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Promoter-, cell-, and ligand-specific transactivation responses of the VDRB1 isoform ☆

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Abstract

The vitamin D receptor (VDR) mediates the effects of $1,25(OH)_2D_3$, the active form of vitamin D. The human VDRB1 isoform differs from the originally described VDR by an N-terminal extension of 50 amino acids. Here we investigate cell-, promoter-, and ligand-specific transactivation by the VDRB1 isoform. Transactivation by these isoforms of the cytochrome P450 CYP24 promoter was compared in kidney (HEK293 and COS1), tumor-derived colon (Caco-2, LS174T, and HCT15), and mammary (HS578T and MCF7) cell lines. VDRB1 transactivation in response to $1,25(OH)_2D_3$ was greater in COS1 and HCT15 cells (145%), lower in HEK293 and Caco-2 cells (70–85%) and similar in other cell lines tested. By contrast, on the cytochrome P450 CYP3A4 promoter, $1,25(OH)_2D_3$-induced VDRB1 transactivation was significantly lower than VDRA in Caco-2 (68%), but comparable to VDRA in HEK293 and COS1 cells. Ligand-dependence of VDRB1 differential transactivation was investigated using the secondary bile acid lithocholic acid (LCA). On the CYP24 promoter LCA-induced transactivation was similar for both isoforms in COS1, whereas in Caco-2 and HEK293 cells VDRB1 was less active. On the CYP3A4 promoter, LCA activation of VDRB1 was comparable to VDRA in all the cell lines tested. Mutational analysis indicated that both the $1,25(OH)_2D_3$ and LCA-regulated activities of both VDR isoforms required a functional ligand-dependent activation function (AF-2) domain. In gel shift assays VDR:DNA complex formation was stronger in the presence of $1,25(OH)_2D_3$ than with LCA. These results indicate that regulation of VDRB1 transactivation activity is dependent on cellular context, promoter, and the nature of the ligand.

Keywords: Vitamin D receptor; Transcription factor; Nuclear receptor; Cytochrome P-450; Lithocholic acid

The active hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], regulates calcium and phosphate homeostasis and has physiological effects on cell growth and differentiation and regulation of the immune system [1]. It acts through binding of a heterodimer of the vitamin D receptor (VDR) and the retinoid X receptor (RXR) to a vitamin D response element (VDRE), which typically comprises direct repeats of a consensus hexamer motif separated by three nucleotides and termed a DR3 element [2]. Members of the superfamily of nuclear receptors (NR) of ligand-inducible transcription factors [3], including VDR and RXR, share a similar modular structure, including a DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD), linked by a flexible hinge. A ligand-dependent activation function, or AF-2, domain at the

☆ *Abbreviations:* VDR, vitamin D receptor; DBD, DNA binding domain; LBD, ligand binding domain; AF, activation function; PCR, polymerase chain reaction.

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C-terminus of the LBD is critical for ligand-induced receptor transactivation [4,5]. The A/B region has little or no homology among the NRs, reflecting the existence of N-terminal variants as well as differences in length between superfamily members, ranging from 23 amino acids in the VDR [6] to more than 600 amino acids in the mineralocorticoid receptor [7]. The A/B region is proposed to play an important role in cell-, developmental stage-, and promoter-specific action of different isoforms of NRs including estrogen, retinoid, glucocorticoid, and other receptors [8–12]. The most consistent feature of the A/B region of the nuclear receptors is the presence of a ligand-independent autonomous activation function, or AF-1 domain, as initially identified in the glucocorticoid and estrogen receptors [13,14]. This domain interacts and synergizes with the C-terminal AF-2 domain to enhance ligand-induced transactivation [15–19].

