Both Coactivator LXXLL Motif-independent and -dependent Interactions Are Required for Peroxisome Proliferator-activated Receptor γ (PPARγ) Function*  

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Nuclear receptor activation is dependent on recruitment of coactivators, including CREB-binding protein (CBP/p300) and steroid receptor coactivator-1 (SRC-1). A three-dimensional NMR approach was used to probe the coactivator binding interface in the peroxisome proliferator-activated receptor γ (PPARγ) ligand binding domain (LBD). In the presence of a CBP peptide, peaks corresponding to 20 residues in helices 3, 4, 5, and 12 of the LBD were attenuated. Alanine mutants revealed that K301A, V315A, Y320A, L468A, and E471A were required for binding of both CBP and SRC-1 and for cell-based transactivation. Several additional amino acids in helix 4 of the PPARγ-LBD were defective with respect to CBP recruitment, but retained relatively normal SRC-1 recruitment. Thus these amino acid residues may be important determinants of specificity for nuclear receptor LBD interactions with discrete coactivator molecules.

Nuclear receptors are a large family of ligand-activated transcription factors that control important cell functions. Nuclear receptors contain a central DNA binding domain and a COOH-terminal ligand binding domain (LBD)† with an activation function domain (AF2). Ligand-induced transactivation is mediated through interactions with members of a growing family of coactivator proteins, such as CBP (1–4), SRC-1 (3, 5–9), PGC-1(10), PBP (11, 12), TRAP220 (13), and ARA70 (14). A short sequence motif LXXLL which is shared by most coactivators has been shown to be required to mediate the ligand-induced receptor-coactivator interaction through AF2 (15–17). Mechanisms that confer the specificity for the nuclear receptor-coactivator interaction have become a central focus of investigation in this field. Different nuclear receptors have been found to preferentially interact with different coactivators and corepressors (18), suggesting that interactions besides LXXLL-AF2 binding per se play important roles. Darimont et al. (16) and McInerney et al. (17) have shown that the amino acids immediately adjacent the LXXLL motifs impart specific binding to selected nuclear receptors. Here, we identified the LBD residues of PPARγ that are required for both CBP and SRC-1 binding; in addition, we also identified several amino acids that differentially affect binding to CBP and SRC-1. These results suggest that certain residues within the LBD of nuclear receptors have an important role in mediating functional specificity by allowing for differential interactions with individual coactivators.

EXPERIMENTAL PROCEDURES

Materials—Compound A is 5-(4-[2-methyl-2-pyridylamino]ethoxybenzyl)thiazolidin-2,4-dione (19). Compound B is 5-(4-[2-(2-hydroxy-4-oxazoly)-2-hydroxyethyl]-4-oxazolyl)benzyl)-2-thiazolidin-2,4-dione (19). Compound C is 5-(4-[3-(7-propyl-3-neopentylbenzoxazol-6-yl)propylamino]-3-chlorophenylacetic acid (20). [3H]Methionine and 35S-labeled dATP were purchased from Amersham Pharmacia Biotech. All the cell culture and cell transfection reagents were from Life Technologies, Inc.

NMR Spectroscopy—For the production of [15N,13C] and D,O-labeled hPPARγ-LBD, BL21 (Stratagene) hosting pGEXhPPARγ-LBD was grown on modified M9 minimal medium (21) with 1 g/liter 15N-ammonium sulfate, 5 g/liter [15C]glucose and 60% D2O. Isopropyl-1-thio-β-D-galactopyranoside-induced culture was used for GST fusion protein purification. Following cleavage with thrombin, hPPARγ-LBD was further purified through a gel filtration chromatography. Samples for NMR spectroscopy were at a concentration of 0.3 mM in a buffer consisting of 25 mM sodium phosphate at pH 7.0 in 90% H2O, 10% D2O. NMR data was collected at 23 °C on a Varian Inova 600 NMR system equipped with a triple-resonance 5-mm probe with a z axis gradient coil. The sample volume was 250 μl and the NMR tubes were fitted with Ustem susceptibility plugs (Wilmad Glass). Because of severe overlap in two-dimensional HSQC experiments, perturbation mapping experiments were performed with three-dimensional HNCO spectra using gradient mode sensitivity enhancement.2 Acquired NMR spectra were processed with nmrPipe (23) and analyzed using NMRView (24).

Assignment of the backbone resonances was accomplished with a suite of five three-dimensional NMR experiments, CT-HNCA, CT-HN(CO/CA) (25), CT-HN(COCA/CA/CB, CT-HN(CA/CB) (26), and HNCO on 90% fractionally deuterated 13C,15N-labeled protein. The experiments were assigned using semi-automated tools in the program NMRView (24), and this will be the subject of a separate publication.3

Construction of Plasmids—pSGS-hPPARγR, which contains the human PPARγ-LBD fused to murine GR-DDD, and pMMTVhuc, which contains the murine mammary tumor virus (MMTV) promoter adjacent to the luciferase (luc) gene, were kindly provided by Dr. Azriel Schmidt (Merck Research Laboratories). pGEXhCBP1–453, which expresses GST fusion protein of human CBP NH2-terminal 1–453 amino acids, and pGEXhSRα6g-780, which expresses human SRC-1 fragment from amino acids 568 to 780 as a GST fusion protein, have been described previously (3). All the PPARγ LBD mutants (alanine replacements) were derived from the pSGS-hPPARγR construct using the Mutan-Gen M13 in vitro mutagenesis kit (Bio-Rad).

