Structural Determinants of the Agonist-independent Association of Human Peroxisome Proliferator-activated Receptors with Coactivators* 

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Lipid homeostasis is controlled by various nuclear receptors (NRs), including the peroxisome proliferator-activated receptors (PPARs, δ, and γ), which sense lipid levels and regulate their metabolism. Here we demonstrate that human PPARs have a high basal activity and show ligand-independent coactivator (CoA) association comparable with the NR constitutive androstane receptor (CAR). Using PPARγ as an example, we found that four different amino acid groups contribute to the ligand-independent stabilization of helix 12 of the PPAR ligand-binding domain. These are: (i) Lys329 and Glu499, mediating a charge clamp-type stabilization of helix 12 via a CoA bridge; (ii) Glu352, Arg425, and Tyr505, directly stabilizing the helix via salt bridges and hydrogen bonds; (iii) Lys347 and Asp503, interacting with each other as well as contacting the CoA; and (iv) His351, Tyr355, His477, and Tyr501, forming a hydrogen bond network. These amino acids are highly conserved within the PPAR subfamily, suggesting that the same mechanism may apply for all three PPARs. Phylogenetic trees of helix 12 amino acid and nucleotide sequences of all crystallized NRs and all human NRs, respectively, indicated a close relationship of PPARs with constitutive androstane receptor and other constitutive active members of the NR superfamily. Taking together, the ligand-independent tight control of the position of the PPAR helix 12 provides an effective alternative for establishing an interaction with CoA proteins. This leads to high basal activity of PPARs and provides an additional view on PPAR signaling.

Dysregulation of lipid levels is characteristic of some of the most prevalent medical disorders, including obesity, cardiovascular disease, and type 2 diabetes. The nuclear receptors (NRs)† peroxisome proliferator-activated receptor (PPAR) α, δ, and γ are prominent players in these diseases because they are important regulators of lipid storage and catabolism (1). NRs form a superfamily of transcription factors (48 human members) and are characterized by their highly conserved DNA-binding domain and structurally conserved ligand-binding domain (LBD) (2). Classical endocrine NRs are the receptors for the agonists estrogen, progesterone, testosterone, cortisol, aldosterone, 1α,25-dihydroxyvitamin D3, 1α,25(OH)2D3, thyroid hormone, and all-trans-retinoic acid. These receptors all show a very selective ligand binding with $K_d$ values in the order of 1 nM or lower (3). Adopted orphan NRs, such as PPARs, bind a variety of structurally diverse compounds with a relatively low affinity (4). Native and oxidized polysaturated fatty acids as well as arachidonic acid derivatives, such as prostaglandins and prostacyclins, selectively bind the PPAR subtypes and stimulate their transcriptional activity (5). PPARγ is the best characterized member of the subfamily because of its prominent role in the regulation of differentiation of cell types with active lipid metabolism, such as adipocytes and macrophage foam cells (6, 7). The importance of this receptor in lipid homeostasis and energy balance is accentuated by the widespread use of synthetic PPARγ ligands, such as the thiazolidinediones rosiglitazone and pioglitazone, as antidiabetic drugs (8).

The LBDs of most NRs is a characteristic three-layer anti-parallel α-helical sandwich formed by 11–13 α-helices. The most C-terminal helix, often called helix 12, serves as a molecular switch by allowing the LBD in its agonistic conformation to interact with coactivator (CoA) proteins, such as steroid receptor coactivator 1 (SRC-1), transcription intermediary factor 2 (TIF2), and receptor-associated coactivator 3 (RAC3) (9). The crystal structure of the agonist-bound conformation of some of these endocrine NRs compared with the apoRXR structure led to the formulation of the "mousetrap" model (10), in which helix 12 should act as a lid to the ligand-binding pocket of the LBD. In general, the conformational flexibility of helix 12 allows a NR to sense the presence of specific ligands, to enhance the selective interaction with CoA and corepressor (CoR) proteins, and ultimately to determine the transcriptional outcome of the NR signaling (11). Interestingly, the apo and holo crystal structures of the LBD of PPARs and other adopted orphan NRs question the mousetrap model because ligand binding to the receptor does not induce any major move of helix 12 (12).
High basal activity and constitutive activity are rather common for adopted orphan NRs. One example with an exceptionally high constitutive activity is the constitutive androstane receptor (CAR) (13). Although the receptor seems to function without a ligand, the imidazothiazole derivative CITCO was shown to be a selective human CAR agonist (14). The stabilization of helix 12 in the active conformation of CAR is mediated by at least four contacts between helix 12 residues and cooperating amino acids in helices 3, 4, and 11 (15, 16). Two of these interactions, the glutamate-lysine charge clamp and the ligand-induced interaction between helices 11 and 12, are rather conserved throughout the NR superfamily, whereas the two other contacts seem to be specific for CAR. Other members of the NR superfamily, such as the retinoid orphan receptors (RORs), also show high basal activity in the absence of ligand (17).

