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The Effect of Antagonists on the Conformational Exchange of the Retinoid X Receptor Alpha Ligand-Binding Domain

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Abstract

The effect of retinoid X receptor (RXR) antagonists on the conformational exchange of the RXR ligand-binding domain (LBD) remains poorly characterized. To address this question, we used nuclear magnetic resonance spectroscopy to compare the chemical shift perturbations induced by RXR antagonists and agonists on the RXR α LBD when partnered with itself as a homodimer and as the heterodimeric partner with the peroxisome proliferator-activated receptor γ (PPAR γ) LBD. Chemical shift mapping on the crystal structure showed that agonist binding abolished a line-broadening effect caused by a conformational exchange on backbone amide signals for residues in helix H3 and other regions of either the homo- or hetero-dimer, whereas binding of antagonists with similar binding affinities failed to do so. A lineshape analysis of a glucocorticoid receptor-interacting protein 1 NR box 2 coactivator peptide showed that the antagonists enhanced peptide binding to the RXR α LBD homodimer, but to a less extent than that enhanced by the agonists. This was further supported by a lineshape analysis of the RXR C-terminal residue, threonine 462 (T462) in the homodimer but not in the heterodimer. Contrary to the agonists, the antagonists failed to abolish a line-broadening effect caused by a conformational exchange on the T462 signal corresponding to the RXR α LBD-antagonist-peptide ternary complex. These results suggest that the antagonists lack the ability of the agonists to shift the equilibrium of multiple RXR α LBD conformations in favor of a compact state, and that a PPAR γ LBD-agonist complex can prevent the antagonist from enhancing the RXR α LBD-coactivator binding interaction.

Keywords

NMR; ¹H, ¹⁵N, ¹³C; RXR α , PPAR γ , retinoid, conformation, exchange

Introduction

Retinoid X receptors RXR α , β , and γ are members of the nuclear receptor (NR) superfamily of ligand-activated transcription factors. RXRs bind to DNA response elements as either homodimers or obligate heterodimeric partners of several other NRs,^[1] such as

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Supplementary Material Available

Chemical shift values in five tables. This material is available free of charge via the Internet at <http://www.interscience.wiley.com/journal/mrc>

peroxisome proliferator-activated receptors (PPARs), liver oxysterol receptors, retinoic acid receptors (RARs), vitamin D receptor and thyroid hormone receptors, and play central roles in diverse physiological processes ranging from embryogenesis and organogenesis to general metabolism.^[2]

RXR α shares NR family structural organization, which consists of an N-terminal A/B domain that contains a ligand-independent activation function 1, a conserved DNA-binding domain (DBD), a flexible hinge or D domain, and a ligand-binding domain (LBD) that contains a ligand-dependent activation function 2 and a dimer interface.^[1] Crystallographic studies comparing the structures of the unliganded (apo) RXR α LBD^[3] with (holo) RXR α LBD complexed with its natural agonist, 9-*cis*-retinoic acid (9cRA),^[4] support a two-state model of ligand-induced transcriptional activation. In this model, binding of 9cRA induces a dramatic change in the position of the LBD C-terminal helix H12 that creates a binding surface for the short NR-interaction motifs (also called boxes)^[5–9] of coactivator proteins. Recruitment of these coactivator proteins leads to the assembly of multiprotein complexes that serve to modify chromatin and initiate transcription at target gene promoters.^[10] This simple two-state model, however, does not explain why synthetic NR ligands exhibit functional activities that range from partial to pure antagonism.^[11]

In fact, further crystallographic studies of the RXR α LBD have shown that helix H12 can assume a wide range of positions relative to the main body of the protein even after binding agonists.^[12–14] Nuclear magnetic resonance (NMR) and fluorescence anisotropy studies suggest that the NR LBDs exist in a dynamic equilibrium that samples a number of conformational states over time.^[15–17] These studies further suggest that the functional consequences of ligand binding are mediated by modulation of the conformational exchange of helix H12 rather than a simple two-state model.

9cRA lacks specificity by binding and activating both RAR and RXR subtypes.^[18–19] Synthetic RXR-selective ligands (rexinoids) are designed to interact selectively with RXR subtypes. Several rexinoids are reported to have anti-cancer, glucose lowering, insulin-sensitizing or anti-obesity effects in animal models.^[20–22] RXR antagonists derived from the scaffold of the 3'-methyl rexinoid agonist SR11273 (Table 1) demonstrated that 3'-*n*-propyl and longer *n*-alkyl groups conferred RXR antagonism.^[23] Thus far, crystallographic analysis of RXR α LBD complexed with such rexinoid antagonists has been hampered by difficulties in their crystallization.^[15,23] The RXR α H12 mobility in two antagonist-RXR α -RAR α LBD heterodimer complexes was recently probed by fluorescence anisotropy measurements on a fluorescein moiety attached to the C-terminal residue threonine 462 (T462).^[15] However, the conformational exchange in the rest of the antagonist-RXR α LBD complex remains poorly characterized.

