Vitamin D-dependent Suppression of Human Atrial Natriuretic Peptide Gene Promoter Activity Requires Heterodimer Assembly*

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Crystallographic structures of the ligand-binding domains for the retinoid X (RXR) and estrogen receptors have identified conserved surface residues that participate in dimer formation. Homologous regions have been identified in the human vitamin D receptor (hVDR). Mutating Lys-386 to Ala (K386A) in hVDR significantly reduced binding to glutathione S-transferase-RXRα in solution, whereas binding of an I384R/Q385R VDR mutant was almost undetectable. The K386A mutant formed heterodimers with RXRα on DR-3 (a direct repeat of AGGTCA spaced by three nucleotides), whereas the I384R/Q385R mutant completely eliminated heterodimer formation. Wild type hVDR effected a 3-fold induction of DR-3-dependent thymidine kinase-luciferase activity in cultured neonatal rat atrial myocytes, an effect that was increased to 8–9-fold by cotransfected hRXRα. Induction by K386A, in the presence or absence of RXRα, was only slightly lower than that seen with wild type VDR. On the other hand, I384R/Q385R alone displayed no stimulatory activity and less than 2-fold induction in the presence of hRXRα. Qualitatively similar findings were observed with the negative regulation of the human atrial natriuretic peptide gene promoter by these mutants. Collectively, these studies identify specific amino acids in hVDR that play a critical role in heterodimer formation and subsequent modulation of gene transcription.

The nuclear hormone receptors are a family of ligand-regulated transcription factors that associate with cognate recognition sequences in close proximity to target gene promoters and through an, as yet, incompletely understood process regulate their transcriptional activity (1, 2). There are two major classes of nuclear hormone receptors. Class I receptors, which encompass the steroid hormone receptors (i.e. receptors for glucocorticoids, mineralocorticoids, progestins, androgens, and estrogens), typically bind as homodimers to palindromic sequences encoding the core recognition sequence. Class II receptors, which include the vitamin D receptor (VDR),3 thyroid receptor (TR), and retinoic acid receptor (RAR), bind to direct repeat (DR) elements as heterodimeric complexes with unliganded retinoid X receptor (RXR) (see below). In contrast to class I receptors that invariably recognize a palindrom spaced by 3 base pairs, the class II receptors bind to DRs spaced by a variable length of nucleotides. This spacing contributes to specificity in the types of receptors that associate with a given recognition sequence (3).

VDR has been shown to interact with canonical recognition elements termed vitamin D response elements (VDREs) in a variety of target genes. In some cases these recognition elements function in a stimulatory mode (e.g. osteopontin (4), osteocalcin (5, 6), calbindin (7), 24-hydroxylase (8), and β3 integrin (9)), whereas in others (e.g. parathyroid hormone (PTH) (10, 11) and parathyroid hormone-related protein (PTHrP) (12, 13)) it is clearly inhibitory. Although there is considerable sequence variation among the stimulatory VDREs, the general structure suggests conservation of two direct repeats of a consensus (A/G)G(G/T)TC spaced by a three-nucleotide spacer (DR-3). VDR typically associates with this element as a heterodimeric complex with RXR prior to effecting changes in transcriptional activity. VDR homodimers have been described (14, 15), most notably with the receptors in an unliganded form (15); however, it is generally accepted that the ligand-dependent assembly of VDR-RXR heterodimeric complexes on the VDRE is the dominant pathway leading to vitamin D-dependent activation of gene expression. Less information is available regarding the inhibitory effects of liganded VDR on gene expression. In the case of PTH and PTHrP, the responsible element contains only one of the two tandem hexameric sites found in VDREs involved in positive gene regulation, and RXR does not appear to be involved in mediating the inhibitory effect (10–13).

As already noted, RXR serves as a heterodimeric partner for a variety of different nuclear receptors (1, 2). In addition, RXR can, in the presence of its cognate ligand 9-cis-retinoic acid (9-cis-RA), assemble as homodimers on a recognition sequence containing two DRs separated by a single nucleotide spacer (DR-1) (3). Thus, there are a number of pathways by which this receptor can regulate downstream transcriptional activity.