Recent studies using microarrays have enlarged the list of potential PPAR target genes in man and rodents. In general, primary NR target genes are defined through the presence of particular binding sites, referred to as response elements (REs), in their promoter regions (18, 19). PPARs, CAR, VDR, or RXR proteins are generated by coupled in vitro transcription/translation using rabbit reticulocyte lysate as recommended by the supplier (Promega, Madison, WI). Protein batches were quantitated in the presence of 35S-methionine. The specific concentration of the receptor proteins was adjusted to −4 ng/ml after taking the individual number of methionine residues/protein into account. Bacterial overexpression of GST-SRC-1Δ1-97–291, GST-TIF2, GST-RAC3, or GST-RAC5 was obtained from the Escherichia coli BL21DE3/pLysS strain (Stratagene) containing the respective expression plasmids. Overexpression was stimulated with 0.25 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, and the proteins were purified and immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's protocol. Proteins were eluted in the presence of glutathione.

**Materials and Methods**

**Compounds**

The PPAR agonist WY14643 was kindly provided by Dr. P. Honkakoski (University of Kuopio). 1α,25(OH)2D3, the PPAR agonist L763483, and the PPAR agonist rosiglitazone were a gift from Drs. L. Binderup and M. W. Madsen (Leo Pharma, Ballerup, Denmark). The CAR agonist CITCO was obtained from Biomol (Copenhagen, Denmark). 1α,25(OH)2D3 was dissolved in 2-propanol, whereas the other compounds were dissolved in dimethyl sulfoxide (Me2SO); further dilutions were made in Me2SO (for cell culture experiments).

**DNA Constructs**

**Protein Expression Vectors**—Full-length cDNAs for human PPARα (26), human PPARβ (27), human PPARγ (28), human CAR (13), human VDR (29), and human RXRα (30) were subcloned into the T7/SV40 promoter-driven pSG5 expression vector (Stratagene) and that for mouse NCoR (31) into the CMV promoter-driven vector pCMX. The point mutants of PPARγ were generated by using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The helix 12 deletion mutant of PPARγ2 was created by introducing a stop codon at amino acid position 492. All mutations were confirmed by sequencing. The same constructs were used for both T7 RNA polymerase-driven in vitro transcription/translation of the respective cDNAs and for viral promoter-driven overexpression of the respective proteins in mammalian cells.

**GST Fusion Protein Constructs**—The NR interaction domains of mouse SRC-1 (spanning from amino acid 597 to 791) (32), human TIF2 (spanning from 646 to 926) (33), and human RAC3 (spanning from 673 to 1106) (34) were subcloned into the GST fusion vector pGEX (Amersham Biosciences).

**Reporter Gene Constructs**—Four copies of the human CPTI gene DR1-type RE (core sequence GTAGGGAAAAAGGTCGA) (35), four copies of the rat atrial natriuretic factor gene DR3-type RE (core sequence AGAGTGCTAGAGGAGCA) (36), and two copies of the rat Pit-1 enhancer DR4-type RE (core sequence GAAGTTCATAGGAGTCGA) (37) were individually fused with the thymidine kinase (tk) minimal promoter driving the firefly luciferase reporter gene.

**In Vitro Translation and Bacterial Overexpression of Proteins**

**In vitro translated** wild type or mutated PPARγ2, PPARα, PPARβ, CAR, VDR, or RXR proteins were generated by coupled in vitro transcription/translation using rabbit reticulocyte lysate as recommended by the supplier (Promega, Madison, WI). Protein batches were quantitated in the presence of 35S-methionine. The specific concentration of the receptor proteins was adjusted to −4 ng/ml after taking the individual number of methionine residues/protein into account. Bacterial overexpression of GST-SRC-1Δ1-97–291, GST-TIF2, GST-RAC3, or GST-RAC5 was obtained from the Escherichia coli BL21DE3/pLysS strain (Stratagene) containing the respective expression plasmids. Overexpression was stimulated with 0.25 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, and the proteins were purified and immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's protocol. Proteins were eluted in the presence of glutathione.

**Gel Shift and Supershift Assays**

Gel shift assays were performed with equal amounts (−10 ng) of appropriate in vitro translated proteins. The proteins were incubated for 15 min in a total volume of 20 μl of binding buffer (10 mM Hepes, pH 7.9, 150 mM KC1, 1 mM dithiothreitol, 0.2 μg/ml poly[d-dC], and 5% glycerol). For supershift experiments 2–10 μg of bacterially expressed GST fusion proteins (or GST alone as a negative control) were added to the reaction mixture. Approximately 1 ng of 32P-labeled double-stranded oligonucleotides (50,000 cpm) corresponding to one copy of the human CPTI DR1-type RE (see above for core sequence), rat Pit-1 enhancer DR4-type RE (core sequence GAAGTTCATAGGAGTCGA) (37), or rat atrial natriuretic factor gene DR3-type RE (core sequence AGAGTGCTAGAGGAGCA) (36) was then added, and incubation was continued for 15 min at room temperature. Protein-DNA complexes were resolved by electrophoresis through 5% nondenaturing polyacrylamide gels in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and visualized on a FLA3000 reader (Fuji, Tokyo, Japan) using ScienceLab99 software (Fuji).

