Combined Simulation and Mutagenesis Analyses Reveal the Involvement of Key Residues for Peroxisome Proliferator-activated Receptor α Helix 12 Dynamic Behavior*

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The dynamic properties of helix 12 in the ligand binding domain of nuclear receptors are a major determinant of AF-2 domain activity. We investigated the molecular and structural basis of helix 12 mobility, as well as the involvement of individual residues with regard to peroxisome proliferator-activated receptor α (PPARα) constitutive and ligand-dependent transcriptional activity. Functional assays of the activity of PPARα helix 12 mutants were combined with free energy molecular dynamics simulations. The agreement between the results from these approaches allows us to make robust claims concerning the mechanisms that govern helix 12 functions. Our data support a model in which PPARα helix 12 transiently adopts a relatively stable active conformation even in the absence of a ligand. This conformation provides the interface for the recruitment of a coactivator and results in constitutive activity. The receptor agonists stabilize this conformation and increase PPARα transcription activation potential. Finally, we disclose important functions of residues in PPARα AF-2, which determine the positioning of helix 12 in the active conformation in the absence of a ligand. Substitution of these residues suppresses PPARα constitutive activity, without changing PPARα ligand-dependent activation potential.

The three peroxisome proliferator-activated receptor isoforms (PPARs)5 α, β/δ, and γ (NR1C1, NR1C2, and NR1C3, respectively (1)) form a distinct subfamily of nuclear hormone receptors (2, 3). They are key regulators of lipid and glucose homeostasis, inflammation, cancer, and tissue repair (4–7). The effect of PPARs on the expression of their target genes results from three events: recognition and binding of the receptor to response sequences in the promoter of the target genes, ligand binding, and co-repressor/co-activator exchange. Two regions exhibit a high degree of similarity in all members of the superfamily, namely the DNA binding domain, which is located toward the N terminus, and the ligand and cofactor binding domain (LBD). Ligand binding by the receptor is generally thought to be the trigger for transcriptional activation, but alternative mechanisms such as modulation of receptor activity by phosphorylation have also been reported (8, 9). Particularly, PPARα shows a high constitutive activity, but the molecular basis and role in vivo of this activity are unclear (10, 11). A wide variety of natural or synthetic compounds, including fatty acids and eicosanoids, was identified as PPAR ligands (12–15). Among the synthetic ligands, the lipid-lowering drugs fibrates and the insulin sensitizers thiazolidinediones are PPARα and PPARγ agonists, respectively; these underscore the important role of PPARs as therapeutic targets. Besides its role in ligand binding and dimerization, the LBD is also the site of the major and ligand-dependent transcriptional activation function of the nuclear receptors, called the activation function 2 (AF-2) domain (16). The PPAR LBD consists of 12 α helices forming the characteristic three-layer anti-parallel α-helical sandwich comprising a small four-stranded sheet, which delimitates a large Y-shaped hydrophobic pocket, the ligand binding cavity (16–25). It is thought that ligands activating PPARs stabilize the LBD in a relatively compact and rigid structure, in which helix 12, the dynamic part of the AF-2, is in a position that promotes binding of co-activator proteins and thus has a critical function in the stimulation of target genes (16–18, 25–27).

In all members of the nuclear hormone receptor superfamily, except Rev-ErbA (28), helix 12 contains a core amino acid sequence motif ΦΦX(D/E)/ΦΦ, with amphipathic α-helix properties, where Φ represents hydrophobic residues and X, in general, denotes residues with long hydrophilic, neutral, or polar chloramphenicol acetyltransferase; GST, glutathione S-transferase; MD, molecular dynamics; wt, wild type; r.m.s.d., root mean square deviation; PPRE, peroxisome proliferator response element.

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5 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; LBD, ligand binding domain; AF-1, -2, activation functions 1 and 2; CAT,
A

| A/B | C | D | E |
|-----|---|---|---|
|     |   |   |   |

AF2 AD

FIGURE 1. Amino acid sequence of the AF-2 domain of PPARs. A, common domain structure of nuclear hormone receptors. A/B, the putative activation function-1 domain (AF-1, ligand-independent); C, DNA binding domain; D, hinge region; E, ligand binding domain (LBD). The position of the activation function-2 domain (AF-2) is indicated with the consensus sequence of the core amino acid motif \( \Phi\Phi X E \Phi\Phi X\Phi\Phi \), where \( \Phi \) represents hydrophobic residues, and \( X \) represents, in general, residues with long side chains of hydrophobic, neutral, or polar nature. B, the 12 C-terminal amino acids of PPARs of each of the three isotypes PPAR\( \alpha \), PPAR\( \beta \), and PPAR\( \gamma \) from X. laevis (x), sea bream (Sparus aurata, so), mouse (m), rat (r), and human (h) are aligned with the identification number of the first and last amino acid shown. The tyrosine (Y) at the right of the alignment represents the C terminus of the proteins. This alignment reveals two overlapping consensus motifs as described in A, which are identified as motif I (gray box) and motif II (white box). There are two overlapping residues between motifs I and II.

side chains. The three PPAR subtypes possess a unique C-terminal sequence that includes two overlapping motifs within helix 12, both conforming to the AF-2 core consensus sequence (Fig. 1). Motif I ends four residues upstream of the C-terminal end of the protein, whereas motif II corresponds to the last six residues of the receptor. Because PPARs are therapeutic targets, it is important to understand the molecular basis of helix 12 constitutive and ligand-dependent dynamics, since this may provide valuable information with regard to drug design. In this report, we have analyzed the role of these C-terminal residues in PPAR\( \alpha \) activity using combined experimental mutant analyses and computational simulations. We have identified important residues for PPAR\( \alpha \), which determine helix 12 regulation in its constitutive and ligand-dependent transcriptional activity. Based on these results, a detailed mechanistic description of the ligand-dependent role of helix 12 is proposed.

