Co-repressor Release but Not Ligand Binding Is a Prerequisite for Transcription Activation by Human Retinoid Acid Receptor α Ligand-binding Domain*

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Nuclear hormone receptors coordinate the activity of genetic networks through the recruitment of transcriptional co-regulators, including co-repressors and co-activators. Allosteric modulation of the ligand-binding domain by hormonal activators shifts the co-factor binding preference by defined structural changes in overlapping docking sites. We report here that mutations at conserved residues within the docking motif of the retinoic acid receptor α cause defects in dimerization, co-repressor association, and transcriptional regulation. Furthermore, although a minimal co-repressor receptor interaction domain is sufficient for receptor binding, flanking sequences appear to stabilize this interaction without interfering with ligand sensitivity. However, ligand sensitivity is changed by the K262A mutation, which requires much higher concentrations of all-trans-retinoic acid to promote co-repressor dissociation. Consequently, K262A functions as a dominant-negative mutant at low concentrations of all-trans-retinoic acid. As a result, transcriptional activation is mechanistically linked to co-repressor release.

Members of the steroid hormone receptor superfamily are hormone-activated transcription factors that control vertebrate development, differentiation, and homeostasis through coordinate regulation of complex genetic networks in multiple target cells (1, 2). Unliganded thyroid hormone (TR) and retinoid acid receptors (RAR) function as potent transcriptional repressors becoming activators upon hormone binding. The repression activity by unliganded RAR and TR is mediated through the recruitment of either the silencing mediator for retinoid and thyroid hormone receptors (SMRT) or nuclear receptor co-repressor complexes that include mSin3A and a variety of histone deacetylases (3–7). In contrast, transcriptional activation by nuclear hormone receptors involves the recruitment of histone acetyltransferase complexes that include CREB-binding protein/p300, p300/CREB-associated factor, and members of the p160 family (SRC-1, GRIP1/TIF2, and ACTR/RAC3/P/CIP) (8–14).

Nuclear receptors contain two conserved modules, the DNA-binding domain and the carboxyl-terminal ligand-binding domain (LBD). DNA-binding domains bind hormone response elements and thus direct receptors to appropriate target genes. LBDs are required for nuclear localization, homodimerization and/or heterodimerization, co-repressor association (including co-repressors and co-activators), and most importantly, ligand binding. The LBDs are composed of 12 helices in which helices 3–5 are the most conserved among receptors and define the nuclear receptor LBD signature motif for co-repressor recruitment. The transcriptional switch of the receptors from repressors to activators involves a ligand-induced conformational change, resulting in an exchange of co-repressors and co-activators. Using site-directed mutagenesis, we and others have previously shown that helices 3–5 of TRβ and peroxisome proliferator-activated receptor γ play a pivotal role in binding co-repressors (15–20). Reciprocally, SMRT and nuclear receptor co-repressor contain two short peptide motifs that are both necessary and sufficient for mediating co-repressor binding to unliganded RARs (15–17). Both motifs contain a hydrophobic core (IL/XX/I/VL). These two motifs, termed coreID I and coreID II (17 and 19 amino acids, respectively), are conserved in both position and in sequence between nuclear receptor co-repressor and SMRT. A third TRβ binding motif aminoterminal to those previously mapped in nuclear receptor co-repressor may also exist (21). Helices 3–5 also interact with co-activators through a LXXLL-containing signature motif. The p160 co-activator proteins contain three putative LXXLL motifs, but the affinity and specificity of the individual LXXLL motifs within p160 proteins toward different receptors are not well characterized.

RARs and RXRs form heterodimers in solution and on re-

trans-retinoic acid; GST, glutathione S-transferase; ID, interaction do-

main(s); DR, direct repeat; TK, thymidine kinase.
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EXPERIMENTAL PROCEDURES

Plasmid Construction—The plasmids pCMX, pCMX-Gal4, pCMX-Gal4-RARα, pCMX-RARα, pCMX-VP16-RXRα, pCMX-Gal4-RRα (LBD), CMX-LacZ, and pMH100-TK-Luc have been described (24, 25). GST-SMRT and GST-ACTR fusion constructs were generated by inserting SMRT and ACTR receptor interaction domain (ID) PCR fragments into the vector pGEX4T-1. Site-directed mutagenesis was carried out using the QuikChange kit according to the manufacturer’s protocol (Stratagene). All of the constructs were verified by double-stranded sequencing to confirm the identity and reading frame.

