COMMONWEALTH OF AUSTRALIA
Copyright Regulation
WARNING
This material has been reproduced and communicated to you
by or on behalf of the University of Sydney pursuant to Part VA
of the Copyright Act 1968 (the Act).
The material in this communication may be subject to copyright under the Act.
Any further reproduction or communication of this material by you
may be the subject of copyright protection under the Act.

Do not remove this notice.
Hereditary 1,25-Dihydroxyvitamin D Resistant Rickets due to a Mutation Causing Multiple Defects in Vitamin D Receptor Function

PETER J. MALLOY, RONG XIU, LIHONG PENG, SARA PELEG, ABDULLAH AL-ASHWAL, AND DAVID FELDMAN

Hereditary vitamin D-resistant rickets (HVDRR) is an autosomal recessive disease caused by mutations in the vitamin D receptor (VDR). We studied a young Saudi Arabian girl who exhibited the typical clinical features of HVDRR, but without alopecia. Analysis of her VDR gene revealed a homozygous T to C mutation in exon 7 that changed isoleucine to threonine at amino acid 268 (I268T). From crystallographic studies of the VDR ligand-binding domain, I268 directly interacts with 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) and is involved in the hydrophobic stabilization of helix H12. We recreated the I268T mutation and analyzed its effects on VDR function. In ligand binding assays, the I268T mutant VDR exhibited an approximately 5- to 10-fold lower affinity for [3H]1,25(OH)$_2$D$_3$ compared with the wild-type (WT) VDR. The I268T mutant required approximately a 65-fold higher concentration of 1,25(OH)$_2$D$_3$ to be equipotent in gene transactivation. Both retinoid X receptor heterodimerization and coactivator binding were reduced in the I268T mutant. Analogs of 1,25(OH)$_2$D$_3$ have been proposed as potential therapeutics for patients with HVDRR. Interestingly, in protease sensitivity assays, treatment with the potent vitamin D analog, 20-epi-1,25(OH)$_2$D$_3$, stabilized I268T mutant proteolytic fragments better than 1,25(OH)$_2$D$_3$. Moreover, 20-epi-1,25(OH)$_2$D$_3$ restored transactivation of the I268T mutant to levels exhibited by WT VDR treated with 1,25(OH)$_2$D$_3$. In conclusion, we describe a novel mutation, I268T, in the VDR ligand-binding domain that alters ligand binding, retinoid X receptor heterodimerization, and coactivator binding. These combined defects in VDR function cause resistance to 1,25(OH)$_2$D$_3$ action and result in the syndrome of HVDRR. (Endocrinology 145: 5106–5114, 2004)

THE BIOLOGICAL ACTIONS of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), including regulation of calcium homeostasis, cellular differentiation, and immune function, are mediated by the vitamin D receptor (VDR), a member of the steroid/nuclear receptor superfamily of ligand-activated transcription factors (1–3). Binding of 1,25(OH)$_2$D$_3$ to the VDR ligand-binding domain (LBD) triggers a series of molecular events leading to the activation of vitamin D-responsive genes. A two-zinc-finger structure in the DNA-binding domain (DBD) near the N terminus of the VDR allows the VDR to bind to vitamin D response elements (VDER) in the promoter regions of target genes. The regulation of gene transcription by the VDR requires binding to VDERs as a heterodimer with the retinoid X receptor (RXR). Initiation of gene transcription also involves the recruitment of coactivator proteins, including the p160 proteins steroid receptor coactivator 1 (SRC1), glucocorticoid receptor interacting protein 1 (GRIP1), and the VDR interacting protein (DRIP)/mediator complex. These coactivator proteins remodel chromatin and act as bridging factors, linking the nuclear receptors to the preinitiation complexes and RNA polymerase II. They bind to the VDR in a ligand-dependent manner and enhance transactivation. A crystallographic study of the VDR LBD bound to 1,25(OH)$_2$D$_3$ has shown that the LBD is composed of 13 $\alpha$-helices and three $\beta$-sheets (4). Ligand binding causes helix H12 in the activation function-2 domain to be repositioned forming a hydrophobic groove with helices H3 and H4. The coactivators bind to the hydrophobic groove on the nuclear receptor surfaces through a conserved LXXLL motif in the nuclear receptor-interacting domains.

