L-764406 Is a Partial Agonist of Human Peroxisome
Proliferator-activated Receptor γ

THE ROLE OF CYS\(^{313}\) IN LIGAND BINDING*

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Insulin-sensitizing thiazolidinedione (TZD) compounds are high affinity ligands for a member of the nuclear receptor family, peroxisome proliferator-activated receptor (PPAR) γ. A scintillation proximity assay for measurement of \(^{3}H\)-radiolabeled TZD binding to human PPARγ under homogeneous conditions was developed. Using this approach, a novel non-TZD compound (L-764406) was shown to be a potent (apparent binding IC\(_{50}\) of 70 nM) PPARγ ligand. Preincubation of PPARγ with L-764406 prevented binding of the \(^{3}H\)TZD, suggesting a covalent interaction with the receptor; in addition, structurally related analogues of L-764406, which would be predicted not to interact with PPARγ in a covalent fashion, did not displace \(^{3}H\)TZD binding to PPARγ. Covalent binding of L-764406 was proven by an observed molecular weight shift of a tryptic PPARγ ligand binding domain (LBD) peptide by mass spectrometry analysis. A specific cysteine residue (Cys\(^{313}\) in helix 3 of hPPARγ2) was identified as the attachment site for this compound. In protease protection experiments, the liganded receptor adopted a typical agonist conformation. L-764406 exhibited partial agonist activity in cells expressing a chimeric receptor containing the PPARγ LBD and a cognate reporter gene and also induced the expression of the adipocyte-specific gene aP2 in 3T3-L1 cells. In contrast, L-764406 did not exhibit activity in cells transfected with chimeric receptors containing PPARα or PPARδ LBDs. The partial agonist properties of L-764406 were also evident in a co-activator association assay, indicating that the increased transcription in cells was co-activator mediated. Thus, L-764406 is a novel non-TZD ligand for PPARγ and is also the first known partial agonist for this receptor. The results suggest a critical functional role for Cys\(^{313}\), and helix 3, in contributing to ligand binding and subsequent agonist-induced conformational changes.

Peroxisome proliferator-activated receptors (PPARs)\(^{1}\) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1, 2). They are currently divided into three subtypes: PPARα, PPARδ (also known as hNUC1 or PPARβ), and PPARγ, with each being encoded by a distinct gene. The presence of at least two promoters in the 5′-flanking region of PPARγ results in the production of two isoforms, γ1 and γ2 (3), where γ2 possesses an additional 30 residues at its amino terminus (4). Like other members of this superfamily, the PPARs exert their effects by regulating gene transcription and all three members bind to DR1 response elements (reviewed in Ref. 1).

Although the three subtypes have been grouped together based on sequence homology, it is clear that they have distinct functions. PPARα is expressed predominantly in the liver and is involved in peroxisome proliferation and regulation of fatty acid catabolism (5). PPARγ plays a pivotal role in fat cell differentiation and lipid storage (6), while PPARδ is expressed in most cell types but its role remains unclear (7). Consistent with their distinct physiological roles, each receptor has been shown to bind a discrete set of ligands, although, in general, these receptors seem to be regulated by fatty acids and eicosanoids (8–10). Thus, for PPARα, selected fatty acids serve as potential natural ligands; in addition, the fibrate class of hypolipidemic drugs and a group of structurally diverse peroxisome proliferators can also function as PPARα ligands and/or activators. The synthetic thiazolidinedione insulin sensitizers (11) and the prostaglandin derivative 15-deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_{2}\) bind and activate PPARγ (12, 13), while several polysaturated fatty acids and eicosanoids can serve to activate PPARδ (7, 9).

These receptors play a central role in lipid homeostasis where imbalances can lead to cardiovascular disease, obesity, and diabetes. Indeed, medications targeted to PPARs have been demonstrated to be effective treatments for hyperlipidemia (fibrates, Ref. 14), and insulin resistance (thiazolidinediones, Ref. 15). Thus, there has been considerable interest in developing new and specific ligands for these receptors (16, 17). It is, therefore, clear that further characterization of the spectrum of natural or synthetic molecules, which can function as PPAR ligands and agonists is an important undertaking. Moreover, a determination of the precise residues in the PPAR ligand binding domains (LBDs), which make contact with agonist ligands would greatly facilitate the subsequent discovery of new and therapeutically useful modulators of these receptors.

