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Vitamin D receptor B1 and exon 1d: functional and evolutionary analysis☆

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Abstract

The vitamin D receptor (VDR) shares a conserved structural and functional organization with other nuclear receptor (NR) superfamily members. For many NRs, N-terminal variant isoforms that display distinct cell-, stage- and promoter-specific actions have been identified. The novel VDR isoform VDRB1, with a 50 amino acid N-terminal extension, is produced from low abundance transcripts that contain exon 1d of the human VDR locus. There is evidence for the conservation of this exon in other mammalian and avian species. The transactivation differences between VDRB1 and the original VDR, clarified here, provide insights into mechanisms that may contribute to functional differences and potentially distinct physiological roles for these two VDR isoforms.

Keywords: Vitamin D; Nuclear receptor; Isoform; Exon 1d; Transactivation

1. Introduction

Active hormonal vitamin D, $1\alpha,25(OH)_2D_3$, plays a vital role in calcium homeostasis and bone physiology. It also regulates keratinocyte differentiation [1,2], haematopoiesis [3,4] and immune function [5–7] at least in part by regulating cellular proliferation, differentiation and apoptosis. These diverse responses are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors. Nuclear receptors share a similar modular structure, including a DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD), linked by a flexible hinge. There is a ligand-dependent activation domain, AF-2, at the extreme C-terminus of the VDR, which is essential for transactivation [8,9]. The less well-conserved N-terminal A/B region of most NRs [10] contains at least one ligand-independent autonomous activation function (AF-1) domain that can interact and synergize with the AF-2 domain to enhance ligand-induced transactivation [11–15]. This region is proposed to play an important role in cell-, developmental stage- and promoter-specific action of different isoforms of other NRs [16–18].

Estrogen, thyroid, peroxisome proliferator-activated, retinoic acid and retinoid X receptor isoforms arise from separate genes, often in concert with alternative promoter and/or exon usage [19–24]. By contrast, variant progesterone receptors are generated through differential start codon usage from a single gene [25,26]. Such variants contribute to functional diversity. For example, the two human progesterone receptor (PR) isoforms differ in promoter specificity, suggesting differential synergies with one another and/or other factors involved in modulation of transcription [27–30]. The PR isoforms also differ in ligand responses, as only the B-receptors can activate transcription in the presence of antiprogestins [31], whereas the A-receptors can dominantly inhibit B-receptors [29,32] and other members of the steroid receptor superfamily [33]. These and other observations indicate that variant receptors may modulate different physiological responses.

Although vitamin D exhibits functional diversity, the VDR differs from other NRs with its very short A/B domain (23aa) and limited structural variability [8,34]. There is only one known VDR gene locus, which in human is on Chr 12, in mouse on Chr 15 and in rat on Chr 7. In a few species

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alternative isoforms have been identified. In flounder, two subtypes of VDR originating from different mRNA species have been described, with nucleotide and predicted amino acid sequence variations not restricted to the N-terminus and with fVDRa showing a more limited tissue distribution than fVDRb [35]. Two chicken VDR isoforms differing only by a 14 amino acid N-terminal extension and generated by alternative translation initiation have been described but functional differences have not been reported [36]. A dominant-negative rat VDR generated by retention of intron 8 [37] also has uncertain physiological significance. A three amino acid N-terminal truncation associated with a common start codon polymorphism in the human VDR locus [38] has been reported to cause elevated transactivation activity [39,40].

In structural studies of the human VDR genomic locus, we identified a number of alternatively spliced transcripts arising from two distinct promoter regions [41]. Of these transcripts, two that contain exon 1d have the potential to encode N-terminally extended VDR isoforms. We have confirmed the existence of one such isoform in human cells and tissue, VDRB1 [42], that is 50 amino acids longer at its amino terminus but otherwise identical to the originally described VDR [34], which we now term VDRA. Here we provide evidence for evolutionary conservation of exon 1d and a recent analysis of the transactivation properties of VDRB1. These findings support the importance of this novel receptor isoform in mediating physiologically diverse responses to vitamin D hormonal regulation.

