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Mutations in the 1,25-Dihydroxyvitamin D_3 Receptor Identifying C-terminal Amino Acids Required for Transcriptional Activation That Are Functionally Dissociated from Hormone Binding, Heterodimeric DNA Binding, and Interaction with Basal Transcription Factor IIB, *in Vitro**

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Peter W. Jurutka‡, Jui-Cheng Hsieh‡, Lenore S. Remus‡, G. Kerr Whitfield‡, Paul D. Thompson‡, Carol A. Haussler‡, Jorge C. G. Blanco§, Keiko Ozato§, and Mark R. Haussler‡¶

From the ‡Department of Biochemistry, College of Medicine, The University of Arizona, Tucson, Arizona 85724 and the §Laboratory of Molecular Growth Regulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892

To investigate a potential ligand-dependent transcriptional activation domain (AF-2) in the C-terminal region of the human vitamin D receptor (hVDR), two conserved residues, Leu-417 and Glu-420, were replaced with alanines by site-directed mutagenesis (L417A and E420A). Transcriptional activation in response to 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) was virtually eliminated when either point mutant was transfected into several mammalian cell lines. Furthermore, both mutants exhibited a dominant negative phenotype when expressed in COS-7 cells. Scatchard analysis at 4 °C and a ligand-dependent DNA binding assay at 25 °C revealed essentially normal 1,25-$(OH)_2D_3$ binding for the mutant hVDRs, which were also equivalent to native receptor in associating with the rat osteocalcin vitamin D responsive element as a presumed heterodimer with retinoid X receptor. Glutathione *S*-transferase-human transcription factor IIB (TFIIB) fusion protein linked to Sepharose equally coprecipitated the wild-type hVDR and the AF-2 mutants. These data implicate amino acids Leu-417 and Glu-420, residing in a putative α-helical region at the extreme C terminus of hVDR, as critical in the mechanism of 1,25-$(OH)_2D_3$-stimulated transcription, likely mediating an interaction with a coactivator(s) or a component of the basal transcriptional machinery distinct from TFIIB.

The actions of the 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)[1] hormonal ligand are mediated by the vitamin D receptor (VDR). This nuclear protein belongs to a large superfamily, which includes receptors for steroids, retinoids, and thyroid hormone (1). As with other members of this superfamily, the VDR possesses an N-terminal DNA binding domain (DBD) that contains two zinc finger DNA binding motifs (2). The C-terminal portion of the VDR contains a ligand binding domain (LBD) that associates with the 1,25-$(OH)_2D_3$ hormone (3). This binding initiates a presumed conformational change in VDR that enhances its interaction with any one of several isoforms of retinoid X receptor (RXR) to form a heterodimer, the apparent active species in recognizing and binding with high affinity to vitamin D-responsive elements (VDREs) located in the upstream promoter region of genes regulated by 1,25-$(OH)_2D_3$ (4, 5). In addition to binding 1,25-$(OH)_2D_3$, the C-terminal LBD of VDR contains residues necessary for heterodimerization with RXRs (6, 7). When the activated, ligand-bound VDR:RXR heterodimer is associated with the VDRE, it stimulates the transcription of downstream target genes (8). Several VDREs have been characterized to date, with the prototypical sequence consisting of an imperfect direct repeat of six nucleotide bases, GGGTGA, separated by a 3-base pair spacer (9–13). VDR has been shown to contact the base pairs in the 3′ half-site of the VDRE, while RXR interacts with the 5′ half-site (14).

The precise mechanism of transcriptional regulation by the activated VDR:RXR heterodimer is not well understood. Functional analyses of members of the nuclear receptor superfamily, including truncation and point mutagenesis studies, have demonstrated the presence of at least two major domains involved in receptor-mediated transcriptional stimulation. The N-terminal regions of several nuclear receptors contain a constitutive activation domain referred to as AF-1 (also designated τ1) (15–17) that can be linked to heterologous DNA binding domains to create functional transcription factors (18–20). Moreover, fusion of the large C-terminal domain of various receptors to the GAL4 or glucocorticoid receptor (GR) DBD produces a chimeric protein capable of activating transcription in response to the cognate ligand (18, 21, 22). A subdomain of this hormone-dependent, C-terminal activation function is known as AF-2 (also termed τ4) (21, 23–25). Unlike the N-terminal AF-1 transcription domain, the C-terminal AF-2 activation unit exhibits a higher degree of homology among the nuclear receptor superfamily, suggesting a common or related mechanism may be involved in ligand-mediated gene regulation. In one study (26), the AF-2 region of the thyroid hormone receptor (TR) was reported to interact with the basal transcription factor IIB (TFIIB), implying that association of nuclear receptors with the basal transcriptional machinery may serve as one means of

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¶ To whom correspondence should be addressed: Dept. of Biochemistry, College of Medicine, The University of Arizona, Tucson, AZ 85724. Tel.: 520-626-6033; Fax: 520-626-9015; E-mail: mrh@ccit.arizona.edu.
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[1] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; (h)VDR, (human) 1,25-dihydroxyvitamin D_3 receptor; DBD, DNA binding domain; LBD, ligand binding domain; RXR, retinoid X receptor; VDRE, vitamin D-responsive element; GR, glucocorticoid receptor; TR, thyroid hormone receptor; TFIIB, basal transcription factor IIB; HEK293, transformed primary human embryonal kidney cell line; GST, glutathione *S*-transferase; ER, estrogen receptor; RAR, retinoic acid receptor; RAF, receptor auxiliary factor; WT, wild-type.

achieving transcriptional control of hormone-stimulated genes.

As part of a preliminary study designed to probe the regions of VDR involved in heterodimerization, 1,25-$(OH)_2D_3$ binding, and transcriptional activation, our laboratory reported previously that truncation of the C-terminal 25 amino acids in hVDR generated a transcriptionally inactive receptor that retained heterodimerization capacity and also partial ligand binding ability (6), suggesting the presence of an AF-2 domain in the extreme C-terminal region of hVDR. In the present study, we have refined the mapping of this domain by identifying specific residues essential for competent, hormone-dependent transcriptional activation that are resolved from other biological activities of the receptor.