Here, we used a radiolabeled thiazolidinedione and recomb-
nant human PPARγ to develop a scintillation proximity assay (SPA) in order to characterize ligands that bind this receptor. Using this approach, a novel non-TZD compound, L-764406, was shown to be a high affinity PPARγ ligand. Several approaches were used to demonstrate that this compound acts as a specific agonist for PPARγ. Importantly, L-764406 was also shown to bind covalently to PPARγ. This phenomenon was exploited in order to determine that the compound bound directly to Cys315 in helix 3 of the LBD of human PPARγ. The identification of Cys315 as the attachment site for L-764406 defines an important role for this residue and for helix 3 in ligand binding and activation of PPARγ.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant PPARγ**—The 1.5-kilobase pair PPARγ insert was released from the pCRII cloning vector by digestion with the restriction enzyme StuI (4) and ligated into the StuI site of the bacterial expression vector pGEX-4T-2 (Amersham Pharmacia Biotech) to produce the vector pGEX-hPPARγ2 containing the full-length human PPARγ2 cDNA fused to glutathione S-transferase. Escherichia coli BL21 cells were transformed with pGEX-hPPARγ2 plasmid DNA. Cells were cultured and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to achieve a protein concentration in the cell lysate of 2 mg per ml. Cells were harvested by centrifugation at 20,000 rpm, 4 °C. Recombinant GST-hPPARγ was isolated batchwise using glutathione-Sepharose as described by the supplier (Amersham Pharmacia Biotech). Typically, 2 mg of GST-hPPARγ at approximately 50% purity was obtained per liter of bacterial culture.

**SPA Binding Assay**—The binding assay was developed for use with microtiter plates (Dynex Technologies, catalog number 011-010-7905) using a total volume of 100 μl of assay buffer: 10 mM Tris-Cl, pH 7.2, 1 mM EDTA, 10% (v/v) glycerol, 10 mM sodium molybdate, 1 mM diithiothreitol, 0.5 mM phenylmethylsulfonfonyl fluoride, 2 μg/ml benzamidine, and 0.1% dry milk powder. Each bottle of protein-A-agarose silicate SPA beads (Amersham Pharmacia Biotech catalog number RPN143) was suspended in 25 μl of assay buffer but omitting the dry milk powder and adding sodium azide to a final concentration of 0.01%. (HI)H5-[3-(2,4-dihydroxyphenyl)-2-oxazolyl]-2-hydroxyethoxy]-benzyl]-2,4-thiazolidinedione (HITZD), 21 Cm mol, was dissolved in ethanol and used at a final concentration of 10 nM. The recombinant GST-hPPARγ preparations were usually used at a dilution of 800 × producing a final concentration of approximately 5 nM. Goat anti-GST antibodies were obtained from Amersham Pharmacia Biotech (catalog number 27-4577-01) and used at a 400-fold final dilution. The GST-hPPARγ, goat anti-GST antibodies, and [35S]HITZD were diluted in assay buffer and combined in a total volume of 70 μl in the microtiter plate. Five μl of test compound was added so that the final concentration of Me3SO did not exceed 2%. Following the addition of 25 μl of protein-A-agarose silicate SPA beads to each well, the plate was incubated at 15 °C for 24 h with shaking. Radioactivity was quantified in a Packard TopCount scintillation counter.

**Plasmids**—Chimeric receptors containing the yeast GAL4 DNA binding domain fused to either human PPARα, PPARβ, or PPARγ were created by insertion of a BamHl/HindIII fragment from pFC DNA binding domain (Strategene) encoding the GAL4 DNA binding domain into the same sites within the mammalian expression vector pcDNA3.1 (+) (Invitrogen) to generate the vector pcDNA3.1-GAL4. The locations of the LBDs for each PPAR receptor were determined by sequence alignment programs from the Wisconsin Sequence Analysis Package (18). The fragments were generated by polymerase chain reaction using appropriate primers, which provided polymerase chain reaction products flanked by BamHI and NotI sites at their 5’ and 3’ ends, respectively. These fragments were digested with BamHI and NotI and ligated into the vector pcDNA3.1-GAL4, which had been digested with the same enzymes. The fragments were then ligated into the vector pGEX-4T-2 (GenBank™ accession number M91298), 167 for PPARγ (GenBank™ accession number M91298), 139 for PPARβ (GenBank™ accession number L07592), and 203 for PPARδ (GenBank™ accession number U63415), and extend to the COOH terminus for each receptor. To confirm accuracy, the DNA sequence for each construction was determined. The reporter plasmid for these GAL4 chimeric receptors (pUAS(5’)-X-k-luc) contains five repeats of the GAL4 response element (UAS) upstream of a minimal thymidine kinase pro

