Crystal Structure of the Ligand Binding Domain of the Human Nuclear Receptor PPARγ*

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The peroxisome proliferator-activated receptors (PPAR) are members of the nuclear receptor supergene family and are considered as key sensors of both lipid and glucose homeostasis. The role of the PPARγ isoform in glucose metabolism is illustrated by the fact that anti-diabetic thiazolidinediones have been shown to be *bona fide* PPARγ ligands. Here we report the crystal structure of apo-PPARγ ligand binding domain (LBD) determined to 2.9-Å resolution. Although the structure of apo-PPARγ-LBD retains the overall fold described previously for other nuclear receptor LBDs, three distinct structural differences are evident. 1) The core AF-2 activation domain of apo-PPARγ LBD is larger and more accessible to the surface in contrast to other LBDs. 2) The proposed ligand binding pocket of apo-PPARγ-LBD is larger and more accessible to the surface. 3) The region of the LBD called the ω-loop is extended in PPARγ and contains additional structural elements. Taken together, the apo-PPARγ-LBD structure is in several aspects different from previously described LBDs. Given the central role of PPARγ as a mediator in glucose regulation, the structure should be an important tool in the development of improved anti-diabetic agents.

The nuclear receptors constitute one of the largest groups of transcription factors known today (1, 2). The members of this protein family are structurally and functionally conserved and have been shown to be expressed in a wide range of metazoan species (3). The nuclear receptors are transcription factors that upon binding of ligand regulate both target gene expression (1, 2) and repression (4). The most conserved domains of this protein family are the centrally located DNA binding domain and the C-terminal part of the receptor, which encompasses the ligand binding domain (LBD). 1

Addition to binding ligand, the LBD is required for efficient homo- and/or heterodimerization as well as interaction with transcriptional co-factors and components of the general transcriptional machinery (5, 6). Although the amino acid identity between certain LBDs is below 25%, such as for the retinoic acid receptor (RAR) and estrogen receptor (ER), the overall three-dimensional structure of different LBDs is highly conserved (7–12). The structural basis for a ligand-dependent transition from an inactive to an active transcription factor has been discussed in the literature, based on the three-dimensional structure of a limited number of nuclear receptor LBDs (7–12). Major structural differences have been observed between the apo-form of retinoid X receptor (RXR) and the holo-forms of RAR, thyroid hormone receptor, ER, and progesterone receptor (PR) that may begin to explain how a ligand causes the receptor to adopt a conformation necessary for transcriptional activation.

The PPAR(s) (peroxisome proliferator-activated receptor(s)) is a subgroup of the nuclear receptor gene family that has been suggested to play important roles in both lipid and glucose homeostasis (13–15). Today three PPAR genes (α, β/δ or FAAR, and γ) have been identified, and recent studies have shown that the different PPAR isoforms appear to have distinct but overlapping ligand binding specificities (15–18). The anti-diabetic thiazolidinediones, such as troglitazone, a specific high-affinity PPARγ (19) ligands (K_d 30–700 nM). In addition, other PPARγ ligands such as 15-deoxy-A_{12,14}-prostaglandin J2 (20, 21), polyunsaturated fatty acids (16, 17), and certain non-steroidal anti-inflammatory drugs (18) have been identified as PPARγ ligands. Thus, in contrast to classical nuclear receptors, such as the estrogen receptor, PPARγ appears to be able to interact with a broad range of different classes of ligands. To elucidate the molecular basis for this apparent broad specificity in ligand binding of PPARγ, we have determined the three-dimensional structure of the human apo-PPARγ-LBD. In this paper, we present this structure, compare it to other LBDs, and discuss the implications for ligand binding and interactions with co-factors.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of hPPARγ-LBD—Human PPARγ-LBD (amino acid residues Leu^{202} to Tyr^{317}) was polymerase chain reaction-amplified from an adipocyte library, introducing a pro tease cleavage site for Genexase™ I (New England Biolabs) upstream and cloned in frame with the His tag in pET15b (Novagen), resulting in the deduced amino acid sequence MGSSHHHHHHHSGLVAVHY(L)^204...Y^{317}. Recombinant protein was produced in shaker flask cultivations of *Escherichia coli* BL21 (DE3), harboring the derived plasmid, in LB medium at 25 °C, according to the recommendations by Novagen. Harvested cells were resuspended in running buffer (50 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM tris(carboxymethyl)phosphine hydrochloride (Pierce)) and subsequently disrupted by sonication at 4 °C. The obtained extract was cleared by centrifugation at 40,000 × g for 15 min. The His_{6}-PPARγ-LBD fusion protein in the supernatant was subjected to a one-step purification using immobilized metal affinity
chromatography on a Talon™ column (CLONTECH), according to the supplier's recommendations. Purified recombinant protein was eluted in running buffer supplemented with 150 mM imidazole and thereafter frozen at $-285^\circ C$. Upon thawing the protein fractions were subjected to centrifugation at 10,000 $g$ for 15 min, and after removing the contaminating protein precipitate, the resulting supernatant was used for crystallization. The engineered protease cleavage site was not used. The protein solution contained 3 mg/ml protein.