2. Materials and methods

2.1. Plasmids

Human VDRB1 and VDRA expression vectors were generated using PCR-generated cDNAs from SAOS-2 osteosarcoma cells extending from nucleotide position -6 upstream of the ATG start codon through the coding region to the termination codon of VDR. Forward primer for VDRA was 5′-GAGTCAAGCTTTCAGGGATGGAGGCAATGGCGG-3′ (nt 110–131 on gb# NM_000376) and for VDRB1 was 5′-GAGTCAAGCTTATGGAGTGGAGGAATAAG-3′ (nt 30–47 of exon 1d; gb# AF080454). The common reverse primer was 5′-GACTCGGGCCCCTAGTCAGGAGATC-TCATTGCCAAAC-3′ (nt 1378–1404 of VDR; gb# NM_000376). PCR products were restricted with *Hin*dIII and *Apa*I enzymes and ligated into compatible sites of the pRC/CMV expression vector (Stratagene, La Jolla, CA). All PCR-generated sequence was confirmed by automated DNA sequencing (ABI PRISM^{TM} 377, Perkin-Elmer). The 24-hydroxylase promoter luciferase reporter construct from the rat 25-hydroxyvitamin D-24-hydroxylase gene [43] was a generous gift from Dr. B. May, Adelaide, Australia.

2.2. Transactivation studies

The COS-1 African green monkey kidney cell line, with low endogenous VDR, was maintained in DMEM with 10% FBS. Cells were transfected in suspension using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Sydney, Australia) with 25 ng pRC/CMV-VDR isoform cDNA expression construct, 250 ng of rat 24-hydroxylase promoter-luciferase reporter and 10 ng pRSV-β-Gal to normalize transfection efficiency, and plated in 24-well plates at a density of 3.0×10^4 cells/cm^2 in DMEM with 2% charcoal stripped FBS. For tests of carrier DNA effects, 230 ng of pRC/CMV, pSG5, pUC18, or pBluescript-SK$^+$ plasmid (Stratagene, La Jolla, CA) was included. Cells were treated 18 h after transfection with 10^{-11} to 10^{-8} M 1,25-$(OH)_2D_3$ or vehicle (isopropanol) and harvested 6 h later. Results are shown as mean ± S.E.M. from four independent experiments.

2.3. Western blotting assay

Lysates of COS-1 cells (10 μg per lane) transfected with plasmids expressing the pCMV-VDR expression constructs were subjected to SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were probed with the anti-VDR rat monoclonal antibody 9A7 (Affinity Bioreagents, Golden, CO) followed by peroxidase-conjugated secondary antibody (Zymed, San Francisco, CA). Immunostaining was visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

3. Results

3.1. Evolutionary conservation of exon 1d

A human VDR transcript that originates from the novel exon 1d encodes the N-terminally extended receptor isoform, VDRB1 (Fig. 1) [42]. VDRB1-like isoforms have not been reported in other species. However, exon 1d appears

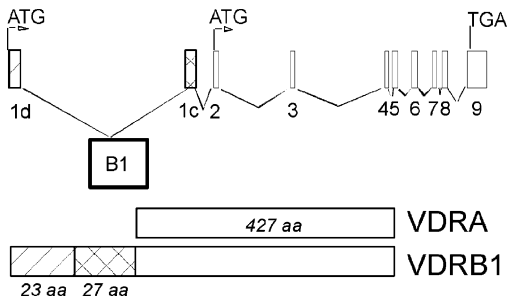

Fig. 1. Exon 1d transcripts of the human vitamin D receptor. The VDRA protein (427 aa) corresponds to the published cDNA sequence; gb# NM_000376. VDRB1 protein is 50 aa larger at the N-terminus. Protein encoded by exons 1d (striped) and 1c (cross-hatched) is indicated.

