Insights into the Mechanism of Partial Agonism

CRYSTAL STRUCTURES OF THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ LIGAND-BINDING DOMAIN IN THE COMPLEX WITH TWO ENANTIOMERIC LIGANDS

The peroxisome proliferator-activated receptors (PPARs) are transcriptional regulators of glucose and lipid metabolism. They are activated by natural ligands, such as fatty acids, and are also targets of synthetic antidiabetic and hypolipidemic drugs. By using cell-based reporter assays, we studied the transactivation activity of two enantiomeric ureidofibrate-like derivatives. In particular, we show that the R-enantiomer, (R)-1, is a full agonist of PPARγ, whereas the S-enantiomer, (S)-1, is a less potent partial agonist. Most importantly, we report the x-ray crystal structures of the PPARγ ligand binding domain complexed with the R- and the S-enantiomer, respectively. The analysis of the two crystal structures shows that the different degree of stabilization of the helix 12 induced by the ligand determines its behavior as full or partial agonist. Another crystal structure of the PPARγ (S)-1 complex, only differing in the soaking time of the ligand, is also presented. The comparison of the two structures of the complexes with the partial agonist reveals significant differences and is suggestive of the possible coexistence in solution of transcriptionally active and inactive forms of helix 12 in the presence of a partial agonist. Mutation analysis confirms the importance of Leu358, Leu469, and Ile472 in the activation by (R)-1 and underscores the key role of Gln286 in the PPARγ activity.

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The on-line version of this article (available at http://www.jbc.org) contains supplementary Figs. 1–5.

1 These two authors contributed equally to this work.

2 To whom correspondence may be addressed: Istituto di Cristallografia, Sede di Monterotondo, Area della Ricerca ROMA 1, CNR, Via Salaria Km 29,300, 0016 Monterotondo Stazione, Roma, Italia. Tel.: 39-06-90672633; Fax: 39-06-90672630; E-mail: giorgio.pochetti@ic.cnr.it.

3 To whom correspondence may be addressed: Laboratorio “Giovanni Galli” di Biochimica e Biologia Molecolare dei Lipidi e di Spettrometria di Massa, Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, 20133 Milano, Italia, and Dipartimento di Chimica Farmaceutica, Università degli Studi di Napoli, 80131 Napoli, Italia, and Dipartimento di Chimica, Ingegneria Chimica e Materiali, Università di L’Aquila, 67010 L’Aquila, Italia

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PPARγ, showing higher potency on PPARα. The S-enantiomer, (S)-1, displays a lower efficacy toward PPARγ and behaves as a partial agonist of this receptor subtype.

We also provide a molecular explanation for their different behavior as full and partial agonists of PPARγ by showing the crystal structures of the complexes of these new ligands with PPARγ ligand binding domain (LBD).

Among the known crystal structures of PPARγ complexed with partial agonists (19–22), PPARγ(S)-1 is the only one in which the ligand interacts directly with the helix 12; the comparison of this structure with that of the complex with the R-enantiomer permits further insights into the molecular basis of partial agonism in PPARs.

Although the (R)-1 ligand induces the canonical transcriptionally active conformation of helix 12, the PPARγ(S)-1 complex shows a suboptimal conformation of this helix, which could be responsible for the partial agonist behavior of the S-enantiomer.

**EXPERIMENTAL PROCEDURES**

*Materials—Wy-14,643, dexamethasone, and isopropyl-β-D-thiogalactopyranoside were purchased from Sigma. Dulbecco’s modified Eagle’s medium/F-12 nutrient (1:1) mixture, fetal calf serum, penicillin, and streptomycin sulfate were purchased from Invitrogen. Invitrogen 49653 (rosiglitazone) was obtained by Hefei Scenery Chemical Co. (Hefei, Anhui, China). GW501516 was purchased from Alexis Corp. (Lausen, Switzerland). Synthesis of compounds (R)-1 and (S)-1 started from the optically active 2-(4-bromophenoxy)-2-methyl-butanolic acids (obtained by resolution of the racemic mixture with (+)- or (−)-1-phenylethylamine) and occurred without affecting the chiral center. The enantiomeric excess was determined by HPLC on Chiralcel OD and estimated as 96% for (R)-1 and 97% for (S)-1. The absolute configuration of (R)-1 and (S)-1 was assigned by chemical correlation with the known (R)- and (S)-2-hydroxy-2-methyl-butanolic acids, from which it was possible to prepare (R)- and (S)-2-(4-bromophenoxy)-2-methyl-butanolic acids, respectively. Achiral compound 2 was obtained following the same procedure starting from 2-(4-bromophenoxy)-2-methyl-propanoic acid. All synthetic details will be reported elsewhere.5*

