Published in final edited form as:
Biochem Biophys Res Commun. 2008 January 4; 365(1): 42–46.

Effect of heterodimer partner RXRα on PPARγ activation function-2 helix in solution

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Abstract

The structural mechanism of allosteric communication between retinoid X receptor (RXR) and its heterodimer partners remains controversial. As a first step towards addressing this question, we report a nuclear magnetic resonance (NMR) study on the GW1929-bound peroxisome proliferator-activated receptor gamma (PPARγ) ligand-binding domain (LBD) with and without the 9-cis-retinoic acid (9cRA)-bound RXRα LBD. Sequence-specific $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and ^{13}CO resonance assignments have been established for over 95% of the 275 residues in the PPARγ LBD monomer. The ^{1}HN, ^{15}N and ^{13}CO chemical shift perturbations induced by the RXR LBD binding are located at not only the heterodimer interface that includes the C-terminal residue Y477, but also residues Y473 and K474 in the activation function-2 (AF-2) helix. This result suggests that 9cRA-bound RXRα can affect the PPARγ AF-2 helix in solution, and demonstrates that NMR is a powerful new tool for studying the mechanism of allosteric ligand activation in RXR heterodimers.

Keywords

PPARγ; RXRα; Heterodimer; Activation; Allosteric communication; NMR

Introduction

Retinoid X receptors (RXRα, β, γ) are members of the nuclear receptor (NR) superfamily of ligand-activated transcription factors that bind to DNA response elements as either homodimers or obligate heterodimer partners with several other nuclear receptors [1] such as peroxisome proliferator-activated receptors (PPARα, β/δ, γ). Both RXR and PPAR consist of an N-terminal A/B domain that contains a ligand-independent activation function-1, a conserved DNA-binding domain, a flexible hinge or D domain, and a ligand-binding domain (LBD) that contains a ligand-dependent activation function-2 (AF-2) at its C-terminal region and a dimerization interface.

PPARs are permissive RXR heterodimer partners so that a RXR agonist can either activate the heterodimer alone or potentiate PPAR agonist activation. Whether allosteric communication across the heterodimer interface facilitates the allosteric ligand activation remains

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controversial, as reported in the crystallographic studies on the estrogen receptor LBD homodimer [2] and retinoic acid receptor-RXR LBD heterodimer [3].

To address this question, we initiated a nuclear magnetic resonance (NMR) study on the agonist GW1929-bound PPARγ LBD with and without the agonist 9-cis-retinoic acid (9cRA)-bound RXRα LBD. Our result suggests that 9cRA-bound RXRα can affect the PPARγ AF-2 helix in solution through the C-terminal residue Y477 at the interface, and demonstrates, for the first time, that NMR is a powerful new tool for studying the mechanism of allosteric ligand activation in RXR heterodimers.

Materials and Methods

Materials

The PPARγ ligand GW1929 and RXRα ligand 9cRA were purchased from Sigma-Aldrich (St. Louis, MO). The PPARγ LBD (a.a. Q203-Y477) cDNA was amplified from human adipose cDNA library and inserted at the *Nde*I/*Xho*I restriction sites of the pET15b expression vector as described [4]. DNA sequencing analysis confirmed the pET15b-PPARγ LBD sequence. The unlabeled RXRα LBD, PPARγ LBD and U-[^{2}H, ^{15}N, ^{13}C]-enriched PPARγ LBD were expressed in *Escherichia coli* BL21-DE3 bacteria, isolated by Talon affinity chromatography, digested by thrombin to remove the N-terminal His_6 tag, and finally purified by gel filtration chromatography as described [4].

Fluorescence Titration

Experiments were performed on 2.25 ml of 1 μM unlabeled PPARγ LBD in gel filtration buffer (50 mM NaCl, 10 mM Tris, pH 8.0, 0.5 mM EDTA, and 10 mM β-mercaptoethanol) containing 0.01% gelatin at 25°C using a fluorometer (Photon Technology International, Birmingham, NJ) by exciting tyrosine residues at 280 nm and monitoring their fluorescence emission at 308 nm before and after adding aliquots (0.4 μl) of 240 μM GW1929 dimethyl sulfoxide solution, as described [5]. To evaluate inner filter effect, experiments were performed on ~1 μM L-tyrosine under the same conditions. Correction for inner filter effect and calculation of the K_d were carried out as described [6]. The reported K_d was the average over three independent measurements.