Hereditary vitamin D-resistant rickets (HVDRR; on line Mendelian Inheritance in Man 277440), also known as vitamin D-dependent rickets type II, is a rare recessive genetic disorder caused by mutations in the VDR. Patients with HVDRR exhibit early-onset rickets, hypocalcemia, and secondary hyperparathyroidism and generally have a history of consanguinity in the family. HVDRR patients also have significantly elevated serum levels of 1,25(OH)$_2$D$_3$, distinguishing this condition from 1a-hydroxylase deficiency, also known as vitamin D-dependent rickets type I. The latter patients present with reduced levels of 1,25(OH)$_2$D$_3$. In some cases, HVDRR patients have total body alopecia. A number of genetic abnormalities in the VDR causing HVDRR have
been described (2). Mutations in the VDR DBD interfere with VDR-DNA interactions, but not with ligand binding, and result in complete loss of transactivation (5–9). Mutations in the VDR LBD affect 1,25(OH)2D3 binding, heterodimerization with RXR and coactivator interaction and result in partial or total hormone unresponsiveness (10–14). Some patients with LBD mutations do not have alopecia. There has also been one report of a patient with HVDRR and alopecia whose fibroblasts were resistant to 1,25(OH)2D3 without a mutation in the VDR (15). The 1,25(OH)2D3 resistance in this patient has been postulated to be due to the overabundance of a hormone response element-binding protein that occupies VDREs and prevents VDR transactivation (16). In this report we describe a novel mutation in helix H5 in the VDR LBD that affects ligand binding, RXR heterodimerization, and coactivator binding and results in the syndrome of HVDRR without alopecia.

**Materials and Methods**

\[ ^{3}H \]1,25(OH)\(_2\)D\(_3\) binding and Western blotting

The wild-type (WT) and mutant VDRs were expressed in COS-7 cells. Cell extracts were prepared in M-PER extraction buffer (Pierce Chemical Co., Rockford, IL) containing 300 mM KCl, 5 mM dithiothreitol, and a complete protease inhibitor tablet (Roche, Indianapolis, IN). Cells were incubated at ambient temperature for 10 min on a rotating mixer. Cell debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C. The crude cell extracts were incubated with \[^{3}H\]1,25(OH)\(_2\)D\(_3\) (Amersham Biosciences, Arlington Heights, IL) with or without a 250-fold excess of radiolabeled 1,25(OH)\(_2\)D\(_3\), as previously described (17). Hydroxylapatite was used to separate bound and free hormone. For Western blotting, cell extracts were denatured in lithium dodecyl sulfate sample buffer at 10 min at 70°C and electrophoresed on 10% NuPAGE gels in 4-morpholinepropanesulfonic acid-sodium dodecyl sulfate running buffer (Invitrogen Life Technologies, Inc., Carlsbad, CA). Proteins were transferred to nitrocellulose and incubated with a mouse anti-VDR monoclonal antibody (DSL Laboratories, Webster, TX) (18) as previously described (19). Protein concentrations were determined by the Bradford method (20).

**Gene amplification and DNA sequencing**

Exons 2–9 of the VDR gene that encode the VDR protein were amplified by PCR and cloned into pBluescript II KS (Stratagene, La Jolla, CA). The PCR products were cloned and sequenced using \[^{3}P\]dideoxy-ATP and Sequenase version 2.0 DNA sequencing kit (Amersham Biosciences).