**that is also sodium sulfate.** The control vector, pCMV-lacZ, contains the CMV promoter adjacent to the galactosidase Z gene. **Cell Culture and PPAR Transactivation—** COS-1 cells were seeded at 12 × 106 cells/well in 96-well cell culture plates in high glucose Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped fetal calf serum (Gemini Bio-Products). Cells were seeded at 100 units/ml penicillin G, and 100 mg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 10% CO2. After 24 h, transfections were performed with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Briefly, transfection mixes for each well contained 0.48 μl of LipofectAMINE, 0.00075 μg of pCMV-hPPARγ expression vector, 0.045 μg of pUAS(5’)-X-k-luc reporter vector, and 0.0002 μg of pCMV-lacZ as an internal control for transfection efficiency. Cells were incubated in the transfection mixture for 5 h at 37 °C in an atmosphere of 10% CO2. The cells were then incubated for ~4 h in fresh high glucose Dulbecco’s modified Eagle’s medium containing 5% charcoal-stripped fetal calf serum, nonessential amino acids, 100 units/ml penicillin G, and 100 mg/ml streptomycin sulfate at increasing concentrations of test compound. Since the compounds were solubilized in Me3SO, control cells were incubated with equivalent concentrations of Me3SO; final Me3SO concentrations were = 0.1%, a concentration that was shown not to affect transactivation activity. Cell lysates were produced using Reporter Lysis Buffer (Promega, Madison, WI) according to the manufacturer’s instructions. Luciferase activity in cell extracts was determined using a Fluoroskan Ascent fluorometer (Promega). The transcription/translation reactions were performed with LipofectAMINE (Promega) in an MLS3000 luminometer (Dynatech Laboratories). β-Galactosidase activity was determined using β-D-galactopyranoside (Calbiochem) as described previously (19).

**Measurement of α2p mRNA—** Confluent 3T3-L1 cells were incubated in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, 1 μM dexamethasone, 150 μM insulin, in the absence or presence of increasing concentrations of test compound for 4 days at 37 °C in 5% CO2 (with one medium change). Total RNA was prepared from cells using the Biotecx Ultraspec™ RNA isolation kit, and RNA concentration was estimated from absorbency at 260 nm. RNA (20 μg) was denatured in formamide/formaldehyde and slot blotted onto Hybond™-N membrane. Prehybridization was performed at 42 °C for 1–3 h in 50% formamide and Thomas solution A containing 25 mM sodium phosphate, pH 7.4, 0.9 μM sodium chloride, 50 mM sodium citrate, 0.1% each of gelatin, Ficol, and polyvinylpyrrolidone, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at the same temperature for 20 h in the same solution with a 32P-labeled α2p DNA probe (2 × 108 cpm/ml). After washing the membranes under appropriately stringent conditions, the hybridization signals were analyzed with a PhosphorImager (Molecular Dynamics).

**Assessment of Receptor Conformation by Partial Protease Digestion—** The protease digestion assays were performed by the method of Allan et al. (20), with previously described modifications (21). The pSG5-hPPARγ plasmid was used to synthesize 32S-radioabeled PPARγ, in a coupled transcription/translation system according to the protocol of the supplier (Promega). Partial protease digested samples were subsequently aliquoted into 22.5-μl volumes, and 2.5 μl of phosphate-buffered saline was added. These mixtures were incubated for 20 min at 25 °C, separated into 4.5-μl aliquots, and 0.5 μl of distilled H2O or distilled H2O-solubilized trypsin were added. The protease digestions were allowed to proceed for 10 min at 25 °C, then terminated by the addition of 95 μl of denaturing gel loading buffer and boiling for 5 min. The products of the digestion were separated by electrophoresis through a 1.5-mm 4–20% polyacrylamide–sodium dodecyl sulfate gel. After electrophoresis, the gels were fixed in 10% acetic acid (v/v)/40% methanol (v/v) for 30 min, treated in ENHANCE for a further 30 min and dried under vacuum for 2 h at 80 °C. Autoradiography was then performed to visualize the radiolabeled digestion products.