EXPERIMENTAL PROCEDURES

**HelA Cell Transfections and Transactivation Assays**

Xenopus laevis PPAR\( \alpha \) mutants were obtained as described previously (29). HeLa cells cultured in 10% delipidated fetal calf serum (Invitrogen) were transfected with pSG5 expression vectors containing full-length wild-type or mutant PPAR\( \alpha \), and either CAT (ACO-A pBL-CAT8+ (30)) or Luciferase (3xAACOPPRE-TK-Luc) reporter plasmids, as indicated in the figure legends. PPARs agonist Wy-14,643 (Chem Syn Laboratories) was added 6 and 24 h after transfection. 48 h after transfection (Lipopectamine 2000, Invitrogen), cell extracts were prepared by freeze-thawing and were assayed for CAT or luciferase activity (Promega, Madison, WI).

**Pull-down Assays**

The GST-p300\(_{2-516}\) (31) fusion protein was expressed in Escherichia coli and purified on a glutathione affinity matrix (Amersham Biosciences). Full-length PPARs were produced with reticulocyte lysates (TnT T7 quick translation/translation system) and labeled with \( ^{35}\)S]methionine. The GST-p300 fusion protein or the GST protein alone (3 \( \mu \)g each) were then incubated with 15 \( \mu \)L of programmed reticulocyte lysate in 500 \( \mu \)L of binding buffer (Tris-HCl, pH 7.4 25 mm, EDTA 1 mm, NaCl 100 mm, Triton X-100 0.1%, phenylmethylsulfonyl fluoride 0.2 mm, protease inhibitor mixture (Roche Applied Science)) supplemented with 0.5% dry milk, during 4 h at 4 °C, in the presence or absence of the PPAR\( \alpha \) ligand Wy-14,643 at 100 \( \mu \)M. Beads were washed three times with binding buffer, and samples were boiled with 40 \( \mu \)L of 2X SDS-PAGE buffer (12.5 mm Tris-HCl, 20% glycerol, 0.002% Bromphenol Blue, 5% \( \beta \)-mercaptoethanol), separated on a 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and exposed to a phosphorimager (PhosphorImager, Storm 840, Amersham Biosciences).

**Computational Methods**

**Deriving the Unliganded PPAR\( \alpha \) Model**—The homology model of the xPPAR\( \alpha \) ligand binding domain (SwissProt accession number P37232) was built using MODELLER v6.2 (32) based on the crystal structure of the human PPAR\( \alpha \) (Protein Data Bank (PDB) (33, 34) code 1K7L (21)). In this structure, helix 12 is in the closed conformation. ClustalW (35) was used to perform a pairwise sequence alignment of the X. laevis and human sequences. The sequence identity is 90%. Default parameters of the homology modeling routine were used. The energy of the model was then minimized using the CHARMM program (36) and the CHARMM19 force field (37, 38), with a dielectric constant of 1 and a 20 Å cutoff. The minimization consisted of 30 steps of steepest descent followed by 30 steps of adopted basis Newton-Raphson. The positions of the Ca atoms were constrained using a mass-weighted harmonic force constant of 10 kcal/(mol Å²).

**Docking of Wy-14,643**—Missing parameters for the ligand, for use in conjunction with the CHARMM22 (39) force field, were derived from the Merck Molecular Force Field (MMFF (40–44)). Based on the PPAR\( \alpha \) model mentioned above, a model of the PPAR\( \alpha \)/Wy-14,643 complex was built using the
EADock evolutionary algorithm, taking account of the solvent effect. Details of the calculations are presented separately (45). Residues of the binding site were flexible during the docking to account for the inherent inaccuracy of coordinates in the PPARα model, and for the induced fit of the protein in the presence of the ligand. These include residues 247, 253, 257, 278–279, 281–283, 285–286, 320, 323–324, 327, 336, 338, 345, 360–361, 446, 450, and 470. The final conformations with the lowest energy were further minimized by 100 steps of steepest descent using the GB-MV2 (46, 47) generalized Born model. The lowest energy conformation was used for the following molecular dynamics (MD) simulations.

MD Simulations—All simulations were performed using the CHARMM program (version c31b1) and the CHARMM22 force field. The starting structure of the R471L/D472N PPARα mutant was obtained from the structure of the wild-type protein by replacing the two native side chains by the mutated ones. MD simulations were performed on four systems, corresponding to the wild-type or mutated PPARα, in the presence (liganded) or in absence (unliganded) of the Wy-14,643 ligand. Each isolated system (no co-activator) was minimized using 250 steps of steepest descent minimization using the GBSW implicit solvent (48) to remove sterical clashes in the structural model. The protein (or complex) was solvated in a cubic box of 80.7 Å³ of TIP3P (49) water molecules that were previously equilibrated at 300 K and 1 atm of pressure. The solvent was equilibrated at 300 K during 20 ps in the presence of the fixed protein. The entire system was then equilibrated at 300 K during 150 ps, in the isothermal isobaric ensemble (NPT) to adjust the solvent density at 1 atm. Finally, the production MD simulation was conducted during 1 ns in the canonical (isovolume isothermal, NVT) ensemble. The MD simulations were performed with periodic boundary conditions. The Verlet leapfrog integrator was used for time propagation with a time step of 0.001 ps. A 12-Å cutoff was applied.

