Identification, Characterization, and Tissue Distribution of Human Peroxisome Proliferator-activated Receptor (PPAR) Isoforms PPARγ2 versus PPARγ1 and Activation with Retinoid X Receptor Agonists and Antagonists*

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We describe the cloning, characterization, and tissue distribution of the two human peroxisome proliferator activated receptor isoforms hPPARγ2 and hPPARγ1. In cotransfection assays the two isoforms were activated to approximately the same extent by known PPARγ activators. Human PPARγ binds to DNA as a heterodimer with the retinoid X receptor (RXR). This heterodimer was activated by both RXR agonists and antagonists and the addition of PPARγ ligands with retinoids resulted in greater than additive activation. Such heterodimer-selective modulators may have a role in the treatment of PPAR/RXR-modulated diseases like diabetes. Northern blot analysis indicated the presence of PPARγ in skeletal muscle, and a sensitive RNase protection assay confirmed the presence of only PPARγ1 in muscle that was not solely due to fat contamination. However, both PPARγ1 and PPARγ2 RNA were detected in fat, and the ratio of PPARγ1 to PPARγ2 RNA varied in different individuals. The presence of tissue-specific distribution of isoforms and the variable ratio of PPARγ1 to PPARγ2 raised the possibility that isoform expression may be modulated in disease states like non-insulin-dependent diabetes mellitus. Interestingly, a third protected band was detected with fat RNA indicating the possible existence of a third human PPARγ isoform.

Peroxisome proliferator-activated receptors (PPARs) are members of the intracellular receptor superfamily. They play a role in lipid metabolism and metabolic diseases. There are three PPAR subtypes with distinct tissue distribution in Xenopus, mice, and humans: PPARα, PPARβ (also called NUC1 or PPARδ), and PPARγ (1–10). PPARγ expression is observed in adipose tissue in rodents. Its expression is induced early in differentiation of 3T3-L1 preadipocytes into adipocytes (11). Two isoforms of mPPARγ resulting from different promoters and alternate splicing have been identified (7, 12, 13). A human isoform, hPPARγ, has been cloned from a human hematopoietic cell line and placenta (14, 15), and another from human fat (16) has been reported. Although a preliminary report on the distribution of PPARγ in human tissues has been published (16), the distribution of PPARγ1 versus PPARγ2 has not been reported.

Thiazolidinediones are high affinity ligands and potent activators for PPARγ. They decrease insulin resistance in insulin-responsive tissues including skeletal muscle (the primary site of insulin-stimulated glucose uptake) in patients with non-insulin-dependent diabetes mellitus (17). It is assumed that PPARγ is the therapeutic target for these compounds; yet the presence of PPARγ has not been conclusively demonstrated in human muscle. The identification of human PPARγ isoforms and their tissue distribution will help in understanding their role in metabolic diseases like non-insulin-dependent diabetes mellitus and obesity.

We undertook to clone and characterize the tissue distribution of human PPARγ1 and PPARγ2 and compare it with that of human PPARα and PPARβ. We compared the ability of PPARγ agonists to activate the two isoforms. A PPARγ antagonist would be a useful tool to dissect PPARγ action and may also block adipocyte differentiation. Such a ligand that competitively antagonizes PPARγ activity has not been reported. An alternative approach would be to block PPARγ/RXR activation with an antagonist of RXR. Surprisingly, an RXR antagonist activated the PPARγ/RXR heterodimer as did an RXR agonist. Greater than additive activation was seen with PPARγ and RXR ligands.

EXPERIMENTAL PROCEDURES

5,8,11,14-Eicosatetraenoic acid and 2-bromopalmitate were purchased from Sigma, and 15-deoxy-12,14-prostaglandin J2 was obtained from Cayman Chemicals. BRL 49653, LG100268, and LG100754 were synthesized at Ligand Pharmaceuticals Inc.

A human heart 5′-stretch cDNA library (Clontech) was screened with a mouse PPARγ (7) probe at low stringency (35% formamide, 5× SSC, 0.1% SDS, 100 µg/ml fish sperm DNA at 37 °C). Several positive clones were isolated and sequenced. Comparison with the mPPARγ sequence indicated that one clone encoded the N terminus and another the C terminus of hPPARγ, and their sequences overlapped by 485 base pairs. The complete hPPARγ-coding region was reconstructed by a triple ligation using pBKCVM (Stratagene) digested with EcoRI and KpnI and utilizing the unique Scal site in the coding region. This plasmid was then digested with NcoI, blunt-ended with Klenow enzyme, and religated with KpnI. The liberated fragment was subcloned into pBKCVM at the XbaI site (blunted with Klenow enzyme) and the KpnI site to give pCMVhPPARγ1.