**Mass Spectrometry—** Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis was performed using a Finnigan TSQ7000, as described previously (22). Mass measurement of receptor-ligand complex was performed as follows. Samples were loaded on a C4 reverse phase column (1 × 100 mm) at a flow rate of 100 μl/min with 1% (v/v) 97.5% aqueous trifluoroacetic acid and eluted with a gradient of 0–20% acetonitrile over 40 min. The effluent was fed directly to the ESI interface of the mass spectrometer. Ions were detected throughout the entire LC gradient over a m/z (mass to charge ratio) range of 500–2,000. Receptor-ligand complex was digested with trypsin (sequence grade, Boehringer Mannheim), and the peptide fragments were analyzed by LC-ESI-MS and LC-ESI-tandem mass spectrometry (LC-ESI-MS/MS) on a Finnigan LCQ.
Construction of C313A PPARγ Mutation—Cys<sup>313</sup> of hPPAR<sub>γ</sub> is located within a 72-base pair restriction fragment bracketed by unique MscI and BsmI sites. The C313A point mutation was made by synthesizing complimentary oligonucleotides containing the appropriate codon and anticodon for Ala at position 313 and flanked with MscI and BsmI sites. The complimentary oligonucleotides were hybridized at room temperature in restriction enzyme buffer and digested with the enzymes MscI and BsmI. The digested oligonucleotides were used to replace the same fragment in the vector pGEX-hPPAR<sub>γ</sub>. The identity of the mutation was confirmed by DNA sequencing. Mutant PPAR<sub>γ</sub> receptor protein was expressed in bacteria and purified as described above. Binding activity was determined using the SPA assay described above.

Co-activator Association Assays—A homogeneous time-resolved fluorescence assay (HTRF) was used to examine the interaction of PPAR<sub>γ</sub> and the mutant receptors with the co-activator CBP (CREB-binding protein). A complete description of this assay has been published elsewhere (23); briefly, 198 μl of reaction mixture (100 mM HEPES, 125 mM KF, 0.125% (w/v) CHAPS, 0.05% dry milk, 1 nM GST-PPAR<sub>γ</sub> LBD or 5 nM GST-PPAR<sub>γ</sub>, 2 nM anti-GST-(Eu)K, 10 nM biotin-CBP1–453, 20 nM SAXL665) were added to each well, followed by addition of 2 μl of test compound or vehicle (Me<sub>2</sub>SO) in appropriate wells. Plates were mixed by hand and covered with TopSeal. The reaction was incubated overnight at 4 °C, followed by measurement of fluorescence reading on a Discovery instrument (Packard). Data were expressed as the ratio, multiplied by a factor of 10<sup>4</sup>, of the emission intensity at 665 nm to that at 620 nm.

RESULTS

Characterization of the SPA Assay—The major advantage of the scintillation proximity assay over other approaches for measuring ligand binding is that it is a single step, homogeneous assay format, so there is no need to separate bound isotope from free. This technical innovation was achieved through the development of beads impregnated with scintillator. Once the receptor is attached to the bead, and ligand is bound, they are close enough to allow the β emission from the tritium to be absorbed by the scintillator which will then shift this energy to produce light (Fig. 1). β emissions from unbound tritiated ligand will dissipate in the buffer. The background in the assay, as determined by the number of counts/min obtained in the presence of 100-fold excess unlabeled TZD. Solid squares, total counts/min; solid diamonds, nonspecific counts/min; solid triangles, specific counts/min. Each point represents the mean ± S.D. for triplicate determinations, and the entire experiment was repeated with similar results. B, Scatchard analysis of data from A. K<sub>d</sub> = 11 nM.
Molecular structures of L-764406 and related compounds

| Molecular Structure | L-number | Binding Activity (%) |
|---------------------|----------|----------------------|
| ![Structure 1](image1) | L-764406 | 95 |
| ![Structure 2](image2) | L-763780 | 0 |
| ![Structure 3](image3) | L-764475 | 0 |
| ![Structure 4](image4) | L-273422 | 0 |

sis, we estimate that >20% of the protein was active in binding.

Known thiazolidinedione PPARγ ligands, including TZD, BRL49653, and CS-045 (troglitazone), were titrated in the SPA assay. IC50 values were determined to be 13, 314, and approximately 1,700 nM, respectively (data not shown). These IC50 values cover 2 orders of magnitude and agree well with previously published binding activities determined using dextran/gelatin-coated charcoal to separate bound from free ligand (4).

Furthermore, the rank order of these compounds is reflected in both transactivation assays and in their in vivo glucose lowering activity (4).

L-764406 Is a Novel PPARγ Ligand—Using the SPA assay approach, L-764406 was shown to be a potent PPARγ ligand. Thus, titration of this compound revealed an apparent IC50 of 70 nM (results not shown). Compared with known synthetic thiazolidinedione ligands for PPARγ, the molecular structure and relatively small size of L-764406 (Table I) suggested that this compound might interact covalently with the receptor. This hypothesis was supported by additional results demonstrating that a structurally related deschloro compound, shown in Table I, did not bind to PPARγ. In particular, the O-methyl derivative, L-273422, would occupy a similar volume to L-764406, thereby indicating that the bulk provided by the chlorine residue at this position is not a critical determinant for binding.