**RFLP analysis**

Exons 7 and 8 of the VDR gene were amplified from the patient’s DNA using oligonucleotide primers 7a and 8b as previously described (17). PCR products were digested with the restriction endonuclease Ddel at 37°C according to the manufacturer’s directions (New England Biolabs, Beverly, MA) and were analyzed on 2% agarose gels in Tris-acetic acid-EDTA buffer. Gels were stained with ethidium bromide, visualized by UV light, and photographed.

**Site-directed mutagenesis and plasmid construction**

Site-directed mutagenesis of the WT VDR cDNA was performed using the pALTER system (Promega Corp., Madison, WI) as previously described (12). The mutant oligonucleotide used was 5’-TCA AGT GCC ACT GAG GTC ATC. Clones were sequenced to confirm the presence of the point mutation. The mutant VDR cDNA was subcloned into the mammalian expression vector pEG5 (Stratagene).

**Transactivation assays**

COS-7 cells were grown to 60–80% confluence in 12-well tissue culture plates. Cells were transfected with 0.125 µg WT or I268T mutant VDR expression plasmid and 0.25 µg rat 24-hydroxylase promoter VDRE-luciferase plasmid using Polyfect (Qiagen, Valencia, CA). A Renilla-luciferase plasmid, pRLnull (0.01 µg), that served as an internal control for transfection efficiency was included in each assay. A human RXRα expression vector was cotransfected in some experiments. After a 16-h transfection, the cells were incubated in DMEM containing 1% fetal bovine serum with or without 1,25(OH)\(_2\)D\(_3\). In some assays cells were treated with 20-µl 1,25(OH)\(_2\)D\(_3\) (MCI288, Leo Pharmaceuticals, Ballerup, Denmark). Twenty-four hours after transfection, the cells were washed and prepared for dual luciferase assays according to the manufacturer’s instructions (Promega Corp.). Luciferase activities were determined using a luminometer (Turner Design, Sunnyvale, CA).

**Glutathione-S-transferase (GST) pull-down**

GST fusion proteins (RXRa, SRC-1, and DRIP205) were expressed in *Escherichia coli* BL21 (DE3) after induction with 0.1 mM isopropyl-\(\beta\)-D-thiogalactoside for 3 h at 37°C. Proteins were extracted by incubating the cells in B-PER extraction reagent (Pierce Chemical Co.) containing 100 mM KCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol, 10% glycerol, and a complete protease inhibitor tablet (one tablet per 50 ml) (Roche Molecular Biochemicals) for 10 min at ambient temperature with gentle shaking. Cell debris was removed by centrifugation at 12,500× g for 20 min at 4°C. WT and I268T mutant VDR were labeled with \[^{35}S\]methionine (Amersham Biosciences) by in vitro transcription/translation using the TNT-coupled system (Promega Corp.). For binding assays, *E. coli* extracts containing GST fusion proteins were mixed with glutathione agarose at 4°C for 16 h and then washed. GS-Labeled VDRs and 1,25(OH)\(_2\)D\(_3\) were added to the beads in GST binding buffer [50 mM Tris buffer (pH 7.5) containing 100 mM KCl, 10 mM MgCl\(_2\), 0.3 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol] and incubated at 4°C for 16 h. The agarose beads were then washed three times with binding buffer. Bound proteins were eluted in 2× lithium dodecyl sulfate sample buffer, heated at 70°C for 5 min, and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (15%). Gels were fixed in 50% methanol/10% acetic acid for 10 min, incubated in Amplify (Amersham Biosciences) for 15 min, dried, and exposed to Hyperfilm (Amersham Biosciences) at −80°C. Nonspecific binding was determined using extracts containing GST alone.

