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Role of residues 143 and 278 of the human nuclear Vitamin D receptor in the full-length and Δ165-215 deletion mutant☆

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Abstract

Most of the actions of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] are mediated by binding to the Vitamin D nuclear receptor (VDR). The crystal structure of a deletion mutant (Δ165-215) of the VDR ligand-binding domain (LBD) bound to 1,25$(OH)_2D_3$ indicates that amino acid residues tyrosine-143 and serine-278 form hydrogen bonding interactions with the 3-hydroxyl group of 1,25$(OH)_2D_3$. Studies of VDR and three mutants (Y143F, S278A, and Y143F/S278A) did not indicate any differences in the binding affinity between the variant receptors and the wild-type receptor. This might indicate that the 3-hydroxyl group binds differently to the full-length VDR than the to deletion mutant. To further investigate, four deletion VDR mutants were constructed: $VDR_{\Delta 165\text{-}215}$, $VDR_{\Delta 165\text{-}215}$ (Y143F), $VDR_{\Delta 165\text{-}215}$ (S278A), $VDR_{\Delta 165\text{-}215}$ (Y143F/S278A). There were no significant differences in binding affinity between the wild-type receptor and the deletion mutants except for $VDR_{\Delta 165\text{-}215}$ (Y143F/S278A). In gene activation assays, VDR constructs with the single mutation Y143F and the double mutation Y143F/S278A, but not the single mutation S278A required higher doses of 1,25$(OH)_2D_3$ for half-maximal response. This suggests that there are some minor structural and functional differences between the wild-type VDR and the Δ165-215 deletion mutant and that Y143 residue is more important for receptor function than residue S278.

Keywords: Vitamin D; Vitamin D receptor; Nuclear receptor

1. Introduction

The hormone, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], mediates a wide variety of biological effects. These include regulation of calcium homeostasis by actions on intestine, kidney and bone, and also other effects on many systems including inducing differentiation and inhibiting proliferation of several types of cells. Many of these effects are mediated through interactions of 1,25$(OH)_2D_3$ with its nuclear receptor (VDR) [1,2]. The cDNA for the VDR was cloned and sequenced in 1988 [3]. It is a member of a superfamily of structurally and functionally related ligand dependent nuclear receptors that include receptors for glucocorticoids, progesterone, estrogen, aldosterone, androgens, hormonal forms of Vitamins A and D, thyroid hormone, peroxisome-proliferator activators and many orphan receptors. Crystal structures of 16 nuclear receptor ligand-binding domains (LBDs) have been determined including retinoid X receptor (RXR) [4,5], retinoic acid receptors (RAR) [6,7], estrogen receptors (ER) [8–10], peroxisome proliferator-activated receptors (PPAR) [11,12], progesterone receptor (PR) [13], and androgen receptor (AR) [14,15]. These structures have been very important in providing information on the structural basis of receptor action.

A double deletion variant of the VDR has also been recently crystallized and a high-quality structure of the LBD was obtained [16]. The LBD consists of twelve α-helices and one β sheet that are folded in three layers to form a hydrophobic binding pocket for the hormone. When ligand binds, conformational changes are induced in the receptor that increase its ability to modulate gene transcription [17]. The most dramatic conformational change involves repositioning of helix 12 creating new surfaces required for interaction with RXR and additional nuclear factors called coactivators [18]. The three hydroxyl groups of 1,25$(OH)_2D_3$ form hydrogen bonds with polar amino acid residues in the hydrophobic binding pocket. Mutational analysis of the residues that contact the 1-hydroxyl (1-OH) group (S237, R274) and the 25-hydroxyl group (H305, H397) indicate reduced binding affinity compared to the wild-type receptor but mutational analysis of the residues that contact the 3-hydroxyl (3-OH) group (Y143, S278)

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did not result in reduced binding affinity. The region of the ligand-binding domain that contacts the 3-hydroxyl group is close to the region deleted in the crystal structure, suggesting that the conformation of that region of the receptor might be different in the full-length receptor. A series of mutations in the Δ165-215 deletion mutant of VDR were constructed to further address this issue.

2. Materials and methods

2.1. Site-directed mutagenesis

The cDNA for the hVDR was cloned into the *Eco*RI and *Nsi*I sites in pcDNA1.1 (Invitrogen, CA) [19]. The two single point mutations and double point mutations were incorporated into the cDNA using QuikChange site-directed mutagenesis kit (Stratagene, CA) and the following primers: Y143F forward (5′-CCATAAGACCTTCGACCCCACCTAC-3′), Y143F reverse (5′-GTAGGTGGGGTCGAAGGTCTTATGG-3′), S278A forward (5′-CGCTCCAATGAGGCCTTCACCATGG-3′), and S278 reverse (5′-CCATGGTGAAGGCCTCATTGGAGCG-3′).