**Limited Protease Digestion Assay**

**In vitro translated** 35S-labeled PPARγ, CAR, and VDR (20 ng) were incubated with Me30SO, 10 μM rosiglitazone, 10 μM TIF2, or 1 μM 1α,25(OH)2D3 for 15 min at room temperature in a total volume of 10 μl. Trypsin (Promega, final concentration 100 ng/μl) was then added, and the mixtures were further incubated for 30 min at room temperature. The digestion reactions were stopped by adding 1 μl of 1 μg/ml protein gel loading buffer (0.2 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromphenol blue). The full-length and digested proteins were denatured for 3 min at 95 °C, resolved by electrophoresis through 15% SDS-polyacrylamide gels, and visualized on a FLA3000 reader using ScienceLab99 software (Fuji).

**Transient Transfection and Luciferase Reporter Assays**

MCF-7 human breast cancer or HEK293 human embryonic kidney cells were seeded into 6-well plates (200,000 cells/well) and grown overnight in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 5% charcoal-stripped fetal bovine serum. Plasmid DNA containing liposomes were formed by incubating 1 μg of an expression vector for wild type or mutant PPARγ2 and 1 μg of reporter plasmid with 10 μg of DOTAP (Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl of.
phenol red-free Dulbecco’s modified Eagle’s medium, the liposomes were added to the cells. Phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 500 μl of 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time, NR ligands or solvent was also added. The cells were lysed 16 h after the onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics), and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Groningen, The Netherlands). The luciferase activities were normalized with respect to protein concentration.

RESULTS AND DISCUSSION

Active Conformation of the ApoPPAR Structures—We inspected the apo crystal structures of the LBDs of the NRs PPARγ (PDB code 2GWX) (38), PPARγ (1PRG) (12), liver receptor homolog 1 (LRH-1, 1PK5) (39), and estrogen-related receptor γ (ERR3, 1TFC) (40) for the position of their helix 12 (pink) in the crystal structures of human apoPPARδ (2GWX), human apoPPARγ (1PRG), mouse apoLRH-1 (1PK5), and human ERR3 (1TFC) was determined by measuring the distance of the conserved glutamate in helix 12 and its charge clamp partner lysine in helix 3 (A). The constitutive activity of ERR3 is confirmed by co-crystallization of a CoA peptide (orange). The crystal structure of human apoPPARγ (1PRG, green) was superimposed on its holo structure (2PRG, blue) (B). The interaction of the charge clamp residues Glu499 and Lys329 with the CoA peptide (orange) is shown in detail (C).

Fig. 1. Active conformation of the apoPPAR structures. The position of helix 12 (pink) in the crystal structures of human apoPPARδ (2GWX), human apoPPARγ (1PRG), mouse apoLRH-1 (1PK5), and human ERR3 (1TFC) was determined by measuring the distance of the conserved glutamate in helix 12 and its charge clamp partner lysine in helix 3 (A). The constitutive activity of ERR3 is confirmed by co-crystallization of a CoA peptide (orange). The crystal structure of human apoPPARγ (1PRG, green) was superimposed on its holo structure (2PRG, blue) (B). The interaction of the charge clamp residues Glu499 and Lys329 with the CoA peptide (orange) is shown in detail (C).
whose position is tilted by ~5° between the two structures, and the region between helices 2 and 3 (Fig. 1B). This observation is in agreement with the crystallographic temperature factors, which are higher in the lower part of the LBD PPARγ structure and lead to less rigid regions of the LBD (12). A second region of differences is the coil between helices 11 and 12. Interestingly, in the hololBD helix 12 is shifted by 1–1.2 A relative to its position in the apoLBD (Fig. 1B). However, this does not prevent maintenance of the active charge clamp and allows the receptor to remain in the active conformation and to interact with CoA peptide (Fig. 1C). In detail, in the apoLBD Lys329 interacts with the carbonyl group of Thr639 of the CoA, and Glu499 contacts the amido group of the CoA residue Leu633 and the hydroxyl group of Ser630 (green in Fig. 1C). In the hololBD the amino group of Lys329 makes hydrogen bonds with the carbonyl oxides of Thr639 and Leu636 of the CoA and the carbonyl group of Glu499 contacts the amido groups of the CoA residues Leu633 and Leu637 (blue in Fig. 1C).