EXPERIMENTAL PROCEDURES

Construction of Mutant hVDR Plasmids and Expression in Transfected Cells—The hVDR expression vector, pSG5hVDR (27), was employed in synthesizing point mutants by *in vitro* site-directed mutagenesis (28). Two residues in the extreme C terminus of hVDR, Leu-417 and Glu-420, were separately altered to alanines (mutants designated as L417A and E420A, respectively). These mutations were confirmed by dideoxy sequencing.

Transfection of Cultured Cells and Transcriptional Activation Assay—COS-7 monkey kidney epithelial cells (700,000 cells/60-mm plate) were transfected with 0.1 μg of wild-type or mutant hVDR expression plasmid and 10 μg of a reporter plasmid ($(CT4)_4$-TKGH) containing four copies of the rat osteocalcin VDRE (13) inserted upstream of the viral thymidine kinase promoter-growth hormone reporter gene (Nichols Institute, San Juan Capistrano, CA) by the calcium phosphate-DNA coprecipitation method as described previously (29). The pTZ18U plasmid was used as carrier DNA, and each transfection contained a constant amount of total DNA (20 μg). The transfected cells were washed, then grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and various concentrations of 1,25-$(OH)_2D_3$ in ethanol vehicle. After 24 h of incubation at 37 °C, the level of growth hormone secreted into the culture medium was assessed by radioimmunoassay using a commercial kit (Nichols Institute). Transfections and treatments of HeLa cells and a transformed primary human embryonal kidney cell line (HEK293) were carried out similarly except these cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. Cellular lysates prepared from transfected cells were analyzed for VDR expression by immunoblotting using the 9A7γ monoclonal anti-VDR antibody as described previously (30).

Gel Mobility Shift Assay—Heterodimeric DNA binding activity was assessed by the gel mobility shift assay essentially as described elsewhere (7). In ligand-independent studies, cellular extracts from COS-7 cells transfected with either pSG5 (control) alone, pSG5 containing wild-type cDNA, or pSG5 containing mutant hVDR cDNAs were incubated with ^{32}P-labeled rat osteocalcin VDRE (5′-AGCTGCACTGGGTGAATGAGGACATTACA-3′; half-sites comprising an imperfect direct repeat are underlined). In experiments employing the 1,25-$(OH)_2D_3$ ligand (31), COS-7 cells were transfected with 50 ng of hVDR expression plasmids, and the extracts from these cells were initially incubated with 10^{-8} M 1,25-$(OH)_2D_3$ for 25 min at 22 °C followed by incubation with the rat osteocalcin VDRE for 25 min at 22 °C.

1,25-$(OH)_2D_3$ Ligand Binding Assay—COS-7 cells transfected with 50 ng of wild-type or mutant hVDR expression plasmids were lysed in KETZD-0.3 buffer (0.3 M KCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3 mM $ZnCl_2$, 5 mM dithiothreitol) containing 0.5% Triton X-100 and supplemented with protease inhibitors (2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 50 μg/ml trypsin inhibitor). Lysates were tested for 1,25-$(OH)_2D_3$ binding activity as described previously (31).

GST Fusion Protein Binding Assay—Human transcription factor IIB (hTFIIB)-GST fusion protein was expressed from pGEX-2T-hTFIIB (26), and GST alone was expressed from pGEX-4T, both in *Escherichia coli* strain DH5α. The overexpressed proteins were coupled to glutathione-Sepharose (1 μg of protein/μl of resin) according to the protocol of the manufacturer (Pharmacia Biotech Inc., Uppsala, Sweden) and stored as a 50% slurry in KETZD-0.3 containing 30% glycerol at −20 °C. COS-7 cells transfected with either 20 μg of control, wild-type, or mutant hVDR expression plasmids were resuspended in 550 μl of KETZD-0.2 (0.2 M KCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3 mM $ZnCl_2$, 5 mM dithiothreitol, 1.0 mg/ml bovine serum albumin, 0.2% Tween 20, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 50 μg/ml trypsin inhibitor) and sonicated on ice. The sonicates were clarified by centrifugation for 15 min at 16,000 × *g* at 4 °C, and 500 μl were incubated with either 20 μl (50% slurry) of hTFIIB-GST-Sepharose or GST-Sepharose alone for 1 h at 4 °C on a rocker tray. The Sepharose beads were then washed four times with 1 ml each of KETZD-0.2, resuspended in 40 μl of 2 × final sample buffer (2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, 20% glycerol) and boiled for 3 min. These samples, along with 25 μl of original lysate (5% of input) were analyzed for VDR content by immunoblotting (30).

