Mutations in Retinoid X Receptor That Impair Heterodimerization with Specific Nuclear Hormone Receptor*

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Retinoid X receptor (RXR) serves as a promiscuous heterodimerization partner for many nuclear receptors through the identity box, a 40-amino acid subregion within the ligand binding domain. In this study, we randomly mutated two specific residues within the human RXRα identity box region previously identified as important determinants in heterodimerization (i.e. Ala416 and Arg421). Interestingly, most of these mutants still retained wild type interactions with thyroid hormone receptor (TR), retinoic acid receptor, peroxisome proliferator-activated receptor α, small heterodimer partner, and constitutive androstane receptor. However, RXR-A416D and R421L were specifically impaired for interactions with TR, whereas RXR-A416K lost both TR and retinoic acid receptor interactions. Accordingly, RXR-A416D did not support T3 transactivation in mammalian cells, whereas RXR-A416K was not supportive of transactivation by retinooids or T3. These results provide a basis upon which to further design mutant RXRs highly selective in heterodimerization, potentially useful tools to probe nuclear receptor function in vivo.

The nuclear receptor superfamily is a group of transcriptional regulatory proteins linked by conserved structure and function (reviewed in Ref. 1). The superfamily includes receptors for a variety of small hydrophobic ligands such as steroids, T3, and retinoids, as well as a large number of related proteins that do not have known ligands, referred to as orphan nuclear receptors. The receptor proteins are direct regulators of transcription that function by binding to specific DNA sequences named hormone response elements in promoters of target genes. Nearly all the superfamily members bind as dimers to DNA elements. Whereas some apparently bind only as homodimers, thyroid hormone receptors (TRs),1 vitamin D receptor, retinoic acid receptors (RARs), the peroxisome proliferator-activated receptors (PPARs), and several orphan nuclear receptors bind their specific response elements with high affinity as heterodimers with the retinoid X receptors (RXRs) (reviewed in Ref. 2). Based on this high affinity binding, such heterodimers have been considered to be functionally active forms of these receptors in vivo. These heterodimers display distinct hormone response element specificities to mediate the hormonal responsiveness of target gene transcription, in that distinct hormone response elements are comprised of direct repeats (DRs) of a common half-site with variable spacing between repeats playing a critical role in mediating specificity (2, 3). For instance, RARs activate preferentially through DRs spaced by two or five nucleotides, whereas TRs activate through DRs spaced by four nucleotides. Response elements composed of palindromes as well as inverted palindromes referred to as direct repeats have also been shown to mediate transcriptional activation by RXR-RAR and RXR/TR heterodimeric complexes (3). Such DNA binding flexibility stands in contrast to the steroid hormone receptors, which bind exclusively as homodimers to inverted repeats spaced by three nucleotides (4).

A dimerization interface has previously been identified within the DNA binding domains of RXXs, RARs, vitamin D receptor, and TRs that selectively promotes DNA binding to cognate DRs (5–10). An additional dimerization interface that mediates cooperative binding to DNA, referred to as the identity box (I-box), has also been mapped to a 40-amino acid region within the carboxyl-terminal ligand binding domains (LBDs) of RAR, TR, chicken ovalbumin upstream promoter transcription factor, and IXX (11). These two dimerization domains appear to work in sequence and led to a two-step hypothesis for the binding of heterodimers to DNA (11). According to this model, the LBD dimerization interface initiates the formation of solution heterodimers that, in turn, acquire the capacity to bind to a number of differently organized repeats. However, formation of a second dimer interface within the DNA binding domain restricts receptors to bind to DRs (11).

We have previously shown that the RXR I-box, when transferred to the homodimeric orphan nuclear receptor hepatocyte nuclear factor 4 (HNF4), directs the chimeric receptor to heterodimerize with RAR and TR (12). In particular, the carboxy-terminal 11-amino acid region of the RXR I-box was sufficient for the dimerization selectivity switch. Furthermore, we identified the human RXXa amino acids Ala416 and Arg421 of the 11-amino acid subregion as the most critical determinants of the heterodimeric interactions, based on the findings that these two residues directed heterodimerization with RAR and TR when incorporated into the corresponding positions in HNF4 (12). Similarly, the HNF4 I-box was also transferable to other receptors such as RXR, allowing the resulting chimeric receptor to interact with the wild type HNF4. However, the carboxyl-
terminal 11-amino acid region of the HNF4 I-box alone was not sufficient to direct homodimeric interactions, in contrast to the results with the RXR I-box (12).

