Distinct retinoid X receptor activation function-2 residues mediate transactivation in homodimeric and vitamin D receptor heterodimeric contexts

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ABSTRACT

The vitamin D receptor (VDR) stimulates transcription as a 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-activated heterodimer with retinoid X receptor (RXR). RXR also forms homodimers to mediate 9-*cis* retinoic acid (9-*cis* RA)-induced gene expression. Both receptors possess a C-terminal hormone-dependent activation function-2 (AF-2), a highly conserved region that binds coactivators to transduce the transcriptional signal. By replacing single amino acids within the AF-2 of human RXRα (hRXRα) or mouse RXRβ (mRXRβ), the contribution of these residues to transactivation by the RXR-VDR heterodimer and the RXR-RXR homodimer was evaluated. In 9-*cis* RA-responsive homodimers, the second and fourth positions of the AF-2 (leucine and glutamate respectively) are essential. However, in the context of an RXR-VDR heterodimer activated by $1,25(OH)_2D_3$, alteration of these two RXR residues has little effect. Instead, AF-2 residues located towards the C-terminus, such as the penultimate position (L455 in hRXRα or L441 in mRXRβ), are crucial for RXR-VDR heterodimers. Indeed, L455A mutant RXR exerts a dominant negative effect on RXR-VDR transcriptional responsiveness to $1,25(OH)_2D_3$. Further experiments with a mutant hRXRα (F313A) which elicits 9-*cis* RA-independent transactivation as a homodimer demonstrate that, when heterodimerized with VDR, this RXR mutant is incapable of activating the RXR-VDR heterocomplex in the absence of the VDR ligand. Taken together, these results indicate that RXR is a subordinate, yet essential transcriptional partner in RXR-VDR-mediated activation of gene expression. Furthermore, a functional switch in RXR AF-2 signaling occurs between RXR residues in the homodimeric versus the heterodimeric states, likely reflecting different interactions between subregions of the AF-2 and coactivator(s).

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INTRODUCTION

The bioactivity of the hormonal metabolite of vitamin D, namely 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), extends beyond its classical calcium/phosphate translocating target tissues of intestine, kidney and bone, to include the cells of the immune, neural, epithelial and endocrine systems (Haussler *et al*. 1998). The plethora of cellular responses elicited by $1,25(OH)_2D_3$ are mediated by a specific nuclear protein, termed the vitamin D receptor (VDR), a member of the superfamily of ligand-activated transcription factors that includes the steroid, retinoid, and thyroid hormone receptors (Mangelsdorf *et al*. 1995). Upon binding its cognate ligand, VDR forms a stable heterodimeric complex with the retinoid X receptor (RXR) (MacDonald *et al*. 1993), the nuclear receptor for 9-*cis* retinoic acid (9-*cis* RA) (Heyman *et al*. 1992). It is this RXR-VDR complex that translates the $1,25(OH)_2D_3$ hormonal signal into a transcriptional response through high affinity binding of the heterodimer to a specific DNA element, termed the vitamin D-responsive element (VDRE), that has been identified within the promoter region of vitamin D-regulated target genes (Haussler *et al*. 1998). Thus, VDR belongs to the sub-group of

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nuclear receptors including the thyroid hormone receptors (TRs), peroxisome proliferator-activated receptors (PPARs) and retinoic acid receptors (RARs), which all heterodimerize with RXR on their respective DNA responsive elements (Mangelsdorf & Evans 1995).

The precise mechanism by which the $1,25(OH)_2D_3$-activated, DNA-bound heterodimer controls the transcription of vitamin D-regulated genes is not clearly understood. While a number of nuclear receptors have been shown to possess a constitutive activation sequence located within the N-terminal domain, termed AF-1, functional analyses have also revealed that a highly conserved region found within helix 12 near the C-terminus of nearly all the members of the superfamily represents a hormone-dependent activation function, known as AF-2 (Leng *et al*. 1995, Parker & White 1996). Studies of the three-dimensional crystal structures of RAR, RXR, TR and VDR (Renaud *et al*. 1995, Wagner *et al*. 1995, Darimont *et al*. 1998, Nolte *et al*. 1998, Egea *et al*. 2000, Rochel *et al*. 2000) have supported the proposed 'mouse trap' hypothesis, according to which binding of ligand induces a repositioning of helix 12 such that it covers the opening of the ligand binding pocket (Renaud *et al*. 1995, Moras & Gronemeyer 1998, Egea *et al*. 2000). In addition to its obvious importance for retaining ligand, this reconfiguration of helix 12 also results in a presentation of key AF-2 residues as an exposed interface that can potentially interact with transcriptional coactivators (Moras & Gronemeyer 1998, Nolte *et al*. 1998, Egea *et al*. 2000). A number of studies support the involvement of the AF-2 in the early stages of transcriptional activation by RXR-VDR-$1,25(OH)_2D_3$, including the functional mapping of the human VDR (hVDR) AF-2 to the extreme C-terminus (Jurutka *et al*. 1997), as well as the demonstration that residues 408–427 of VDR can exhibit autonomous transcriptional activity when placed into a heterologous context (Masuyama *et al*. 1997). Mutation of the highly conserved L417 and E420 residues in the hVDR AF-2 abolishes ligand-dependent transcriptional activity (Jurutka *et al*. 1997) and also eliminates interactions between hVDR and the p160 class of transcriptional coactivators, like SRC-1 (Masuyama *et al*. 1997). The hVDR AF-2 has also been shown to participate in the ligand-dependent recruitment of other members of the p160 class of coactivators that effect derepression via histone acetyl transferase activity, such as GRIP1 (Hong *et al*. 1997) and ACTR (Chen *et al*. 1997). Finally, the AF-2 of VDR subsequently contacts the DRIP/mediator complex (Rachez *et al*. 1999) that interacts with RNA polymerase II and ultimately leads to the stimulation of target gene transcription.

Given this crucial role of the AF-2 region in VDR, what is the contribution, beyond DNA element binding, of RXR in transactivation by the RXR-VDR heterodimer? Studies attempting to define the role of the RXR partner and its 9-*cis* RA ligand in the context of RXR-containing heterodimers have suggested that such heterocomplexes are either 'permissive' or 'non-permissive' with respect to the contribution of liganded RXR to the overall transcriptional activity of the heterodimer (Vivat *et al*. 1997). In permissive heterodimers, such as those with PPAR, NGFI-B, and liver X receptor (LXR), RXR functions as a fully active partner, largely independent of the ligand status of the primary partner (Forman *et al*. 1995, Willy & Mangelsdorf 1997). For non-permissive heterodimers, however, such as those with RAR and TR, the primary partner actively influences the ability of RXR to stimulate transcription in response to RXR-specific ligands. For example, studies with RXR-RAR heterodimers have indicated that, while liganded RAR and RXR both participate in transcription, RAR silences RXR activity in the absence of an RAR ligand (Vivat *et al*. 1997, Dilworth *et al*. 1999). Then, upon RAR ligand binding, a conformational change is induced in the RXR partner (Vivat *et al*. 1997), allowing it to interact with a transcriptional coactivator such as SRC-1 (Westin *et al*. 1998). Finally, the presence of the RXR-ligand has been reported to further boost transcription through a presumed synergism with the RAR ligand (Chen *et al*. 1996, Minucci *et al*. 1997). Likewise, for heterodimers containing unliganded TR, the RXR partner is unable to respond to its cognate ligand (Forman *et al*. 1995). However, in the presence of 9-*cis* RA in combination with thyroid hormone (triiodothyronine, T_3), both repressive (Lehmann *et al*. 1993) and additive/synergistic (Kakizawa *et al*. 1997) effects on RXR-TR-mediated activity have been observed. Thus, the effect of 9-*cis* RA on the T_3 response, while generally 'nonpermissive', appears to be influenced by multiple parameters that include the thyroid hormone responsive element, and the isoform of the RXR heteropartner (Walfish *et al*. 1996).

In the case of $1,25(OH)_2D_3$-mediated signaling via VDR, examinations of the RXR partner and its cognate ligand have produced apparently conflicting results. Previous data from our laboratory indicated that 9-*cis* RA, at relatively high concentrations, had an inhibitory effect on the $1,25(OH)_2D_3$-mediated transcriptional response of the rat osteocalcin promoter (MacDonald *et al*. 1993), as well as on the $1,25(OH)_2D_3$-dependent binding of the RXR-VDR heterodimer to the rat osteocalcin VDRE

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(Thompson *et al*. 1998). Similar findings have been communicated by Freedman and associates (Lemon & Freedman 1996), who utilized the mouse osteopontin VDRE. However, it has also been observed in other experimental systems that the presence of an RXR ligand, in combination with $1,25(OH)_2D_3$, results in a synergistic transcriptional response (Kato *et al*. 1995, Sasaki *et al*. 1995, Kephart *et al*. 1996, Li *et al*. 1997). Moreover, a C-terminally truncated RXR lacking the AF-2 domain has been reported to exert a dominant negative effect on VDR-mediated transactivation, suggesting that an intact RXR AF-2 region is required for $1,25(OH)_2D_3$-stimulated transcription (Blanco *et al*. 1996). Interpretation of these latter results warrants caution, however, as the generation of such C-terminal deletions could also conceivably expose a constitutive corepressor binding site (Schulman *et al*. 1996), thereby eliciting the observed dominant negative phenotype in RXR.