To address this question, we embarked on chemical shift analyses of the RXR α LBD homodimer complexed with synthetic rexinoids having transcriptional agonist or antagonist activity in comparison with 9cRA.^[16] To address the concern that a proposed RXR α monomer-homodimer exchange might complicate the conformational analysis, we then analyzed the heterodimer complex of the rexinoid-RXR α LBD and the PPAR γ LBD complexed with a potent selective PPAR γ transcriptional agonist GW1929.^[24] This is because the RXR α -PPAR γ LBD heterodimer is stable and preferred over the RXR α LBD homodimer.^[25] Finally, we examined the interaction of the agonist- and antagonist-RXR α LBD complexes with a glucocorticoid receptor-interacting protein 1 (GRIP1) NR box 2 coactivator peptide by a one-dimensional (1D) lineshape analysis of the peptide and a 2D analysis of the RXR C-terminal residue threonine 462 (T462), which is a useful probe for 9cRA ligand binding-induced recruitment of the GRIP1 peptide.^[17] The results suggest that the antagonists lack the ability of the agonists to shift the equilibrium of multiple RXR α

LBD conformations in favor of a compact state, and that the PPAR γ LBD-agonist complex can prevent the antagonist from enhancing the RXR α LBD-coactivator binding interaction.

Experimental

Materials

9cRA and 2-[2-(pyrid-2-ylcarboxy)phenyl]amino-3-[4-(2-methylpyrid-2-ylamino)ethoxyphenyl]propanoic acid (GW1929) were from Sigma-Aldrich (St. Louis, MO), and [9,10- $^3\text{H}_2$]9cRA from GE Life Sciences (Piscataway, NJ). Rexinoids SR11173, SR11179, BI-1003 and BI-1005 were synthesized as described.^[23] GRIP1 NR box 2 peptide, $^{686}\text{KHKILHRLLQDSS}^{698}$, of greater than 95% purity was from Biomolecules Midwest (Waterloo, IL). Stable isotope-labeled reagents for NMR studies were from Cambridge Isotope Laboratories (Andover, MA). Unlabeled human RXR α LBD (a.a. T223–T462), unlabeled human PPAR γ LBD (a.a. Q203–Y477) and uniformly (U)-[^2H , ^{15}N , ^{13}C]- or U-[^2H , ^{15}N]-enriched RXR α LBD were prepared as described.^[16,25]

Competitive Binding Assays

Binding affinities of 9cRA and synthetic rexinoids in competition with 7.5 nM [11,12- $^3\text{H}_2$]9cRA were determined in duplicate after 14–16 h incubation at 4 °C as described.^[23] Nonspecific [^3H]9cRA binding in the presence of 10 μM unlabeled 9cRA was typically less than 10% of total bound radiolabel. Specific binding was calculated as: (total bound cpm minus nonspecific bound cpm)/(control cpm) $\times$ 100.

Fluorescence Titration

Experiments were performed on PPAR γ and RXR α LBD in gel-filtration buffer G [50 mM NaCl, 10 mM Tris, pH 8.0, 0.5 mM EDTA and 10 mM β -mercaptoethanol (β -ME)] containing 0.01% gelatin using a fluorescence spectrometer (Photon Technology International, Birmingham, NJ) as described.^[25,26]

Rexinoid Transcriptional Activity Assays

CV-1 cell culture, cell cotransfection with the (TREpal) $_2$ -*tk*-CAT reporter vector, RAR α , β , or γ or RXR α expression vector, β -galactosidase expression vector and carrier DNA pBluescript, and subsequent rexinoid treatment were as described.^[23,27] Chloramphenicol acetyl transferase (CAT) activity was expressed relative to β -galactosidase activity to normalize for transfection efficiency and expressed relative to 9cRA.

NMR Sample Preparation

Purified uniformly labeled RXR α LBD homodimer or labeled RXR α -unlabeled PPAR γ LBD heterodimer complex that was prepared as described^[25] was concentrated in an ultrafiltration cell (Amicon, Danvers, MA) under nitrogen at 4 °C to 10 μM to 40 μM , which were estimated by UV measurements. The concentrations of all homodimeric and heterodimeric samples in this work were expressed in term of the RXR α LBD monomer. The sample was added with an aliquot (1.1–1.2 molar equivalent) of 13–50 mM 9cRA-, rexinoid- or GW1929-DMSO- d_6 stock solution, and was further concentrated and then exchanged 4 or 5 times with 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 -ME- d_6 and 5% D $_2\text{O}$). For use in resonance assignments, each final NMR sample contained 0.5–1.0 mM apo-, 9cRA-, SR11173-, BI-1003- or SR11179-U-[^2H , ^{15}N , ^{13}C]-enriched RXR α LBD alone or combined with the GW1929-unlabeled PPAR γ LBD binary complex in 500 μl of NMR buffer.

For the chemical shift perturbation and GRIP1 peptide-binding studies, the buffer exchange resulted in 960- μ l of 0.29 mM apo-, 9cRA-, SR11173-, BI-1003-, SR11179- or BI-1005-U-[^2H , ^{15}N]-enriched RXR α LBD homodimer or RXR α -PPAR γ LBD (complexed with GW1929). First, the oligomerization status and protein concentration of each sample were determined by gel-filtration chromatography, protein assays (BioRad, Hercules, CA) and sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Next, a 0.2-LBD-molar equivalent of each ligand was added to its respective holo-LBD sample to ensure 99% pocket occupancy. Adding excess of ligand at this point was not included in the previous sample preparation.^[17] Finally, one half of the homodimer or heterodimer sample was added with one- or two-RXR α LBD-molar equivalents of 25-mM GRIP1-DMSO- d_6 stock solution, respectively. The other half was added with an equal volume of DMSO- d_6 to serve as a control. The final 500- μ l NMR samples contained 0.26 mM labeled RXR α LBD homodimer or PPAR γ LBD heterodimer plus 20 μ l of DMSO- d_6 , which was kept constant in all samples to minimize the solvent effect.