Calculation of Side-chain Contributions to the Conformational Stability—To estimate the role of the residue side chains on the protein stability, we used the approach developed by V. Zoete and M. Meuwly (52). The method is based on the notion that the binding free energy (∆G_{stat}) corresponding to the alchemical complexation of a given side chain (considered as a “pseudo-ligand”) into the rest of the protein (considered as a “pseudo-receptor”) reflects the importance of this side chain to the thermodynamic stability of the protein (52).

The binding free energy was estimated according to the MMGBSA approach,

$$\Delta G_{\text{bind}} = \langle E_{\text{vdw}} \rangle + \langle E_{\text{elec}} \rangle + \langle \Delta E_{\text{intra}} \rangle + \langle \Delta G_{\text{elec,desolv}} \rangle$$

$$\quad + \langle \Delta G_{\text{np,desolv}} \rangle - T(\Delta S) \quad \text{(Eq. 1)}$$

where the brackets, ⟨ and ⟩, indicate an average of these energy terms over 250 frames regularly extracted from the 1-ns MD simulation trajectory of the complex described above (one every 4 ps). Terms relative to a given isolated partner were also calculated using frames extracted from the MD simulation of the complex, after removing the other partner. This single trajectory method was found to lead to important cancellation of errors (53). E_{vdw} and E_{elec} are the van der Waals and electrostatic pseudo-ligand:pseudo-receptor interaction energies, respectively. ∆E_{intra} is the variation of the internal energy of both partners upon complexation. Because we use the single trajectory method, we have in fact ∆E_{intra} = 0. ∆G_{elec,desolv} and ∆G_{np,desolv} are the electrostatic and non-polar desolvation energies, respectively. ∆G_{elec,desolv} is calculated according to the GB-MV2 generalized Born model. ∆G_{np,desolv} is assumed to be proportional to the solvent-accessible surface area that is buried upon complexation (54, 55); i.e. ∆G_{np,desolv} = σ × ΔSASA, with σ = 0.0072 kcal/(mol Å²) (56–58). We neglected the entropy term −TΔS.

The binding free energy has been calculated for all side chains, except for proline residues. Actually, the role of a proline side chain on the protein stability, which may arise from the covalent bridge between the Ca and N atoms, is not expected to be assessed by the present method, which only takes account of the non-bonded interactions between the side chain and its environment.

RESULTS

Integrity of Motifs I and II of PPARα Helix 12 Is Required for the Full Transcriptional Activity of the Receptor—Using X. laevis PPARα (PPARα) as a model (30), we studied whether motifs I and II of helix 12 are both necessary for the function of the PPARs. Mutants were generated in which 1, 2, 4, or 12 C-terminal residues were deleted (Δ1, Δ2, Δ4, and Δ12, respectively). Of these, Δ1, Δ2, and Δ4 affected motif II residues, whereas Δ12 affected the entire helix 12. The mutants were tested for their ability to activate a PPRE-driven reporter construct using transient transfections in HeLa cells in the presence or absence of Wy-14,643, a selective PPARα ligand (59) (Fig. 2A). The mutations did not affect the ectopic expression level of these proteins relative to wt PPARα (data not shown). As expected, deletion of both motif I and II (Δ12) resulted in total loss of transcriptional activity (Fig. 2A). Interestingly, total loss of activity was also observed with mutant Δ4, which lacks motif II, suggesting that motif I on its own is unable to sustain transactivation. The integrity of motif II as a prerequisite for full transactivation was confirmed with mutants Δ1 and Δ2, which exhibited decreased transcriptional activity. These first experiments also confirmed that PPARα has a relatively high constitutive activity in the absence of any exogenous ligand (10, 59, 60). A PPARα deletion mutant lacking 84 N-terminal residues exhibited similar constitutive activity to the wt PPARα protein, suggesting that the contribution of AF-1 in this case is minor (data not shown).

The requirement of an intact α-helical structure for the AF-2 function was further demonstrated by the site-directed substitution of either Arg-471 or Asp-472 with a Pro, a residue that would destabilize the PPARα helix 12 secondary structure (mutants R471P and D472P, respectively, Fig. 2B). Constitutive receptor activity was reduced in the R471P mutant and was totally absent in the D472P mutant, whereas neither mutant exhibited ligand-induced activity (Fig. 2B).

To further investigate which residues are responsible for the transactivation properties of the receptor, point mutations on helix 12 residues were generated. The acidic residue centrally located within the AF-2 core motif of estrogen receptor, thyroid hormone receptor, and retinoic acid receptor, was shown to be

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FIGURE 2. Transcriptional activity of deletion and point mutants in the helix 12 motifs I and II of PPARα. Mutations introduced in the C-terminal wt PPARα sequence are indicated on each panel. The truncated forms are indicated as “Δx” with the number of amino acids removed (A). The substitutions introduced in this sequence are indicated with arrows (B–D). A, HeLa cells were transfected with the CAT reporter plasmid and expression vectors for the indicated wild-type and truncated PPARα or the empty pSG5 expression vector as a control. The cells were treated with 100 μM Wy-14,643. Each bar represents the average of at least three experiments. Relative CAT activity of 100 corresponds to the normalized CAT value obtained with wt PPARα activated with the ligand. B–D, HeLa cells were transfected with the CAT (B) or luciferase (C and D) reporter plasmid, expression vectors for the indicated wild-type or point mutant PPARα, or the empty pSG5 expression vector as a control. The cells were treated with 100 μM Wy-14,643. Each bar represents the average of at least three experiments. Relative CAT or luciferase activity of 100 corresponds to the normalized value obtained with wt PPARα activated with the ligand. *, significant p value (Student’s t test) between activated wt and mutant PPARα; **, significant p value (Student’s t test) between constitutive activity of wt and R471L/D472N PPARα.