2.2. Creation of deletion mutants

The Δ165-215 deletion was engineered by inverse PCR using phosphorothioate-containing primers: primer 1 (5′-GGAGGGTCTGTG**ACCC**TAGAGCTG-3′) and primer 2 (5′-CACAGACCCTCC**ACCA**TCATTCAC-3′), bold residues contain phosphorothioate. The resulting PCR fragment was digested with T7 gene 6 exonuclease for 1 h at 37 °C to create 12 base, single-stranded 3′-complementary ends. The 3′ ends self-hybridize creating nicked circular DNA that was used to transform XL1-blue supercompetent cells (Stratagene, CA). Clones were selected and sequenced using fmole sequencing kit (Promega, WI).

2.3. Cell culture and transient transfection of COS-7 cells

COS-7 monkey kidney cells were maintained in continuous culture in Dulbecco's modification of Eagle's medium (DMEM) (Mediatech Inc., VA) with 10% fetal bovine serum (FBS) (BioWhittaker, MD) at 37 °C in a humidified atmosphere with 5% CO_2. For transfection, cells were seeded at 3×10^5 cells per 150 mm tissue culture dish (Corning Inc., NY). After 24 h incubation, phosphate buffered saline (PBS)-washed cells were treated with 1 mg/ml diethylaminoethyl (DEAE)–dextran (Sigma, MO) in PBS for 9 min. The DEAE–dextran was removed by aspiration and cells were washed twice with PBS then incubated for 30 min with 10.6 μg DNA in PBS/dish. Cells were then incubated in DMEM supplemented with 10% FBS and 80 μM chloroquine (Sigma, MO) for 4 h followed by incubation in the same medium without chloroquine for 24 h. Cells were harvested 72 h post-transfection.

2.4. Saturation binding analysis

Aliquots of lysates from COS-7 cells transfected with cDNA for VDR or mutant VDR were incubated with increasing concentrantions of [^{3}H]-1,25(OH)$_2$D$_3$ (specific activity, 90–93 Ci/mmol, Amersham Pharmacia Biotech, IL) in the presence or absence of a 200-fold excess of nonradiolabeled 1,25(OH)$_2$D$_3$. Hormone bound to receptor was separated from free hormone using hydroxylapatite batch assay as previously described [20]. Specific binding was calculated by subtracting non-specific biding from total binding.

2.5. Gene transactivation assays

COS-7 cells were seeded at 3×10^5 cells per well in 24-well plates (Corning Inc., NY) and co-transfected as described above with 0.5 μg per well pcDNA1.1 VDR plasmid and 1.5 μg per well pSEAP–VDRE plasmid, containing the osteocalcin gene Vitamin D response element (VDRE) linked to secreted alkaline phosphatase reporter gene (SEAP). Twenty-four hours post-transfection, the cell medium was supplemented with 1,25(OH)$_2$D$_3$ in 0.1% ethanol, final concentration. At 30 h after hormone treatment, the cell medium was harvested and assayed for SEAP activity using Phospha-Light kit (Tropix, MA).

3. Results

3.1. Effects of mutations on ligand binding

The crystal structure of the double deleted ligand-binding domain of the VDR has been solved at high resolution. The data from the crystal structure indicates that the 3-OH group makes hydrogen bonding contacts with residues Y143 and S278. Previous mutational analysis of the VDR did not indicate these residues as important for ligand binding [21]. Originally, we constructed these two single mutants and the double mutation in the wild-type receptor to assess the importance of these residues for binding. Representative graphs of the saturation binding analysis are shown in Fig. 1. As seen in Table 1, changing the residues individually or together had no effect on ligand binding. From these results, it was not clear whether the 3-OH group was anchored differently in the full-length receptor compared to the deleted receptor of the crystal structure. Therefore, we synthesized four Δ165-215 deletion mutants: wild-type, single point substitutions at 143 and 278 as well as the double point mutant. The full-length VDR, Y143F, and S278A displayed similar binding affinities to their deleted counterparts, $VDR_{\Delta 165\text{-}215}$, $Y143F_{\Delta 165\text{-}215}$, $S278A_{\Delta 165\text{-}215}$, respectively. Only the double mutant with the deletion, $Y143F/S278A_{\Delta 165\text{-}215}$, had decreased binding affinity for hormone that was significantly different from both the full-length VDR and $VDR_{\Delta 165\text{-}215}$. This suggests that residues 143 and 278 are not very

