The Tetramerization Region of the Retinoid X Receptor Is Important for Transcriptional Activation by the Receptor*

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The retinoid X receptor (RXR), a member of the superfamily of hormone nuclear receptors, is a ligand-inducible transcription factor that is activated by the vitamin A derivative 9-cis-retinoic acid. We previously showed that RXR self-associates into tetramers with a high affinity and that ligand binding induces rapid dissociation of receptor tetramers to smaller species. Here, the RXR region that is responsible for mediating tetramer formation is identified. It is shown that this interface, which we term the “tetramerization domain,” critically contains two consecutive phenylalanine residues located at the C-terminal region of the receptor. Mutation of these residues is sufficient to disrupt RXR tetramers without affecting the overall fold of the protein or interfering with ligand binding, dimer formation, or DNA binding by the receptor. Nevertheless, the tetramer-impaired mutant was found to be transcriptionally defective. The newly characterized tetramerization domain and the previously identified main dimerization interface of RXR act autonomously to affect separate inter-subunit interactions that, overall, lead to formation of tetramers. Protein-protein interactions mediated by the tetramerization domain, but not those that involve the dimerization interface, are disrupted following ligand binding by RXR. Overall, these data attest to the specificity of the interaction and implicate the tetramerization interface in playing a direct role in regulating transcriptional activation by RXR.

Retinoic acids are lipophilic hormones that can modulate the rates of transcription of a variety of genes and play important roles in cellular proliferation, differentiation, and apoptosis (1–3). Transcriptional regulation by retinoic acids is mediated by two types of transcription factors: the retinoic acid receptors (RARs), which can bind and are activated by all-trans and 9-cis retinoic acids (tRA and 9cRA), and the retinoid X receptors (RXRs), which respond exclusively to 9cRA (4–8). These proteins belong to the superfamily of nuclear receptors, which also includes the vitamin D receptor (VDR), the thyroid hormone receptors (TR), the peroxisome proliferator-activated receptors (PPAR), and a large number of orphan receptors (for review, see Refs. 9–12). DNA recognition sequences (response elements (RE)) for these receptors consist of an inverted, inverted, or direct repeat of the consensus motif PuG(G/T)TCA, with a variable number of base pairs between the two half-sites (15–15).

As suggested by the repeat structure of their REs, most hormone nuclear receptors bind to cognate DNA as dimers. RXR can bind to cognate DNA with a high affinity as a homodimer (16–18), and, in the context of at least some promoters, RXR homodimers efficiently regulate transcription (19–23). In contrast, homodimers of other members of the hormone nuclear receptor family, such as RAR, VDR, PPAR, and TR, bind to cognate DNA weakly and are inefficient transcriptional regulators. Instead, these receptors form heterodimers with RXR and their activities seem to be exerted mainly via these heterodimers (24–30). The precise role of RXR within heterodimers is not completely understood at the present time. It has been proposed that RXR within RXR-RAR heterodimers does not associate with its ligand and thus serves as a transcriptionally silent partner (31, 32). In conflict with this notion, it has been demonstrated that neither the equilibrium binding affinity nor the kinetic parameters of the interactions of RXR with its ligand are affected by formation of RXR-RAR heterodimers regardless of whether the heterodimers are in solution or bound to cognate DNA (33, 34). In accordance with the later observations, in vivo transactivation data in a number of systems revealed that there exists functional synergy between RAR- and RXR-specific agonists (25, 35–38) and that both RAR and RXR are required for 9cRA-mediated inhibition of activation-induced apoptosis (39, 40).

It is well documented that ligands are important for transcriptional regulation by hormone nuclear receptors, but the molecular mechanisms by which ligands modulate the activities of their receptors have only recently begun to be elucidated. Binding of ligands to RAR and to TR results in the release of a corepressor that associates with the unliganded receptors (41–46). This corepressor associates only very weakly with RXR, suggesting that ligand-dependent activation of RXR operates via a different mechanism (42, 44–46). Activation of RXR is likely to involve several coactivators and general transcription factors that have been shown to associate with this receptor in a ligand-enhanced fashion (47–54). Another important clue regarding the mechanism of activation of RXR emerged from the recent reports that RXR self-associates to form tetramers with a high affinity and that the oligomeric state of the receptor is regulated by its ligand (55–57). Several lines of evidence suggested that ligand-dependent modulation of the oligomeric state of RXR might serve to regulate the activity of the receptor. It was demonstrated that, in the absence of ligand, RXR tetramers comprise the predominant species of the receptor at concentrations that are higher than about 70 nm. It was shown...
further that upon addition of ligand, RXR tetramers rapidly dissociate to dimers and monomers (18, 56). These observations led to the suggestion that RXR serves as its own “corepressor,” i.e. that tetramer formation by this receptor functions to sequester active species, i.e. dimers and monomers, into a transcriptionally inactive, tetrameric complex. Ligand binding induces a conformational change that results in dissociation into dimers and monomers, thereby activating both the homodimeric and the heterodimeric RXR signaling pathways. It was therefore proposed that ligand-induced dissociation of RXR tetramers is an essential step in transcriptional activation mediated by this receptor.