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essential for the ligand-dependent activation function of these receptors (61–66). Therefore, we first targeted Glu at position 468 (motif I) and Asp-472 (motif II), which were replaced by Gln and Asn, respectively (E468Q and D472N). The E468Q mutant was totally inactive in the transactivation assay, in the presence or absence of ligand (Fig. 2B). The D472N mutation had a less dramatic effect, because the receptor retained 35% of its constitutive and 60% of the induced activity of the wt PPARα. One feature distinguishing motif II from motif I in all known PPARs is the presence of a positively charged residue, Arg or Lys, preceding the centrally located Asp residue (Fig. 1). Replacement of Arg-471 with Leu (R471L) resulted in a disproportionately decrease of the constitutive relative to the induced activity (Fig. 2C). The simultaneous substitution of residues Arg-471 and Asp-472 to Leu and Asn, respectively (R471L/D472N), resulted in further decrease in the ligand-dependent but in no cumulative decrease in the constitutive activity, compared with the single mutant D472N (Fig. 2C).

Tyr-470, a potential target of phosphorylation by various kinases, with Phe (Y470F, Fig. 2B). This mutation resulted in a receptor retaining 80% of wt PPARα activity, indicating that phosphorylation of this residue, if it takes place, is not crucial for PPARα activity. Together, the above results suggest that the PPARα AF-2 function depends on the integrity of both motifs I and II and confirm the crucial role of the acidic residues Glu-468 (motif I) and Asp-472 (motif II).

Point mutants were then designed according to the data obtained after free energy simulations of the wt PPARα LBD. As presented in detail later in this report, an in silico approach was used to determine the contribution of each residue in the LBD to the protein structural stability. It showed that in helix 12, Leu-466, Met-473, and Tyr-474 are associated with large negative ΔG value and that they most probably strongly stabilize the active conformation of the helix (Table 1). Each of these residues was mutated into the neutral residue Ala (L466A, M473A, and Y474A). A double mutation of the C-terminal residues Met-473 and Tyr-474 was also generated (M473A/Y474A), and the activity of these mutants was tested in the transactivation assay (Fig. 2D). Mutation of Leu-466 almost totally suppressed the activity of the receptor. M473A and Y474A retained 30 and 80% of wt activity, respectively. Interestingly, M473A/Y474A lost constitutive activity but retained 30% of the ligand-inducible activity, like the Δ2 deletion mutant. Transactivation assays thus confirmed the in silico prediction according to which Leu-466, Met-473, and Tyr-474 are important residues for the activity of the receptor. Taken together, all our mutagenesis results argue for a role of both motif I and II residues in the stabilization of helix 12 in a position that is optimal to provide an interface for efficient interactions with co-activators.

Molecular Characterization of the PPARα Mutants—Except for the Δ12 mutant, all mutants retained almost intact their capacity to heterodimerize with retinoid X receptor and to bind to PPRE (data not shown). Thus, binding to retinoid X receptor and to DNA cannot account for the differences in transcriptional activity observed in transactivation assays. Therefore, their effect on the PPARα-dependent transcription must reflect a distorted interface that does not allow efficient protein-protein interactions with co-activators. To test this hypothesis, the ability of wt, Δ1, Δ2, Δ4, Δ12, and R471L/D472N PPARα to
recruit the coactivator p300 was assessed by pull-down assays. The deletion mutants showed progressive loss of transcriptional activity in parallel with progressive deletion of motifs I and II (Fig. 2A). The profile of p300 recruitment by these mutants is consistent with their respective activities in transactivation assays (see Fig. 2A for transactivation assays and Fig. 3A for p300 recruitment). Indeed, significant or total loss of transcriptional activity correlates with a loss of p300 recruitment upon ligand binding for Δ2, Δ4, and Δ12. wt and R471L/D472N receptors showed high ligand-inducible transcriptional activity, whereas only wt PPARα showed significant constitutive activity (Fig. 2C). Analyzing the p300 recruitment by these two receptors provided indirect information about the placement of helix 12 with regard to constitutive versus ligand-dependent activity. The wt PPARα was able to significantly recruit p300 in the absence of a ligand, and the recruitment of the co-activator was further enhanced upon ligand binding (Fig. 3B). In contrast, the double mutant did not bind to p300 in the absence of ligand, but its ability to recruit p300 was intact in the presence of the ligand. Thus, in both cases, the ability to recruit p300 parallels the level of constitutive and ligand-dependent transcriptional activity. These data indirectly suggest that helix 12 is able to adopt an active conformation in the unliganded wt PPARα, but not in the unliganded R471L/D472N PPARα, and that in both wt and mutant receptors, the ligand induces the active position of helix 12. We addressed this hypothesis using MD modeling approaches, to study the stability of the active conformation of helix 12 from a structural, thermodynamic, and dynamic point of view.

