Characterization of Transcriptional Activation and DNA-Binding Functions in the Hinge Region of the Vitamin D Receptor†

Paul L. Shaffer,‡ Donald P. McDonnell,§ and Daniel T. Gewirth*,‡

Department of Biochemistry and Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Received October 26, 2004; Revised Manuscript Received December 8, 2004

ABSTRACT: The vitamin D receptor (VDR) is a ligand-responsive transcription factor that forms active, heterodimeric complexes with the 9-*cis* retinoic acid receptor (RXR) on vitamin D response elements (VDREs). Both proteins consist of an N-terminal DNA-binding domain, a C-terminal ligand-binding domain, and an intervening hinge region. The length requirements of the hinge for both transcriptional regulation and DNA binding have not been studied to date for any member of the steroid hormone superfamily. We have generated a series of internal deletion mutants of the VDR hinge and found that deletion of as few as five amino acids from the C-terminus of the hinge significantly reduces transcriptional activation in vivo. Replacing deleted residues in the C-terminus of the hinge with alanines restored activity, indicating that this section of the hinge acts as a sequence-independent spacer. The hinge region of VDR forms a long helix, and the geometric consequences of this structure may explain the requirement of the hinge region for transcriptional activity. Interestingly, all of the deletion mutants, even those that do not activate transcription, bind VDREs with equal and high affinity, indicating that the defect in these mutants is not their ability to bind VDREs. In contrast to VDR, constructs of RXR containing deletions of up to 14 amino acids in the hinge region exhibit near wild-type transcriptional activity. The ability to delete more of the RXR hinge may be related to the additional plasticity required by its role as the common heterodimer partner for nuclear receptors on differing DNA elements.

The vitamin D receptor (VDR)[1] (*1*) is a ligand-activated transcriptional regulator belonging to the steroid and nuclear hormone receptor superfamily that plays a key role in calcium homeostasis and has been implicated in regulating diverse biological functions, including cellular proliferation and differentiation (*2*−*5*). The superfamily has 48 members in humans, including receptors for thyroid hormone (TR) and 9-*cis* retinoic acid (RXR) (*6*, *7*). Like all members of the superfamily, the VDR protein possesses a modular configuration consisting of four distinct domains. The VDR contains a very short, 23-amino acid, N-terminal AB domain, followed by the DNA-binding domain (DBD) connected to the C-terminal ligand-binding domain (LBD) by the hinge region.

VDR mediates the response to $1,25(OH)_2D_3$ by binding as a heterodimer with the 9-*cis* retinoic acid receptor (RXR) to specific vitamin D response elements (VDREs) located upstream of target genes (*8*, *9*). Most VDREs are bipartite, consisting of two hexameric half-sites arranged as a direct repeat separated by a spacer of three base pairs (DR3) (*6*, *10*). Binding to the response element is accomplished via the DBD that consists of a highly conserved 66-residue core made up of two zinc-nucleated modules that fold into a unified globular domain (*11*, *12*). The structure of the VDR DBD has been solved as a homodimeric complex bound to various naturally occurring and canonical VDREs (*13*). The structure of the core VDR DBD is very similar to the previously studied hormone receptor DBDs (*14*−*18*), but the VDR structures also provided insight into the structure of the hinge region, or C-terminal extension (CTE), of the DBD. In particular, residues 97−121 are observed to form a continuous α-helix, nearly identical in trajectory to the CTE helix of the TR DBD (*14*).

Classically, the DBDs of steroid and nuclear hormone receptors are thought to recapitulate the dimerization and DNA-binding properties of the full-length receptor. For example, the isolated TR and RXR DBDs form a heterodimeric complex in the presence of a TR response element, the same behavior as observed in the intact receptors. The isolated DBDs of VDR and RXR do not form a DNA-bound heterodimer in solution however (*13*), a violation of these classic models. While the presence of a strong heterodimerization interface between the ligand-bound VDR and RXR LBDs will increase the local concentration of the DBDs, it still seems likely that the DBDs associate to achieve specific binding to DR3 response elements. This hypothesis was called into question, however, with the recent determination of the structure of a VDR DBD−RXR DBD−

† Supported in part by Grant DK58857 to D.T.G. and Grant 5U19-DK-62434 to D.P.M.

* To whom correspondence should be addressed. E-mail: gewirth@duke.edu.

‡ Department of Biochemistry.

§ Department of Pharmacology and Cancer Biology.

[1] Abbreviations: VDR, vitamin D receptor; TR, thyroid hormone receptor; RXR, 9-*cis* retinoic acid receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDREs, vitamin D response elements; DR3, direct repeat with spacer of 3 bp; CTE, C-terminal extension; hVDR, human vitamin D receptor; hRXRα, human 9-*cis* retinoid acid receptor alpha; DR1, direct repeat with spacer of 1 bp; RAR, *all-trans* retinoid acid receptor.

10.1021/bi0477182 CCC: $30.25
Published on Web 01/21/2005

DNA complex. The structure of this complex revealed that crystal-packing forces were capable of reversing the polarity of the DNA-bound complex (*19*), with the VDR DBD bound to the upstream half site and the RXR DBD bound independently to the downstream half site. This reinforces the growing notion that the RXR and VDR DBDs associate only very weakly, if at all. This led to our current investigation of the hinge region as a possible source of additional stability in the DNA bound heterodimeric complex.

Biochemical studies have identified residues in the hinge region as necessary for transcriptional activity and VDRE binding. Within the context of the full-length receptor, some hinge residues are necessary for dimerization (*13*), while others play a role in DNA binding (*20*−*22*). Interestingly, chimeric receptors that contain the DBD and hinge region of VDR along with the hinge region and LBD of TR respond to thyroid hormone but bind to vitamin D response elements, demonstrating that ligand-binding and DNA-binding activities can be isolated (*23*). Further, this illustrates that the linker between the DBD and LBD of hormone receptors has some plasticity; here it can double in length while maintaining the ability to bind DNA and activate transcription. Since the linker could double in length and still result in receptor activity, this raised the corollary question: What was the minimal length requirement in the hinge regions of the hVDR and hRXRα needed to mediate transcriptional regulation and specific VDRE binding?