Inherent in the hypothesis that RXR tetramers serve to silence the transcriptional activity of the receptor is that a region in RXR that is necessary for transcriptional activation is masked within tetramers. One region of RXR that is expected to be masked within tetramers is the interface that mediates tetramer formation. The present work was undertaken to identify this region and to explore whether it is important for the transcriptional activity of the receptor. The results of this work led to the identification of the RXR region that mediates tetramer formation. It is demonstrated that mutation of two amino acid residues within this region results in disruption of RXR tetramers without affecting the overall fold of the receptor and without impairing its ligand binding, dimerization, or DNA binding functions. Nevertheless, it is shown that disruption of the tetramerization interface diminishes the ability of RXR to activate transcription of a reporter gene. These results implicate the tetramerization region in playing a direct role in transcriptional activation by RXR.

EXPERIMENTAL PROCEDURES

Generation of Truncated and Mutant Proteins—All Polymerase Chain Reaction (PCR) amplifications were performed with Vent-polymerase using the following cycling times: 90 s at 94 °C, 120 s at 55 °C, and 180 s at 72 °C.

Truncated Proteins—mRXRa-LBD, mRXRaAB, and mRXRa-LBDH12 were prepared by PCR amplification of the respective regions of mRXRs. Primer sequences are available upon request. Purified PCR fragments were subcloned either into the Nde−I/XhoI sites of pET15b or into the NdeI-XhoI sites of pET28a. Plasmids were initially transfected into the Escherichia coli strain DH5 and, following verification and amplification, into E. coli BL21 (DE3) for protein expression.

Site-specific Mutants—For preparation of site-specific mutants of mRXRaAB, two separate, partially overlapping PCR fragments were generated using either wild-type mRXRa or mutant mRXRaAB as a template. The sequences of the primers used for PCR are available upon request. Mutant sequences were verified by automated sequencing carried out at the Cornell Biotechnology Center.

Proteins—Proteins were obtained by over-expression in E. coli and isolated as described previously (55, 58). Concentration of antibiotics was 100 g/ml ampicillin for usage of pET15b and 30 g/ml kanamycin for usage of pET28a. All isolated proteins were found to be at least 95% pure based on Coomassie Blue staining following SDS-PAGE. Ligand binding characteristics were determined using fluorescence-based binding assays (34, 56, 58). All proteins used in the current study exhibited equilibrium dissociation constants ($K_d$) for 9cRA in the range of 4–26 nM and a number of ligand binding sites in the range of 0.65–0.9 mol/mol of protein, i.e. all mutant proteins possessed wild-type affinity for this ligand. Purified proteins were dialyzed against a buffer containing 10 mM Hepes, pH 8.0, 0.1 mM EDTA, 0.4 mM dithiothreitol, 400 mM KCl, and 5% glycerol and were stored at −20 °C in 50% glycerol. Protein concentrations were determined with the Bradford Assay (Bio-Rad) using bovine serum albumin as a standard.

Antibodies were generously provided by Pierre Chambon (Stobbehouse).

Non-denaturing gel electrophoresis was carried out as described previously (55).

Electrophoretic Mobility Shift Assays (EMSA)—Formation of receptor-DNA complexes was investigated by EMSA in the presence of oligonucleotides containing the DR-1 RE with the sequence: 5′-TGGAGGTTAGGG-TGGAGGTTACTGCCTGGA-3′. Oligonucleotides were end-labeled with [32P]ATP by filling in with Klenow fragments, and free nucleotides were removed with the Qiagen nucleotide removal kit. 1 μl of labeled oligonucleotide (50–200 nM; final concentration, 2.5–10 nM) and 1 μl of 2.4 mg/ml di-4C were mixed with the indicated amounts of RXRΔAB in 18 μl of 10 mM Hepes, pH 8.0, 0.1 mM EDTA, 0.4 mM dithiothreitol, 100 mM KCl, and 15% glycerol. 9cRA was added from a concentrated solution in ethanol to a final concentration of 1 μM, and mixtures were incubated for 15 min at room temperature. Protein-DNA complexes were resolved by electrophoresis in 5% polyacrylamide gels (0.5 × TBE, 2–3 h pre-run at 100 volts, 2 h run at 25 mA/gel), the gel was dried, and protein-DNA complexes were visualized by autoradiography. During electrophoresis, the gel was cooled with circulating water at 12 °C.