Molecular Dynamics Simulation of Helix 12 in the Liganded wt PPARα—Because the experimental structure of PPARα associated with Wy-14,643 was not available, we first obtained the model structure of the complex, which showed the binding mode (position, orientation, and conformation) of the ligand in the binding pocket of the PPARα LBD (EADock program (45)). Overall, the hydrogen bond pattern between wt PPARα and Wy-14,643 was similar to that observed for the human PPARα and its ligands (21, 67) and after theoretical docking of ligands in the PPARγ ligand binding pocket (68, 69) (Fig. 4). The MD simulation was then performed to analyze the dynamic behavior of PPARα LBD, and more particularly the local movements of helix 12 during 1 ns, starting from the active conformation. As shown in Fig. 5A, this starting position of the helix 12 was very stable during the simulation of the wt PPARα LBD associated with Wy-14,643. Indeed, the calculated root mean square deviation (r.m.s.d., backbone 1.5 Å, all heavy atoms 2.0 Å) showed that helix 12 stayed close to its starting position. The positioning of the ligand was also very stable (r.m.s.d., heavy atoms 0.7 Å), and the hydrogen bond network mentioned above between the protein and its agonist remained intact during the simulation. In addition, a transient hydrogen bond formed between the aniline function of the ligand and the backbone carbonyl O atom of Cys-282 (helix 3) during 14% of the MD simulation, whereas a transient hydrogen bond between Arg-471 (helix 12) and Asp-472 (helix 12) existed during only 4% of the frames. In addition to the canonical α-helix hydrogen bonds between backbone atoms of helix 12, we also found strong hydrogen bonds between the backbone atoms of Ile-469 (helix

| Table 1 - Contributions of the LBD residue side chains to the stability of the fold calculated from the MD simulation of the wt PPARα-Wy-14,643 complex |
|-----------------|----------------|----------------|----------------|----------------|
| Helix 12 residues | (F_pvdw) | (F_elec) | (ΔGstab) | (ΔGstab) |
| Leu-465 | −5.87 | 2.80 | 3.26 | −1.85 | −1.65 |
| Leu-666 | −11.98 | 3.17 | 3.31 | −1.94 | −7.44 |
| Gln-467 | −7.77 | −0.42 | 11.29 | −1.88 | −2.22 |
| Gla-468 | −6.65 | −2.99 | 90.44 | −1.69 | 2.11 |
| Ile-469 | −9.26 | 3.89 | 2.13 | −1.97 | −5.31 |
| Tyr-470 | −15.07 | −12.79 | 15.19 | −1.97 | −2.67 |
| Arg-471 | −8.17 | −91.33 | 98.04 | −1.83 | 0.70 |
| Asp-472 | −1.56 | −45.75 | 51.44 | −1.26 | 2.87 |
| Met-473 | −14.75 | 3.13 | 3.51 | −2.10 | −10.20 |
| Tyr-474 | −11.81 | −16.35 | 12.99 | −2.59 | −17.75 |

Most important residues, not in helix 12

| residue | (F_pvdw) | (F_elec) | (ΔGstab) |
| Met-360 | −15.10 | 3.08 | 2.04 | −2.06 | −12.03 |
| Phe-324 | −16.26 | 2.23 | 3.67 | −2.42 | −12.78 |
| Phe-349 | −15.25 | 2.87 | 4.15 | −2.49 | −13.43 |
| Phe-279 | −15.26 | −0.06 | 4.10 | −2.43 | −13.64 |
| Phe-303 | −17.22 | 2.33 | 3.40 | −2.37 | −13.86 |
| Met-336 | −14.37 | 3.18 | −0.73 | −2.14 | −14.06 |
| Thr-252 | −3.13 | −2.17 | 12.76 | −1.63 | −14.16 |
| His-422 | −7.12 | −126.81 | 121.79 | −2.04 | −14.19 |
| Arg-440 | −8.69 | −131.95 | 128.36 | −2.43 | −14.71 |
| Ser-286 | −3.03 | −17.92 | 7.46 | −2.35 | −14.77 |
| Phe-365 | −18.24 | 0.71 | 4.72 | −2.35 | −15.15 |
| Phe-384 | −16.92 | 2.09 | 2.00 | −2.35 | −15.18 |
| Phe-296 | −17.58 | 0.98 | 3.51 | −2.39 | −15.56 |
| Tyr-221 | −14.89 | −11.75 | 12.20 | −2.62 | −17.06 |
| Arg-277 | −12.19 | −85.81 | 82.42 | −2.66 | −18.24 |
| Tyr-320 | −15.06 | −14.20 | 13.21 | −2.44 | −18.49 |
| Arg-347 | −9.26 | −131.24 | 121.98 | −2.58 | −21.10 |
| His-447 | −8.01 | −101.15 | 88.17 | −1.91 | −22.90 |
| Arg-394 | −16.25 | −145.62 | 140.00 | −2.46 | −24.25 |
| Glu-321 | −3.14 | −148.12 | 126.03 | −1.82 | −27.05 |
| Asp-380 | −5.80 | −139.99 | 114.85 | −1.55 | −32.49 |
12), Arg-471 (helix 12), and Asp-472 (helix 12) on the one hand and the Lys-316 (helix 4) side chain on the other hand, and a weak hydrogen bond between the Met-473 (helix 12) backbone carbonyl CO and the Tyr-317 (helix 4) side chain.

In summary, helix 12 was stable in the active conformation in the MD simulation of the liganded wt PPAR. Thus, the PPAR LBD behaved as expected from experimental data, which is an excellent validation of the proposed model.

