets to the subendothelium. Adhesion requires the binding of platelet membrane glycoprotein (GP) Ib-IX-V to von Willebrand factor (vWF) following binding of vWF to the subendothelial matrix (Lopez et al., 1987). GPIb-V-IX binds different ligands, but its crucial role in primary haemostasis relies on its ability to interact with vWF, a multimeric adhesive protein associated with collagen in the vessel wall. The vWF binding sites are located in the N-terminal portion of the GPIbα subunit (Blockmans et al., 1995). vWF is the only subendothelial component able to efficiently capture platelets from flowing blood at sites of vessel wall injury and to initiate platelet deposition and thrombus formation (Luo et al., 2007a, 2007b). GPIb-V-IX also plays roles in thrombin-induced platelet activation, as a counter-receptor for P-selectin and interacts with endothelial cells during the inflammatory response (Adam et al., 2003; Canobbio et al., 2004; Romo et al., 1999; Simon et al., 2000). In murine, platelets can roll on inflamed endothelium through the specific interaction of P-selectin with GPIb-V-IX (Frenette et al., 2000). The GPIb-IX-V complex is composed of four single-span transmembrane protein subunits, including GPIbα (CD42b), GPIbβ (CD42c), GPIX (CD42a) and GPV (CD42d) with stoichiometry of 2:4:2:1 (Du et al., 1987; Modderman et al., 1992; Qiao et al., 2014). GPIb consists of two disulphide-linked subunits such as a GPIb α chain and a GPIb β chain (Phillips and Agin, 1977). The GPIbα, GPIbβ and GPIX subunits interact with one another through their transmembrane and extracellular domains to form the tightly integrated GPIb-IX complex (Luo and Li, 2008; Luo et al., 2007a, 2007b; McEwan et al., 2011; Mo et al., 2006, 2009, 2010; Qiao et al., 2014). However, GPIX cannot be expressed alone on the surface of transfected CHO cells. Its expression is significantly enhanced when co-expressed with GPIbβ because GPIbβ interacts with and presumably stabilises GPIX (Blockmans et al., 1995; Phillips and Agin, 1977). In addition, GPV does not express well on the plasma membrane of transfected cells when expressed alone, but it does when co-expressed with GPIb-IX (Kahn et al., 1999; Li et al., 1995; Meyer and Fox, 1995). This is similar to the aforementioned dependence of GPIX surface

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expression on GPIbβ, suggesting that efficient surface expression of GPV depends on its interaction with GPIb-IX. The GPIbβ molecule of the GPIb-IX-V complex has been suggested to be necessary for surface-membrane expression and function. To date, GPIbβ, as well as the GPIb-IX-V complex, has been cloned in mammals, and their function has been characterised in mice and humans. However, mammalian GPIbβ orthologues have not been reported in fish, although the nucleotide and amino acid sequences of several fish GPIbβ molecules have been deposited in the GenBank database. Almost nothing is known about the haemostasis mechanism in teleosts; thus, this report provides an effective approach to understand haemostasis in lower vertebrates. Here, we first describe the molecular identification and characterisation of teleost CD42c (GPIbβ) from rock bream (*Oplegnathus fasciatus*). Furthermore, the tissue-specific distribution of RbCD42c (GPIbβ) and the expression response following bacterial and viral infection were also investigated.

2. Materials and methods

2.1. Molecular cloning of RbCD42c

A partial sequence of RbCD42c cDNA was identified by expressed sequence tags analysis in the cold shock-stimulated rock bream red blood cell library (unpublished data). Full-length RbCD42c cDNA was obtained with a 3′ RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) and RbCD42c RACE-PCR primer (RbCD42c RACE-PCR-F: 5′-GTCCTGGAGTCCTGCCACTA-3′) according to sequence information from the obtained fragment. The signal peptide and functionally important domain of RbCD42c were analysed using the online SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and SMART programmes (http://smart.embl-heidelberg.de/), respectively. The deduced amino acid sequence of the RbCD42c gene was compared with those of known CD42 genes in the GenBank database using the ClustalW programme. A phylogenetic tree analysis based on the entire CD42 amino acid sequence in rock bream and other species was constructed by the neighbour-joining algorithm in MEGA software ver. 4.0. Bootstrap sampling was performed with 2000 replicates.