The sequence of biotinylated SRC1 (steroid receptor coactivator 1) peptide spanning amino acids 676–700 was donated by the University of Athens, Greece. The expression vector for the LBD of human PPARγ was expressed as N-terminal His-tagged protein using a PET28 vector and purified as previously described (24). In brief, freshly transformed *E. coli* BL21 DE3 were grown in LB medium with 30 µg of kanamycin/ml at 37 °C to an OD of 0.6. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and further incubated at 18 °C for 20 h. Cells were harvested and resuspended in a 20 ml/liter culture of Buffer A (20 mM Tris, 150 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8) in the presence of protease inhibitors (Complete Mini EDTA-free; Roche Applied Science). Cells were sonicated, and the soluble fraction was isolated by centrifugation (35,000 × g for 45 min). The supernatant was loaded onto an Ni²⁺-nitrilotriacetic acid column (GE Healthcare) and eluted with a gradient of imidazole 0–300 mM in Buffer A with a PU980 HPLC system (Jasco, Lecco, Italy). The fractions containing the protein were collected, quantitated with a Bradford assay, and analyzed on 12% SDS-PAGE (supplemental Fig. 1A). The protein was then dialyzed over Buffer C (20 mM Tris, 20 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8) to remove imidazole, and it was cleaved with thrombin protease (GE Healthcare) (10 units/mg) at room temperature for 2 h. The cleavage was monitored by SDS-PAGE (supplemental Fig. 1A) and by histidines and the cleavage site for thrombin protease upstream of the LBD of PPARγ in the pET28 bacterial expression vector. These plasmids were kindly prepared by Dr. Krister Bamberg (AstraZeneca, Mölndal, Sweden). The vectors expressing the Gal4 fusion proteins with the ligand binding domains of RARα, 9-cis-retinoic acid receptor α, RAR-related orphan receptor, thyroid hormone receptor α, glucocorticoid receptor, Nur1, and farnesoid X receptor were generated by fusing the LBDs to the DNA-binding domain of Gal4 and inserting them into pCDNA3 (Invitrogen) by standard cloning techniques. Assays were validated using known ligands. The expression vector for Gal4-hepatocyte nuclear factor 4 LBD was provided by Dr. Ian- nis Talianidis (Institute of Molecular Biology and Biotechnol- ogy Foundation for Research and Technology Hellas, Herak- leion, Crete, Greece). Q286G, I472A, L465A, and L469A mutants were prepared using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by automated sequencing.

**Cell Culture and Transfections—Human hepatoblastoma cell line HepG2/C3A (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium/F-12 nutrient (1:1) mixture containing 10% heat-inactivated fetal calf serum, 100 units of penicillin G/ml, and 100 µg of streptomycin sulfate/ml at 37 °C in a humidified atmosphere of 10% CO₂. For transactivation assays, 10⁵ cells/well were seeded in 24-well plates in triplicate, and transfections were performed with a modification of the calcium-phosphate method (23). After transfection, cells were treated with the indicated ligands for 20 h. Luciferase activity in cell extracts was determined with a luciferase detection kit (Promega, Milan, Italy) using a luminometer (Sirius, Berthold Detection Systems, Pforzheim, Germany). β-Galactosidase activity was determined as previously described (23). Each experiment was repeated three times.**

**Protein Expression and Purification—**The LBD of human PPAR γ was expressed as N-terminal His-tagged protein using a PET28 vector and purified as previously described (24). In brief, freshly transformed *E. coli* BL21 DE3 were grown in LB medium with 30 µg of kanamycin/ml at 37 °C to an OD of 0.6. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and further incubated at 18 °C for 20 h. Cells were harvested and resuspended in a 20 ml/liter culture of Buffer A (20 mM Tris, 150 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8) in the presence of protease inhibitors (Complete Mini EDTA-free; Roche Applied Science). Cells were sonicated, and the soluble fraction was isolated by centrifugation (35,000 × g for 45 min). The supernatant was loaded onto an Ni²⁺-nitrilotriacetic acid column (GE Healthcare) and eluted with a gradient of imidazole 0–300 mM in Buffer A with a PU980 HPLC system (Jasco, Lecco, Italy). The fractions containing the protein were collected, quantitated with a Bradford assay, and analyzed on 12% SDS-PAGE (supplemental Fig. 1A). The protein was then dialyzed over Buffer C (20 mM Tris, 20 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8) to remove imidazole, and it was cleaved with thrombin protease (GE Healthcare) (10 units/mg) at room temperature for 2 h. The cleavage was monitored by SDS-PAGE (supplemental Fig. 1A) and by