Analytical Gel-Filtration Chromatography

A 12- μ l aliquot of each 0.26–0.29 mM NMR sample diluted into 500 μ l of buffer G was rechromatographed over a Superdex 200 HR 10/30 column (Amersham, Piscataway, NJ) at 4 °C in a ProStar HPLC system (Varian Analytical, Palo Alto, CA) to verify that the predominant oligomerization state was homodimeric or heterodimeric.

NMR Spectroscopy

All NMR experiments were conducted at 25 °C on either a 600- or 700-MHz four-channel Varian Inova spectrometer equipped with pulsed-field gradient triple resonance probes and employed the transverse relaxation-optimized spectroscopy (TROSY) scheme.^[28] For sequence-specific resonance assignments of SR11173-, BI-1003- and SR11179-RXR α LBD complexes, and apo-, 9cRA-, SR11173- and BI-1003-RXR α -GW1929-PPAR γ heterodimer complexes, 2D ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) and 3D HNCO spectra were collected. In addition, 3D HNCA^[29] and HNCACB^[30] spectra were collected on SR11173- and BI-1003-RXR α LBD homodimer complexes, and 3D HNCA spectra were collected on the 9cRA-heterodimer complex. Data acquisition and processing and spectral display were as described.^[16]

Assignments were performed using a simple similarity search protocol that started with the backbone ^1HN , ^{15}N and ^{13}CO chemical shift assignments for the 9cRA-LBD complex as templates and included three steps: (i) Automatic search within the limits of ^1HN 0.03 ppm; ^{15}N 0.3 ppm; and ^{13}CO 0.2 ppm. (ii) Automatic search of the remaining unassigned data within the ranges of ^1HN , 0.03–0.05 ppm; ^{15}N , 0.3–0.4 ppm; and ^{13}CO , 0.2–0.3 ppm. (iii) Manual similarity search of the remaining unassigned data without specific limits. The cutoff between small and moderate chemical shift perturbations was set as: $\Delta\delta_{\text{RMSD}} = 0.13$ ppm or 0.08 ppm if the ^{13}CO chemical shift was not available,^[25] where the root-mean-squared difference (RMSD) of chemical shift ($\Delta\delta_{\text{RMSD}}$) = $(\Delta\delta_{\text{HN}})^2 + (0.154\Delta\delta_{\text{N}})^2 + (0.341\Delta\delta_{\text{CO}})^2$ with $\Delta\delta_{\text{HN}}$, $\Delta\delta_{\text{N}}$ and $\Delta\delta_{\text{CO}}$ being the ^1HN , ^{15}N and ^{13}CO chemical shift differences' respectively.^[31]

To assess GRIP1 peptide binding, first, 1D ^1H spectra were recorded on 0.26 mM peptide alone or peptide plus apo- or holo-RXR α LBD using the 600-MHz spectrometer and the Watergate pulse sequence,^[32] 10205-Hz spectral width, 18558 complex data points, 32 scans and 1.5-s relaxation delay parameters and were processed with digital solvent suppression and the 3-Hz line broadening window function. Next, 2D HSQC spectra were recorded on the same homodimer or heterodimer samples with and without the peptide and processed as described.^[17] For comparison, the intensity of the peptide-free T462 signal in

the presence of the peptide was normalized to that of the T462 signals in the absence of the peptide in order to minimize effects of small variations in sample concentrations and T462 transverse relaxation times.^[16–17] The sum of intensities was used for normalization in case of multiple T462 signals. Uncertainty in signal intensity measurements was defined as the mean of positive spectral noises plus three times the standard deviation. Uncertainty in signal intensity ratios was estimated by error propagation assuming that intensity measurements were independent.

Results and Discussion

Ligand-Binding Affinities and Transcriptional Activities of Rexinoid Antagonists

Design of RXR transcriptional antagonists 4-[(3'-*n*-butyl-5',6',7',8'-tetrahydro-5',5',8',8'-tetramethyl-2'-naphthalenyl)(cyclopropylidene)methyl]benzoic acid (SR11179) and two 3'-*n*-propyloxy analogues (BI-1003 and BI-1005) was based on the scaffold of the corresponding 3'-methyl analogue SR11173, an RXR-selective transcriptional agonist (Table 1).^[23] Transient transactivation experiments revealed that SR11179, BI-1003 and BI-1005 displayed at most only 1% of the transactivation observed with the parent compound at 10 μ M. Of the three ligands, SR11179 exhibited the strongest antagonist activity in terms of suppressing activation induced by 0.1 μ M 9cRA, whereas BI-1005 exhibited minimal activity.

Assays measuring displacement of radiolabeled [11,12-³H₂]9cRA from His₆-tagged RXR α LBD revealed that the IC₅₀ values for rexinoid agonist SR11173 and antagonist BI-1003 were similar to that for 9cRA. The IC₅₀ values for rexinoid antagonists SR11179 and BI-1005 were about 7- and 18-fold greater, respectively (Table 1). Fluorescence titration experiments were also performed to measure binding of the rexinoid antagonists to the RXR α LBD homodimer (Figure 1). The dissociation constant (K_d) values for SR11173, BI-1003 and SR11179 were similar to that of 9cRA, whereas that of BI-1005 was 17-fold greater (Table 1). Except for SR11179, the relative binding affinities determined by both methods were similar.

