Retinoid X receptor α (RXRa) Helix 12 Plays an Inhibitory Role in the Recruitment of the p160 Co-activators by Unliganded RXRa/Retinoic Acid Receptor α Heterodimers*

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The nuclear hormone receptors comprise a large family of transcription factors that regulate animal development, cell proliferation, and cell differentiation through the control of a network of genes involved in these processes (1, 2). Nuclear receptors harbor two evolutionarily conserved domains, DNA binding domains (DBDs)† and ligand binding domains (LBDs). Whereas the DBD recognizes specific DNA sequences, the LBD dictates transcriptional activity by nuclear receptors through recruitment of co-repressors such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor or co-activators, including p160 family proteins (3, 4).

Nuclear hormone receptors have been further subdivided into four classes based on their mode of action and DNA binding status (1). Retinoic acid receptors (RARs) and Retinoid X receptors (RXRs) are class II receptors that mediate retinoid signaling by regulating a complex gene network. RARs primarily form heterodimers with RXRs on DR5 (direct repeat spaced by 5 base pairs) or DR1-containing sequences. Conversely, RXRs are capable of forming homodimers on DR1 or heterodimers with other class II nuclear hormone receptors (1). The ability of RXRs to form homodimers or heterodimers with other receptors generates a complex mechanism by which the ligands of RXR may or may not exert effects on target genes, depending on the dimerization partner of the receptor (5). Receptors such as RARs and TRs (thyroid hormone receptors) dimerize with RXRs, generating a non-permissive complex in which RXR ligands are not capable of activating heterodimers. In such heterodimeric complexes, RXRs are referred to as silent partners. The mechanisms underlying this observation are currently unclear, but it has been proposed that RXRs within these heterodimers cannot bind their agonists (6, 7). In contrast, permissive complexes between peroxisome proliferating activator receptors (PPARs) or vitamin D receptors and RXRs can be activated solely by RXR ligands.

Despite divergence in the primary sequences of LBDs of nuclear receptors, the structures of several LBDs are remarkably conserved (8, 9). Structural comparison between RXRa apo-LBD (hormone-free) (10) and RXRγ holo-LBD (hormone-bound) (11) indicates that ligand binding triggers a conformational change within the receptors in which helix 12 of the RXR LBD makes a lid to a cavity containing helices 3, 4, and 5. Recent crystal and molecular studies have suggested that such a conformational change by helix 12 creates an interface for co-activator binding (12–14). Although the complex structure of the antagonist-bound PPARα and co-repressor peptide has been solved (15), co-crystal structures between co-activators and apo-receptors are unavailable. Nonetheless, biochemical and molecular studies indicate that the binding pockets for

all-trans-retinoic acid; Y2H, yeast two-hybrid assay; Y3H, yeast two-hybrid assay; M2H, mammalian two-hybrid assay; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; RID, receptor interaction domain; RARE, retinoic acid response element; 9-cis-RA, 9-cis-retinoid acid; ACTR, activator for thyroid hormone and retinoid receptor; T3, thyroid hormone.
co-repressors and co-activators largely overlap (16–19). It is widely believed that, physiologically, class II receptors form high affinity DNA binding transcription factors as heterodimers, rather than as monomers (1). The high resolution structure of the liganded RXRa/RARs heterodimeric complex is now available (20) and provides insight into the molecular mechanism of dimerization; however, the crystal structure of the apo-heterodimer is currently unavailable, likely due to the unstructured helix 12. Therefore, understanding the roles of the helix 12 of dimerization partners in co-regulator recruitment is key to elucidating the molecular basis of transcriptional regulation by class II receptors.

In this report, we map the critical residues in the helix 12 of RARs and RXRs for co-regulator association and transcriptional regulation by both receptors. Interestingly, although several mutants exhibit ligand-independent association with the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that LG268, a known RXR-specific ligand, promotes co-activator dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor. Our data indicate that the co-activator, these mutants also acquire ligand-insensitive dissociation from the co-repressor.