To probe the affects of hinge length on VDR and RXR activity, we constructed VDR and RXR mutants containing internal deletions of the hinge region. These mutants were then tested for their ability to activate transcription in vivo from a promoter containing a VDR or RXR response element. Select recombinant mutant proteins were expressed in bacteria, purified, and used to precisely determine VDRE binding affinity using fluorescence anisotropy. These two sets of experiments allow us to define residues of the hinge regions that are required for activation and specific high affinity VDRE binding. We found that even short deletions in the VDR hinge region reduced transcriptional activity by more than half, while deletion of up to 14 residues, half of the hinge region, of RXR resulted in retention of greater than 60% of transcriptional activity. Remarkably, VDR and RXR mutants that were transcriptionally inactive were able to form heterodimers and bind VDREs with high affinity in vitro, indicating that the defect in these mutants is not their ability to bind to response elements.

MATERIALS AND METHODS

Cell Culture and Transfection. CV-1, COS, and HeLa cells were maintained in minimum essential medium (MEM) plus 10% fetal bovine serum, 0.1 mM essential amino acids, and 1 mM sodium pyruvate in a humidified 37 °C incubator with 5% CO_2. For transient transfections, cells were split into 24-well plates 16 h before transfection. Lipofectin (Life Technologies, Inc.)-mediated transfection has been described in detail previously (*24*). A DNA-lipofectin mixture containing a total of 3000 ng of DNA in each triplicate sample was incubated with cells for 6 h, and transfection was stopped by replacing the transfection mix with fresh medium (MEM without phenol red) containing 10% charcoal-stripped serum. Receptor ligands were added to the cells 24 h before the assay. Luciferase and β-galactosidase activities were measured as described (*24*). For a typical triplicate testing of VDR mutant activation, CV-1 cells were transfected with 1000 ng of p24OHase-Luc reporter plasmid, 50 ng of pCMV-hVDR expression vector, 100 ng of pCMV-βgal, and the balance, 1650 ng, was made up using pBluescriptII. For RXR mutant activation triplicate assays, HeLa cells were transfected with 1500 ng of DR1-Luc reporter plasmid, 500 ng of pRS-hRXRα expression plasmid, 100 ng of pCMV-βgal, and 900 ng of pBSII. All triplicate activation assays were performed on at least three different days; data shown are the results of a single, representative triplicate experiment.

Western Blot Analysis. Western blotting was performed with whole cell extracts isolated from COS cells transfected with plasmid containing either VDR or RXR together with pCMV-βgal for normalization purposes. Whole cell extracts were prepared by freeze−thaw lysis of cells scraped and resuspended in ×400 buffer (20 mM HEPES pH 7.4, 20% glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). After centrifugation of the sample at 16000*g* for 10 min, total protein concentration was determined by Bradford assay. A total of 30 μg of each extract was separated on a 12.5% SDS−polyacrylamide PhastGel (Pharmacia) and transferred using the PhastTransfer kit to a nitrocellulose membrane (Bio-Rad Laboratories). The blots were probed with either rabbit anti-VDR polyclonal antibody (PA1-711, Affinity BioReagents) or rabbit anti-hRXRα polyclonal antibody (catalog no. 39054, Active Motif) and a rabbit anti-βgal antibody (Cortex Biochem). The commonly used VDR monoclonal antibody 9A7γ was not used because its epitope lies in the hinge region and therefore would not recognize some of the deletion mutants. The immunocomplexes were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Reagents and Plasmids. The pCMV-hVDR plasmid was a gift from L. Freedman, the p24OHase-Luc reporter plasmid was a gift from J. Pike, and plasmids expressing the VDR and RXR mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). 1,25$(OH)_2D_3$ and 9-*cis* RA were purchased from Sigma.

hVDR Protein Purification. Wild-type and mutant hVDRΔAB constructs, residues 16−427, were cloned into the NdeI and BamHI sites of the pET15b expression vector that contains an N-terminal histidine tag. Protein was expressed in *Escherichia coli* BL21(DE3)pLysS cells grown in LB media containing 100 μg/mL ampicillin and 50 μg/mL chloramphenicol to an OD_{600} of 1.0. The temperature was reduced to 18 °C and after 1 h, the cells were induced with 0.5 mM IPTG at 18 °C for approximately 8 h. Cell pellets were resuspended in 10 mL of lysis buffer (20 mM Tris pH 7.6, 500 mM NaCl, 10 mM βME, 10 mM imidazole, 0.1% deoxycholate, 0.5% Triton X-100) per gram of cells and lysed using a MicroFluidics M-110L cell cracker. After centrifugation at 33800*g* for 15 min at 4 °C, polyethylenimine was added to a final concentration of 0.05% by dropwise addition of 10% polyethylenimine solution (pH 7.0) while stirring the solution gently. The precipitant was removed by centrifugation at 33800*g* for 45 min at 4 °C, and the supernatant was loaded onto a Ni-NTA agarose (Qiagen) column that had been equilibrated with lysis buffer minus detergents. The column was washed with 10 column volumes of lysis buffer minus deoxycholate, followed by 50 column

volumes of lysis buffer minus both detergents. Protein was eluted with a 10 column volume 10−250 mM imidazole gradient. Fractions containing VDR were identified by SDS−PAGE on a 12.5% polyacrylamide PhastGel and Coomassie blue staining. Peak fractions were pooled and concentration determined by UV absorbance. The salt concentration of the pooled elution was lowered to 150 mM by addition of SP buffer (50 mM HEPES pH 7.4, 5 mM DTT). A 10% molar excess of $1,25(OH)_2D_3$ was added to the solution. Because of its limited solubility in water, ligand was added in small aliquots corresponding to no more than a 300 nM concentration of ligand for each addition. The liganded protein solution was loaded onto an SP Sepharose (Pharmacia) column that had been equilibrated with SP buffer containing 50 mM NaCl. The column was washed with 5 column volumes of SP buffer containing 50 mM NaCl and eluted with 10 column volumes of SP buffer containing 500 mM NaCl. Fractions containing pure protein were identified and homogeneity assessed by SDS−PAGE. Fractions containing VDR were pooled and concentration determined by UV absorbance. Purified protein was stored in single use aliquots at −80 °C after freezing in liquid N_2.