**CoA Interaction of the Unliganded and Liganded PPARγ**—We have shown previously that the constitutive activity of CAR derives from ligand-independent interactions of this NR with CoAs (15, 46). To investigate whether this was also the case for the PPARs, supershift assays were performed using in vitro translated PPAR-RXR heterodimers, bacterially produced NR interaction domains of the p160 CoA family members SRC-1, TIF2, and RAC3 as GST fusion proteins, and a 32P-labeled double-stranded oligonucleotide carrying the sequence of the human CPT1 DR1-type RE (Fig. 2A). To exclude possible effects of RXR homodimers that have been shown to bind DR1-type REs (21), experiments with RXR alone were done to act as a negative control (Fig. 2A, lanes 1–8). All three PPAR subtypes formed effective heterodimers with RXR on the DR1-type RE in a ligand-independent fashion (compare lanes 9, 17, and 25 with lanes 13, 21, and 29). Of the three tested CoAs, RAC3 interacted most efficiently with all three PPAR subtypes irrespective of the presence of subtype-selective agonists (lanes 12, 16, 20, 24, 28, and 32). However, a direct comparison of the three CoAs is difficult because of the imponderable amounts of active protein in the respective fusion protein fraction. A direct comparison of the three PPAR subtypes with the same CoA is easier because equal amounts of in vitro translated proteins were used. The results showed that the interaction of RAC3 with PPARγ was weaker than with PPARα and γ. For comparison, under the rather stringent conditions chosen here only PPARα bound TIF2 (lanes 11 and 15) and PPARγ complexed weakly with SRC-1 (lanes 18 and 22). Because of efficient interaction of RAC3 with all three PPAR subtypes, we concentrated for the remaining experiments of this study on PPARγ2 and RAC3.

In supershift assays PPARγ2-RXR, CAR-RXR, and VDR-RXR heterodimers were compared in the absence and presence of their agonists rosiglitazone, CITCO, and 1α,25(OH)2D3, respectively, at graded amounts (0–10 μg) of RAC3 (Fig. 2B). In absence of agonist VDR showed no interaction with the CoA (lanes 26–30), but in the presence of 1α,25(OH)2D3 the receptor bound RAC3 already at the lowest RAC3 concentration (lanes 31–36). In contrast, with CAR already low concentrations of RAC3 induced a supershift in an agonist-independent fashion (lanes 14–18 and 20–24). The RAC3 interaction profile of PPARγ2 was shown to be between these two extremes. PPARγ2 was able to interact with the CoA in the absence of agonist (lanes 4–6) at slightly higher RAC3 concentrations than in the presence of rosiglitazone (lanes 9–12). Therefore, an amount of 2 μg of RAC3 seems to be optimal to observe the ligand-independent CoA association of the PPARs, and this amount was chosen for the following supershift experiments (see Figs. 4E, 5E, and 6E).

The limited protease digestion assay, in which interaction of a nuclear receptor with ligand protects the LBD against protease digestion (47), has proven to be a powerful method for characterizing functional NR conformations (48). For this purpose comparable amounts of in vitro translated, 35S-labeled PPARγ2, CAR, and VDR in absence and presence of saturating concentrations of their agonists rosiglitazone, CITCO, and 1α,25(OH)2D3, respectively, were digested for a limited time period with trypsin (Fig. 2C). In the presence of agonist all three NRs were stabilized in their agonist-specific active conformation c1, i.e. a respective fragment of the LBD was resistant to protease digestion. The impact of an additional conformation c2 of the PPARγ and CAR LBD has not yet been investigated in detail, but in the case of VDR the conformation c3 represents a silent, nonagonistic state of the LBD (49). With VDR both conformations were only stabilized in the presence of agonist as reported previously (48, 50), whereas with PPARγ at least a clearly weaker amount as in the presence of specific ligand was found. However, in the case of CAR the addition of ligand showed no significant effect on the stabilization of c1. Taken together, this indicates that the binding of specific ligand is necessary for the stabilization of the LBD of the endocrine NR VDR, not needed for that of CAR, and of limited effect for the LBD of PPARγ.

**Basal Activity of PPARs in Living Cells**—To monitor the basal activity of the three PPAR subtypes in relation to that of CAR (as a positive control for constitutive activity) and VDR (as a negative control for ligand-dependent activation), we transiently transfected MCF-7 human breast cancer and HEK293 cells with expression vectors for the respective human NRs (Fig. 3). Luciferase reporter gene assays showed that in MCF-7 cells the basal activity of PPARα had the same elevated level as that of CAR, whereas the activity of PPARγ and PPARα was found to be even 5 and 28 times higher, respectively (Fig. 3A). In contrast, the overexpression of VDR reduced the basal activity more than 2-fold, which is a known phenomenon and related to the increased attraction of CoR proteins in the absence of an antagonistic ligand (51). In kidney-derived HEK293 cells (Fig. 3C), which represent a more typical PPAR target tissue, the effects were not as drastic as in MCF-7 cells. However, the basal activity of PPARα still exceeded 2–2.5-fold that of PPARγ and PPARα showing a profile comparable with CAR. In this cell line, the overexpression of VDR had a minor supplementary effect. In addition, the level of ligand inducibility the PPAR subtypes resembled more the adopted orphan NR CAR than the endocrine NR VDR (Fig. 3, B and D). Although 1α,25(OH)2D3 induced the activity of VDR 36- and 74-fold, WY14643, L763483, and rosiglitazone reached in maximum only a 3.5-fold induction of their PPAR subtype target, and CITCO could stimulate human CAR not more than 1.8-fold. Interestingly, whereas the overexpression of the CoR NCoR reduced the basal activity by 50–75%, it increased the response to the PPARγ ligand rosiglitazone up to a 6-fold induction.