**MATERIALS AND METHODS**

**Plasmid Construction**—The plasmids, pGBT9-SMRT (RID), pGBT9-ACTR (RID), pACTII-RXRa, pCMX, pCMX-RARa, pCMX-RRXa, pCMX-TRβ, pCMX-Gal4, pCMX-Gal4-SMRT (RID), pCMX-Gal4-ACTR (RID), pCMX-Gal4-RARa, pCMX-Gal4-RRXa (LBD), pCMX-VP16, pCMX-VP16-RARa, pCMX-LacZ, βRAR-TR-Luc, γSMRT-TR-Luc, αSMRT-TR-Luc, and wild-type βRARα were translated from cDNA of wild-type human RXRa. Histidine tagged GST fusion proteins were added, followed by an additional 30 min of incubation. The reaction mixtures were loaded on a 5% polyacrylamide (29:2:0.8) non-denaturing gel followed by electrophoresis in 0.5% TBE buffer (50 mM Tris, 50 mM boric acid, 25 mM EDTA pH 8.0). After electrophoresis, the gel was dried and subjected to autoradiography.

**RESULTS**

Association of RARα Helix 12 Mutants with Co-repressors—To investigate the roles of helix 12 in nuclear receptor function, we focused on helix 12 of RARα and RXRa. Fig. 1A shows an alignment of helix 12 sequences from several class II nuclear receptors. In this study, mutants were generated in the conserved residues of RARα helix 12. We first tested whether these mutants bound SMRT by EMSAs. GST-SMRT (receptor interaction domain (RID)) fusion protein was expressed in bacteria and subsequently affinity-purified (17). EMSAs were conducted using a [32P]-labeled DNA duplex containing a DR-1 element of the retinoic acid response element (bRARE) of human RARβ2 gene. Both RXRa and wild-type or mutant RXRa were translated in vitro and incubated with the [32P]-labeled probe and purified GST-SMRT in the absence or presence of increasing concentrations of AT-RA. Wild-type RXRa displayed a dose-dependent hormone-induced dissociation from SMRT (Fig. 1B, lanes 1–4), whereas P408L (lane 9) and E412A (lane 17) bound SMRT weakly, and this binding was dramatically decreased in the presence of 10 μM AT-RA (lanes 10 and 18). Furthermore, RARα Δ403 (lanes 5–8), L409A (lanes 13–16), and M413A (lanes 21–24) did not fully dissociate SMRT, even at 1 μM AT-RA. These data indicate that Pro-408 and Gla-412 play an important role in co-repressor binding and that Leu-409 and Met-413 are critical for hormone-dependent dissociation of co-repressor.

We next analyzed the association of co-repressor with helix 12 deletion (Δ403) and point mutants by mammalian two-hybrid (M2H) assays using Gal4-SMRT (RID) and VP-RARα (LBD) along with a reporter construct (pMH100) containing multiple Gal4 binding sites upstream of a TK basal promoter and the firefly luciferase gene. As shown in Fig. 1C, all helix 12 mutants showed high reporter activity, suggesting that these mutants bound SMRT in the absence of all-trans-retinoic acid (AT-RA), although the degree of association varied among the mutants. In the presence of 10 nM AT-RA, wild-type RARα and several of the mutants displayed a dramatic reduction of the reporter activity. In contrast, significant amounts of the reporter activity were detected for RARα Δ403, L409A, and M413A in the presence of 10 nM AT-RA, and 50% of RARα Δ403 was still associated with SMRT at 1 μM AT-RA. These results suggested that RARα Δ403 acquired aberrant ligand-insensitive co-repressor binding. Similarly, the reporter activity was diminished for point mutants L409A and M413A only at 1 μM AT-RA, suggesting that complete dissociation of SMRT required more than 1 μM AT-RA. In contrast, P408L and E412A displayed less reporter activity, indicating that they bound SMRT more weakly than that of wild-type RARα, but this binding was readily disrupted in the presence of 10 nM AT-RA. These results are consistent with those of EMSAs.
Hormone-dependent Co-activator Association with RARα Helix 12 Mutants—The ability of RARα helix 12 mutants to recruit co-activators such as ACTR was examined by EMSAs and M2H assays. EMSAs were carried out as described in Fig. 1B except that GST-ACTR (RID) fusion protein was used (Fig. 2A). Wild-type RARα exhibited a hormone-dependent association with ACTR, whereas mutants Δ403 (lanes 5–8), L409A (lanes 13–16), and E412A (lanes 17–20) completely lost ACTR binding activity. Unexpectedly, we found that unliganded P408L bound weakly to ACTR (lane 9) and no further ACTR binding was induced with or without increasing concentration of AT-RA by mammalian two-hybrid (M2H) assays. The reporter activity of Gal4-SMRT with VP16 alone was defined as one. Activation was calculated as the ratio of the reporter activity of Gal4-SMRT ID I + II with VP16-RARα/the reporter activity in the presence of Gal4-SMRT ID I + II with VP16 alone.