In this paper, each of the human RXRα residues Ala^{416} and Arg^{421} was randomly mutated in an effort to construct RXR mutants that are altered in heterodimerization selectivity, and...
we have found three RXR mutants that are selectively impaired in interactions with TR and/or RAR. These results lay a foundation upon which to further design mutant RXRs highly selective in heterodimerization.

**EXPERIMENTAL PROCEDURES**

**Hormones, Yeast Cells, and Plasmids—**T3, 9-cis-RA, and all-trans-RA were obtained from Sigma. EGY48 cells (MATa/α- his3 trp1 ura3 leu2::pLexa-op6 leu2/ΔasalLεu2), the lexA-β-galactosidase reporter construct, the LexA and B42 parental vectors were as reported (10, 11). Gal4, LexA, or B42 fusions to the SRC-C-C2 fragment (i.e., the SRC-1 residues 759–1141), the full-length RAR, small heterodimer partner, and the LBDS of PPARα, RXR, constitutive androstane receptor (CAR), and TR; glutathione S-transferase (GST) or VP16 fusions to the full-length RAR and TR; and the Gal4-TK-Luc reporter construct were as described previously (14–18). It is notable that the LBDS of all the receptors described throughout this work denote the complete D, E, and F domains. All the RXR LBDS constructs contain the human RXRα residues 198–462, for instance. Two-step polymerase chain reactions were employed to construct all the mutant RXRs within the context of B42-RXR LBDS by using a set of oligonucleotides containing NN(G/C) at the target sites, as described previously (12). All of the resulting RXR mutants were confirmed by DNA sequencing for the directed mutations as well as against unwanted mutations. For mammalian expression of Gal4 fusion and in vitro transcription/translation, the resulting mutant RXR LBDS were inserted into EcoRI and XhoI restriction sites of pCMXGal4N (11) and pcDNA3, respectively.

**Yeast β-Galactosidase Assays—**The cotransformation and transactivation assays in yeast were performed as described previously (13). For each experiment, at least three independently derived colonies expressing chimeric receptors were tested.

**GST Pull-down Assays—**GST fusion proteins were produced in *Escherichia coli* and purified using glutathione-Sepharose affinity chromatography essentially as described (15, 16). GST proteins were bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) in binding buffer (50 mM KPO4 (pH 6.0), 100 mM KCl, 10 mM MgCl2, 10% glycerol) and allowed to incubate with GST or GST fusions to the full-length TR and RAR, either in the absence or presence of 0.1 μM T3 or all-trans-RA, as indicated. Beads were washed, and specifically bound material was eluted with reduced glutathione and resolved by SDS-polyacrylamide gel electrophoresis. C, the yeast transformation and the β-galactosidase readings were essentially done as described (13), except that cells were grown either in the absence or presence of 0.1 μM 9-cis-RA. SRC-C encodes the SRC-1 residues 759–1141 (16). The B42 fusions encoded the LBDS of various RXRs. Fold-activations by each B42 chimera were calculated by defining the reporter activity in the presence of B42 alone as 1. The result is the average of at least three different experiments, and the standard deviations are less than 5%.