The present study was undertaken in order to decipher the comparative functional importance of the AF-2 of RXR in mediating both $1,25(OH)_2D_3$ and 9-*cis* RA transcriptional responses, using a series of single point mutations within the reported AF-2 domains of both human RXRα (hRXRα) and mouse RXRβ (mRXRβ). These AF-2 mutations were employed, in combination with other altered forms of RXR that adopt active configurations in the absence of 9-*cis* RA, to evaluate the functional significance of the AF-2 of RXR and its cognate ligand when RXR is part of a heterodimer with hVDR, as compared with the activity of RXR as a homodimer. The present results are consistent with VDR functioning as a nonpermissive primary partner, similar to TR and RAR, with the AF-2 of RXR playing an important, yet subordinate role in transactivation that, in the context of the current experimental conditions, does not require the presence of 9-*cis* RA.

MATERIALS AND METHODS

Construction of mRXRβ and hRXRα mutant plasmids

The expression vectors, pSG5 mRXRβ and pSG5 hRXRα (MacDonald *et al*. 1993) were employed in synthesizing point mutants by *in vitro* site-directed mutagenesis. Alterations of specific residues were created using previously described single-stranded (Hsieh *et al*. 1991) and double-stranded (Jurutka *et al*. 1997) DNA protocols to generate single residue replacements of five highly conserved residues in, or immediately adjacent to, the consensus AF-2 region of both mRXRβ (F436A, L437A, E439K, L441A, E442K) and hRXRα (F450A, L451A, E453K, L455A, E456K). A point mutation was also generated within the ligand-binding domain of both mRXRβ (F299A) and hRXRα (F313A) to produce 'constitutive' mutant forms (Vivat *et al*. 1997) which are structurally modified such that the hormone binding pocket is occupied by an ubiquitous, non-retinoid, fatty acid ligand (most likely oleic acid) (Bourguet *et al*. 2000). In addition, the F299A oligonucleotide was employed to introduce this mutation in the context of each mRXRβ AF-2 point mutant. All mutations were confirmed by dideoxy sequencing. The construction of the mutant R391C hVDR has been described previously (Whitfield *et al*. 1996).

Transfection of cultured cells and transactivation assays

The ability of each RXR mutant to mediate a transcriptional response to 9-*cis* RA as a homodimer from a retinoid X responsive element (RXRE)-containing reporter vector was evaluated using a reporter plasmid, $(RXRE)_4$TK-GH, containing four tandem copies of the RXRE from the rat cellular retinol binding protein II (CRBP II) gene (Mangelsdorf *et al*. 1991), inserted into the HindIII site upstream of the thymidine kinase promoter of pTK-GH (Nichols Institute, San Juan Capistrano, CA, USA). COS-7 cells (700 000 cells per 60 mm plate) were transfected by the calcium phosphate coprecipitation method (Jurutka *et al*. 1993) with 7 μg of the reporter plasmid in combination with 2 μg of the expression vector pSG5 mRXRβ, pSG5 hRXRα (MacDonald *et al*. 1993), or one of their respective mutant forms. Transfected cells were treated with 9-*cis* RA (10^{-6} M) for 48 h. Cell media were then assayed for human growth hormone (GH) by RIA (Nichols Institute) and the results were expressed as μg GH produced/plate. For evaluating activity of RXR mutants in a heterodimeric setting, a reporter plasmid $(CT4)_4$TK-GH, containing four copies of the rat osteocalcin VDRE (Terpening *et al*. 1991), was utilized. COS-7 cells were transfected as described above, with 7 μg of this plasmid in combination with either the pSG5-based expression vector for hVDR (1–2 μg) or for the R391C hVDR mutant (4·5 μg). In all transfection experiments, the plasmid pTZI8U was included to maintain a constant level of 30 μg DNA per plate. Whole cell lysates of transfected cells were subjected to Western blot analysis with the mRXRβ specific antibody MOK13.17 as previously described (MacDonald *et al*. 1993).

Ligand-dependent gel mobility shift assay

COS-7 cells (700 000 cells per 60 mm plate) were transfected with an expression vector for wild-type

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mRXRβ or for the indicated mutant mRXRβ. Cellular lysates were prepared at 36 h post transfection as previously described (Jurutka *et al*. 2000*b*), then 10 μg total protein aliquots were taken for ligand-dependent gel mobility shift assays (Thompson *et al*. 1998) using a final adjusted KCl concentration of 170 mM. After incubation for 50 min in the presence of the indicated ligands, radiolabeled CRBP II RXRE (0·8 nM) was added and the reaction allowed to proceed for a further 40 min, followed by non-denaturing gel electrophoresis and autoradiographic analysis, as described previously (Nakajima *et al*. 1994). The retinoid ligands, LG100364, an RXR selective ligand or 'rexinoid', and TTNPB, an RAR specific ligand, were generous gifts from Dr E A Allegretto, Ligand Pharmaceuticals Inc., La Jolla, CA, USA.

RESULTS

Amino acids positioned centrally within the AF-2 are most significant for the transcriptional integrity of the RXR-RXR homodimer

Figure 1 depicts seven amino acids constituting the conserved AF-2 region of selected members of the nuclear receptor superfamily, including five in hRXRα and mRXRβ that were chosen for evaluation via site-directed mutagenesis. The resulting mutants in hRXRα are denoted F450A, L451A, E453K, L455A and E456K, and alteration of the analogous residues in mRXRβ yielded mutants F436A, L437A, E439K, L441A and E442K. Mutations were also generated within the ligand binding domain of hRXRα (F313A) and mRXRβ (F299A), both of which correspond to an activated form of mRXRα (F318A) that exhibits 9-*cis* RA-independent transcriptional activity (Vivat *et al*. 1997). Figure 2 illustrates the effects of each of the AF-2 mutations on the transcriptional activation properties of mRXRβ homodimers in response to 9-*cis* RA, using an RXRE-containing reporter vector. The data in Fig. 2 show that mutation of the second and the fourth (central) AF-2 residues in mRXRβ (L437A and E439K) leads to the greatest impairment in transcriptional responsiveness to 9-*cis* RA (83% and 87% reductions, respectively, compared with wild-type mRXRβ). Mutation of the initial residue in the AF-2 (F436A) also results in a marked decrease in the transcriptional activity of the mRXRβ homodimer. Conversely, mutants in which either of the two C-terminal AF-2 residues are altered (L441 and E442) are least affected in their transcriptional activation properties. Indeed, the L441A mutation, while displaying a 30% reduction in its fold transcriptional response to

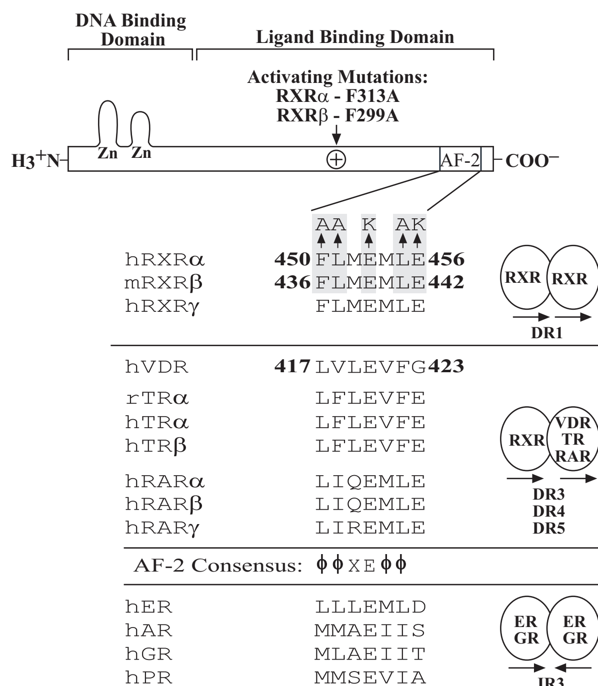

FIGURE 1. Amino acid sequences of the C-terminal ligand-dependent AF-2 region in various nuclear receptors. The top three sequences represent RXR isoforms, two of which are tested herein. Point mutations which were generated in the AF-2 regions of hRXRα and mRXRβ are indicated by the gray vertical boxes. The receptors in the middle section of the figure include hVDR, TRs and RARs, all of which form heterodimers with RXR on direct repeat response elements (DR3, DR4 and DR5 respectively). The bottom four sequences represent receptors that homodimerize on inverted repeat type responsive elements (IR3s), including the estrogen (ER), androgen (AR), glucocorticoid (GR) and progesterone (PR) receptors. The AF-2 consensus (a Glu residue flanked by hydrophobic (ϕ) amino acids) is shown below the sequence alignment for heterodimerizing receptors. Also depicted at the top are activating mutations (⊕) which were generated within the ligand binding domain of hRXRα (F313A) and mRXRβ (F299A). These alterations, first described by Vivat *et al*. (1997), render the receptor responsive to an endogenous fatty acid ligand and therefore independent of 9-*cis* RA binding for activity (Egea *et al*. 2000).

ligand, exhibits a level of ligand-stimulated transcriptional activity similar to that of wild-type mRXRβ. Western blot analysis reveals that protein expression levels for the mRXRβ AF-2 mutants (using the mRXRβ-specific monoclonal antibody MOK 13.17; lower portion of Fig. 2) are similar to, if not greater than, that of the wild-type isoform, thus arguing against the possibility that the