M2H assays were conducted as described in Fig. 1C using pCMX-ACTR (RID) and VP-RARα (LBD). The association of RARα with ACTR was measured in the presence of increasing concentrations of AT-RA. In the absence of AT-RA, no reporter activity was detected, suggesting no association with ACTR (RID) was detected for wild-type or any of the mutant RARαs (LBD) (Fig. 2B). In the presence of AT-RA, the reporter activity was increased, indicating that wild-type RARα bound ACTR in a dose-dependent manner. However, we found that all mutants exhibited much lower reporter activity than that of the wild-type even in the presence of 1 μM AT-RA. These data suggested that RARα Δ403, L409A, and E412A possess defects in co-activator binding. Similarly, P408L and M413A dramatically lost their ability to bind ACTR. The aberrant association of these mutants with SMRT and ACTR suggested that RARα helix 12 mutants have a defect in hormone-dependent transcriptional regulation. Transient transfection assays were employed to examine the ability of helix 12 mutants to regulate transcription by RARα. Wild-type and mutant RARα were fused to the yeast Gal4 DNA-binding
Fig. 2. Association of ACTR and mutants of RARα helix 12. A, association of RARα helix 12 mutants with ACTR on DR5 elements. ACTR binding was assayed by EMSA assays on DR5 in the presence of increasing concentrations of AT-RA similar to that of Fig. 1B. Ligand-dependent association of RARα with ACTR. Association of ACTR (RID) with RARα mutants was determined with or without increasing concentrations of AT-RA by mammalian two-hybrid (M2H) assays. The reporter activity of Gal4-SMRT with VP16 alone was defined as one. The -fold activation was calculated as the ratio of the reporter activity of Gal4-SMRT with VP16-RARα/helix 12 mutants with ACTR binding/cis-AT-RA similar to that of Fig. 1C. Assays were carried out similar to that of Fig. 1C.

Association of RARα Helix 12 Mutants with Co-regulators—Having determined the effects of mutations of RARα helix 12 on co-regulator binding and transcriptional activity, we next examined the properties of RARα helix 12 mutants. We first determined whether RARα helix 12 is critical for co-regulator recruitment in yeast two-hybrid (Y2H) assays. Because RXRs are capable of dimerizing with a set of nuclear receptors in mammalian cells and because yeast does not express nuclear receptors, Y2H assays provide a system to examine co-regulator association that excludes possible bridging effects by other receptors. Y2H expression vectors, pACTII-RXRα (LBD) and pGBT9 (Gal4 DBD)-SMRT (RID) or pGBT9-ACTR (RID), were co-transformed into yeast. The ability of these mutants to interact with co-regulators was measured in the absence or presence of ligand. In the absence of RXR agonists, β-galactosidase was expressed in cells co-transformed with pGBT9-SMRT (RID) and pACTII-RXRα (LBD) (Fig. 4A), whereas little β-galactosidase activity was detected in experiments with only one of the proteins. Although addition of LG268 did not have an effect on the interaction, 9-cis-RA moderately induced interaction between RARα and SMRT. These data suggest that, in contrast to RAR ligands, which induce dissociation of corepressors from RAR, RXR ligands do not disrupt RARα-corepressor association.

Mutations at the conserved residues within RARα helix 12 displayed varying effects on SMRT binding (Fig. 4B). Although mutants F450A and M454A/L455A completely abolished association with SMRT, mutant L451A increased SMRT binding activity, and this interaction was partially inhibited in the presence of 9-cis-RA or LG268. Deletion of helix 12 (∆443) strongly enhanced hormone-independent co-repressor interaction, whereas 9-cis-RA or LG268 reduced the interaction by
Our results suggest that helix 12 plays an important role in co-repressor recruitment by RXR. Similar assays were also carried out to examine association between ACTR and wild-type or mutant RXR. Expression of pGBT9-ACTR or pACTII-RXR alone did not activate -galactosidase expression with or without ligands (data not shown). In the absence of 9-cis-RA or LG268, a high level of -galactosidase activity was measured in cells expressing pGBT9-ACTR and pACTII-RXR, indicating that unliganded RXR interacts with ACTR (Fig. 4C). Moreover, 9-cis-RA and LG268 induced -galactosidase expression an additional 3- to 4-fold, suggesting that both 9-cis-RA and LG268 were capable of promoting further association between RXR and ACTR. Mutations or deletion of helix 12 completely abolished ligand-independent and ligand-induced RXR-ACTR interactions. These results indicate that helix 12 is essential for both hormone-independent and -dependent recruitment of co-activators by RXR in yeast.