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5 F. Loiodice, P. Tortorella, A. Laghezza, G. Fracchiolla, A. Lavecchia, E. Novel- lino, F. Mazza, G. Pochetti, C. Godio, N. Mitro, D. Caruso, A. Galmozzi, S. Scurati, and M. Crestani, manuscript in preparation.
mass spectrometry, and we observed a complete digestion, as assessed by the reduction of 1751 Da of the molecular mass of the recombinant protein, corresponding to the loss of 17 amino acids (supplemental Fig. 1B). The identity of the native and digested protein was determined on the basis of the molecular weight (supplemental Fig. 1B) and of the sequencing of the tryptic peptides obtained by liquid chromatography-electrospray ionization mass spectrometry and tandem mass spectrometry (data not shown) (LTQ; ThermoElectron Co., San Jose, CA), respectively. The digested mixture was reloaded onto an Ni2+-nitrilotriacetic acid column to remove the His tag and the undigested protein. The flow-through was loaded onto a Q-Sepharose HP column (GE Healthcare) and eluted with a gradient of NaCl 0–500 mM in Buffer B (20 mM Tris, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8). The protein was then dialyzed over Buffer B and kept frozen in aliquots at a concentration of 1 mg/ml.

**Co-activator Recruitment Assay**—His-PPARγ-LBD (20–50 nM) was incubated in black flat-bottom 384-well microplates for 1 h at 37 °C with biotinylated peptide (500 nM), europium-labeled anti-histidine antibody (1 nM), and allophycocyanin-labeled streptavidin (100 nM) in assay buffer (50 mM KCl, 50 mM Tris pH 7.5, 0.1 mg/ml fatty acid-free bovine serum albumin, 1 mM dithiothreitol). Ligands were diluted in Me2SO, starting at the indicated concentration for each ligand. Time-resolved fluorescence was measured on an Analyst GT multimode reader (Molecular Devices, Sunnyvale, CA). Excitation was at 340 nm, and the results are expressed as the ratio of allophycocyanin fluorescence (λ = 665 nm) to europium fluorescence (λ = 620 nm) multiplied by 10,000. The final results represent this ratio minus the background (Me2SO ratio) of six independent experiments. EC50 and Kd values of the ligands were calculated using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA).

**Competition Binding**—The scintillation proximity assay was performed as previously described (24). Briefly, the assay was performed in 96-well plates (PerkinElmer Life Sciences) using 100 μl of buffer containing 20 mM Tris (pH 7.5), 80 mM NaCl, 2 mM Tris(2-carboxyethyl)phosphine, 0.125% CHAPS, and 10% glycerol. Each well contained 0.1 mg of polylisine-coated yttrium silicate beads (GE Healthcare), 1 μg of His-PPARγ-LBD, and 40 nM [3H]rosiglitazone (American Radiolabeled Chemicals) at 50 Ci/mmol. The amount of protein did not deplete ligand concentration. Cold ligands were tested in 10-point concentration-response curves starting at the indicated concentration. All components were added simultaneously and incubated with gentle shaking for 1 h at room temperature. Scintillation counts were determined in a Microbeta 1450 Wallac Trilux counter (PerkinElmer Life Sciences), reading each well for 1 min. Wells devoid of competitor represented 100% binding. Nonspecific binding was measured by leaving PPARγ protein out of the scintillation proximity assay reaction. Experiments were repeated three times. Kd values of the ligands were calculated using GraphPad Prism version 4.0c for Macintosh (GraphPad Software).

**Protein Crystallization**—Crystals of apo-PPARγ (25) were obtained by vapor diffusion at 20 °C using sitting drop made by
and \( a = 93.54 \, \text{Å}, \, b = 60.91 \, \text{Å}, \, c = 118.35 \, \text{Å}, \, \beta = 103.1^\circ \) for that with the \( S \)-enantiomer (crystal soaked 30 days). The asymmetric unit is formed by one homodimer (53% solvent).

Structure Determination—X-ray data were collected at 100 K under a nitrogen stream using synchrotron radiation (beamline XRD1 at Elettra, Trieste). The diffracted intensities were processed using the programs DENZO and SCALEPACK (26). Refinement was performed with CNS (27) using the coordinates of apo-PPAR\( \gamma \) (28) (Protein Data Bank code 1PRG) as a starting model. All data between 8 and 2.1 Å (8–2.25 Å for the \( S \)-enantiomer) were included with \( \sigma \) cut-off of 2.0. The statistics of crystallographic data and refinement are summarized in Table 1.

**RESULTS**

(R)-1 and (S)-1 Activate PPAR\( \gamma \)-mediated Transcription—The activity of the two enantiomers of a novel compound deriving from the ureidofibrate class (Fig. 1) was evaluated on a panel of nuclear receptors in a Gal4-based assay. The results in Fig. 2A show that (R)-1 and (S)-1 only activate the transcription of PPAR\( \alpha \) and PPAR\( \gamma \), whereas the activity of the other nuclear receptors, including the PPAR\( \beta/\delta \) subtype, is not affected by these two molecules.

