Interactions of RXR with Coactivators Are Differentially Mediated by Helix 11 of the Receptor's Ligand Binding Domain†

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Received September 5, 2001; Revised Manuscript Received November 9, 2001

ABSTRACT: RXR is a nuclear hormone receptor that is activated by the vitamin A metabolite 9-*cis*-retinoic acid. Previously, it was shown that, in the absence of a cognate ligand, RXR self-associates into tetramers, thereby silencing its own transcriptional activity. It was also shown that the tetramerization region of RXR critically contains two of three consecutive phenylalanine residues found in helix 11 (H11) of the receptor's ligand binding domain. Mutation of these residues abolishes the ability of RXR to form tetramers but also results in a receptor that is defective in its ligand-induced transcriptional activity. These observations suggest that the region may be involved in the association of RXR with transcriptional coactivators. Here, it is demonstrated that mutation of the H11 phenylalanine residues diminishes the ability of RXR to associate with the p160 coactivators TIF2 and p/CIP, but has little effect on ligand-dependent interactions of the receptor with the unrelated coactivator TIF1. It is further shown that a peptide comprised of the H11 sequence effectively competes with RXR for binding of TIF2 but not of TIF1. Finally, transactivation assays demonstrate that the defective transcriptional activity of the H11 mutant can be rescued by ectopic expression of TIF1 but not of TIF2. Taken together, the results indicate that H11 is directly involved in stabilizing the interactions of RXR with p160 coactivators, but is not required for association with TIF1. This region is thus a novel coactivator interaction surface which selectively mediates the association of RXR with transcriptional coactivators.

Nuclear hormone receptors comprise a superfamily of transcription factors that mediate signaling by small hydrophobic hormones such as steroids, vitamin D_3, thyroid hormone, and retinoic acids. These proteins associate with DNA response elements located in the promoter regions of target genes and modulate transcriptional rates in response to their specific cognate ligands (*1*, *2*). The retinoid X receptor (RXR)[1] is a member of the nonsteroid branch of the nuclear receptor superfamily which is activated by the 9-*cis* isomer of retinoic acid (9cRA). RXR can bind to cognate DNA and regulate transcription as a homodimer (*3*−*7*). In contrast, other proteins within this branch, e.g., the thyroid hormone-, vitamin D-, peroxisome proliferator-activated, and retinoic acid receptors (TR, VDR, PPAR, and RAR, respectively), self-associate weakly, and they exert their activities via high-affinity heterodimers with RXR (*4*−*6*). The ability of RXR to serve as a common binding partner for various other receptors thus allows it to regulate multiple signaling pathways that converge at the genome.

Transcriptional regulation by nuclear receptors involves ligand-induced recruitment of multiple coregulatory proteins (*8*−*10*). It has been demonstrated that, in the absence of cognate ligands, some nuclear receptors, e.g., RAR and TR, associate with accessory proteins that act to repress transcription (*11*−*15*). Ligand binding induces structural rearrangements that result in dissociation of the corepressors and recruitment of different accessory factors, termed coactivators, that, in turn, activate target gene transcription. Multiple types of coactivators have been identified to date (*8*−*10*, *16*). Among them are members of the p160 family which comprises at least three distinct members: SRC-1 (steroid receptor coactivator 1), p/CIP (p300/CBP interacting protein), and TIF2 (transcription intermediary factor 2). Two other, unrelated, coactivators, TIF1 (transcription intermediary factor 1) and RIP140 (receptor interacting protein 140), have been reported to interact with nuclear receptors in a ligand-dependent fashion. The association of all of these coactivators with receptors is mediated by multiple helical LXXLL motifs present in distinct regions of the coactivators, termed NR boxes (*17*, *18*). More recently, a different class of coactivators, alternatively called DRIP (VDR interacting proteins), TRAP (TR-associated protein), or mammalian Mediator, have been identified (*16*, *19*−*22*). These proteins form a multicomponent complex that mediates between the receptors and the general transcription machinery, thereby activating transcription.

Ligand-dependent activation of nuclear receptors is mediated by the carboxyl terminal region of the receptors, termed the ligand-binding domain (LBD), which coordinates many of the functions of these proteins. The LBD contains the ligand binding pocket as well as regions that mediate multiple protein−protein interactions, including association with

† This work was supported by Grant CA68150 from the NIH.

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[1] Abbreviations: RXR, retinoid X receptor; TR, VDR, and PPAR, thyroid hormone-, vitamin D-, and peroxisome proliferator-activated receptors, respectively; 9cRA, 9-*cis*-retinoic acid; H11 and H12, helix 11 and helix 12, respectively; EMSA, electrophoretic mobility shift assay; LBD, ligand binding domain; SEM, standard error of the mean.