The effect of helix 12 mutations on transcriptional regulation of RXR was examined by transient transfection assays as described in Fig. 3A. Reporter activity was measured in the absence or presence of an RXR-specific agonist, LG268. Fig. 4D shows that, in the absence of ligand, Gal4-RXR weakly activated transcription. Mutations at the conserved residues had little effect on unliganded RXR activity, except A443 and L451A, which possessed moderate repression activity, consistent with their strong association with SMRT in yeast. In the presence of LG268, Gal4-RXR was able to activate transcription, but mutation or deletion of helix 12 dramatically reduced this activation. These data are consistent with the protein-protein interaction data from Y2H assays and suggest that helix 12 is critical for hormone-mediated transcriptional activation by Gal4-RXR.

**RXR Helix 12 Is Required for AT-RA-mediated Activation by RXR/RAR**—The primary sequence of RXR helix 12 is similar to that of other class II receptors, yet the role of RXR helix 12 in transcriptional regulation by class II nuclear receptors is unclear. AT-RA is an RAR-specific ligand and is thought to be permissive in AT-RA-mediated transactivation by RXR/RAR heterodimers. In contrast, RAR-specific ligands such as LG268 do not activate transcription by RXR/RAR and therefore RXR is a non-permissive partner (1, 5). These observations argue that RXR plays a minor role in AT-RA-mediated transcriptional activation. To examine the role of RXR helix 12 in transcriptional regulation by RXR/RAR, RXR helix 12 mutants were used to test the transcriptional activity of mutant RAR helix was assayed on a reporter construct containing the retinoic acid response element present in the promoter of human RAR gene (β-RARE). The reporter activity of Gal4 DBD transfected samples were used to normalize for calculation of -fold repression and activation.
tion activity of RXRα/RARα on a βRARα-derived (DR5) reporter construct with or without hormone. Fig. 5A shows that AT-RA increased reporter activity in the absence of transfected RXRα (~9-fold). Ectopic expression of wild-type RXRα stimulated AT-RA-induced activation by an additional 10-fold. Mutations in RXRα helix 12 reduced the AT-RA-induced activation observed with RXRα/RARα by 50–70%. In particular, exogenous expression of RXRα Δ443 completely abolished AT-RA-induced activation above the endogenous background. These data indicate that helix 12 of RXRα is essential for optimal AT-RA-induced transcriptional activation by RXRα/RARα heterodimers.

The decreased reporter activity noted in Fig. 5A with the helix 12 mutants could be due to the inability of mutant RXRα/RARα heterodimers to recruit co-activators or the inability of RXRα/RARα to dissociate from co-repressors. To address this issue, we examined the association profiles of RXRα/RARα with co-repressors or co-activators by EMSAs. We first tested disso-
Citation of SMRT from wild-type or mutant RXRα/RARα heterodimers with increasing concentrations of AT-RA (Fig. 5B). We found that mutant heterodimers with L451A (lane 9) or M454A/L455A (lane 13) display a slightly higher SMRT binding activity than that of wild-type RXRα/RARα. Furthermore, AT-RA-induced dissociation of SMRT was partially impaired in these mutants (lane 14). The ability of mutant heterodimers to recruit ACTR was also examined in the presence or absence of hormone (Fig. 5C). We found that the AT-RA-induced ACTR association with RXRα/RARα was slightly affected by the RXRα helix 12 point mutations.

Deletion of RXR Helix 12 Reveals a Novel Role in Recruitment of Co-regulators by RXRα/RARα—We further examined whether deletion of RXRα helix 12 had effects on co-regulator recruitment by RXRα/RARα heterodimers. Fig. 6A shows an EMSA in which, as expected, wild-type RARα/RXRA heterodimers recruit ACTR (lanes 2–4) and dissociate from SMRT (lanes 6–8) in an AT-RA dose-dependent manner. To our surprise, heterodimeric RXRαΔ443/RARα acquired a hormone-independent co-activator binding activity (lanes 1 and 9), and this binding could be further induced by AT-RA (lanes 10–12). Moreover, RXRαΔ443/RARα had better SMRT binding activity...
Fig. 6. Effects of RXRa helix 12 deletion on co-regulator recruitment. EMSAs were carried out according to “Materials and Methods.” A, effects of helix 12 deletion on the recruitment of co-activators by RXRa/RARa heterodimers on DR5 probe. B, effects of LG268 on co-regulator association with RXRa/RARa heterodimers on DR5 probe. C, effects of helix 12 deletion on co-regulator recruitment by RXRa/RARa heterodimers on DR1. D, effects of LG268 on co-regulator association with RXRa/TRβ heterodimers on DR4. RXR homodimers do not bind these elements under these assayed conditions (data not shown).