We have recently demonstrated that formation of VDR-RXR heterodimers is important for activation of a DR-3-dependent reporter in cultured neonatal rat atrial myocytes (16). However, whereas VDR-dependent inhibition of ANP promoter ac-
tivity is amplified by cotransfection with RXR, the dependence of this inhibition on heterodimerization of these two receptors remains unclear. A VDR mutant (L262G), which demonstrates impaired heterodimerization with RXR (17), retains the ability to suppress human atrial natriuretic peptide (hANP) promoter activity (16) in transfected myocytes. Thus, the dependence of hANP promoter suppression on heterodimer formation remains open to question.

The crystallographic structures of RXRs (18) and the estrogen receptor (ER) (19) imply an important role for several surface residues within helix (H) 10 (for RXR) and 11 (for ER) in homodimer formation. This region resides within the 9th heptad repeat proposed for the TR (20) and has been shown to be highly conserved in other receptors of this class, suggesting conservation of the structural determinants that govern dimerization in this family of regulatory proteins. In fact, mutations have already been reported in this region of VDR (21), RXR (22), and TRβ (23) that appear to interfere with dimer formation. However, based on the available structures (e.g. that for TRα), a number of these mutations target residues placed internally in the receptor molecule (24) where they might easily effect disruption of receptor folding and structural integrity. We (25) have recently shown that mutation of selected surface residues in the 9th heptad of TR interferes with dimer formation yet preserves other receptor functions such as ligand binding, DNA binding, and coactivator interactions. Based on analogy to TR, we have placed homologous surface mutations in human (h) RXR and hVDR in positions predicted to interfere selectively with dimer interactions but not with binding to DNA, ligand, or the relevant coactivators. We have examined the effects of these mutations on dimer formation and functional activity in a transfected atrial myocyte model.

MATERIALS AND METHODS

Plasmids—Expression vectors for human VDR (26) and RXRs (27) have been described previously. Mutations K386A (28), I384R/L419R (29), K386A/L419A (29), L262G (17), and L262G/L420G (30) have been described previously. The reporter plasmid containing the hANP promoter suppression on heterodimer formation reflection was 1100 hANP CAT (30) have been reported previously. The reporter plasmid containing the synthetic VDR response element has two copies of the DR, spaced by three nucleotides (AGGGGAAGGTCA) (DR-3), cloned immediately upstream from a minimal (−32/−45) thymidine kinase promoter and linked to luciferase coding sequence. GST-VDR was constructed by isolating an EcoRI fragment from pSG5hVDR (26) followed by ligation into pGEX2T. The structures of all new constructs were verified by DNA sequencing.

GST Pull-down Assay—Wild type or mutant pSG5 hVDR vectors and wild type or mutant pEthinRXRs vectors were used to produce radiolaabeled full-length receptors in vitro using the TNT-Coupled Reticulocyte Lysate System (Promega, Madison, WI) and [35S]methionine. GST-hVDR, GST-hRXRα, and GST-GRI F1 fusion proteins were prepared using conventional protocols (29). Briefly, the plasmids were transformed into HB101, amplified in culture, pelleted, resuspended in buffer IPAB-80 (20 mM HEPES, 80 mM KCl, 6 mM MgCl2, 10% glycerol; pH 7.6; 0.25 mM EDTA; 0.5 mM MgCl2; 5% glycerol) for 20 min at room temperature in the presence or absence of 100 nM of the appropriate ligand. The reaction mixtures were separated on 5% nondenaturing polyacrylamide gels in TEA buffer (67 mM Tris, pH 7.5, 10 mM EDTA; 33 mM sodium acetate). The gel was run at 240 V for 3 h at 4 °C, washed extensively with 30% methanol and 10% glacial acetic acid, and amplified for 30 min (Amplifier; Amersham Pharmacia Biotech), dried, and exposed for autoradiography.

Cell Culture and Transfection—Atrial cells were obtained from 1- to 2-day-old neonatal rat hearts by alternate cycles of trypsin digestion and mechanical disruption as described previously (30). The cells as described previously (30) were transfected by electroporation (280 V and 250 μF) using the plasmids indicated. All transfections were normalized for equivalent DNA content with PUC18. After transfection, cells were resuspended in Dulbecco's modified Eagle's medium H21 containing 10% bovine calf serum (HyClone, Logan, UT) and cultured for 24 h. At that time medium was changed to Dulbecco's modified Eagle's medium/serum substitute (32), and the cultures were treated with 10 nM 1,25-dihydroxyvitamin D3. Alternatively, cultures were incubated for 24 h in the presence or absence of unlabeled 1,25-(OH)2-23,24[3H]vitamin D3 (98 Ci/mmol; Amersham Pharmacia Biotech) overnight at 4 °C in the presence or absence of unlabeled 1,25-dihydroxyvitamin D3 (100-fold molar excess). Bound and free ligand were separated with dextran-coated charcoal (Sigma) using the method of Dokoh et al. (34). Scatchard analysis was carried out using the Graphpad Prism program.