None of the rexinoids bound to the PPAR γ LBD, except for BI-1005 (K_d = 170 nM). The K_d values for the remaining rexinoids bound to RXR α LBD when partnered with the PPAR γ LBD-GW1929 complex were the same as those determined for the rexinoids bound to the RXR α LBD homodimer (data not shown).

Rexinoid Antagonist-Induced Chemical Shift Perturbations in the RXR α LBD Homodimer

Although recombinant RXR α has been reported in various oligomerization states ranging from monomer to dimer to tetramer,^[13,33,34] the NMR samples of apo-RXR α LBD and RXR α LBD complexed with 9cRA were recovered predominantly as homodimers.^[16] As shown in Figure 2(a), the NMR samples of 0.29 mM RXR α LBD complexed with the rexinoid agonist SR11173 and the two related antagonists were also recovered predominantly as homodimers after gel-filtration chromatography. The SDS-PAGE analysis confirmed that the concentrations of these samples were roughly identical (Figure 2(b)).

The effects of agonist SR11173 and the two antagonists, SR11179 and BI-1003, on the NMR spectra were initially screened by collecting ¹H-¹⁵N TROSY-based HSQC data on the above samples and comparing the spectra with that of the apo-LBD (Figures 2(c)–2(g)). In our previous NMR study, we assigned backbone amide resonances corresponding to 192 of 226 expected residues in the 9cRA-RXR α LBD complex.^[16] We have now developed a simple similarity search protocol that was primarily based on the 3D HNCO data set for backbone resonance assignments of the rexinoid-RXR α LBD complexes, as described in Experimental Procedures. Using this approach, amide resonances corresponding to 173, 157

and 151 residues of 226 expected residues were assigned in the 3D HNCO spectra of 1.0 mM SR11173-, BI-1003- and SR11179-LBD complexes, respectively. HSQC spectra on 0.26 mM BI-1003- and SR11179-LBD complexes provided 9 and 7 more residues, respectively. Chemical shift values for the rexinoid-LBD complexes are listed in Supplementary Tables S1–S3.

Chemical shift perturbations that were common to binding of both agonists (9cRA and SR11173) and antagonists (BI-1003 and SR11179) included moderate chemical shift perturbations (highlighted in red in Figures 2(h)–2(k)) in resonances corresponding to S312, R316 and I318 in H5, G329 in loop S1-S2, D347 and V349 in the N-terminal half of H7 in the complexes. All of these residues are located in the vicinity of the ligand-binding pocket. Moderate chemical shift perturbations common to binding of both agonists and antagonists were also observed in resonances corresponding to residues not located in the ligand binding pocket and, therefore, not in close contact with the bound ligand. These residues included K245, T246, A252, N253 and D263 in loop H1–H3, which would be consistent with the dramatic differences between the crystal structures of the apo- and 9cRA-LBDs in these regions.^[3–4] Other residues included E239-A241 and E243 in H1, V280 and A283 in the C-terminal half of H3, E352-S355 and M357 in the C-terminal half of H7, A372, F376 and N377 in H8, L418 and R426 in H10.

In the crystal structure, the homodimer interface comprises residues in helices H7, H9 and H10, and the loop H8-H9.^[3] No differences between the chemical shift perturbations corresponding to these residues were observed for either agonist (9cRA and SR11173) or antagonist (BI-1003 and SR11179) complexes with exception of V396 and Y397 in H9, which were undetectable in both the apo- and SR11179-LBD homodimer complexes.

The residues corresponding to crosspeaks that were missing in the apo-LBD but observed after binding ligand are mapped in orange in Figures 2(h)–2(k). Comparison of the HNCO spectra of the RXR α LBD homodimer complexed with 9cRA or one of the three rexinoids revealed that fewer resonances emerged on binding of the rexinoids than on binding of 9cRA (specifically crosspeaks corresponding to V265, C269, Q270, A272-K274, L276-T278, E281 and W282 in H3, L451-L455 in H12 were absent in the three rexinoid complexes (highlighted in gray in Figures 2(h)–2(j)) but were present in the 9cRA complex (highlighted in orange in Figure 2(k))). Inspection of the HNCO spectra also demonstrated that fewer resonances emerged after binding of antagonists than agonists (specifically crosspeaks corresponding to T266-I268 and Q275 in H3, R302 in H4, and W305, L308, F313 and S314 in H5 were absent in the two antagonist complexes (highlighted in gray in Figures 2(h)–2(i)) but were present in the two agonist complexes (highlighted in orange in Figures 2(j)–2(k))). Four of these residues (I268, Q275, W305 and F313) are predicted to be in close contact (~ 4.2 Å) with bound 9cRA on the basis of the RXR α LBD-9cRA crystal structure (PDB: 1fby).^[4]

In summary, the chemical shift analysis showed that contrary to the two agonists, the two antagonists lack the ability to abolish line-broadening effect on the signals for residues in discrete regions, such as helix H3 that is distal from the dimer interface and is a component of the coactivator-binding surface. As described in the following sections, a differential modulation of a conformational exchange process by the agonist and antagonist likely contributed to the distinct line-broadening effects, which correlated with different receptor-coactivator peptide binding interactions in the agonist and antagonist complexes. Although the line-broadening pattern is consistent with the H12 mobility model, other more local conformational exchange models cannot be ruled out. This is because the absence of resonances made it difficult to measure the chemical shift difference, which might otherwise confer information about the structural difference between the apo and holo complexes.