Transient Transfection—CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin G, and 50 µg/ml of streptomycin sulfate at 37 °C in 7% CO2. For Gal4-RARα, CV-1 cells (60–70% confluence, 48-well plate) were co-transfected with 16.6 ng of pCMX-Gal4 and pCMX-VP16 for (mammalian two-hybrid assays) fusion constructs, 100 ng of pMH100-TK-Luc, and 100 ng of pCMX-LacZ in 200 µl of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum by the N-[1-(2,3-dioleoyl)propyl]-N,N,N-trimethylammonium methysulfate-mediated procedure (7). For DR5-TK-Luc (see Fig. 5), the cells were transfected with 0.33 ng of pCMX-RARα, 100 ng of β-RARE-TK-Luc, and pCMX-LacZ. The amount of DNA in each transfection was kept constant by the addition of pCMX. The CV-1 cells were transferred to charcoal-stripped serum after adding DNA. After 24 h, the medium was replaced with or without all-trans-retinoic acid. The cells were harvested and assayed for luciferase activity 36–48 h after transfection. The luciferase activity was normalized to the level of β-galactosidase activity. Each transfection was performed in triplicate and repeated at least three times.

Electrophoresis Mobility Shift Assays—GST fusion proteins were expressed in Escherichia coli DH5α strain and affinity purified by glutathione-Sepharose 4B beads. Immobilized GST fusion proteins were eluted by 20 µM glutathione and dialyzed against 1× phosphate-buffered saline. RARα and RXRα synthesized in vitro (Promega) were incubated with a 32P-labeled probe containing a DR5 element derived from the βRARE promoter containing the sense strand sequence 5′-GTT-AGG-GGT-TAC-CGA-AAG-TTC-AC-T-C3′ with or without 1 µM AT-RA. DNA-protein binding was conducted in a reaction mixture containing 20 mM Hepes, pH 7.4, 50 mM KCl, 1 mM β-mercaptoethanol, and 10% glycerol. After 30 min of incubation at 25 °C, the purified GST-SMRT or GST-ACTR fusion proteins were added followed by an additional 30 min of incubation. The final reaction mixtures were loaded onto a 5% polyacrylamide (29:2.08) nondenaturing gel followed by electrophoresis in 0.5× TBE buffer. After electrophoresis, the gel was dried and subjected to autoradiography.

RESULTS

Identification of RARα Residues Critical for Dimerization with RXRα—To determine the residues that are critical for RARα function, we generated mutations on conserved and diverged residues within helices 3 and 4. Fig. 1A shows the nuclear receptor signature motif and the targets for site-specific mutations in this study. Primarily, alanine substitutions were generated and tested for their ability to dimerize with RXRα (Fig. 1B). The ability of RARα mutants to dimerize with wild-type RXRα was evaluated by mammalian two hybrid (2H) assays and electrophoresis mobility shift assays (EMSA) on a template containing a DR5. M2H assays were conducted using Gal4-RXRα and VP-RXRα (LBD) along with a reporter construct (pMH100) containing multiple copies of Gal4-binding sites upstream of the thymidine kinase (TK) promoter fused with luciferase gene. Induction of the luciferase activity is an indicative of interaction of the partners. As a control, Gal4-RXRα with VP16 alone only gave a basal activity (Fig. 1B). In the presence of VP16-RXRα, the reporter activity is induced, suggesting an association between RXRα and RARα. In the absence of AT-RA, all of the mutants except F249A and Q257A retain at least 75% of wild-type activity. As expected, the addition of 1 µM AT-RA increased the reporter activity of the wild-type RARα, probably because of the recruitment of co-activators, which further increase transcriptional activation by Gal4-RXRα/RARα. Interestingly, most of the RARα mutants gave lower reporter activity than that of the wild type, indicating that these mutants have lower activation activity. This result suggests that these mutants might be defective in co-activator binding. Although unliganded F249A binds RXRα much less efficiently than wild type, the reporter activity of G4-RXRα/RARα (F249A) increased dramatically upon addition of 1 µM AT-RA. To test the heterodimerization ability of wild-type or mutant RARα with RXRα on DNA, in vitro synthesized RARα and RXRα were mixed with a radiolabeled DNA duplex containing a DR5 element derived from the promoter of βRARE