To gain more insights on the potency and efficacy of these new compounds on the PPAR\( \gamma \) subtype, we carried out transfection assays with the human Gal4-PPAR\( \gamma \)-LBD expression vector and compared the curves obtained with increasing concentrations of (R)-1 and (S)-1 with the corresponding data obtained with rosiglitazone, here used as a reference compound. As shown in Fig. 2B, (R)-1 is able to activate PPAR\( \gamma \), but its potency is clearly lower than that of rosiglitazone (Table 2). (S)-1 is less potent than the \( R \)-enantiomer toward PPAR\( \gamma \), and in addition its efficacy is markedly lower than that of rosiglitazone and (R)-1 (Table 2).

Co-activator recruitment assays (Fig. 2C) are consistent with the results of cellular assays and show that (S)-1 is less potent and has lower efficacy than (R)-1 and rosiglitazone (Table 2). The determination of binding constants by a scintillation proximity assay confirms that (S)-1 is a lower affinity PPAR\( \gamma \)
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ligand, whereas the affinity of (R)-1 is comparable with that of rosiglitazone (Fig. 2E).

**(S)-1 Behaves Like a PPARγ Partial Agonist**—Since the efficacy of (S)-1 in cell-based assays is 50%, and that in the co-activator recruitment assay is about 25% of that of rosiglitazone (Table 2), we wanted to test whether this molecule could behave as a partial agonist of PPARγ. The co-activator recruitment assay (Fig. 2D) shows that when 1 or 10 μM rosiglitazone is incubated with increasing concentrations of (S)-1, the percentage of efficacy decreases to about 25%, a value corresponding to the maximal efficacy reached by (S)-1. These results suggest that (S)-1 is a partial agonist of PPARγ.

**Overall Structure of the LBD**—Having observed the different behavior of the two enantiomers, we solved the crystal structure of PPARγ complexed with (R)-1 and (S)-1. The structure of PPARγ-LBD in both complexes is very similar to that of the apo-form, with an r.m.s. deviation between Cα atoms of 0.31 Å (225 Cα pairs). The r.m.s. deviation between the structures of the two complexes is only 0.20 Å (267 Cα pairs), the largest deviation regarding the terminal part of helix 12 (Cα 473–476). The LBD domain consists of 12 helices and a small β-sheet of four strands. The electron density corresponding to residues 260–275, belonging to the so-called omega loop, is badly defined or missing. The residues of this region were not considered in the refinement. A homodimer forms the asymmetric unit in both structures. The two monomers, denoted as A and B, contain one ligand molecule. Helix 12 of monomer A is in its active conformation. As observed in other PPAR structures, helix 12 in monomer B does not adopt the active conformation, despite the presence of the ligand (24). This is because H12 of monomer B is engaged in several contacts with a symmetry-related molecule of monomer A. Particularly, it occupies the hydrophobic groove into which the coactivator peptide binds (nuclear receptor-box) (28, 30, 31), thus mimicking the two-turn amphipathic coactivator α helix (supplemental Fig. 2).

**TABLE 2**

Potency and efficacy of tested ligands toward PPARγ as determined in Gal4-based assays (top) and in cell-free co-activator recruitment assays (bottom)

Values are mean ± S.D. Efficacy values were calculated as a percentage of the reference compound rosiglitazone.

| Tested ligand | EC$_{50}$ | Efficacy |
|---------------|-----------|----------|
| Rosiglitazone | 3.8 ± 1.5 | 100.0 ± 10.5 |
| (R)-1         | 73.3 ± 49.9 | 116.1 ± 9.0 |
| (S)-1         | 593.0 ± 114.1 | 50.4 ± 5.1 |

| Tested ligand | $K_d$ | $\Delta_{max}$ |
|---------------|-------|---------------|
| Rosiglitazone | 14.8 ± 6.6 | 100.0 ± 4.9 |
| (R)-1         | 684.8 ± 106.8 | 70.5 ± 2.3 |
| (S)-1         | 1,978 ± 523.5 | 23.3 ± 3.2 |

**FIGURE 3.** A, 2Fo – Fc electron density map calculated around the R-enantiomer (shown in yellow) and contoured at 1σ; B, 2Fo – Fc electron density map calculated around the S-enantiomer (shown in cyan) and contoured at 0.9σ.

**FIGURE 4.** A, hydrogen bonding network between the R-enantiomer (yellow) and the LBD of PPARγ (the triad His$_{423}$, His$_{449}$, and Tyr$_{473}$ is colored in orange); B, hydrogen bonding network between the S-enantiomer (cyan) and the LBD of PPARγ (the triad and Ser$_{469}$ are in purple).
cavity, there making hydrophobic contacts with the surrounding protein residues. In the region of the distal cavity, the final Fourier difference map shows residual electron density that was attributed to several interlinked water molecules, suggesting that this region could still accommodate additional substituents. In fact, the PPAR ligand binding pocket is larger than that of other nuclear receptors, explaining its capability to accept a variety of naturally occurring and synthetic lipophilic acids.