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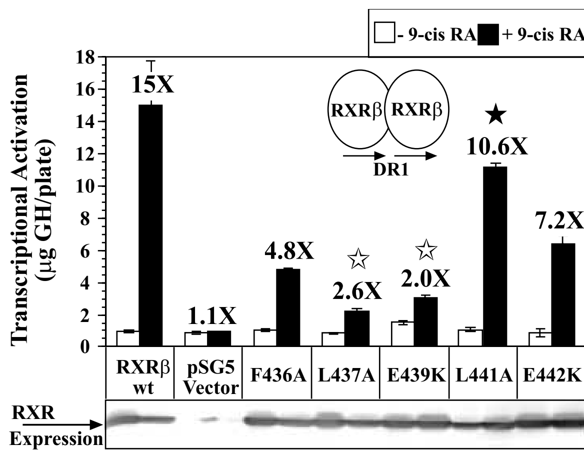

FIGURE 2. Effect of mutational changes within the AF-2 region of RXRβ on the direct repeat-1 (DR1) RXRE-driven transcriptional response to 9-*cis* retinoic acid as mediated by the RXR homodimer. COS-7 cells were transfected with the reporter plasmid $(RXRE)_4$TK-GH in combination with the expression vector pSG5 mRXRβ, or mutated versions of pSG5 mRXRβ, as indicated. Transfected cells were treated with 9-*cis* RA (10^{-6} M) or ethanol vehicle (each treatment in triplicate) for 48 h, and cell media were then assayed for human growth hormone (GH) as described in Materials and Methods. Values are expressed as means ± S.D., and the results presented are the average of four independent experiments with $n=3$ in each assay. The calculated fold induction by 9-*cis* RA is given at the top of the solid bar in each treatment group. Depicted at the far left is the transcriptional response to 9-*cis* RA of wild-type (wt) mRXRβ, followed by the pSG5 vector control lacking the mRXRβ insert. The balance of the figure illustrates the responses of RXRβ point mutants; a solid star indicates the mRXRβ mutant that is least affected, and open stars denote the two mutants that are most impaired in transactivation. Shown below each lane are the results of Western blot analysis in which the corresponding whole cell lysates of the transfected samples were probed with an mRXRβ-specific antibody, MOK 13.17.

observed transcriptional inhibition pattern for RXR mutants is the result of deficiencies in protein expression. Finally, an analogous set of transcription activation experiments was performed using the homologous set of AF-2 mutations generated with hRXRα. The profiles (data not shown) were generally similar, especially in that mutants altered in the second and fourth AF-2 positions were the most impaired in their transcriptional responses. Again, the mutant in which the penultimate AF-2 residue was changed (L455A) was the least affected. Thus, the functional activities of individual AF-2 residues in the RXRα-RXRα and RXRβ-RXRβ homodimers seem qualitatively consistent. Furthermore, gel shift experiments utilizing RXR isoform-specific antibodies indicate that the major endogenous RXR in COS-7 kidney cells is the α-isoform (data not shown), although the β-isoform is also expressed at lower levels (Mangelsdorf *et al*. 1992). Consequently, to minimize potential interference from endogenous RXRα, RXRβ and its mutants were the focus of the balance of RXR homodimer investigations.

Mutations within the RXR AF-2 do not attenuate ligand-dependent receptor binding to an RXRE

Although no ligand contact residues lie in the AF-2/helix 12 domain of RXR (Egea *et al*. 2000), proper helix 12 pivoting would appear to be crucial for sealing the binding pocket and conforming the receptor for transactivation (Renaud *et al*. 1995, Moras & Gronemeyer 1998, Egea *et al*. 2000). Therefore, it was necessary to ensure that the loss of transactivation in RXR AF-2 mutants (Fig. 2) was not caused by defects in ligand-retention and/or subsequent dimerization-dependent DNA binding. To evaluate the effects of these mutations on homodimer formation on an RXRE in response to ligand, gel mobility shift assays were performed, and the results are depicted in Fig. 3. Wild-type mRXRβ, the series of mRXRβ AF-2 mutations, and also the activated F299A mRXRβ, were examined for their ability to form ligand-dependent DNA binding complexes on the CRBP II RXRE. Lane 1 of Fig. 3A shows that there is one moderately intense, apparently non-specific background band present, but as revealed in lane 2, no major new DNA binding complexes are generated by the addition of a RXR-specific ligand (LG100364) to a lysate from cells transfected with the pSG5 expression vector without the mRXRβ insert (mock). Also, no major new complex is generated when the COS-7 lysate is derived from cells transfected with the mRXRβ expression vector, either in the presence of ethanol vehicle (lane 3) or the RAR-specific ligand TTNPB (lane 4). However, a major DNA binding complex, migrating close to but slightly faster than the nonspecific band, is observed when the same COS-7 lysate is incubated with the RXR specific ligand LG100364 (lane 5), and this complex is supershifted in the presence of the mRXRβ-specific monoclonal antibody MOK 13.17 (lane 6; +Ab). The right portion of Fig. 3A (lanes 7–11) indicates that the formation of the RXR homodimer-RXRE complex is dependent upon the concentration of LG100364, with a dose-dependent effect between 10^{-8} and 10^{-5} M ligand. These data demonstrate that mRXRβ, when in the context of a

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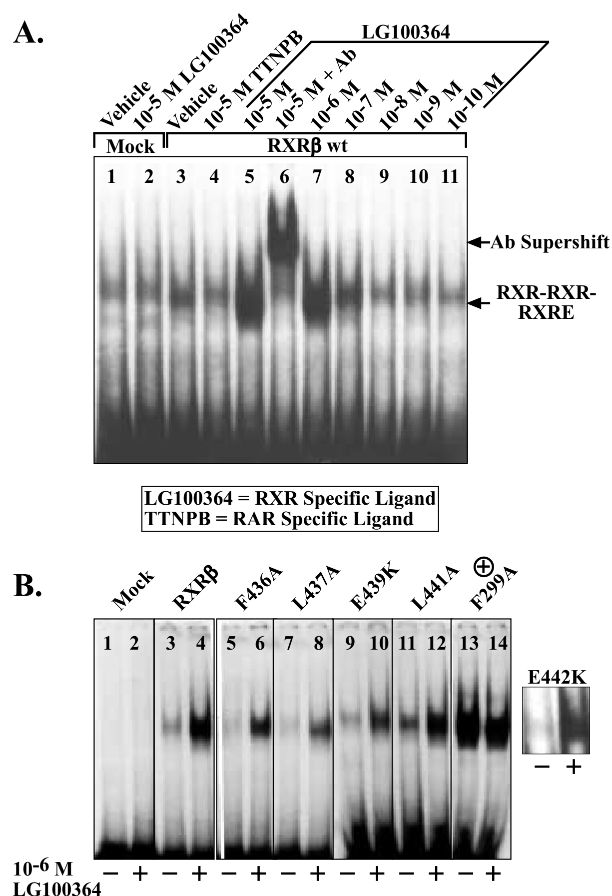

FIGURE 3. Ligand-dependent gel mobility shift analysis of wild-type and mutant mRXRβ proteins binding to an RXRE probe. (A) The binding of wild-type (wt) mRXRβ to the CRBP II RXRE in the presence of the RXR-specific ligand, LG100364. A major DNA binding complex is produced when a COS-7 lysate from cells transfected with the mRXRβ expression vector is incubated with LG100364 (10^{-5}-10^{-7} M, lanes 5, 7 and 8), but neither with the RAR-specific ligand TTNPB (10^{-5} M, lane 4), nor with vehicle-treated lysate (lane 3), nor with lysate from cells transfected without the mRXRβ insert (lanes 1 and 2). The anti-RXRβ antibody MOK 13.17 was used to confirm the presence of RXRβ in the shifted complex (lane 6, antibody (Ab) supershift). (B) Binding of mRXRβ AF-2 mutants to the CRBP II RXRE in the absence or presence of LG100364. The far right of the main panel (lanes 13 and 14) depicts DNA complexes formed by a lysate from cells containing the F299A mRXRβ mutant that is activated by an endogenous fatty acid and is therefore rexinoid-independent (Bourguet *et al*. 2000). The inset at the right shows results obtained with the E442K mRXRβ mutant. The data depicted are representative of three independent experiments. The apparent nonspecific band in the mock transfected samples that appears in lanes 1 and 2 of (A) does not occur in (B), and is therefore probably not significant.

cellular extract, displays a marked dependency on the presence of an RXR-specific ligand in forming DNA-binding homodimeric complexes on its target RXRE. The experiment depicted in Fig. 3B illustrates the DNA binding complexes formed in the absence and presence of LG100364 by lysates from COS-7 cells transfected with expression vectors for wild-type mRXRβ (RXRβ) or the indicated mRXRβ AF-2 mutants. The mRXRβ AF-2 mutants (lanes 5–12 and inset at right) all display the phenotype of wild-type mRXRβ (lanes 3 and 4) in responding to the presence of ligand to form DNA binding complexes, indicating that these mutants are not impaired in their ability to homodimerize on the RXRE in response to ligand. Finally, DNA complexes formed by a lysate from cells containing mRXRβ with the activating mutation, F299A, are strong both in the absence and presence of the LG100364 ligand (compare lanes 13 and 14).