hRXRα Protein Purification. Wild-type and mutant hRXRα ΔAB constructs, residues 130−462, were cloned into the NdeI and XhoI sites of the pET28b expression vector containing an N-terminal histidine tag. Protein was expressed in *E. coli* BL21(DE3)pLysS cells grown in TB media containing 50 μg/mL kanamycin to an OD_{600} of 3.0. The temperature was lowered to 20 °C for 15 min, and cells were induced with 0.5 mM IPTG at 20 °C for approximately 8 h. Cell pellets were resuspended in 10 mL of lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 10 mM βME, 10 mM imidazole) per gram of cells and lysed using a MicroFluidics M-110L cell cracker. After centrifugation of the sample at 33800*g* for 45 min at 4 °C, the supernatant was applied to a Ni-NTA agarose (Qiagen) column equilibrated with lysis buffer. The column was washed with 10 column volumes of lysis buffer containing 0.1% Triton X-100, followed by 50 column volumes of lysis buffer. The protein was eluted with a 10 column volume 10−300 mM imidazole gradient. Fractions containing RXR were identified by SDS−PAGE on a 12.5% polyacrylamide PhastGel and Coomassie blue staining. Peak fractions were pooled and the salt concentration lowered by dialysis in MWCO 8,000 tubing for at least 8 h at 4 °C against dialysis buffer (10 mM HEPES pH 7.4, 200 mM NaCl, 2 mM DTT). The dialyzed sample was spun at 15000*g* for 20 min at 4 °C, and the supernatant loaded onto a SP Sepharose FastFlow (Pharmacia) column that had been equilibrated with dialysis buffer. The column was washed with 5 column volumes of dialysis buffer and eluted with 10 column volumes of dialysis buffer containing 800 mM NaCl. Peak fractions were identified by UV absorbance and loaded onto a Superdex 200 (Pharmacia) column equilibrated with S200 buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT). The purified protein eluted in two peaks from the column, corresponding to different oligomeric states of the receptor. The smaller species contained the monomeric RXR protein, and fractions from this peak were pooled and concentration was determined by UV absorbance. Homogeneity was assessed by SDS−PAGE, and purified protein was stored in single use aliquots at −80 °C after freezing in liquid N_2.

In Vitro DNA Binding. DNA binding was assayed at room temperature by fluorescence anisotropy on a Panvera Beacon-2000 instrument, as described (*25*). Four read cycles were averaged per measurement in single-blank mode. Samples of VDR−RXR heterodimers were made by mixing stoichometric amounts of each component in FA buffer (25 mM HEPES pH 7.4, 250 mM NaCl, 10 mM DTT). Appropriate dilutions of the heterodimeric solution were made into FA buffer to a final volume of 180 μL per sample and 20 μL of 100 nM fluorescein-labeled DNA was added to each sample (10 nM final DNA concentration). Each sample was mixed thoroughly, transferred to a disposable cuvette, overlaid with mineral oil (125 μL) to slow oxidation of the protein, and allowed to equilibrate overnight at room temperature. The DNA sequence, 5′-CAC<u>AGGTCA</u>CGA<u>AGGTCA</u>CAC-3′ (half-sites underlined) and its complement were commercially prepared (Keck Oligonucleotide Synthesis Facility at Yale University). The complementary sequence was synthesized with a fluorescein label covalently linked to the free 5′-hydroxyl position of the anti-sense strand. The anti-sense strand was prepared by precipitation in ethanol. The sense strand was purified and detritylated on a reverse phase column (Rainin Dynamax-300 Å PureDNA). The purified strands were annealed by heating a stoichometric mixture of the two strands to 95 °C and slowly cooling to room temperature. Each equilibrium binding study was performed in triplicate, and data were fit using nonlinear least-squares analysis. Error between triplicate measurements was at most ±3 nM.

RESULTS

Functional Characterization of hVDR Hinge Truncation Mutants. To identify the minimal length of the VDR hinge region that retains in vivo transcriptional activity, we deleted amino acids from the C-terminal end of the hinge, creating molecules that contained an intact DBD and LBD connected by increasingly shorter linkers. Structures of the VDR LBD (*26*−*29*), residues 118−427, showed that residue Arg121 hydrogen bonds to the core of the LBD and Pro122 forms the start of helix 1. Therefore, residue Leu120 was chosen as the C-terminal limit of these truncations. Transcriptional activation was monitored by a luciferase based reporter gene assay in CV-1 cells. CV-1 cells, which are derived from African green monkey kidney cells, have little or no endogenous VDR, but appreciable amounts of RXR (*30*). As shown in Figure 1A, decreasing the linker region by five (Δ116−120), seven (Δ114−120), or nine (Δ112−120) amino acids resulted in receptors with less than 50% of the transcriptional activity of the wild-type, but still significantly more activity than background. Deletion of 13 amino acids (Δ108−120) however results in a receptor with no activity greater than background controls. Further truncations (Δ106−120, Δ104−120, Δ102−120, Δ100−120) also exhibited no detectable activity by reporter gene assay. Ligand dose response curves indicate that the ligand binding affinity is not altered in these mutants (Figure 2). Additionally, Western blot analysis of whole cell extracts indicates that the wild-type, Δ114−120, and Δ108−120 mutants are expressed at the same level (data not shown). Increasing the amount of VDR expression plasmid used in each transfection did not alter transcriptional activity either (data not shown). Together these indicate that differences in protein stability or degrada-