Ligand Binding—Binding of ligand was monitored as described previously (56) by following the decrease in the intrinsic fluorescence of RXR (Aex = 280 nm, Aem = 340 nm) upon titration with ligand. Protein (1 μM) was titrated with 9cRA from a concentrated solution in ethanol. The dissociation constant and number of binding sites were calculated by fitting the fluorescence data to an equation derived from simple binding theory. Non-linear least squares regressions were carried out using the software Origin (MicroCal, Inc.).

Retinoid Transactivation Assays—DNA encoding RXR (or mutant), CAT reporter vector, and pCH110 (ratio 1:20:20) at a final concentration of 20 mg/100-mm plate was used to transfet HeLa cells following a standard calcium phosphate-mediated transfection protocol (59, 60). Cells with DNA precipitates were incubated at 37 °C with 5% CO2 for 17–20 h, washed with PBS, and refed with Dulbecco’s modified Eagle’s medium supplemented with 5% delipidated serum. After several hours of growth, cells were transfected with various concentrations of 9cRA in the range of 10−9−10−6 M and incubated at 37 °C for another 18 h. Cells were scraped into 1 ml of cold PBS, transferred to a 1.5-ml Eppendorf tube, and kept on ice. Cells were pelleted by centrifugation at 3,000 rpm, 4 °C, for 5 min in a Beckman GPXRR centrifuge. Supernatants were discarded, and pellets were drained for several minutes. Drained pellets were resuspended in 250 ml of cold 100 mM Tris-HCl, pH 7.5. Cells lysates were prepared by three consecutive freeze-thaw cycles (three min on dry ice, 5 min at 37 °C). Cellular debris was pelleted at 15,000 rpm in an Eppendorf microfuge for 10 min at 4 °C. 200 μl of cell extracts were transferred to a 96-well microtiter plate. Retinoid efficacy was measured by the concentration of induced CAT gene product in the extracts from transfected cells, using CAT enzyme-linked immunosorbent assay kit (5 Prime → 3 Prime, Inc., Boulder CO). CAT activity was routinely normalized for transfection efficiency by the β-galactosidase activity.

RESULTS

The Ligand Binding Domain of RXR Self-associates into Tetramers—We previously suggested that ligand binding activates RXR by inducing dissociation of receptor tetramers into dimers and monomers (18, 55). According to this model, RXR dimers and monomers are monomers capable of activating transcription, while tetramers are transcriptionally silent. This model proposes further that the inability of RXR tetramers to activate transcription might be due to masking of region(s) necessary for transcriptional activation within the tetrameric structure. One region of RXR that is expected to be masked within tetramers is the interface that mediates the formation of tetramers. We thus set out to localize the region responsible for tetramerization of RXR.

A scheme outlining the domain structure of mRXRs and detailing the amino acid residues that are of particular interest in the context of the present study is shown in Fig. 1. The search for the region in RXR that is involved in tetramer formation by the receptor could be somewhat narrowed based on our previous observations that the N-terminal A/B region of RXR is not required for tetramerization (18, 55). Accordingly, the tetramerization interface of RXR is likely to reside within domains C–E of the receptor.

Dimer formation by retinoid receptors is known to be mediated by a strong dimerization function in the ligand binding domain (LBD) and a weaker dimerization function in the DNA binding domain (DBD) of these proteins (61–63). The main homodimerization function of RXR was shown by x-ray crystallography to be located within helix 10 of the LBD (64). Formation of tetramers often requires protein-protein interac-
tions involving two protein regions, such that one of these regions is involved in formation of dimers while the second region acts to attach two dimers together. In principle, these two regions might operate independently. Helix 10 of the LBD is thus expected to participate in tetramer formation by RXR as it contains one of the interaction interfaces.

The second interaction surface necessary for tetramer formation could potentially be located within the RXR-DBD, a domain that by itself forms dimers when bound to cognate DNA (61). To examine whether the RXR-DBD contributes to tetramer formation, an RXR deletion mutant lacking this domain was prepared. The mRXRα-LBD was expressed in E. coli as a histidine-tagged protein and purified, and its oligomeric state was examined by electrophoresis under non-denaturing conditions (Fig. 2). It was previously shown that truncated RXR lacking the A/B domain (RXRΔAB) is resolved by native PAGE into three distinct bands corresponding to protein monomers, dimers, and tetramers, where the dimeric and tetrameric bands display a high intensity and the monomeric band is faint and, in some cases, not observed (55). Binding of ligand by the receptor induces dissociation of RXR tetramers leading to a significant decrease in the intensity of the slower moving, tetrameric band and to an enhancement in the faster moving, dimeric band (56). The data in Fig. 2A show that the RXR-LBD was similarly resolved by native PAGE into two bands with differential mobilities and that the intensity of the slower moving band significantly decreased while the faster moving band was enhanced upon addition of an RXR ligand. A similar pattern was observed at RXR-LBD concentrations as low as 1 μM. Ferguson analysis of the two bands revealed that their mobilities corresponded to molecular masses of 52 and 112 kDa (data not shown), verifying that the two bands reflect the presence of RXR-LBD dimers and tetramers (calculated molecular masses of 56 and 112 kDa, respectively). As previously noted for RXRΔAB (56), ligand binding resulted in a conformational change in receptor dimers leading to a somewhat higher mobility of liganded versus unliganded dimers. Ligand binding by the LBD thus elicits a response that is similar to the response of RXRΔAB, suggesting that both interaction interfaces responsible for tetramerization of RXR are located within the LBD.