**Yeast two-hybrid assay**

Plasmids pAS2-WT VDR, pAS2-I268T mutant, and pGAD-RXRa were transformed into *Saccharomyces cerevisiae* strains CG1945 or Y187 cells carrying FROZEN-EZ yeast transformation kit (ZYMO Research, Orange, CA). Diploid colonies were obtained by mating CG1945 with Y187 cells on medium lacking leucine, tryptophan, and histidine and containing 5 mM 3-aminotriazole. Clones were grown overnight at 30°C in selective medium, then diluted 1:20 in fresh medium with or without 1,25(OH)\(_2\)D\(_3\) and grown for an additional 16–24 h. \(\beta\)-Galactosidase activity was determined using the yeast \(\beta\)-galactosidase assay kit (Pierce Chemical Co.). One unit of \(\beta\)-galactosidase is defined as the amount of enzyme that hydrolyzes 1µmol O-nitrophenyl \(\beta\)-D-galactopyranoside/m at 30°C. \(\beta\)-Galactosidase activity was normalized to the cell culture density determined by measuring the OD\(_{600}\) at the time of harvest.

**DNA binding**

DNA binding was assessed by gel mobility shift assays. The human osteopontin VDRE was end-labeled using \[^{32}P\]ATP and polynucleotide kinase. WT and I268T VDRs were expressed in COS-7 cells. Cell extracts were prepared by incubating the cell pellet in M-PER extraction buffer containing 300 mM KCl, 5 mM dithiothreitol, and a protease inhibitor tablet for 10 min. Cell extracts were incubated with vehicle (ethanol) or 10 mM 1,25(OH)\(_2\)D\(_3\) in buffer containing 0.25 µg/ml polynucleosome-deoxyribonuclease for 20 min. The \[^{32}P\]labeled osteopontin-VDRE probe was added for an additional 20 min. All incubations were performed at ambient temperature. The final concentration of salt in the binding assay was 150 mM KCl. For supershift assays, a monoclonal antibody against the C terminus of the VDR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added before addition of the probe. The samples were then electrophoresed on 5% polyacrylamide gels (acrylamide/bis-acrylamide, 29:1) in 0.5× Tris-borate buffer at 180
V for 90 min at ambient temperature. The gel was then dried and subjected to autoradiography at −80°C.

Protease sensitivity assays

WT and mutant VDRs were synthesized and labeled in vitro with [35S]methionine (1000 Ci/mmol) using the TNT-coupled transcription/translation system (Promega Corp.). The translated receptor preparations were incubated with 1,25(OH)₂D₃ or 20-epi-1,25(OH)₂D₃ for 10 min at ambient temperature. Next, trypsin was added to a concentration of 20 μg/ml, and the mixtures were incubated for 10 min. The digestion products were then separated by SDS-PAGE and detected by autoradiography.

Results

Case history

Informed consent for this study was obtained from the patient's parents using Stanford University institutional review board-approved protocols. The parents declined to provide their own DNA samples and would not consent to a skin biopsy of the affected child. The patient, a young Saudi girl, first presented to her local hospital at age 11 months with evidence of severe rickets, inability to walk, multiple fractures, and recurrent pulmonary infections. Her family has a history of consanguinity. The parents did not have bone disease; however, two older children died at ages 5 and 6 yr of a condition similar to that of the younger girl. None of the affected children had alopecia. The patient's blood tests at that time showed a calcium of 1.89 mmol/liter (normal, 2.1–2.6), phosphate of 1.4 mmol/liter (normal, 1–1.5), and alkaline phosphatase of 5080 U/liter (normal, 60–300). Her 1,25(OH)₂D₃ level was 168 pmol/liter (normal, 39–102), and PTH was 368 pg/ml (normal, 10–65). X-rays showed severe rickets, chest deformities, and evidence of restrictive lung disease. She was treated with oral calcitriol, calcium, and phosphate and discharged.