Effect of activating mutations on transcriptional responsiveness of RXR AF-2 mutants

The transcriptional properties of the hRXRα (F313A) and mRXRβ (F299A) activating mutants are depicted in Fig. 4A, panels 2 and 4 respectively. In comparison with the transactivation properties of wild-type hRXRα (panel 1) and mRXRβ (panel 3), these mutants exhibit high levels of transcriptional activity in the absence of 9-*cis* RA, nearly equivalent to the transcriptional levels displayed by the respective wild-type counterparts in the presence of this ligand. The addition of 9-*cis* RA results in a small additional fold increase in the transcriptional activity of these mutants in the range of 1·3- to 2·2-fold (see also Fig. 4B). These data indicate that the LG100364-independent DNA binding displayed by these mutants (as shown for mRXRβ F299A in Fig. 3B) is also associated with potent transcriptional activity. The elucidation of the crystal structure for the corresponding mutation in mRXRα (F318A) has revealed the unexpected presence of a fatty acid accommodated within the binding cavity of this receptor as a result of the additional space generated by this mutation (Bourguet *et al*. 2000). The presence of this fatty acid can evidently induce a transcriptionally active configuration in this RXR mutant, even in the absence of its natural cognate ligand, 9-*cis* RA.

In addition to comparing the transcriptional profile of wild-type mRXRβ to that of mRXRβ F299A, similar comparisons are depicted in Fig. 4B between the transcriptional activities of the mRXRβ AF-2 mutants F436A, L437A, E439K and L441A

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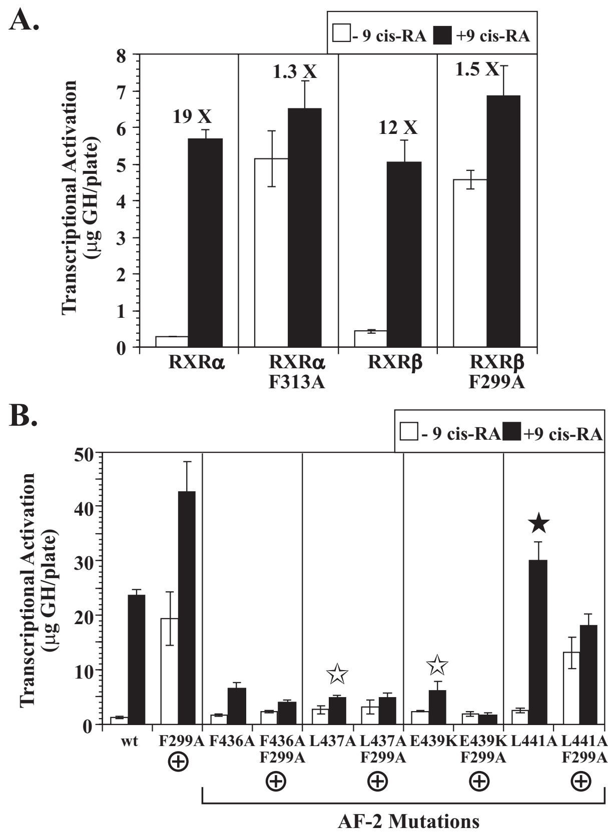

FIGURE 4. The generation of an activating mutation in RXR and its effect on transcriptional responsiveness to 9-*cis* RA in RXRs with altered AF-2 residues. (A) Analysis of activating point mutations in RXRα and RXRβ. COS-7 cells were cotransfected with the reporter plasmid $(RXRE)_4$ TK-GH in combination with an expression vector for one of the following: wild-type hRXRα, mRXRβ, or the activated mutants F299A (mRXRβ) or F313A (hRXRα). Replicate plates were then treated with 9-*cis* RA (10^{-6} M) or vehicle for 48 h and GH assays were performed as described in Materials and Methods. The data shown (means ± S.D.) are representative of three independent experiments, and the numbers over the bars indicate the fold-effect of hormone treatment in each case. (B) Introduction of the RXRβ activating mutation F299A in the context of AF-2 mutations, either in the absence or presence of 9-*cis* RA. The transcriptional profile of the mRXRβ AF-2 mutants F436A, L437A, E439K and L441A (left half of each grouping), and those of their respective counterparts possessing the F299A mutation in addition to the specific AF-2 mutation (right half of each grouping), are shown. The results are presented as means ± S.D. from two independent experiments. As in Fig. 2, the L437A and E439K mutants (open stars) display the most blunted activity, whereas the L441A (solid star) mutant is least affected.

(left half of each grouping) and receptors containing these same mutations along with the F299A mutation (right half of each grouping). In the case of those mutants for which transcriptional activity is significantly impaired, i.e. the N-terminal (F436A) and the second and fourth residues (L437A and E439K, denoted by open stars), little or no ligand-independent activity is discernible. However, the L441A mutant (as denoted by a solid star), which possesses transactivation levels comparable to wild-type mRXRβ, assumes the activated phenotype with the introduction of the F299A mutation. This double mutant (L441A/F299A) displays a high level of 9-*cis* RA-independent transcriptional activity that increases only slightly in the presence of this ligand. The overall transcriptional levels of this mutant are, however, generally lower than that of RXRβ F299A, despite both receptors exhibiting equal levels of expression in this experiment, as detected by Western blotting (data not shown). Transcriptional properties similar to L441A/F299A are also exhibited by the E442K/F299A double mutant (data not shown). These results confirm that L441 of mRXRβ (and presumably its hRXRα homolog, L455) are not active participants in the RXR homodimer-mediated transcriptional response. However, the fact that the introduction of the activating F299A mutation does not overcome the loss of transcriptional activity associated with the mRXRβ AF-2 mutants F436A, L437A and E439K, emphasizes the importance of these residues in ensuring the full transcriptional competency of the RXR homodimer, likely through interactions with transcriptional coactivators.

The activating F313A hRXRα mutation, in the context of the VDR heterodimer, cannot drive transcription in the absence of $1,25(OH)_2D_3$

As described above, RXRα is the major endogenous, and apparent natural heteropartner for VDR in COS-7 cells as well as in intestine, kidney and skin, the primary target tissues of $1,25(OH)_2D_3$ action (Mangelsdorf *et al*. 1992). In addition, data published on the vitamin D-24-hydroxylase VDRE indicate that RXRα, but not RXRβ, serves as the VDR heterodimeric partner in the activation of this gene (Kephart *et al*. 1996). Therefore, experiments probing the RXR-VDR heterodimer described below will, for the most part, involve RXRα and its mutations. Accordingly, the activating F313A mutation in hRXRα was next tested in the context of the VDR heterodimer (Fig. 5A). For these experiments, the rat osteocalcin-VDRE-containing reporter vector, $(CT4)_4$TK-GH, was transfected into COS-7 cells in combination with an expression vector for hVDR and, where indicated, hRXRα. Following transfection, the cells were treated with either ethanol vehicle or $1,25(OH)_2D_3$. As shown in

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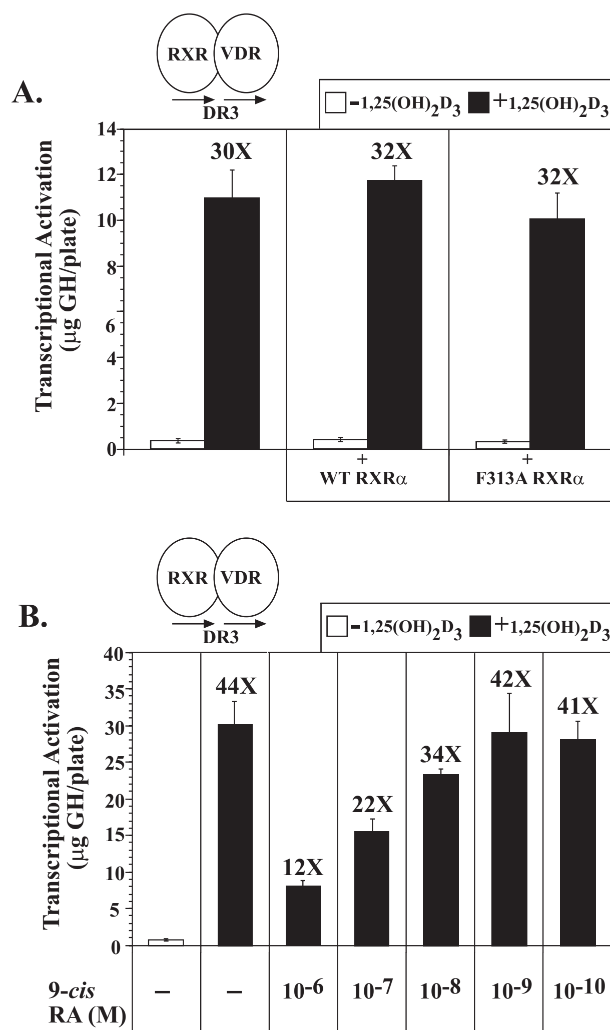

FIGURE 5. $1,25(OH)_2D_3$-stimulated transactivation mediated by the RXR-VDR heterodimer: influence of activated RXR or 9-*cis* RA. (A) Lack of effect of an hRXRα activating mutation. COS-7 cells were transfected with 7·0 μg of the rat osteocalcin VDRE-containing reporter vector $(CT4)_4$TK-GH in combination with 1·0 μg of the hVDR expression vector pSG5 VDR alone (left panel), or in combination with 1·0 μg of the expression vector for wild-type (WT) hRXRα (center panel), or the F313A activated RXRα mutant (right panel). Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) for 48 h. The depicted experiment is representative of three independent experiments. (B) Dose-dependent inhibition by 9-*cis* RA. COS-7 cells were transfected with $(CT4)_4$TK-GH reporter and the hVDR expression vector. Cells were treated for 48 h with $1,25(OH)_2D_3$ (10^{-8} M) alone, or in combination with 9-*cis* RA at the indicated concentration. Aliquots of culture medium were then assayed for human GH as described in Materials and Methods. Results are expressed as means ± S.D. with $n=3$, and the data pictured are representative of three replicate experiments. The numbers over the bars indicate the fold-effect of hormone treatment in each case.