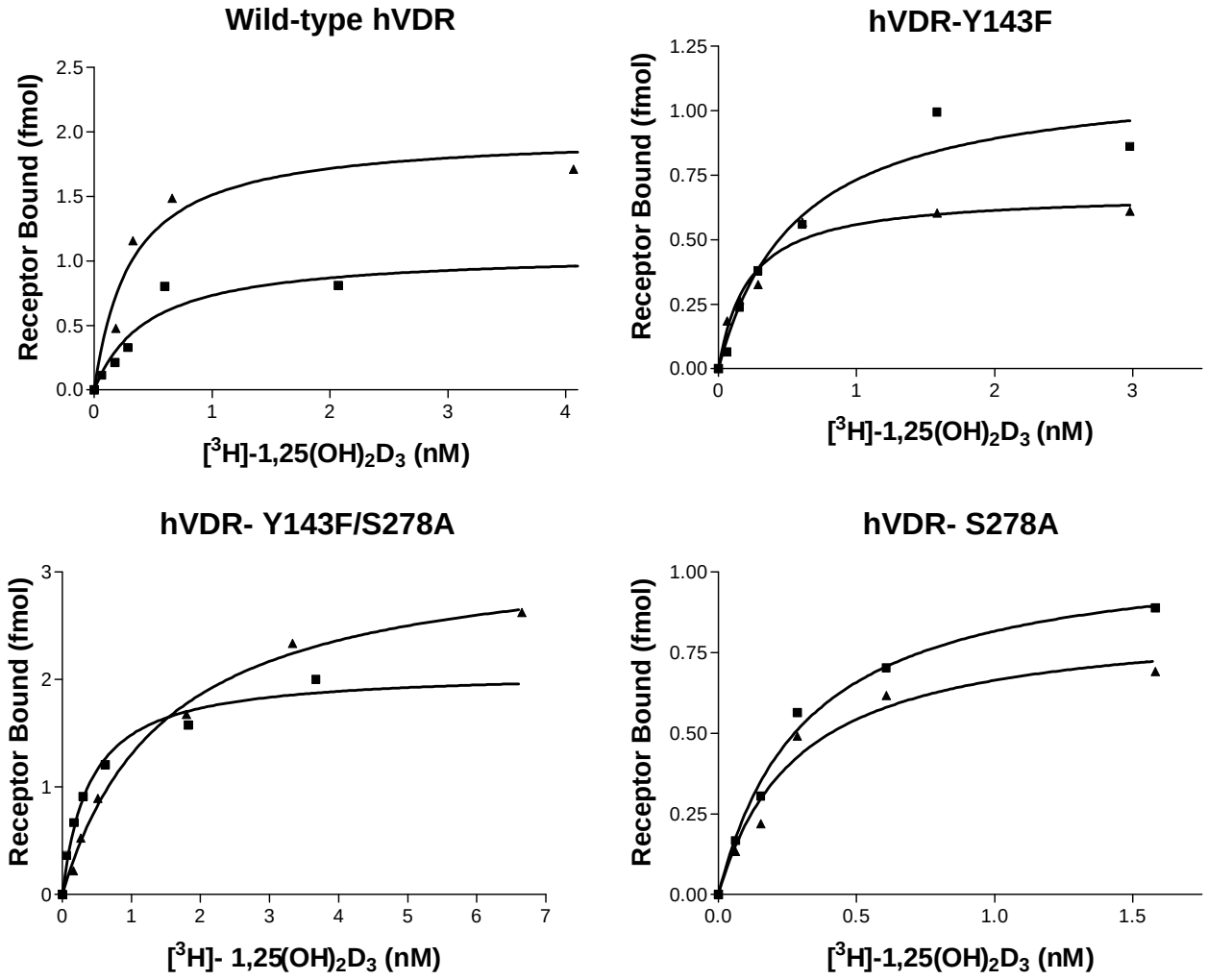

Fig. 1. Saturation binding analysis: increasing concentrations of radiolabeled $1,25(OH)_2D_3$ were added to aliquots of cell homogenates transfected with wild-type or mutant VDR cDNA in the presence or absence of 200-fold excess non-radioactive $1,25(OH)_2D_3$. Hormone bound to receptor was separated from free hormone using hydroxylapatite. Specific binding was calculated by subtracting non-specific binding from total binding. The data for total binding are the mean of triplicate points and for non-specific binding are the mean of duplicate points.