10.1021/bi011764+ CCC: $22.00
Published on Web 01/29/2002

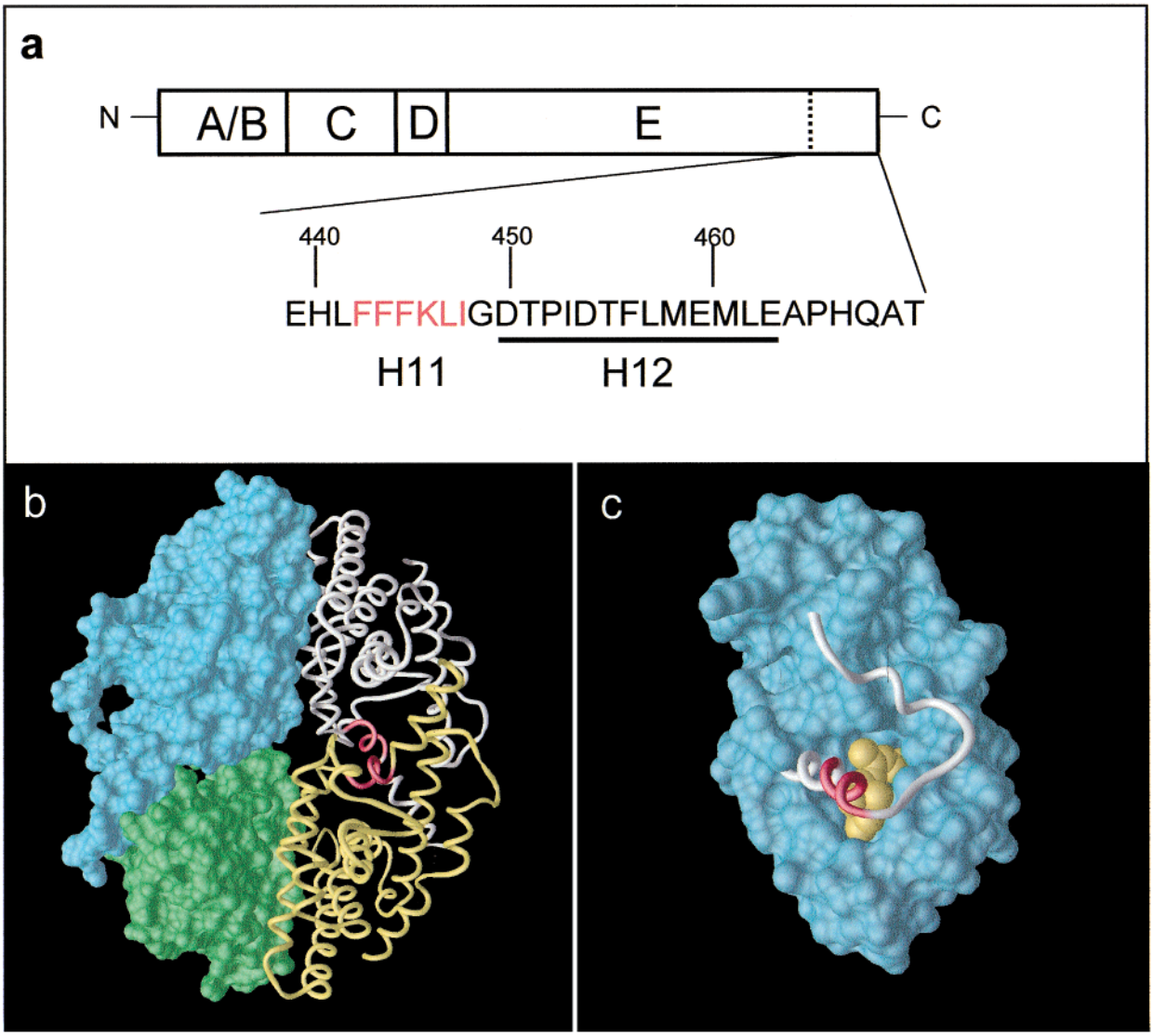

FIGURE 1: Structure of RXR. (a) Domain structure of mRXRα. The sequence of the C-terminus of the receptor, including helices 11 (red) and 12 (underlined), is shown. (b) Three-dimensional structure of the RXR tetramer (*42*). The tetramer is composed of two RXR dimers; one monomer in each dimer is shown as a space-filling model (top, blue; bottom, green). Ribbon structures of the remaining monomers are shown to emphasize the involvement of H11 (red) in stabilizing dimer−dimer association. (c) Three-dimensional structure of liganded RXR (*59*). One monomer is shown. The C-terminus of the protein, including H11 (red), is folded over the entrance to the ligand binding pocket. Bound 9cRA is depicted in yellow.

coactivators, dimerization, and, in the case of RXR, formation of tetramers. The LBDs of nuclear receptors are far less conserved than their DNA binding domains. Nevertheless, all of the LBDs for which the three-dimensional crystal structures have been described share a common fold (*23*−*25*). They consist of 12 α-helices (numbered from the amino to the carboxyl terminus) and a *β*-turn arranged as an antiparallel "sandwich". Comparison between apo- and holo-LBD structures revealed that the most significant conformational change induced by ligand binding involves repositioning of the amphipathic α-helix of the AF-2 core at the C-terminus of the LBD and led to the suggestion of a "mousetrap" model which describes how a series of ligand-induced conformational changes may lead to receptor activation (*26*). According to this model, ligand binding by nuclear receptors induces the C-terminal helices of the LBD, helix 11 and helix 12 (H11 and H12, respectively; Figure 1), to undergo a large conformational change whereby H12 shifts from its extended position in the absence of ligand to a position where it folds over the entrance to the ligand binding pocket as a "lid" (Figure 1c). In its folded conformation, H12 participates in the formation of a surface that mediates the interactions of the receptors with coactivators, a function that renders H12 critical for the ligand-dependent transcriptional activity of the receptors (*27*−*29*). Recently, the three-dimensional structures of several complexes of LBDs with short coactivator peptides containing the NR recognition motif LXXLL have been reported (*30*−*32*). The structure of liganded TR*β* associated with a 13-residue peptide containing the receptor interaction domain of GRIP1 revealed that the peptide is accommodated by a shallow hydrophobic groove on the surface of the receptor which is formed by residues donated by H3−H5 and H12. A similar hydrophobic groove was found to comprise the coactivator interaction surface of the hERα LBD (*30*). Hence, in addition to H12, the coactivator binding region of the nuclear receptor involves contributions from several helices across the LBD.