PPAR γ LBD-Induced Chemical Shift Perturbations

We observed that complexes of 0.29 mM PPAR γ LBD-GW1929 and RXR α LBD-9cRA or a rexinoid were uniformly recovered as heterodimers (see Figure 3(a)) except in the case of SR11179. Concentration caused the SR11179-heterodimer complex to almost completely precipitate out of the buffer. Thus, study of the SR11179 sample was not pursued. The SDS-PAGE analysis confirmed that the RXR α and PPAR γ LBD concentrations of these NMR samples were roughly identical (Figure 3(b)).

Analysis of signals in the 3D HNCO spectra of apo-, 9cRA-, SR11173- and BI-1003-heterodimer complexes led to the identification of 117, 186, 165 and 147 RXR α LBD residues, respectively. Ten more residues were identified in the HSQC spectrum of the BI-1003-heterodimer complex. Chemical shift values for the spectra of these complexes are listed in Supplementary Tables S1-S5.

Partnering the RXR α LBD with PPAR γ LBD (complexed with GW1929) in the heterodimer instead of with itself in the homodimer resulted in chemical shift perturbations in a subset of crosspeaks (see Figures 3(c)–3(f)). Moderate chemical shift perturbations (red in Figures 3(g)–3(j)) were observed for amide crosspeaks corresponding to residues located at the dimer interface in the crystal structures of RXR α LBD partnered with itself and with the PPAR γ LBD.^[3,13,36] These included crosspeaks corresponding to E352, S355 and K356 in H7, S380 in loop H8-H9, V389, E394, L400 and E401 in H9, A416, A424 and L425 in H10 of RXR α . Moderate chemical shift perturbations were also observed for crosspeaks corresponding to RXR α residues that are remote from the dimer interfaces in the crystal structures (PDBs: 1g1u and 1fm6). These RXR α residues included A226, E228-M230 in the LBD N-terminal region, E233, E237 and A238 in H1, I299 and L301 in H4, G323 in β -strand S1, M360 and D363 in loop H7-H8, I373, V374, F376 and N377 in the C-terminal half of H8, C404, K405 and Y408 in the C-terminal half of H9.

The emergence of RXR α LBD resonances corresponding to residues located in the vicinity of the ligand-binding pocket and in the activation helix, H12, was observed in the spectra of the RXR α LBD-PPAR γ LBD(GW1929) heterodimer compared to spectra of the corresponding RXR α LBD homodimer (orange in Figures 3(g)–3(j)). These residues included (i) W305, L308, L309, I310 and F313 in H5 in the antagonist BI-1003-bound heterodimer but not in its corresponding homodimer; (ii) L276, E281 or W282 in H3 in the heterodimer bound by either antagonist BI-1003 or agonist SR11173 compared to each's corresponding homodimer; and (iii) M452, E453 or M454 in H12 of the heterodimer without an RXR ligand or bound with either the rexinoid agonist or the antagonist.

Fewer resonances emerged after binding of BI-1003 than either 9cRA or agonist SR11173. Specifically, resonances corresponding to T266-I268 and Q275 in H3, R302 in H4, and W305, L308, F313 and S314 in H5 of the heterodimer emerged after binding by either agonist 9cRA or SR11173, but not after binding by antagonist BI-1003. As noted above, residues I268, Q275, W305 and F313 are located within the ligand-binding pocket of the RXR α LBD-9cRA crystal structure.^[4]

In summary, the antagonist BI-1003 showed the same inability to abolish the line-broadening effect on the signals for RXR H3, H4 and H5 residues in heterodimer as in homodimer, indicating that a conformational exchange within the antagonist-RXR α LBD subunit rather than a monomer-dimer exchange was the primary cause for such a line-broadening effect.

Effect of Rexinoid Antagonist on GRIP1 Peptide Binding by the RXR α LBD Homodimer

GRIP1 peptide binding to the deuterium-labeled RXR α LBD was first monitored by recording 1D ^1H spectra on the same samples that were used to record the 2D HSQC spectra. In principle, a small peptide such as GRIP1 peptide tumbles rapidly in solution, giving rise to sharp NMR signals with narrow line-widths. On interacting with a large protein, its tumbling rate decreases, thereby giving rise to broader, less intense signals. Consequently, coactivator peptide binding by the RXR α LBD induced by ligand can be determined by monitoring decreases in free peptide signals. Examination of fluorescein-tagged coactivator peptide binding by the RXR α LBD using fluorescence anisotropy is based on the same phenomenon.^[15,37]

By using U- ^{2}H , ^{15}N -enriched RXR α LBD, in which deuterons replace aliphatic protons (> 99% D), free GRIP1 peptide ^1H signals were not obscured and could be monitored. A stack plot of 1D ^1H spectra of the peptide alone and in the presence of apo-, SR11179-, BI-1003-, SR11173- and 9cRA-LBDs is shown in Figure 4. Positions of free peptide signals (asterisks in Figure 4) remained constant, whereas their intensities decreased from top to bottom. These results suggest that peptide binding occurred in a slow exchange regime on the chemical shift timescale and that the decline in free-peptide signal intensities correlated with peptide-LBD binding.^[17] New signals indicating bound peptide (arrows in Figure 4) were observed in the spectra for the agonist-bound complexes with 9cRA and SR11173 but not for those with bound antagonist (BI-1003 and SR1179).