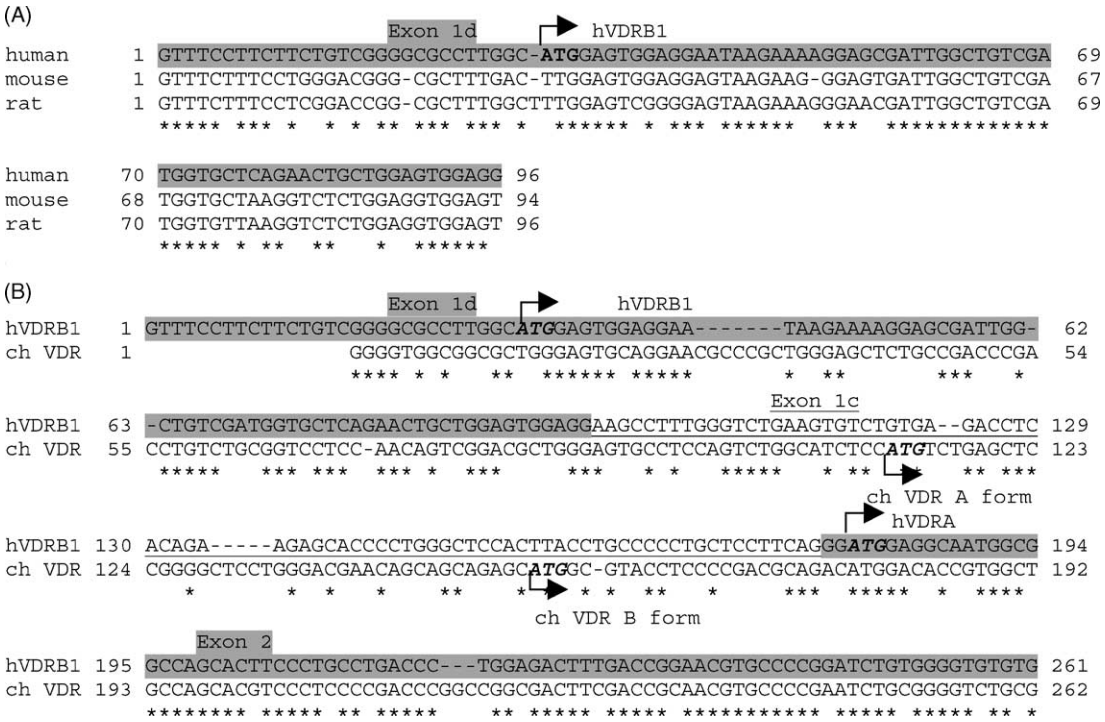

Fig. 2. Exon 1d nucleotide sequence is present in non-human vertebrates. (A) Multiple alignment of human exon 1d (gb# AF080454) with related sequences in mouse (gb# AC134554) and rat (gb# AC119476). (B) Alignment of the 5′ ends of human VDRB1 and chicken VDR (gb# AF011356). There is over 50% identity in exon 1d, 40% in exon 1c and over 80% in subsequent exons (only 5′ residues of exon 2 shown here). Arrows indicate 'ATG' start codons and stars mark identical residues. Exons 1d and 2 are shaded and exon 1c is underscored.

to be evolutionarily conserved, as it is present in mouse and rat genomes (Fig. 2A). The relative position of this 1d-like exon is the same in rodents and humans, between exons 1a and 1c. Nucleotide identity with human exon 1d is over 75% for both mouse and rat. Unlike the human variant, however, the typical 'ATG' initiation codon is replaced in rodents by 'TTG', an alternative start codon (Fig. 2A). In chicken, the VDR transcript that encodes an N-terminally extended isoform [44] exhibits more than 50% identity to human exon 1d at its 5′ end (Fig. 2B). However, this 1d-like sequence lacks a putative initiation codon and does not encode the known chicken N-terminal variant extension. Despite this lack of sequence conservation, the presence of an N-terminal variant chicken VDR isoform may indicate functional conservation.

3.2. Transactivation by VDRB1

VDRB1 transactivation of the VDRE-containing rat CYP24 reporter was greater than that of VDRA at increasing 1,25-$(OH)_2D_3$ concentrations in transient transfections (Fig. 3). As this pattern was the reverse of our earlier observations [42] we analyzed differences between past and current experimental protocols to determine the source of this variation. With inclusion of pRC/CMV empty vector as carrier, transactivation by VDRB1 was consistently lower than VDRA, in association with a marked reduction in transfection efficiency (Fig. 4A). Inclusion of pSG5 vector also abolished the VDRB1 transactivation advantage, whereas inclusion of either pUC18 or pBluescript-SK^+ vector as carrier did not affect the relatively greater activity of VDRB1, despite similar overall reductions in transfection efficiency. Interestingly, pRC/CMV and pSG5 carrier reduced VDR expression levels, whereas the other carrier DNAs did not (Fig. 4B). Duration of ligand treatment (6 h versus 24 h), degree of culture confluence at time of ligand treatment, and other procedural differences were not sources of variability in the VDRB1-VDRA comparison (data not shown). Hence the presence in high copy numbers of a strong viral promoter had marked effects on the relative activities of VDRB1 and VDRA.