than that of wild-type RXRa/RARa (lanes 13–16), and SMRT dissociation was less sensitive to AT-RA than that of wild-type RXRa/RARa (lanes 5–8).

Because co-repressor release is a pre-requisite for co-activator binding and subsequent transcriptional activation (17), we hypothesized that, although RXR agonists promote co-activator association, they may not be able to induce dissociation of co-repressors. To address this question, we used EMSAs to test whether LG268 is capable of promoting recruitment of co-activators and release of co-repressors. As shown in Fig. 6B, addition of AT-RA (lanes 2 versus 3) or LG268 (lanes 2 versus 4) strongly induced association with ACTR with the wild-type heterodimer. However, whereas AT-RA completely disrupted SMRT binding (lanes 5 versus 6) with the wild-type heterodimers, LG268 did not (lanes 5 versus 7). In the context of the RXRa Δ443/RARα heterodimer, AT-RA was still capable of promoting ACTR association (lanes 9 and 10). Interestingly, addition of LG268 weakly inhibited ACTR association (lanes 9 versus 11) and did not have an effect on SMRT binding (lane 14). We also examined whether these results can be recapitulated using a DR1 probe, which is also a known response element for RXRa/RARα. Experiments were carried out in the same manner as Fig. 6B, except that the probe was a DR1-containing element derived from the human CRABPII gene. Fig. 6C demonstrates that, whereas AT-RA strongly induced ACTR association and SMRT dissociation on DR1 with the wild-type heterodimer (lanes 2 versus 3 and 6), LG268 was unable to promote dissociation of SMRT (lanes 5 versus 7). Similar to the DR5 probe, LG268 inhibited recruitment of ACTR by the RXRa Δ443/RARα heterodimers on DR1 probe (lanes 9 versus 11).

We further tested whether this observation was unique to RXRa/RARα heterodimers or common to other non-permissive heterodimers such as RXRa/TRβ. The effect of LG268 on recruitment of co-activators by RXRa/TRβ was further examined using the thyroid hormone response element, DR4, derived from the human DI gene (Fig. 6D). While thyroid hormone (T3) weakly promoted ACTR binding (lanes 2 and 3), LG268 significantly enhanced ACTR association (lanes 2 and 4). We also found that T3 induced significant dissociation of SMRT from RXRα/TRβ, but LG268 did not (lanes 5–7). In the context of RXRa Δ443/TRβ heterodimers, T3 had a more pronounced effect on promoting ACTR binding compared with wild-type heterodimers (lanes 2 and 3 versus 9 and 10). Similar to that of RXRa Δ443/RARα heterodimers, LG268 weakly inhibited ACTR binding (lanes 9 versus 11) to RXRa/TRβ. Our results indicate that helix 12 of RXRa has an inhibitory effect on co-activator recruitment by unliganded heterodimers but is critical for the agonistic effect of LG268. Moreover, the failure of LG268 to induce dissociation of co-repressors from receptors is common to RXRa/RARα and RXRa/TRβ heterodimers.

Hormone-independent Activation by RXRa Δ443/RARα Heterodimers in Yeast—We next tested whether our in vitro observations with EMSAs can be recapitulated in vivo using yeast three-hybrid (Y3H) assays. Plasmids expressing Gal4-DBD-ACTR (RID), pGAD-RARα (LBD), and RXRa Δ443 were prepared and transformed into yeast strain Y190. Based on our EMSA data (Fig. 6), we anticipated that expression of the β-galactosidase activity induced by the formation of ACTR (RID)/RXRa Δ443/RARα trimeric complex will be higher than that of ACTR (RID)/RXRa/RARα. As shown in Fig. 7A, expression of Gal4-DBD-ACTR (RID)-RXRa and RARα moderately induced expression of β-galactosidase activity. Replacement of wild-type RXRa with RXRa Δ443 further increased the reporter activity 2-fold, suggesting that mutant RXRa Δ443/RARα heterodimers bind ACTR better than wild-type RXRa/
RARα. Addition of AT-RA promoted association of ACTR with both RXRα/RARα and RXRα Δ443/RARα and activated reporter activity, suggesting that deletion of RXRα helix 12 does not prevent AT-RA-induced recruitment of ACTR. Taken together, these data suggest that helix 12 of RXRα is inhibitory to the recruitment of co-activator by unliganded RXRα/RARα.