Theoretical Determination of the Contribution of Helix 12 Residues to the Stability of the Protein Structure—As briefly mentioned above, the MD simulation of the liganded wt PPAR enabled us to identify the residues that make a significant and favorable contribution to the structural stability of the LBD. The calculated contribution of the side chains of the LBD residues is given in Table 1. In helix 12, four residues were of particular interest: Leu-466, Tyr-470, Met-473, and Tyr-474. The contribution of the latter was due in part to the network of hydrogen bonds that it formed with Glu-321 (helix 4/helix 5 junction), Arg-394 (loop between helix 8 and helix 9), and Arg-440 (helix 11), and to the favorable van der Waals interactions with residues Tyr-317 (helix 4), the aliphatic part of Arg-440 (helix 11), Met-473 (helix 12), and Thr-444 (helix 11). Met-473 made favorable hydrophobic contacts with the aliphatic part of Lys-316 (helix 4), as well as with the Tyr-317 (helix 4), Tyr-318 (helix 4), Ala-447 (helix 11), Ile-469 (helix 12), Tyr-470 (helix 12), and Tyr-474 (helix 12) side chains. Tyr-470 made a hydrogen bond with the carboxylate function of the ligand, and van der Waals contacts with Tyr-320 (helix 4/helix 5 junction), Val-443 (helix 11), Ala-447 (helix 11), Val-450 (helix 11), Leu-462 (loop between helix 11 and helix 12), Leu-465 (helix 12), and Tyr-470. Finally, Leu-466 made favorable hydrophobic contacts with the aliphatic part of Gln-283 (helix 3) and Ser-286 (helix 3), as well as with Tyr-320 (helix 4/helix 5 junction), Leu-462 (loop between helix 11 and helix 12), Leu-465 (helix 12), and Tyr-470. Among the residues that contribute most favorably to the stability of the protein, we also found the residues involved in the hydrogen bond network made by the residue pairs Glu-321 (helix 4/helix 5 junction) and Arg-394 (helix 4), Met-326, Val-338, and Val-340 of the β sheet between helices 5 and 6; Met-361 of helix 7; and Val-450 of helix 11 (data not shown). The figure was produced using the VMD program. (92). The hydrogen bonds are in green dotted lines.

FIGURE 3. Recruitment of the coactivator p300 by the wt, truncated, and R471L/D472N PPARα. wt, truncated (A), and R471L/D472N (B) mutant PPARα were incubated with purified GST-p3002–516 fusion protein, in the presence of 100 μM Wy-14,643 or vehicle only. A shows one representative experiment with the truncated PPARα mutants, with the percentage of material pulled down compared with the respective input (top panel) and the picture of the corresponding gel (bottom panel). The truncated forms are indicated by “Δ” with the number of amino acids removed. B shows the percentage of material pulled down compared with the respective input calculated as a mean of three independent experiments. The value for wt PPARα in the presence of agonist was set to 100. GST protein alone was used as control.

FIGURE 4. Three-dimensional representation of hydrogen bonds between PPARα and Wy-14,643. The polar carboxylate function of Wy-14,643 makes hydrogen bonds with the side chains of Ser-286 (helix 3), Tyr-320 (helix 4/helix 5 junction), His-446 (helix 11), and Tyr-470 of helix 12. An additional hydrogen bond takes place between the NH function of Wy-14,643 and the side-chain OH function of Ser-286. The hydrophobic tail of Wy-14,643 extends into the hydrophobic pocket of the LBD, where it makes van der Waals contacts with Phe-279, Cys-282, Thr-285, and Thr-289 of helix 3; Met-323, Phe-324, and Leu-325 of helix 5; Met-336, Val-338, and Val-340 of the β sheet between helices 5 and 6; Met-361 of helix 7; and Val-450 of helix 11 (data not shown). The figure was produced using the VMD program (92). The hydrogen bonds are in green dotted lines.
In summary, the theoretical determination of the contribution of helix 12 residues to the stability of the LBD structure suggested that Leu-466, Tyr-470, Met-473, and Tyr-474 strongly stabilize the active conformation of PPARα/H9251 LBD by making contacts with most helices in the LBD. Consistently, mutation of these residues significantly impaired the transcriptional activity of PPARα (Fig. 2).

Loss of Constitutive Activity of the R471L/D472N Mutant Correlates with Changes in the Dynamic Behavior of Helix 12—We then compared the dynamic behavior of helix 12, in wt and R471L/D472N PPARα, in the absence (unliganded) or the presence (liganded) of the ligand Wy-14,643, during 1-ns MD simulations (Fig. 5). The helix 12 of unliganded and liganded wt PPARα LBD, and of liganded R471L/D472N LBD, showed similar behavior (Fig. 5, A–C). In all three cases, helix 12 was stable in the active conformation during the 1-ns period of time. In contrast, the active position of helix 12 was unstable during the MD simulation of the unliganded R471L/D472N PPARα (Figs. 5D and 7). The shift in the position of helix 12 took place at the beginning of the simulation, the new position remaining globally unchanged during the rest of the trajectory (Fig. 5D). In this new position, the hydrogen bonds between the side chain of Lys-316 (helix 4) and the backbone of helix 12 (residues 469, 471, and 472) were lost, which contributed to helix 12 instability (Fig. 7). This suggests that the active starting position is not thermodynamically favorable in the unliganded R471L/D472N PPARα, which is in agreement with experimental data (72) and with the loss of constitutive activity and p300 recruitment of the unliganded R471L/D472N shown in the present study.