[3H]TZD Cannot Displace L-764406 in the SPA Assay—The SPA assay was used to determine whether a TZD could displace L-764406 from PPARγ. The experiment was designed to also ensure that PPARγ binding activity could still be detected after 48 h. Thus, recombinant receptor was incubated with 10 nM [3H]TZD and submaximal amounts of either L-764406 or unlabeled TZD. After 24 h, the amount of [3H]TZD was increased to 50 nM. Under these conditions, the amount of specific binding should increase with time as the system progresses toward equilibrium (Fig. 2A), but only if binding sites are still available. As seen in Fig. 3A, this only occurs with the samples preincubated with either 15 or 20 nM TZD. Those samples preincubated with either 150 or 300 nM L-764406 did not exhibit an increase in specific counts, suggesting that there were no free binding sites available to be occupied by the increased amount of [3H]TZD. This would be expected if L-764406 was a covalent ligand, since binding equilibrium would not be achieved. These results were confirmed by preincubation of PPARγ with L-764406 at a maximally effective concentration of 4 μM (Fig. 3B). Under these conditions, no [3H]TZD binding activity could be detected after 24 h, while a second sample incubated in parallel with vehicle alone retained binding activity.

Identification of the Binding Site for L-764406—Electrophoretically pure hPPARγ LBD (amino acid residues 204–505, GenBank™ accession number U63416) was used to identify the specific binding site for L-764406 on the receptor. This fragment includes all residues from the hinge domain to the COOH terminus of the receptor as well as 2 residues, glycine and serine, which are encoded at the NH2 terminus after insertion of a BamHI site. The purified fragment was incubated with L-764406 or vehicle alone and analyzed by high performance liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) using a C8 1.0 × 50-mm column. The results indicated that all PPARγ protein in the reaction formed a complex with L-764406, since no free PPARγ protein was observed in the mass spectrum (Fig. 4A). To identify the binding site of L-764406, a tryptic digest was performed using a sample of PPARγ that was preincubated with L-764406. Mass mapping of the digest by LC-ESI-MS revealed a single peptide with a size of 1,282 Da that was 283 Da greater than the predicted tryptic fragment (Table II). This molecular mass difference corresponds exactly to the additional mass provided by the compound (with the concomitant loss of HCl), indicating covalent binding of L-764406 to this peptide fragment of PPARγ. LC-ESI-MS/MS was performed on this peptide. Analysis of the resultant MS/MS spectrum revealed that L-764406 was linked to the single cysteine residue within this fragment (Fig. 4B and Fig. 5). This cysteine corresponds to Cys<sup>313</sup> in the sequence of full-length hPPARγ<sub>2</sub> (4) and is located within helix 3 of the receptor’s LBD.

Mutation of Cys<sup>313</sup> Prevents Binding of L-764406 to hPPARγ—Recombinant GST-hPPARγ protein with a C313A point mutation was purified as described above and used in the SPA assay. As expected, L-764406 (10 μM) did not compete with the [3H]TZD for binding to the mutated receptor (data not shown). Binding activity for TZD was also greatly reduced so that the number of specific cpm was decreased by approximately 75% (data not shown) and with a rightward shift in the IC<sub>50</sub> (Fig. 6). Thus, Cys<sup>313</sup> is necessary for binding of L-764406 and dramatically reduces the binding of TZD as well.

L-764406 Is a Partial PPARγ Agonist—Several experiments were performed to determine whether L-764406 could function as a PPARγ agonist. It has been shown for several members of the nuclear receptor family that binding of agonist ligands induces a conformational change in the LBD. In the case of estrogen receptor α, a major component of this conformational change has been shown to involve folding of the AF-2 domain into the core of the LBD, which results in a more compact structure (25). This conformational change is reflected by the increased resistance of the receptor LBD to partial digestion by proteases. Furthermore, distinct protease digestion patterns can be identified upon binding of agonists versus antagonists (25). A typical agonist-like protease protection pattern (21) was obtained when L-764406 was bound to hPPARγ (Fig. 7). The most obvious feature of this pattern is the major 27-kDa core fragment, which could also be seen with the thiazolidinedione ligand BRL49653 (4), indicating that L-764406 induces an agonist-like conformational change in PPARγ.