In an attempt to examine the molecular basis of co-regulator association with RARα, we have generated mutations within both conserved and diverged residues within RARα helices 3 and 4 and systematically analyzed the properties of these mutants. We have identified critical residues for RARα functions including dimerization, co-regulator association, and transcriptional activity. Our results suggest that the transcriptional activity of RARα depends primarily on its ability to dissociate from co-repressors.

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gene. Consistent with M2H assays, F249A and Q257A failed to heterodimerize with RXRα productively on DR5 (data not shown; see below). A likely possibility is that these two mutants failed to form heterodimer efficiently.

Helices 3 and 4 of RARαs Are Critical for Co-repressor Association—The ability of the unliganded RARα mutants to bind SMRT was tested by M2H assays. M2H assays were conducted using Gal4-SMRT ID I + II (amino acid 2064–2307 of mouse SMRT, a fragment containing both ID I and II) and VP-RARα (Fig. 2A). Our data indicate that unliganded mutants V240A, F249A, Q257A, I258A, and L261A dramatically lost their SMRT binding activity (lanes 3, 6, and 8–10). In the presence of 100 nM AT-RA, RARα and SMRT interaction is completely abolished in all cases except mutant K262A (lane 11). We also tested whether these RARα mutants bind SMRT in EMSAs.

Using the same SMRT ID I + II fragment used in M2H assays, we constructed pGEX-4T-1-SMRT ID I/H11001/H9251 or pGEX-4T-1-SMRT coreID II fusion proteins. In vitro synthesized RARα and RXRα were mixed with a radiolabeled DNA duplex containing a DR5 element derived from the promoter of the human βRARE gene. EMSAs were performed with or without purified GST-SMRT ID I + II (Fig. 2B). Consistent with M2H assays, RXRα/RARα heterodimers with RARα mutants V240A, F249A, Q257A, I258A, and L261A, significantly lost the ability to bind SMRT. The SMRT binding activity of mutants S232A, K244A, G248A, S232A, and K262A is moderately reduced. As with M2H assays, the addition of 100 nM AT-RA completely blocked SMRT ID association with RARα except for mutant K262A (data not shown). These data indicate that the LBD signature motif is critical for co-repressor binding. We also noted that F249A and Q257A failed to heterodimerize with RXRα productively on DNA. This result is consistent with M2H data. A likely possibility is that these two mutants failed to bind DNA because they do not form heterodimers efficiently.

We next measured the association between RARα LBD mutants with coreID II (Fig. 2C) and found that SMRT coreID II domain alone is highly sensitive to mutations within RARα LBD. Three patterns of association were identified. Most of the mutants, with the exception of I254A and K262A, failed to bind SMRT coreID II. In another group, S232A, K244A, and G248A bound ID I + II but not the coreID II alone. Finally, association of coreID II and ID I + II with RARα LBD was abrogated in a third group consisting of V240A, F249A, Q257A, I258A, and L261A. Based on these results, we conclude that these residues are critical for co-repressor association and that sequences flanking coreID II may play a role in the association with RARα LBD.