Interestingly, when the resolution of the gel was improved, two distinct bands moving at the approximate position of the dimers could be observed (denoted dimers in Fig. 2B). As the two bands display comparable intensities and mobilities, and as monomeric bands have been shown to be much fainter or not observed at all, the two bands are likely to represent two different types of dimers. One dimeric band could reflect a dimer that is formed via interactions between the dimerization interfaces of two monomers, while the other band could reflect dimers in which monomer-monomer interactions are mediated by the second, “tetramerization,” interface. The observations in

![Fig. 2](image243x577 to 558x729)

**Fig. 2. Analyses of the mRXRα-LBD by non-denaturing gel electrophoresis.** The RXR-LBD was electrophoresed on a 8% polyacrylamide gel under non-denaturing conditions (running gel, pH 8.8, no stacking gel) for 5–8 h at 12 °C. Protein bands were visualized by Coomassie Blue staining. A, non-denaturing gel electrophoresis of the RXR-LBD (600 pmol) in the absence (lane 1) or presence (lane 2) of the RXR-specific ligand LG1069 (2000 pmol). B, non-denaturing gel electrophoresis of increasing amounts of RXR-LBD (800, 1200, and 1600 pmol, lanes 1–3, respectively). Fig. 2B thus further support the premise that the tetramerization interface is located within the RXR-LBD.

The Tetramerization Interface of RXR Maps to Consecutive Phenylalanine Residues in Helix 11—To further narrow down the search for the RXR region that contains the tetramerization interface, we attempted to generate various deletion mutants of the LBD. We found that RXR-LBD lacking the C-terminal helix (H12 according to the nomenclature used by Renaud et al. (65)) was able to form tetramers in solution (data not shown), indicating that the interface is not present in this region. However, we encountered difficulties in obtaining other deletion mutant proteins in pure and soluble forms. Alternatively, we considered the reported crystal structure of the dimeric RXR-LBD (64) and attempted to assess which region within this domain may interact with another dimer without compromising symmetry (see below). We concluded that four helices could potentially serve to create a symmetric tetramer. Using the nomenclature of Renaud et al. (65), these were H6, H9, H10, and H11. Within these helices, we focused on residues that are located at the surface of the protein and that are potentially involved in protein-protein interactions.

Various mutants of mRXRαΔAB containing single or dual site-specific mutations were subsequently generated by PCR and expressed in E. coli as histidine-tagged proteins. The protein construct of choice for generating these mutants was RXRΔAB. We previously found that not only RXRΔAB dimers but also RXRΔAB tetramers efficiently associate with oligonu-
cleotides containing a single DR-1 RE. Tetrameric RXR-DNA complexes thus formed were found to contain two oligonucleotides, i.e. each dimer within the tetramer associated with DNA separately (18). This mode of DNA binding by RXR tetramers is different from the reported association of multiple RXR subunits with DNA containing multiple half-sites (66, 67), and its physiological significance remains to be clarified. However, the ability of both dimers and tetramers of RXRΔAB to associate with DNA allows for examination of the oligomerization properties of the various mutants by EMSA. Surface amino acid residues with functional groups that are frequently involved in protein-protein interactions, i.e. hydrophobic (a leucine in H6) or charged (a glutamate in H9, a lysine in H10) residues were targeted. In H11, the presence of three sequential phenylalanine residues raised suspicion, and two of them were mutated.

The DNA binding patterns of the site-directed mutants were investigated by EMSA in the presence of an oligonucleotide containing the consensus DR-1 response element (see "Experimental Procedures" for sequence details) and are shown in Fig. 3A. The wild-type protein formed two distinct complexes with the DR-1 RE (lane 1). These complexes were previously shown to reflect that RXRΔAB as well as the full-length receptor bind to cognate DNA as dimers and tetramers (18). The DNA binding patterns of RXR containing mutations in H6 (L335A, lane 2) or in H9 (E399Q, lane 3) were similar to that of the wild-type receptor except that a mobility shift was observed in the dimer-DNA complex of E399Q. The origin of this shift, which was not observed with the tetrameric complex of the mutant, is not clear and might reflect the charge change or a conformational rearrangement accompanying the mutation.

