Molecular Characterization of Peroxisome Proliferator-Activated Receptor Isoforms from the Baikal Seal (*Pusa sibirica*)

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Abstract—The peroxisome proliferator-activated receptor (PPAR) is a member of the ligand-activated nuclear hormone receptor superfamily. The present study attempts to clarify the molecular characterization of PPARβ/δ in Baikal seals. We successfully cloned a full-length cDNA, encoding PPARβ/δ from the liver of Baikal seals, which has a deduced open reading frame of 441-amino acid residues with a predicted molecular mass of 49.7 kDa. Comparison of the amino-acid sequence of Baikal seal PPARβ/δ with that of other mammalian PPARβ/δs showed considerable similarities with PPARβ/δ of dog (99%), human (96%), mouse (91%) and rat (90%). Moreover, to investigate the interaction of Baikal seal PPARβ/δ with its agonist GW0742, glutathione-*S*-transferase-tagged recombinant Baikal seal PPARβ/δ protein was expressed using a wheat germ cell-free protein synthesis system, and surface plasmon resonance (SPR) analysis was performed. We could detect SPR signals from the interaction of Baikal seal PPARβ/δ protein immobilized on the sensor chip with GW0742, suggesting a possibility of screening Baikal seal PPARβ/δ ligands using this biosensor system.

Keywords: peroxisome proliferator-activated receptor β/δ, surface plasmon resonance, wheat germ cell-free protein synthesis technology, Baikal seal

INTRODUCTION

The peroxisome proliferator-activated receptor (PPAR) is a member of the ligand-activated nuclear hormone receptor superfamily that regulates the expression of multiple target genes (Juge-Aubry *et al*., 1997). To date, it is known that three PPAR isoforms, α, β/δ and γ are involved in metabolic pathways associated with lipidogenesis, adipose tissue accumulation and fatty-acid oxidation, and in disorders such as type-II diabetes (Issemann and Green, 1990; Dreyer *et al*., 1992; Tontonoz *et al*., 1994). In previous studies, PPAR isoforms have been cloned and characterized in some experimental animals, but little is known about the presence of PPAR and contaminant-induced signaling modulation via PPAR in wildlife.

The Baikal seal (*Pusa sibirica*) accumulates high levels of environmental contaminants, polychlorinated dibenzo-*p*-dioxin (PCDDs), dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs) (Nakata *et al*., 1995; Iwata *et al*., 2004). We have previously suggested that these pollutants are likely to modulate aryl hydrocarbon receptor (AhR) signaling pathway including cytochrome P450 1A (CYP1A) induction (Kim *et al*., 2005: Hirakawa *et al*., 2007). Furthermore, we have also shown that contamination by some PCB congeners and DDTs [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane and its metabolites] can alter the signal transduction mediated by constitutive androstane receptor (CAR) in Baikal seal (Sakai *et al*., 2006, 2009). In addition to these contaminants, our recent study demonstrated the induction of hepatic expression levels of PPARα mRNA and CYP4A protein in wild Baikal seals contaminated with perfluorochemicals (PFCs), such as perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) (Ishibashi *et al*., 2008a). We also constructed an *in vitro* reporter gene assay system consisting of the Baikal seal PPARα expression vector, and showed the PPARα-mediated transactivation potencies by PFCs (Ishibashi *et al*., 2008b). These results indicate that PPARα-mediated response may be a useful biomarker to evaluate potential biological effects of PFCs in wildlife. However, there is no information on other PPAR isoforms, such as PPARβ/δ, of this species.

In this study, we isolated and sequenced a full-length PPARβ/δ cDNA from the liver of Baikal seals, and investigated the molecular characterization of this receptor. Further, a surface plasmon resonance (SPR) assay using the Baikal seal PPARβ/δ protein expressed with an *in vitro* wheat germ cell-free protein synthesis system was developed for assessing the interaction of Baikal seal PPARβ/δ with its agonist, GW0742.

MATERIALS AND METHODS

cDNA cloning and sequencing

One microgram of poly(A)$^+$ RNA isolated from the liver tissue of Baikal seals was reverse-transcribed with random hexamers using the GeneAmp® RNA PCR Kit (Applied Biosystems, Foster City, CA) following manufacturer's instruction. For the full-length cDNA cloning of PPARβ/δ from the seal liver, specific sense and antisense primers were designed from the highly conserved nucleotide sequences of mouse (DDBJ accession No. U10375), rat (U40064), dog (DQ648277) and human (NM_006238) PPARβ/δ cDNAs. The full-length PPARβ/δ cDNA of Baikal seal was cloned with reverse transcription-polymerase chain reaction (RT-PCR) as described previously (Ishibashi *et al*., 2008b). The purified cDNA fragment was ligated into pGEM®-T Easy Vector (Promega) and transformed into *E. coli* DH5α cells. After the transformation, positive colonies were selected and sequenced using BigDye Terminator Cycle Sequencing kit (PE Biosystems, Foster City, CA) and ABI PRISM^{TM}3130 automatic sequencer. The Baikal seal PPARβ/δ amino acid sequence was aligned using ClustalW version

1.7. For determination of the full-length Baikal seal PPARβ/δ sequence, at least ten clones of each PCR product were sequenced.