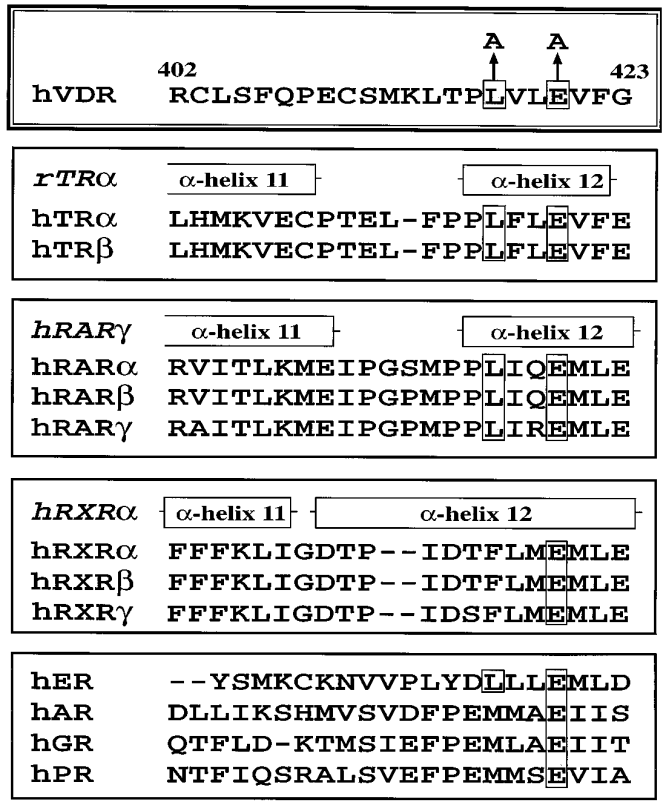
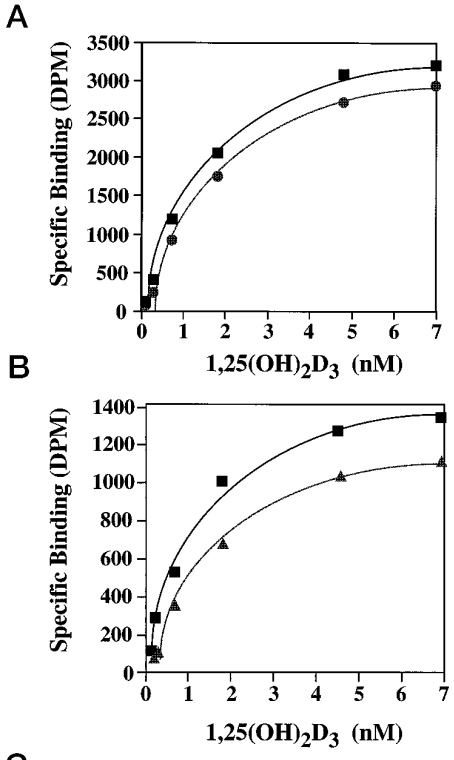


FIG. 1. **Amino acid sequence comparison of the extreme C-terminal region between VDR and other members of the nuclear receptor superfamily.** The deduced amino acid sequence of hVDR between residues 402 and 423 is shown (*top*) along with the homologous region in other nuclear receptors. Full-length hVDR contains 427 amino acids, with residues 424–427 being Asn-Glu-Ile-Ser. *Boxed* and *shaded* are the highly conserved leucyl and glutamyl residues (at positions 417 and 420 in hVDR, respectively), which were individually mutagenized to alanine. Corresponding α-helical regions in the recently elucidated crystal structures of rTRα (33), hRARγ (34), and hRXRα (35) are indicated with *open bars*. *AR*, androgen receptor; *PR*, progesterone receptor.

RESULTS

Conservation of Residues Near the C Terminus of Nuclear Receptors and Selection of Amino Acids for Site-directed Mutagenesis—The C-terminal LBD of the nuclear receptor superfamily possesses several regions of high homology (32). One such subdomain, located at the extreme C terminus of the LBD, is depicted in Fig. 1. Examination of this region of hVDR reveals Leu-417, which is conserved in the VDR subfamily of nuclear receptors (VDR, TR, and retinoic acid receptor (RAR)), as well as in the estrogen receptor (ER), and Glu-420, which is positionally conserved throughout the superfamily. These two amino acids probably reside in an α-helical segment of the VDR based on the recently elucidated three-dimensional crystal structures of the homologous regions in rat TRα (33), human RARγ (34), and human RXRα (35) LBD. Thus, these residues were individually altered to alanine in hVDR to preserve the potential α-helical character in this region and to probe their potential functional significance.



C

Apparent K_d (x 10^9 M)

Receptor	Exp. 1	Exp. 2
WT	0.9	1.7
L417A	1.6	2.3
E420A	2.0	5.0

FIG. 2. **Saturation ligand binding and Scatchard analysis of hVDR point mutants.** Cellular extracts from COS-7 cells transfected with WT (■) or mutant hVDRs were incubated with increasing concentrations of 1,25-$(OH)_2$[^{3}H]D_3 for 16 h at 4 °C. Two representative saturation binding curves, one for L417A (*A*, ●) and one for E420A (*B*, ▲), are shown. Saturation binding data from two independent experiments were transformed and plotted by the method of Scatchard to yield the indicated K_d values (*C*).

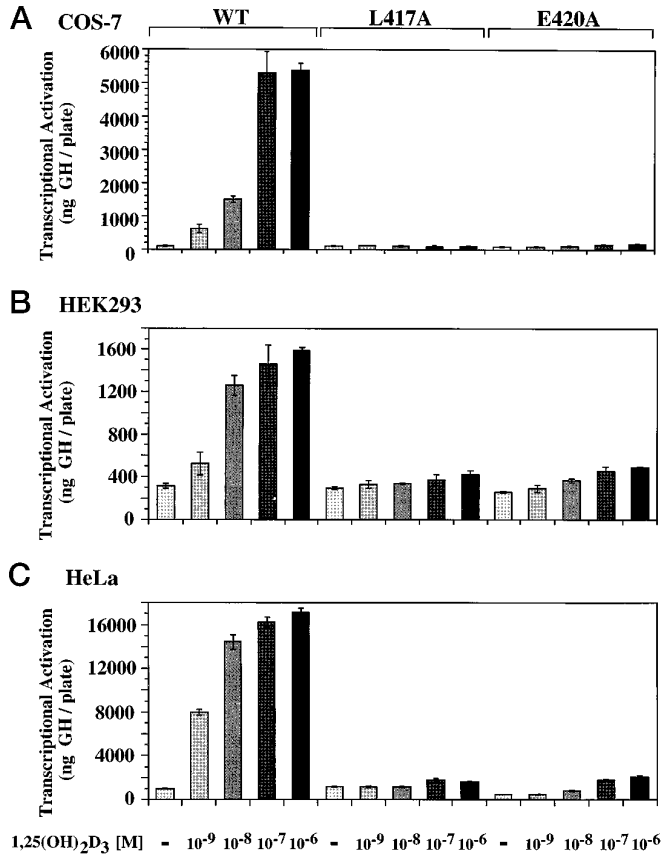


FIG. 3. **Functional assessment of hVDR C-terminal point mutants.** *A*, COS-7 cells were cotransfected by calcium phosphate-DNA coprecipitation with expression vectors for either the wild-type (*WT*) or mutant hVDRs and a reporter plasmid containing four osteocalcin VDREs linked to the human growth hormone gene. Cells were treated for 24 h post-transfection with the indicated amounts of 1,25-$(OH)_2D_3$ or ethanol vehicle. The level of growth hormone secreted into the culture medium, which serves as an index of transcriptional activity, was assessed by radioimmunoassay. The transcriptional activity of each receptor species was similarly monitored in a transformed primary human embryonal kidney cell line, HEK293 (*B*), and in HeLa cells (*C*).