In contrast, the distribution of protein-DNA complexes of the H11 mutant F443S/F444S was strikingly different than that of the wild-type protein (Fig. 3A, lane 4). Whereas the predominant RXR-DNA complex of the wild-type protein was tetrameric, the F443S/F444S mutant bound to DNA almost exclusively as a dimer. It seems then that conversion of the two consecutive phenylalanines within helix 11 into serines was sufficient to completely disrupt tetramer formation. The same result was obtained when the phenylalanines were mutated into alanines (Fig. 3B, lane 5), indicating that the effect was specific to the removal of the phenylalanines. Interestingly, mutating the three phenylalanines of H11 individually had little effect on the oligomeric state of the receptor (Fig. 3A, lanes 5–7). Taken together, the data suggest that the second interaction interface necessary for the formation of the tetrameric structure of RXR consists of at least two consecutive phenylalanines within H11 of the receptor.

To examine whether the Phe-443/Phe-444 mutant was able to efficiently interact with 9cRA, the equilibrium dissociation constants of the complexes of the wild-type mRXRΔAB and the corresponding F443S/F444S mutant with 9cRA were measured by fluorescence titrations (34, 56). Representative titrations are shown in Fig. 4. The $K_d$ values characterizing the interactions of the wild-type and the mutant receptors with 9cRA were found to be $20 \pm 3$ nM ($n = 3$) and $13 \pm 5$ nM ($n = 3$), respectively, verifying that the mutations did not reduce the ligand binding affinity of the receptor. Previous data from this laboratory demonstrated that binding of 9cRA by RXR leads to rapid dissociation of receptor tetramers as well as causes a conformation change, resulting in a more compactly folded protein (18, 56). This conformational change is reflected by a slight increase in the mobility of the DNA-bound RXR dimer upon binding of 9cRA (Fig. 3B, lanes 1 and 2). The ligand-induced mobility shift of the RXR dimer could also be observed for the Phe-443/Phe-444 mutants (Fig. 3B, lanes 3–6), further demonstrating that these mutants are able to efficiently bind ligand. Interestingly, although the ligand bound to mutant receptors with high affinity, it did not induce further dissociation of receptor dimers. This observation suggests that the ligand-induced change in oligomerization state of the wild-type receptor is at least partially mediated by Phe-443 and Phe-444.

To examine whether the mutations affected the stability or the global folding of the protein, the sensitivities of the wild-type mRXRΔAB and the corresponding F443A/F444A mutant to urea-induced unfolding were examined. As shown in Fig. 5 (inset), urea-induced unfolding of RXR is associated with a shift in the fluorescence emission spectrum of the protein. The progressive unfolding of the proteins at increasing urea concentrations could thus be followed by monitoring the shift in fluorescence emission maxima. The data in Fig. 5 demonstrate
that the behavior of the mutant upon exposure to urea was very similar to that of the wild-type receptor, such that the unfolding process was 50% complete at a urea concentration of about 5.5 M in both cases. Thus, mutating the phenylalanine residues did not affect the global folding of RXR.

Overall, the mutations in Phe-443 and Phe-444, which completely abolished the ability of RXR to self-associate into tetramers, did not change the overall folding of RXR (Fig. 5) and did not disrupt the ligand binding (Fig. 4), dimer formation (Fig. 2), or DNA binding (Fig. 3) functions of the receptor. These amino acid residues thus seem to be specifically and critically involved in formation of receptor tetramers and in ligand-induced changes in the oligomeric state of the receptor.

The Interaction Interfaces within Helices 10 and 11 Can Function Autonomously—The data reported above suggest that self-association of RXR into tetramers is mediated by two separate regions: helix 10, which is involved in formation of receptor dimers, and helix 11, which provides an additional interaction surface. The data further indicate that the interactions mediated by helix 11, but not those involving helix 10, are broken upon ligand binding by the receptor. To further investigate this issue, proteins containing site-specific mutations in amino acid residues that were shown to be important for protein dimerization via helix 10 (64) were generated.

The results of EMSAs carried out with a number of these mutants is shown in Fig. 6. A mutation in Lys-422 (K422A), a residue that is involved in hydrophilic interactions between receptor monomers (64), resulted in a significant weakening of both tetrameric and dimeric binding to DNA (Fig. 6A, lanes 3 and 4) in accordance with the premise that H10 serves as an interface in both dimeric and tetrameric RXR. A double mutant containing the K422A mutation and an additional mutation in Leu-435 (L435A), a residue that stabilizes dimer formation by hydrophobic interactions (64), showed little further weakening of tetrameric and dimeric DNA binding (Fig. 6A, lanes 5 and 6). When the three amino acids comprising the core of the H10 dimerization interface (Lys-422, Leu-424, and Pro-428) were mutated simultaneously, both protein-DNA complexes corresponding to tetrameric and dimeric binding to DNA disappeared. Instead, a different band with a slightly slower mobility than the original DNA-bound dimer became apparent (Fig. 6A, lane 7), though visualization of this band required higher receptor concentrations. The similarity between the mobilities of this band and the original DNA-bound dimer, coupled with the likelihood that the three mutations have completely abolished the H10 interface (68), suggest that it might represent a DNA-bound dimer that is stabilized by the H11 interface containing Phe-443 and Phe-444. As discussed above, interactions mediated by this interface are disrupted upon ligand binding. Thus, if the dimer that is revealed following disruption of the helix 10 dimerization surface indeed is stabilized by Phe-443/Leu-435, then addition of ligand to this mutant should result in its dissociation into monomers. As RXR monomers have a very low affinity for DNA (18), ligand binding is expected to signif-
significant reduce the DNA binding affinity of the mutant. Indeed, addition of ligand to the protein containing the mutations in H10 significantly decreased the intensity of the band, indicating dimer dissociation (Fig. 6A, compare lanes 7 and 8).