NMR Sample Preparation

The unliganded PPARγ-RXRα heterodimer was prepared by mixing equal molar amounts of the purified LBDs and adjusting to a 1:1 molar ratio according to the gel filtration chromatogram of the complex. A NMR sample containing 0.27 mM unliganded (apo), labeled PPARγ LBD monomer, 1.0 mM GW1929-bound, labeled PPARγ LBD monomer, or 1.0 mM heterodimer of GW1929-bound, labeled PPARγ LBD with 9cRA-bound, unlabeled RXRα LBD in a NMR buffer (20 mM potassium phosphate, pH 7.4, 50 mM KCl, 0.05% NaN_3, 0.5 mM EDTA-d_{16}, 8 mM β-mercaptoethanol- d_6 and 5% D_2O) was prepared as described [4].

NMR Spectroscopy

Experiments were conducted at 25°C in either a 600- or 700-MHz four-channel Varian Inova spectrometer equipped with a pulsed-field gradient triple resonance probe. For sequence-specific resonance assignments, a 2-dimensional (2D) ^{1}H-^{15}N-heteronuclear single quantum correlation (HSQC) spectrum and six 3D- and 4D-triple resonance through-bond experiments were performed on the 1.0 mM liganded PPARγ LBD monomer sample as described [4]. For analyzing the chemical shift perturbation induced by heterodimerization with the liganded RXRα LBD, 2D HSQC and 3D HNCO experiments were performed on the 1.0 mM heterodimer sample. Details on the experimental parameters, data processing and chemical

shift-derived secondary structure were as described [4]. The weighted root-mean-squared deviation of chemical shifts between the monomer and heterodimer was defined as: $\Delta\delta_{RMSD} = \sqrt{(\Delta\delta_{HN})^2 + (0.154\Delta\delta_N)^2 + (0.341\Delta\delta_{CO})^2}$ [7].

Results

The PPARγ LBD was purified as monomer to more than 95% chemical homogeneity by gel filtration chromatography. The dissociation constant K_d of GW1929 binding to the PPARγ LBD was 19 ± 10 nM by fluorescence titration. This value was similar to the reported half-maximal activation EC_{50} of 9 ± 7 nM for GW1929 [8], suggesting that the purified PPARγ LBD was functional. The gel filtration chromatography of a heterodimer sample and the subsequent SDS-PAGE analysis of the elution fractions showed that the two LBDs were co-eluted in a 1:1 molar ratio (Supplementary Fig. 1), suggesting that the heterodimer (59.0 kDa) was predominant over the PPARγ LBD monomer (31.8 kDa) and RXRα LBD homodimer (54.4 kDa).

Sequence-specific $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$ resonance assignments have been established for 262 of the 275 residues (> 95%) in the GW1929-bound PPARγ LBD monomer using a previously described method [4]. The chemical shift values are listed in Supplementary Table 1 and have been deposited in BioMagResBank with accession number BMRB15518. The 2D-HSQC spectra of the apo- and liganded-LBD are shown in Fig. 1. The backbone amide crosspeaks of 244 residues (> 92%) have been identified from a total of 264 expected residues after excluding the 11-proline residues in the liganded-LBD. In contrast, at least 62 backbone amide crosspeaks were missing in the 2D-HSQC spectrum of the apo-LBD sample, including those in the AF-2 helix and the C-terminal region. This result suggests that GW1929 binding stabilizes the PPARγ LBD conformation.

The $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$chemical shift deviations from the random coil values after correction for the deuterium isotope effect [4] and the secondary structure determined by the chemical shift index (CSI) program [9] are shown in Fig. 2. The CSI consensus reveals that the PPARγ LBD has 12 α-helices, which match those in the crystal structure of the PPARγ LBD bound to GI262570 (farglitazar) and a coactivator peptide [10] and are usually shortened by up to one turn on either end of the helix except for helices H2′, H3 and H8. The analysis of helices H3 and H8 may be affected by the missing resonance assignments and helix H3′, which is not part of the canonical NR LBD structure, is not detected here either. Although the CSI consensus reveals only one β-strand S4, the $^{13}C^{\alpha}$ chemical shift changes support the existence of β-strands S2 and S3 but not S1.