In contrast with RAR and TR, the interactions of apo-RXR with known transcriptional corepressors are weak (*11*, *13*, *14*, *33*). These observations suggest that ligand-dependent activation of RXR may be controlled by a different mechanism. We previously demonstrated with regard to this that, in the absence of a cognate ligand, RXR self-associates into tetramers with a high affinity, suggesting that it exists as a tetramer at physiological concentrations (*34*−*39*). Because binding of a cognate ligand to RXR tetramers induces their rapid dissociation into dimers and monomers, we proposed that tetramer formation serves to silence the transcriptional activity of RXR, and that the first step in the activation of this receptor involves ligand-induced tetramer dissociation (*37*, *38*). This model was supported by mutagenesis analyses that indicated that two residues within the ligand binding pocket of RXR, mRXRα-R321 and mRXRα-F318, are important for communicating between the binding pocket and the tetramerization region at the surface of the protein. It was shown that an R321A mutant of mRXR forms tetramers with a wild-type affinity, but these tetramers fail to dissociate following ligand binding (*39*). As predicted by the model described above, this mutant displays a low transcriptional activity even under saturating ligand concentrations. In contrast, the RXRα-F318A mutant is unable to form tetramers, although it self-associates into dimers with a wild-type affinity (*39*). In accordance with the notion that tetramer formation allows RXR to act as an autosilencer, this mutant was found to display constitutive, ligand-independent activity (*39*−*41*).

The core of the tetramerization region of RXR was localized to H11 of the receptor LBD, a region that contains three consecutive phenylalanine residues (*38*). Recently, the three-dimensional structure of the RXR tetramer was determined by X-ray crystallography, and the emerging model revealed that, in addition to the H11−H11 interface, the tetrameric structure is also stabilized through an H3−H3 interface, and by interactions between the AF2 domain of one dimer and the coactivator binding groove of another (*42*; see Figure 1b). The involvement of the latter in stabilizing the interactions between adjacent RXR dimers will physically exclude binding of coactivators thereby contributing to the autosilencing function of the RXR tetramer (*42*). Surprisingly, we found that mutation of the Phe residues at the core of the teramerization region not only hinders the ability of the receptor to form tetramers but also inhibits its activity (*38*). These observations were unexpected because H11 was not previously implicated in contributing to the transcriptional activity of RXR. We thus proposed that H11 may play a dual function. In the absence of ligand, it stabilizes tetramer formation; upon addition of ligand and tetramer dissociation, it may be involved in the receptor's transcriptional activity, perhaps by mediating the interations of RXR with some transcriptional coactivators (*38*, *39*). Here, we examined this hypothesis by investigating the role of RXR H11 in stabilizing the interations of the receptor with coactivators. The results of this study show that the region is critical for the association of RXR with the p160 coactivators p/CIP and TIF2, but not for binding of TIF1. The data suggest that different structural determinants underlie the association of RXR with different coactivators, and implicate H11 of the receptor in direct interations with accessory proteins.

EXPERIMENTAL PROCEDURES

Ligands. 9cRA was purchased from ICN. LG1069 was a gift from Ligand Pharmaceuticals (San Diego, CA). Stock solutions in ethanol or DMSO were stored in amber vials at −80 °C. Experiments with 9cRA were carried out under yellow light to minimize isomerization and degradation.

Bacterial Expression Vectors. GST-tagged mRXRαΔAB and mRXRαΔAB-F443/4S were generated by PCR amplification of residues 140−467 of mRXRα using mRXRα and mRXRα-F443/4S as templates. mRXRαΔAB-ΔH12 was generated by PCR of amino acids 140−448 of mRXRα. Constructs were verified by sequencing and subcloned into the bacterial expression vector pGEX4T-1 (Pharmacia Biotechnologies).

Site-Directed Mutants. mRXRα mutants were generated by the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using mRXRα as a template. Internal primers containing the mutant sites were as follows: F443S/F444S, 5′-GGAGCACCTGTTCTCCTCCAAGCTCATCGG; F442S/F443S/F444S, 5′-GGAGCACCTGTCCTCCTCCAAGCTCATCGG; F442A, 5′-TGCCTGGAGCACCTGGCCTTCTTCAAGCTCATC; and F442A/F443A, 5′-TGCCTGGAGCACCTGGCCGCCTTCAAGCTCATC.

Coactivators. Expression vectors for hTIF1α and TIF2 were gifts from H. Gronemeyer and P. Chambon. The expression vector for p/CIP was provided by C. Glass.

Proteins. GST-tagged proteins were expressed and purified from *Escherichia coli* (JM109). Briefly, bacteria were grown at 37 °C to an OD_{600} of 0.6−0.8. Protein expression was induced with 0.5 mM IPTG, and bacteria were grown for an additional 3 h, harvested, lysed, and centrifuged. Supernatants were incubated with pre-equilibrated glutathione−agarose beads for 2 h, and the beads were extensively washed with $HEDK_{100}$ [10 mM HEPES (pH 8.0), 0.1 mM EDTA, 0.4 mM DTT, 100 mM KCl, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin] containing 0.1% NP40. The purity of the matrix-bound proteins was determined by SDS−PAGE and Coomassie blue staining. Matrix-bound proteins were stored at 4 °C until they were used. Histidine-tagged proteins were similarly expressed and purified using chelating affinity chromatography as previously described (*39*).