Fig. 5A (right panel), addition of exogenous F313A has little effect on the transcriptional response to $1,25(OH)_2D_3$, comparing it to the profiles obtained with pSG5 hVDR and endogenous RXR (left panel) or plus exogenous wild-type hRXRα (center panel). In particular, the elevation of basal transcription in the absence of ligand, a conspicuous feature of the F313A mutant in a homodimeric context (Fig. 4A), is totally lacking in the setting of an RXR-VDR heterodimer.

9-*Cis* RA is an inhibitor of VDR-mediated transcriptional activity, likely by diverting RXR to form homodimers

Figure 5B illustrates an experiment evaluating the role of the RXR ligand, 9-*cis* RA, in the VDR heterodimer-mediated transcriptional response to $1,25(OH)_2D_3$. Under the same experimental conditions as described in Fig. 5A, it is observed that none of the tested concentrations of 9-*cis* RA, in combination with $1,25(OH)_2D_3$, elicit an increase in the transcriptional response beyond that obtained with $1,25(OH)_2D_3$ alone. In fact, the data in Fig. 5B demonstrate that relatively high concentrations of 9-*cis* RA are inhibitory, with 10^{-6} and 10^{-7} M 9-*cis* RA producing reductions of 73% and 50% respectively. Thus, in this context, the natural ligand for RXR is not synergistic with $1,25(OH)_2D_3$, but rather inhibitory, especially at relatively high doses. This effect is probably the result of 9-*cis* RA (at higher concentrations) binding to, and diverting, endogenous RXR to form RXR homodimers (MacDonald *et al*. 1993, Lemon & Freedman 1996, Thompson *et al*. 1998).

Full transcriptional competency of the VDR heterodimer requires an intact AF-2 domain of RXR

Because endogenous RXR levels in COS-7 cells are usually sufficient to mediate optimal signaling via the RXR-VDR heterodimer, it was necessary to utilize a 'rescue' assay featuring a mutated hVDR that is partially impaired in its heterodimerization function in order to evaluate the relative transcriptional abilities of RXR AF-2 mutants in the context of the VDR heterodimer. As depicted in the first two panels of Fig. 6, the addition of exogenous hRXRα to transfections containing intact hVDR and the reporter plasmid results in only a very small increment in transcriptional activity by the RXR-VDR heterodimer, indicating that endogenous RXR(s) is largely sufficient to serve as the partner for wild-type VDR. However, if a natural hVDR

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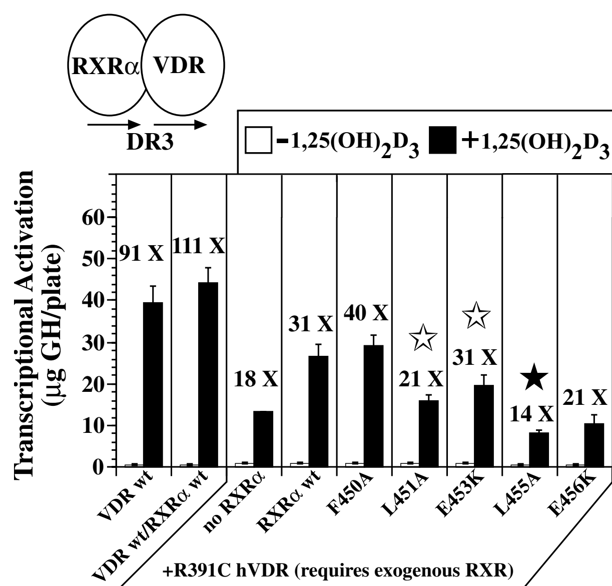

FIGURE 6. Effect of AF-2 mutations in RXRα on $1,25(OH)_2D_3$-stimulated transcription as mediated by the RXR-VDR heterodimer. COS-7 cells were cotransfected with the reporter plasmid $(CT4)_4$TK-GH, in combination with the pSG5 hVDR expression vector (2·0 µg), or the expression vector for the R391C mutant hVDR (4·5 µg) and, where indicated, 0·5 µg of the appropriate expression vector for wild-type (wt) hRXRα or the designated hRXRα mutant. The rationale for using hVDR-R391C is explained in the text. Transfected cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) and media were assayed for human GH as described in Materials and Methods. Data presented are the average of three independent experiments (± S.D.), with each experiment having an n=3 for each tested condition. Corresponding to the L441A RXRβ mutant in Fig. 2, the L455A RXRα mutant is marked with a solid star indicating lack of inhibition in a homodimeric context, although in this new setting it is the *most* impaired in transactivation. Conversely, mutants L451A and E45K, marked with open stars and originally showing (Fig. 2) maximal impairment in the RXR homodimeric context, now display a mild (L451A) or minimal (E453K) inhibition in the new setting.

mutant, R391C, is used in which the heterodimerization function of VDR is impaired (Whitfield *et al*. 1996), the addition of exogenous RXRα elicits a readily detectable increase in transcriptional activity by the heterodimer (compare the $1,25(OH)_2D_3$ effect in the third versus the fourth panel of Fig. 6).

Results obtained utilizing hRXRα AF-2 mutants in this rescue assay are shown in the remaining panels of Fig. 6, and similar data were observed from experiments employing mRXRβ AF-2 mutations (data not shown). In both cases, the mutation with the most serious consequences for RXR activity in this heterodimeric context is in the penultimate AF-2 residue, namely L455A in hRXRα (marked by a solid star in Fig. 6) or L441A in mRXRβ. It is noteworthy that this same residue is the *least* affected when mutated in the homodimeric context (Figs 2 and 4B; also denoted by a solid star). In a similar flip-flop of activities, alteration of the central Glu residue in the AF-2 of both hRXRα (E453K) (denoted by an open star in Fig. 6) and mRXRβ (E439K) reveals this amino acid to be relatively functionally unimportant in the RXR-VDR heterodimer, even though its mutation severely affected the activity of the RXR homodimer (Figs 2 and 4B). Therefore, it is clear that the AF-2 residues important for RXR-mediated transactivation in the RXR-VDR heterodimer are distinct from those that determine RXR activity in the homodimer.

The L455A hRXRα (L441 in mRXRβ) AF-2 mutation exerts a reversible dominant negative effect on RXR-VDR-mediated transcription

Given the apparent functional importance of the penultimate RXR AF-2 residue (Leu) in the RXR-VDR heterodimer, a mutant containing an alanine substitution of this residue was tested for its ability to confer a dominant negative phenotype. This L455A hRXRα mutant was examined in cotransfection experiments in competition with either endogenous RXRs (Fig. 7A) or exogenous hRXRα (Fig. 7B). As a control, the F450A mutant was utilized, for which the transcriptional activity in a heterodimeric context is, if anything, enhanced over wild-type RXRα (Fig. 6, middle panel). As illustrated in Fig. 7A (left panel), the addition of 0·2 µg pSG5-hRXRα results in approximately a 42% increase in the fold transcriptional response to $1,25(OH)_2D_3$ compared with that obtained with hVDR and endogenous RXR. An even greater increase (79%) in the transcriptional response is observed with the cotransfection of 0·2 µg of the F450A hRXRα vector (Fig. 7A, middle panel), consistent with earlier tests of this mutant (Fig. 6). The addition of higher amounts of wild-type and F450A hRXRα does not elicit any further increase of the GH reporter gene transcription, implying saturation of the heterodimer with exogenous RXR. A different response is obtained, however, using the L455A hRXRα expression plasmid (Fig. 7A, right panel). Addition of 0·2 µg pSG5 vector encoding this mutant does not significantly increase the $1,25(OH)_2D_3$-dependent transcriptional activity over that obtained with hVDR in combination with endogenous RXR. Further, cotransfection of

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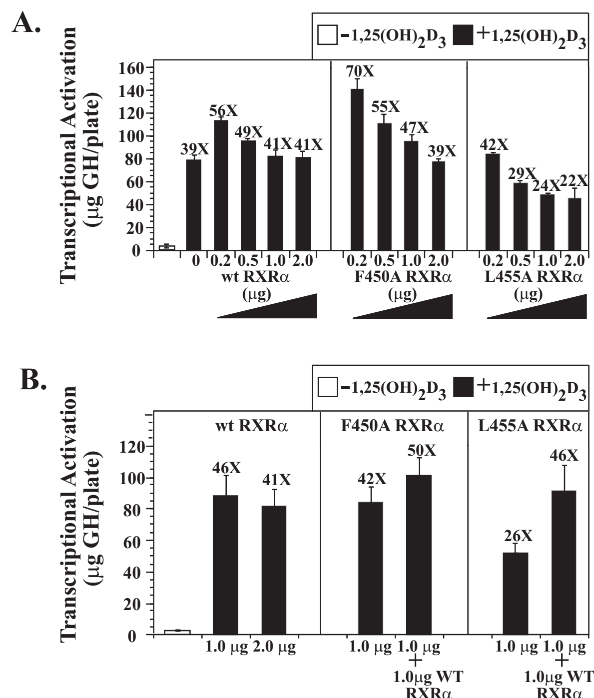