RESULTS

Crystallographic structures of RXR and ER have identified surface residues that participate in receptor dimerization. The majority of these residues lie in helix 10 of RXR (18) and helix 11 of ER (19). These, as well as homologous regions from the ligand-binding domains of RAR, TR, and VDR are aligned in Fig. 1 for comparison. We have made mutations in several surface residues in RXRs and homologous residues in VDR (identified by shaded boxes) to evaluate their roles in generating dimeric complexes in vitro and regulating transcription in vitro.

By using a GST pull-down assay, a method that identifies...
protein-protein interactions in solution, independent of the presence of a DNA recognition element, we examined the ability of wild type and mutant VDRs to form complexes with RXR. As shown in Fig. 2A, GST-VDR showed little propensity to self-associate with either of the VDR mutants. There was, however, a small amount of homodimeric complex formed with wild type VDR, and this association increased modestly with the addition of 1,25-dihydroxyvitamin D3. GST-RXR strongly associated with wild type VDR, and again, this interaction was ligand-dependent. There was a reduced level of interaction with the K386A mutant of VDR (ligand-dependent) but virtually no interaction with the I384R/Q385R mutant. In each instance there was a modest increase in the presence of ligand. Of note, both the wild type and the mutant VDR proteins associated with GST-GRIP to an equivalent degree and in a ligand-dependent fashion, indicating that overall structure and function of these mutants were preserved. Thus, both VDR mutants appear to selectively impair heterodimerization with RXR in solution.

Similar findings were obtained when the RXR mutants were examined (Fig. 2B). Wild type RXR bound to GST-VDR in a 1,25-dihydroxyvitamin D3-dependent fashion. The R421A and L419R/L420R mutants each displayed impaired capacity to associate with VDR, and in both instances this limited interaction was ligand-dependent. The L419R/L420R RXR mutant also demonstrated poor heterodimerization with TRβ and peroxisome proliferator-activated receptor γ (data not shown).

Since the mutants seemed to disrupt dimerization differentially and because DNA may provide support for heterodimer interactions (35, 36), we decided to test the ability of these mutations to disrupt homo- and heterodimerization on DNA using conventional electrophoretic gel mobility shift assays (EMSA). As shown in Fig. 3A, wild type RXRα effectively formed ligand-dependent homodimers and hTRβ1-dependent heterodimers on a conventional DR-4 element. Selective mutation at position 421 (R421A) in RXRα resulted in a loss of homodimeric complexes while, if anything, it increased heterodimer formation. Mutation at positions 419 and 420 (L419R/L420R) abolished formation of wild type RXRα.
homodimers on DR-1, as well as the weak TRβ1-RXRα heterodimers noted above. Again, as in Fig. 2, it was substituted for wild type VDR. The I384R/Q385R VDR mutant, on the other hand, proved incapable of interacting functionally with either the wild type RXRα or the RXRα mutants.

The ANP gene promoter has been shown previously to be a target for the liganded VDR (16, 27, 38, 39). 1,25-Dihydroxyvitamin D₃, as well as a number of non-hypercalcemic analogues of vitamin D, effects a VDR-dependent reduction in hANP promoter activity. With this in mind, we examined the ability of the various VDR and RXRα mutants to impact on this inhibitory activity. As shown in Fig. 6, liganded VDR effected ~50% inhibition in hANP-CAT reporter activity, whereas liganded RXRα produced only a 20% reduction, levels that are in agreement with those previously reported (27). The VDR K386A mutant was slightly less effective than wild type VDR in promoting the inhibition, whereas the I384R/Q385R mutant was virtually devoid of activity. Neither of the RXRα mutants proved capable of inhibiting hANP promoter activity.