Fluorescence Titrations. Equilibrium dissociation constants (K_d) characterizing the association of RXR with 9cRA were measured by fluorescence titrations. Ligand binding was monitored by following the changes in the intrinsic fluorescence of the proteins (λ_{ex} = 280 nM; λ_{em} = 340 nM) as previously described (*35*). Titration curves were corrected for inner filtering and analyzed by using an equation from simple binding theory to yield the K_d and the number of ligand binding sites (*43*). Nonlinear least-squares regressions were carried out using Origin (MicroCal, Inc.).

Eletrophoretic Mobility Shift Assays (EMSAs). EMSAs were carried out in the presence of an oligonucleotide containing a DR-1 response element with the sequence 5′-TCGAGGGTAGGGGTCAGAGGTCACTCGTCGA-3′ as previously described (*37*).

Coupled in Vitro Transcription and Translation. ^{35}S-labeled TIF1, TIF2, or p/CIP was synthesized by the TNT Quick-coupled reticulocyte lysate system (Promega) in the

presence of [^{35}S]methionine according to the manufacturer's protocol.

Coprecipitation Assays. Beads containing a GST−mRXRαΔAB, GST−F443/4SΔAB, or GST−mRXRαΔABΔH12 fusion protein or GST alone (150−300 pmol) were incubated with 4−10 μL of ^{35}S-labeled coactivator in the presence or absence of ligand (1.5−2 μM) at 4 °C for 2 h. One hundred to one hundred fifty microliters of beads was used in a total reaction mixture of 1 mL of $HEDK_{100}$ buffer containing 0.2 mM PMSF. Following incubation, beads were precipitated by centrifugation and washed extensively with $HEDK_{100}$ containing 0.1% NP40 and 0.2 mM PMSF. Bead-bound proteins were then resolved by SDS−PAGE and visualized by autoradiography. Bands were quantitated using ScionImage software.

Peptide Competition Assay. Purified histidine-tagged proteins (150 pmol) were spotted onto an Immobilon-P PVDF transfer membrane (Millipore Corp., Bedford, MA) using a 96-well microsample filtration minifold system (Schleicher & Schuell, Inc., Keene, NH). Membranes were then incubated in a buffer containing 25 mM HEPES (pH 7.7), 25 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT for 1 h at room temperature, and blocked in the same buffer containing 5% nonfat dry milk for 30 min, followed by a second 30 min block in a buffer containing 1.0% nonfat dry milk. They were then cut into several pieces which were sealed separately in plastic bags and incubated at 4 °C for 1 h in a buffer containing 25 mM HEPES (pH 7.7), 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.05% NP-40, and 1.0% nonfat dry milk in the presence or absence of the RXR-selective ligand LG1069. ^{35}S-labeled in vitro-translated coactivators and the appropriate competing peptide were then added in the absence or presence of 2.0 μM LG1069. Following an overnight incubation at 4 °C, membranes were washed and air-dried, and coactivators were visualized by autoradiography.

Transactivation Assays. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transfected with the DR-1-tk-luciferase reporter vector (0.3 μg), and an expression vector encoding the appropriate RXR (0.3 μg). Cells were also transfected with the vector pCH110, a β-galactosidase expression plasmid (0.2 μg, internal control). Transfections were carried out using Fugene (Roche Diagnostics Corp.) according to the protocol of the manufacturer. Twenty-four hours after transfection, the medium was replaced by DMEM without serum and ligands were added from a concentrated DMSO solution. Following treatment for 24 h, cells were lysed and assayed for luciferase activity using the luciferase assay system (Promega). Luciferase activity was corrected for β-galactosidase activity which was measured by standard procedures.

RESULTS

The RXR Tetramerization Core Is Required for Binding of the Receptor to TIF2 and p/CIP, but Not for Interactions with TIF1. To investigate whether the Phe residues in H11 that were shown to be critical for RXR tetramerization, F443 and F444, are also important for association of the receptor with coactivators, a series of coprecipitation experiments were carried out. GST-labeled RXR lacking the A/B domain (GST−RXRαΔAB fusion protein) and a corresponding protein in which these residues are substituted (GST−RXRαΔAB-F443/4S fusion protein) were bacterially expressed. As it is well-established that the main coactivator interaction region of nuclear receptors resides in their carboxy terminal helix, H12, a corresponding receptor lacking H12 (GST−RXRαΔABΔH12 fusion protein) was also generated and used as a control. The proteins were expressed in *E. coli* and purified by glutathione affinity chromatography. To verify that the mutations did not result in alterations of the overall folding of the receptor, the ligand binding affinities of RXR and its mutants for 9cRA were measured. Equilibrium dissociation constants (K_ds) were measured by fluorescence titrations, a method that we and others used in the past to examine ligand binding by a variety of retinoid binding proteins, including RXR (*34*−*36*). Due to the extensive overlap of the fluorescence emission spectra of proteins (displaying a maximum at 340−350 nM) and the absorption spectrum of RA (peaking at 380 nM), ligand binding by RXR is accompanied by a significant decrease in the intrinsic fluorescence of the protein, a phenomenon that can be used to construct a titration curve. Proteins were placed in a cuvette and titrated with 9cRA as previously described (*34*−*36*). Resulting titration curves were analyzed to obtain the K_ds characterizing the association of RXR and its mutants with 9cRA. K_d values for all three proteins were found to be in the range of 15−25 nM (mean of three independent measurements for each protein, SEM did not exceed 10% for any of the proteins). These values are in agreement with the previously reported equilibrium dissociation constants of RXR (*34*−*36*) and of RXR lacking H12 (*44*). Hence, the ligand binding affinity of RXR was not altered by the mutations, demonstrating that the overall folding of the mutants was intact.