When the GAL4/hPPARγ Chimeric Receptor and the Reporter Construct, 5×UAS-TK-luc, were cotransfected into
COS-1 cells, we found that L-764406 served to induce PPARγ-dependent transcription of the luciferase gene (Fig. 8). The compound was specific for the PPARγ LBD, since it failed to induce transcription mediated by either GAL4/hPPARα or GAL4/hPPARδ. The EC₅₀ for transcriptional activation of GAL4/hPPARγ in COS-1 cells was 69 nM, which is in good agreement with the IC₅₀ of 70 nM obtained with the SPA binding assay. Interestingly, maximal transcriptional activation attained using L-764406 was only 25% of that obtained with thiazolidinediones, indicating that this compound was a partial agonist under these conditions. The above findings were confirmed in experiments designed to measure the ability of endogenous wild type PPARγ to regulate a classic PPARγ adipocyte-specific target gene, adipose fatty acid-binding protein.

Fig. 3. [³H]TZD cannot displace L-764406 bound to PPARγ. A, using the SPA format, L-764406 and cold TZD were incubated with PPARγ at two concentrations close to their IC₅₀ values for 24 h with 10 nM [³H]TZD followed by measurement of bound [³H]TZD (0 h, white bars). Subsequently, additional [³H]TZD was added to a final concentration of 50 nM, and the amount of radioactivity bound was determined 0.75 and 25 h later. Results are expressed as percent of maximum where maximum binding activity was determined by incubation with 100-fold excess of unlabeled TZD. B, 1-μg aliquots of purified recombinant hPPARγ LBD were incubated for 24 h with Me₂SO alone or Me₂SO with 4 μM L-764406 (total volume: 0.5 ml). Subsequently, aliquots of these incubations were obtained and used in the SPA assay to determine [³H]TZD (10 nM) binding activity in the presence (+TZD) or absence (–TZD) of unlabeled TZD (1 μM). Results are individual determinations from two experiments run in parallel and are expressed as counts/min of bound [³H]TZD.
Using murine 3T3-L1 preadipocytes, we assessed the ability of L-764406 to promote adipocyte differentiation as measured by induction of aP2 mRNA expression. As depicted in Fig. 9, treatment of 3T3-L1 preadipocytes with L-764406 resulted in a substantial increase in aP2 mRNA expression with an EC50 value of between 100 and 1,000 nM. As seen in transfected COS-1 cells, this effect of L-764406 was submaximal, achieving approximately 25% of the highest activity obtained with TZD.

Since transcriptional activation is mediated through the interaction of nuclear receptors with co-activators (2), we used a co-activator association assay (23) to show that L-764406 induced binding of hPPARγ to CBP. Preincubation of the hPPARγ LBD with 4 μM L-764406 induced binding of the receptor to CBP, however, as with the transcriptional activation of aP2 gene in 3T3-L1 cells and the luciferase reporter in COS-1 cells, this activation was limited to approximately 25% of that seen with TZD (Fig. 10A). Furthermore, this activation could not be increased by subsequent addition of the more potent TZD, indicating that the L-764406 could not be displaced by TZD and that TZD was unable to activate hPPARγ when the binding pocket was occupied by this relatively small covalent agonist. Conversely, an excess of the high affinity TZD ligand can protect against the alkyating effect of L-764406 (Fig. 10A). As was expected from the results of the SPA binding assay (results are not shown), TZD was not able to promote the association of the C313A mutation of hPPARγ with CBP (Fig. 10B).

**DISCUSSION**

We developed a novel SPA-based binding assay for the nuclear receptor, PPARγ. This is a simple assay that does not require separation of bound and free radiolabeled ligands. Us-

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**Table II**

| Sample | Predicted | Observed | Difference |
|--------|-----------|----------|------------|
| PPARγ + Me2SO | 34594.2 | 34590.4 | Δ 3.8 |
| PPARγ + L-764406 | 34877.2 | 34876.6 | Δ 0.6 |