Mutagenesis of the Leu<sup>465</sup>, Leu<sup>469</sup>, and Ile<sup>472</sup> Residues of PPARγ—The importance of these residues of the C-terminal portion of PPARγ in the activation mediated by (R)-1 was studied by mutagenesis analysis, which shows that the basal activity of these mutants is severely impaired (Fig. 6). Neither rosiglitazone nor (R)-1 could reach activity levels comparable with those of the activated wild type receptor (Fig. 6).

Binding of the S-Enantiomer in the PPARγ-LBD—The electron density of the S-enantiomer in the PPARγ ligand cavity (Fig. 3B) is interrupted at the central part of the molecule, and it is generally less well defined than that of its enantiomer. The location of the polar head is about 1 Å away from the triad His<sup>323</sup>, His<sup>449</sup>, and Tyr<sup>473</sup> and from the helix 12, as compared with that of the R-enantiomer (Fig. 7). However, the fused ring systems of the two ligands, almost perpendicular to each other, and their long aliphatic chains maintain unshifted positions in the cavity because of different torsion angles involving the central aromatic ring and the hexocyclic nitrogen atom. The 1-Å shift adopted by the S-enantiomer is probably required to reduce the steric clash between its ethyl group and the Gln<sup>286</sup> backbone belonging to helix 3 (Fig. 7). Such ligand displacement gives rise to a different, noncanonical, hydrogen bonding network of its polar head. As can be seen in Fig. 4B, one of the ligand carboxylate oxygens forms three hydrogen bonds of 2.4, 2.7, and 3.1 Å, respectively, with Tyr<sup>473</sup> OH, His<sup>323</sup>, and His<sup>449</sup> N<sup>ε2</sup> atoms. The other carboxylate oxygen is engaged in a hydrogen bond of 2.4 Å with the Ser<sup>289</sup> OH. Moreover, the hydrophobic interactions between the ligand and the Leu residues in the loop 11/12 and in H11 and H12 helices reduce to only one favorable contact of 4.0 Å with the Leu<sup>453</sup> methyl and the methyl on the asymmetric carbon atom, as shown in Fig. 5B.

A comparison between the two complexes reveals that, although the His<sup>323</sup> and His<sup>449</sup> residues maintain the same orientation, a rearrangement for the backbone atoms of the critical residue of H12, Tyr<sup>473</sup>, occurs in the complex with the S-enantiomer, so that this helix is slightly shifted from its active conformation (Fig. 7). Fig. 8A shows the orientation of the Tyr<sup>473</sup> side chain that is supported by the polarization interactions between the aromatic π-cloud and the surrounding methyl groups of Val<sup>450</sup>, Leu<sup>453</sup>, Leu<sup>476</sup>, and Ile<sup>472</sup>, occurring in
the complex with the R-enantiomer. Fig. 8B shows the same view for the complex with the S-enantiomer. In this case, the Tyr\textsuperscript{473} side chain interacts only with the Leu\textsuperscript{453} methyl group, which causes an evident destabilization of H12, also denoted by the increase of the B values (20–30%) of residues 472–474 of the complex with the S-enantiomer.

Another Crystal Structure of the PPAR\textgamma-S-Enantiomer Complex—The structure of the complex between PPAR\textgamma and the S-enantiomer, obtained from crystals of the apo-form soaked for only 6 h in the ligand solution, was also determined and compared with that previously reported, resulting from crystals soaked for more than 30 days. Intriguingly, the comparison of the two structures reveals significant differences in the electron density for the ligand and several protein residues and gives additional insights into the conformational changes induced by accommodating the ligand in the LBD. It is likely, therefore, that we have observed, by freezing the crystal at different soaking times, two different thermodynamic states of the ligand bound into the cavity. The hypothesis of equilibrium of multiple coexisting conformations of nuclear receptors in the presence of a partial agonist needs to be supported by further molecular dynamics simulations. However, the ligand binding comparison between the R- and the S-enantiomer, previously described, has been made, choosing the structure of the complex with the S-enantiomer showing a suboptimal, probably inactive, conformation of H12, which belongs to the crystal soaked for 1 month.