Interactions of WT-RXRαΔAB and its F443/4S and ΔH12 mutants with three different coactivators (p/CIP, TIF1, and TIF2) were then examined by coprecipitation assays. It may be noted that coprecipitation assays do not allow for measurements of absolute values of equilibrium dissociation constants. However, this methodology will provide reliable information about relative binding affinities, i.e., about the effects of the F443/4S and ΔH12 mutations on the ability of RXR to associate with a particular coactivator. Receptors (200 nM), immobilized on glutathione−agarose beads, were incubated with in vitro-transcribed and -translated ^{35}S-labeled coactivators in the absence or presence of the RXR-selective ligand LG1069. Mixtures were centrifuged; beads were extensively washed, and coactivators that coprecipitated with the receptors were visualized by autoradiography (Figure 2). As expected, WT-RXR associated with all three coactivators in a ligand-dependent manner. Also, as expected, deletion of H12 abolished the ability of RXR to bind to the three coactivators in accordance with the essential role of this region in receptor−coactivator interactions. Interestingly, while the F443/4S mutation had only a small effect on the interactions of RXR with TIF1 (Figure 2c), it all but abolished the ability of the receptor to bind either p/CIP or TIF2 (panel a or b of Figure 2, respectively). These observations suggest that H11 may comprise a critical part of the surface region of RXR that mediates its interactions with TIF2 and p/CIP, but that this region is not involved in binding of TIF1.

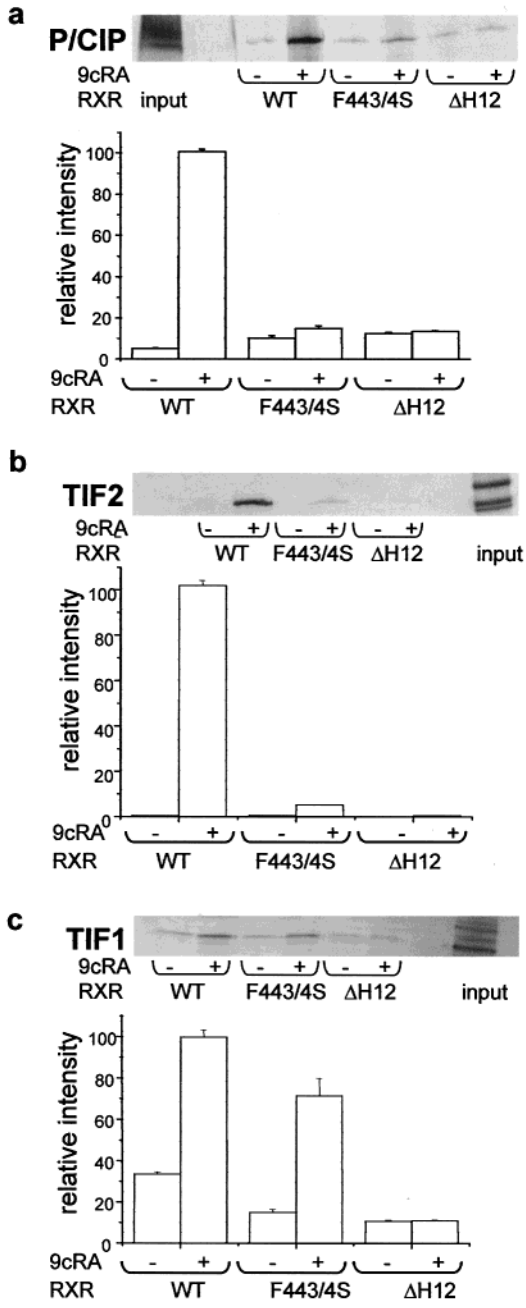

FIGURE 2: Interactions of coactivators with mRXRαΔAB and its mutants. Coprecipitation assays were carried out using a bacterially expressed GST−mRXRαΔAB or GST−mRXRαΔAB-F443S/F444S fusion protein or mRXRαΔABΔH12 as bait. The receptors were immobilized on glutathione beads. Immobilized proteins (200 nM) were mixed with in vitro-transcribed and -translated ^{35}S-labeled coactivators and incubated in the absence or presence of the RXR-selective ligand LG1069 (1 μM). Beads were centrifuged and washed, and proteins that coprecipitated with the receptors were resolved by SDS−PAGE and visualized by autoradiography. Experiments were carried out with the coactivators (a) p/CIP, (b) TIF2, and (c) TIF1. In each panel, the top shows a representative gel and the bottom quantitation of band intensities. Points are means ± SEM of band intensities derived from three independent experiments.

A Peptide Containing the Amino Acid Sequence of RXR H11 Competes with RXR for Binding to TIF2 but Not to TIF1. The observations that the F443/4S mutation inhibits the association of RXR with TIF2 and p/CIP suggest a direct involvement of H11 in mediating the association of the receptor with these coactivators. It could be argued, however, that the mutation may have resulted in misfolding of the C-terminal region of RXR, thereby hindering proper association with TIF2 and p/CIP. It should be noted with regard to this that the observations that the mutation did not alter the ligand binding affinity of the receptor, and our previous findings that the urea sensitivity of RXR-F443/4S is identical to that of the wild-type receptor (*38*), support the conclusion that this double mutation does not result in alterations of the global structure of the protein. In addition, the observation that the mutant associated with TIF1 in a ligand-dependent fashion (Figure 2) suggests that it is folded correctly for interaction with at least one coactivator. Nevertheless, to further clarify whether H11 is specifically and directly involved in the RXR−TIF2 association, we studied the ability of a peptide containing the amino acid sequence of this region to compete with RXR for coactivator binding. If H11 is essential for binding of TIF2 but not of TIF1, we can expect that such a peptide will compete with RXR for binding of the former but not the latter coactivator. If the Phe residues of H11 are important for the RXR−TIF2 interaction, it can be further expected that substitution of these residues within the peptide will hamper its ability to compete with RXR for binding of the coactivator. In these experiments, an additional peptide comprised of the amino acid sequence of H12 was used as a control. This peptide is expected to effectively compete with RXR for binding of both TIF1 and TIF2.