Crystallization and Structural Determination—Crystals were grown by the hanging drop diffusion method and appeared in 3–8 days. The 1-ml well solution contained 3.4M sodium formate, 0.1 M Tris/HCl buffer, pH 9.5, and 1% polyethylene glycol 6000. Crystallization drops contained 3 $\mu l$ each from protein and well solutions. The crystals grew to an approximate size of 0.15 $\times$ 0.15 $\times$ 0.1 mm. They were mounted in glass capillaries and diffracted to 2.9 Å. All data were collected using a Rigaku RU300 rotating anode with Molecular Structure Corp. mirrors and an RaxisIV image plate detector. The data were processed with DENZO and Scalepack (22). Programs in the Collaborative Computational Project 4 Suite (23) and from the Uppsala Software factory (24) were extensively used for the structure determination. The crystals were of space group P4$_3$2$_1$2 with cell dimensions $a = b = 67.3$ Å and $c = 156.8$ Å. There is one protein molecule per asymmetric unit, and the solvent content is 54%. SDS-polyacrylamide gel electrophoresis chromatography performed on dissolved crystals indicated that the protein was intact and had not been cleaved.

The structure was solved using multiple isomorphous replacement. Three different heavy atom derivative crystals were obtained by adding 0.1 $\mu l$ of saturated solutions of UO$_2$Ac$_2$, HgAc$_2$, and Hg(CN)$_2$/HgCl$_2$ to the respective crystal containing drops. Only two unique sites were found, one for the uranyl compound and one for the mercury compounds. Anomalous data were used from the two first derivatives. The heavy atom positions were determined by difference Patterson function inspection and difference Fourier syntheses. Mlphare (25) was used for initial phasing to 4.0-Å resolution to determine and evaluate the heavy atom sites and to determine the hand and space group ambiguities. The programs Sharp (26) and Solomon (27) were used for final phasing with phase extension to 2.9 Å. The resulting phases were of sufficient quality for synthesis of an easily interpretable electron density map.

The model of PPARγ-LBD was built in O (28). Refinement was carried out with the program CNS (29), using a simulated annealing protocol with bulk solvent correction, followed by conjugate gradient minimization and isotropic B-factor refinement. The quality of the

| Data set | Native | UO$_2$Ac$_2$ | HgCl$_2$/Hg(CN)$_2$ | HgAc$_2$ |
|----------|--------|-------------|---------------------|----------|
| Resolution (Å) | 2.9 | 4.0 | 4.0 | 4.0 |
| Completeness (%) | 96 | 100 | 71 | 96 |
| $R_{	ext{sym}}$ | 0.07 | 0.14 | 0.22 | 0.14 |
| $R_{	ext{free}}$ | 0.47 | 0.73 | 0.78 | 0.72 |
| Phasing power | 1.7 | 1.7 | 2.0 | 2.0 |
| Number of sites | 1 | 1 | 1 | 1 |
| Mean figure of merit | 51.1% | | | |

$R_{\text{sym}} = \frac{\sum_i |I(h) - \langle I(h)\rangle|}{\sum_i \langle I(h)\rangle}$, where $\langle I(h)\rangle$ is the average intensity of reflection h, $\Sigma_i$ is the sum over all reflections, and $\Sigma_h$ is the sum of all measurements of reflection h.