FIGURE 7. Influence of transfected RXRα expression plasmid on $1,25(OH)_2D_3$-stimulated transactivation as mediated by the RXRα-VDR heterodimer. (A) Effect of excess F450A or L455A hRXRα mutants. COS-7 cells were transfected with $(CT4)_4$TK-GH in combination with the expression vectors for hVDR (2·0 μg) and, where indicated, 0·2–2·0 μg of wild-type (wt) hRXRα (left panel), F450A hRXRα (middle panel), or L455A hRXRα (right panel). Appropriate amounts of the parent vector pSG5 were included to maintain a constant level of expression vector (4 μg total) for each treatment. Cells were exposed to $1,25(OH)_2D_3$ (10^{-8} M) and relative transcription levels were assessed as described in Materials and Methods. Results are representative of two independent experiments with means ± S.D. and n=3 for each individual assay. (B) The dominant negative effect of L455A hRXRα on the $1,25(OH)_2D_3$ transcriptional response can be reversed by the addition of wt hRXRα. COS-7 cells were transfected with $(CT4)_4$TK-GH and pSG5 hVDR (2·0 μg) in combination with 1·0 μg expression vector for wt hRXRα (left panel), F450A hRXRα (middle panel), or L455A hRXRα (right panel). Where indicated, an additional 1·0 μg wt hRXRα was included in each of the three transfection groups. Data are expressed as in (A), and a constant overall level of expression vector (4 μg) was maintained by the inclusion, where appropriate, of pSG5.

increasing amounts results in a clear inhibition of the $1,25(OH)_2D_3$-induced transcriptional response. The magnitude of the inhibition reached approximately 44% when 2·0 μg of the L455A expression plasmid were added to the transfection. This level of inhibition, combined with the observation that addition of similar amounts of either wild-type hRXRα or the F450A mutant fail to inhibit $1,25(OH)_2D_3$-mediated transcription, constitutes clear evidence for dominant negative activity of L455A hRXRα. As a final confirmation of the ability of the L455A mutant to competitively inhibit the activity of other RXRs in the context of a RXR-VDR heterodimer, it was reasoned that addition of excess wild-type hRXRα would abolish the inhibitory (i.e. dominant negative) effect on $1,25(OH)_2D_3$-stimulated transcription. The left panel of Fig. 7B indicates that, as was observed in Fig. 7A, the presence of 2·0 μg wild-type hRXRα does not result in a significant change in the $1,25(OH)_2D_3$ transcriptional response from that obtained using 1·0 μg hRXRα expression vector. In a similar fashion, the addition of 1·0 μg wild-type hRXRα, in combination with 1·0 μg F450A hRXRα does not significantly alter the levels of transcriptional activity observed using 1·0 μg F450A hRXRα alone (middle panel of Fig. 7B). In contrast, the blunted transcriptional activity consistently observed following the cotransfection of 1·0 μg L455A hRXRα can be overcome by the co-addition of 1·0 μg hRXRα (right panel of Fig. 7B). Taken together, the results in Fig. 7 demonstrate that L455A hRXRα functions as a dominant negative inhibitor of RXR-VDR-mediated transcriptional activity, and that this inhibitory activity can be overcome by supplying excess wild-type hRXRα.

DISCUSSION

Role of AF-2 residues in the RXR homodimer-mediated transcriptional response to ligand

X-ray crystallographic structures for both the apo homodimeric (Bourguet *et al*. 1995) and holo monomeric (Egea *et al*. 2000) ligand binding domain (LBD) of hRXRα have provided a three dimensional view of the role of the terminal (twelfth) α-helix (H12), representing the core AF-2 region, in RXR homodimer-mediated transactivation (Moras & Gronemeyer 1998). These structures support a mechanism for nuclear receptor action in which binding of agonist induces a repositioning of H12 to provide a surface for interaction with transcriptional coactivators (Renaud *et al*. 1995, Darimont *et al*. 1998, Moras & Gronemeyer 1998, Torchia *et al*. 1998, Egea *et al*. 2000). In addition, there have been a number of reports demonstrating, via site-directed mutagenesis, that the AF-2 domain in the RXR homodimer is essential for mediating 9-*cis* RA-dependent

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interactions with transcriptional comodulators. For example, mutations within the AF-2 core motif of mRXRα abolish interactions with the transcriptional coregulators TIF1 and TIF2 (GRIP 1) (Le Douarin *et al*. 1995, vom Baur *et al*. 1996, Voegel *et al*. 1996), leading to a transcriptionally inactive receptor. In agreement with these previous results, we observe that residues in the N-terminal half of the AF-2, such as F436, L437 and E439 of mRXRβ and L451 and E453 in hRXRα, are essential for the full transcriptional responsiveness of the RXR homodimer to ligand (Fig. 2). Referring to the holo hRXRα LBD X-ray structure for the exact positions of these residues, E453 appears to be exposed to solvent and presumably available for direct contact with coactivators, while the second AF-2 residue (L451) turns inward to form tight hydrophobic contacts with residues in α-helix 3 (H3) and α-helix 5 (H5) that function to seal H12 in place over the mouth of the ligand-binding cavity (Egea *et al*. 2000). Thus, in the context of the RXR homodimer, it would seem that the N-terminal half of the AF-2 region is critical for transcriptional activity and ligand retention. Extending this concept, we report herein that residues located towards the C-terminal end of the AF-2, in particular L441 of mRXRβ and its equivalent in hRXRα, L455A, are relatively uninvolved in mediating transcriptional activity in the homodimer.

The AF-2 of RXR, unlike homologous domains within TR and RAR, can be deleted in RXR without abolishing ligand binding and dimerization functions (Tate *et al*. 1994, Leng *et al*. 1995). The present data are consistent with this observation, since none of the current series of RXR AF-2 point mutants are impaired in their ability either to homodimerize on the RXRE in response to ligand (Fig. 3B) or to heterodimerize with VDR in solution (data not shown). It was also observed that the mRXRβ F299A mutant displays a striking rexinoid-independence in binding to DNA (Fig. 3). The homologous mutation in mRXRα (F318A) was originally reported to exhibit 'constitutive' activity (Vivat *et al*. 1997). However, it has recently been determined that this rexinoid-independent activity is actually accounted for by the presence of an endogenous fatty acid that can be accommodated in the binding pocket of the alanine substitution mutant, but not of wild-type RXR (Bourguet *et al*. 2000). The present results in no way contradict this proposed scenario. Further, the observation that the F299A mutation does not confer a ligand-bound 'active' phenotype when introduced in the context of the mRXRβ AF-2 mutants F436A, L437A and E439K implies that the impaired transcriptional responsiveness to 9-*cis* RA of these mutants cannot be attributed to diminished homodimeric association, binding of DNA, or ligand binding/retention, but instead results primarily from weakened interactions with transcriptional coactivators.

Functional switching of RXR AF-2 residues crucial for transcriptional potency in the context of RXR homodimer versus RXR-VDR heterodimer

It has recently been shown that H12 of RXR has an important role in sterically preventing binding of corepressor to apo-RXR (Zhang *et al*. 1999). Such unmasking of the RXR corepressor site may be responsible for the reported dominant negative phenotype exhibited by an RXR AF-2 deletion mutant in inhibiting VDR-mediated, $1,25(OH)_2D_3$-dependent transcription (Blanco *et al*. 1996). To exclude the possible repressor activity that would result from the use of an RXR total AF-2 deletion mutant, the contribution of unliganded RXR to the overall transcriptional responsiveness of the RXR-VDR heterodimer was assessed in the current study using a series of RXR AF-2 point mutations. The data presented herein indicate that there appears to be a functional switch in the AF-2 residues that mediate transactivation when RXR participates in the context of an RXR homodimer compared with that of an RXR-VDR heterodimer. In marked contrast to the homodimeric situation, RXR AF-2 residues for which alteration is most deleterious to transactivation by the RXR-VDR heterodimer (Fig. 6) occur in the C-terminal portion of the AF-2 region (i.e. L455 in hRXRα or L441 in mRXRβ). The importance of the C-terminal AF-2 residues in RXR for transcriptional competency of RXR-VDR is further validated by the partial dominant negative activity exhibited by the L455A hRXRα mutant on the transcriptional response of the heterodimer to $1,25(OH)_2D_3$ (Fig. 7). Conversely, the N-terminal half of the RXR AF-2, which is crucial to the activity of RXR homodimer (Fig. 2), does not appear to be as important to transcriptional activation by RXR in a heterodimer with VDR (Figs 6 and 7). This switching of functional roles for AF-2 residues does not appear to be a consequence of the use of different RXR isoforms. Although mRXRβ was employed for the present homodimer studies, and hRXRα was utilized as a heterodimeric partner, a full test was performed of the mutant mRXRβ as well as the hRXRα AF-2 mutant panels in both the homodimeric and heterodimeric contexts. In all such experiments (data not shown), the overall profile, particularly with regard to the least versus most transcriptionally impaired mutations, was similar.