LG268 Is Able to Activate Transcription by RXR/RAR in Yeast—Our EMSA data also suggested that LG268 is capable of promoting co-activator recruitment by RXRα/RARα in the absence of co-repressors. We reasoned that LG268 is incapable of activating RXRα/RARα in mammalian cells, because LG268 does not promote co-repressor dissociation from the heterodimers. Thus, we predicted that LG268 would promote co-activator association and subsequent transcriptional activation by RXRα/RARα in cells that lack SMRT and nuclear receptor co-repressor, such as yeast. To test this hypothesis, the transformed yeast cells were grown in the absence or presence LG268, and the reporter activity was measured. As shown in Fig. 7B, LG268 was able to promote association between ACTR and RXRα/RARα heterodimers and activate reporter activity (lanes 1 and 2). However, the LG268-induced reporter activity relies on the integrity of RXRα helix 12, because LG268 did not promote association between ACTR and RXRα Δ443/RARα (lanes 3 and 4). These data suggest that RXRα/RARα is able to respond to LG268 and activate transcription in the absence of co-repressors.

DISCUSSION

In this study, we have examined the role of helix 12 of RARα and RXRα on co-repressor recruitment and transcriptional regulation by RXRα/RARα. Our results indicate that mutations at conserved residues within helix 12 of both RXRα and RARα as well as wild-type, but AT-RA barely caused co-repressor dissociation. Indeed, at 1 μM AT-RA, residual amounts of SMRT still bound RXRα/RARα (M413A) on DR5. Additionally, M413A acquired hormone-independent association with ACTR and AT-RA to promote further ACTR association in a dose-dependent manner. This mutant is reminiscent of the K262A mutant we described previously (17), suggesting that these two residues serve common functions, preventing hormone-independent co-activator association and promoting hormone-induced dissociation of co-repressors. The third group includes Δ403 and L409A, which possessed two defects, hormone-dependent dissociation of co-repressors and hormone-dependent recruitment of co-activators. Although mutant M413A was capable of interacting with ACTR in the absence of AT-RA in EMSAs (Fig. 2A), it did not interact with ACTR in M2H assays (Fig. 2B). Presumably, M413A remains associated with co-repressors in mammalian cells in the absence of AT-RA. Indeed, Gal4-RARα (M413A) only weakly repressed transcription (Fig. 3A). These data suggest that transcriptional regulation by RARα depends primarily on dissociation of co-repressors and release of co-activators, which is a prerequisite for activation.

Although our data indicate that replacement of the conserved residues of RARα helix 12 effects co-activator binding, we cannot exclude the possibility that the defect in some of the mutations was due to an inability of these mutants to bind hormone. Structural studies have indicated that the only residue in the helix 12 of RARα in close proximity to AT-RA is Ile-410 (−4.1 Å) (10), whereas the closest residue in the helix 12 of RXRα to 9-cis-RA is Leu-451, but a direct interaction is unlikely given the substantial distance between Leu-451 and 9-cis-RA (−7.1 Å) (11). Indeed, it has been shown that deletion of helix 12 caused only a moderate decrease on AT-RA binding, suggesting RARα helix 12 plays a minor role in AT-RA binding (23). Furthermore, none of the residues within helices 12 of ligand-bound RXRα/RARα heterodimers are within the range for an interaction with the synthetic ligands (20). Our data are...
also consistent with the structure of T3-bound TRβ/GRIP1 complex (12) in which Leu-454, Glu-457, and Val-458 of TRβ (corresponding to residues Leu-409, Glu-412, and Met-413 of RARα) directly contact the LXXLL-containing α-helix from GRIP1 and are not located near the T3 binding site. There is no structure data available for apo-RARα complexed with co-repressor peptides, but studies from antagonist-bound PPARα/SMRT complex suggested that the helix 12 of PPARα lies parallel to the SMRT peptide at a distance too great for an interaction between helix 12 and the co-repressor peptide (>10 Å) (15). The SMRT peptide is held by helices 3–5 in the structure. However, it is possible that helix 12 of antagonist-bound PPARα adopts a distinct conformation that is not present in apo-RARα or apo-RXα. Further structural studies will help to address this issue.