At 20 months of age she was severely ill and presented to the local hospital with severe osteopenia, inability to walk, and fractures. She had chest deformities, restrictive lung disease requiring oxygen, and failure to thrive. On admission at that time her calcium was 1.5 mmol/liter, phosphate was 0.9 mmol/liter, alkaline phosphatase was 1570 U/liter, 25-hydroxyvitamin D [25(OH)D] was less than 9 nmol/liter (normal, 22–116), indicating severe vitamin D deficiency, 1,25-(OH)₂D was 150 pmol/liter (normal, 39–102), and PTH was 840 pg/ml.

The patient received a total of 1.2 million IU vitamin D by injection over 12 wk that brought her 25(OH)D levels almost to normal range. However, she did not show clinical improvement with normalization of 25(OH)D and oral calcium. She received up to 10 μg calcitriol twice daily with no response. When she did not improve on oral calcium and calcitriol, she was started on high dose calcium iv infusions of 1000 mg/m² nightly plus total parenteral nutrition. Over calcitriol, she was started on high dose calcium iv infusions

After repeated infections from her indwelling catheter, the iv therapy was discontinued, and she was discharged from the hospital on oral calcium (4 g elemental calcium daily and phosphate). However, after several months off iv infusions, her rickets began to recur. Her PTH rose to 620, alkaline phosphatase to 1136, and x-rays again showed osteopenia. She has been readmitted for repeat iv calcium infusion therapy every 6–8 wk as signs of rickets recur when the infusion is discontinued, heralded by rising alkaline phosphatase and PTH levels.

DNA sequence analysis and genotype

In our initial studies we examined the patient's DNA for mutations in the VDR gene and identified a novel T to C missense mutation in exon 7. The mutation occurs in helix H5 in the VDR LBD and changes isoleucine to threonine at amino acid 268 (I268T; Fig. 1). The mutation generated a unique Ddel restriction site in exon 7. We amplified exons 7–8 by PCR and used Ddel to determine whether the patient was homozygous for the mutation. As shown in Fig. 2, Ddel digestion of exons 7–8 from a normal control showed two bands of 306 and 180 bp (the smaller size fragments are not visible on the figure). Ddel digestion of exons 7–8 from the patient showed a smaller size fragment of 271 bp and the 180-bp fragment consistent with the presence of a new Ddel site. Because the normal WT fragment is not present, the data demonstrate that the patient is homozygous for the mutation.

DNA samples from the parents or siblings were not available for analysis.

We recreated the I268T mutation in the WT VDR cDNA by site-directed mutagenesis and expressed the mutant VDR cDNA in COS-7 cells. We then examined the effects of the

![A. Nucleotide Sequence](image_url)

**Patient's VDR (264)**

**KSSATEVIM**

**wt VDR (264)**

**KSSAIEVIM**

![B. Amino Acid Sequence](image_url)

**Mutation**

![C. VDR Ligand-Binding Domain](image_url)

Fig. 1. Sequence analysis of the patient's VDR uncovers a missense mutation in exon 7. Exon 7 was amplified from the patient's DNA by PCR and cloned into pBluescript II KS⁺. A, Sequence of the sense strand with the T to C mutation indicated. B, The mutation changed isoleucine to threonine at amino acid 268 (I268T). C, Schematic representation of the VDR LBD and location of the I268T mutation. α Helices are shown as rectangles, with the helix number inside.
I268T mutation on VDR function to determine whether the mutation is the molecular cause of the 1,25(OH)2D3 resistance in this patient.

Transactivation

Because the mutation was expected to cause a loss or reduction in VDR function, we first determined the effects of the I268T mutation on VDR transactivation. As shown in Fig. 3, in transactivation assays the I268T mutant VDR required approximately 65-fold more 1,25(OH)2D3 to induce gene transcription from a 24-hydroxylase promoter reporter compared with the WT VDR (average EC50, \(10^{-8}\) M for I268T mutant vs. \(10^{-9}\) M for WT VDR). Western blot confirmed equal expression of the VDR proteins (data not shown). These results demonstrate that the I268T mutation causes 1,25(OH)2D3 resistance that can be overcome with very high concentrations of ligand.