A third positive clone was isolated and sequenced. This sequence
overlapped that of hPPARγ by 1268 base pairs but had a unique N terminus. The technique of crossover polymerase chain reaction was utilized to create pcMVhPPARδ. The sequences of the two external primers were 5′-TGATGTCGATCCGATATCTGGTAATACAGGC-3′ and 5′-GATCCTAGCTGCTGATCTGCACTGCG-3′. The internal primers were 5′-CATTACGGAGAGAGATCCAC-3′ and 5′-ATGGTTGACACAGAGATG-3′. The polymerase chain reaction product was cloned into the Smal site of pCRII vector (Invitrogen). This was linearized in vitro with EcoRI and the 1-kilobase pair fragment isolated. This DNA was linearized with NcoI and subcloned into pGEM 3Zf(−) (Promega). The baculovirus/Sf21 cell system was used to express the hPPARδ. The internal primers were 5′-ATGGTTGACACAGAGATG-3′ and 5′-TGAGTCAGCTCGAGATATCAGTGTGAATTACA-3′. The polymerase chain reaction product was cloned into the pCMVhPPARγ plasmid (18) which contained the NcoI and EcoRI sites of pBKCMV, and the orientation was indicated by a PhosphorImager (Molecular Dynamics).

FIG. 1. Nucleotide and predicted amino acid sequence of hPPARγ2. The first three methionine codons are underlined. The translation stop codon is indicated by a dot and the stop codon upstream and in-frame with the first predicted methionine is indicated by an asterisk.

The positions of the nucleotides and the predicted amino acids in the sequence are indicated on the side. The 30 amino acids at the N terminus unique to PPARα2 are shown in bold type.

Northern Blots—Human multiple tissue Northern blots were purchased from Clontech. Hybridization was done according to the manufacturer’s protocol. The probe for hPPARα has been described (6), pcMVhPPARδ (pcMVhNuc1) (18) was digested with EcoRI and the 500-base pair fragment was isolated. pcMVhPPARγ was digested with Scal and KpnI and the 1-kilobase pair fragment isolated. This probe will recognize hPPARγ1 and hPPARγ2 RNA. All probes were labeled by random priming.

RNease Protection Assays—Four human white fat and two skeletal muscle samples were obtained from the National Disease Research Interchange (NDRI, Philadelphia) or the University of California (San Diego) tissue bank. Total RNA was isolated using standard techniques. A human heart cDNA library was screened with a probe corresponding to the mouse PPARγ (7). Three overlapping clones were identified, purified, and sequenced. The nucleotide sequence is shown in Fig. 1. The longest open reading frame starting from the nucleotide at position 91 coded for a polypeptide of 505 amino acids. There was an in-frame stop codon upstream of this methionine suggesting the translation initiation occurred from this codon. The first and third methionine codons were at positions 29 and 31 in the amino acid sequence. The second methionine translation occurred from this codon. The second and third methionine codons were at positions 29 and 31 in the amino acid sequence.

Co-transfection Assays—Transfections in CV-1 cells were performed as described (6, 23). The reporter plasmid pPPREA3-tk-Luc containing three copies of the PPRE identified in the acyl CoA oxidase (AOX) gene has been described (24). The β-galactosidase expression plasmid pCH110 was used to normalize difference in transfection efficiencies. The normalized response was the luciferase activity of the extract divided by the β-galactosidase activity of the same. Compounds were dissolved in Me2SO (vehicle). Each data point is the mean of triplicate transfections, and the error bars represent the standard error of the mean. Each experiment was repeated at least two times. A representative experiment is shown in each case.

RESULTS

A human heart cDNA library was screened with a probe corresponding to the mouse PPARγ (7). Three overlapping clones were identified, purified, and sequenced. The nucleotide sequence is shown in Fig. 1. The longest open reading frame starting from the nucleotide at position 91 coded for a polypeptide of 505 amino acids. There was an in-frame stop codon upstream of this methionine suggesting the translation initiation occurred from this codon. The first and third methionine codons were at positions 29 and 31 in the amino acid sequence.