Alternative splicing, use of different promoters, and distinct translational start sites generate multiple A/B domains, leading to the expression of various nuclear receptor isoforms (e.g., progesterone receptor isoforms A and B from a single gene [20] and multiple thyroid hormone receptor isoforms from two distinct genes [21]). In humans, an N-terminally extended VDR isoform, VDRB1, generated from a novel transcript [22] is 50 amino acids longer than the originally described isoform, now sometimes termed VDRA [23]. Transactivation by VDRB1 differs from that by VDRA, with some experimental evidence that the relative activities of the two isoforms can vary with changes in the nuclear environment [24]. Factors that naturally influence the relative activities of the two isoforms have not, however, been defined. Such differences in nuclear environment may include variations in transcription factor and cofactor pools, relative VDR isoform levels, promoter context of the VDRE, or the nature of the activating ligand. The natural ligand for VDR is $1,25(OH)_2D_3$, but recently it was observed that the secondary bile acid lithocholic acid (LCA) can activate the VDR [25]. In the present study, we examined the dependence of VDRB1 and VDRA activities on promoter and cell context in mammalian cells in response to these two structurally related ligands.

Materials and methods

Plasmids. Human VDRA and VDRB1 expression vectors were generated using PCR-generated cDNAs from SAOS-2 osteosarcoma cells as previously described [23,24] and cloned using *Hin*dIII and *Apa*I restriction sites into the pRc/CMV expression vector (Invitrogen, Carlsbad, CA). The VDRA and VDRB1 mutants L417S and E420Q were generated by cloning amplified cDNA encoding the AF-2 region modified by oligonucleotides L417S (5′-G CTA ACG CCC TCT GTG CTC GAA G-3′) and E420Q (5′-A TA *G GGC CC*C TAG TCA GGA GAT CTC ATT GCC AAA CAC TTG GAG CAC-3′). Mutated nucleotides are underlined and *Apa*I site on the E420Q primer is italicized. The corresponding amino acid numbers in VDRB1 are L467S and E470Q. All PCR-generated sequences were confirmed by sequencing. The CYP24 promoter luciferase reporter construct from the rat 25-hydroxyvitamin D-24-hydroxylase gene [26] was a generous gift from Dr. B. May, Adelaide. The human CYP3A4 5′-flanking region-luciferase reporter construct was described previously [27].

Transactivation studies. COS1 African green monkey kidney cells and Caco-2 colon carcinoma cells were maintained in DMEM with 10% FBS and HEK293 human embryonic kidney cells in DMEM/F12 with 10% FBS. The rest of cancer cell lines, LS174T and HCT15 from colon, and MCF7 and HS578T from breast, were maintained in RPMI with 10% FBS. Cells were transfected in suspension using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Sydney) with 25 ng pRc/CMV-human VDR isoform cDNA expression construct, 250 ng of promoter-luciferase reporter (rat CYP24 or human CYP3A4), and 10 ng pRSV-β-Gal to normalize transfection efficiency, and plated in 24-well plates at a density of 3×10^4 cells/well in DMEM with 2% charcoal stripped FBS. Cells were treated 18 h after transfection with 1 nM of $1,25(OH)_2D_3$, various concentrations of lithocholic acid (LCA) or vehicle (ethanol) and harvested 6 h later. Luciferase activity was quantified by the luciferase assay system (Promega) and β-galactosidase activity by the galacto light assay system (Applied Biosystems). Transactivation results were from at least three independent transfections, each performed in triplicate, with data corrected for β-galactosidase activity.