Binding of 1,25-$(OH)_2D_3$ by VDR Mutants—Considering that Leu-417 and Glu-420 are located in the LBD, and the residues corresponding to Val-418 and Phe-422 in VDR (Fig. 1) are ligand contact sites in either RAR (34) or TR (33), we tested the 1,25-$(OH)_2D_3$ ligand binding activity of the above generated mutant hVDRs in an equilibrium binding assay performed at 4 °C. Extracts from COS-7 cells transfected with expression plasmids encoding either wild-type or a mutant hVDR were incubated with increasing concentrations of 1,25-$(OH)_2D_3$ overnight at 4 °C. The data from saturation binding curves (Fig. 2, *A* and *B*) were then transformed and plotted by the method of Scatchard, yielding an estimate of the dissociation constant (K_d) for the wild-type and mutant receptors. Results from two independent experiments (Fig. 2*C*) revealed that the apparent K_d for the 1,25-$(OH)_2D_3$ ligand is not significantly altered by replacement of Leu-417 with alanine, at least under the conditions of this standard binding assay. Similarly, only a negligible increase in the apparent K_d occurs when Glu-420 is altered to alanine (Figs. 2, *B* and *C*). These data extend previous results (6) that revealed truncation of the last 25 amino acids in hVDR does not abolish ligand binding but does cause an approximate 10-fold elevation in the K_d. The extreme C terminus of hVDR, therefore, is not absolutely required for relatively high affinity interaction with 1,25-$(OH)_2D_3$, suggesting that this region of the receptor, and more specifically the conserved residues Leu-417 and Glu-420, performs a different function.

Transcriptional Activation Capacity of Mutant hVDRs in Transfected Cells—The transcriptional activity of wild-type and point mutated hVDRs was evaluated in three transfected cell lines. Fig. 3*A* illustrates a 1,25-$(OH)_2D_3$-stimulated, dose-dependent increase in transcription of a rat osteocalcin VDRE-linked reporter gene in transfected COS-7 cells. The maximal hormone-mediated increase in transcription with the wild-type receptor in this cell line is approximately 40-fold (at or above 10^{-7} M 1,25-$(OH)_2D_3$). Under parallel transfection conditions, the L417A and E420A hVDRs do not exhibit detectable transactivation even at the highest dose (10^{-6} M) of 1,25-$(OH)_2D_3$ tested (Fig. 3*A*). The fact that this pharmacological level of 1,25-$(OH)_2D_3$ is ineffective in enhancing transcription (Fig. 3*A*) is consistent with the conclusion that the mutant VDRs are not partially impaired in ligand binding (Fig. 2). Expression of these mutant hVDRs was similar to that of the wild-type receptor (data not shown; see also Figs. 4*C*, 5*B*, and 6*B*), eliminating differential expression as a trivial explanation for these results. To evaluate the possibility that the abrogation of transcriptional activity in these mutant hVDRs is a cell-specific phenomenon, a similar set of experiments was carried out in a transformed primary human embryonal kidney cell line (HEK293; Fig. 3*B*) and in HeLa cells (Fig. 3*C*). The maximal fold increase in transcriptional activity by wild-type hVDR was

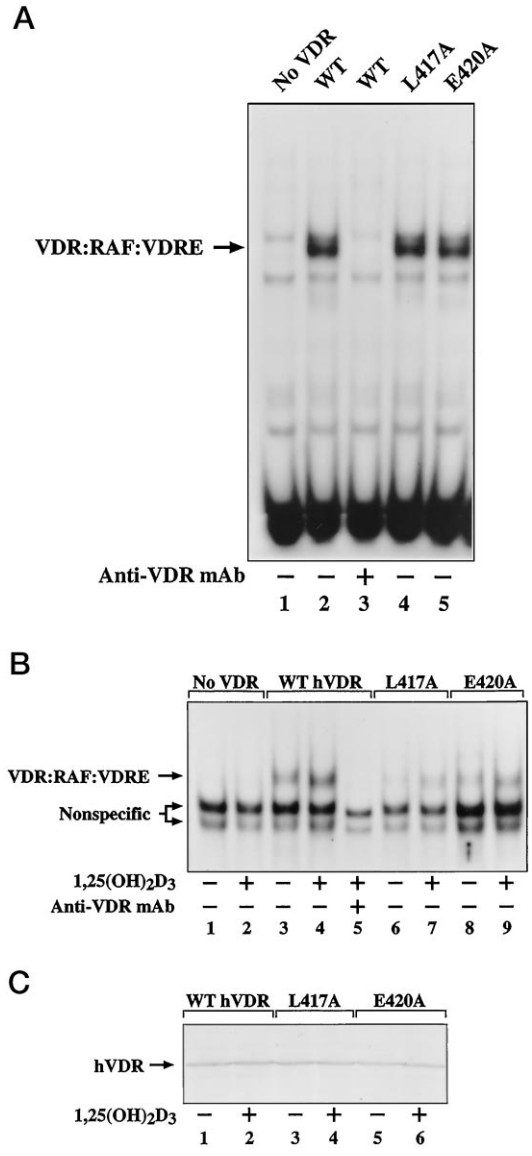


FIG. 4. **DNA binding activity of wild-type and AF-2 point mutant hVDRs.** *A*, a gel mobility shift assay was performed using extracts from COS-7 cells transfected with control or hVDR expression plasmids (1.0 μg) along with a labeled oligonucleotide containing the VDRE from the rat osteocalcin gene as described under "Experimental Procedures." No 1,25-$(OH)_2D_3$ ligand was included in these incubations. *B*, the gel mobility shift assay was performed as in *A* except that the COS-7 cells were transfected with 0.05 μg of the indicated expression plasmid. In addition, the extracts were incubated with the osteocalcin VDRE either in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle. The anti-VDR monoclonal antibody (9A7γ) is directed against an epitope near the DNA binding domain and disrupts the interaction of VDR with the VDRE (60). *C*, Western blot analysis of the extracts employed in *B*.