$R_{\text{free}} = \frac{\Sigma_h |F_{\text{PH}}| - |F_{\text{PH}}|}{\Sigma_h F_{\text{PH}}}$. Where $F_{\text{PH}}$ and $F_{\text{PH}}$ are the derivative and native structure factor amplitudes.

$R_{\text{cullis}}$ = lack of closure/isomorphous difference.

Phasing power = $F_{\text{PH}}$/lack of closure. Acentric reflections.

Values and definitions are from Procheck (30).

**TABLE I Crystallographic data**

**FIG. 1. A stereo image of apo-PPARγ-LBD.** The protein adopts a conformation for helix 12 similar to RARγ and PR, both of which have ligands bound. The figure was made with Molscript (33).
emerging model was monitored by Procheck (30) and O. 92% of modeled residues fall within the most favored regions of the Ramachandran plot as defined by Procheck, with no residues in disallowed regions. The R-factor is 20.9% for all reflections between 15.0 and 2.9 Å. The corresponding free R-factor is 27.1%. The final model contains the introduced protease cleavage site and the complete PPARγ-LBD, except for 11 residues (amino acids 263–273) for which no electron density could be detected. The data collection and refinement statistics are summarized in Table I.

RESULTS AND DISCUSSION

The structure of apo-PPARγ-LBD reveals a new variation on the nuclear receptor fold, where the predominantly α-helical LBD core is conserved (Fig. 1). There are a total of 12 helices and a small β-sheet of four strands (Fig. 2). The helices are numbered in agreement with RXRα-LBD (7), although helix 10 and 11 are in fact one continuous helix in PPARγ. Among the LBD structures available in the Protein Data Bank, apo-PPARγ-LBD is most similar to RARγ-LBD bound to all-trans-retinoic acid, and the two structures can be aligned with a root mean square deviation of 1.45 Å for 200 α-carbons. Four major stretches of homologous structure are identified in this alignment: helix 1, helices 3–5 and strand 2, helices 7–11, and helix 12. The region between helix 1 and helix 3, called the α-loop, has the largest deviation from the canonical fold and contains an insertion of more than 20 amino acid residues relative to most other nuclear receptors. It is made up of two helices, 2a and 2b, separated by a β-strand. There are 11 amino acid residues missing in the density between helix 2b and helix 3, indicating high mobility of the region. In this crystal form of apo-PPARγ-LBD, helix 12 covers the predicted ligand binding pocket and adopts a conformation very similar to that observed in PR and RARγ-LBDs when bound to progesterone and all-trans-retinoic acid, respectively. The α-carbons of the apo-PPARγ-LBD helix 12 show a mean displacement of 2.2 Å from the RARγ-LBD helix 12 in a direction away from the ligand-binding pocket, when the two LBDs are aligned. The interactions of helix 12 with the rest of the receptor are mainly hydrophobic with two exceptions: there is a weak hydrogen bond between His323 and Tyr473 and a salt bridge between Lys319 and Asp475. A similar salt bridge is present in RARγ involving a lysine at the equivalent position and a glutamate at the N-terminal part of helix 12. This salt bridge has been shown to be critical for transcriptional activation in RARγ (8) and is likely to have a stabilizing effect on the transcriptionally active LBD.