Table 1

Receptor construct	Saturation binding KD (nM)	Transactivation EC_{50} (nM)
wt VDR	0.61 ± 0.33 ($n = 7$)	0.27 ± 0.24 ($n = 4$)
$VDR_{\Delta 165\text{-}215}$	0.42 ± 0.08 ($n = 4$)	0.13 ± 0.11 ($n = 4$)
Y143F	0.46 ± 0.16 ($n = 4$)	3.08 ± 2.75 ($n = 4$)[a]
$Y143F_{\Delta 165\text{-}215}$	0.64 ± 0.34 ($n = 6$)	0.88 ± 0.79 ($n = 4$)[b]
S278A	0.43 ± 0.17 ($n = 4$)	0.66 ± 0.68 ($n = 3$)
$S278A_{\Delta 165\text{-}215}$	0.51 ± 0.20 ($n = 6$)	0.27 ± 0.04 ($n = 3$)
Y143F/S278A	0.56 ± 0.17 ($n = 7$)	126 ± 5.7 ($n = 3$)[a]
Y143F/ $S278A_{\Delta 165\text{-}215}$	1.15 ± 0.70 ($n = 5$)[b,c]	30 ± 13 ($n = 4$)[a,d]

Data are expressed as means and S.D. of n independent experiments. One-way ANOVA with Newman–Keul's post test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, CA.

[a] Significantly different than wt VDR, $P < 0.001$.

[b] Significantly different than $VDR_{\Delta 165\text{-}215}$, $P < 0.05$.

[c] Significantly different than wt VDR, $P < 0.05$.

[d] Significantly different than $VDR_{\Delta 165\text{-}215}$, $P < 0.001$.

important for binding affinity but may be more important in the deletion VDR construct than the full-length VDR.

3.2. Gene transactivation studies

The functionality of the various VDR constructs was assessed by gene transactivation assays. Representative data is shown in Fig. 2. As seen in Table 1, full-length wt VDR, Y143F, S278A, and Y143F/S278A displayed similar transcription activation as their deleted counterparts: $VDR_{\Delta 165\text{-}215}$, $Y143F_{\Delta 165\text{-}215}$, $S278A_{\Delta 165\text{-}215}$, $Y143F/S278A_{\Delta 165\text{-}215}$, respectively. All mutants with the Y143F mutation required higher doses of hormone to induce gene transcription than both wt VDR and $VDR_{\Delta 165\text{-}215}$. This suggests that residue 143 is important for gene activation in both the deleted VDR construct and the full-length VDR construct.