The first and third methionine codons in hPPARγ2 were in a context appropriate for translation initiation, i.e. the Kozak context (7). Three overlapping clones were at positions 29 and 31 in the amino acid sequence. The first and third methionine codons were at positions 29 and 31 in the amino acid sequence.

Amino acid sequence comparison indicated 97% identity overall between hPPARγ2 and mPPARγ2. The DNA binding domains were 83% conserved between hPPARγ2 and hPPARα or hPPARβ. Further, three amino acids were present between
the two cysteines in the D-box (amino acids 177–179), a characteristic feature of all PPARs known to date. Based on these observations we believe this human isoform is hPPAR2. Gel retardation reactions were performed with 1 μl of in vitro-translated hPPAR2 and 1 μg of recombinant baculovirus-expressed hRXRα. Oligonucleotides containing PPREs from the bifunctional enzyme (lanes 1, 4, and 7), acyl-coenzyme A oxidase (lanes 2, 5, and 8) and apoA-1 (lanes 3, 6, and 9) genes were used as probes.

PPARγ2 alone did not form a complex with oligonucleotides containing PPRE sequences identified in the promoters from the enoyl-CoA hydratase/Δ3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme), acyl-CoA oxidase, and the A site of the apoA-1 gene (lanes 1–3). Similarly, no complex was observed with RXRα and the PPRE-containing oligonucleotides (lanes 7–9). However, with PPARγ2 and RXRα, retarded complexes are clearly observed (lanes 4–6). Similar results were obtained with hPPAR1 (data not shown). We concluded that hPPAR1 and hPPARγ2 bound to PPREs as heterodimers with RXR. Complexes were also observed between hPPAR1 or hPPARγ2 and RXRβ and RXRγ (data not shown).

The transcriptional response of hPPARγ2 to PPARγ activators was determined in a cotransfection assay (Fig. 4A) and compared with hPPAR1 (Fig. 4B). PPARγ1 and PPARγ2 are activated by BRL 49653 with an EC50 of approximately 100 nM and by 15-deoxy-D12,14-prostaglandin J2 (an endogenous PPARγ ligand) (26, 27) with an EC50 around 3 μM. They are also activated by 5,8,11,14-eicosatetraenoic acid and 2-bromopalmitate. The response of hPPARγ2 to these four activators is very similar to that of hPPAR1. We conclude that both hPPARγ1 and hPPARγ2 are similarly activated by known PPARγ activators.

Since PPARγ2 binds to PPREs as a heterodimer with RXR, we next determined the transcriptional response of the PPARγ2/RXR heterodimer to an RXR ligand. LG100268 (28) is a highly selective RXR ligand (Kida 3 nM). Both BRL 49653 and LG100268 transcriptionally activated the PPARγ2/RXR heterodimer (Fig. 5A), and the transcriptional response observed with both ligands was greater than that observed individually. RXR agonists activated a reporter containing the hydratase (bifunctional enzyme) PPRE. They also induced expression of the hydratase gene in vivo, and increased induction is seen with a combination of RXR and PPAR agonists.2

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Recently LG100754, another high affinity RXR ligand ($K_d \sim 12$ nM), has been described as an RXR/RXR homodimer antagonist on a CRBPII-tk-Luc reporter (29). To determine if the response of PPARγ/RXR to BRL 49653 will be antagonized by LG100754 binding to RXR, a cotransfection assay with PPARγ/RXR was performed (Fig. 5A). Surprisingly, LG100754, like LG100268, is an agonist of hPPARγ/RXR, and activation by the combination of BRL 49653 and LG100754 is greater than the individual compounds. It is also an agonist of hPPARγ1/RXR (data not shown) and hPPARγ2/RXR (30).

Since LG100754 is a high affinity RXR ligand and also activates the hPPARγ/RXR heterodimer, we determined whether LG100754 also activates RXR homodimers using the same reporter used for the PPAR assays (pPPREA3-tk-Luc). Since the consensus PPRE and RXR response element are of the DR-1 type (24), it would be interesting to compare the effect of RXR modulators on the two response elements. LG100268 strongly activated the RXR/RXR homodimer on PPREA3-tk-Luc (Fig. 5B). LG100754 was a very weak activator of the RXR homodimer. Interestingly, it antagonized the activation of RXR/RXR by LG100268 (Fig. 5B). Hence, LG100754 acted as a PPARγ/RXR heterodimer agonist but as an RXR homodimer antagonist on the same response element, a PPRE. This was the first demonstration of an RXR ligand having such dimer-selective effects on the same reporter. This dimer-selective activity is probably not due to LG100754 binding with high affinity to PPARγ since LG100754 displaces labeled BRL 49653 from PPARγ only at very high concentrations in a DNA-dependent ligand binding assay using PPARγ/RXR heterodimers (40) (data not shown).