Electrophoresis mobility gel shift assays. For electrophoretic mobility shift assays (EMSA) human VDR was prepared by in vitro translation using a coupled transcription–translation system (Promega, Madison, WI). Human RXRα protein was provided in nuclear extracts of COS1 cells (7.2×10^5 cells/10 cm plate) transiently transfected (24 h) with 1 μg wildtype pSG5-RXRα expression plasmid [28]. Proteins were incubated for 20 min at room temperature with 50,000 cpm of radiolabelled oligonucleotides in 20 mM Tris (pH 8), 60 mM KCl, 5 mM $MgCl_2$, 100 μg/ml BSA, 4% glycerol, 1 mM dithiothreitol, and 16 μg/ml poly(dI–dC) (Amersham Pharmacia Biotech, Uppsala) [29]. The mixture was then electrophoresed on a 5% polyacrylamide gel in 0.25× TBE buffer (22.5 mM Tris base, 22.5 mM boric acid, and 0.5 mM EDTA). Double-stranded oligodeoxynucleotides were radiolabelled using the Klenow fragment of DNA Polymerase I and [α^{32}P]dCTP (Perkin–Elmer, Boston, MA) for use as probes (sense strand is only shown, with the vitamin D response element hexanucleotides in bold): CYP24 proximal VDRE1 −144 to −130 (5′-CCCCGG**CGCCCT**CAC**TCACCT**CGCTGA-3′); CYP3A4 distal VDRE −7739 to −7713 (5′-GCTGAA**TGAACT**TGC**TGACCC** TCTGCT-3′). Some reactions also included 1 μg of either anti-RXRα antibody D-20 (SG-553, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-VDR rat monoclonal antibody 9A7 (Affinity Bioreagents, Golden, CO) to specifically inhibit VDR:RXR binding to the VDRE. Autoradiography was obtained using Kodak BioMax MR film. Densitometry was performed on a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Ligand response of VDRB1 was compared to that of VDRA by ANOVA (Superanova v1.11), with significance at $p < 0.05$. Results in graphs are means ± SE.

Results

Cell-, promoter-, and ligand-dependent transactivation

Our previous observation that the presence of excess copies of a viral promoter could alter the relative activities of VDRA and VDRB1 [24] suggested that the abundance of a limiting transcriptional regulator might

contribute to differences in VDR isoform activities. To determine if the relative activities of the VDR isoforms are dependent on the cellular context, transactivation by the isoforms was compared in various cell lines from different tissues. We studied cell lines from calciotropic tissues, kidney, and intestine, as well as from non-calciotropic tissues such as breast, as VDR is widely expressed in most tissues [30]. The transactivation capacity of the VDR isoforms was analyzed using the CYP24 promoter gene and cell cultures were treated with 1 nM of $1,25(OH)_2D_3$.

In COS1 cells, the activity of VDRB1 on the CYP24 was higher than VDRA in response to treatment with $1,25(OH)_2D_3$ (Fig. 1). Similarly, the colon cancer cell line HCT15 VDRB1 showed higher activity than VDRA (Fig. 1). In contrast, in the other kidney cell line (HEK293), and in the colon cancer cell line Caco-2, the activity of VDRB1 on the CYP24 promoter was lower than that of VDRA. In three further cancer cell lines, LS174T (colon), MCF7, and HS578T (breast) the activity of both isoforms was comparable (Fig. 1).

Since the fold induction over empty vector observed in the VDR isoforms in the COS1, HEK293, and Caco-2 cells was higher than the rest of cell lines, we used them to further analyze the importance of promoter context by using the CYP3A4 promoter. In contrast with the CYP24 in the kidney cell lines (COS1 and HEK293 cells), on the CYP3A4 the transactivation capacity of both isoforms was comparable. In the Caco-2, the transactivation shown by VDRB1 was again lower than VDRA, as it was on the CYP24 promoter (Table 1). The possibility that VDR isoform activity may differ depending on the activating ligand was investigated using lithocholic acid (LCA). Maximal activity for both isoforms with minimal cellular toxicity was achieved at 10 μM LCA, which is consistent with previously reported values [25] (dose–response data not shown). Both isoforms exhibited comparable responses to LCA on the CYP24 promoter in COS1 cells, whereas in the HEK293 and Caco-2 cells, the activity of VDRB1 was lower (64–81%, respectively) than that of VDRA (Table 1). In a parallel analysis on the CYP3A4 promoter, VDRB1 and VDRA had comparable activity in these three cell lines. However, the fold induction over empty vector was quite low for the LCA and CYP3A4 in all cell lines (1.2- to 2-fold) (not shown).