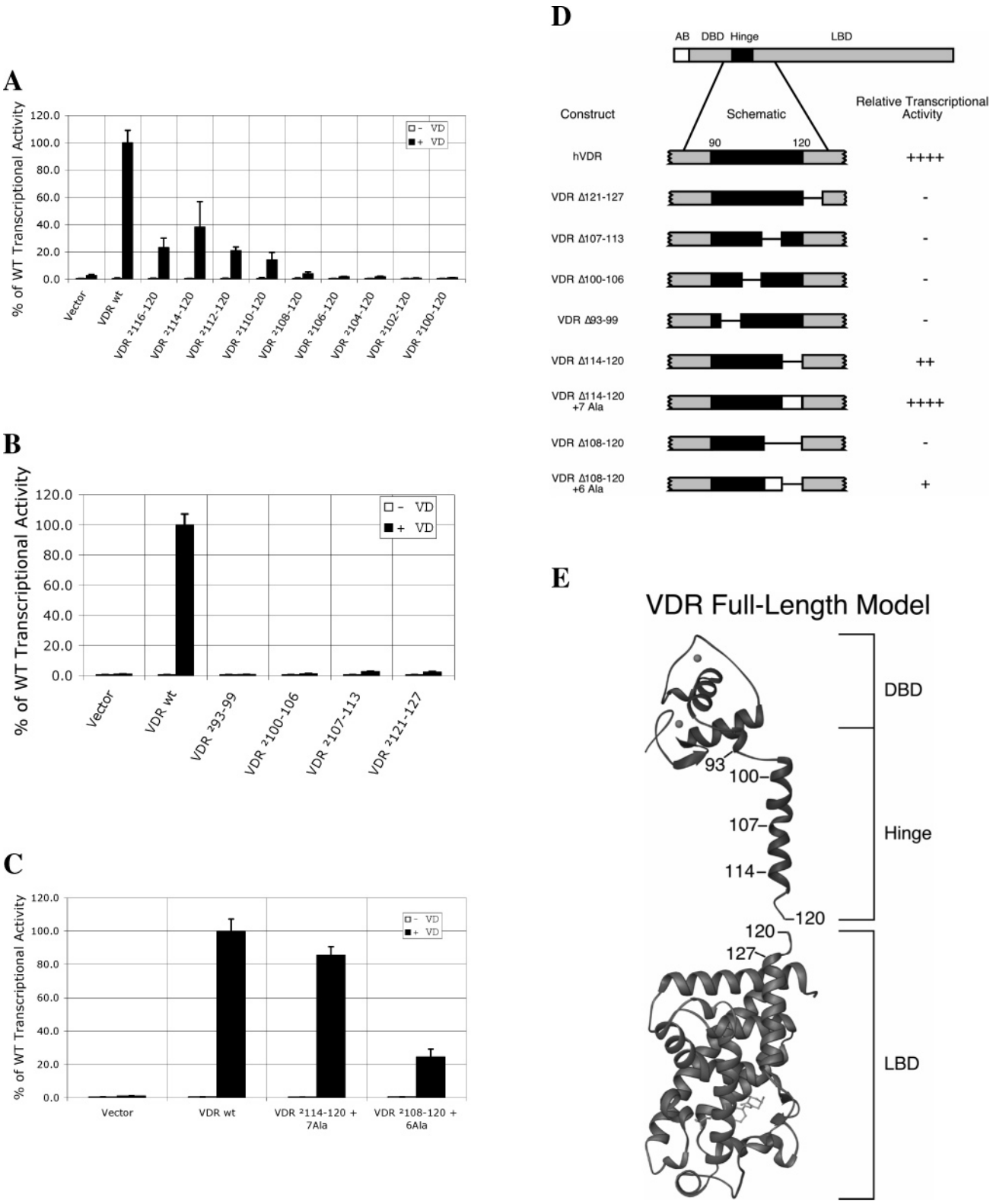

FIGURE 1: Functional characterization of hVDR hinge truncation mutants. (A) Transcriptional activity of initial VDR hinge mutants by luciferase reporter assay. CV-1 cells were cotransfected with p24OHase VDRE reporter plasmid, pCMV-βGAL (transfection efficiency internal control), carrier DNA, and either wt or mutant pCMV-hVDR expression plasmid. Cells were treated with vehicle (− VD) or $1,25(OH)_2D_3$ (10^{-8} M). Luciferase and β-gal activities were measured after 24 h. A representative experiment is shown, and each data point represents the average of triplicate determinations of normalized luciferase activity with error bars indicating the standard deviation. (B) Transcriptional activity of Δ7 VDR hinge mutants. Transcription assays were performed and analyzed as described in panel A. (C) Transcriptional activity of alanine substitution mutants. Transcription assay were performed and analyzed as described in panel A. (D) Summary of VDR mutant transcriptional activity. Constructs are shown schematically with the DBD and LBD as gray boxes, the hinge as a black rectangle, deletions as a line, and alanine substitutions as a white box. Activity is relative to wild-type as follows: ++++, 75−100%; +++, 50−75%; ++, 25−50%; +, 10−25%; −, 0−10%. (E) Model of full-length VDR molecule. Limits of truncations are indicated by sequence numbering. The DBD and hinge region coordinates are from PDB 1KB4 (*13*) and the LBD coordinates are from 1DB1 (*26*). This figure was created using Ribbons (*34*).

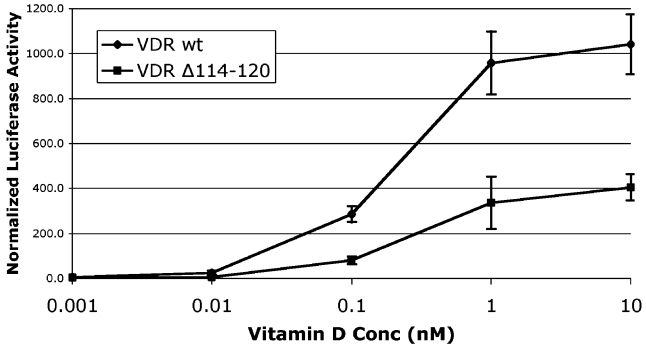

FIGURE 2: VDR hinge mutations do not affect ligand binding. Assays were performed as described in Figure 1. Cells were treated with increasing concentrations (10^{-12}−10^{-8} M) of 1,25$(OH)_2D_3$.

tion are unlikely to be the source of altered activity in the VDR deletion mutants.

In a second series of experiments, we explored deletion of stretches of seven amino acids from other sections of the hinge of VDR. All of these mutants had activity that was at or below background levels (Figure 1B). The most C-terminal of these mutants, Δ121−127, deletes part of the first helix of the LBD, a likely reason for its lack of function. The N-terminal mutant, Δ93−99, deletes residues that are involved in DBD homodimerization and likely involved in heterodimerization with the RXR DBD. The other mutants, Δ100−106 and Δ107−113, delete basic amino acid sequences that have previous been described as necessary for activation (*22*).