Hence, the ability of the following peptides to compete with RXR for association with either TIF1 or TIF2 was investigated: (1) H11 (FFFKLISGRGKLEGD), where the actual H11 sequence is underlined. A hydrophilic extension was added to increase the aqueous solubility of the markedly hydrophobic helix and allow for experiments at peptide concentrations that are necessary for competition. The overall charge of the peptide was not changed by the addition of the extension. (2) Mutant H11 (AAAKLISGRGKLEGD). (3) H12 (DTPIDTFLMEMLE). RXR (150 pmol) was spotted on a PVDF membrane, incubated in the presence or absence of a cognate ligand, and probed with in vitro-transcribed and -translated ^{35}S-labeled coactivators. Assays were carried out in the absence or presence of the appropriate peptides (Figure 3). The data show that the peptide containing the H12 sequence efficiently competed with RXR for binding to both TIF1 and TIF2. The peptide containing the H11 sequence had only a small effect on the RXR−TIF1 interactions, but effectively competed with RXR for binding to TIF2. Mutation of the Phe residues of the H11 peptide significantly hindered its ability to compete with RXR for binding of TIF2, although it did not abolish binding completely (Figure 3a). These observations thus further support the conclusion that H11 is directly involved in mediating the association of RXR with TIF2, but that the region is dispensable for the RXR−TIF1 interaction.

TIF1 Can Rescue the Transcriptional Activity of an RXR Mutant Lacking the Phe Residues of H11, but TIF2 Cannot. We previously reported that mutation of F443 and F444 in H11 results in a protein that is unable to self-associate into tetramers and is thus dimeric even in the absence of a cognate ligand. It was further found that this mutant is defective in its transcriptional activity when transfected into HeLa cells (*38*). The observations depicted in Figures 2 and 3 suggest, however, that the transcriptional activities of the H11 mutant

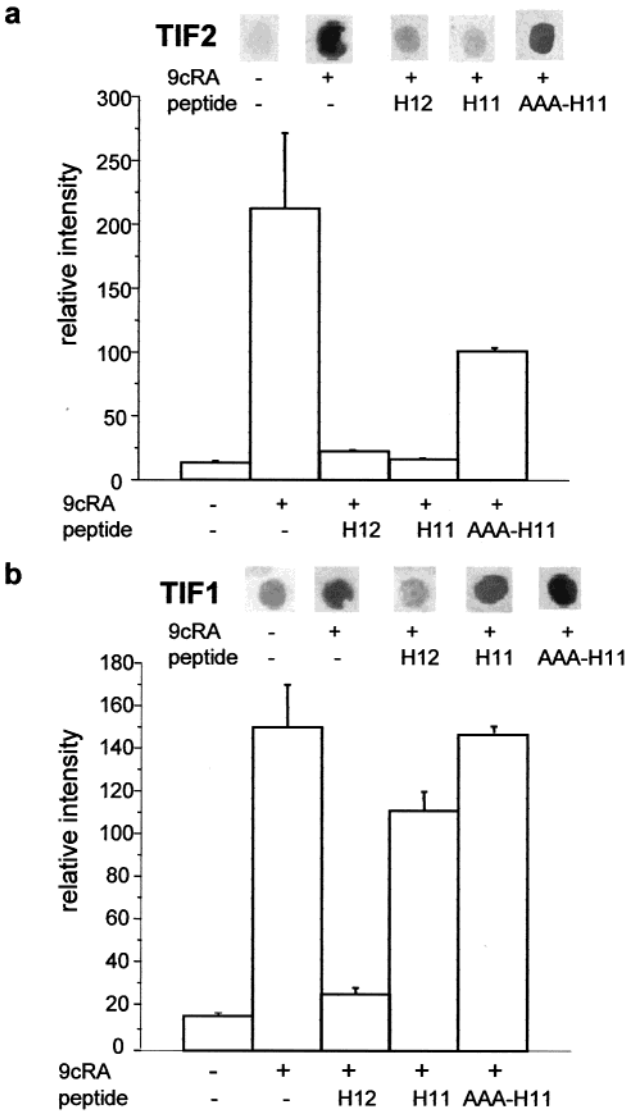

FIGURE 3: Competition between mRXRαΔAB and peptides comprising the sequences of H11 and H12 for binding to coactivators. Histidine-tagged mRXRαΔAB (150 pmol) was spotted onto PDVF membranes. Membranes were incubated with in vitro-transcribed and -translated ^{35}S-labeled TIF2 (a) or TIF1 (b). Incubations were carried out as described in Experimental Procedures in the absence or presence of the RXR-selective ligand LG1069 (2 μM). The ability of RXR to interact with the coactivators was examined either in the absence or in the presence of 1 mM peptides comprised of the sequences of H12, H11, or H11 in which the Phe residues are substituted (AAA-H11). The top is a representative gel; the bottom shows quantitation of spot intensities. Points are means ± SEM of spot intensities derived from three independent experiments.