The Role of RXα Helix 12 in Retinoid Signaling—Using Y2H assays, we detected interactions between ACTR and unliganded RARα as well as RXα (Fig. 4). This result is unexpected, because we did not detect interactions between RARα and ACTR in M2H assays (17) or in GST pull-down assays (data not shown). It is likely that there are molecules, either produced by yeast or in yeast medium, mimicking receptor ligands. Alternatively, receptors may interact with co-regulators through bridging factors in yeast. Our Y2H data indicate that, in the absence of RARα, RXα Δ443 not only gains co-repressor binding activity but also loses ligand-mediated co-activator recruitment, indicating that helix 12 is critical for recruitment of co-regulators by RXα. Consistent with this observation, helix 12 mutations had severe effects on ligand-induced transcriptional activation by Gal4-RXα. We show that RXα Δ443 does not potentiate AT-RA-mediated activation of the DB5 reporter construct (Fig. 5A). However, our EMSA data indicated that at 100 nM AT-RA, RXα Δ443/RARα completely released SMRT and was able to recruit co-activator (Fig. 6A). This discrepancy is likely due to the fact that the concentration of GST-ACTR is much higher than that of GST-SMRT in our EMSA assays. Furthermore, the ratio of the [co-repressors]/[co-activators] in the cells may also determine whether RXα Δ443/RARα represses or activates transcription.

Our data demonstrate that deletion of helix 12 appears to create a better co-repressor interaction surface for RXα (Fig. 4) as well as RXα/RARα and RXα/TRβ, an observation similar to that of two previous reports (24, 25). Interestingly, our
data indicated that mutations in the helix 12 of RXRs have either positive (Leu-451 and M454A/L455A) or negative effect on the association of RXRs with co-repressor. This result cannot be explained by a model in which the length, rather than the specific sequence of the RXRs helix 12, masks the co-repressor binding pocket (25).

Intriguingly, we note that RXRα Δ443 or unliganded RARs did not bind co-activator individually. However, DNA-bound RXRα Δ443/RAR heterodimers acquired a hormone-independent co-activator binding activity. Our Y2H assays also verified that RXRα Δ443/RARα gained a better binding affinity to ACTR than wild-type RXRα/RARα (Fig. 7). Furthermore, AT-RA is capable of inducing further interaction between RXRα Δ443/RARα and ACTR. These results suggest: 1) deletion of helix 12 of RXRs did not effect hormone binding activity of RARα; 2) helix 12 of RXRs blocks co-activator binding and therefore plays an inhibitory role in the recruitment of co-activator by unliganded RXRα/RARα heterodimers; 3) helix 12 of RXRα partially overlaps with the co-activator binding pocket within the heterodimers. The fact that deletion of helix 12 of RXRα creates a better co-repressor and co-activator binding surface for RXRα/RARα heterodimers further strengthens the notion that the co-repressor and co-activator binding pockets are partially overlapped within the heterodimers. Together, these data pose an additional complexity of the allosteric effects mediated by dimerization of the partners. Future studies on the three-dimensional structure of unliganded heterodimers will advance our understanding of the molecular data.

Our finding that LG268 is able to induce recruitment of co-activators to heterodimers raises an important question. Do co-activators bind the liganded or unliganded RAR within the heterodimers? Further studies will be required to address this question. Our EMSAs showed that, although AT-RA was able to promote recruitment of the co-activator by RXRα Δ443/ RARα and RXRα Δ443/TRβ, LG268 was not. Indeed, addition of LG268 moderately inhibited co-activator binding to RXRα Δ443/RARα and RXRα Δ443/TRβ, implying that binding of LG268 causes a conformational change, either within RXRα or RARα, such that it generates a poor co-activator binding pocket, thus acting as an antagonist. However, it remains to be seen whether this observation is common to all RXR agonists.

Why Are RXRs Silent Partners in Non-permissive Heterodimers?—The reasons why RXR agonists are unable to activate transcription by RXR/RAR heterodimers are unclear. It was proposed that only RARs can bind ligand; the RXR partner provide an alternative mechanism by which RXRs function as silent partners for RXR/RAR and RXR/TR heterodimers.