We next determined the tissue distribution of human PPARγ RNA (using a probe common to PPARγ1 and PPARγ2) and compared it with that of hPPARα and hPPARβ by Northern blotting (Fig. 6A). Human PPARα was found predominantly in skeletal muscle, liver, heart, and kidney, a distribution similar to that reported for mPPARα. PPARβ RNA was more ubiquitously expressed with maximal expression in placenta and skeletal muscle. One band approximately 2 kilobases in length was observed with the PPARγ probe. Human PPARγ is expressed in the insulin-responsive tissues (skeletal muscle, heart, and liver) and is consistent with the distribution in mice (31).

Since the probe used in the Northern blot experiments could not distinguish between PPARγ1 and PPARγ2 RNA, we developed an RNase protection assay to distinguish the two isoforms. The majority of insulin-stimulated glucose uptake occurs in skeletal muscle, therefore, we determined the expression of PPARγ2 versus PPARγ1 in muscle. Since mPPARγ2 expression is restricted to fat (32), and the commercial blot used in the Northern analysis did not have a sample of fat RNA, we included human fat RNA in the study (Fig. 6B). Two bands (78 and 252 nucleotides long) were observed in all adipose tissue samples arising from protection of the probe by PPARγ1 and PPARγ2 RNA, respectively, as shown. In contrast to the findings in mice (31), PPARγ1 was expressed at higher levels in all human fat samples studied. Quantitation of the band intensities indicated that the ratio of PPARγ1 to -γ2 varied in the human samples (from 2 (lane 5) to 10 (lane 7)).

With RNA from human skeletal muscle, we observed the protected fragment due to hPPARγ1 but not from PPARγ2 in all three samples. This was not observed with yeast RNA, which was used as a negative control.

To test whether PPARγ1 RNA observed in muscle was solely due to fat contaminating the muscle samples, we performed RNase protection assays with the muscle RNA and a mouse aP2 probe and compared that with a sample of fat RNA. aP2 (adipocyte protein 2) gene expression is fat-specific (33). Very little specific protection of the probe was seen with 10 μg of muscle RNA (Fig. 6C, lanes 5–7) while an intense band is seen with only 2 μg of fat RNA (lane 4). The autoradiogram was

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** hPPARγ2 and RXR cooperatively activate transcription. A, CV-1 cells were transfected with the expression plasmids pCMVhPPARγ, pBsmRXRβ (38), and pPPREA3-tk-Luc as reporter. BRL 49653 was added to a final concentration of 100 nM, and LG100268 (LG 268) or LG100754 (LG 754) to 1 μM, respectively. B, CV-1 cells were transfected with pShRXRα (39) and pPPREA3-tk-LUC. LG100268 was added to a final concentration of 100 nM where indicated, and the concentration of LG100754 is shown. vehicle, MeSO4.
deliberately overexposed (see lane 3) to reveal any protected bands in lanes 5–7. The smear observed in lanes 5–7 was probably due to nonspecific hybridization between human RNA and the mouse probe and is also seen with yeast RNA (lane 8). We concluded that PPARγ was expressed in human skeletal muscle and PPARγ1 was the predominant isoform in this tissue. In contrast, both PPARγ1 and PPARγ2 were expressed in human fat and at much higher levels compared with muscle.

Interestingly, a third protected fragment (170 nucleotides long) was also observed (denoted by an asterisk) in all four fat samples but not in the muscle samples (Fig. 6B). This could be simply due to RNase digestion in regions of imperfect hybridization. However, the ratio of the intensity of this fragment compared with PPARγ2 varied in the different fat samples, hence, it is unlikely that this was due to breakdown of the larger protected fragment. We therefore hypothesized a third isoform of PPARγ in humans that may arise due to alternate splicing and promoter usage.