**Impact of the Charged Residues on the Stabilization of Helix 12 in PPARγ**—To determine the structural basis for the ligand-independent CoA interaction of PPARγ2, we created a series of point mutants of critical amino acids. First, we addressed the CoA-contacting lysine-glutamate charge clamp (46, 52), which is formed between Lys329 in helix 3 and Glu499 in helix 12 (Fig. 4A, see also Fig. 1C). This amino acid pair has a distance of 19.6 Å bridging the LLXXL NR interaction motif of CoS (53) and allowing their docking to the LBD (Fig. 4B). This distance is preserved in the structure of ligand-bound PPARγ (1FM6/D) as well as in apoPPARγ (1PRG_A) and apoPPARγ (2GWX, Fig. 1A). In relation to the crystal structures shown in Fig. 1A, this holo-apo comparison suggests that PPARγ and
probably also the two other subtypes (see Fig. 7), is able to interact with CoAs in the absence of agonist (12).

To monitor the basal and ligand-induced activity of the receptor, we transiently transfected HEK293 cells with expression vectors for wild type and point-mutated PPARγ and a luciferase reporter gene construct containing for copies to the

**FIG. 2.** CoA interaction of the unliganded and liganded PPARγ. Supershift experiments (A and B) were performed with equal amounts of in vitro translated wild type RXRα, PPARα, PPARγ, CAR, or VDR with RXRα protein and one copy of 32P-labeled human CPTI DR1-type RE (for PPARs), rat Pit-1 DR4-type RE (for CAR), or rat atrial natriuretic factor DR3-type RE (for VDR). RXR homodimers were preincubated with 1 μM 9-cis-retinoic acid, PPAR-RXR heterodimers with 10 μM WY14643, 10 μM L73348, or 10 μM rosiglitazone, respectively, CAR-RXR heterodimers with 10 μM CITCO and VDR-RXR heterodimers with 1 μM 1α,25(OH)2D3. Equal amounts (2 μg, A) of bacterially expressed GST (as a control), GST-SRC1597–791, GST-TIF2646–926, and GST-RAC3673–1106 or graded concentrations of GST-RAC3673–1106 (0, 0.5, 2, 5, and 10 μg, B) were then added. Protein-DNA complexes were resolved from the free probe through 8% nondenaturing polyacrylamide gels. Protein-DNA complexes were separated from free probe through 8% nondenaturing polyacrylamide gels. Representative gels are shown. NS indicates nonspecific complexes. Limited protease digestion assays were performed by preincubating in vitro translated 35S-labeled PPARγ, CAR, and VDR with Me2SO (as solvent control) or 10 μM rosiglitazone, 10 μM CITCO, or 1 μM 1α,25(OH)2D3 (C). After digestion with trypsin, the ligand-stabilized NR conformations c1, c2, and c3 and full-length controls were electrophoresed through 15% SDS-polyacrylamide gels. Representative experiments are shown.
expression vectors for the respective NRs. Cells were treated for 16 h with 10 μM L783483 for PPARα, 1 μM rosiglitazone for PPARγ, 10 μM WY14643 for CAR and 1 μM L110110 for VDR, and expression vectors for the respective NRs. Cells were treated for 16 h with 10 μM L783483 for PPARα, 1 μM rosiglitazone for PPARγ, 10 μM WY14643 for CAR and 1 μM L110110 for VDR. The data were normalized to the activity with pSG5 (empty vector) transfected cells (A