Ligand binding

From the crystallographic studies of the holo-VDR, I268 has been postulated to interact with 1,25(OH)2D3 and be involved in the repositioning of helix H12 for coactivator interaction (4). A model depicting the location of the amino acids contacting 1,25(OH)2D3 is shown in Fig. 4. I268 forms van der Walls interactions with C22 in the side-chain of 1,25(OH)2D3. Also, I268 forms hydrophobic interactions with residues in helix H12. Based on these data, we next determined the effects of the mutation on ligand binding using [3H]1,25(OH)2D3 binding assays and coactivator binding using GST pull-down assays. Figure 5 shows the result of a representative Scatchard analysis of the ligand binding data. The I268T mutant VDR bound [3H]1,25(OH)2D3 with a dissociation constant (Kd) of \(8 \times 10^{-10}\) M assayed at the same time. This is an approximately 5-fold lower affinity than the WT VDR Kd of \(8 \times 10^{-9}\) M assayed at the same time.

Coactivator binding

We also determined whether coactivator binding to the VDR was compromised by the I268T mutation. As shown in Fig. 6, in GST pull-down assays the WT VDR bound SRC-1 and DRIP205 in a ligand-dependent manner. In contrast, the I268T mutant VDR exhibited reduced binding to both SRC-1 and DRIP205. At 1–10 nM 1,25(OH)2D3, the I268T mutant VDR was unable to bind to SRC-1 or DRIP205, in contrast to the WT VDR that bound these coactivators at these 1,25(OH)2D3 concentrations. However, as the 1,25(OH)2D3 concentration was raised to 100 nM, the mutant receptor was able to bind the coactivators. The I268T mutant VDR required approximately 100-fold higher concentrations of 1,25(OH)2D3 for binding to occur compared with the WT VDR. These results coupled with the transactivation data demonstrate that the I268T mutation causes a defect in coactivator binding that reduces the receptor’s ability to activate gene transcription in addition to its effects on ligand binding.

RXR heterodimerization

The I268T mutation is also located near the E1 domain (amino acids 244–263), which has been shown to be involved in VDR-RXR heterodimerization (21, 22). To determine
whether the I268T mutation affects VDR-RXR heterodimerization, we used GST pull-down and yeast two-hybrid assays. As shown in the GST pull-down assay, WT VDR binds RXR in the absence of ligand and shows an increase in RXR binding concomitant with increasing concentrations of 1,25(OH)2D3 (Fig. 7). The I268T mutant VDR also binds RXR in the absence of 1,25(OH)2D3; however, RXR binding did not increase with incremental ligand until the ligand concentration was raised to 10 nM 1,25(OH)2D. We also examined VDR-RXR interaction using the yeast two-hybrid assay (23). In these assays the I268T mutant VDR consistently bound RXR slightly better than the WT VDR in the absence of 1,25(OH)2D3 (Fig. 7). However, as the concentration of 1,25(OH)2D3 was increased, the I268T mutant did not show an increase in RXR binding until the concentration was greater than 10 nM 1,25(OH)2D3. We also examined VDR-RXR interaction using the yeast two-hybrid assay (23). In these assays the I268T mutant VDR consistently bound RXR slightly better than the WT VDR in the absence of 1,25(OH)2D3 (Fig. 7). However, as the concentration of 1,25(OH)2D3 was increased, the I268T mutant did not show an increase in RXR binding until the concentration was greater than 10 nM 1,25(OH)2D3. In addition, the I268T mutant VDR was not able to achieve the maximal activity that the WT VDR exhibited at the concentrations tested. As a control we also included an F251C mutant VDR that we have previously shown to have reduced affinity for 1,25(OH)2D3 and a significant defect in RXR binding even at high concentrations of 1,25(OH)2D3 (13). We also determined whether the transactivation function of the I268T mutant VDR could be restored by the addition of excess RXR. As shown in Fig. 7, coexpression of RXR slightly augments the transactivation activity of the mutant VDR; however, it did not shift the dose-response curve to WT levels.
DNA binding