5-fold (Fig. 3*B*) and 17-fold (Fig. 3*C*) in HEK293 and HeLa cells, respectively. The dose-response profile of wild-type hVDR in these additional cells lines was somewhat similar to that observed in COS-7 cells, although 10^{-8} M 1,25-$(OH)_2D_3$ elicited a near-maximal transcription effect in HEK293 and HeLa cells, and 10^{-9} M ligand approximated the ED_{50} in HeLa cells. Importantly, neither the L417A nor the E420A mutant hVDR mediates significant ligand-dependent transactivation in these other two cell lines upon exposure to 10^{-9} M or even 10^{-8} M 1,25-$(OH)_2D_3$.

Thus, taken together, the data in Fig. 3 identify residues 417 and 420 in the transactivation function of VDR. A minor but significant repression of basal transcription is detected in the case of E420A hVDR, especially in HeLa cells (Fig. 3*C*), which is relieved by addition of increasing concentrations of 1,25-$(OH)_2D_3$. This observation is consistent with the findings that unoccupied nuclear receptors associate with a transcriptional corepressor that is released by binding of the cognate ligand (36) and that corepressor interaction may be enhanced by mutation of the receptor AF-2 domain (37). These results support a model (38) of mutually exclusive binding of a corepressor and coactivator to overlapping regions in the C termini of nuclear receptors, a domain that includes Leu-417 and Glu-420.

Transcriptionally Defective VDRs Possess Normal Heterodimeric DNA Binding Activity—We next evaluated the ability of the transcriptionally defective mutant hVDRs to interact with a prototypical rat osteocalcin VDRE as a heterodimeric complex with endogenous RXR in transfected COS-7 cells. Extracts from cells transfected with the wild-type hVDR expression plasmid form an intense shifted complex when incubated with the VDRE probe in a gel mobility shift assay (Fig. 4*A, lane 2*). This retarded band is referred to as VDR:RAF:VDRE because the exact identity of the receptor auxiliary factor (RAF) as one of the RXRs is not defined for COS-7 cells. This complex is absent when extracts from cells transfected with the expression vector lacking the VDR insert are utilized (*lane 1*) or when wild-type VDR-containing extracts are preincubated with anti-VDR monoclonal antibody 9A7γ (*lane 3*). When extracts from cells transfected with either the L417A or E420A mutant are incorporated into this assay (*lanes 4* and *5*), a shifted complex of equal intensity and migration position compared with the wild-type receptor is observed. Thus, there is no evidence to this point that either mutation in hVDR affects its heterodimerization activity, with the caveats that the VDR-containing extracts used in the experiments pictured in Fig. 4*A* were obtained from COS-7 cells expressing large amounts of each receptor protein and, additionally, these cells were not treated with the 1,25-$(OH)_2D_3$ hormone. Thus, a similar analysis was performed with extracts from COS-7 cells expressing limiting amounts of VDR, which more closely approximate levels found in 1,25-$(OH)_2D_3$ target tissues, such as bone, intestine, or kidney (39). Extracts from these cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle and used in a hormone-dependent gel shift assay (31). Under these near physiologic conditions, wild-type and both hVDR mutants formed a VDR-containing shifted complex that was enhanced by the addition of the 1,25-$(OH)_2D_3$ ligand (Fig. 4*B*, compare *lanes 4* with *3*, *7* with *6*, and *9* with *8*). Significantly, the level of augmentation by 1,25-$(OH)_2D_3$ (2-fold) based on densitometric scanning of the images shown in Fig. 4*B* (data not shown) was similar for the wild-type receptor and mutant hVDRs. Also, the level of expression of each receptor was essentially equivalent and unmodified by sterol ligand as monitored by immunoblotting (Fig. 4*C*). The apparent decrease in VDRE binding exhibited by the L417A mutant (Fig. 4*B, lanes 6* and *7*) is actually the result of less total protein loaded into these lanes as demonstrated by the decreased intensity of the nonspecific bands migrating below the hVDR-containing complex. The nonspecific binding of the two lower complexes was deduced by their appearance in non-VDR-transfected cells (Fig. 4*B, lanes 1* and *2*) as well as their lack of elimination by the VDR antibody (Fig. 4*B, lane 5*). The level of L417A-containing complex formation approaches that of the wild-type band when corrected for this loading difference (as assessed by densitometric scanning), and repeat experiments (not shown) indicate that similar levels of hVDR complexes are formed on the VDRE by all three hVDRs tested (see also Fig. 4*A*). Taken together, the results illustrated in Fig. 4 strongly suggest that the alteration of Leu-417 or Glu-420 to alanine does not attenuate heterodimeric DNA binding by the VDR protein, and such a phe-