DISCUSSION

We have cloned the cDNA for a second isoform of the human PPARγ, hPPARγ2. Sequence comparison with mPPARγ2 revealed 97% amino acid identity. Human PPARγ2 bound to PPREs as a heterodimer with RXRs as do all the PPARs known to date. Human PPARγ1 and hPPARγ2 have similar activation profiles in response to BRL 49653 and 15-deoxy-D12,14-prostaglandin J2. Interestingly, there was only 63% identity in the N-terminal 30 amino acids between human and mouse PPARγ2, far less than in the rest of the polypeptide (98%). This suggests that the N terminus coded by a different exon (13) has diverged more rapidly than the rest of the protein during evolution. The function of these amino acids is unclear.

The hPPARγ2/RXR and hPPARγ1/RXR heterodimers were activated by the RXR modulators LG100268 and LG100754. They increased the transcriptional response seen with the PPARγ agonist BRL 49653. This is consistent with our previous studies showing that RXR modulators increase the responsiveness of the PPARα/RXR heterodimer (6, 24). LG100754 was interesting because it is an agonist of PPARγ/RXR but an RXR/RXR antagonist. Binding of LG100754 to RXR may lead to distinct conformational changes of the receptor dimer such that PPARγ/RXR is read as an activator by the transcriptional machinery, but the RXR/RXR homodimer is transcriptionally silent. Such compounds like LG100268, LG100754, and BRL 49653 may therefore modulate distinct but overlapping sets of target genes and might have a role in the treatment of PPAR/RXR-modulated diseases like diabetes.

The tissue distribution of hPPARγ is important for the therapeutic activity of drugs targeting the PPARγ/RXR heterodimer. Thiazolidinediones act as insulin sensitizers in skeletal muscle and are high affinity PPARγ ligands (34). Structure
activity relationship indicates a good correlation between in vivo potency and in vitro activity of thiazolidinediones (35), implicating PPARγ as the therapeutic target for these compounds. However, earlier data indicated PPARγ is expressed at high levels, specifically in adipose tissue in rodents (11, 32), and is essentially undetectable in muscle where approximately 80% of insulin-stimulated glucose uptake occurs (36). It was not clear how PPARγ expressed almost exclusively in adipose tissue could have the effect of insulin sensitization in skeletal muscle and raised the possibility that insulin sensitization by thiazolidinediones in skeletal muscle was not mediated by PPARγ-dependent mechanisms.

Our data demonstrated that PPARγ was expressed in human skeletal muscle, fat, and heart, tissues where the majority of insulin-stimulated glucose uptake occurs. Further, while both PPARγ1 and PPARγ2 are expressed in human fat, the dominant isoform in human muscle is PPARγ1. These findings are consistent with recently published data in mice (31) and suggest that PPARγ1 might be the relevant target for thiazolidinediones in human skeletal muscle. The close conservation of sequence, subtype, and tissue distribution of PPARγ between mice and humans is consistent with the observation that thiazolidinediones act as insulin sensitizers in both species (17, 37). However, we do not yet know the distribution of PPARγ1 versus PPARγ2 protein in these tissues.

The ratio of the intensities of the PPARγ1 and -γ2 isoforms varied in the four individual fat samples, hinting that isoform expression may be modulated. Further, only PPARγ1 was detected in muscle, not PPARγ2, pointing to differential expression of PPARγ isoforms in tissues. Therefore, an analysis of PPARγ isoform distribution in skeletal muscle and fat in normal, obese, and diabetic individuals might yield valuable information and is currently underway.

We used a commercially available Northern blot to determine PPARγ distribution. Although we did not observe hybridization to placenta and lung RNA on this blot we note that PPARγ expression was observed in these tissues (15). We cannot explain this other than as a variation between individuals. For an accurate determination it was important to assay expression levels in several individuals as was done in our RNase-protection assays.

Thiazolidinediones appear to act as insulin sensitizers in vivo through activation of PPARγ/RXR. Our data indicate the presence of PPARγ in insulin-responsive tissues in humans. One may speculate that thiazolidinediones bind to and activate PPARγ altering the expression of key genes in target tissues rendering them more responsive to insulin, increasing glucose uptake, lowering hepatic glucose output, and lowering hyperglycemia. Since RXR modulators are also able to activate the PPARγ/RXR heterodimer, they could activate a set of thiazolidinedione-responsive genes and may therefore either alone or in combination with thiazolidinediones have utility in the treatment of non-insulin-dependent diabetes mellitus.

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