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Molecular cloning and characterization of olive flounder (*Paralichthys olivaceus*) peroxisome proliferator-activated receptor γ

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that play key roles in lipid and energy homeostasis. Olive flounder (*Paralichthys olivaceus*) *PPARγ* cDNA (*olPPARγ*) was isolated by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The full-length cDNA is 1667-bp long and encodes a polypeptide with 532 amino acids containing a C4-type zinc finger and a ligand-binding domain. Quantitative RT-PCR revealed that *olPPARγ* transcription was detected from 7 days post-hatching, and its expression was increased under a starved condition. Overexpression of olPPARγ stimulated PPAR response element (PPRE) activity, and treatment with rosiglitazone, a PPARγ agonist, augmented olPPARγ-stimulated PPRE activity in HINAE olive flounder cells. Cotransfection of olPPARγ and olRXRβ, in the absence or presence of rosiglitazone and ciglitazone, produced a synergistic effect on PPRE transactivation in 3T3L1 adipocytes. Moreover, olPPARγ, in the presence or absence of rosiglitazone, regulated the expression of lipid synthesis- and adipogenesis-related proteins in NIH3T3 and 3T3L1 cells. Taken together, these results suggest that olPPARγ is functionally and evolutionarily conserved in olive flounder and mammals.

1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ), as its isotypes α and β, are members of the nuclear hormone receptor superfamily. PPARγ is activated by natural ligands such as arachidonic acid metabolites and fatty acid-derived components, and by rosiglitazone (Ro), a thiazolidinedione (TZD; Spiegelman, 1998). PPARγ is a critical transcription factor in adipogenesis, and its expression is greatly increased during adipocyte differentiation (Rosen et al., 2002; Gregoire et al., 1998). By activating PPARγ, Ro promotes adipocyte differentiation *in vitro* (Hutley et al., 2003; Shao and Lazar, 1997). The overexpression of PPARγ in fibroblasts induces adipogenesis, whereas PPARγ-null embryonic stem cells and fibroblastic cells from PPARγ-deficient mouse embryos cannot differentiate into adipocytes *in vitro* (Rosen et al., 2000; Kadowaki, 2000; Lee et al., 2003).

Transcriptional activation by PPARs requires the presence of PPAR response elements (PPREs) in the promoter of the target gene. PPREs are DR1-type direct repeat elements (direct repeat spaced by 1 bp). PPARs bind PPREs as heterodimers with any one of three retinoid X receptor (RXR) isotypes (α, β, or γ), which function as receptors for the vitamin A metabolite 9-cis-retinoic acid (Mangelsdorf et al., 1995; Chambon, 1996; Desvergne and Wahli, 1999). PPAR target genes for which a functional PPRE has been identified include *acyl-CoA synthase* (*ACS*), *adipocyte lipid binding protein* (*ALBP/aP2*), *fatty acid transport protein* (*FATP*), and *liver fatty acid binding protein* (*L-FABP*) (Schoonjans et al., 1995; Tontonoz et al., 1994; Frohnert et al., 1999; Issemann et al., 1992).

PPARs have recently been discovered in several fish species, including tarafugu (Kondo et al., 2007), zebrafish (Ibabe et al., 2005), salmon (Leaver et al., 2007), goldfish (Mimeault et al., 2006), grey mullet (Raingeard et al., 2006), rainbow trout (Liu et al., 2005), sea bass (Boukouvala et al., 2004), plaice, and sea bream (Leaver et al., 2005). Although these reports studied tissue- and/or developmental stage-specific gene expression, the regulation and function of each PPAR remain unknown. The aim of the present study was to clone and characterize PPARγ from olive flounder (*Paralichthys olivaceus*) in order to address its function in the regulation of lipid homeostasis in fish.

2. Materials and methods

2.1. Reagents

Rosiglitazone and ciglitazone (PPARγ ligands) was purchased from Cayman Chemical (Michigan, USA). The transfection reagents

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SuperFect and PolyFect were purchased from Qiagen and JetPEI was purchased from PolyPlus Transfection. All other reagents were purchased from Sigma.

2.2. cDNA sequences of olive flounder PPARγ (olPPARγ)

Initial PCR was performed with specific primers to obtain the fragment sequences of olive flounder PPARγ (P1: 5′-GCC ATC CTC TCT GGG AAG ACC G -3′, P2: 5′-CAG CGC CAT GTC ACT GTC GTC C-3′). 5′-, 3′-Rapid Amplification cDNA Ends (RACE) were performed using SMART™ RACE cDNA amplification kit (Clontech), following the manufacturer's instruction to obtain olive flounder PPARγ cDNA full sequences. Based on the partial PPARγ sequences, internal primers were designed (P3: 5′-GCA ATT AAT GAA CTG CTC TCC TTC C-3′, P4: 5′-AGC TGT CGT CCA GCT CCG AGA G-3′, P5: 5′-CAT GAC GCG GGA GTT CCT CAA G-3′, P6: 5′-GTC AGA TGA TGG AAC CAA AGT TTG AG-3′) and were used in combination with the universal primer supplied with the kit to amplify the 5′- and 3′-end of olPPARγ transcript. DNA sequencing was performed with the universal and the internal primers using an ABI 3100 autosequencer. The full-length of the olPPARγ cDNA sequence was obtained by combining the DNA sequences of the partial sequences and 5′-, 3′-RACE PCR products.