Association of Co-activators with RARα Mutants—Because helices 3 and 4 of TRβ and peroxisome proliferator-activated receptor γ have also been shown to be critical for co-activator association (18–20), we tested the interaction between RARα helices 3 and 4 mutants and p160 co-activator proteins. M2H assays were carried out to determine in vivo association of p160 co-activator proteins with RARα using Gal4-ACTR (SRC-1 or GRIP1) ID (receptor interaction domain) and VP16-RARα (LBD). Among the three known p160 family members, RARα bound ACTR/RAC3/PCIP the best, with SRC-1 less well and GRIP1 the least (Fig. 3A), whereas TRβ bound SRC-1 better than ACTR and GRIP1. M2H assays were used to determine in vivo association of ACTR and wild-type or mutant RARα. The data in Fig. 3B show that the association between ACTR and RARα is severely compromised for mutants V240A, K244A, F249A, Q257A, I258A, and L261A. Furthermore, we found that the interactions between ACTR and RARα are ligand concentration-dependent (data not shown; see below). We then tested the effect of RARα mutations on ACTR binding by EMSA. EMSA was conducted as described in Fig. 2 with or without GST-ATCR in the presence of 100 nM AT-RA. We found that GST-ATCR RID was able to supershift DNA-bound wild-type.
heterodimers RXRα/RARα efficiently. However, ACTR binding activity is dramatically impaired for mutants V240A, K244A, F249A, Q257A, I258A, and L261A, indicating the loss of ACTR binding activity of these mutants.

**Co-regulator Association with RARα Correlates with Transcriptional Activity**—The ability of these RARα mutants to regulate transcription was examined by transient transfection assays. Wild-type and RARα mutants were fused to the yeast Gal4 DNA-binding domain to generate Gal4-RARα expression plasmids. Transient transfection assays were conducted in the presence or absence of 100 nM of AT-RA. In the absence of AT-RA, the degree of transcriptional repression activity of RARα varies among these mutants. Notably, V240A, F249A, Q257A, I258A, and L261A lose more than 80% of their repression activity (Fig. 4A). The repression activity correlates with the ability of these mutants to interact with SMRT. Furthermore, the activation activity of mutants K244A, F249A, Q257A, L261A, and K262A was dramatically impaired at 100 nM AT-RA (Fig. 4B). We noted that liganded V240A, I258A, and L261A bind ACTR less efficiently than the wild-type RARα, but their ligand-dependent transcriptional activation is only partially inhibited. We propose that this observation may reflect the loss of SMRT binding activity by V240A, I258A, and L261A.

In addition, although K262A and wild type bound ACTR equally well in the presence of 100 nM, K262A only moderately activated transcription in transient transfection assays. We interpret this to mean that K262A is still associated with co-repressor complexes in the presence of 100 nM AT-RA, as shown in Fig. 2 (see “Discussion”).

**Distinct Activities of RARα Helices 3 and 4 Mutants F249A and K262A**—During our analyses for ligand concentration-dependent association/dissociation of RARα mutants and co-factors, we found that the association/dissociation profile of K262A with co-factors was distinct from those of other helices 3 and 4 mutations. Fig. 5A is an EMSA with wild-type RARα and mutant K262A in the presence of GST-SMRT with or without increasing concentration of AT-RA. Similar to other mutants, K262A acquires reduced SMRT association activity (Fig. 5A, compared the ratio of SMRT-bound/unbound RARα). We noted that at 1 μM residue SMRT still remains associated with DNA-bound RXRα/RARα heterodimer. M2H assays were also carried out to determine ligand-dependent dissociation between SMRT and wild-type RARα or K262A (Fig. 5B). Consistently, M2H assays demonstrated that dissociation between SMRT and K262A requires at least 1 μM AT-RA as opposed to 100 nM for SMRT and wild type RARα. In contrast, heterodimer RXRα/RARα (K262A) binds ACTR in the absence of ligand in vitro (EMSA), indicating a better ACTR binding activity than that of the wild-type RARα (Fig. 5C). However, in vivo association of K262A with ACTR requires the presence of AT-RA.
Although K262A binds to ACTR equally as well as that of wild type at 100 nM AT-RA, K262A binds less tightly than wild type at 1 μM AT-RA in M2H assays (Fig. 5D). Under the same conditions, F249A can only associate with ACTR in the presence of 1 μM AT-RA.