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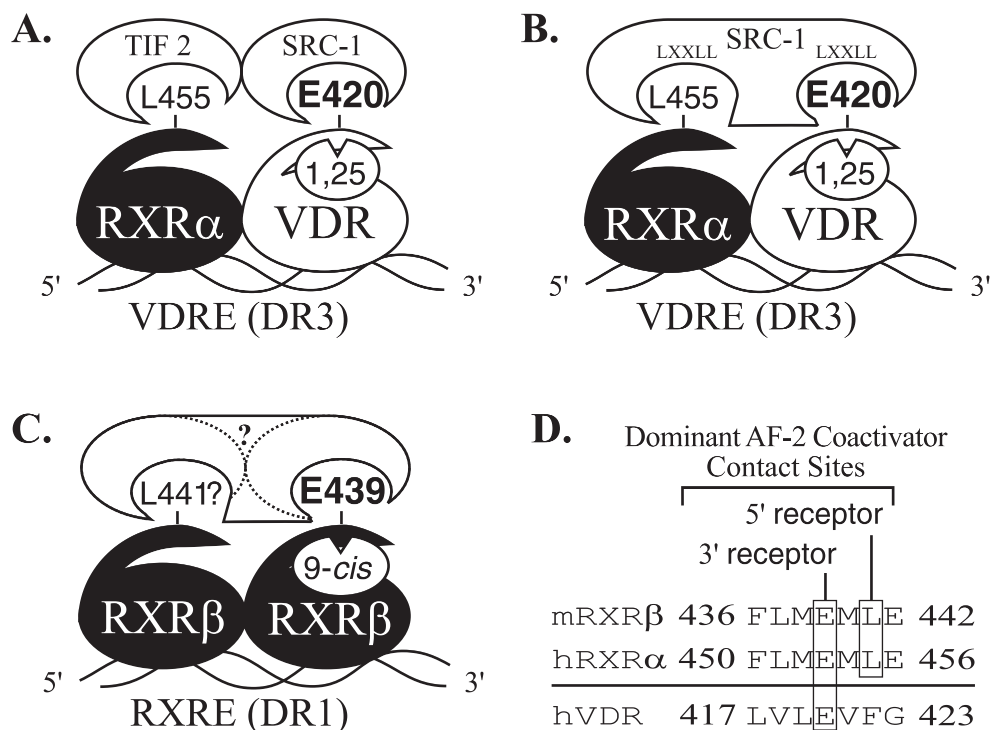

FIGURE 8. Model for the varying roles of the RXR AF-2 as a contact site for coactivator(s) in the context of an RXR-VDR heterodimer (panels A and B) versus that of an RXR-RXR homodimer (panel C). The transcriptional activity of the RXR-VDR heterodimer is proposed to consist of a 'primary' contribution from the AF-2 of VDR that resides on the 3′ half-site of the VDRE, plus a 'secondary' contribution from the AF-2 of the RXR partner on the 5′ half-site. Residue E420 within the VDR AF-2 has been postulated to represent a vital site of interaction with a coactivator, such as SRC-1 (Jurutka *et al*. 1997, Masuyama *et al*. 1997). In this context, the RXR partner bound to the 5′ half-site appears to utilize L455 of hRXRα, or the corresponding L441 of mRXRβ, to contact coactivator(s). The model illustrates two possible mechanisms by which the AF-2 of RXR contributes to the overall transcriptional activity of the RXR-VDR heterodimer: (A) the AF-2 of the RXR partner may contact a different, but still important subsidiary coactivator such as TIF-2 (GRIP 1); (B) alternatively, the RXR AF-2 may contact a second, less critical, LXXLL motif within the same primary SRC-1 coactivator. In the context of RXR-RXR homodimers, as depicted in (C), the RXR associated with the 3′ half-site of the RXRE assumes the role of the primary partner, while the 5′ RXR plays the part of the secondary partner. Thus, an RXR-RXR homodimer can be considered as behaving functionally like a heterodimer. In keeping with this model, the central AF-2 Glu residue (E439 in mRXRβ or E453 in hRXRα) supplies a crucial contact site for coactivator in the AF-2 of the RXR residing on the 3′ (dominant) half-site. The RXR positioned on the 5′ (secondary) half-site then provides a less crucial, but still important contact for coactivator that apparently interacts with the penultimate Leu (L441 or L455), as it did in the case of the VDR-RXR heterodimer. (D) Summary of the positions of the proposed dominant AF-2 coactivator contact sites in the context of the 3′ primary receptor partner (either VDR or RXR) and of the 5' secondary receptor (RXR only).

An hypothesis for explaining this apparent 'switch' proposes that the transcriptional activities of both the RXR homodimer and RXR-VDR heterodimer consist of a 'primary' AF-2 contribution from the receptor residing on the 3′ half-site of the VDRE, and also a 'secondary' AF-2 contribution from the receptor (RXR) that binds to the 5′ half-site (Fig. 8). The AF-2 of the primary receptor contains vital sites of interaction between the primary partner and coactivator(s), such as SRC-1 (Oñate *et al*. 1995, Masuyama *et al*. 1997, Darimont *et al*. 1998, Kraichely *et al*. 1999), GRIP1 (Hong *et al*. 1997, Nolte *et al*. 1998), and ACTR (Chen *et al*. 1997). In the case of the RXR

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homodimer (see Fig. 8C), the primary receptor (the RXR on the 3′ half-element of a DR-1 type RXRE) binds coactivator(s) via the central Glu residue (E439 or E453). The primary receptor in the RXR-VDR heterodimer, namely hVDR on the 3′ half-site of the VDRE, likewise has been shown to bind coactivators via the conserved central residue, E420 (Jurutka *et al*. 1997, Masuyama *et al*. 1997). This E420 residue is not only vital for transactivation, but is also positionally conserved in nearly all known nuclear receptors. There is, in fact, direct evidence that the conserved central glutamate in hPPARγ (Nolte *et al*. 1998) and hTRβ (Darimont *et al*. 1998) directly contacts coactivator, suggesting that such interaction may be a general role of this AF-2 residue in the nuclear receptor superfamily.

The hPPARγ study, performed with an SRC-1 fragment (Nolte *et al*. 1998), and the hTRβ study, in which the hTRβ ligand binding domain was cocrystallized with peptides derived from GRIP1 (Darimont *et al*. 1998), also agree in demonstrating participation in transcription by the initial AF-2 residue, corresponding to F436/450 in RXR and L417 in hVDR. Indeed, there is evidence that L417 is important for correct AF-2 positioning in hVDR (Jurutka *et al*. 1997, Masuyama *et al*. 1997, Rochel *et al*. 2000), and the present study reveals a significant role for the homologous residue (F436) in mRXRβ homodimer-mediated transactivation (Fig. 2). Interestingly, the residue in the AF-2 of hVDR that is immediately C-terminal of L417, and is positionally equivalent to RXR L437/L451, namely V418, also participates in the transactivation function of hVDR, and it does so by latching the ligand binding pocket closed, just as the L437/L451 residue in RXR has been shown to do (Egea *et al*. 2000). However, V418 in hVDR accomplishes this, not by interacting with other helices in the hVDR ligand binding domain, but by contacting the $1,25(OH)_2D_3$ ligand itself (Rochel *et al*. 2000). Thus, the first two AF-2 residues (L417/V418 in hVDR, F450/L451 in hRXRα and F436/L437 in mRXRβ) appear to play receptor-specific roles in transactivation. This diversity of function is also reflected in their evolutionary conservation, which is discernible only in the preservation of hydrophobic character at these two positions (see Fig. 1).

The RXR residing on the 5′ half-element of both the VDRE and the RXRE is postulated to serve as a secondary partner in recruiting coactivators. In this subordinate role, the AF-2 residues that interact with coactivator are distinct from those AF-2 residues that are crucial in the primary receptor, even when the receptor in both cases is RXR (Fig. 8C). As depicted in the model in Fig. 8A, in the context of the heterodimer, a potential role for the secondary AF-2 may be in contacting a different but still important coactivator (Jurutka *et al*. 2000*a*), represented by TIF-2 (GRIP 1) (Voegel *et al*. 1996) in the hypothetical model presented in Fig. 8A. A number of reports have suggested that the transcriptional activity of RXR-containing heterodimers may occur by each subunit contacting different components of a common activation complex. Indeed, differential interactions have been described for RXR, RAR, and VDR in their contacts with TIF1 versus SUG1. Thus, while RXR can interact with TIF1, but not SUG1, VDR and RAR demonstrate equally strong affinities for both of these comodulators (vom Baur *et al*. 1996). A recent report (Yang *et al*. 2000) has revealed that RXR and PPARγ interact selectively with p160 proteins and DRIP 205 in a ligand-dependent fashion. Significantly, RXR-specific ligands promoted specific binding of RXR to p160, while PPARγ-specific ligands elicited the selective recruitment of DRIP 205 by PPARγ. This selectivity was observed exclusively in the presence of the PPARγ responsive element, suggesting allosteric contributions from DNA. Furthermore, there is evidence that VDR and RXR, when in the context of an RXR-VDR heterodimer, may interact with distinct components of the transcriptional complex, as provided by the observation that $TAFII_{135}$ augments AF-2 dependent transactivation by VDR (and also TR and RAR) but not by RXR (Mengus *et al*. 1997).