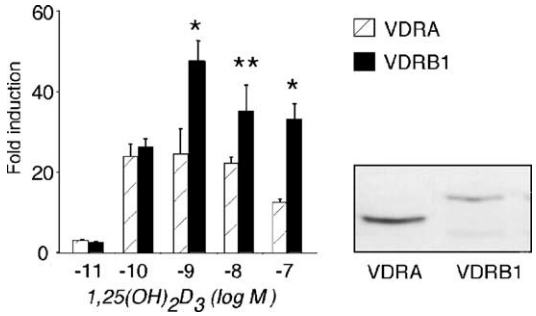

Fig. 3. Transcriptional activation of rat 24-hydroxylase reporter construct was determined for both isoforms in COS-1 cells. Transcriptional activity was assessed after treatment with vehicle or 1,25-$(OH)_2D_3$ (10^{-11} to 10^{-7} M) for 6 h. Western blot analysis confirmed production of VDR proteins (VDRA striped bars, VDRB1 black bars). $^*P < 0.05$; $^{**}P < 0.01$.

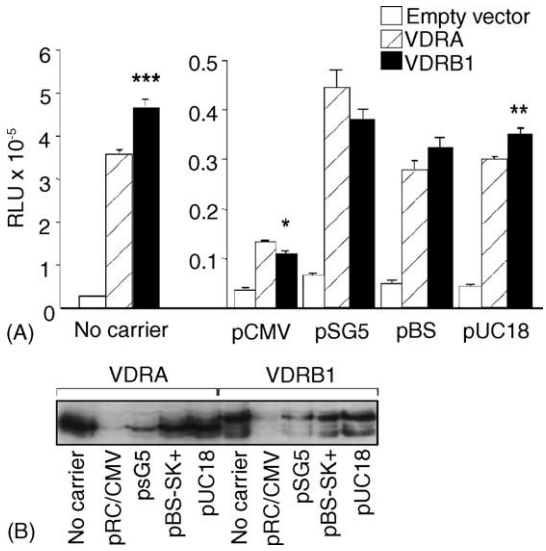

Fig. 4. (A) Effect of carrier DNA (230 ng) on VDRB1 transactivation (black bars) relative to VDRA (striped bars) and empty vector (white bars) after 1,25-$(OH)_2D_3$ treatment. Note change in Y axis scale ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.002$). (B) Western blot of whole cell extracts from transfected cultures in panel A probed with anti-VDR antibody. Longer exposure confirmed the presence of VDR protein in the pRC/CMV lanes (data not shown).

4. Discussion

Amino-terminal variant isoforms of other NRs differ in transactivation properties depending on tissue, cell type or promoter context [16–18], and N-terminal variants of the human and chicken vitamin D receptors have been described [42]. Genomic sequence comparisons indicate conservation of the VDRB1-characteristic exon 1d sequence, with homology decreasing as evolutionary distance increases. However, to date there is no evidence confirming N-terminal variants of the VDR or exon 1d-containing transcripts in non-human mammals. Interestingly, all upstream exons reported for human, including exon 1d [41], are conserved with 98–100% identity in chimpanzee (WGS Whole Genome Shotgun reads #194003216). Further investigation of VDRB1-like receptors in non-human species will provide important insights into the prevalence and potential importance of this VDR isoform.

The extended N-terminus of the human VDRB1 isoform affects transcriptional activity. VDRB1 transactivation was greater than that of VDRA in transient transfections on the CYP24 promoter in COS-1 cells. These findings differed from our earlier experiments, which had indicated lower transactivation by VDRB1 on the same promoter and in the same cell line [42]. Potential procedural reasons for this difference were explored systematically. The use of carrier DNA including the CMV (human cytomegalovirus) immediate early promoter in the earlier transfection experiments appears to be the source of this variability. In support of this likelihood, pSG5 with its strong viral SV40 early promoter also abolished the VDRB1 advantage, whereas omission of carrier DNA or use of carrier DNA lacking a viral promoter did not modify the greater transactivation function of VDRB1 relative to VDRA.

The greater susceptibility of VDRB1 transactivation to competition by pRC/CMV and pSG5 carrier DNA may relate to transcription complexes forming on the viral promoters. For example, the CMV promoter, containing three functional retinoic acid response elements, may compete for RXR and other relevant transcription factors or cofactors [45] and, in principle, squelch a VDRB1-assembled transcription complex more than a VDRA complex. The low VDR protein levels in the transfections with viral promoters may also contribute to this effect. These observations indicate that changes in availability of transcriptional regulators may alter the relative activities of VDRB1 and VDRA. Greater understanding of any competition for RXR-associated or other transcriptional regulators could elucidate the composition of VDRB1-containing complexes and suggest a mechanistic basis for enhanced transactivation by VDRB1 on vitamin D target promoters. This mechanism and the variations of relative transactivation by VDRB1 on different target promoters in different cell types are the subject of ongoing investigations.

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