To examine the effect of heterodimerization and RXR ligand binding on the PPARγ LBD, we analyzed crosspeak displacements between the monomer and heterodimer in the 2D-HSQC and 3D-HNCO spectra, as shown in Fig. 3A and 3B. Using $\Delta\delta_{RMSD}$ = 0.13 ppm as the cutoff, which corresponds to $\Delta\delta_{HN}$ = 0.05 ppm, $\Delta\delta_N$ = 0.4 ppm and $\Delta\delta_{CO}$ = 0.3 ppm, or $\Delta\delta_{RMSD}$ = 0.08 ppm when $\Delta\delta_{CO}$ is excluded due to unavailable ^{13}CO resonance assignment in the heterodimer, we mapped residues with chemical shift perturbations above and below the cutoff in red (moderate) and blue (small), respectively, on the crystal structure [10], as shown in Fig. 3C The red residues are primarily located in H9 and H10, the H8-H9 and H9-H10 loops and the C-terminal residue Y477, all of which are components of the heterodimer interface. Residues Y320 in H4 and E324 in H5 (Fig. 3A) that are sequentially remote from the interface are also red. A close inspection of the crystal structure reveals that these two residues and other six residues including R397 (Fig. 3A) in the H8-H9 loop, R443, V446, T447 and V450 in H10, and L476 in the C-terminal region are in close contact (≤4.2 Å) with Y477 (Fig. 3D). Consistently, all eight residues are red upon heterodimerization. Intriguingly, residues Y473

(Fig. 3B) and K474 in the AF-2 helix are also red, suggesting that 9cRA-bound RXRα can affect the PPARγ AF-2 helix in solution.

Discussion

In this study, we have established sequence-specific $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and ^{13}CO resonance assignments for over 95% of the 275 residues in the GW1929-bound PPARγ LBD monomer. The chemical shift-derived secondary structure generally agrees with the crystal structure of the PPARγ LBD-GI262570-coactivator peptide ternary complex [10]. Both GW1929 [8] and GI262570 [10] are N-aryl tyrosine derivatives that were designed to mimic the interactions between the PPARγ H3, H5, H10 and AF-2 helices and the insulin-sensitizing thiazolidinedione (TZD) drug rosiglitazone head group in the crystal structure [11]. Previous NMR study [12] on the PPARγ LBD without (apo) and with rosiglitazone binding showed that the 3D HNCO spectra of the apo-LBD had less than half of the expected crosspeaks, and rosiglitazone binding restored the missing crosspeaks including those in the AF-2 helix and the C-terminus Y477. This is similar to our finding on GW1929 binding to the PPARγ LBD. Therefore, both NMR studies support the crystallographic findings that both rosiglitazone and N-aryl tyrosine directly interact with the AF-2 helix.

Our chemical shift perturbation analysis shows that heterodimerization with 9cRA-bound RXR causes moderate backbone ^{1}HN, ^{15}N and ^{13}CO chemical shift changes of the PPARγ LBD in areas that generally coincide with the heterodimer interface in the crystal structure, and in residues that are in close contact with the C-terminus Y477. Intriguingly, residues Y473 and K474 in the AF-2 helix also exhibit moderate chemical shift changes. This is unexpected from the crystal structures because the conformations of the AF-2 helix H12 and the C-terminal segment D475-L476 in both homodimer [13] and heterodimer [10] configurations are very similar (Fig. 3E). The results from gel filtration chromatography and NMR lineshape analysis suggest that the PPARγ LBD is mainly a monomer in solution regardless of ligand binding. The chemical shift perturbation on the AF-2 helix could be the result of the displacement of the Y477 side chain, a small backbone conformational change in the segment Y473-Y477 or both, the latters of which would affect ligand-dependent PPARγ activation. Further NMR and mutational studies should determine the precise structural mechanism. A parallel study on the RXRα LBD subunit should identify other structural determinants of allosteric ligand activation.

Finally, we demonstrate, for the first time, that it is feasible to monitor residue-specific structural and dynamic changes of a RXR LBD heterodimer in response to ligand binding in solution, and NMR is a powerful new tool for studying the mechanism of allosteric ligand activation in RXR heterodimers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from the National Institutes of Health (NIH) DK59501 (E. Li), Washington University Digestive Diseases Research Core Center (DK 52574), a pilot/feasibility award (J. Lu) from the Washington University Center for Human Nutrition (DK 56341). We thank Mr. Weidong Wen for preparing human adipose cDNA library and Mr. Bob McGibbon for assistance in protein purification.