As indicated in Fig. 8B, an alternative mechanism involving the secondary (RXR) AF-2 may be contacting a second, less critical region of the same coactivator. Such a scenario has precedent with other receptors such as RAR. Thus, in the context of the RXR-RAR heterodimer, allosteric inhibition of RXR results from RXR AF-2 contact with the RAR coactivator binding site. Recruitment of SRC-1 to RAR in response to ligand displaces the RXR AF-2 domain (from its association with the AF-2 of RAR), freeing it to bind a secondary LXXLL motif in the same SRC-1 molecule that is bound to RAR (Westin *et al*. 1998). A similar scenario has also been described for hPPARγ (Nolte *et al*. 1998). In the present experimental system, we observe no synergistic activity when the RXR-VDR heterodimer is in the presence of both respective cognate ligands (Fig. 5B); thus it is possible that the VDR primary receptor itself, when liganded, can mediate a reconfiguration of the apo RXR AF-2 so that it is able to interact with a second contact region within the same coactivator (such as SRC-1) molecule, even in the absence of 9-*cis* RA.

In summary, as pictured in Fig. 8D, we conclude that the dominant coactivator contacts in the

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receptor on the 3′ half-element are the central glutamate (plus N-terminal core residues), whereas the receptor (RXR) on the 5′ half-element interacts with coactivator predominantly via the C-terminal leucine within the AF-2. Yet another possible role of the AF-2 region is suggested by recent studies of transcriptional initiation. Following the remodeling of target gene chromatin through the intrinsic histone acetyl transferase activities associated with coactivators, a large 15 subunit assembly, known as the DRIP/mediator complex, is reported to bind to the hormone-occupied receptor through a key subunit designated DRIP 205 (Rachez *et al*. 1999). This 1,25$(OH)_2D_3$-induced complex can then bind to the RNA polymerase II holoenzyme machinery to activate transcription (Chiba *et al*. 2000). It has been shown recently that, although only one of two LXXLL motifs within DRIP 205 is used for binding to the AF-2 of VDR, *in vitro*, both LXXLL motifs are used in the context of the RXR-VDR heterodimer during transcription, *in vivo* (Rachez *et al*. 2000). It is therefore possible that the role of the AF-2 of RXR, in the context of the RXR-VDR heterodimer, is to provide an additional site of interaction for binding with DRIP 205 through the secondary LXXLL motif. Thus, L441/L455 of RXR may represent an important site for contact with DRIP 205, thereby ensuring a full transcriptional response to 1,25$(OH)_2D_3$.

The role of 9-*cis* RA in the context of RXR-VDR-mediated signaling

The present data indicate that relatively high concentrations of 9-*cis* RA have a suppressive effect on the levels of 1,25$(OH)_2D_3$-mediated transcriptional activity from a rat osteocalcin VDRE-containing reporter construct (Fig. 5B). The mechanism for this suppression is presumed to involve diversion of RXR, in response to its ligand, from the VDR-containing heterodimer to form RXR homodimers instead (MacDonald *et al*. 1993, Lemon & Freedman 1996, Thompson *et al*. 1998). The observation that the dose-dependence of this effect (between 10^{-9} to 10^{-6} M) resembles that of RXR homodimeric binding to an RXRE (Fig. 3A) is consistent with this interpretation. In addition, herein it is shown that the active phenotype of the F313A hRXRα mutant, triggered by the presence of a common fatty acid, is silenced when in the context of an RXR-VDR heterodimer. Such silencing has also been reported for F318A mRXRα complexed with unliganded RAR (Vivat *et al*. 1997). These data signify that, in the present experimental system, RXR-mediated 9-*cis* RA signaling through RXR-VDR is silenced and rendered subordinate to the ligand status of the primary receptor (VDR). This is consistent with previous *in vitro* data, which have indicated that the ligand status of the primary receptor (VDR) can allosterically regulate the response of its RXR partner to 9-*cis* RA (Thompson *et al*. 1998). Such intra-heterodimeric subordination of the RXR AF-2 as a result of allosteric action by the ‘primary’ partner again mirrors the RXR-RAR heterodimer, where, as discussed above, RXR signaling is inactive unless the RAR partner is liganded, and can only operate through synergy with the RAR ligand (Apfel *et al*. 1995, La Vista-Picard *et al*. 1996, Chiba *et al*. 1997, Minucci *et al*. 1997, Vivat *et al*. 1997). Although synergism by both cognate ligands of the RXR-VDR heterodimer has been reported (Kato *et al*. 1995, Sasaki *et al*. 1995, Kephart *et al*. 1996, Li *et al*. 1997), we observe no such synergistic activity by 9-*cis* RA at any concentration in the test systems described in this report. Nevertheless, such transcriptional synergism probably does exist under the proper set of parameters that may include cell specific factors, different RXR heteropartner isoforms, and/or the influence of *cis*-acting DNA elements within natural gene promoters regulated by vitamin D.

RXR is a versatile player in the control of transcription by nuclear receptors

Taken in the context of the X-ray crystallographic structures of nuclear receptors including RXR and VDR, the present results permit a generalization on the possible biological and evolutionary implications of the structure/function of RXR in transcriptional control. Because RXR can serve as either the primary receptor in a homodimeric setting, or as a versatile secondary heteropartner with numerous other nuclear receptors that respond to their cognate ligands, it is not surprising that it possesses several unique properties with respect to the AF-2/helix 12 domain. Unlike other nuclear receptors for which there are discrete ligand contact amino acids in the core AF-2 (Whitfield *et al*. 1999), no ligand contact residues exist in the RXR AF-2 when cocrystallized with 9-*cis* RA (Egea *et al*. 2000). Furthermore, when acting as the secondary receptor in the liganded RXR-RAR cocrystal, the RXR helix 12 remains in the open (antagonist) mode (Bourguet *et al*. 2000). This intimates that, if the RXR AF-2 participates in signaling in a heterodimeric context, it may require structural stabilization through the association of a coactivator with the RXR heterocomplex (Jurutka *et al*. 2000*a*, Rachez *et al*. 2000). We postulate that the primary mechanism of activating an AF-2 for transcriptional signaling in

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nuclear receptors is for the ligand to pull the helix 12 'door' shut by direct contact with AF-2 core residues. This would not occur efficiently with the binding of the 9-*cis* RA natural ligand to RXR, because of the lack of direct AF-2 ligand contact, rendering it the subordinate receptor in most heterodimeric settings. Consequently, in this secondary role, RXR likely depends upon allosteric activation by the liganded primary partner followed by coactivator recruitment to create a stable supercomplex that remains bound to the DNA recognition site (Blanco *et al*. 1996, Jurutka *et al*. 2000*a*, Rachez *et al*. 2000).

From the current data and the above speculation, we conclude that N-terminal AF-2 hRXRα/mRXRβ residues L451/437 are functionally analogous to AF-2 ligand contacts such as V418 in hVDR, but participate in intramolecular interactions of the AF-2 with other helices in the LBD, such as H3 and H5, without the aid of direct ligand contact. Such interactions are apparently important for displaying the conserved central glutamate (E453/439) on the surface for coactivator attraction when RXR is acting as the primary receptor. Conversely, when RXR operates as the secondary partner, C-terminal AF-2 hRXRα/mRXRβ residues L455/441, which are positionally conserved with the F422 ligand contact in the hVDR AF-2, become important to transcriptional signaling. Although leucine 455/441 could be integral to intramolecular association of the AF-2 with the LBD in this case (Egea *et al*. 2000), a more likely scenario is that this C-terminal H12 amino acid establishes intermolecular interactions between RXR and coactivators, as is actually observed for hPPARγ and SRC-1 (Nolte *et al*. 1998) (Fig. 8B). Thus, the AF-2 of the secondary RXR is envisioned as mobile, being capable of intermolecularly repressing the AF-2 of the primary partner in the unliganded state (Westin *et al*. 1998), but also competent to generate coactivator contact when the primary receptor is liganded. Perhaps the most interesting extension of this theory occurs in instances when the secondary RXR partner is transformed into the dominant 'primary' player in a heterocomplex, such as in the case of the activation of RXR-LXR by selective rexinoids (Repa *et al*. 2000), some of which may actually contact RXR AF-2 core amino acids and conformationally transfer this ligand binding signal to the partner (LXR) receptor. Clearly, RXR is a flexible nuclear receptor that can adopt different motifs, thus acquiring the capability either of transducing molecular information received as a subordinated secondary receptor, or of assuming itself the dominant role as a master heteropartner for other nuclear receptors.

In conclusion, the present study provides evidence that RXR does not act merely as a silent DNA-binding partner for VDR, but rather makes an important transcriptional contribution to the overall potency of the RXR-VDR heterodimer in responding to $1,25(OH)_2D_3$. A detailed understanding of the allosteric communication that exists between these two subunits and how the role of the transcriptionally active RXR heteropartner may be modulated by such factors as DNA element sequence, cell-specific VDR- and RXR-interacting proteins, and natural and synthetic ligands for both receptors, will provide further insights into the molecular mechanisms that generate the wide spectrum of bioresponses to $1,25(OH)_2D_3$.

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