Pull-down Assay—GST-CBP, GST-SRC-1, or GST-RXR were purified and used to precipitate radiolabeled hPPARγR wild type and mutant

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† The abbreviations used are: LBD, ligand binding domain; SRC, steroid receptor coactivator; PPAR, peroxisome proliferator-activated receptor; GST, glutathione S-transferase; MMTV, murine mammary tumor virus; GR, glucocorticoid receptor; CBP, CREB-binding protein; RXR, retinoid X receptor; PAGE, polyacrylamide gel electrophoresis.

2 B. Johnson, Y. Li, D. Moller, R. Smith, and G. Zhou, submitted for publication.
3 B. A. Johnson, et al., manuscript in preparation.
forms with [35S]methionine (using TNT coupled in vitro transcription/translation system from Promega) (3).

Cell Culture and Transactivation Assay—For transfection, COS1 cells were switched to Dulbecco’s modified Eagle’s medium medium containing charcoal-stripped fetal bovine serum and seeded in 96-well plates at a concentration 1.5 × 10^4/well 17 h prior to transfection. Transfections were performed using LipofectAMINE (Life Technologies, Inc.). Each well was transfected with 20 ng of hPPARγ GR plasmid, 40 ng of GR-responsive reporter gene MMTV/luciferase and 25 ng of β-galactosidase expression vector for the control of transfection efficiency. After 6 h, cells were treated with compound C at the concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM. Cell lysis was produced 48 h later using reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were measured.

Ligand Binding—Extracts from COS1 cells expressing transfected hPPARγ/GR were used for radioligand binding assay using [3H]-labeled compound B (19).

RESULTS AND DISCUSSION

Characterization of the PPARγ LBD Coactivator Binding Site via Differential NMR Shift Maps—NMR spectroscopy can be used to identify contact interfaces for protein-ligand and protein-protein interfaces (27, 28).

The binding site of a 14-amino acid CBP peptide, containing an LXXLL motif, was mapped by comparison of NMR spectra of the PPARγ LBD in the presence and absence of the peptide. In both spectra the PPARγ LBD was bound to a potent known agonist (compound A) (19). The most significant perturbation of the spectra observed in the presence of CBP peptide was a reduction in intensity or disappearance of a specific set of peaks. However, the majority of the peaks remained relatively unchanged in position and intensity indicating that peptide binding results in only a local perturbation of the structure. Fig. 1a illustrates four representative peaks that were assigned to a six-residue stretch of the protein. The position and intensity of a peak corresponding to Val-307 was essentially unchanged by CBP peptide binding, whereas that corresponding to Asp-310 was attenuated significantly and those corresponding to Leu-311 and Asn-312 were no longer observable after addition of peptide. The most likely explanation for these results is a broadening and signal loss due to chemical exchange of the peptide on and off the protein. The perturbed peaks can be used to map the location of the binding site of the CBP peptide onto the three-dimensional structure of the PPARγ LBD as determined by x-ray crystallography. Fig. 1b illustrates the mapping of the perturbed cross-peaks onto a ribbon diagram of the PPARγ LBD. The perturbed residues form a contiguous surface formed by residues from helices 3, 4, 5, and 12.

Alanine Mutagenesis Defines the Role of Selective Amino Acids in PPARγ Function—Based on the NMR differential map, each of 20 perturbed amino acids was replaced with the neutral and small amino acid, alanine. Thr-297 and Val-307, which were not associated with an observed NMR peak shift, were chosen as control in this study. Each mutant was examined for its function in four assays: agonist-induced coactivator interaction, ligand binding (22), agonist-stimulated transcriptional activation and dimerization with its partner RXR. The results are summarized in Table I. None of the mutants were shown to ablate PPARγ dimerization with RXR in a GST-RXR pull-down assay using [35S]Met-labeled PPARγ (data not shown).

The data in Table I can be used to classify the mutants into four groups. The first group, including V293A, Q294A, E295A, T297A, E298A, V307A, D310A, Q314A, L317A, L318A, V322A, and Q470A, did not abolish receptor function in all experiments performed. Thus, the chemical shifts of peaks corresponding to these amino acids in differential NMR studies may represent an indirect influence from the surrounding protein structure environment.

The second group of mutants, including K301A, V315A, Y320A, L468A, and E471A, abolished the interaction of PPARγ with either CBP or SRC-1 in vitro and were associated with deficient transcriptional activation function in cells (Table I and Fig. 2). The third group, L311A, N312A, and T316A, selectively abolished PPARγ interaction with CBP but maintained interaction with SRC-1 (Table I and Fig. 3).