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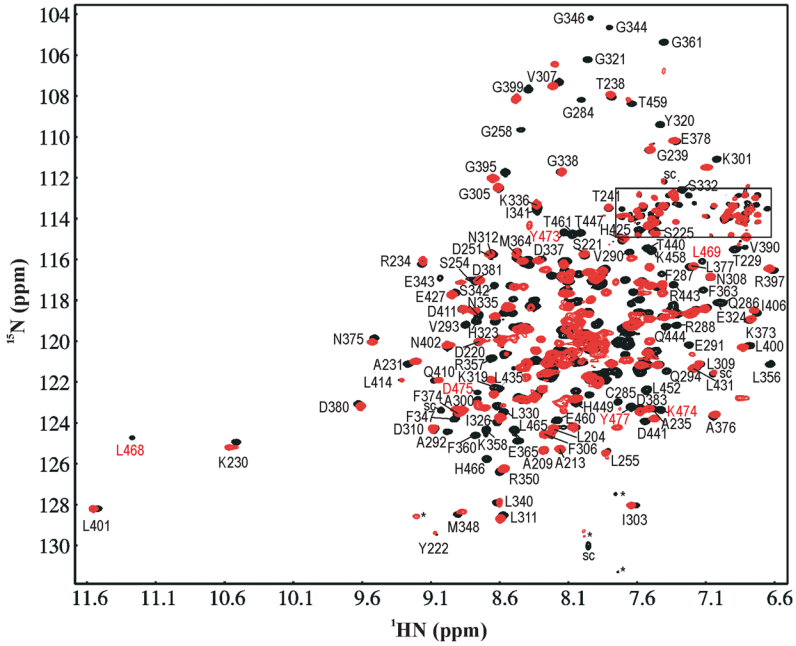

Fig. 1.

GW1929 binding stabilizes the PPARγ LBD conformation. The 2D-HSQC spectrum of a 0.27 mM apo-PPARγ LBD sample (red) was superimposed onto that of a 1.0 mM GW1929-bound PPARγ LBD sample (black). Crosspeaks in less crowded regions of the GW1929-bound sample are labeled with the amino acid one-letter code and the residue number. All labels are black except for those in the AF-2 helix and the C-terminal region (red). Side chain crosspeaks are either enclosed in a box or labeled with “sc”. Asterisks designate unknown crosspeaks, most of which are likely from minor aggregates.