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![Spectral analyses of the PPARγ LBD-L-764406 complex. A, LC-ESI-MS deconvoluted mass spectra of hPPARγ LBD before and after incubation with L-764406. The result suggests that all PPARγ protein in the reaction formed a complex with L-764406. B, LC-ESI-MS/MS spectrum of a 1282-Da peptide generated from trypsin digestion of hPPARγ-LBD-L-764406 complex. Analysis of this spectrum shows that L-764406 is linked to this peptide at the cysteine residue.](image)
ing this approach, L-764406 was shown to function as a potent ligand for hPPAR\(\gamma\). Based upon the chemical structure of this compound, we suspected that it might bind covalently to the PPAR\(\gamma\) LBD. This concept was also suggested by the observation that three closely related analogue compounds lacking the chlorine found in L-764406 were inactive in the PPAR\(\gamma\) SPA binding assay. Experimental evidence in favor of this hypothesis includes the fact that preincubation of PPAR\(\gamma\) with this compound abrogated the ability of a potent TZD ligand to displace L-764406 binding. Moreover, covalent binding of L-764406 to PPAR\(\gamma\) was definitively proven by mass spectral analysis of PPAR\(\gamma\) LBD incubated with this compound; a molecular mass increase in one peptide fragment was detected, which corresponded precisely to the molecular weight of L-764406, minus 35 Da for the loss of chlorine.

Fig. 5. Binding site of L-764406. The peptide sequences for the LBDs of hPPAR\(\gamma\) (GenBank\(\text{TM}\) accession number U63415), hPPAR\(\alpha\) (GenBank\(\text{TM}\) accession number L02932), and hPPAR\(\delta\) (GenBank\(\text{TM}\) accession number L07592) are shown with the cysteine at residue 313 marked in bold. The numbering system in the figure uses the same numbering for amino acid residues as marked for hPPAR\(\gamma\) (u63415). The peptide sequences for all three LBDs were aligned using the Pileup multiple sequence program from the GCG sequence analysis package (18) at its default settings. For proper alignment, the program introduced a gap marked by a dash at position 226.

Fig. 6. Cys\(^{313}\) of hPPAR\(\gamma\) is necessary for binding of L-764406. Recombinant GST-hPPAR\(\gamma\) wild type (solid squares) or protein with a C313A point mutation (open circles) were used in the SPA assay at approximately equal protein concentrations. Results are expressed as percent of maximum, where maximum binding activity was determined by incubation with 100-fold excess of unlabeled TZD. L-764406 and TZD were each used at a concentration of 1 \(\mu\)M.

Fig. 7. L-764406 induces an agonist-like conformational change in PPAR\(\gamma\). \(^{35}\text{S}\)-hPPAR\(\gamma\)1 was synthesized in vitro in a coupled transcription/translation system. It was subsequently preincubated with 0.1\% Me2SO (Control) or 10 \(\mu\)M L-764406, then incubated with distilled H\(\text{2O}\) or increasing concentrations of trypsin. Digestion products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. An asterisk indicates the 27-kDa protease-resistant fragment of hPPAR\(\gamma\)2.
trations greater than 1 μM, it was only capable of producing partial agonist activity at these concentrations when its ability to induce PPARγ-mediated gene transcription was assessed. This was noted using either GAL4/hPPARγ chimeric receptors in COS-1 cells, and luciferase activity in the cell extract was determined after 48 h of incubation in the presence of increasing concentrations of L-764406. Results were normalized to luciferase activity induced by 1 μM TZD, which was considered to be a full agonist.

Fig. 8. L-764406 is a partial agonist of PPARγ-mediated transcription in COS-1 cells. GAL4/hPPARγ (solid triangles), GAL4/hPPARδ (solid squares), and GAL4/hPPARγ (solid circles) chimeric genes were cloned into the expression vector pcDNA 3.1 (+). The reporter construct p5×UAS-tk-luc contained five tandem UASs linked to the thymidine kinase minimal promoter. The constructs were transfected into COS-1 cells, and luciferase activity in the cell extract was determined after 48 h of incubation in the presence of increasing concentrations of L-764406. Results were normalized to luciferase activity induced by 1 μM TZD, which was considered to be a full agonist.

Fig. 9. L-764406 increases aP2 mRNA expression in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were incubated in medium containing 10% fetal bovine serum, 1 μM dexamethasone, and increasing amounts of L-764406 for 72 h. Total RNA samples were prepared and used for slot blot analysis with an aP2-specific probe. The results are shown as normalized aP2 levels of triplicate determinations from two independent experiments.

Although the molecular basis for partial agonist activity is not well understood, it could be due to the production of a receptor conformation with a reduced affinity for co-activators. McDonnell et al. (25) have suggested that the estrogen receptor...
can assume conformations distinct from those of the normal ligand-bound receptor when bound to ligands with varying agonist activities. Their studies also demonstrate that these activities are dependent on cellular and promoter contexts; thus, it is possible that L-764406 might behave as a full agonist under different experimental conditions. In protease protection experiments, L-764406 produced a trypsin digestion profile that was indistinguishable from the thiazolidinedione agonist BRL49653. Although estrogen receptor antagonists are known to produce a distinct protease protection pattern from that of 17β-estradiol (20, 25), we conclude that possible conformational differences responsible for the partial agonist activity of L-764406 may be too subtle for detection by the protease protection assays performed here.