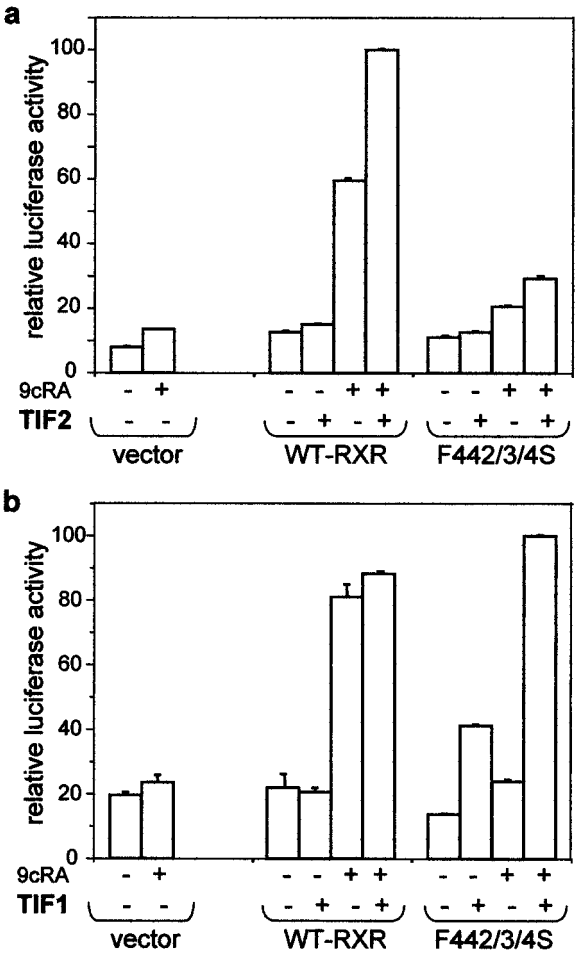

FIGURE 4: Effects of coactivators on the transcriptional activity of RXR and its F442/3/4S mutant. Transactivation assays were carried out in COS-7 cells. Cells were cotransfected with a luciferase reporter construct driven by a DR-1 response element, a pCH110 vector encoding β-galactosidase (used to control for transfection efficiency), and an expression vector for either WT-RXR or its F442/3/4S mutant. To examine the effects of coactivators on the transcriptional activities of the receptors, cells were cotransfected with an expression vector for either TIF2 (a) or TIF1 (b) or with an empty vector (control). Cells were treated with 9cRA for 24 h, lysed, and assayed for luciferase and β-galactosidase activity. Luciferase activity normalized to the activity of β-galactosidase is shown. Bars represent means ± SEM of three independent experiments.

may depend on the nature of the particular coactivators that are present in cells, i.e., that the mutant may be silent in cells that express coactivator(s) that critically require the H11 Phe residues for interactions with RXR, but active in the presence of a coactivator that interacts with RXR in an H11-independent fashion. To examine this notion, we investigated the effects of ectopic expression in COS-7 cells of either TIF1 or TIF2 on the ability of RXR carrying H11-Phe mutations to activate transcription of a luciferase reporter driven by a RXR response element (Figure 4). Addition of 9cRA to cells ectopically expressing WT-RXR resulted in a 5-fold increase in the receptor activity, while the mutant displayed a very low transcriptional activity (Figure 4a). Ectopic expression of TIF2 enhanced transcriptional activation mediated by WT-RXR but had only a small effect on the transcriptional activity of the mutant, which remained low. In contrast, cotransfection of TIF1 fully restored the transcriptional activity of the mutant (Figure 4b).

DISCUSSION

RXR is unique among nuclear receptors in that it serves as a common binding partner for numerous receptors and thus regulates the transcriptional activities of multiple signaling pathways converging at the genome. RXR is also unique in that, unlike other nuclear receptors, it self-associates into tetramers, thereby silencing its own transcriptional activity in the absence of a cognate ligand. The first step in the activation of RXR is thus believed to comprise ligand-induced dissociation of the inactive tetrameric struc-

ture into dimers and monomers (*39*, *42*). In turn, RXR homodimers can regulate transcription through RXR response elements, while RXR monomers may associate with partner receptors to regulate transcription of different sets of target genes. Studies of the structural determinants that underlie tetramer formation by RXR indicated that H11, close to the C-terminus of RXR, is at the core of the region that mediates dimer−dimer association of the unliganded receptor, and that the association is also stabilized through H3 of the RXR−LBD and by interactions of the AF-2 helix with the coactivator binding site of an adjacent dimer (*38*, *42*). Inspection of the sequence of H11 reveals the presence of three consecutive phenylalanine residues which, importantly, are conserved in RXRs in all reported species and in all RXR isotypes. It has been shown that two of these (F443 and F444 in mRXRα) are critical for tetramer formation and that their substitution results in a protein that is unable to form tetramers (*38*). Interestingly, it was found further that mutation of these residues not only abolishes tetramer formation but also results in a dimer that is defective in its transcriptional activity (*38*). These observations suggest that, while H11 assists in stabilization of tetramers of apo-RXR, the region may also be important for the transcriptional activity of the receptor, perhaps through involvement in the receptor interactions with transcriptional coactivators.