Next, GRIP1 binding to the RXR α LBD homodimer was probed by monitoring RXR α LBD resonances. We focused primarily on changes in the C-terminal T462 resonances for monitoring the interaction of the C-terminal region with the coactivator peptide.^[17] As shown in Figure 5(a), the T462 amide resonance appeared as a single intense crosspeak in the 2D HSQC spectrum of the apo-RXR α LBD homodimer. Binding of 9cRA corresponded with the appearance of an additional minor T462 crosspeak with a relative intensity of 19:1 (major:minor) in the HSQC spectra. This minor crosspeak was not detected without an excess of 9cRA in the sample.^[17] Binding of SR11173 corresponded with the appearance of two additional crosspeaks with relative intensities of 10:8:5. Because the SR11173-LBD complex was predominantly a homodimer under the condition (Figure 2(a)), the three T462 crosspeaks likely corresponded to three different conformational states. In contrast, after binding of antagonist SR11179 or BI-1003 the crosspeak signal corresponding to T462 was not significantly altered in the resulting HSQC spectrum compared to that of the apo-RXR α homodimer.

The addition of a molar equivalent of GRIP1 coactivator peptide to the apo-RXR α LBD homodimer, as well as to the homodimer complexed with agonist 9cRA or SR11173 or antagonist BI-1003 or SR11179 resulted in significant attenuation of the dominant peptide-free crosspeak corresponding to T462 (see Figure 5(b)). The peptide-free signals in the spectra of the antagonist SR11179- and BI-1003-LBD complexes were attenuated to a greater extent than that in the spectrum of the apo-LBD. The intensity ratios before and after addition of the GRIP1 peptide revealed a following attenuation order: apo < SR11179 ~ BI-1003 < SR11173 ~ 9cRA (Figure 5(c)), which agreed with the decline in free-peptide signal intensities shown in Figure 4.

Addition of the GRIP1 peptide in the absence (apo) or presence of 9cRA and agonist SR11173 resulted in the emergence of new T462 crosspeaks corresponding to the peptide-bound complex (Figure 5(b)), but the crosspeak in the apo-LBD was much weaker than those in the presence of the agonists. In contrast, addition of the GRIP1 peptide in the presence of the antagonists did not result in the emergence of a new crosspeak corresponding to the peptide-bound complex. This result suggests that the line-broadening

effect was caused by a conformational exchange within the antagonist-LBD-peptide ternary complex rather than the peptide binding exchange because the latter exchange would have led to a more severe line-broadening effect on T462 for the apo-LBD-peptide binary complex than the antagonist-LBD-peptide ternary complex.

In summary, the results suggest that antagonists and agonists differentially modulate the conformational exchange of T462 in the binary and ternary complexes, and that the two antagonists enhanced peptide binding to the RXR α LBD homodimer, but to a less extent than that enhanced by the agonists.

Effect of Rexinoid Antagonist on GRIP1-Peptide Binding by RXR α LBD-PPAR γ LBD Heterodimer

As noted above, resonances corresponding to several residues in H12 of the RXR α LBD emerged when partnered with GW1929-PPAR γ LBD, regardless of the RXR α ligand. When partnered with GW1929-PPAR γ LBD, only a single T462 crosspeak was observed in the 2D HSQC spectrum of the apo-RXR α LBD or when the holo complex with 9cRA, agonist SR11173, and antagonist BI-1003 (Figure 6(a)). The same degree of attenuation of signal was observed after the addition of GRIP1 peptide to the homodimer and heterodimer complexed with 9cRA or the agonist (Figure 6(b)). However, less attenuation of signal was observed in the heterodimer compared to the homodimer complexed with antagonist BI-1003. In fact, the intensity ratio before and after addition of the GRIP1 peptide to the SR11179-heterodimer complex is the same as that for the apo-homodimer (Figure 6(c)). A similar analysis of the heterodimer-SR11179 complex was not conducted because it precipitated. In contrast, a greater degree of attenuation by GRIP1 peptide was observed in the apo-heterodimer compared to the apo-homodimer. Therefore, the results suggest that the binding of the GW1929-PPAR γ LBD to the RXR α LBD may restrict the conformational flexibility of T462 and residues in helix H12, and prevent the antagonist from enhancing the RXR α LBD-coactivator binding interaction.

Conclusion

This present study represents our analysis of the effect of antagonists on this dynamic equilibrium using NMR spectroscopy of RXR α LBD complexes. This approach provides an opportunity to probe the conformational exchange of the RXR α LBD along the whole length of the protein sequence and over a range of time scales. A number of investigators including this laboratory have observed that binding of agonists by NRs is associated with the emergence of formerly missing crosspeaks in their NMR spectra.^[16,38–39] The most likely explanation for the missing crosspeaks and their subsequent emergence after ligand binding is that the peak intensities of the missing residues were reduced by line broadening caused by conformational exchange prior to ligand binding. Under the conditions of the NMR experiments, the RXR α LBD alone formed a homodimer and in the presence of the PPAR γ LBD formed a heterodimer. While we cannot exclude ongoing association and dissociation of the RXR α LBD subunits to form monomers and tetramers, the exchange processes likely represent intra-subunit exchanges rather than inter-subunit ones. We conclude that RXR agonists, but not RXR antagonists, restricted conformational exchanges involving residues located on helices H3, H4 and H5.