To understand how co-regulator association correlates with the transcriptional activity of K262A, transient transfection assays were employed. Unliganded wild-type Gal4-RARα represses transcription efficiently (Fig. 5E, lane 5), whereas K262A moderately repressed basal transcription (lane 13). The reduced repression activity is consistent with the reduced SMRT binding activity shown in Fig. 5 (A and B). In the presence of 10 nM AT-RA, wild-type RARα dramatically activated transcription (lane 6, 31.3-fold), whereas K262A only activated transcription 3-fold compared with basal (lane 14). Of note, transcriptional activation by K262A at 1 μM AT-RA was
lower than that of the wild type at 10 nM AT-RA (lanes 6 and 16). Surprisingly, F249A strongly activates transcription at 1 μM AT-RA. This result is consistent with the observation that F249A acquires co-activator association at 1 μM AT-RA shown in Fig. 5D.

We further examine the activity of the mutants on a DR5-containing reporter construct (Fig. 5P). In the presence of AT-RA, the expression of the reporter activity is induced in the absence of exogenousRARα. Presumably, this activity is derived from the endogenous RARs (lanes 2–4). Exogenous expression of the wild-type RARα further increased the expression of the reporter activity (lanes 6–8). Intriguingly, we found that although F249A binds ACTR weaker than the wild-type RARα at 1 μM AT-RA as shown in Fig. 5D, the transcription activity of F249A is comparable with that of wild-type RARα. Furthermore, at lower AT-RA concentration, expression of K262A resulted in lower reporter activity than that in the absence of AT-RA (lanes 14 and 15 compared with lanes 2 and 3), suggesting that K262A inhibits endogenous RAR activity and that K262A functions as a dominant-negative mutant of the wild-type RARα.

**DISCUSSION**

Site directed mutagenesis was used to dissect the mechanistic links between co-repression, co-activation, and ligand binding. Our results indicate that residues within the receptor signature motif contribute in specific ways to both co-repressor and co-activator (Table I and Fig. 6). One unusual mutation is K262A, which results in increased co-activator and reduced co-repressor binding but functionally acts as a dominant-negative mutation. This implies an altered co-repressor off rate leading to the suggestion that co-repressor release is dominant to co-activator binding. This in turn implies that the principle role of ligand is to induce co-repressor release, enabling the signature motif to attract the co-activator. Taken together, these data strongly suggest that transcriptional repression and activation are mechanistically linked.

Recent structural studies have demonstrated that helices H7, H9, H10, and H11 of RARα are involved in heterodimerization with RXRα (Ref. 26 and Fig. 6A). Our results are consistent with their findings because helices 3 and 4 mutants possessed at least 60% of the heterodimerization activity in M2H assays of the wild-type RARα. The exceptions were F249A and Q257A, which are absolutely conserved within class II nuclear receptor (Fig. 1A). We also note that both F249A and Q257A fail to bind SMRT. It is possible that the global structure of mutants F249A and Q257A is dramatically disturbed, so that they lose most of the LBD-associated activities. Gln257 is located at the middle of helix 4, and both of its amide groups can donate hydrogen bonds, whereas the carbonyls of Leu252, Thr250, and Phe249 can accept the hydrogen bond (not shown). Phe249 is positioned in the middle of the loop connecting helices H3 and H4. Thus, mutations of either Gln257 to Ala or Phe249 to Ala will abolish these hydrogen bonds and very likely destabilize helices 3 and 4 and the whole structure of the molecule. Both F249A and Q257A mutations create large cavities (Fig. 6, C–E), and as a consequence the protein structure may tend to relax. This could result in significant structural rearrangements, indirectly altering the dimerization interface involving helices H7, H9, H10, and H11. In addition, mutation of Phe249 to Ala would probably result in rotation of the Gln257 side chain as modeled by Swiss-PdbViewer (Fig. 6E) and also very likely destabilize the loop connecting helices H3 and H4. One might speculate that Phe249 and Gln257 are part of the folding nucleus. Folding nuclei are known to include conserved amino acid residues (29–31). Mutation of the residues critical for the formation of the nucleation core could also result in folding defects. However, the exact structural changes caused by Gln257 to Ala as well as Phe249 to Ala mutations have yet to be determined. Although M2H assays indicated that F249A did not interact with RXRα, EMSAs showed that F249A was able to associate with RXRα, albeit with much less affinity than that of wild type. This discrepancy could be due to the fact that RXRα LBD was used in M2H assays, whereas full-length RXRα was used for the EMSA assays. One possibility is that the RXRα DNA-binding domain may contribute to heterodimerization with RXRα on DNA.