Based on comparisons of the current structure with the structures of LBDs with bound ligand, possible ligand binding sites of PPARγ can be predicted (Fig. 3). A number of LBD structures have been published recently, where the ligand is bound in a buried pocket (8–11). The corresponding region of PPARγ can be divided into two interconnected cavities, both of which extend into a wide surface accessible groove parallel to helix 3. This groove is created by a separation of helix 3 and the β-sheet. In the holo-forms of RARγ, ERα, and PR the β-sheet tightly covers up this side of the pocket. The first cavity of PPARγ corresponds to the described ligand binding pockets of PR, ERα, and RARγ. The bottom of this cavity is made up of side chains from helices 3, 5, 10, 11, and 12 and displays a remarkably polar surface created by His349, Tyr352, Lys367, His349, and Tyr473. A second cavity extends toward helix 1 and the β-sheet and is delimited by Phe226, Pro227, Ile296, and Met329. It is covered from the surface by a salt bridge made up by Glu295 and Arg478 on helix 3. The cavities and the groove are predominantly hydrophobic, which would be expected if natural ligands such as 15-deoxy-Δ12,14-prostaglandin J2 (20, 21) and polyunsaturated fatty acids (16, 17) were to be accommodated. One can argue that the groove may serve as an alternative entry point for ligands, as opposed to an entrance created by the putative displacement of helix 12 suggested for RARγ (8). The observed position of helix 12 in apo-PPARγ-LBD, obstructing the ligand binding pocket, also raises the possibility that an alternative entry point for ligands is needed. The thyroid hormone receptor has a similar structure for the region around the α-loop and the β-sheet, and it has been proposed that a displacement of its helix 2 may create an entry point for a ligand (9). The 11 amino acids that are disordered in apo-PPARγ-LBD are in close proximity of the groove and may also have an effect on ligand binding.

PPARγ does not form functional homodimers, although in this crystal form there is a crystallographic 2-fold present relating two receptor molecules. This symmetric crystal contact creates a hydrophobic pocket bordered by Val293 and Thr297 on helix 3; Leu311, Val315, and Leu318 on helix 4; Leu468 and Ile472 on helix 12 from the two molecules. This region contains the core AF-2 activation domain on helix 12 as well as side chains in the conserved signature motif of helix 3 and 4. The hydrophobicity of this surface is conserved for all nuclear receptors and mutational analysis suggests that this is a likely interaction surface with a group of co-activator proteins (31). It is not surprising that this area of the LBD is involved in intermolecular contacts in the crystal, if its natural function is to recruit and bind hydrophobic co-activators.

The conformation of helix 12 is likely to be critical for transcriptional activation, and large differences have been observed in the position of this helix for agonist and antagonist-bound forms of the estrogen receptor (10). The structure of apo-PPARγ-LBD adopts a conformation more similar to the ligand bound nuclear receptor structures than to the structure of RXRα-LBD without a ligand. This raises the question whether any of these two apo-receptors represent a true native conformation. In both cases, helix 12 are involved in crystal contacts and may therefore be forced into artificial conformations. It is possible that ligand binding in PPARγ induces more subtle conformational changes than what has been presumed for RXRα and that the two structures represent two variations on ligand free nuclear receptors. One of the crystal structures of ERe in complex with estradiol furthermore demonstrates that a bound agonist ligand does not necessarily entail the recruitment of helix 12 to cover the ligand binding pocket (12). In the absence of apo- and holo-receptor crystal structures for identi-
FIG. 3. A comparison of the ligand binding pockets of holo-RARγ (a), holo-PR (b), and apo-PPARγ (c). A cavity search was performed with the program Voidoo (34). The surfaces displayed represent accessible surfaces of cavities using a 1.8-Å probe. In PR and RARγ, the cavities are closed, whereas for PPARγ it is accessible from the outside of the protein (Protein Data Bank entries: 2LBD for RARγ and 1A28 for PR). The figure was made with O (28).
cal receptors in multiple crystal forms, we cannot with certainty distinguish true conformational change from structural variations between receptors and crystal artifacts.

Note Added in Proof—Recently Nolte and coworkers (Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V., (1998) Nature 395, 137–143) published structures of PPARγ-LBD with and without a ligand and a coactivator peptide. Their structures are both found in the canonical dimer form, not present in the current structure.

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