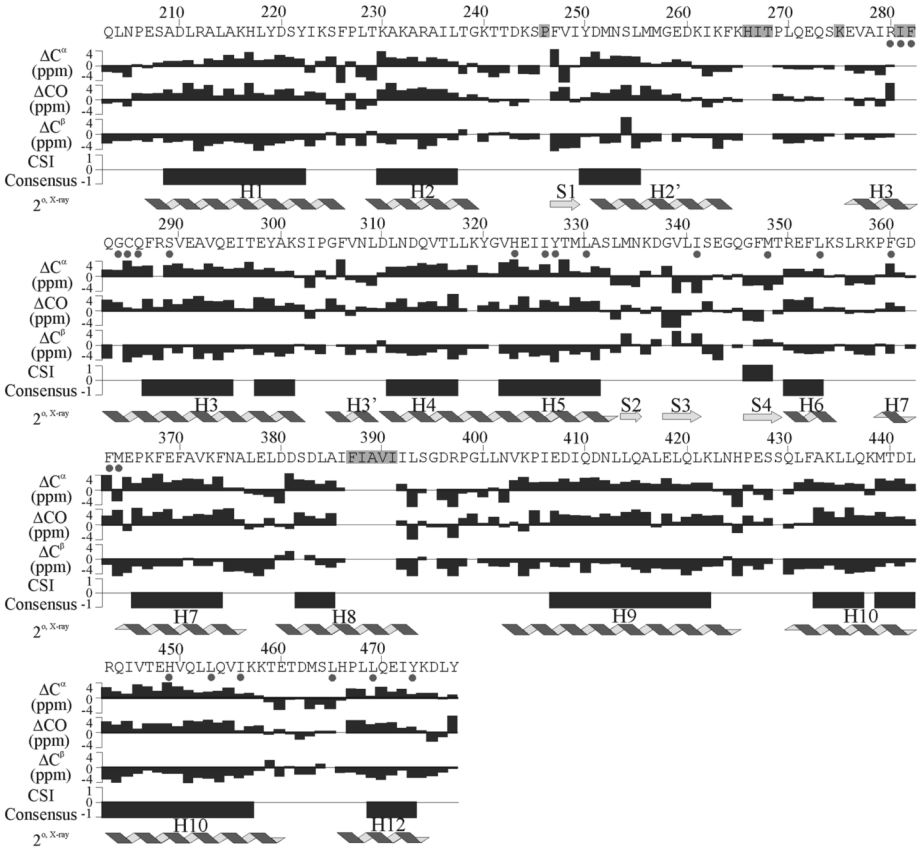

Fig. 2.

Chemical shift-derived secondary structure of GW1929-bound PPARγ LBD monomer. The $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$ chemical shift deviations between the measured and random coil values and the chemical shift index (CSI) consensus are plotted versus the primary sequence at the top. The three CSI values 1, 0 and −1 designate α-helix (H1-H12), random coil and β-sheet (S1-S4), respectively. The ribbon diagrams depict the secondary structure in the crystal structure of the PPARγ LBD-GI262570-coactivator peptide ternary complex (PDB: 1FM9). The primary sequence is annotated as followed: the shaded letters are unassigned residues; filled circles are residues in close contact (≤4.2 Å) with GI262570.

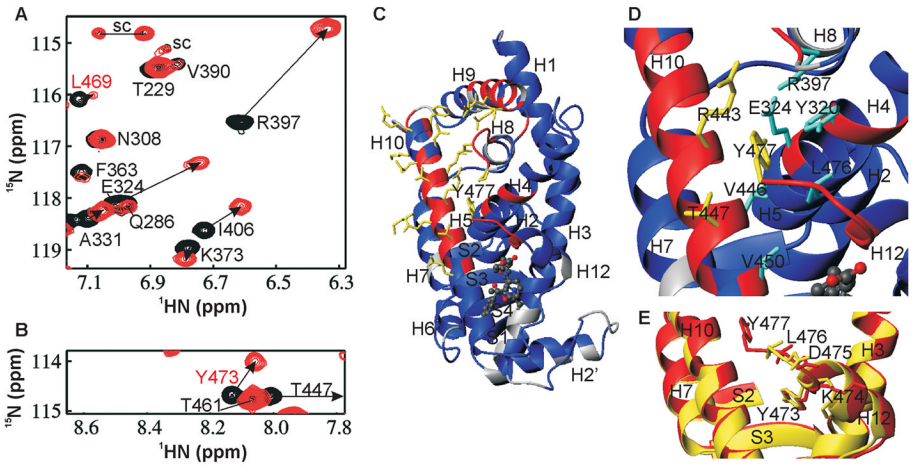

Fig. 3.

Chemical shift perturbation induced by heterodimer partner 9cRA-bound RXRαLBD. (A), (B) Overlays of the expanded regions in the 2D-HSQC spectra recorded on either 1.0 mM GW1929-bound PPARγ LBD monomer (black) or its heterodimer with 9cRA-bound and unlabeled RXRα LBD (red). The annotation is the same as that described in Fig. 1, with arrows indicating crosspeak displacement. (C) Chemical shift perturbation mapping on the crystal structure of the PPARγ LBD-GI262570 (ball-and-stick model with C, N and O atoms in dark, blue and red, respectively)-coactivator peptide (not shown) ternary complex. Color schemes: red and blue, moderate and small chemical shift changes, respectively; gray, proline residues and unassigned residues in either monomer or heterodimer; yellow, side chains of residues at the heterodimer interface. (D) Zooming onto the C-terminal residue Y477 and the eight residues in close contact with it. The side chains outside the heterodimer interface are in light blue. (E) Superimposition of the crystal structure (PDB: 1I7I) of the AZ242-bound PPARγ LBD homodimer (yellow) onto that of the PPARγ-RXRα LBD heterodimer (red) bound to ligands (GI262570 and 9cRA) and coactivator peptides showing the similarity between the two AF-2 helices. Residue Y477 is missing in the crystal structure of the homodimer. Helices H2′, H4 and H5 have been removed for clarity.