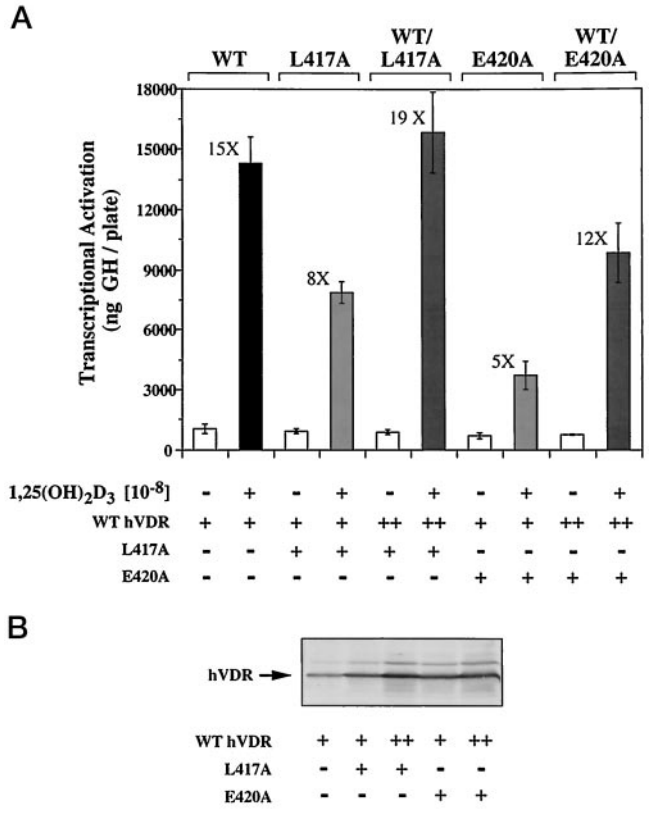


FIG. 5. **Analysis of the dominant negative effect of L417A and E420A.** *A*, COS-7 cells were cotransfected with 10 μg of the $(CT4)_4$-TKGH reporter vector and 0.1 μg (+) WT hVDR expression plasmid in combination with 0.5 μg (+) of a vector encoding the L417A or E420A mutant hVDRs as described under "Experimental Procedures." In rescue experiments, the level of WT hVDR expression plasmid transfected was increased to 0.35 μg (++). All cells were transfected with a constant total amount of pSG5 parent expression vector. Cells were treated for 24 h post-transfection with 10^{-8} M $1,25\text{-}(OH)_2D_3$ or ethanol vehicle. The level of growth hormone secreted into the culture medium serves as an index of transcriptional activity and was assessed by radioimmunoassay. The suppression in transactivation elicited by either mutant receptor can be reversed by expression of additional wild-type hVDR (++). *B*, Western blot of cellular extracts prepared from cells transfected in *A*. Note that the migration position of L417A or E420A is the same as WT hVDR and that the signal increases with transfection of additional amounts of hVDR expression plasmid.

nomenon cannot therefore account for the paucity of transactivation by these mutants. Additionally, the essentially equivalent enhancement in VDRE binding elicited by the $1,25\text{-}(OH)_2D_3$ hormone is consistent with the similar K_d values exhibited by the wild-type and mutant receptors (Fig. 2*C*).

L417A and E420A hVDRs Act as Effective Dominant Negative Mutants—Because the above described DNA binding studies were carried out at nonphysiologic temperatures, *in vitro*, with cell extracts, it is not possible to conclude rigorously that the transcriptionally inactive hVDR mutants actually bind to the VDRE in intact cells (40, 41). Therefore, we determined whether the two mutant hVDRs under study could act in a dominant negative fashion in transactivation assays. Cotransfection experiments in COS-7 cells were designed utilizing the rat osteocalcin VDRE reporter vector and 0.1 μg of wild-type hVDR expression plasmid (similar to the experiment shown in Fig. 3). Under these conditions, the $1,25\text{-}(OH)_2D_3$ hormone results in about a 15-fold increase in transcription of the GH reporter gene (Fig. 5*A, WT*). Additional cotransfection of a 5-fold excess (0.5 μg) of L417A or E420A expression plasmid reduced ligand-dependent transcription by approximately 50% (L417A) and 66% (E420A), respectively. These results are analogous to those obtained previously in which a mutant hVDR possessing an altered T-box region was shown to be an effective dominant negative at ratios of mutant to wild-type hVDR expression plasmid of five and greater (42). In rescue experiments (Fig. 5*A*), the level of transfected mutant expression vector was maintained at 0.5 μg, while the amount of wild-type hVDR expression plasmid was increased to 0.35 μg (designated ++ in Fig. 5*A*). The corresponding increase in wild-type hVDR expression restores $1,25\text{-}(OH)_2D_3$-dependent transcriptional activation to fold effects approaching (*WT/E420A, 12X*), or even slightly exceeding (*WT/L417A, 19X*), the fold effects observed with transfection of only the wild-type control (*WT, 15X*). As expected, immunoblotting of extracts from these transfected cells revealed a close correlation between the total amount of VDR receptor plasmid transfected (denoted schematically by the total number of + symbols in each lane, Fig. 5*B*) and the level of receptor expression. Because both L417A and E420A mutant hVDRs retain wild-type levels of hormone and heterodimeric DNA binding, *in vitro* (Figs. 2 and 4), and act in a dominant negative fashion in transfected cells (Fig. 5), we conclude that these AF-2 mutants exhibit $1,25\text{-}(OH)_2D_3$-dependent binding to the VDRE, *in vivo*, but as transcriptionally *inactive* heterodimers. As demonstrated in Fig. 5*A*, the expression of additional wild-type VDR effectively competes with the mutant receptor for RXR association, and subsequent binding of a normal heterodimer to the response element leads to enhanced activation. In contrast, overexpression of RXRβ does not reverse the dominant negative effect (data not shown), presumably because the mutated VDR in the heterocomplex contains an inactive AF-2 domain.

Wild-type and Mutant hVDRs Interact with Human TFIIB, in Vitro—Recently, it has been reported that the VDR interacts physically and functionally with the general transcription factor IIB in a $1,25\text{-}(OH)_2D_3$ ligand-independent fashion (43, 44). In one of these reports, it was suggested that the extreme C-terminal portion of VDR may represent a contact domain for TFIIB (44), an observation also reported for TFIIB interaction with TR (26). Consequently, we probed the potential interaction of L417A and E420A with TFIIB, *in vitro*, to determine if a lack of TFIIB association could explain the transcriptionally defective and dominant negative phenotypes of these mutant receptors. Extracts from COS-7 cells transfected with expression plasmids for each receptor protein were incubated in the absence of the $1,25\text{-}(OH)_2D_3$ ligand (Fig. 6*A*) with hTFIIB-GST fusion protein linked to glutathione-Sepharose beads (*GST-TFIIB-S*) or GST-Sepharose (*GST-S*) as a control (Fig. 6*A*). After extensive washing, the presence of VDR protein coprecipitated by the GST-TFIIB-S or GST-S complex was detected by immunoblotting using an anti-VDR monoclonal antibody. The results illustrate that wild-type VDR (Fig. 6*A, lane 4*), as well as both mutant hVDRs (Fig. 6*A, lanes 6* and *8*), interact efficiently and specifically with TFIIB; similar results were obtained in the presence of 10^{-8} M $1,25\text{-}(OH)_2D_3$ (data not shown). The levels of VDR protein were essentially equivalent as verified by Western blot analysis (Fig. 6*B*) using 5% of the total extracts from the experiment illustrated in Fig. 6*A*. These data argue against a role for Leu-417 and/or Glu-420 in contacting TFIIB.