The agonist activity of L-764406 is apparently specific for PPARγ, since no activity was noted with GAL4 chimeric receptors containing either PPARα or PPARβ LBDs. A multiple sequence alignment of the LBDs for hPPARγ, hPPARα, and hPPARδ (Fig. 6) shows that the single cysteine in PPARγ (Cys313) is conserved in the other two receptors. Although PPARγ has only one cysteine in the LBD, the PPARα LBD contains two additional cysteine residues flanking the conserved cysteine, with one of these additional cysteines also being present in PPARδ. Thus, despite the availability of potential attachment sites in the LBDs of hPPARα and hPPARδ, L-764406 does not activate these receptors (Fig. 5).

Cys313 in the LBD of PPARγ is within a predicted helical domain2 that corresponds to helix 3 as previously diagrammed in the crystal structures of the RARγ (26) and ERα (27) LBDs. Importantly, residues within helix 3 of the ERα LBD have been shown to interact with the A-ring of 17β-estradiol (28), as well as the ER antagonist raloxifene (27). The specific residues of the ERα LBD that interact with 17β-estradiol include Leu345, Thr345, and Glu352 and are not conserved in hPPARγ. Although a homologous cysteine has not been identified in the estrogen receptors, a cysteine corresponding to Cys313 is conserved in the LBDs of human RXRα, -β, and -γ, but not in RARα, -β, and -γ. In addition to providing contacts with its ligand, all-trans-retinoic acid, helix 3 from the LBD of RARγ forms intramolecular interactions with helix 12 and thus may be involved in the orientation of the AF-2 domain (26). This relationship suggests a pathway whereby ligand interactions at helix 3 are translated into interactions with co-activators through the AF-2 domain. Indeed, we have shown that the partial agonist L-764406 exhibits a diminished interaction with CBP (Fig. 10). Since this interaction could not be augmented by subsequent addition of a more potent agonist, TZD (Fig. 10), it suggests that despite the relatively small size of L-764406, the modified Cys313 disrupts the ligand binding pocket sufficiently to prevent activation by TZD. We know that Cys313 is not an absolute requirement for TZD binding since the C313A mutation is still active in this regard, albeit at a dramatically reduced level. Together these experiments suggest that the activity of L-764406 as a partial agonist is mediated by a limited (versus full agonists) interaction with co-activators. However, it should be noted that although L-764406 functions as a partial agonist in the cell-based transactivation assays (Figs. 8 and 9) and in the in vitro co-activator association assay (Fig. 10), it is possible that this compound could function as a full agonist in another context.

Although RARs do not possess a cysteine residue homologous to the one found in the LBD of PPARγ, RARα does have a serine residue at this position. In fact, the replacement of Ala225 for Ser332 is the only difference between the LBDs of RARβ and RARα and has been shown to account for their ligand specificity (26). This shows that the residue at this position in helix 3 is involved in the determination of ligand specificity for other nuclear receptors.

Since the completion of our studies, Nolte et al. (29) reported their results where x-ray crystallography was used to determine the PPARγ LBD structure. Their findings indicate that Cys313 is indeed located within helix 3 and that helix 3 forms an important component of the ligand binding pocket when occupied by the TZD, BRL49653. Flanking Cys313 are two residues, Phe310 and Glu314, which form part of a hydrophobic pocket occupied by the sulfur atom of the TZD ring in BRL49653 (29). Cys313 is believed to form part of a narrow pocket occupied by the central benzene ring of BRL49653 (29). Our results obtained using a novel covalent ligand provide an independent line of evidence which shows the involvement of helix 3, and in our case, Cys313, in forming critical components of the PPARγ ligand binding pocket.

In summary, L-764406 was shown to possess agonist activity in cells and produced an LBD protease protection pattern that was similar to that caused by a known TZD; thus, it is likely that the interaction of ligands with helix 3 (and Cys313) is important for the induction of conformational changes which mediate co-activator recruitment and activation of transcription. L-764406 is also unique among known PPARγ ligands in that it functions as a partial agonist (in co-activator association, transactivation, and adipogenesis). This finding supports the notion that PPARγ ligands, which might exhibit more restricted (tissue- or even gene-specific) effects, and hence different therapeutic or toxicity profiles, await discovery.

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