In the structure of crystal soaked 6 h, the S-enantiomer carboxylate group participates in a hydrogen bonding network with the His\textsuperscript{323}, His\textsuperscript{449}, and Tyr\textsuperscript{473} almost identical to that found for the R-enantiomer. However, the ligand ethyl substituent gives rise to repulsive interactions with the Gln\textsuperscript{286} backbone of helix 3 (Fig. 9). In fact, missing and weak electron density at the expected positions of the ethyl group and Gln\textsuperscript{286} side chain, respectively, have been observed (supplemental Fig. 3A). Modeling the ethyl group gives rise to repulsive interactions in all cases. Moreover, the electron density around the Tyr\textsuperscript{473} side chain, positioned as in the complex with the R-enantiomer, is well defined (supplemental Fig. 4A), and the water molecule, here hydrogen-bonded to one of the ligand carboxylate oxygens, forms short contacts with one of the methyls of Leu\textsuperscript{453} (2.8 Å) and of Leu\textsuperscript{460} (3.3 Å). At variance with this situation, the observed displacement of about 1 Å found in the complex obtained after ~30 days of soaking reduces the steric clash between the ligand ethyl group and the Gln\textsuperscript{286} residue, as evidenced by well defined electron density maps in the regions of the above groups (supplemental Fig. 3B). Simultaneously, the Tyr\textsuperscript{473} side chain is rotated as shown in Fig. 9, and the water molecule approaching the Leu\textsuperscript{460} residue forces its side chain to a different conformation. This conformational change is transmitted, through the Met\textsuperscript{463} side chain, to the Phe\textsuperscript{282} aromatic ring, resulting in a general rearrangement of the loop 11/12 (supplemental Fig. 5).

Mutagenesis of the Gln\textsuperscript{286} Residue of PPAR\textgamma—To validate the functional importance of the steric clash between the ethyl group of (S)-1 and Gln\textsuperscript{286}, we attempted to eliminate it by substituting Gln\textsuperscript{286} with a glycine. However, this substitution reduces dramatically the basal activity and prevents the activation of the receptor by (S)-1 (Fig. 10A). Conversely, rosiglitazone activates the mutated receptor to levels comparable with the wild type although only at micromolar concentration (Fig. 10A). Since the Q286G mutant did not allow us to demonstrate the functional relevance of this residue in the steric clash, we synthesized the achiral compound 2 bearing the shorter methyl group in place of the ethyl group (Fig. 1). As shown in Fig. 10B, compound 2 is more potent than (S)-1, but the efficacy is lower than that of rosiglitazone. This result strongly argues for the presence of the steric clash between the ethyl group of (S)-1 and Gln\textsuperscript{286}.

**DISCUSSION**

We have identified two ureidofibrate-like enantiomers that display different behavior toward PPAR\textgamma.
The \( R \)-enantiomer is a more potent agonist than the corresponding \( S \)-enantiomer, which has the typical behavior of a PPAR\( \gamma \) partial agonist, since its efficacy is lower than that of rosiglitazone and the \( R \)-enantiomer, and it decreases the co-activator recruitment on PPAR\( \gamma \) induced by rosiglitazone. It should be noted that the differences of the values obtained in the cell-based reporter gene assay and in the \textit{in vitro} co-activator recruitment assay (Table 2) may be explained by the fact that the recruitment assay measures the binding of a peptide containing a single nuclear receptor box to the LBD of PPAR\( \gamma \). Therefore, the recruitment of ligands in this type of assay may underestimate the ability of ligand-induced co-activator recruitment to induce transcription in a cell-based reporter gene assay. In addition, as opposed to the cell-free co-activator recruitment assay, in the cellular environment more co-activators are present at the same time, and they may cooperate in activating gene transcription.

\textbf{Structural Basis for the Mechanism of Partial Agonism—}The transmission of information through structural changes is fundamental to the function of many proteins. It is well known that in nuclear receptors, the dynamic behavior of helix 12 plays a key role in coactivator recruitment. Ligand binding to the LBD modulates the stabilization of H12 in different ways; it functions as a trigger that acts directly, stabilizing its active conformation (32), or indirectly, influencing the H12 stability through additional structural changes, such as repositioning of H11 or different conformations of the loop 11/12. H12 stability is also promoted by the action of the ligand as LBD global stabilizer (24, 33).

Full agonists shift the equilibrium between active and inactive conformation of helix 12 toward the active state, leading to coactivator recruitment. Partial agonists are incapable of shifting this equilibrium in favor of the active conformation to the same extent as full agonists; they are not able to stabilize the H12 in the proper position, due to the lack of a few key interactions, and even a slight mispositioning of H12 may result in an attenuated transcriptional response (6, 17).

Previous work on estrogen receptors showed that a high affinity ligand can induce a low affinity coactivator binding site, behaving as a partial agonist, by selecting for suboptimal conformation of H11 that, in turn, destabilizes the agonist position of H12 through loss of hydrophobic or electrostatic contacts (34).

Moreover, in a structural study on the human mineralocorticoid receptor, it has been proposed that the residues of the loop 11/12 might be compared with a zip fastener, in which each residue of the loop 11/12 is a link, which helps to promote the precise positioning of H12 (35).