**RESULTS AND DISCUSSION**

**Random Mutations of the RXR I-box Residues—**We have previously identified two residues of the human RXRα I-box, i.e., Ala416 and Arg421, as determinants that impart the heterodimerization potential of RXR when constructed into the corresponding positions in homodimeric HNF4 (12). These results were self-evident from the fact that these two residues are absolutely conserved between RXR and its *Drosophila* homologue ultraspireacle (20) but not HNF4 (21) (Fig. 1A). Notably, RXR is a highly promiscuous heterodimerization partner molecule, exhibiting associations with a large number of nuclear receptors, including TR, RAR, PPARs, and CAR, with similar affinities (2). In this work, we attempted to construct RXR mutants that are less promiscuous (i.e. more selective) in choosing heterodimerization partners. For this end, we decided to randomly mutate, within the context of B42-RXR LBDS, the RXR residues Ala416 and Arg421 by using two-step polymerase chain reactions in which a set of oligonucleotides containing NN(G/C) at the target site were utilized. As shown in Fig. 1B, series of 15 and 10 different mutants were isolated for the positions of RXR Ala416 and Arg421, respectively. Interestingly, most of these mutants retained wild type interactions with many nuclear receptors when tested in the yeast two-hybrid-based assays, suggesting that only a single amino acid change in these positions is not generally enough to change the RXR heterodimerization character, consistent with our previous results (12). The receptors tested included TR, RAR, small heterodimer partner, and PPARα (2, 17, 18). Surprisingly, however, A416D and R421L were specifically abolished for TR interactions, whereas A416K lacked both the TR and RAR interactions (Fig. 1B). As a control, we have also mutated the RXR Lys417 position, which was suggested to be a nonessential determinant for heterodimerization (12). Indeed, all of the 12 mutants obtained in this position were indistinguishable from the wild type receptor in interactions with different receptors (Fig. 1B). Overall, these results clearly indicate that mutations of specific residues within the I-box could, in principle, lead to a change in the receptor selectivity of RXR.

**Specific Dimerization Defects of RXR-A416D and RXR-A416K—**Quantitative interaction data for the two mutant
RXRs, RXR-A416D and RXR-A416K, are presented in Fig. 2A. Transactivation directed by LexA fusion to RAR was enhanced by B42 fusions to the wild type RXR and RXR-A416D but not RXR-A416K in yeast. Interestingly, the LexA-RAR-directed interactions with B42-RXR and B42-RXR-A416D were moderately enhanced by the presence of 0.1 μM all-trans-RA. Transactivation directed by LexA-TR was stimulated only by B42-RXR but not by B42-RXR-A416D and B42-RXR-A416K. This yeast interaction of LexA-TR with B42-RXR was further enhanced by the presence of 0.1 μM T3. In contrast, the LexA-CAR transactivation was responsive to all three of these B42-RXR fusion proteins. Essentially identical results were also obtained in the mammalian two-hybrid tests (Fig. 2, B and C). These results are consistent with recent reports in which basal interactions of RXR-RAR and RXR-TR were shown to be further stimulated by ligand (22, 23). Given these results, we wanted to make certain that the differential interaction profiles of these RXR mutants are not the result of different protein expression levels. It is notable that pG4–4–5, the parental vector to express B42 fusions in yeast, contains the translation start codon ATG followed by sequences encoding nuclear localization signal from SV40 T antigen, B42 transactivation domain, and Flu-epitope and multiple cloning sites, as described previously (13, 19). Thus, we have executed Western analysis in which Flu monoclonal antibody was used to detect various B42-RXR proteins that were employed in the yeast two-hybrid tests. As shown in Fig. 3A, however, both RXR-A416D and RXR-A416K were comparably expressed as the wild type RXR in yeast. Next, these interactions were further confirmed in the GST pull-down assays. Consistent with recent reports (22, 23), radiolabeled wild type RXR interacted with GST fusions to TR and RAR in a ligand-dependent manner but not with GST alone, whereas radiolabeled luciferase interacted with none of these GST proteins (Fig. 3B). In contrast, IXR-A416D interacted with RAR only in the presence of ligand and IXR-A416K interacted with neither GST-TR nor GST-RAR, consistent with the yeast and mammalian two-hybrid-based interaction results (Fig. 2). Although we have not directly tested whether these two RXR mutants bind their cognate ligand 9-cis-RA, the following results clearly demonstrated their ligand binding capacities. First, these mutants interacted with SRC-C (16), the SRC-1 fragment containing its receptor interaction domain, in a 9-cis-RA-dependent manner (Fig. 3C). Second, the interactions with the orphan nuclear receptor small heterodimer partner were also 9-cis-RA-dependent for both RXR-A416D and RXR-A416K (results not shown), like the wild type RXR (17). From these results, we concluded that these RXR mutants are specifically impaired for their dimerization potential but not for either ligand binding or protein expression level.