Phylogenetic analysis

The amino-acid sequences of PPARs from Baikal seal and other species were aligned using ClustalW analysis. A phylogenetic tree of PPAR amino-acid sequences was constructed with the neighbor-joining method in Mac Vector 7.2.3 program. Bootstrap values based on 1000 samplings are shown above each branch. Positions with gaps were excluded, and corrections were made for multiple substitutions.

Plasmid construction and recombinant protein synthesis

The Baikal seal PPARβ/δ-ligand-binding domain (LBD) cDNA was subcloned into pEU-E01-GST-TEV-MCS-N2 expression vector (CellFree Sciences Co., Ltd., Ehime, Japan). Messenger RNA was prepared by *in vitro* transcription with SP6 RNA polymerase. Glutathione-*S*-transferase (GST)-tagged Baikal seal PPARβ/δ-LBD protein was expressed by an *in vitro* cell-free wheat germ protein synthesis system, with some modifications (Endo and Sawasaki, 2005). Purification of GST-tagged Baikal seal PPARβ/δ-LBD protein was performed with glutathione sepharose^{TM} 4B (GE Healthcare, Tokyo, Japan) following manufacturer's instruction, and was confirmed by electrophoresis on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).

Surface plasmon resonance (SPR) assay

All experiments were performed using ProteOn XPR36 instruments (Bio-Rad Laboratories, Inc., Tokyo, Japan). GST-tagged Baikal seal PPARβ/δ-LBD protein was dissolved in a coupling buffer (10 mM sodium acetate buffer, pH 4.0) and immobilized on a GLH sensor chip with *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) by amine coupling method (Bravman *et al*., 2006). For the SPR assay in this study, the baseline of SPR signal was equilibrated with a continuous flow of running buffer (10 mM Hepes, 150 mM sodium chloride, 3 mM EDTA, 4 mM DTT, 0.1% (v/v) tween 20, pH 7.6) through the sensor chip for 1 hr. The interaction of Baikal seal PPARβ/δ with a PPARβ/δ agonist GW0742 was tested at six graded concentrations. For kinetic analysis, each set of five reference-subtracted sensorgram signals was globally fit into a 1:1 interaction model (Morton and Myszka, 1998), giving a local parameter for surface capacity (R_{max}), an association rate constant (k_a), and a dissociation rate constant (k_d). Using the ratio of rate constant (k_d/k_a) equilibrium dissociation constant (K_D) was obtained.

RESULTS AND DISCUSSION

Identification of Baikal seal PPARβ/δ cDNA

From the liver of a Baikal seal, the full-length PPAR cDNA with ATG start
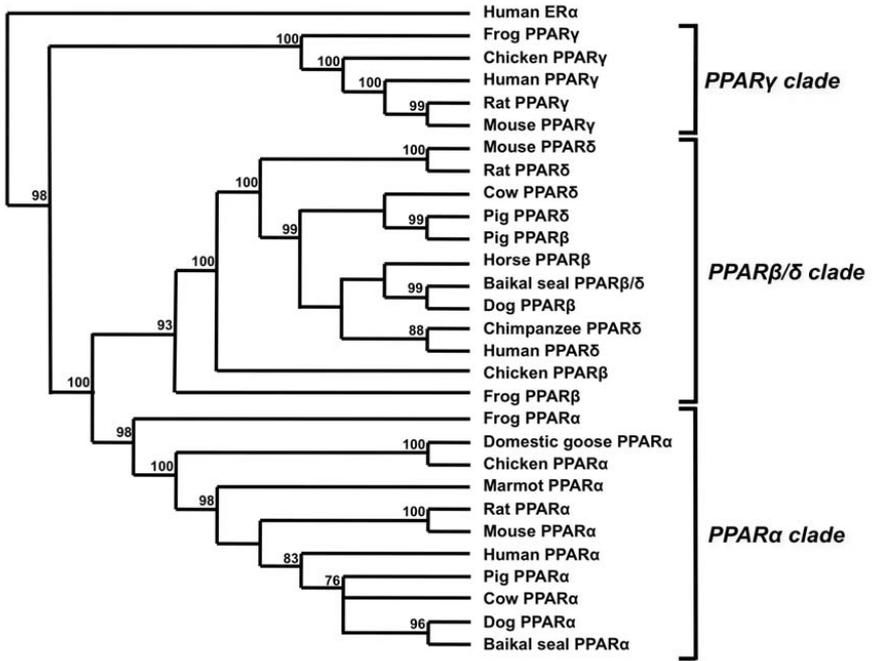
Fig. 1. Phylogenetic analysis of amino-acid sequences of PPAR isoforms from vertebrate species. The sequence of human estrogen receptor α (ERα) was used as an out-group.
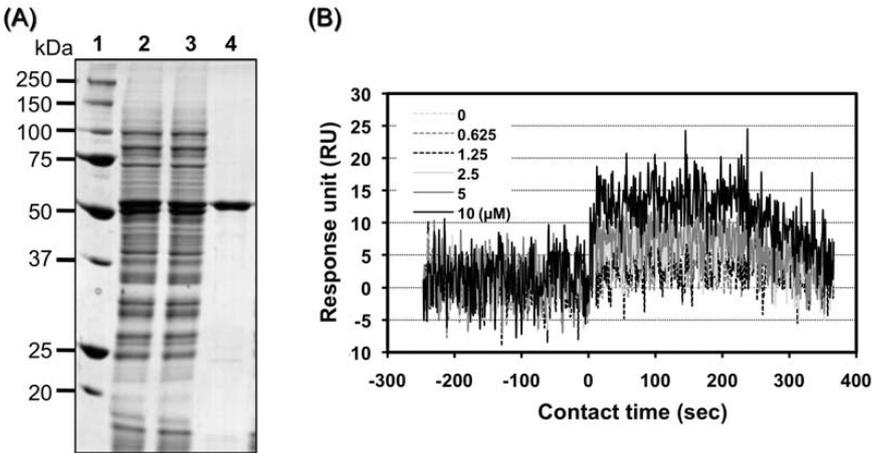