2.3. Bioinformatic analysis

Analyses of potential open reading frames (ORFs) and comparison of amino acid sequences (or nucleotide sequences) were performed with the ORF finder and BLAST programs on the National Center for Biotechnology Information website. The multiple sequence alignments and the construction of phylogenetic trees (using the neighbor-joining method) were performed with the Mega 3.1 (http://www.megasoftware.net).

2.4. Fish rearing condition

Artificially fertilized flounder eggs were stocked in a tank with a flow through system of filtered seawater. A total of 98% of the eggs hatched 3 days later. Feeding program was modified from Sakakura (2006). Enriched L-type rotifers (*Brachionus plicatilis* complex) were fed from day 3 to day 14; enriched *Artemia franciscana nauplii* were supplied from day 13 to day 28; commercial fish diets (Maruwa Co., Ltd.; crude protein: 48–54%, crude fat: 9–12%) were offered from day 21. Feeding was given six times per day for ensuring sufficient food supply. Temperature on rearing tanks was maintained at 18 °C.

2.5. Starvation protocol

Fish (approximately 16 cm in size) were randomly divided into two experimental groups (10 fish each). Starvation protocol was modified from Salem et al. (2005). Control group was fed a commercial fish diets (Suhyup Feed; crude protein: 52%, crude fat: 11%) twice per day. Experimental group was subjected to a starvation regimen for 30 days. At the end of the experimental period, several tissues were collected from each group.

2.6. Quantitative real-time RT-PCR analysis

Total RNA was prepared from cell lines or tissues using TRIzol® reagent (Invitrogen) following the manufacturer's instructions. The sizes of flounders which were used for tissue sampling during early development are approximately 3.7 mm at D7, 8 mm at D18, and 12 mm at D33. One microgram of total RNA was DNase treated, and cDNA was synthesized using the Advantage® RT-for-PCR kit (BD Biosciences). The dilution factor of the cDNA used quantitative RT-PCR is 1. Quantitative real-time PCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Roche) and the following forward and reverse primers: olPPARγ F, 5′-GCC ATC CTC TCT GGG AAG ACC G-3′, olPPARγ R, 5′-CAG CGC CAT GTC ACT GTC GTC C-3′, ol18S RNA F, 5′-ATG GCC GTT CTT AGT TGG TG-3′, ol18S RNA R, 5′-CAC ACG CTG ATC CAG TCA GT-3′, mFASN F, 5′-GCT GTG CTT GCA GCT TAC TG-3′, mFASN R, 5′-CGG ATC ACC TTC TTG AGA GC-3′. mActin F, 5′-GAC TAC CTC ATG AAG ATC-3′, mActin R, 5′-GAT CCA CAT TTG CTG GAA-3′. Following an initial 10-min *Taq* activation step at 95 °C, LightCycler PCR was conducted via 40 cycles under the following cycling conditions: 95 °C for 15 s, 60 °C for 5 s, 72 °C for 10 s, and fluorescent reading. Immediately after the PCR, the machine performed a melting curve analysis by gradually (0.1 °C/s) increasing the temperature from 65 to 95 °C, with a continuous registration of changes in fluorescent emission intensity.

2.7. Construction of the expression plasmid

Amplification of the open reading frame (ORF) of the olive flounder PPARγ was carried out using the Ex Taq DNA polymerase (TaKaRa) and primers specific to the 5′ (starting at the ATG initiator codon) and 3′ ends of the olPPARγ cDNA based on nucleotide sequence. The primers used were designed so that the amplified DNA would contain EcoRI and XbaI restriction endonuclease sites at its 5′ and 3′ ends, respectively. The primer sequences were as follows: forward, 5′-AAG AAT TCA TGG TGG ACA CCC AGC AG-3′; reverse, 5′-CCT CTA GAC TAA TAC AAG TCC TTC ATG ATC TC-3′. The amplified cDNA fragment was cloned into pcDNA3-HA vector. The construct was confirmed by DNA sequencing.

2.8. Cell culture

HINAE flounder embryonic cells, a gift from Takashi Aoki, were maintained in Leibovitz L-15 medium (L-15; GIBCO BRL) with 10% heat-inactived fetal bovine serum (FBS; GIBCO BRL) and 1% (v/v) penicillin–streptomycin (PS; GIBCO BRL) at 20 °C. 3T3L1 and NIH3T3 cells were propagated in growth medium consisting DMEM, 10% FBS and 1% PS at 37 °C in humid atmosphere 5% CO_2. Medium was changed every second day in all experiments.

2.9. Transient transfection and luciferase assay

The PPRE-driven luciferase reporter vector J3-TK-Luc containing the three copies of J-site (−737 to −715 site) of the human apoA-II gene promoter upstream of the thymidine kinase (TK) promoter and expression vector pSV SPORT1-mPPARγ1 were kindly gifted from Bruce M. Spiegelman. And expression vector pcDNA3-RXRα was kindly gifted from Hueng-Sik Choi. Cells were seeded in 24-well culture plate and transfected with reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmids using SuperFect or Polyfect (Qiagen). Total amounts of expression vectors were kept constant by pcDNA3.1 (Invitorgen). Twenty-four hours after transfection, cells were incubated in the presence or absence of rosiglitazone (Ro) and ciglitazone (Ci) for 24 h. After 48 h of transfection, the cells were lysed in the cell culture lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were performed at least in triplicate.

2.10. SDS–PAGE and Western blot analysis

The cells were prepared by washing with cold-PBS and lysed. The protein concentration was determined using Bradford reagent