Because the I268T mutant VDR was defective in heterodimerization, we examined whether the I268T mutation also affected DNA binding. As shown in Fig. 8, in gel mobility shift assays, no band shifts were observed in the absence of VDR (pSG5 vector alone), in the absence of ligand, or in the presence of 0.1 nM 1,25(OH)2D3 in either WT VDR or I268T mutant VDR. However, both WT and I268T mutant exhibited VDR complex formation as the concentration of ligand increased from 1 to 100 nM 1,25(OH)2D3. Addition of an anti-VDR antibody caused the complexes to supershift, demonstrating the presence of the VDR in the complex. These results indicate that the I268T mutation does not alter the DNA binding function of the mutant receptor.

Sensitivity to proteolytic digestion

We have previously shown that the ability of a ligand to stabilize WT or mutant VDR against proteolytic digestion by trypsin may correlate better with the VDR’s ability to interact with coactivators in vitro and better with its transcriptional activity in cells than with its affinity for VDR (24). Furthermore, we have shown that potent vitamin D analogs may restore stabilization against proteolytic digestion and transcriptional activity of VDRs from HVDRR patients with mutations in the LBD that affect ligand binding (24). The results described above demonstrate that the decrease in affinity of the I268T VDR (5-fold) for 1,25(OH)2D3 is less remarkable than its decrease in ability to be transactivated or to interact with p160 coactivator in a hormone-dependent manner (a

Fig. 7. The I268T mutant VDR binds RXRa. A, Analysis of VDR-RXR binding using GST pull-down assays. In vitro 35S-labeled I268T mutant and WT VDRs were incubated with GST-RXRα bound to glutathione agarose beads and increasing concentrations of 1,25(OH)2D3. After washing, the bound proteins were eluted and subjected to SDS-PAGE and autoradiography. B, Analysis of VDR-RXR binding using the yeast two-hybrid system. Diploid strains were obtained by mating S. cerevisiae CG-1945 containing pAS2-VDRs and S. cerevisiae Y187 containing pGAD-RXRα. The diploid cells were grown overnight at 30 °C with or without 1,25(OH)2D3. VDR-RXR interactions were determined by analyzing β-galactosidase activity. An F251C mutant VDR defective in RXR binding was used as a control. Error bars represent ±SD. C, Co-expression of RXRα increases the transactivation activity of the I268T mutant VDR. VDR and RXRα expression plasmids were cotransfected into COS-7 cells and incubated with increasing concentrations of 1,25(OH)2D3. Error bars represent ±SD.

Fig. 8. The I268T mutant VDR binds to the osteopontin VDRE. COS-7 cells extracts containing I268T mutant and WT VDRs were incubated with 32P-labeled osteopontin VDRE with and without 1,25(OH)2D3. The bound complexes were then resolved on nondenaturing gels. In some samples, a monoclonal antibody against the VDR was added to supershift the complex.
100-fold decrease). Therefore, we investigated whether the I268T mutation affects the ability of the VDR to be stabilized in a transcriptionally active conformation by 1,25(OH)2D3 and whether this function can be rescued by the potent analog, 20-epi-1,25(OH)2D3. As shown in Fig. 9, a major protease-resistant band from the WT VDR was stabilized by 10 nM 1,25(OH)2D3 and 10 nM 20-epi-1,25(OH)2D3. In contrast, protease-resistant bands from the I268T mutant VDR were protected when 10 nM 20-epi-1,25(OH)2D3 was added, but not with 1,25(OH)2D3. When the mutant VDR was tested over a range of different ligand concentrations, the natural hormone protected a smaller molecular weight fragment only when 1000 nM 1,25(OH)2D3 was added. Lower concentrations of 1,25(OH)2D3 were ineffective in protecting the I268T mutant VDR from proteolysis. In contrast, addition of 20-epi-1,25(OH)2D3 protected two major fragments, the smaller fragment observed with 1,25(OH)2D3 and a higher molecular weight fragment observed in the WT VDR. These fragments were protected over a wide range of 20-epi-1,25(OH)2D3 concentrations (Fig. 9). These data demonstrate that the vitamin D analog, 20-epi-1,25(OH)2D3, stabilized the I268T mutant receptor at least 100 times more effectively than 1,25(OH)2D3.