Differences in the hydrophobic packing of this loop may contribute to different H12 dynamics (34). However, to date there is still little structural information to explain the reduced efficacy observed with high affinity partial agonist ligands (such as genistein for estrogen receptor \( \beta \)) (34, 36).

To gain further insights at the molecular level into the behavior of a ligand as a partial agonist, we solved the structures of PPAR\( \gamma \) complexes with the two enantiomeric forms of a ureidofibrate-like derivative, both active toward PPAR\( \gamma \), the \( R \)-enantiomer behaving as a full agonist and the \( S \)-enantiomer as a partial agonist (Table 2, Fig. 2). These model compounds are particularly suitable to study the mechanism of partial agonism,
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because they differ only for the switching of a methyl with an ethyl group on the asymmetric carbon atom, a small structural change that causes a significant difference in the pharmacological profile.

Although helix 12 is in a similar conformation in both complexes, there are some important differences. In the crystal complex PPARγ-(R)-1, the active conformation of H12 is stabilized by the following interactions: (a) both carboxylate oxygens of the ligand engage canonical hydrogen bonds with the three residues His323, His449, and Tyr473 involved in the receptor activation (Fig. 4A); (b) the appropriate position of the Tyr473 aromatic side chain is ensured by polarization interactions with Ile472 and Leu476 on one side and with Val450 and Leu453 on the other side (Fig. 5A); (c) the ligand methyl and ethyl groups form several favorable hydrophobic interactions with Leu453 of H11, Leu469 of H12, and Leu465 of the loop 11/12 (Fig. 5A). Thus, the potency of the R-enantiomer is a direct consequence of a very effective stabilization of the helix 12, through hydrophobic and electrostatic interactions. Moreover, helix 12 is here stabilized in the proper conformation to recruit the coactivator, the same observed in other crystal structures of complexes with full agonists (24, 25, 37–39).

In the complex with the S-enantiomer (crystals soaked for 30 days), a 1-Å shift of the ligand away from helix 12 is observed. This is probably caused by a steric clash between the ligand ethyl group and the Gln286 side chain of the key residue Gln286, belonging to H3. Leu465 also interacts with the ring of Tyr473, stabilizing the active conformation of this residue.

**Effect of L465A and L469A Mutations on the Potency of (R)-1**—As expected, the shortening of the side chain at positions 465 and 469 shows a reduction of activity of (R)-1 due to the lack of the important hydrophobic contacts between the methyl and ethyl substituents of the ligand and the methyl groups of the leucines (Fig. 5A). However, the strong reduction of activity observed with the mutated apo-receptor could also be ascribed to the role played by these two leucines in stabilizing H12 through direct contacts with H3. In fact, they form favorable contacts with the side chain of the key residue Glu286, belonging to H3. Leu465 also interacts with the ring of Tyr473, stabilizing the active conformation of this residue.

**Effect of I472A on the Potency of (R)-1**—Also, in this case there is a dramatic reduction of the potency of (R)-1 toward the I472A mutant. This could be determined by the loosening of the contact with Tyr473 and by the lack of a short hydrophobic contact made by a methyl of Ile472 with a carbon atom of the H323 ring (3.4 Å). This contact helps to stabilize the correct orientation of this very important residue, involved in hydrogen bonds with the Tyr473 ring and the carboxylate oxygens of the ligand. This hydrophobic contact is not present in the structure with the (S)-1 ligand (4.4 Å), where the Ile472 and His323 side chains slightly changed their orientations.

**Effect of the Q286G Mutation on the Potency of (S)-1**—Fig. 11 is a superposition among the crystal complexes formed by PPARγ with rosiglitazone, (R)-1, and (S)-1, respectively. There, the relative positions adopted by the three ligands in the binding cavity can be deduced. In the complex with rosiglitazone, the Gln286 NH2 group forms a hydrogen bond with the ligand CO, and it is at 3.1 Å from one of the Leu465 methyls, working as a bridge between the ligand and the loop 11/12 preceding helix 12. If the Gln286 side chain maintained the same position in the complexes with (R)-1 and (S)-1, the methyl of (R)-1 and the ethyl of (S)-1 would form short contacts with the Gln286 Cγ atom (2.9 and 1.8 Å, respectively). To release these repulsive interactions, a reorientation of the Gln286 side chain takes place in both complexes. In the new position that the Gln286 side chain adopts in the complexes with (R)-1 and (S)-1, its NH2 forms a hydrogen bond of 3.2 and 3.0 Å, respectively, with the carbonyl oxygen of Ser464 belonging to loop 11/12. Moreover, it is at 3.1 and 3.0 Å from one of the Leu465 methyls. From these observations, it is evident that in the three complexes, the Gln286 residue plays a key role in stabilizing helix 12, and the Q286G mutation has a dramatic effect on the receptor activity. Because it is not possible to confirm the functional relevance of the steric clash between Gln286 and the ethyl group of (S)-1 by mutating this residue, we tested a ligand with the shorter methyl group replacing the ethyl group. As expected, this substitution improves the potency of this ligand as compared with that of (S)-1, thus confirming that this steric clash does play a role in determining the behavior of the S-enantiomer.