The fourth group, K319A and L469A, were shown to exhibit substantially defective radioactive ligand binding. Although K319A mutant failed to exhibit significant radioligand binding, at higher ligand concentrations, it was able to bind coactivators in vitro and to mediate transactivation, suggesting that the
Differential Interactions of PPARγ with Its Coactivators

Functional assessment of PPARγ LBD mutants

| Pull-down     | Ligand binding | Transactivation | Group |
|---------------|----------------|-----------------|-------|
| CBP           | SRC            |                 |       |
| Wild type     | 100            | 100             | 1     |
| Val-293       | 60             | 36              | 1     |
| Gln-294       | 61             | 80              | 1     |
| Glu-295       | 175            | 205             | 1     |
| Thr-297       | 68             | 276             | 1     |
| Glu-298       | 186            | 218             | 1     |
| Lys-301       | 0              | 0               | 1     |
| Val-307       | 115            | 91              | 1     |
| Asp-310       | 110            | 115             | 1     |
| Leu-311       | 0              | 49              | 1     |
| Asn-312       | 17             | 61              | 1     |
| Gln-314       | 74             | 59              | 1     |
| Val-315       | 0              | 0               | 1     |
| Thr-316       | 0              | 170             | 1     |
| Leu-317       | 73             | 93              | 1     |
| Leu-318       | 82             | 311             | 1     |
| Lys-319       | 0              | 38              | 1     |
| Tyr-320       | 0              | 0               | 1     |
| Val-322       | 120            | 120             | 1     |
| Leu-468       | 0              | 0               | 1     |
| Leu-469       | 0              | 0               | 1     |
| Gln-470       | 67             | 99              | 1     |
| Glu-471       | 0              | 0               | 1     |

a binding is less than 10% of the wild type receptor.
b The wild type and all mutant receptors were transfected into COS1 cells together with luciferase reporter gene and titrated with compound A and C from 0.1 to 10 μM. Similar results were obtained with either compound A or compound C. The percentage luciferase activity of maximal wild type receptor activity at 10 μM compound C is expressed in the table.

Fig. 2. Binding of mutant forms to CBP or SRC-1. GST-CBP containing amino acids 1–450 of CBP or GST-SRC-1, including 1–780 amino acids of SRC-1, was used to pull down [35S]methionine-labeled hPPARγ/GR wild type and mutant forms in the presence of 5 μM compound A or C, followed by SDS-PAGE. Mutants shown here are the representatives from total 22 mutants listed in Table I. Upper and middle panels show the binding of radiolabeled hPPARγ/GR forms to CBP and SRC-1, respectively. Lower panel shows radiolabeled receptor forms from 2 μl of TNT mixture. The first lane is the control of WT hPPARγ/GR binding to CBP or SRC-1 in the absence of compound.

Fig. 3. Differential interactions of mutant L311A, N312A, and T316A to CBP and SRC-1. Protein complex was separated by SDS-PAGE. Labeled proteins that bind to CBP or SRC-1 were detected by autoradiography (A) and quantitated by phosphoimage (B). In B, binding of L311A, N312A, and T316A to CBP or SRC-1 were graphed as percentage of WT. Error bars represent the S.D. from duplicate data.

The main effect of this mutation was a relative defect in ligand binding affinity (Fig. 4).

Amino Acids in Group Two Form the LXXLL Binding Pocket—The in vitro pull-down assay showed that mutants K301A in helix 3, V315A in helix 4, Y320A in helix 5, and L468A and E471A in helix 12 had completely lost ligand-induced coactivator interaction function, while their radioligand binding abilities were retained (Table I, Figs. 2 and 4). With the exception of a single LXXLL motif, the 14-amino acid CBP peptide used in this experiment displayed little similarity with the SRC-1 fragment (from amino acid 568 to 780; containing three LXXLL motifs) used for the pull-down assay. The fact that the group two mutants, which were identified via CBP peptide NMR mapping, abolished PPARγ interactions with both CBP and SRC-1 strongly suggests that these five amino acids are directly involved in the LXXLL motif binding. This is consistent with the recently reported “charge-clamp” model based on
specific interactions between nuclear receptors and distinct coactivators. Ligand-induced conformational change (stabilized conformation) creates a binding surface for coactivators. The coactivator LXXLL motif is oriented via a charge clamp formed by a conserved lysine residue and a glutamate residue such that LXXLL binding forms a bridge between helix 12 and helix 3. As a result, two additional interactions follow: (a) first, side chains of leucine residues are allowed to pack into a hydrophobic pocket formed by helices 3, 4, 5, and 12 (including Val-315, Tyr-320, and Leu-468); (b) second, the interaction between amino acids COOH-terminal to LXXLL motif in the coactivator and LBD residues in helix 4 (including Leu-311, Asn-312, and Thr-316 in PPARγ) takes place. This second interaction, which determines the specificity, is also required for nuclear receptor function.

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