The rexinoid antagonists used in this study differ from the parent 3'-methyl-substituted agonist by having longer 3'-alkyl or alkoxy substituents.^[23] Fewer resonances, either within or removed from the ligand-binding pocket, emerged after binding of antagonists by the RXR α LBD homodimer and the RXR α -PPAR γ heterodimer than after binding of agonists. These results suggest that binding of antagonists did not restrict conformational exchange involving residues in H3, H4 and H5 to the extent observed for the agonists. A prior

computational study had suggested that the position of the tetrahydrotetramethylnaphthalenyl (TTN) ring of SR11179 underwent a 180° flip on docking to the ligand-binding pocket.^[23] Such a rotation may partly account for the persistent line broadening of the signals corresponding to residues predicted to directly contact the bound ligand. However, the persistent line broadening observed for residues that are not predicted to directly contact the bound ligand suggests that the effect on conformational exchange is more global.

Our previous NMR studies of 9cRA binding to the RXRα LBD homodimer revealed that, while binding of 9cRA stabilized the ligand-binding pocket as evidenced by the emergence of crosspeaks corresponding to residues within the ligand-binding pocket, the effect of 9cRA binding on the C-terminal T462 resonances was minimal.^[16] The dynamics of the T462 residue was significantly restricted only in the LBD-9cRA-peptide ternary complex based on the appearance of T462 crosspeaks in the NMR spectra and direct measurements of backbone dynamics.^[17] In the present study, the intermediate state(s) between the loose (apo) and compact (ternary) states for T462 were observed in the 9cRA- and SR11173-LBD binary complexes when the ligand-binding pocket was fully occupied. These intermediate states were all converted to the compact state in the ternary complexes. In contrast, the two antagonists SR11179 and BI-1003 failed to induce the intermediate and compact states for T462 in the binary and ternary complexes, respectively. These observations were consistent with fluorescence anisotropy studies on how agonists and antagonists differentially modulated the conformational exchange of the RXRα LBD C-terminal helix H12 onto which a fluorescein label had been attached.^[15] Together, these studies suggest that the antagonists lack the ability of the agonists to shift the equilibrium of multiple RXRα LBD conformations in favor of a compact state.

The measured transcriptional antagonistic activity of BI-1003 correlated with the result from the lineshape analyses on heterodimer but not on homodimer. The discrepancy is likely attributed to a smaller homodimer interface^[3,13] than the heterodimer interface^[36] and the agonist GW1929 bound to the PPARγ LBD subunit of the heterodimer. Future NMR studies using a PPARγ antagonist, a RXRα LBD mutant with an enhanced homodimerization activity^[40] and a RXR construct that includes both the DBD and LBD so that the receptors, ligands, the cognate DNA and the coactivator peptide can be assembled in a test tube will resolve the discrepancy and shed new light on RXR transcriptional antagonism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used are

1D, 2D and 3D

1-, 2- and 3-dimensional

9cRA

9-*cis*-retinoic acid

β-ME	β-mercaptoethanol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
HNCA, HNCACB, HNCO	backbone amide proton (H) and nitrogen (N), alpha (CA), beta (CB) and carbonyl carbon (CO) correlation
HSQC	heteronuclear single quantum correlation
K_d	dissociation constant
LBD	ligand-binding domain
LBP	ligand-binding pocket
NMR	nuclear magnetic resonance
NR	nuclear receptor
PPAR	peroxisome proliferator-activated receptor
RAR	retinoic acid receptor
RMSD	root-mean-squared difference
RNA	ribonucleic acid
RXR	retinoid X receptor
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TROSY	transverse relaxation-optimized spectroscopy

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Figure 1.

Dissociation constant (K_d) values were determined by fluorescence titration measurements. RXR LBD tryptophan fluorescence emissions at $\lambda = 321$ nm were recorded before and after addition of aliquots of the synthetic rexinoid transcriptional antagonist BI-1003 (squares) or BI-1005 (diamonds), corrected for inner filter effects and normalized to that of the LBD before ligand addition (apo). Curves were calculated using the one-site-per-LBD binding equation.

Figure 2.