In conclusion, the stability of the active conformation of helix 12 during the simulation is consistent with high constitutive and ligand-dependent activity of wt PPARα. It also correlates well with high ligand-induced activity of R471L/D472N. Finally, the unstable active position of helix 12 reflected the absence of constitutive p300 binding and transcriptional activity of this unliganded mutant.

Correct Positioning of Residues Is Important for the Co-activator Recruitment Interface in the R471L/D472N Mutant—It is important to note that, because our approach was based on the MD simulations done for the PPARα LBD in the absence of a co-activator, the results presented here provide information about the role of the side chains of the residues in the LBD itself in the absence of any possible stabilizing effect of co-activators. Therefore, they are not expected to provide information about mutations that would affect interactions with the co-activators. However, interesting features of the wt and R471L/D472N
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PPARα LBDs were noticed with regard to the position of the two charge clamp residues Glu-468 (helix 12) and Lys-298 (helix 3) (Fig. 8). With a proposed optimal distance of 18–20 Å between the corresponding residues in the PPARγ receptor, these residues form a charge clamp that places and orients the co-activators in a binding site cleft (25, 70). Based on available structural data, we calculated a distance of 19.72 and 20.71 Å between the two corresponding residues in the liganded human PPARα in the presence (1K7L in the Protein Data Bank (PDB)) (33, 34)) or absence (1I7G in the PDB) of a co-activator, respectively. In the present study, we found distances of 19.72, 20.49, and 22.66 Å between these two residues in the averaged structures over the MD simulations for the liganded and unliganded wt, and for liganded and unliganded R471L/D472N receptors, respectively. These observations suggest that the positioning of Glu-468 and Lys-298, which provides the interface for co-activator recruitment, was intact over the 1-ns simulation in the unliganded and liganded wt and the liganded R471L/D472N PPARα, whereas it was distorted in the unliganded R471L/D472N PPARα. These observations are in agreement with the p300-recruiting ability of these two receptors as described above.

**DISCUSSION**

The activity of PPARs is generally thought to be triggered mainly by ligand binding and recruitment of co-activators. However, mechanisms such as modulation of receptor activity by phosphorylation have also been reported (8, 9). AF-1 domain and interaction with retinoid X receptor also participate in PPAR activity (10, 11, 31, 73). However, with regard to drug design, understanding the dynamic behavior of helix 12 represents a cornerstone in our knowledge of the mechanisms behind PPAR functions in gene regulation (74–77). Moreover, regulation of helix 12 activity per se is a very interesting example of a subtle molecular switch that determines interactions with partners in a ligand-dependent manner. Therefore, our study was designed to address the molecular basis of the AF-2 contribution to PPARα constitutive and ligand-dependent activity, independently of AF-1 or retinoid X receptor interactions.
To reveal the key principle behind helix 12 regulation, we combined the functional study of PPARα mutant transcriptional activity and MD simulations. These two approaches used very different observation time windows, the former analyzing the steady-state receptor activity, and the latter local changes at the nanosecond scale. The findings of the MD simulations have triggered functional validation experiments, and, reciprocally, the nanosecond scale. The findings of the MD simulations have definitely important for the protein stability among the numerous residues that make interactions in the core of the protein. In addition, it determines the nature of the role played by these residues in terms of interaction energies. The experimental mutagenesis results were in remarkable agreement with the simulations, the Ala mutants on these four residues showing a notable decrease in constitutive and ligand-induced capacity for transcriptional activation. The deletion of Tyr-474 (Δ1), or of both Tyr-474 and Met-473 (Δ2), also decreased the constitutive and ligand-dependent activity by ∼50 and 70%, respectively, whereas the mutation of Met-473 or Tyr-474 to Ala decreased the ligand-induced activity by 70 and 20%, respectively. The L466A mutation had a dramatic effect, because it totally suppressed the activity of the receptor, a result that is in excellent agreement with the molecular modeling findings. The Y470F mutation only decreased the activity of the protein by 20%, showing that the hydrogen bond between this residue...
show a noticeable theoretical contribution to the stability of helix 12 active conformation. However, this does not rule out the possibility that such residues might have a role in helix 12 regulation through an indirect mechanism. Indeed, another mutation that had dramatic functional effects targeted Glu 468, which is highly conserved in nuclear receptor AF-2 domains (>85%) and is known to be necessary for transactivation in receptors other than PPARs. From structural studies in PPARγ and other nuclear hormone receptor, it is known that this Glu, together with a Lys in helix 3 of the LBD forms the charge clamp that orients and places co-activators in the binding cleft formed by helices 3, 4, 5, and 12 (25, 78). Therefore, a modification at that position is expected to alter transcriptional activation. Mutation of this Glu and one nearby Leu both to Ala significantly reduced PPARγ transcriptional activity, due to impaired recruitment of co-activators, and silenced basal transcription via co-repressor recruitment (79). Our E468Q PPARα mutant indeed suppressed transactivation, suggesting that this residue was likely to have a charge clamp function also in PPARα. Consistently, the model showed that the distance between the Cα atoms of Lys-298 and Glu-468 was in the range of a functional charge clamp (25, 70) in the unliganded and liganded wt PPARα.