Here, we examined the involvement of the Phe residues of RXR H11 in mediating the interactions of the receptor with three coactivators (p/CIP, TIF1, and TIF2). Coprecipitation assays showed that mutation of two of the Phe residues hinders the association of the receptor with TIF2 and with p/CIP in the presence of an RXR agonist. In contrast, the mutant retained its ability to bind to TIF1 (Figure 2). These observations imply that H11 serves to stabilize the interactions of the receptor with some coactivators but not with others. This notion was further supported by investigating the ability of a peptide comprised of the H11 sequence to compete with RXR for interactions with coactivators. Such a peptide effectively competed with holo-RXR for binding of TIF2 but not for association with TIF1 (Figure 3). Hence, while H11 of RXR is directly involved in the interactions of the receptor with TIF2, it does not appear to participate in the formation of the RXR−TIF1 complex. In agreement with these in vitro observations, the findings of this work further show that overexpression of TIF1, but not of TIF2, restores the defective transcriptional activity of an RXR mutant in which the H11 Phe residues have been substituted. The observations that the mutant displayed wild-type activity in the presence of a coactivator that can bind to it, such as TIF1, further verify that the mutations did not disturb the overall structure of RXR but specifically interfered with the receptor's ability to interact with particular coactivators. Taken together, the data demonstrate that H11 mediates binding of specific coactivators by RXR. Additional studies will be required to unravel the significance of this selectivity in coactivator binding for RXR function.

The reported three-dimensional structures of RXR in its apo and holo forms reveal that H11 undergoes an important "twist" upon ligand binding. In the apo configuration, one face of the helix, including two of the Phe residues (F437 and F438 in hRXRα, corresponding to F442 and F443, respectively, in mRXRα), is positioned inward while the other face, including the third Phe residue of hRXRα, F439 (corresponding to mRXRα F444), is exposed to the solvent. Following ligand binding, the helix reverses the directions of its faces, thereby positioning mRXRα F444 toward the ligand binding pocket and rendering mRXRα F442 and F443 accessible to the bulk solvent (*45*). These structural features imply that of the three Phe residues of H11, only mRXRα F444, which is at the surface of the protein in the apo state, may be directly involved in stabilizing the tetrameric structure of the unliganded receptor. In turn, only mRXRα F442 and F443, which are at the surface of the receptor in the ligated state, may directly mediate the interactions of the receptor with coactivators. However, it was previously reported that individual mutations of each of the H11 Phe residues are not sufficient to hamper the ability of apo-RXR to form tetramers, and that tetramers are unaffected unless both mRXRα F443 (which is directed inward in apo-RXR) and mRXRα F444 (positioned at the surface of the receptor in its unliganded state) are replaced (*38*). These observations suggest that F443 may indirectly enable tetramer formation, perhaps by correctly positioning H11 for mediating the self-association of apo-RXR. Similarly, in the presence of a cognate ligand, F444 may act indirectly by stabilizing a configuration that positions H11 correctly for interactions with p160 coactivators. In addition, the observation that an H11 peptide in which all three Phe residues have been substituted retains a partial ability to inhibit TIF-2−RXR association (Figure 3) suggests that H11 residues other than the phenylalanines may also be involved in coactivator binding. Hence, while H11 plays critical roles both in tetramer formation and in coactivator binding by RXR, the functions of individual residues within the helix remain to be better determined.

The results of this study indicate that RXR employs differential modes of interactions with different coactivators. Specifically, in view of the high degree of homology found among p160 family coactivators, the data suggest that H11 of RXR serves as a coactivator binding site for p160 coactivators (e.g., TIF2, SRC1, and p/CIP) but not for the unrelated coactivator TIF1. This conclusion is surprising because the interactions of both TIF1 and p160 coactivators with nuclear receptors are believed to be mediated through the common coactivator NR interaction motif LXXLL. TIF1 was found to possess one, while p160 coactivators contain multiple such sequences with three of these contributing to receptor−coactivator association (*29*, *46*−*48*). The structural features through which TIF2 associates with the H11 region of RXR thus remain to be clarified. It may be worth noting that, in addition to its differential mode of interactions with RXR reprorted here, TIF1 is different from p160 coactivators in a number of ways. For example, while p160 coactivators function to stimulate nuclear receptor-mediated hormone-dependent transcription (*49*−*51*), the role of TIF1 is not as clear, and it has been reported that this accessory protein may act as a repressor in some settings (*52*, *53*). It was also reported that, unlike other coactivators, TIF1 is directly associated with chromatin in P19 cells (*54*).

The reported three-dimensional structures of cocrystals of nuclear receptors with peptides comprised of an NR interaction region of p160 coactivators revealed that the coactivator peptide binds to a shallow hydrophobic groove at the surface of the receptors, and that this groove is formed by residues donated from H3−H5 and H12. H11 was not

implicated in these interactions. It should be noted, however, that these studies utilized short interacting coactivator peptides rather than full-length coactivators (*30*−*32*). It is possible, therefore, that the reported coactivator binding determinants only reflect a partial interaction interface. In addition, available structural information about coactivator−receptor interactions is based on studies carried out with TR, ER, and PPAR, receptors that, unlike RXR, do not contain consecutive phenylalanine residues in their H11 sequences. H11 of RXR thus appears to comprise a novel coactivator binding interface. An hypothesis suggested by this work is that H11 may also function in coactivator binding by other receptors, such as COUP-TF1, ARP-1, EAR2, HNF-4, and SEVEN UP, that share with RXR extensive homology in this region, including two of the three phenylalanine residues (*55*−*58*).

ACKNOWLEDGMENT

We are grateful to Richard Gillilan for generating the pictures presented in Figure 1. We also thank Pierre Chambon and Christopher Glass for constructs.

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BI011764+