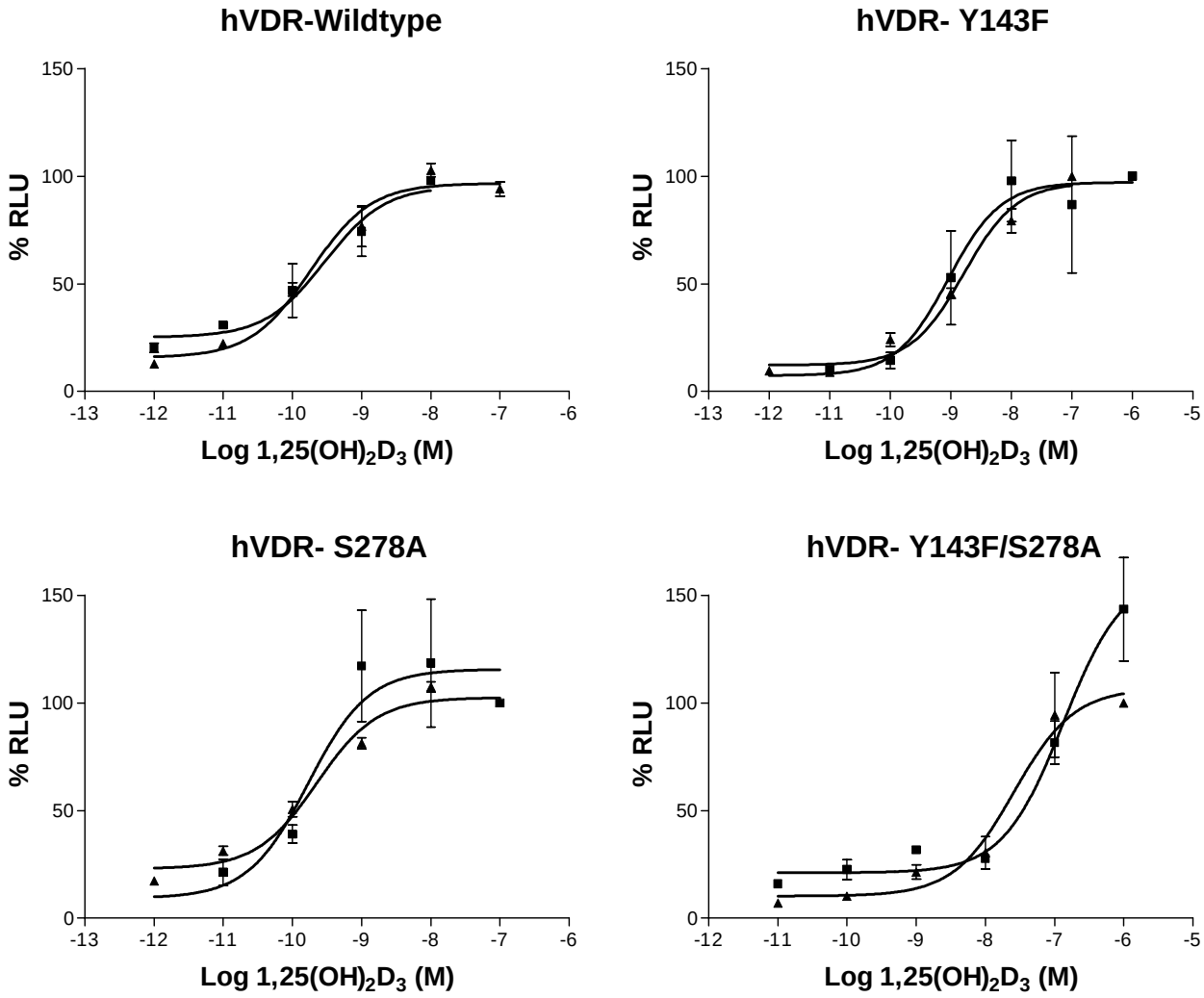

Fig. 2. Gene transactivation assay: COS-7 cells were co-transfected with the osteocalcin VDRE fused to secreted alkaline phosphatase gene and a cDNA for VDR (wild-type or mutant). using DEAE–dextran. After 24 h, cells were treated with increasing doses of $1,25(OH)_2D_3$ or ethanol. Forty-eight hours later, culture medium was collected and secreted alkaline phosphatase activity was determined by chemiluminescence. Each point is the average of triplicate transfections.

4. Discussion

Understanding the structure–function relationships of the VDR is important for understanding its actions and for designing analogs with selective biological activity. The only structural data on the VDR is from crystal structures of the ligand-binding domain with a 50-residue deletion. While binding studies and functional assays indicated that this deletion had very little effect on the activity of the receptor [22], there were questions about receptor interactions with the 3-OH group. No earlier studies using VDR mutants identified residues 143 or 278 as important residues for binding [21]. Initially we synthesized these mutants in the full-length receptor and found no significant differences in binding affinity between any of the constructs, so we synthesized the same mutants in VDR but also with the Δ165-215 deletion. The only construct with a significant decrease in binding affinity compared to the other constructs was the double point mutation with the deletion, $Y143A/S278A_{\Delta 165\text{-}215}$. There are several possible explanations. The 3-OH is not as critical for high affinity binding as the other two hydroxyl groups of the hormone. This was shown by steroid competition binding assays using analogs that were missing one of the hydroxyl groups [1]. Therefore, residues that interact with the 3-OH group may not contribute very much to the overall binding affinity, so changing those residues may have little or no affect on binding affinity. Another possibility is that the deletion changed the region of the binding pocket that interacts with the 3-OH group and different residues interact with the 3-OH group in the full-length receptor. If this were true, we might have expected to see more of a difference in binding affinity between the deletion constructs than the full-length constructs. Since there was a slight decrease in binding affinity for the $Y143A/S278A_{\Delta 165\text{-}215}$

construct, there may be slight conformational differences between the full-length and $VDR_{\Delta 165\text{-}215}$. Additionally, the crystal structure indicates the presence of several water molecules surrounding the A ring of the hormone in the ligand-binding domain. These water molecules could substitute for the missing hydroxyl groups in the mutant receptors and make hydrogen bonding interactions with the 3-OH of the hormone. More detailed structural analysis studies will have to be done to distinguish between these possibilities.

The gene transactivation assays show that residue 143 and 278 are important for receptor function since single point mutations of 143 required significantly higher doses of $1,25(OH)_2D_3$ to achieve maximal response and, in combination with a mutation at 278, required even higher doses of hormone.

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