Role of Helix 12 Dynamic Behavior and Charge Clamp Residues in the Constitutive and Ligand-inducible Activity of PPARα—Additional experimental data showed that residues Arg-471 and Asp-472 also play a role in helix 12 function. In the human PPARγ, the residue corresponding to Asp-472 probably interacts with a Lys in helix 4 of the LBD, as well as with co-activators (70). Consistent with a similar role of Asp-472 in PPARα, the substitution of this residue with Asn reduced the constitutive activity by ~65% (D472N). The R471L/D472N double mutation did not further suppress the constitutive p300 binding and transactivation, whereas the ligand-induced p300 recruitment and transcriptional activation were further decreased compared with the two single mutants. To explore in more detail the concerted function of residues Arg-471 and Asp-472 in helix 12 dynamics, we performed 1-ns MD simulations of the wild-type and R471L/D472N PPARα with or without a ligand. These MD simulations illustrate very short term and local movements of helix 12 in the LBD, whereas quantification of co-activator recruitment and transcriptional activity reflect the final outcome of structural changes in the whole protein. Despite these major differences in the approaches and observation time windows, comparison of the four simulations showed that the predicted stability of helix 12 in the active conformation and the experimental data obtained with the wt and R471L/D472N PPARα are in excellent agreement. Moreover, comparison of these simulations revealed a possible mechanism accounting for the constitutive versus ligand-dependent co-activator recruitment and transcriptional activity of PPARα. The conformational changes observed during the simulation of the R471L/D472N in the absence of Wy-14,643 revealed an unstable active conformation of helix 12 and a disruption of the charge clamp between Lys-298 and Glu-468 (25, 78), as depicted in Fig. 8. The distance between the Cα atoms of Lys-298 and Glu-468 observed at the end of the simulation (23 Å) was out of the range reported to be optimal for interaction with the co-activator (18–20 Å), whereas high constitutive activity of the wt PPARα correlated with a functional charge clamp and stable helix 12 active position. To assess the reproducibility of these results, an additional 1-ns MD simulation was performed for each of the four systems. They all showed behaviors similar to that described above (data not shown).

The molecular basis underlying the contribution of Arg-471 and Asp-472 to the dynamic behavior of helix 12 remains unclear, mainly because the description of their interactions with other residues varies depending on the experimental structure. Asp-472 makes electrostatic interactions with a lysine of helix 4 only in some structures (67), suggesting that this interaction is not crucial to stabilize the active conformation of helix 12. This interaction is absent in the MD simulations of the wt PPARα, despite the stable active conformation of helix 12. Arg-471 (or the corresponding lysine in PPARγ or PPARβ/δ) makes hydrogen bonds with the glutamine and glutamate residues of helix 12 (67). However, whether this hydrogen bond network, internal to helix 12 and situated on the solvent face of AF-2, stabilizes significantly helix 12 in its active conformation remains unclear. Indeed, this hydrogen bond network is present in the MD simulations of the wt PPARα but does not exist in the MD simulation of the liganded R471L/D472N PPARα, where the helical character and active conformation of AF-2 are both conserved. In addition, it is absent in some experimental structures where helix 12 is in the active conformation (80). Thus, these interactions of Asp-472 with helix 4 and of Arg-471 with other residues in helix 12 probably contribute to, but do not seem to be crucial for, helix 12 stability. Therefore, they can only partially explain the destabilization and altered function of helix 12 after replacement of Arg-471 and Asp-472 in the double mutant R471L/D472N.

CONCLUSIONS

Like other members of the nuclear hormone receptor family, such as CAR (81–84), Nurrl1 (85), and ERR3 (86), PPARα and PPARγ display a basal constitutive activity (10, 59, 60, 79). Constitutive activity was thought to be due to helix 12 being able to adopt the active conformation in the absence of a ligand (22, 24, 25, 70, 72). A ligand would stabilize this active conformation allowing stronger activation of the receptor (87). In addition to the structural and biochemical studies mentioned above (see Ref. 26, for a review), our data clarify the differences between ligand-free and ligand-occupied PPARs for which helix 12 appears to be a critical player. In the absence of a ligand, helix 12 can adopt the active position, although less efficiently than the liganded receptor, and thus is able to recruit one or more co-activators, which result in constitutive activity. Most likely, the level of this activity will depend on the relative amounts of co-regulators in a given cell type and on the dynamic exchange between co-activators and co-repressors (88). Based on such considerations, it has been proposed that receptors present different degrees of dynamic behavior in accordance with their constitutive activity and that ligand-dependent receptor activation “is not like a regular on-off light switch, but is more like a dimmer switch” (26, 87). In the case of AR, single point mutations were shown to alter helix 12 dynamics and to result in androgen insensitivity in patients, showing that impaired helix...
12 mobility may have a major physiological impact (89). These observations seem particularly pertinent to the roles of PPARs in modulating energy homeostasis and other vital processes, which depend on very fine-tuned mechanisms (5, 6). In fact, PPARγ mutants with unstable helix 12 were identified in patients with severe insulin resistance (72, 90). How the dynamic behavior of PPAR helix 12 contributes to the different steps through which it finally modulates vital processes in vivo deserves further investigation. Finally, understanding the mechanisms responsible for PPAR constitutive and ligand-dependent activity, as well as describing the mechanisms behind helix 12 dynamics and co-activator recruitment, provides valuable information with regard to drug design. In practice, the utilization of PPAR agonists as therapeutic agents is associated with various side effects (for review see Ref. 91) that could be overcome with agonists having a restricted activation potential. At the molecular level, the specificity of action of these selective PPAR modulators would result from the recruitment of a specific subset of PPAR cofactors, for which an understanding of the molecular basis of helix 12 activity is of prime importance.

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