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REFERENCES
1. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 441–450
2. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., and Chambon, P., and Evans, R. M. (1995) Cell 83, 835–883
3. Glass, C. K., and Rosenfeld, M. G. (2000) Genes Dev. 14, 121–141
4. McKenna, N. J., Lanza, R. B., and O'Malley, B. W. (1999) Endocr. Rev. 20, 321–344
5. Leblanc, B. P., and Stunnenberg, H. G. (1995) Genes Dev. 9, 1811–1816
6. Ruan, R. M., Umesono, K., Chen, J., and Evans, R. M. (1995) Cell 84, 541–550
7. Kurokawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gless, B., Rosenfeld, M. G., Heyman, R. A., and Glass, C. K. (1994) Nature 371, 528–531
8. Greschik, H., and Moras, D. (2003) Curr. Top. Med. Chem. 3, 1573–1599
9. Renaud, J. P., and Moras, D. (2000) Cell Mol. Life Sci. 57, 1748–1769
10. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) Nature 376, 577–582
11. Egea, P. F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. (2000) EMBO J. 19, 2592–2601
12. Darmoni, B. D., Wagner, R., L. C., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fleitierick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3345–3356
13. McIverney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Notte, B. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) Genes Dev. 12, 3357–3368
14. Shuai, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, A. D., and Greene, G. L. (1998) Cell 95, 927–937
15. Xu, H. E., Stanley, T. B., Montana, V. G., Lambert, M. H., Shearer, B. G., Cobb, J. E., McKe, D. D., Galardi, C. M., Plummet, K. D., Notte, B. T., Parks, D. J., Moore, J. T., Klierer, S. A., Willson, T. M., and Stimmel, J. B. (2002) Nature 415, 813–817
16. Hu, and Lazar, M. A. (1999) Nature 402, 93–96
17. Kao, H. Y., Han, C. C., Korma, A., and Evans, R. M. (2003) J. Biol. Chem. 278, 7966–7973
18. Nagy, L., Kao, H. Y., Love, J. D., Li, C., Banayo, E., Gooch, J. T., Krishna, V., Chatterjee, K., Evans, R. M., and Schwabe, J. W. (1999) Genes Dev. 13, 3209–3216
19. Perissi, V., Staszewski, L. M., McIverney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999) Genes Dev. 13, 3198–3208
20. Bourguet, W., Vivat, V., Wartz, J. M., Chambon, P., Gronemeyer, H., and Moras, M. (2000) Mol. Cell. 5, 289–298
21. Kao, H. Y., Ordentlich, P., Kayano-Nakagawa, N., Yang, Z., Downes, M., Kintner, C. R., Evans, R. M., and Kadesch, T. (1998) Genes Dev. 12, 2269–2277
22. Schulman, I. G., Chakravarti, D., Jugulon, H., Rono, A., and Evans, R. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8288–8292
23. Lefebvre, B., Rabche, C., Formetache, P., and Lefebvre, P. (1995) Biochemistry 34, 5477–5485
24. Ghosh, J. C., Yang, X., Zhang, A., Lambert, M. H., Li, H., Xu, H. E., and Chen, J. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5842–5847
25. Zhang, J., Hu, X., and Lazar, M. A. (1999) Mol. Cell. 19, 6448–6457
26. Li, D., Li, T., Wang, F., Tian, H., and Samuels, H. H. (2002) Mol. Cell. 22, 5782–5792
27. Castillo, A. I., Sanchez-Martinez, R., Moreno, J. L., Martinez-Iglesias, O. A., Palacios, D., and Aranda, A. (2004) Mol. Cell. 24, 502–513
28. Lee, G., Eldwo, F., McNeil, J., Weiszmann, J., Lindstrom, M., Amaral, K., Nakamura, M., Miao, S., Cas, P., Learmont, R. M., Chen, J. L., and Li, Y. (2002) J. Biol. Chem. 277, 19649–19657
29. Zamir, I., Zhang, J., and Lazar, M. A. (1997) Genes Dev. 11, 835–846
30. Germain, D., Iyer, J., Zechel, C., and Gronemeyer, H. (2002) Nature 415, 187–192

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Retinoid X Receptor α (RXRα) Helix 12 Plays an Inhibitory Role in the Recruitment of the p160 Co-activators by Unliganded RXR α/Retinoic Acid Receptor α Heterodimers

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