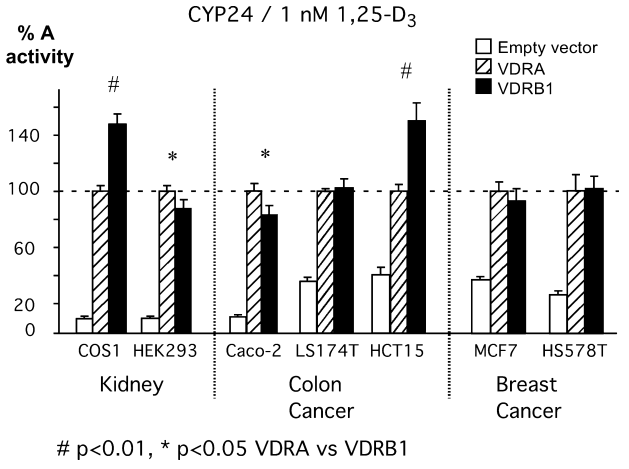

Fig. 1. Transactivation by the VDR isoforms of CYP24 promoter in various cell lines in response to $1,25(OH)_2D_3$. Data of transcriptional activation by human VDR isoforms on CYP24 reporter constructs are presented as percentage over VDRA activity. Luciferase activity was determined in response to $1,25(OH)_2D_3$ in the kidney cell lines, COS1 and HEK293; colon carcinoma cell lines, Caco-2, LS174T, and HCT15; and breast cancer cell lines, MCF7 and HS578T. Significant differences are obtained from pooled analysis from triplicates of three to five independent experiments.

Table 1
Relative transactivation activity of the VDR isoforms

	COS1	HEK293	Caco-2
$1,25(OH)_2D_3$			
CYP24	B1 > A 148%	B1 < A 87%	B1 < A 83%
CYP3A4	B1 = A	B1 = A	B1 < A 68%
LCA			
CYP24	B1 = A	B1 < A 64%	B1 < A 81%
CYP3A4	B1 = A	B1 = A	B1 = A

The VDR isoforms (A, VDRA; B1, VDRB1) were analyzed in three cell lines, on two cytochrome P450 promoters (CYP24 and CYP3A4) and in response to two ligands, its natural form $1,25(OH)_2D_3$ and the secondary bile acid lithocholic acid (LCA). Where there were significant differences in the activity of VDRB1 and VDRA, the percentage of the VDRB1 isoform over VDRA activity is shown. 'B1 = A' indicates no difference in isoforms activity.

AF-2 dependence of LCA activation

To validate the LCA transactivation response, VDR isoforms mutated at residues essential for AF-2 function [31,32] were tested on the rat CYP24 promoter after $1,25(OH)_2D_3$ and LCA treatment. Transactivation by either ligand was markedly reduced by the AF-2 mutations L417S and E420Q in each isoform (Fig. 2). Thus, despite ligand structural differences, VDR isoform-mediated transactivation by $1,25(OH)_2D_3$, and LCA shared an AF-2 functional requirement.

Ligand-dependent VDR–DNA interactions on the CYP24 and CYP3A4 promoters

In gel shift mobility assays, both VDR isoforms bound VDRE1 (proximal DR3) of CYP24 in the presence of nuclear extracts from hRXRα-transfected COS1 cells, with the interaction enhanced by the addition of either $1,25(OH)_2D_3$ or LCA (Fig. 3A). For both VDR isoforms, this DNA–VDR:RXR complex, which was eliminated by the 9A7 VDR antibody, appeared