Fig. 2. Purified Baikal seal PPARβ/δ-LBD protein (A) and the sensorgrams of the interaction with a PPARβ/δ agonist GW0742 on the SPR sensor chip (B). The proteins were separated on 12% SDS-PAGE and detected by Coomassie brilliant blue staining. Lane 1, molecular marker; lane 2, soluble fraction; lane 3, flowthrough fraction; and lane 4, purified fraction.

and TAA termination sites was isolated. The deduced open reading frame (ORF) of PPAR encoded 441-amino acid residues with a predicted molecular mass of 49.7 kDa. To confirm the isoform of Baikal seal PPAR cDNA isolated, we constructed a phylogenetic tree using amino-acid sequences of the PPARα, β/δ and γ from a variety of vertebrate species. The phylogenetic analysis demonstrated that the isolated PPAR cDNA was positioned in the PPARβ/δ clade, and belonged to the same group as dog PPARβ/δ (Fig. 1). To our knowledge, this is the first report on the PPARβ/δ cDNA from aquatic mammalian species.

Comparison of the amino-acid sequence of Baikal seal PPARβ/δ with that of other mammalian PPARβ/δs showed high identities with PPARβ/δ from dog (99%), human (96%), mouse (91%) and rat (90%). The regions coding the putative LBD of PPARβ/δ was conserved more; the amino-acid sequence of Baikal seal PPARβ/δ-LBD was 99%, 98%, 93% and 92% identical to dog, human, mouse and rat PPARβ/δ-LBDs, respectively. The greatest similarity among the sequences was found within the conserved basic helix-loop-helix, P-box and AF-2 domains, which are critical for the DNA binding, PPARβ/δ/RXR dimerization, ligand binding, and transcriptional activation. High conservation of PPARβ/δ protein sequences between the Baikal seal and other mammalian species indicates that this seal species also expresses the orthologous protein of PPARβ/δ.

Interaction and kinetic analysis of Baikal seal PPARβ/δ with GW0742

To investigate the interaction of Baikal seal PPARβ/δ with its agonist GW0742, GST-tagged Baikal seal PPARβ/δ-LBD protein was expressed using an *in vitro* wheat germ cell-free protein synthesis system. After purification of the protein, a single band (approximately 52 kDa) was detected in the SDS-PAGE analysis (Fig. 2A).

The *in vitro* synthesized and purified LBD protein of Baikal seal PPARβ/δ was immobilized on SPR sensor chip by amine coupling method, and coupled at 18000 RU. The SPR analyses revealed the interaction of GW0742 with Baikal seal PPARβ/δ in a dose-response manner (Fig. 2B). The binding of GW0742 to the immobilized PPARβ/δ showed an association rate constant of $k_a = 5.64 \times 10^4$ $M^{-1} s^{-1}$ and an dissociation rate constant of $k_d = 7.12 \times 10^{-3} s^{-1}$. The equilibrium dissociation constant K_D (k_a/k_d) could thus be calculated as 127 nM. GW0742 is a PPARβ/δ agonist ($EC_{50} = 0.001 \pm 0.002\ \mu M$) that exhibits 1000-fold selectivity over PPARα and γ (Sznaidman *et al.*, 2003). These results indicate that functional PPARβ/δ proteins were successfully expressed in the wheat germ cell-free protein synthesis system. Further investigations are necessary to screen the potential ligands of Baikal seal PPARβ/δ with this SPR biosensor system.

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