26548

for PPAR

C

and HEK293

A

sition of helix 12 in an indirect fashion via a CoA bridge (Fig. 4, A

270

these values. The PPAR

mutants K329A and E499A showed low basal activities (~30% of wild type levels), which were comparable with the remaining activity of a helix 12 deletion mutant (25%, Fig. 4C). This result suggests that p160 CoAs contribute 75% to the basal activity of PPARγ, but the remaining 25% of the activity may be the result of the interaction with other CoAs contacting, e.g. the AF-1 domain in the N terminus of the receptor. Consistent with this, the CoA interaction of the helix 12 deletion mutants, K329A and E499A, was clearly reduced in supershift assays (Fig. 4E, lanes 6, 10, and 14). Please note that the presence of GST-RAC3, but not of GST alone, is reducing the amount of PPARγ-RXR heterodimer formation. The nature of this effect is not known, but it is equally affecting all PPARγ mutants. The agonist inducibility of K329A and E499A was nearly as low as that of the helix 12 deletion mutants (25%, Fig. 4C). This result suggests that p160 CoAs contribute 75% to the basal activity of PPARγ, but the remaining 25% of the activity may be the result of the interaction with other CoAs contacting, e.g. the AF-1 domain in the N terminus of the receptor. Consistent with this, the CoA interaction of the helix 12 deletion mutants, K329A and E499A, was clearly reduced in supershift assays (Fig. 4E, lanes 6, 10, and 14). Please note that the presence of GST-RAC3, but not of GST alone, is reducing the amount of PPARγ-RXR heterodimer formation. The nature of this effect is not known, but it is equally affecting all PPARγ mutants. The agonist inducibility of K329A and E499A was nearly as low as that of the helix 12 deletion (Fig. 4D), and a faint ligand effect was detectable in the supershift assay supporting this finding (Fig. 4E, lanes 12 and 16). Although the Lys

297

Glu

499

amino acid pair stabilizes the position of helix 12 in an indirect fashion via a CoA bridge (Fig. 4, A and B), a detailed inspection of the PPARγ crystal structure (1FM6 D) suggested that a salt bridge-type direct interaction between Lys

347

in helix 4 and Asp

499

in helix 12 may have an impact on the stabilization of helix 12 (Fig. 4A). Interestingly, in reporter gene assays the mutants K347A and D503A showed a low basal activity comparable with K329A and E499A (Fig. 4C). This was confirmed by supershift experiments (Fig. 4E, lanes 18 and 22). However, the ligand inducibility of the K347A mutant was comparable with that of wild type PPARγ, and the agonist response of the D503A mutant was even higher (Fig. 4D). Accordingly, recovery of CoA binding after the addition of agonist was observed in the supershift experiment (Fig. 4E, lanes 20 and 24). This observation suggests that the Lys

347

Asp

499

interaction affects only the ligand-independent positioning of helix 12. In addition to their salt bridge interaction, Lys347 and Asp499 also interact directly with the CoA protein. For example, the formation of a hydrogen bond between Lys

347

of the receptor and His

687

of SRC-1 is possible (12). The counterpart of Asp

499

in human and mouse CAR is the negatively charged C terminus, which is known to interact with Lys

395

and Lys

705

(15, 16, 42, 54). In summary, in addition to the established charge clamp amino acid pair Lys

329

Glu

499

that stabilizes helix 12 indirectly, the Lys

347

Asp

499

salt bridge allows a direct ligand-independent stabilization of helix 12.

Fixation of Helix 12 in PPARγ2 by Residues Involved in the Heterodimerization with RXR—The positively and negatively charged amino acids Glu

352

and Asp

424

in helix 5, Asp

471

in helix 11 form an interaction network (Fig. 5A). In detail, the pairs Asp

344

Arg

471

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and Arg425-Glu352 form salt bridges that support the tertiary structure of PPARγ (Fig. 5B). All four amino acids are strictly conserved in heterodimerizing NRs and define a signature motif (55). Moreover, Arg425 and Glu352 form hydrogen bonds with Tyr505 at the C terminus of helix 12 (Fig. 5B). Therefore, this interaction may also contribute to the stabilization of helix 12.
The mutants E352A, D424A, R425A, R471A, Y505A, and Y505F were created to challenge this hypothesis. All mutants showed basal activities that were significantly decreased by 25–65% compared with wild type PPARγ2 (Fig. 5C). At the same time, the ligand inducibility of E352A was slightly increased, whereas that of D424A, R425A, and R471A was in-
increased by 80–150% (Fig. 5D). In contrast, the mutants Y505A and Y505F showed ligand inducibilities identical to that of wild type PPARγ2. The supershift assays confirmed in their tendency the reduced basal activities of all mutants (Fig. 5E, lanes 6, 10, 14, 18, 22, and 26) and the increased ligand inducibilities of the mutants (D424A, R425A, and R471A; lanes 12, 16, and 20, respectively). However, it has to be noted that the supershifts with these PPARγ mutants are because of their diffuse appearance difficult to quantify and represent only tendencies.

Interestingly, although the Asp424-Arg471 and the Arg425-Phe422 in human VDR (52). However, in the case of VDR, the reduced basal activities of all mutants (Fig. 5F, lanes 13, 18, 22, and 26), decreased ligand inducibility (−45%, Fig. 6D), and lower CoA binding ability compared with PPARγ2 (Fig. 6E, lanes 6, 8, 10, 12, 18, and 20). In contrast, the mutant H477A led to a significantly lower basal activity (Fig. 6C) paired with increased ligand inducibility (Fig. 6D). In addition, the CoA interaction of H477A was reduced but could be restored by ligand binding (Fig. 6E, compare lanes 14 and 16). The contact between His351 and Tyr501 is comparable with that of His397 and Phe291 in human VDR (52). However, in the case of VDR, the 25-hydroxyl group of the agonist 1α,25(OH)2D3 is in contact with His397, which gives this residue a direct role in controlling the ligand inducibility of the receptor (41). In contrast, in the case of PPARγ, the agonist is directly in contact with helix 12 but not His477, which excludes the latter residue from directly influencing the response to a ligand. Taken together, via a hydrogen bond network the amino acids His351 and His477 directly and the residue Tyr355 indirectly contact Tyr501, stabilize helix 12, and thus affect the basal activity of PPARγ2.