2.2. Detection of RbCD42c gene expression in healthy fish

Peripheral blood leukocytes (PBLs) and red blood cells (RBCs) were isolated by Percoll (Sigma-Aldrich, St. Louis, MO, USA) density gradient, as described previously (Hwang et al., 2013). Distilled water was added to the isolated PBL fraction and gently mixed several times with a pipette to lyse the mature RBCs. The PBLs, RBCs, kidney, spleen, liver, intestine, gill and muscle were isolated from healthy rock bream. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). cDNA was synthesised from the RNA template using a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan). The real-time polymerase chain reaction (PCR) was performed with SYBR Green Master Mix (TaKaRa) following the manufacturer's protocol. Real-time PCR was carried out with cDNA templates of each organ and specific RbCD42c primer sets (RbCD42c-F: 5′-GTCATGGCAGTCAGGTGAAC-3′, RbCD42c-R: 5′-GAGGGAGGTCAAATCATCCA-3′). Relative expression levels of RbCD42c mRNAs were determined using the rock bream β-actin gene (β-actin – F: 5′-TCATCACCATCGGCAATGAGAGGT-3′, β-actin – R: 5′-TGATGCTGTTGTAGGTGGTCTCGT-3′) as an internal reference by the comparative Ct ($2^{-\Delta\Delta Ct}$) method according to the Thermal Cycler DICE Real-Time System (TaKaRa). Significant differences in gene expression among tissues were determined by analysis of variance (ANOVA) compared to values for muscle.

2.3. Effects of Streptococcus iniae, Edwardsiella tarda and red seabream iridovirus (RSIV) infection on RbCD42c gene expression

Healthy rock bream (approximate body length: 11–13 cm) were challenged with an intraperitoneal injection of pathogenic *S. iniae*, *E. tarda* or RSIV, which were adjusted to 5×10^6, 3×10^6 cell/fish and 1×10^6 copies/fish, respectively, in phosphate-buffered saline (PBS) (Hwang et al., 2013). Control fish were injected with PBS alone. Bacteria- and virus-infected fish were kept in seawater at 23 °C. At 1, 6, 24 and 48 h post-infection, kidney, spleen, liver and gill were collected from three fish in each group and pooled. RNA extraction, cDNA synthesis and real-time PCR were performed as described earlier. Relative expression levels of the RbCD42c gene were normalised to expression of the β-actin gene and expressed as fold changes relative to the control values. The results were subjected to one-way ANOVA, followed by Fisher's protected least significant difference test using SPSS ver. 17 (SPSS, Inc., Chicago, IL, USA). A $P < 0.05$ was considered significant. All data are presented as the mean ± standard deviation.

3. Results and discussion

3.1. Characterisation of RbCD42c cDNA

The full-length RbCD42c cDNA was 2007 bp long and contained an open reading frame of 621 bp that encoded 207 amino acid residues with a predicted molecular mass of 22.9 kDa (GenBank accession no. BAM36379).

RbCD42c consisted of the predicted signal peptide (1–20 aa), LRR-NT (20–54 aa) domain and LRR-CT (84–138 aa) domain. A comparison of other known CD42 amino acid sequences showed that the LRR-NT, LRR-CT domain and transmembrane region (aa 146–168) were highly conserved between mammals and fish (Fig. 1A). The integrin Mac-1 [$\alpha_M\beta_2$] is expressed on leukocytes and is involved in the adhesive interaction with endothelial cells during the inflammatory response (Flick et al., 2004). Mac-1 also regulates cell migration, phagocytosis, oxidative burst, and signalling (Zarbock and Ley, 2008). The interaction is specific and involves the Mac-1 I domain (which is analogous to the vWF A1 domain) and the GPIbα LRR and C-terminal flanking regions (Simon et al., 2000).

A phylogenetic analysis was conducted using the neighbour-joining distance method to investigate the molecular evolution between RbCD42c and other known CD42 genes. The phylogenetic analysis showed that RbCD42c clustered with the teleost CD42cs and showed the closest relationship to tilapia CD42c (Fig. 1B).

3.2. Gene expression of RbCD42c

RbCD42c was significantly expressed in RBCs, spleen, head kidney and kidney (762.7-, 42.5-, 33.0- and 24.1-fold, respectively) but weakly expressed in heart and eye (19.5- and 15.9-fold, respectively) of healthy fish, as compared to expression in the stomach (Fig. 2A).

In mammals, CD42 genes are well-known specific markers for circulating blood platelets and are only expressed on the platelet surface. CD42 expression has been highly detected in spleen and bone marrow of mice, which are major haematopoietic organs (Fujita et al., 1998). RbCD42c gene expression in healthy rock bream was similar to that of mice. RbCD42c was highly expressed in the spleen and head kidney, which are important haematopoietic organs in teleosts. Furthermore, RbCD42c was markedly expressed in rock bream RBCs, but not in those of mammals. GPIb is a major platelet membrane receptor in mammals, and about 25,000 GPIb molecules/cell exist. The RbCD42c expression level in RBCs was approximately 760-fold higher than that in other tissues, suggesting that the GPIb-IX-V complex is present in teleost RBCs. The differential expression