To investigate whether the length or the sequence of the hinge was a requirement of the VDR hinge region, two final mutants were constructed that replaced deleted VDR residues with alanines (Figure 1D). The polyalanine substitution is still capable of forming an α-helix, mimicking the geometry of the wild-type receptor, while abolishing any potential side-chain-mediated contacts. The first mutant added seven alanines back into the VDR Δ114−120 mutant to create a protein with a full-length hinge. The second mutant took the transcriptionally inactive VDR Δ107−120 mutant and added six alanines back to create a protein with the length of the VDR Δ114−120 mutant. The VDR Δ114−120+7Ala mutant regained near wild-type transcriptional activity, indicating the hinge length, but not sequence, is required (Figure 1C). The VDR Δ107−120+6Ala mutant did not regain the activity of the VDR Δ114−120 mutant, indicating that both the length and the sequence in the 107−113 region are necessary.

Transcriptional Activity of hRXRα Hinge Mutants. We also evaluated a series of hinge region truncations mutants of hRXRα and tested for their ability to activate transcription as a homodimer from a DR1 response element in vivo. For the purposes of these mutations, the C-terminal end of the hinge was defined as residue Asp229, since Met230 packs into the core of the LBD and Pro231 forms the start of helix 1 (*31*). Mutants deleting 7, 14, 21, or 28 amino acids were tested, and the results are shown in Figure 3. Mutants lacking 7 (Δ223−229, Δ216−222) or 14 (Δ216−229) amino acids at the C-terminal end of the hinge retained greater than 60% of the wild-type activity. However, all constructs with deletion of seven or more amino acids in the N-terminal half of the hinge, residues 202−215, (Δ202−208, Δ209−215, Δ209−222, Δ209−229, Δ202−229) resulted in a construct

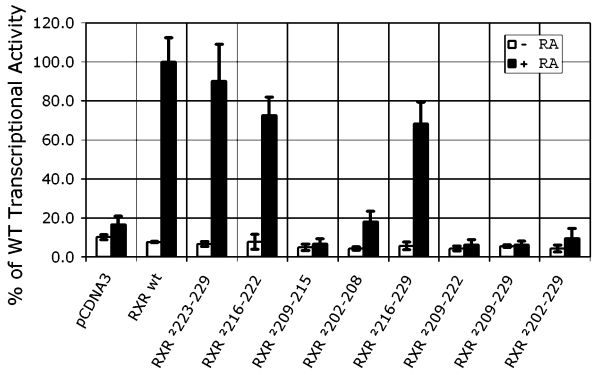

FIGURE 3: Functional characterization of hRXRα hinge mutants by luciferase reporter assay. HeLa cells were cotransfected with DR1-Luc reporter plasmid, pCMV-βGAL, carrier DNA, and either wt or mutant pRS-hRXRα expression plasmid. Cells were treated with vehicle (− RA) or 9-*cis* retinoic acid (10^{-7} M). Luciferase and β-gal activities were measured after 24 h. A representative experiment is shown, and each data point represents the average of triplicate determinations of normalized luciferase activity with error bars indicating the standard deviation.

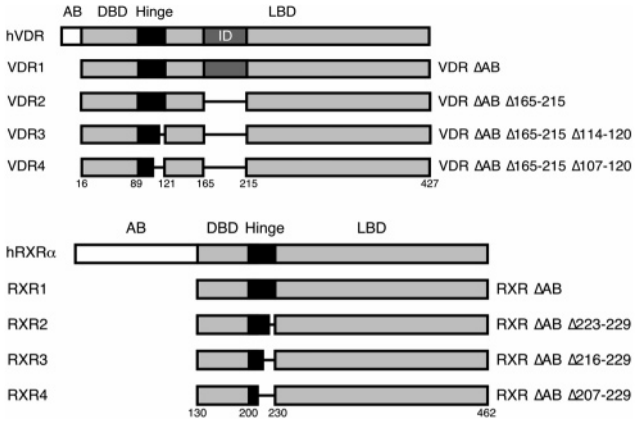

FIGURE 4: Schematic diagram of the hVDR and hRXRα constructs purified. Constructs VDR1−4 and RXR1−4 were purified from bacterial cells for use in VDRE binding assays. All constructs lack the AB domain. The insertion domain (ID) in the LBD of VDR was deleted in all hinge truncation mutants.

that lacked any activity above background. Western blot analyses indicated that constructs Δ216−229 and Δ209−229 are expressed at the same levels as the native protein (data not shown).

Quantification of VDRE Binding Affinity of wt and Mutant VDR/RXR Heterodimers. Several of the VDR and RXR hinge truncation mutants were cloned into bacterial expression vectors and purified from *E. coli* (Figure 4). VDR mutants Δ107−120 and Δ114−120 were cloned into a VDR construct lacking the insertion domain of the LBD, residues 165−215. This region has previously been shown to be unnecessary for in vivo transcriptional activation (*32*), a result that was recapitulated in the current system (data not shown). The VDR construct lacking just the insertion domain was also tested in these experiments to investigate its in vitro VDRE binding properties. During the purification of the VDR constructs 1,25$(OH)_2D_3$ was added to the protein. Stoichometric amounts of purified RXR and purified, liganded VDR were mixed together. These proteins formed a heterodimeric complex, as described previously (*33*) and confirmed by gel filtration chromatography (Figure 5). This heterodimeric complex was used in fluorescence anisotropy