We have previously shown that SMRT coreID association is highly sensitive to mutations within the LBD of TRβ (16). All of the TRβ mutants examined failed to bind SMRT ID. Our data show that most RXRα helices 3 and 4 mutants, with the exception of I254A and K262A, failed to bind SMRT coreID II (Fig. 2C). These results imply the existence of a common co-repressor-interacting surface between the LBD of RXRα and TRβ. Intriguingly, we found that some of these mutants, such as S232A, K244A, and G248T, failed to bind coreID II and could still associate with SMRT ID I + II. These data suggest that the sequence flanking coreID II contacts these residues and stabilizes the interaction between SMRT and RXRα. However, mutants including V240A, F249A, Q257A, I258A, and L261A failed to bind both SMRT coreID II or SMRT ID I + II. One common characteristic of these residues is that they are highly conserved among nuclear receptors. These data suggested that
these conserved residues are absolutely essential for co-repressor interaction.

Among all of the mutants analyzed, K262A displays characteristics that are distinct from others. First, K262A binds SMRT more poorly than wild-type RARα both in EMSA and M2H assays. Consistently, Gal4-RARα (K262A) repressed transcription to a lesser extent than the wild-type RARα. Second, K262A did not respond to ligand properly. Dissociation of K262A with SMRT required a high concentration of AT-RA (1 μM). This aberrant ligand responsiveness could be derived from a differential ligand binding affinity of K262A. However, previous studies on RARγ and RXRα suggested that ligand binding, dimerization, and DNA binding of this mutant were not affected (23, 27). Intriguingly, unliganded K262A binds ACTR better than that of wild-type RARα. This mutant did not activate transcription at 50 nM AT-RA. However, our results indicate that even though ACTR association of F249A is not as strong as that of the wild-type RARα, the ligand-dependent transcriptional activation by F249A (Fig. 2). A similar observation has been reported for mutant F249A (28). Similar to our findings, this mutant did not activate transcription at 50 nM AT-RA.

Fig. 6. Ribbon diagrams of RXRα and RARα based on the crystal structure of liganded RXRα/RARα heterodimer (Protein Data Bank code 1DKF) (26). A, the dimeric arrangement of RXRα/RXRα heterodimer is viewed perpendicular to the dimer axis. Mutated residues of RARα are represented in red and gray (Gly248 and Phe249 depicted in gray are behind Ile254 in this view) and denoted with residue number (van der Waals radii of the side chains are shown). RARα helix H5 is depicted in blue, helix H4 is in pink, and helix H12 is in yellow. RXRα helices H7, H9, H10, and H11 forming in part the heterodimer interface are depicted in light green. RXRα helices H7, H9, H10, and H11 as well involved in heterodimer interface are depicted in banana green. Wire frame models of bound ligands are depicted in gray. Unmodeled in 1DKF region between H1 and H3 is not shown. B, structural model of RARα rotated ~90° relative to A. Mutated residues are denoted as in A; helix H3 is depicted in blue, helix H4 is in pink, and helix H12 is in yellow. C, structural model of RARα. Orientation is the same as in B. Only Gln257 and Phe249 are depicted by their side chain van der Waals radii and residue number. D, structural model of RARα viewed as in C. Gln257 is mutated to Ala. E, structural model of RARα viewed as in C. Phe249 is mutated to Ala. Molecular simulations were performed using GlaxoSmithKline R & D Deep View v3.7 Swiss-PdbViewer.
5E). We interpret this to mean that in M2H assays, K262A is strongly associated with co-repressor complexes in the absence of ligand. Indeed, at 100 nM, K262A still remained associated with SMRT (Fig. 5, A and B) and functioned as a dominant-negative mutant (Fig. 5F). Although the co-regulator association property is not identical to that of PML-RARα, the transcription activity of K262A is reminiscent of PML-RARα at low concentrations of AT-RA. In summary, we conclude that helices 3 and 4 of the RARα LBD play a critical role in RARα functions and that co-repressor release is a prerequisite for co-activator association and consequently transcriptional activation.

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