In an additional set of experiments, we examined the DNA binding pattern of a mutant protein in which both the dimerization interface of H10 and the interaction surface of H11 were simultaneously disrupted. EMSA of this mutant did not reveal any protein-DNA complexes (Fig. 6B, lane 3). These observations further support the suggestion that self-association of RXR requires the presence of interacting residues in both H10 and H11 and that mutations in these residues lead to complete dissociation of RXR tetramers into monomers. Taken together, the data provide evidence that tetramers are formed by two independently interacting interfaces originating from the two neighboring helices H10 and H11.

Although EMSA is a useful method for examining the oligomerization state of RXR, the observed DNA binding patterns may have also reflected, in addition to variations in the oligomeric state, changes in DNA binding affinity resulting from the mutations. The oligomeric state of the various mutants was therefore also investigated in the absence of a DNA template. Fig. 7 shows the resolution of several of the mutant proteins by PAGE under non-denaturing conditions. In accordance with our previous observations (55), wild-type RXRΔAB resolved into two distinct bands, corresponding to tetramers and dimers (Fig. 7, lane 1). The protein containing the mutations in helix 11 (F443A/F444A) moved as a single band at the position of the RXR dimer (Fig. 7, lane 2), further substantiating the conclusion that disruption of these residues abolishes the ability of the receptor to form tetramers. The protein containing the three mutations that abolish the dimerization interface of helix 10 (K422A/L424A/P428S, Fig. 7, lane 3) displayed a band of higher mobility as compared with that of the wild-type dimers and the F443A/F444A dimers. This band might reflect a dimer that is stabilized by interactions between H11 of the two monomers and thus may have a different conformation from dimers that are stabilized by the H10 dimerization interface. The increased mobility of the K422A/L424A/P428S dimer in comparison with the F443A/F444A or the wild-type dimer may also be ascribed to the net loss of one positive charge, resulting in increased mobility. The receptor containing the combined mutations in H10 and H11 exhibited a further increase in mobility, most likely reflecting complete dissociation of the receptor into monomers (Fig. 7, lane 4). These observations indicate that the DNA binding patterns of the various mutant receptors, as visualized by EMSA, correspond to the oligomerization state of the proteins in solution, as revealed by native PAGE.

Phe-443/Phe-444 Are Recognized by a Monoclonal Antibody

Directed against the RXR-LBD—Additional support for the suggestion that Phe-443 and Phe-444 are involved in tetramer formation by RXR came from a different line of experiments. A monoclonal antibody that was raised against the D-E regions of E. coli mRXRα (4RX-1D12–1; Refs. 69 and 70), efficiently and completely supershifted receptor dimers bound to a DR-1 RE. However, DNA-bound receptor tetramers could not be completely supershifted by this antibody (Fig. 8, lanes 1 and 2). These observations suggest that the epitope that is recognized by the antibody is at least partially masked within the tetrameric but not within the dimeric structures of RXR. As shown above, RXR in which Phe-443 and Phe-444 are mutated does not form tetramers but binds to DNA almost exclusively as a dimer. Interestingly, the antibody that efficiently interacts with dimeric DNA complexes of wild-type RXRΔAB failed to supershift the dimeric DNA complex of the F443A/F444A mutant (Fig. 8, lanes 3 and 4) and did not recognize this mutant in immunoblots (data not shown). It seems then that the epitope that is recognized by the antibody has been deleted in the mutant, i.e. that the antibody is, at least partially, directed against Phe-443 and Phe-444. The data thus indicate that, within the tetramer, Phe-443 and Phe-444 are involved in protein-protein interactions important for epitope recognition and support the conclusion that these residues play an important role in tetramer formation.
shown in Fig. 9. The data clearly demonstrate that the two point mutations significantly impaired the ability of RXR to activate transcription of the reporter gene in response to the cognate ligand 9cRA.