**Molecular Adaptation Caused by the Entry of the Partial Agonist**—Two crystal structures with the S-enantiomer, collected after different soaking times, offer two snapshots depicting different nuclear receptor conformations provoked by the binding of a partial agonist in the LBD. The comparison of the two structures shows that in one of these (6 h of soaking), the helix 12 is in the canonical transcriptionally active conformation, whereas in the other, H12 shows a suboptimal, probably inactive conformation. In the structure from crystals...
soaked for 6 h, a steric hindrance between the ethyl group of the ligand and the backbone of H3 (Gln286) is present. In the structure from crystals soaked for 1 month, the ligand, in the attempt to reduce the steric hindrance with Gln286, displaces its position, weakening the hydrogen bond with Tyr473. This residue, consequently, changes its position; a water molecule, bound to Tyr473, shields a zone of hydrophobic interactions between ligand and Leu453 of the loop 11/12, forcing the Leu453 side chain to interact with that of Met463. This, in turn, changes its position, influencing that of Phe282 of H3. The relevance of a bulky, properly oriented, hydrophobic residue at this site in stabilizing the loop, and indirectly H12, was highlighted in a recent work on RAR, where a mutation at the corresponding position (W225A in RAR) created a constitutive repressor that failed to bind co-activator (29). PPARγ Phe282 occupies a key position to form van der Waals contacts with several residues in the loop 11/12 and in H11 (supplemental Fig. 5).

The final result of this general rearrangement of the LBD leads to a loss of hydrophobic interaction between the ligand and the apolar residues of H12, H11, and loop 11/12, destabilizing helix 12, as shown by the lack of well defined density of its crucial residue Tyr473 (supplemental Fig. 4, A and B). This residue is now oriented in a less productive way, losing the important hydrophobic interaction with Val450 (Figs. 8B and 12). The importance of this site is well highlighted in the work on RAR discussed above (29), where a mutation at this position (A392R in RAR) led to a constitutively active receptor. In that case, the larger Arg side chain, with respect to that of Ala, could form several new interactions with helices 4 and 12, stabilizing the active conformation of H12.

Even in the crystal structure of PPARγ complexed with the weak partial agonist GW0072 (22), which does not interact at all with helix 12, a similar orientation of the Tyr473 aromatic ring, lacking the important contact with Val450, has been observed. Fig. 12 represents a superposition of the receptor backbones adopted in the crystal complexes with our R- and S-enantiomers and with GW0072. There, a progressive reorientation of the Tyr473 side chain can be noted. Its aromatic ring is stabilized by polarization interactions with both Val450 and Leu453 residues in the complex with the almost full agonist R-enantiomer, whereas it faces only the Leu453 residue in the complex with the partial agonist S-enantiomer and the weak partial agonist GW0072. In the complex with GW0072, there is also an evident displacement of helix 11, which further destabilizes H12. The three crystal complexes can represent different states of stability of the helix 12, with the PPARγ GW0072 clearly representing the less stable one. A similar behavior has been observed for the structures of the complexes with the other known PPARγ partial agonists (19–21).

Conclusions—In the present work, we argue that the partial agonist behavior of the S-enantiomer could be ascribed to a destabilization of the active conformation of helix 12. A suboptimal conformation of this helix was observed in one of the two structures of the complex with the same partial agonist, suggesting the coexistence in solution of transcriptionally active and inactive forms and probably explaining the dramatic lack of efficacy in co-activator recruitment and in transactivation activity.

Finally, it will be interesting to test whether the PPARγ partial agonist (S)-1 can recruit a different set of co-activators. This feature, along with the capacity to activate also the PPARα subtype, may aid the identification of Selective PPARγ modulators and will help the design of new agents characterized by improved pharmacological properties and at the same time reduced side effects typical of other known PPARγ agonists.

Acknowledgments—We thank Drs. John Schwabe (Medical Research Council, Cambridge, UK) and Enrique Saez (Genomics Institute of the Novartis Research Foundation, San Diego, CA) for critically reading the manuscript and for helpful discussion. We are grateful to Drs. Iannis Talianidis, Kristir Bamberg for kindly providing the pGal4-PPARγ, and -pHis-PPARα, and p5xGal4UAS vectors and to Elda Desiderio Pinto for administrative assistance.

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