DISCUSSION

Previously, we reported that the C-terminal 25 amino acids of hVDR appeared to be vital for hormone-dependent transactivation (6). It is now demonstrated that two well conserved amino acids within this region of hVDR (Fig. 1), Leu-417 and Glu-420, are essential for $1,25\text{-}(OH)_2D_3$ ligand-stimulated transcription (Fig. 3) and likely define the AF-2 function in the VDR. Because mutation of these residues does not affect hor-

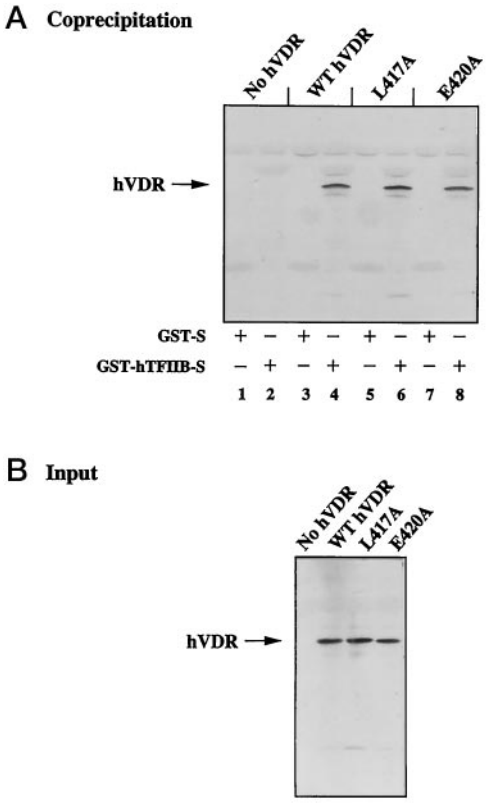


FIG. 6. **Interaction of hVDR AF-2 mutants with human transcription factor IIB (*hTFIIB*).** *A*, extracts from COS-7 cells transfected with control or hVDR expression plasmids (5.0 μg) were incubated with either a glutathione *S*-transferase (*GST*)-hTFIIB fusion protein bound to Sepharose beads (*GST-hTFIIB-S*) or GST alone bound to Sepharose (*GST-S*) for 1 h at 4 °C. The beads were then washed extensively, and the amount of precipitated WT or mutant hVDR protein was detected by electrophoresis of denatured samples extracted from the beads, followed by immunoblotting. The amount of extract analyzed by Western blotting as input (*B*) was 5% of the amount used in the coprecipitation reactions (*A*).

mone (Fig. 2) or DNA binding (Fig. 4) to any significant degree, these amino acids are thought to participate directly in the mechanism of transcriptional activation following the recruitment of ligand-bound VDR:RXR heterodimers to the VDRE. Such a mechanism might involve the interaction of VDR with basal or TATA-binding protein-associated factors, thus facilitating the formation of an active transcriptional complex. VDR also physically interacts with TFIIB (43), and inferences from studies utilizing TR and VDR (26, 44) suggested that the C terminus of these receptors might be involved in TFIIB interaction. However, the fact that the wild-type receptor and both mutant VDRs interact similarly with TFIIB, *in vitro* (Fig. 6), argues against any interaction between these residues and TFIIB. Rather, the present results suggest that Leu-417 and Glu-420 represent contact sites for the interaction of VDR with another coactivator protein(s) required for ligand-dependent transcriptional stimulation. Further studies of wild-type and AF-2 mutant VDRs employing methodologies such as the yeast two-hybrid system will be required to identify proteins which physically and functionally associate with this region of VDR.

Other members of the nuclear receptor superfamily also possess a transactivation domain in the C-terminal region. The chicken TRα has been shown to contain a ligand-dependent activation function in the last 35 amino acids of the receptor (45), and within this domain several acidic and hydrophobic residues were found to be critical for transcriptional activity. Interestingly, this region of the protein is absent in the transcriptionally defective v-*erb*A protein, the viral homolog of cTRα. Similar results have been reported for the human TRβ1, where mutations in the two residues corresponding to Leu-417 and Glu-420 generated receptors with wild-type hormone and DNA binding activity but abrogated transactivation (46, 47), a phenotype shared by the present hVDR mutants. In addition, point mutagenesis of the mouse RARα1 and truncation analysis of mouse RXRα have also revealed the importance of this domain in gene activation in these and other receptors (25, 48), an observation consistent with the relatively high level of conservation of this region among the nuclear receptor superfamily (Fig. 1). In fact, it appears that the homologous amino acids identified in the present study for VDR may be essential for transactivation even in the more distantly related ER and GR (23, 49), although in one study (23), mutation of the residue in mouse ER (E546A) corresponding to Glu-420 in hVDR only modestly affected transcriptional activation. In contrast, an E546A mutant that also lacked the ER AF-1 domain could not stimulate transcription. These results suggest that the previously described synergism between AF-1 and AF-2 (19) might depend on the presence of Glu-546 in ER. If the VDR, with its relatively small N terminus, lacks an AF-1 region, this could explain the almost complete abolishment of transcriptional activity when Glu-420 is mutated in hVDR, while the ER E546A mutant still possesses significant activity. The importance of the AF-2 region in the nuclear receptor superfamily is further supported by the observation that ER and RXR truncation mutants (41, 49) and a deletion mutant of RARα (48), all of which are missing amino acids homologous to Leu-417 and Glu-420 in hVDR, act as effective dominant negative receptors similar to the hVDR mutants described herein (Fig. 5). The concept of a dominant negative phenotype for AF-2 mutants (34), including those in the VDR, is in agreement with wild-type hormone and dimeric DNA binding properties exhibited by these altered receptors, since this would allow the mutants to form inactive dimers that compete with the wild-type protein for binding to the cognate responsive elements, thereby suppressing gene stimulation.