These observations demonstrate that the two amino acid residues in helix 11 that are critical for tetramer formation by RXR are also important for the transcriptional activity of the receptor. These amino acid residues are thus likely to have a direct role in mediating transcriptional activation by RXR.

**Proposed Structure of the RXR Tetramer**—One criterion employed in the present study for targeting the region that is likely to comprise the tetramerization domain of RXR was that it will be at the surface of the protein. The other criterion was that the region will be at a location that permits formation of a symmetric tetramer. A symmetric arrangement was deemed necessary because the interactions between the four subunits that comprise receptor tetramers evidently allow for simultaneous contacts via both the tetramerization interface and the dimerization interface of helix 10. It is therefore difficult to envision how two types of surfaces in four identical protein molecules can be placed such that they will allow for two independent sets of interactions to occur simultaneously unless symmetry is observed. The identification of critical residues in the tetramerization interface, together with the published structure of the RXR dimer (64), allow for a more detailed consideration of the possible structure of RXR tetramers. Using the published coordinates of the asymmetric unit (monomer) of RXR allows for generation of the dimer by application of a 2-fold rotation axis that is an element of the reported P6322 space group of the crystal. If an additional dimer is placed upside down in such a way that its 2-fold axis is coincident with the 2-fold axis of the original dimer, rotation of one dimer relative to the other about this common axis can bring residues Phe-437–439 (the hRXRa equivalent of the mRXRα Phe-442–444) on opposing monomers into close proximity. The results of this approximate manual alignment are shown in Fig. 10A as a space-filling model. A backbone trace of the protein in which the phenylalanine residues are visible is shown in Fig. 10B. Interestingly, within the structure proposed in Fig. 10, expansive hydrophobicatches meet at the putative tetramerization interface, suggesting that such a tetramer may be solvated better than the separate dimers.

**DISCUSSION**

RXR self-associates in solution to form tetramers that respond to binding of ligand by rapidly dissociating into dimers and monomers. Based on these and other observations, we previously proposed that RXR tetramers serve as an inactive storage pool of the receptor and that ligand-induced dissociation of RXR tetramers is an important mechanism by which the receptor is activated by its ligand (18, 55–57). An extension of this hypothesis suggests that RXR tetramers may be transcriptionally silent because a region that is important for the receptor’s transcriptional activity is sequestered within tetramers. Tetramer dissociation following binding of ligand will unmask this region, allowing for ligand-induced transactivation. One region that will clearly be masked within receptor tetramers is the receptor domain that mediates the formation of tetramers. Here, we set out to identify this region, which we termed “the tetramerization domain,” and to examine whether it might be directly involved in the transcriptional activity of RXR.

The data presented above show that the RXR-LBD by itself forms tetramers that respond to the cognate ligand similarly to the response of the full-length protein, i.e. that upon ligand binding, LBD tetramers dissociate to dimers and monomers concomitantly with a conformational change resulting in a more compact structure. It was therefore concluded that the tetramerization domain of RXR is located within the LBD. Possible regions within this domain that might serve for formation of tetramers were then considered. Aided by available information on the crystal structure of the apo-RXR-LBD (64), amino acid residues that were deemed to comprise potential interaction sites were targeted. Site-specific mutagenesis of these residues and investigation of the oligomeric state of the mutant proteins mapped the region that mediates tetramer formation to helix 11 of the RXRα-LBD, where three consecutive phenylalanines are located. The data show that while introducing mutations in the three phenylalanines individually was of little consequence, synchronous mutations in two of these residues (Phe-443 and Phe-444 in mRXRα) obliterated the ability of the protein to self-associate into tetramers. These mutations did not affect the overall folding of the protein and had no influence on the receptor’s ability to bind ligand, form homo- and heterodimers (data not shown), or to bind to DNA, indicating that they did not cause global structural alterations but specifically targeted the tetramerization domain.

It is not clear at present whether Phe-442, the first phenylalanine in the array of three, contributes to tetramerization. However, the conservation of the three phenylalanines in RXRs of all reported vertebrate species and in all RXR isoforms supports the notion that all three residues participate in the interaction. The array of three phenylalanines in the C-terminal portion of the RXR-LBD is not found in nuclear receptors such as RAR, VDR, TR, and PPAR. However, a number of RXR-related receptors including COUP-TF1, ARP-1, EAR2, TR2, and TR4 share extensive homology in the region around H11, including conservation of two out of the three phenylalanine residues (71–76). To our knowledge, it is unknown at present whether any of these receptors is capable of forming tetramers.

One question that arises is how do the phenylalanines in
helix 11 mediate intersubunit interactions in RXR? The pronounced cooperativity in formation of RXR tetramers (57) suggests that RXR tetramers are remarkably stable. It could be envisioned that interactions between phenylalanines in individual protein monomers (or monomers that are subunits of a dimer) will be driven by ring stacking between the aromatic
rings of the phenylalanine side chains. The model presented in Fig. 9 suggests that the tetrameric structure is further stabilized via expansive hydrophobic patches in the region comprising the dimer-dimer interface. Conclusive verification of this model will have to await the elucidation of the crystal structure of tetrameric RXR.