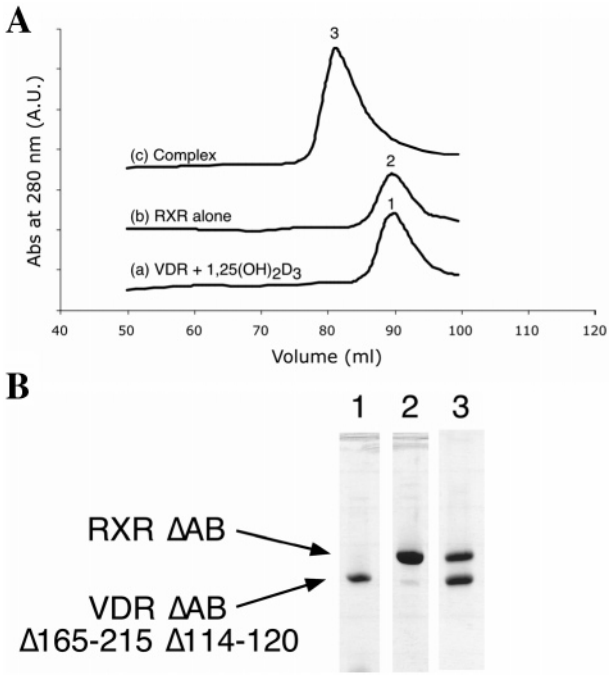

FIGURE 5: Formation of VDR/RXR heterodimers. (A) Analysis of VDR/RXR interaction using size exclusion chromatography. VDR ΔABΔ165−215Δ114−120, RXR ΔAB alone or as a mixture were incubated on ice and separated on a Superdex 200 16/60 gel filtration column. The protein elution was monitored by absorbance at 280 nm. Traces are VDR alone (a), RXR alone (b), and a mixture of VDR and RXR at a 1:1 molar ratio (c). (B) SDS−PAGE analysis of the protein(s) in each of the eluted peaks. Lane 1, peak from trace (a); lane 2, peak from trace (b); lane 3, peak from trace (c).

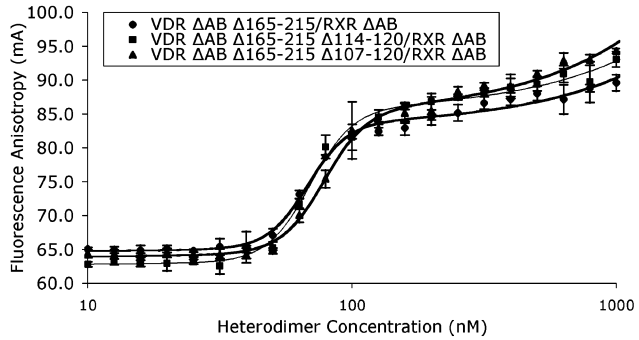

FIGURE 6: Fluorescence anisotropy measurements of VDR/RXR heterodimeric binding to DR3 DNA. Various purified, liganded VDR hinge truncations mutants, in complex with purified RXR ΔAB, were used to titrate a fluorescently labeled DR3 DNA duplex. Anisotropy measurements were taken at room temperature in 25 mM HEPES pH 7.4, 250 mM NaCl, 10 mM DTT. The concentration of DR3 DNA in each sample was 10 nM. Each point represents the average of three independent experiments with error bars indicating the standard deviation. The data were fitted using nonlinear least-squares analysis.

experiments to determine the complex's binding affinity for DR3 DNA, an idealized VDRE. Examples of the binding isotherms generated in these experiments are shown in Figure 6 and the binding constants for the various combinations of RXR and VDR constructs are summarized in Table 1. All combinations of RXR and VDR, including, surprisingly, mutants that were nonfunctional in vivo, were found to have binding constants between 64 and 92 nM. By contrast, RXR or VDR alone were found to have K_D values greater than 500 nM, indicating that the RXR/VDR heterodimeric complex is the high-affinity species, as expected. There is a small

Table 1: Summary of VDRE Binding Constants of VDR/RXR Heterodimers[a]

		VDR construct			
		VDRΔAB	VDRΔAB Δ165−215	VDRΔAB Δ165−215 Δ114−120	VDRΔAB Δ165−215 Δ107−120
RXR construct		VDR1	VDR2	VDR3	VDR4
RXRΔAB	RXR1	71	68	70	86
RXRΔAB Δ223−229	RXR2	66	70	68	78
RXRΔAB Δ216−229	RXR3	79	68	71	64
RXRΔAB Δ209−229	RXR4	92	88	71	71

[a] Binding constants (nM) were determined in 250 mM NaCl at room temperature and are the average of three measurements.

trend of slightly weakened binding with constructs of mismatched linkers, such as RXRΔAB with VDRΔABΔ165−215Δ107−120 or RXR ΔABΔ209−229 with VDR ΔABΔ165−215. However, these binding constants differ by less than a factor of 2 and can thus be considered equivalent. Thus, the similarity in DNA binding constants indicates that all the mutants, even those that fail to activate transcription in reporter gene assays, are able to bind to DR3 response elements. We therefore conclude that the defect that abrogates transcriptional regulation is not loss of DNA binding affinity.

DISCUSSION

The Entire Hinge Region of hVDR Is Required for Full Transcriptional Activation. Truncation of the hinge region of hVDR and hRXRα was used in the current experiments to gain insight into the specific residues that are necessary for in vivo transcription activation and in vitro VDRE binding. Comparing the sequence of the hinge region of VDR from various species reveals that this region is highly conserved, supporting the concept that this region is important to receptor function and more than merely a linker between the DBD and LBD (Figure 7). In contrast, the insertion domain of the LBD of VDR (residues 165−215 in the human sequence) is not conserved in sequence or length, suggesting that while VDR is found only in higher eukaryotes, sufficient time has passed for evolutionary divergence. The hypothesis that the hinge region is more than a flexible linker is supported by the current research because deletion of as few as five amino acids from the hinge region of VDR results in a construct that has significantly impaired ability to drive transcriptional activation.

Models of a RXR−VDR DBD complex based on the structure of the TR/RXR DBD heterodimer bound to a DR4 DNA target hypothesized that the CTE helix of VDR adopts a different trajectory from TR to bind to the shortened DR3 geometry of a VDRE (*14*). To accomplish this rearrangement and also to expose residues thought to be involved in heterodimerization, the authors of that study remodeled the protein backbone in the T-region (residues 90−97) of VDR. Since that TR/RXR study was published, we have solved the crystal structure of the VDR DBD homodimer (*13*) and a reverse-polarity VDR/RXR heterodimeric complex (*19*), both bound to DR3 DNA elements. These structures of the VDR DBD show that residues 97−121 form a continuous α-helix (*13*) that is nearly identical to the CTE helix observed

FIGURE 7: Comparison of the amino acid sequence of the hinge region of VDR from various species. Sequences begin with the universally conserved "GM" amino acid pair at the end of the core DBD and extend through the first four residues of helix 1 in the LBD.