In addition, His351, Tyr355, and Tyr501, but not His477, control the ligand inducibility of the receptor.

**Impact on All Three PPAR Subtypes**—The amino acids that are crucial for the stabilization of helix 12 in PPARγ2 can be divided into four groups (Fig. 7A). According to the NR mutation survey (receptors.ucsf.edu/NR/mutation/Rec__page/PPAT_HUMAN.mut.html) none of the mutants highlighted in this study has been studied before in detail. The residues of the first group, Lys329 and Glu499, are on the surface of the LBD and are in direct contact with the CoA. They mediate an indirect stabilization of helix 12 via a CoA bridge. The residues of the second group, Glu352, Arg425, and Tyr505, are involved in a direct stabilization of helix 12 via ionic interactions and hydrogen bonds. The third group includes the amino acids Lys347 and Asp503, which display a mixture of direct and indirect stabilization of helix 12 by both interacting directly with each other and in addition contacting the CoA protein. The amino acids of the fourth group, His351, Tyr355, His477, and Tyr501, form a hydrogen bond network with the ligand and are responsible for both ligand-dependent and -independent stabilization of helix 12. Finally, in the case of rosiglitazone, there is a direct ligand-helix 12 interaction, which is not observed with every PPAR ligand.

A structural alignment of helices 3–12 of the LBD of all three PPAR subtypes (Fig. 7B) using the vector alignment search tool (VAST) algorithm of NCBI demonstrated that critical amino acids Lys329, Lys347, Glu352, Asp424, Arg425, Arg471, His477, Glu499, Tyr501, Asp503, and Tyr505 (red in Fig. 7B) are conserved. Lys329, Lys347, Glu352, Arg425, and His477 contribute directly to the stabilization of helix 12 (Fig. 7A), whereas the effects of the Asp424-Arg471 amino acid pair are only indirect. This suggests that helix 12 of PPARα and PPARδ is stabilized in a very similar way as shown in this study for PPARγ2. In contrast, amino acids His351 and Tyr355, which specifically contact rosiglitazone, are not conserved among the three PPAR subtypes (green in Fig. 7B). Comparison of the crystal structures of the apo form of PPARβ/δ (2GWX, Fig. 7C, left) with its eicosapentaenoic acid-bound form (3GWX, Fig. 7C, right) indicated an interaction network formed by His387, Phe291, His413, and Tyr437, which is very comparable with that formed by the homologous residues His351, Tyr355, His477, and Tyr501 in PPARγ (see Fig. 6B). The difference is Phe291 in PPARγ at the position of Tyr355 in PPARβ/δ. The formation of a hydrogen bond between Phe291 and Tyr437 is impossible, however the two phenyl groups can form a hydrophobic interaction (Fig. 7C). This observation suggests that also in PPARδ helix 12 may be stabilized by mechanisms very similar to those in PPARγ.

A similar structural comparison is not possible for PPARα because this receptor has not yet been crystallized in apo form. However, the PPARα homolog to Tyr355 is Phe318, and a situation similar to that for PPARγ applies.

The structure-function relationship of NRs correlates directly with the dynamics of the LBD, which at the end reflects on the level of the activity of the receptor. Taking into account different functions, such as ligand inducibility and ligand-independent CoA association, PPARs may be placed between VDR and CAR (Fig. 7D).