Effects of ligand binding on oligomeric status and chemical shift perturbations of RXRα LBD signals. (a) Chromatograms of 0.29 mM apo-LBD, uniformly (U)-[^2H , ^{15}N]-enriched RXRα LBD and ligand-(U)-[^2H , ^{15}N]-enriched RXRα LBD at 1.2 ligand-to-LBD molar ratios are shown stacked to demonstrate that RXR homodimer formation was favored regardless of ligand binding status. Samples (12 μl) were diluted into 0.5 ml of buffer G immediately before high-performance chromatographic analyses. (b) SDS-PAGE of equal volumes (0.6 μl per lane) of these samples followed by Coomassie staining of protein bands shows that their protein concentrations are equivalent. (c) Overlay of 600-MHz 2-dimensional heteronuclear single-quantum correlation (HSQC) spectra for 0.26 mM apo-

(magenta) and SR11173-LBDs (black). Spectra here and in Fig. 4 were processed and are displayed in the same manner. (d)-(g) Expanded regions of the HSQC spectra, shown boxed in (c), for 0.26 mM SR11173-, 9cRA-, SR11179- or BI-1003-LBDs (black), respectively, superposed on that for the apo-LBD (magenta). Peaks are designated by amino acid one-letter codes and human RXR α sequence numbers. (h)-(k) Chemical shift perturbations induced by SR11179, BI-1003, SR11173 and 9cRA binding, respectively, to the RXR α LBD in solution were mapped onto the ribbon diagram of the crystal structure of the RXR α LBD complexed with 9cRA (space-filling format) (PDB: 1fby). Helices H1 and H3-H12, β -strands S1 and S2, the N-terminus and P458 are labeled. P458 is the last visible C-terminal residue in this crystal structure. The invisible (disordered) segment K245-N262 between H1 and H3 is replaced by a gray curve. Color coding: red and blue, residues having moderate and small changes in chemical shift positions, respectively; orange, residues assigned only in the retinoid-LBD binary complex; gray, proline residues and unassignable residues in both apo- and ligand-LBDs; gray and red balls, C and O atoms in 9cRA, respectively. Molecular images were generated using MOLMOL.^[35]

Figure 3.

Chemical shift perturbations of RXRα LBD signals induced by heterodimerization with the agonist GW1929-PPARγ LBD complex. (a) Chromatograms recorded for samples containing 0.29 mM apo- or liganded-(U)[²H, ¹⁵N]-enriched RXRα LBD combined with GW1929-unlabeled PPARγ LBD complex at 1.2 ligand-to-LBD molar ratios are shown stacked to demonstrate only minimal levels of RXR self-oligomerization existed. Samples (12 μl) were diluted into 0.5 ml of buffer G and immediately chromatographed. (b) SDS-PAGE of equal volumes (0.6 μl per lane) of the samples in (a) demonstrating their equivalency of protein concentration. (c)–(f) Overlays of expanded regions of the HSQC spectra for 0.26 mM apo-, 9cRA-, SR11173- and BI-1003-RXRα LBDs alone (magenta)

and in the presence of GW1929-unlabeled PPAR γ LBD complex (black). (g)–(j) Chemical shift perturbations induced by GW1929-PPAR γ LBD binding to the apo-, BI-1003-, SR11173- and 9cRA-RXR α LBDs, respectively, were mapped onto the crystal structure of the RXR α LBD complexed with 9cRA (space-filling model) (PDB: 1fby) and are labeled and color-coded as in Figure 3, except that orange residues are those assignable only in the presence of the holo-PPAR γ LBD and gray residues also include those unassignable in the presence, but assignable in the absence, of the holo-PPAR γ LBD.

Figure 4.

GRIP1 coactivator peptide binding by the RXR α LBD monitored by 1D ^1H NMR. Stack plot of aliphatic regions of 1D ^1H spectra for 0.26 mM GRIP1 peptide alone and combined with a molar equivalent of apo-, SR11179-, BI-1003-, SR11173- and 9cRA-U-[^2H , ^{15}N]-enriched RXR α LBDs. Asterisks and arrows indicate LBD-free and LBD-bound peptide signals, respectively. A curly line indicates truncation of DMSO- d_6 solvent signal at 2.65 ppm.

Figure 5. RXRα C-terminus, threonine 462 (T462), signals act as sensors for GRIP1 peptide binding. (a) and (b) T462 crosspeak(s) in 2D HSQC spectra of 0.26 mM apo-, SR11179-, BI-1003-, 9cRA- and SR11173- $U\text{-}[^2\text{H}, ^{15}\text{N}]$ -enriched RXRα LBDs alone (a) and with (b) an equivalent molar amount of GRIP1 peptide. 1D ^1H slices were taken at each peak. Numbered, peptide-free and peptide-bound crosspeaks for T462 are labeled with 1–3, f and b, respectively. Asterisks designate artifacts. (c) T462 intensity ratios between the intensities of f-crosspeaks in (b) and the sum of intensities in (a) measured in present (gray) and prior (black) studies.^[17] Error bars were calculated from spectral noise as described in Experimental Procedures.

Figure 6.

Agonist GW1929-PPAR γ LBD complex blocks GRIP1 coactivator peptide binding to the antagonist BI-1003-RXR α LBD complex. (a) and (b) T462 crosspeaks in 2D HSQC spectra for 0.26 mM apo-, BI-1003-, 9cRA- and SR11173- $U\text{-}[^2\text{H}, ^{15}\text{N}]$ -enriched RXR α LBD samples containing a molar equivalent of GW1929-unlabeled PPAR γ LBD complex alone (a) and combined with (b) 2.0 molar equivalents of GRIP1 peptide. 1D ^1H slices were taken at the peak of each T462 signal. Peptide-free and peptide-bound crosspeaks for T462 are labeled f and b, respectively. (c) T462 signal intensity ratios between intensities of f-crosspeaks in (b) and crosspeak sums in (a) measured for apo- and holo-RXR α LBDs (gray) and apo- and holo-RXR α -GW1929-PPAR γ (black) complexes.