for TR. Moreover, in contrast to the TR-based modeling study, the structure of the T-region, outside the core DBD, is in fact invariant. In every VDR DBD structure as well as in several other DBD complexes on direct repeat elements including RXR homodimeric (*16*) and RXR/RAR heterodimeric (*15*) complexes on a DR1 elements, it forms a single turn of helix. Given that RAR, RXR, VDR, and TR adopt the same conformation in this region, the rearrangement suggested for VDR in the model of the heterodimeric complex is unlikely. Interestingly, the VDR and TR CTE helices adopt nearly identical trajectories, contrary to the model based on the TR structure. These trajectories do not prevent binding to a DR3 response element, eliminating the rationale for rearrangement of the T-region in the DR3 model. Finally, the conformation of the VDR hinge region has been observed in three independent crystal settings; upstream and downstream in the homodimeric complex, as well in the nonphysiological heterodimeric complex. In each of these cases, the hinge has adopted an identical conformation, indicating this is not an artifact of its environment within the protein−DNA complex or crystal.

While the entire VDR hinge region is necessary for full transcriptional activity, three distinct sections can now be delineated. The most N-terminal section of the hinge, residues 90−97, consists of the T-region. As mentioned above, this region adopts a conserved fold and also contains residues that are necessary for dimerization. In particular, residues Glu92 and Phe93 participate in the VDR DBD homodimer interface and residues Glu92, Phe93, and Ile94 have been identified by mutagenesis as necessary for heterodimerization with the RXR DBD (*20*, *21*). Because of the conserved structure and role in dimerization, this region is necessary for VDRE binding and activation.

The middle section of the hinge, residues 98−113, contains two stretches of three basic amino acids in a row, residues 102−104 and 109−111, that had been shown to be necessary for transcriptional activation and VDRE binding by alanine mutagenesis (*22*). However, in that study, these residues have a much larger impact on transcriptional activity than on VDRE binding. We have confirmed and extended part of these observations here, as the VDR mutants lacking any part of this region were transcriptionally inactive. However, these mutants were able to bind VDREs. This suggests that these residues have a role in VDR function beyond simple modulation of DNA affinity. Further, adding alanines back into the deletion restores length, but does not restore transcriptional activity, indicating that the authentic sequence is required. Interestingly, the side chains of these residues are largely disordered in the crystal structure of the VDR DBD, suggesting that they are mobile, and do not make specific contacts with the DNA. However, the exact role these residues play in transcriptional activation has yet to be determined.

The C-terminal section of the hinge, residues 114−120, is the primary focus of this investigation, and our results show that it forms a sequence-independent spacer. These residues can be replaced by an equal number of alanines without significant effect on transcriptional activation. Because these residues are not required for VDRE binding, the purpose of this section is not likely to be DNA clearance or direct interaction. Instead, it appears that the purpose of this region is to establish a specific binding geometry between the DBD and LBD that is necessary for co-regulator interactions. Deletion of these residues disrupts this geometry and subsequent interactions.

Significant Regions of the RXR Hinge Can Be Deleted While Maintaining in Vivo Activity. Unlike VDR, the hinge region of RXR can be truncated significantly without impairment of transcriptional regulation. The RXR DBD that has been used in crystal structures to date has not included most of the hinge region, so the structure of this region is unknown. However, insight can be drawn from the biological role of RXR as the common binding partner of many nuclear hormone receptors on numerous direct repeat response elements with widely varying spacing between the half-sites (*6*). The hinge region in RXR is likely to be more flexible to accommodate various binding geometries, and this flexibility may allow for deletion of significant portions of the hinge without drastic reduction in transcriptional activation.

The Hinge Region of TR Is Likely To Be Necessary for Activity. The hinge region of TR adopts a long helix that is nearly identical in structure and trajectory to the helix observed in the VDR structures. This similarity in structure of the VDR and TR CTE helices suggests that they might serve a similar role. On the basis of this structural similarity, it is likely that even short truncations of the TR hinge will result in receptors with severely impaired transcriptional regulation, as seen in VDR. Interestingly, there are several stretches of basic amino acids in the CTE of TR, some of which have been shown to make base or backbone contacts in the minor groove of the response element. However, these basic stretches do not align in sequence or structure with the basic residues in VDR, suggesting that while these basic residues in TR might be necessary for function, they may play different roles in transcriptional activation.

VDR and RXR Mutants Form Heterodimers and Bind DNA with High Affinity. In the presence of $1,25(OH)_2D_3$, the LBDs of VDR and RXR form a high-affinity, stoichometric complex (*33*). While the heterodimeric interface in the LBDs drives assembly of the complex, DNA-binding affinity and specificity are dictated by interactions of the DBDs and hinge regions. Extensive truncation of the hinge region of RXR

and VDR results in receptors that are still capable of forming heterodimers and binding DNA with high affinity, even though they cannot activate transcription in vivo. Therefore, it appears that the defect in these receptors that prevents full activity is not formation of a heterodimer or that heterodimer's ability to bind DNA. Further, the role of the mid-hinge region between residues 108 and 114 lies beyond merely linking, separating, or positioning of the DBD and LBD, as replacement of the authentic sequence with alanines does not restore activity. The reason for this sequence requirement in the middle section of the VDR hinge has yet to be determined, but might indicate that the hinge region makes significant interactions with transcriptional co-regulators.

ACKNOWLEDGMENT

We thank Ching-yi Chang and Rachid Safi for technical assistance on tissue culture assays.