When used in combination, wild type VDR and RXRα effected a >90% inhibition of ~1150 hANP CAT activity (Fig. 7). RXRα R421A was less active than wild type in amplifying VDR activity, whereas inhibition in the presence of I384R/Q385R was reduced to ~50%, the level seen with wild type VDR alone (see above). In agreement with the observations made with DR-3 TKCAT (see above), the combination of VDR and the RXR mutants resulted in a stepwise loss of ANP promoter inhibition that varied as a function of the “severity” of the mutation. The most significant loss of inhibitory activity was seen with the combination of RXRα L419R/L420R and VDR I384R/Q385R. This combination had virtually no effect on the ~1150 hANP CAT reporter. These findings support the hypothesis that residues critical for heterodimerization in these two nuclear receptors are also critical for maintenance of transcriptional regulatory activity (in this case, either stimulatory or inhibitory in nature).

A trivial explanation of these findings arises from the possibility that the mutations, which we assume are selectively
targeted to the dimer interface, actually lead to global changes in receptor structure. Since these mutations are positioned in the ligand-binding domain of the receptor, alterations in ligand binding could account for both the loss of heterodimerization and the impairment in functional activity. To address this question, we examined the ligand binding properties of both the wild type and mutant VDRs in a cell-free system. As shown in Fig. 8, affinity of the receptors for $[^{3}H]$dihydroxyvitamin D$_3$ was almost identical for each of the three receptors while, if anything, total binding capacity was modestly increased with the mutants. This, together with the observation that each of the VDRs bound equivalently to GRIP-1 (Fig. 2A), argues against major structural changes as accounting for the loss of functional activity in the mutants and implies that the latter results from selective impairment in the ability of these mutants to form heterodimers.

We employed a double mutation to probe the VDR heterodimerization function to maximize the probability of interfering with the dimer interface. One of the amino acids mutated here (Gln-385) has previously been shown to reduce VDR interaction with an auxiliary factor (presumably RXR) present in COS-7 cells (21). To address the selective role of Ile-384 in the dimerization process, we introduced a site-directed mutation at this position, and we examined the effects of this perturbation on the RXR binding and functional properties of VDR. I384R, like the double mutant (I384R/Q385R), was ineffective in activating DR-3-TK-Luc (Fig. 9A) or inhibiting 21150 hANP CAT (Fig. 9B) in atrial myocytes. In addition, this mutant displayed a markedly reduced affinity for RXRa in the GST pull-down assay (Fig. 9C). Placed in the context of the earlier results of Nakajima et al. (21), it would appear that both Ile-384 and Gln-385 play equivalently important roles in heterodimer assembly.

**DISCUSSION**

Recent crystallographic analyses of individual nuclear receptors suggest conservation of structural features involved in dimer assembly. Specifically amino acid residues in helix 10 of RXRa (18) (these residues are located in helix 11 in hTR and hER) and helix 11 of hTRβ (24) and hER (19) appear to play a
A number of mutations in helices 10 and 11 have been shown previously to be involved in dimerization of VDR (Leu-419 and Leu-420), which structural studies place on the surface of RXR \( \alpha \) and VDR, respectively.

On the DR-4 template, RXR \( \alpha \) R421A demonstrated impaired formation of RXR \( \alpha \) homodimers, whereas heterodimer formation with TR \( \beta 1 \) was relatively normal. If anything, the latter was modestly increased, perhaps reflecting diversion of the mutant RXR \( \alpha \), incapable of assembling with itself, into complexes with a heterodimeric partner. The double mutation (L419R/L420R) further upstream completely eliminated both homo- and heterodimer assembly with RXR \( \alpha \). Noteworthy, the double mutation still permitted monomer binding to the DR-4 template, implying that the DNA binding function, per se, is not perturbed in this mutant.

We noted no VDR homodimer formation on the DR-3 template in the EMSA and only minimal interaction in the GST pull-down assay. VDR homodimers have been identified in \textit{in vitro} binding studies by others (14, 15), and in the unliganded form they may function as suppressors of gene transcription (15). However, most studies suggest that the functionally relevant complex in transducing the positive vitamin D signal in the target cell is the liganded VDR-RXR heterodimer (39). VDR I384R/Q385R completely disrupted heterodimeric pairings with either wild type RXR \( \alpha \) or the RXR mutants. Mutations in this region of the hVDR molecule have been reported previously to interfere with heterodimer formation (21). Specifically, VDR mutations K382E, M383G, Q385K, and L390G reduced assembly of a VDR/accessory factor complex on VDRE. Studies with the Q385K mutant (21) support our findings with I384R/Q385R. Additional studies focusing specifically on Ile-384 (mutation I384R) indicate that this residue, as well, plays an important role in heterodimer formation. Thus, it would appear that both Ile-384 and Gln-385 participate directly in dimer assembly. Lys-386, on the other hand, despite its contiguous location on the receptor surface, does not appear to play a critical role in this process.