Another issue that should be considered is that the RXR-LBD was reported to crystallize as a dimer (64), although a major fraction of the protein exists in solution as a tetramer (55). One possible explanation for the discrepancy is that crystallization conditions employed in the study of Bourguet et al. (64) led to dissociation of protein tetramers. In that study, crystals were grown in the presence of a detergent. It is reasonable to suggest that electrostatic or hydrophilic interactions, such as those that help stabilize the RXR dimer, might be resistant to the presence of a detergent. In contrast, protein-protein interactions that are stabilized exclusively by hydrophobic interactions, such as tetramer formation, are likely to be disrupted in the presence of a detergent.

The proposed structure of the RXR tetramer is further supported by the observations that protein-protein contacts via the tetramerization interface in H11 are disrupted upon binding of ligand. Comparison between the crystal structures of liganded RAR and unliganded RXR suggests that ligand binding by retinoid receptors results in migration of helix 12 from its extended position in the apo-receptor, toward the entrance of the ligand binding site, which it consequently covers as a “lid.” Concurrent with the migration of H12, H11 is repositioned in continuity with H10, shifting the main axis of the helix by nearly 90° (65). Thus, while H11 in the apo-receptor is aligned in parallel to the putative plane of interaction, it shifts upon ligand binding to a position directly perpendicular to it. It may be envisioned that ligand-induced shift in the position of H11 will prevent contacts with a corresponding H11 in the neighboring subunit and thus abolish inter-subunit interactions via this region.

The close proximity between the H11 interface and the entrance to the ligand binding pocket of RXR raises the question whether tetramer formation interferes with the entry of ligand into the pocket. We previously found that the ligand binding affinity of RXR tetramers is significantly lower than that of the dimers and that this differential affinity is correlated with a pronounced positive cooperativity in ligand binding by the receptor. This feature of the interactions of RXR with its ligand was taken to reflect the regulatory role of tetramer formation (57). The data reported here point at a possible structural basis for the reduced ligand binding affinity of RXR tetramers versus dimers. The location of the tetramerization domain in close proximity to the entrance of the ligand binding pocket is consistent with the suggestion that formation of tetramers will lead to a lower accessibility of the binding pocket and to a reduced binding affinity. Ligand binding by RXR, which leads to dissociation of inter-subunit interactions via H11, will allow freer access to the ligand binding pocket and thus to the higher ligand affinity displayed by receptor dimers.

Our model proposes that a ligand-dependent activation function of RXR is sequestered within RXR tetramers. It was suggested that this function (AF-2) of RXR may involve helix 12 of the RXR-LBD (75–77). The tetramer model presented above suggests that the region of helix 12, which is expected to be involved with interactions with accessory proteins, i.e. the side of the helix facing the solution, is not masked within tetramers. This is supported by the observation that the point mutations in the tetramerization interface of RXR, which disrupted the tetramerization function of the receptor, did not interfere with the ability of the ligand to induce a conformational change, resulting in a more compactly folded protein (Fig. 3). As discussed above, this ligand-induced conformational change is believed to result from movement of helix 12 from its extended position in the unliganded protein to a folded position in the liganded receptor. Overall, the present findings suggest that tetramer formation does not interfere with the function of helix 12. Instead, the present findings suggest that the tetramerization domain in helix 11, which is buried inside the tetrameric structure and becomes unmasked upon ligand-induced tetramer dissociation, is directly involved in ligand-dependent activation of RXR. One mechanism by which helix 11 could participate in transcriptional activation is by directly interacting with other proteins that serve as coactivators.

In summary, the present study led to the identification of a novel protein-protein interaction interface in RXR. This interface, which we term the tetramerization domain, is located in helix 11 at the C-terminal region of the receptor’s LBD and critically contains two to three consecutive phenylalanine residues. The newly characterized region, in conjunction with the previously identified main dimerization interface of RXR (64), mediates self-association of the receptor into tetramers. The two interfaces act autonomously to affect separate intersubunit interactions within RXR tetramers. Protein-protein interactions via the tetramerization domain are disrupted following ligand binding by RXR, indicating that this region is responsible for mediating the ligand-induced dissociation of receptor tetramers previously reported (18, 56). Mutation of only two amino acid residues within the tetramerization region is sufficient to disrupt RXR tetramers without disrupting the overall fold of the protein or interfering with ligand binding, dimer formation, or DNA binding by the receptor. Nevertheless, the tetramer-impaired mutant was found to be transcriptionally defective. Overall, these data attest to the specificity of the interaction and implicate the tetramerization interface in playing a direct role in regulating transcriptional activation by RXR.

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