The recently described crystal structures for the hormone binding domains of rTRα (33), hRARγ (34), and hRXRα (35) indicate that all three receptors share a large degree of α-helical content, with many of the helices being generally conserved within the overall structure of the protein. In particular, the terminal α-helix in all three molecules (Fig. 1, *α-helix 12*) encompasses the conserved region that contains Leu-417 and Glu-420. It has been postulated that this α-helical segment projects away from the core of the LBD and that hormone binding induces a conformational change in the receptor causing the repositioning of helix 12 so that it essentially covers the opening of the ligand binding pocket. This hypothesis, termed the "mouse trap" model (34), further predicts that Glu-414 in hRARγ (homologous to Glu-420 in hVDR) forms a crucial salt bridge with Lys-264 (homologous to Lys-264 in hVDR) which is thought to "lock" helix 12 in a position over the ligand binding pocket following interaction of the receptor with its cognate hormone. Additionally, this conformational change appears to place helix 12 in a position to effectively interact with a coactivator protein. Mutation of either partner in this putative salt bridge in hRARγ (34) abolishes transactivation and results in a receptor with the dominant negative phenotype. In preliminary experiments with hVDR,[2] a theoretical hVDR salt bridge "reversal" double mutant (K264E-E420K), which would be predicted to preserve the putative electrostatic bond, was found to be inactive transcriptionally. Thus, Lys-264 in hVDR does not

[2] P. W. Jurutka, L. S. Remus, and M. R. Haussler, unpublished observations.

appear to form a salt bridge with Glu-420, but this does not exclude potential electrostatic bonds between Glu-420 and other positively charged VDR residues or the existence of a novel salt bridge capable of positioning the VDR AF-2 to seal the ligand binding pocket.

The proposed role of the AF-2 region in providing a protein-protein interaction surface for contacting a coactivator is supported not only by the three-dimensional crystal structures of hRARγ, rTRα, and hRXRα as discussed above, but also by the recent identification of candidate coactivator proteins for various nuclear receptors. The ER, for example, has been shown to interact with a 160-kDa protein in an estrogen- and AF-2-dependent manner (50). Another protein, SPT6, appears to physically interact with the C-terminal portion of ER and to enhance ER-dependent transactivation (51). Moreover, the nuclear protein RIP140 also associates with and enhances ER activity in the presence of estrogen but not the antagonist, 4-hydroxytamoxifen (52). In this latter study, the interaction of hormone-occupied ER with RIP140 was abolished by mutations in the ER AF-2 that abrogated transcriptional activation (in the absence of AF-1), including a point mutant at residue 546 (homologous to hVDR Glu-420). Other candidate coactivators include GRIP1 (53), which interacts with ER, GR, and the androgen receptor, and steroid receptor coactivator-1 (54), which stimulates the transcriptional activity of several nuclear receptors. In each case, the interaction between the putative coactivator and the receptor protein requires residues in the AF-2-containing LBD. Preliminary data[3] with hVDR AF-2 mutants indicate that both 1,25-$(OH)_2D_3$ ligand and an intact AF-2 are required for squelching of dexamethasone/GR/glucocorticoid responsive element-mediated transcription in COS-7 cells. These results suggest that the AF-2 domain of hVDR (Fig. 1, *α-helix 12*), including residues Leu-417 and Glu-420, likely represents a docking site for a general nuclear receptor coactivator, possibly including one or more of those proteins described above. In addition, given that some amino acid variation in this region exists between the nuclear receptors (Fig. 1), several residues in this domain may constitute a "coactivator code" that dictates receptor-specific coactivator interactions in certain cell types.

Analogous to other ligand-activated nuclear receptors, VDR apparently exists in a monomeric, inactive conformation with the C-terminal AF-2 presumably extended away from the hormone binding cavity. We propose that upon binding 1,25-$(OH)_2D_3$ in target cell nuclei (55), VDR assumes an active conformation as the AF-2 is repositioned for both ligand retention and coactivator contact, with Leu-417 and Glu-420 being critical for this latter association. In addition, the hormone facilitates interaction of VDR and RXR through a stabilized heterodimerization interface mediated by other regions within the VDR C-terminal hormone binding domain (6, 7). The VDR partner, RXR, also possesses an AF-2 (Fig. 1 and Ref. 25) that participates in transcriptional activation by 1,25-$(OH)_2D_3$ through association with a distinct coactivator. This interpretation is supported by the fact that C-terminally truncated RXRs function as dominant negative partners in VDR-mediated transcription (41). Additionally, based upon a previous study (5), it is speculated (56) that the AF-2 in RXR has the ability to be positioned for transactivation in the absence of its 9-*cis*-retinoic acid ligand, likely via allosteric modulation by 1,25-$(OH)_2D_3$-occupied VDR in the RXR:VDR heterodimer. Therefore, the mechanism of VDR action is proposed to be similar to that of TR (57, 58), where liganding of only the primary receptor and not the RXR heteropartner is sufficient to elicit maximal transcriptional stimulation via the AF-2s of both receptors. This differs from the function of RXR:RAR heterodimers (59), in which the liganding of both partner receptors yields responsive element occupation, *in vivo*, and full transcriptional activation by the cooperation of AF-2s. The availability of VDR AF-2 mutants presented herein will facilitate the isolation of VDR-specific coactivators, which should help to define further the mechanistic diversity of action among the VDR/TR/RAR subfamily.

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[3] P. W. Jurutka, L. S. Remus, and M. R. Haussler, unpublished observations.

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