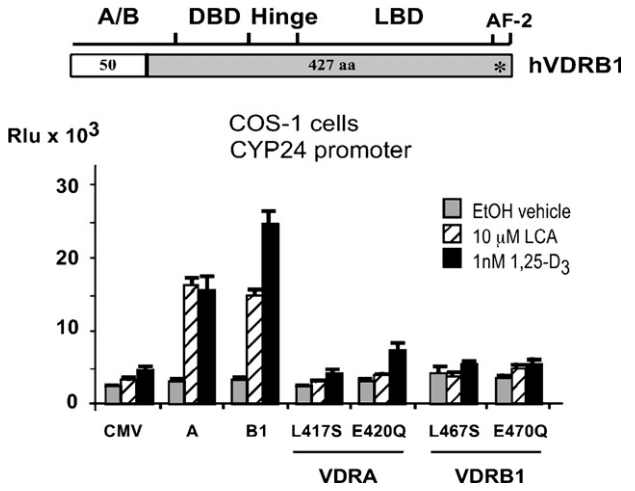

Fig. 2. LCA transactivation requires functional AF-2 domain in both VDR isoforms. VDRA and VDRB1 AF-2 transactivation of CYP24 promoter reporter construct after $1,25(OH)_2D_3$ (1 nM) or LCA (10 μM) treatment of transiently transfected COS1 cells. Both L417S and E420Q mutations (coordinates referring to VDRA molecule, which are residues L467S and E470Q of VDRB1) abolished the activation induced by either ligand in either VDR isoform. Vehicle treatment gray bars, 10 μM LCA striped bars, and 1 nM $1,25(OH)_2D_3$ black bars.

to be stronger in the presence of $1,25(OH)_2D_3$, than with LCA (Fig. 3A). There was a moderate $1,25(OH)_2D_3$ dose-responsive increase in the amount of shifted VDRA–CYP24–VDRE1 complex formed using COS1 expressed hRXRα, whereas the VDRB1 complex on this probe was high at both $1,25(OH)_2D_3$ concentrations tested. Ligand-dependent increases in DNA–VDR binding were also observed with the distal DR3 of the human CYP3A4 promoter (Fig. 3B). There was a sharp dose-responsive increase in the amount of VDRB1–CYP3A4–DR3 complex in response to $1,25(OH)_2D_3$ that was not evident for VDRA on this probe. As for VDRE1 of CYP24, stronger binding was observed with $1,25(OH)_2D_3$ than with LCA.

Discussion

We previously determined that the N-terminal extended VDRB1 isoform had greater transactivation capacity of the rat CYP24 promoter than the originally described VDR molecule (sometimes termed VDRA isoform), in transient transfections in COS1 cells [24].