The EMSA analyses indicate that both VDR K386A and RXR R421A retain the capacity to interact with heterodimeric partners (RXR \( \alpha \) in the case of VDR and TR \( \beta 1 \) in the case of RXR \( \alpha \)) at near wild type levels. The conclusion, at least as it applies to the RXR mutant, R421A, stands in contrast to those of Lee et al. (22) who identified this residue as critical for heterodimerization. This difference remains unexplained since both EMSA and the functional analyses indicated that this particular mutant displays close to wild type activity in our system. It should be noted, however, that the GST pull-down assays showed impaired heterodimeric interactions of these two mutants (i.e. VDR K386A and RXR R421A) (see Fig. 3). This discrepancy (GST pull-down versus EMSA) likely reflects differences in the end points being addressed in these two assays. The GST pull-down assay assesses the ability of proteins to associate in solution. Such associations, by definition, have to be of sufficient affinity to preclude disruption during the washing procedure used to reduce “nonspecific” protein-protein interactions. The EMSA is carried out in the presence of DNA template. Positioning of nuclear receptors next to each other on DNA may promote dimer contacts between the DNA binding domains of the subunits (35, 36), and receptor-DNA contacts may further stabilize the dimer complex. Thus, the GST pull-down assay is probably a more sensitive method to detect subtle impairment of protein-protein interactions that might otherwise be obscured when the same proteins are bound to DNA. With reference to the current study, although VDR K386A and RXR \( \alpha \) R421A displayed obvious impairment in their capacity to establish protein-protein interactions in solution, the impairment was not seen when they were permitted to assemble on DNA, and the latter, rather than the former, is probably most reflec-
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tive of their functional activity in the intact cell (see Figs. 4 and 7).

Collectively, our data suggest that the surfaces involved in homo- versus heterodimerization overlap (i.e. impairment of both homo- and heterodimerization is seen with the double mutants). They also reveal a critical role for selected residues in dimer assembly. Disruption of the hydrophobic residues in both VDR (Ile-384 and Gln-385) and RXR (Leu-419 and Leu-420) abolishes heterodimerization on DNA, whereas disruption of the charged residue flanking this hydrophobic patch (Lys-386 or Arg-421, respectively) has no effect on this process. Homodimerization appears to be equally affected by any of these mutations suggesting that homodimer assembly is less stable than that of heterodimers, a finding that is in agreement with the fact that heterodimers form preferentially (over homodimers) in solution or on DNA (see Figs. 2–4) (2).

We have shown previously that the liganded VDR exerts anti-hypertrophic activity and suppresses ANP gene transcription in cultured neonatal rat atrial (16, 38, 39) and ventricular (27) myocytes. This effect was clearly amplified by cotransfection with RXXRα (16, 27); however, a VDR mutant (L262G) with impaired capacity for heterodimer formation (17) was found to retain the ability to suppress the hANP gene promoter (16). This places into question the inferred requirement for VDR heterodimerization in generating the inhibitory effect. Our studies with the I384R/Q385R mutant clearly demonstrate that capacity for heterodimerization closely parallels the ability of the receptor to suppress hANP gene promoter activity. By inference, this would suggest that the L262G mutant described above retains the capacity to interact with a heterodimeric partner, albeit not RXXRα (17), as a prelude to initiating its biological activity.

In summary, VDR and RXR mutations which, based on RXR and ER structural studies, would be predicted to disrupt protein-protein interactions involved in receptor dimerization do, in fact, demonstrate impairment in dimerization in two independent in vitro assays. This is accompanied by a commensurate reduction in functional activity, assessed through transactivation of a DR-3-dependent promoter or suppression of an hANP dependent reporter, in transiently transfected rat atrial myocytes. The studies provide support for the suggested conservation of structure-function relationships across different members of the nuclear receptor family and highlight the role of heterodimer formation in vivo as a prerequisite for functional activity of VDR in activating or repressing target gene expression.

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