**Phylogenetic Trees for Helix 12**—Finally, we addressed the functionality and evolution of helix 12 by the calculation of phylogenetic trees (Fig. 8). First, for all NRs for which a crystal structure was available in the Protein Data Bank (www.pdb.org), a structural alignment using VAST was performed. From each receptor the eight amino acids that correspond to Pro341 to Ser348 of human CAR were taken as a profile for the calculation of the distance matrices and construction of a phylogenetic tree using the Vector NTI AlignX module and NJplot.
Interestingly, the tree demonstrates that helix 12 of all three PPAR subtypes is the closest to CAR, suggesting a similar function and a comparable high constitutive activity. According to this phylogenetic tree, the next closest NRs to this cluster are the liver X receptors, farnesoid X receptor, Nur-related factor 1, ERR3, and the RORs. The last three receptors are known for their high constitutive activity, which is comparable with that of CAR (59–61). At the opposite end of the phylogenetic tree, and therefore most dissimilar to PPARs and CAR, are the classical endocrine NRs. These are characterized by a high ligand inducibility and low basal activity, which are qualities opposite to constitutive NRs. This com-
FIG. 7. Structural alignment of the LBDs of the three PPAR subtypes. A, schematic representation of the interaction of helix 12 amino acids with their counterparts in other helices. The α-helical part of helix 12 (Pro495 to Tyr501) is highlighted in purple. Positively charged or polar amino acids are in blue, negatively charged or polar residues are in red, and tyrosines are highlighted in orange. B, structural alignment of the amino acid sequence of helices 3–12 of the LBDs of human PPARα (1I7G_A), human PPARδ (1GWX_A), and human PPARγ (1NYX_A) using the VAST service of NCBI. Conserved amino acids crucial for the stabilization of helix 12 are highlighted in red and not conserved amino acids responsible for the ligand-specific effects of PPARγ are in green. Lowercase letters indicate nonaligned amino acids. Cylinders and arrows above the sequence indicate the position of α-helices and β-sheets, respectively, according to the PPARγ crystal structure (12). A detailed view on the interaction network formed by His287, Phe291, His413, and Tyr437 of PPARδ (C, left, apo receptor (2GWX); right, holo receptor with eicosapentaenoic acid (3GWX)), which is very comparable with the network formed by the homologous residues His351, Tyr355, His377, and Tyr401 in PPARγ (see Fig. 6B). Nitrogen atoms are indicated in blue, oxygen atoms in red, and hydrogen bonds by green dashed lines (distance below 3.4 Å). The position of PPARs within the NR superfamily between VDR and CAR is depicted schematically (D).
parison suggests that helix 12 of constitutive NRs contains conserved amino acids required for high basal activity.

To support this finding further, an alternative phylogenetic tree was constructed on the basis of the nucleotide sequence corresponding to the first eight amino acids of helix 12 of 46 human members of the NR superfamily (Fig. 8B). Rev-ErbA and Rev-ErbB were excluded because they do not have a helix 12 and do not interact with CoA proteins (62). This nucleotide sequence-based tree showed a perspective similar to that of the structure-based tree (Fig. 8A) in that all three PPAR subtypes are the closest neighbors to CAR and the three RORs. Taken together, both phylogenetic trees suggest that helix 12 of the three PPARs, CAR, and the three RORs are structurally and functionally conserved.

Conclusions—In this study we demonstrate that in the absence of high affinity ligands PPARs interact with CoA proteins, stabilize the agonistic LBD conformation c1, so that the addition of agonist provides only a rather weak increase in complex formation and conformation stabilization, and show high basal activity in reporter gene assays. In all these aspects PPARs resemble more CAR than the endocrine NR VDR. It cannot be excluded that the LBDs of the PPARs and of CAR have already captured a ubiquitous endogenous ligand, such as cholesterol or palmitic acid in the case of RORα and hepatocyte

Fig. 8. Phylogenetic trees for helix 12. A structural alignment of all NRs available in the Protein Data Bank was performed using the VAST service of NCBI and a structurally based phylogenetic tree of the first eight amino acids of helix 12 was calculated (A). An evolutionary based phylogenetic tree was calculated on the basis of the nucleotide sequence corresponding to the first eight amino acids of helix 12 of all 48 human NRs (B). ClustalW service was used for sequence alignment and NJplot software allowed displaying the phylogenetic trees.
High Basal Activity of PPARs

nuclear factor-α, respectively (63, 64). However, PPARγ and δ LBDs did not attract such ligands during their purification and crystallization (12, 38), although they may have dissociated during rigorous isolation procedures. However, the overexpression of NCoR leads in MCF-7 and HEK293 cells to a significant reduction of the basal activity, i.e. a massive increase of CoR protein in relation to constant endogenous CoA proteins apparently leads to an exchange of CoAs against CoRs as PPAR-associated proteins and the basal activity is reduced. This process may even happen in the presence of an endogenous fatty acid within the ligand-binding pocket of the PPARs, but it is not largely influenced by it. In contrast, the addition of specific ligand increased the reporter gene activity by 100–250% (at endogenous cofactor levels) and by up to 500% in the presence of massive amounts of NCoR. This indicates that a specific ligand has the potency to overcome repressing settings caused by high CoR amounts, whereas endogenous fatty acid ligands are not able to do so. Finally, the strongest argument may come from the mutant Y501A, in which the direct contact caused by high CoR amounts, whereas endogenous fatty acid specific ligand has the potency to overcome repressing settings is not largely influenced by it. In contrast, the addition of process may even happen in the presence of an endogenous associated proteins and the basal activity is reduced. This 1ently leads to an exchange of CoAs against CoRs as PPAR-protein in relation to constant endogenous CoA proteins appar- tion of NCoR leads in MCF-7 and HEK293 cells to a significant (12, 38), although they may have dissociated crystallization (12, 38), although they may have dissociated

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Structural Determinants of the Agonist-independent Association of Human Peroxisome Proliferator-activated Receptors with Coactivators
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