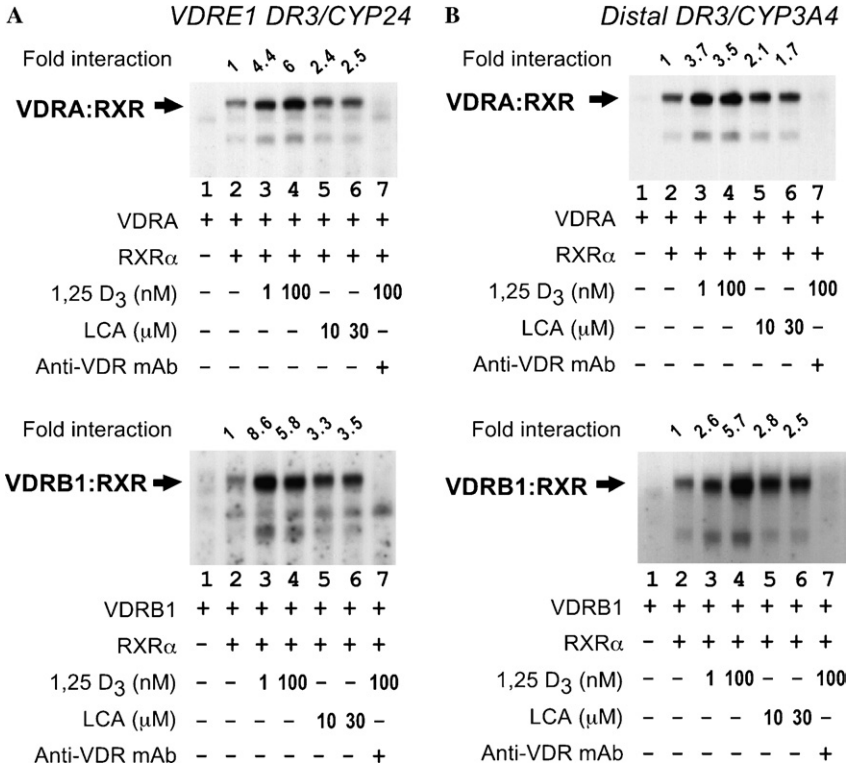

Fig. 3. CYP24 and CYP3A4 vitamin D responsive element binding to the VDR isoforms. Electrophoretic mobility shift assay of VDREs from CYP24 (left panels, A) and CYP3A4 (right panels, B) were performed as indicated under Materials and methods. Radiolabelled double-stranded oligonucleotide was incubated with VDR alone (control, lane 1), or in the presence of the heterodimerization partner RXRα in COS1 nuclear extracts (lanes 2–7). In the absence of ligand (lane 2) there was formation of complex that was greatly enhanced, as shown by the fold interaction, by treatment with $1,25(OH)_2D_3$, (lanes 3 and 4; 1 and 100 nM, respectively) and at a lower level by LCA treatment (lanes 5 and 6; 10 and 30 μM, respectively). Treatment with anti-VDR monoclonal antibody 9A7 prevented the appearance of the complex (lane 7). Similar results were observed in repeated EMSA experiments.

Studies have clearly demonstrated that nuclear receptor A/B domains possess promoter- and cell context-dependent activities [33–35], suggesting that nuclear receptor N-termini may interact with cell-specific cofactors. Further examination of VDRB1 activity in cell lines from tissues involved in calcium homeostasis (kidney; COS1 and HEK293 and intestine; Caco-2, LS174T, and HCT15) as well as from non-calciotropic tissues (breast cell lines; HS578T and MCF7) was undertaken to study whether its activity was cell context-dependent. We examined the response to $1,25(OH)_2D_3$ of the promoter for the ubiquitously expressed CYP24 gene, which encodes a cytochrome P450 enzyme that metabolically inactivates $1,25(OH)_2D_3$ [36].

VDRB1 activity relative to VDRA differed according to the cell type. VDRB1 transactivation of the CYP24 promoter after $1,25(OH)_2D_3$ treatment was higher than that of VDRA in COS1 and HCT15, but it was lower in HEK293 and Caco-2 cells, while the activity of the VDR isoforms was similar in the other cell lines under study. Surprisingly, the differential activity for VDRB1 was not consistent for a particular tissue of origin, but rather it responded to individual cell lines, regardless of the originating tissue, thus for the kidney cell lines, in COS1 VDRB1 showed higher transactivation capacity than VDRA, while on HEK293, VDRB1 was less active. Thus, among the colon cancer cell lines, VDRB1 was more active than VDRA in HCT15, but less active in Caco-2 and was similar to VDRA in LS174T. In the breast cancer cell lines, VDRA and VDRB1 had similar activity in both cell lines analyzed (MCF7 and HS578T). The difference in these patterns presumably relates to promoter requirements for different transcriptional cofactors and their availability in specific nuclear environments.

Higher activity of the VDR isoforms in COS1, HEK293, and Caco-2 cells, as the fold induction over the empty vector was 8- to 10-fold (in contrast with lower fold inductions of 3- to 5-fold in the other cell lines), prompted further analyses in these cell lines. The transactivation responses of the VDR isoforms were analyzed on another $1,25(OH)_2D_3$ responsive promoter from the CYP3A4 gene, encoding the most abundant cytochrome P450 in the liver and intestine, which metabolizes drugs and xenobiotics [37]. VDRB1 activity relative to VDRA differed according to promoter. VDRA transactivation of the CYP3A4 promoter in response to $1,25(OH)_2D_3$ treatment was higher than VDRB1 in Caco-2 cells, while it was comparable to VDRB1 in the kidney cell lines (COS1 and HEK293). The activity of the CYP3A4 promoter induced by the VDR isoforms was lower than that of CYP24 (about 2-fold in the kidney cell lines, and up to 6-fold in Caco-2 cells—data not shown). Consistent with its ubiquitous expression pattern, the CYP24 promoter appears to be highly responsive in all cell lines. In contrast, CYP3A4 promoter activity is lower (particularly in the kidney cell lines which was only about 2-fold over empty vector), perhaps reflecting its more restricted and specialized role in drug detoxification and bile acid metabolism in liver and intestine [37,38]. It has previously been reported that the CYP3A4 promoter functions better in HepG2 than COS1 cells [27], and in the colon carcinoma cell line Caco-2 there is up to 6-fold induction of this promoter by both VDR isoforms. Together these findings are consistent with liver- and intestine-specific transcription factors important in regulation of CYP3A4 gene expression.