REFERENCES

1. Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., and O'Malley, B. W. (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor, *Proc. Natl. Acad. Sci. U.S.A. 85*, 3294−3298.
2. Abe, E., Miyaura, C., Sakagami, H., Takeda, M., Konno, K., Yamazaki, T., Yoshiki, S., and Suda, T. (1981) Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3, *Proc. Natl. Acad. Sci. U.S.A. 78*, 4990−4994.
3. Bouillon, R., Okamura, W. H., and Norman, A. W. (1995) Structure-function relationships in the vitamin D endocrine system, *Endocr. Rev. 16*, 200−257.
4. Feldman, D., Glorieux, F. H., and Pike, J. W. (1997) *Vitamin D*, Academic Press, San Diego, CA.
5. DeLuca, H. F., and Zierold, C. (1998) Mechanisms and functions of vitamin D, *Nutr. Rev. 56*, S4−10; discussion S54−75.
6. Mangelsdorf, D. J., and Evans, R. M. (1995) The RXR heterodimers and orphan receptors, *Cell 83*, 841−850.
7. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995) The nuclear receptor superfamily: the second decade, *Cell 83*, 835−839.
8. Sone, T., Ozono, K., and Pike, J. W. (1991) A 55-kilodalton accessory factor facilitates vitamin D receptor DNA binding, *Mol. Endocrinol. 5*, 1578−1586.
9. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements, *Cell 67*, 1251−1266.
10. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors, *Cell 65*, 1255−1266.
11. Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA, *Nature 352*, 497−505.
12. Khorasanizadeh, S., and Rastinejad, F. (2001) Nuclear-receptor interactions on DNA-response elements, *Trends Biochem. Sci. 26*, 384−390.
13. Shaffer, P. L., and Gewirth, D. T. (2002) Structural basis of VDR-DNA interactions on direct repeat response elements, *EMBO J. 21*, 2242−2252.
14. Rastinejad, F., Perlmann, T., Evans, R. M., and Sigler, P. B. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats, *Nature 375*, 203−211.
15. Rastinejad, F., Wagner, T., Zhao, Q., and Khorasanizadeh, S. (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1, *EMBO J. 19*, 1045−1054.
16. Zhao, Q., Chasse, S. A., Devarakonda, S., Sierk, M. L., Ahvazi, B., and Rastinejad, F. (2000) Structural basis of RXR-DNA interactions, *J. Mol. Biol. 296*, 509−520.
17. Zhao, Q., Khorasanizadeh, S., Miyoshi, Y., Lazar, M. A., and Rastinejad, F. (1998) Structural elements of an orphan nuclear receptor-DNA complex, *Mol. Cell 1*, 849−861.
18. Shaffer, P. L., and Gewirth, D. T. (2004) Vitamin D receptor-DNA interactions, *Vitam. Horm. 68*, 257−273.
19. Shaffer, P. L., and Gewirth, D. T. (2004) Structural analysis of RXR-VDR interactions on DR3 DNA, *J. Steroid Biochem. Mol. Biol. 89*−*90*, 215−219.
20. Quack, M., Szafranski, K., Rouvinen, J., and Carlberg, C. (1998) The role of the T-box for the function of the vitamin D receptor on different types of response elements, *Nucleic Acids Res. 26*, 5372−5378.
21. Towers, T. L., Luisi, B. F., Asianov, A., and Freedman, L. P. (1993) DNA target selectivity by the vitamin D3 receptor: mechanism of dimer binding to an asymmetric repeat element, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6310−6314.
22. Hsieh, J. C., Whitfield, G. K., Oza, A. K., Dang, H. T., Price, J. N., Galligan, M. A., Jurutka, P. W., Thompson, P. D., Haussler, C. A., and Haussler, M. R. (1999) Characterization of unique DNA-binding and transcriptional-activation functions in the carboxyl-terminal extension of the zinc finger region in the human vitamin D receptor, *Biochemistry 38*, 16347−16358.
23. Miyamoto, T., Kakizawa, T., Ichikawa, K., Nishio, S., Takeda, T., Suzuki, S., Kaneko, A., Kumagai, M., Mori, J., Yamashita, K., Sakuma, T., and Hashizume, K. (2001) The role of hinge domain in heterodimerization and specific DNA recognition by nuclear receptors, *Mol. Cell. Endocrinol. 181*, 229−238.
24. Norris, J., Fan, D., Aleman, C., Marks, J. R., Futreal, P. A., Wiseman, R. W., Iglehart, J. D., Deininger, P. L., and McDonnell, D. P. (1995) Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers, *J. Biol. Chem. 270*, 22777−22782.
25. Ozers, M. S., Hill, J. J., Ervin, K., Wood, J. R., Nardulli, A. M., Royer, C. A., and Gorski, J. (1997) Equilibrium binding of estrogen receptor with DNA using fluorescence anisotropy, *J. Biol. Chem. 272*, 30405−30411.
26. Rochel, N., Wurtz, J. M., Mitschler, A., Klaholz, B., and Moras, D. (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand, *Mol. Cell 5*, 173−179.
27. Tocchini-Valentini, G., Rochel, N., Wurtz, J. M., Mitschler, A., and Moras, D. (2001) Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands, *Proc. Natl. Acad. Sci. U.S.A. 98*, 5491−5496.
28. Vanhooke, J. L., Benning, M. M., Bauer, C. B., Pike, J. W., and DeLuca, H. F. (2004) Molecular structure of the rat vitamin D receptor ligand binding domain complexed with 2-carbon-substituted vitamin D3 hormone analogues and a LXXLL-containing coactivator peptide, *Biochemistry 43*, 4101−4110.
29. Tocchini-Valentini, G., Rochel, N., Wurtz, J. M., and Moras, D. (2004) Crystal structures of the vitamin D nuclear receptor liganded with the vitamin D side chain analogues calcipotriol and seocalcitol, receptor agonists of clinical importance. Insights into a structural basis for the switching of calcipotriol to a receptor antagonist by further side chain modification, *J. Med. Chem. 47*, 1956−1961.
30. Lemon, B. D., and Freedman, L. P. (1996) Selective effects of ligands on vitamin D3 receptor- and retinoid X receptor-mediated gene activation in vivo, *Mol. Cell. Biol. 16*, 1006−1016.
31. Egea, P. F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. (2000) Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid, *EMBO J. 19*, 2592−2601.
32. Rochel, N., Tocchini-Valentini, G., Egea, P. F., Juntunen, K., Garnier, J. M., Vihko, P., and Moras, D. (2001) Functional and structural characterization of the insertion region in the ligand binding domain of the vitamin D nuclear receptor, *Eur. J. Biochem. 268*, 971−979.
33. Cheskis, B., and Freedman, L. P. (1994) Ligand modulates the conversion of DNA-bound vitamin D3 receptor (VDR) homodimers into VDR-retinoid X receptor heterodimers, *Mol. Cell. Biol. 14*, 3329−3338.
34. Carson, M. (1991) Ribbons 2.0, *J. Appl. Cryst. 24*, 958−961.

BI0477182