The Dimerization Defects of Mutant RXRs Correlated with Altered Transactivation Function—From the above interaction results, we suspected that these RXR mutants would not support T3 and/or all-trans-RA-dependent transactivation. Because cells free of all the RXR isotypes (2) were not available, we were unable to clearly confirm this prediction with the conventional DR4 and DR2/5-based reporter constructs. Thus, we utilized Gal4-TK-Luc reporter gene, whose expression is driven by the upstream Gal4-binding sites. Interestingly, Gal4 fusion to the wild type RXR LBD (i.e. Gal4-RXR), when cotransfected into CV1 or HeLa cells along with TR expression vector, directed repression of the basal level of transcription (Fig. 4A). Consistent with the lack of TR interactions, Gal4 fusions to RXR LBD-A416D and RXR LBD-A416K (i.e. Gal4-A416D and Gal4-A416K) were not as efficient as the wild type RXR in directing this basal repression, even in the presence of additional TR (Fig. 4A). In addition, Gal4-RXR efficiently induced transactivation by T3, whereas Gal4-A416D and Gal4-A416K were inert. Similarly, Gal4-RXR as well as Gal4-A416D directed basal repression in the presence of co-expressed RAR in CV1 and HeLa cells, whereas Gal4-A416K was not able to direct this basal repression, consistent with its lack of interaction with RAR (Fig. 4B). Accordingly, Gal4-RXR and Gal4-A416D directed efficient induction by all-trans-RA, whereas Gal4-A416K was inert. As expected from the interaction results, co-expression of CAR was able to direct the previously described constitutive transcriptional activities (18) with all three of these Gal4-RXR fusion proteins (Fig. 4C). These results suggest that basal repression by TR and RAR, known to involve the receptor corepressors NCoR/SMRT (24, 25), also occurs when these receptors are indirectly recruited to DNA via interaction with RXR fused to the heterologous DNA-binding protein Gal4, consistent with the previously reported results (26). We also concluded that the dimerization defects of these RXR mutants are accordingly translated into altered transactivation function in vivo.

Overall, it was rather surprising that two specific positions...
RXRs with Altered Heterodimerization Potential

within the major dimerization interface of RXR (i.e., RXR Ala and Arg), previously shown to be essential for heterodimerization (12), were able to host a series of different mutations without significantly affecting the heterodimerization potential of RXR. However, these results are consistent with our previous findings that a single amino acid change within the I-box region is not generally sufficient to change the specificity of heterodimerization (12). From the recently completed structural studies of RXR and other receptors in which the dimerization interfaces were shown to include other helices as well as the intervening sequences (27, 28), it will probably require additional mutations to fully alter the heterodimerization selectivity of RXR. For instance, simultaneous introduction of a few mutations may allow the resulting mutant RXR to specifically recognize TR but not other receptors, and those inert RXR mutants described in Fig. 1B may also exhibit more heterodimerization-selective phenotypes, if coupled with other mutations. With regard to this idea, it is an interesting possibility that combinatorial mutations of RXR-A416 and RXR-R421 together may have better results in changing the heterodimerization selectivity of RXR. To understand in detail how changes of these two mutated RXRs (i.e., A416D and A416K) affect the receptor interaction profiles, both more mutational and structural studies will be required. Finally, it is notable that five new families were recently diagnosed with thyroid hormone not caused by mutations in the TRβ gene (29, 30), the major genetic alterations in resistance to thyroid hormone (reviewed in Ref. 31). These patients were suspected to have defects in the TRβ signaling pathways, such as transcription cofactors for TRβ. Consistent with this hypothesis, mice deficient in SRC-1 were recently found to show syndromes associated with resistance to thyroid hormone (32). Specific mutations of RXR resulting in impaired interactions with TR, such as RXR-A416D and RXR-R421L described in this work, may serve as a novel cause for resistance to thyroid hormone. Thus, these or similar RXR mutations could be found to be associated with those patients having resistance to thyroid hormone not caused by mutations in the TRβ gene (29, 30).

In conclusion, we have isolated point-mutated RXRs with altered selectivity in choosing heterodimerization partners. In particular, RXR-A416D and RXR-R421L were specifically impaired for interactions with TR, whereas RXR-A416K lost both TR and RAR interactions. Overall, these results provide the possibility of further constructing mutant RXRs that are highly selective in heterodimerization, potentially useful tools to probe nuclear receptor function in vivo.

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