The nature of the ligand also determined the relative transactivation activities of VDRB1 and VDRA, with the response patterns of VDRB1 and VDRA to the secondary bile acid, LCA (also cholesterol derivatives, like $1,25(OH)_2D_3$) different from the $1,25(OH)_2D_3$ responses noted above. The two VDR isoforms transactivated the CYP24 promoter to similar levels in COS1 cells after LCA treatment, but in HEK293 and Caco-2 cells VDRB1 transactivation was 19–36% lower (Table 1). By contrast, on the CYP3A4 promoter the activity of VDRB1 was comparable than that of VDRA in response to LCA in the three cell lines analyzed. Different activating ligands can cause different transactivation responses by NRs [34], possibly related to interaction between the amino terminus and the C-terminal ligand binding domain [16–19]. There is evidence of such an interaction in VDRB1 [39], and the data presented here suggest that the N-terminal extension of VDRB1 may regulate this interaction, depending on ligand as well as cellular context.

Distinct ligand binding pocket residues are required for activation of the VDRA isoform by $1,25(OH)_2D_3$ and LCA [40]. Here, we determined that mutation of two functionally critical residues in the AF-2 domain essential for $1,25(OH)_2D_3$-induced cofactor interactions, L417S and E420Q [31,32], also abolished the LCA response of both VDR isoforms (Fig. 2). Transcription cofactors may stabilize ligand-dependent interactions between amino- and carboxy-termini and enhance transactivation function of other NRs [16,19]. The present observations suggest a similar mechanism that may mediate the cell line related differences in VDRA and VDRB1 transactivation, as the VDRB1 A/B domain exhibits stronger cofactor binding than the shorter A/B region of VDRA [39].

Interestingly, the EMSA assays also revealed differences between the two VDR isoforms. Rat CYP24 contains three VDREs between nucleotides −262 and −130 from the transcription initiation site, of which VDRE1 (at position −150 to −136) has greater transactivation response to $1,25(OH)_2D_3$ treatment [41]. The human CYP3A4 promoter contains six consensus VDREs, of which a distal direct repeat (DR3-type element) and a proximal everted repeat with a spacer of

6 bp (ER6) are functional [42]. On each promoter the VDR isoform that exhibited constant high levels of complex formation (Fig. 3) also exhibited greater transactivation activity in transient transfection assays (Fig. 1), suggesting that the effect of the extended N-terminus of VDRB1 on transactivation may relate to the formation of DNA–protein complexes at lower $1,25(OH)_2D_3$ concentrations. Apparently, this effect was specific for $1,25(OH)_2D_3$, as VDRB1 exhibited greater complex formation than VDRA with LCA on both VDRE probes (Fig. 3).

The present data thus provide new evidence for functional differences between the two VDR isoforms and indicate that these are determined by the promoter, the cellular context, and the ligand activating the receptors. Each of these contributing factors may in turn be determined by or contribute to differential cofactor interactions with the VDRB1 A/B region, as observed in two-hybrid assays [39]. In this model, the relative levels of the two VDR isoforms, the complement of cofactors present in a tissue environment, and the concentrations of receptor-activating ligands may determine the physiological VDR mediated activation of target promoters, resulting in changes in gene expression and physiological responses.

Acknowledgments

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