Transcriptional activity of the I268T mutant in response to 20-epi-1,25(OH)2D3

To determine whether the better ability of the 20-epi-1,25(OH)2D3 analog to stabilize conformation of I268T VDR against proteolytic digestion was reflected in rescue of transcriptional activity of this mutant, we performed transfection experiments. As shown in Fig. 10, when COS-7 cells were transfected with the WT VDR and the 24-hydroxylase promoter luciferase reporter gene and WT or mutant VDR expression vectors. Left panel, WT VDR treated with 1,25(OH)2D3 (○) or 20-epi-1,25(OH)2D3 (□). Middle panel, I268T mutant VDR treated with 1,25(OH)2D3 (■) or 20-epi-1,25(OH)2D3 (▲). Right panel, Comparison of WT VDR treated with 1,25(OH)2D3 (○) with I268T mutant VDR treated with 20-epi-1,25(OH)2D3 (■). Each transfection experiment was performed in triplicate, and each titration was performed twice. A representative experiment is shown. Error bars represent ± SD.

WT VDR treated with 1,25(OH)2D3 [EC50, 0.18 nM for 20-epi-1,25(OH)2D3 for I268T and 0.1 nM for 1,25(OH)2D3 for WT VDR]. These results demonstrate that the analog exhibits increased potency in the mutant and is able to achieve transactivation potency similar to that of 1,25(OH)2D3 in the WT VDR (Fig. 10).

Discussion

The young girl described here exhibited the classical clinical pattern of HVDRR. She initially presented with early-onset rickets, secondary hyperparathyroidism, hypocalcemia, and elevated serum 1,25(OH)2D3 levels, but no alopecia. In analyzing the VDR from this patient, we identified a novel mutation, I268T, as the molecular cause of her vitamin D resistance. Our studies showed that the I268T mutation affects ligand binding, RXR heterodimerization, and coactivator binding. Although we were unable to obtain DNA from the parents, we presume each parent to be heterozygous for the mutation, because this is a recessive disease. The family has a history of consanguinity.

From crystallographic studies of the VDR and other members of the steroid receptor superfamily, the LBDs of these receptors are composed of 11 or 12 α-helices forming a hydrophobic core that is occupied by the ligand (4). In the VDR, the ligand binding pocket is formed by helices H1, H3, H4, H5, H7, H8, H9, H10, and H11 (4). The I268T mutation described here occurs in the LBD and is located in helix H5. Figure 4 shows the amino acids in the VDR LBD that interact with the ligand. The amino acids V234, I268, H397, and Y401, which directly interact with 1,25(OH)2D3, are also involved in the hydrophobic stabilization of helix H12 (4). The fact that these residues participate in both ligand binding and helix H12 repositioning suggests that the ligand may control helix H12 repositioning through these amino acids.
The repositioning of helix H12 is a critical event that occurs as a consequence of ligand binding and is essential for transactivation. Repositioning of helix H12 involves both hydrophobic contacts and polar interactions and is essential for creating the correct surface interface for coactivator binding. The amino acids residues involved in the hydrophobic contacts include residues T415, L417, V418, L419, V421, and F422 from helix H12; residues D232, V234, S235, I238, and Q239 from helix H3; residues A267 and I268 from helix H5 and residues H397 and Y401 from helix H11 (4). Replacement of the hydrophobic isoleucine residue I268 with a nucleophilic threonine residue I268T would be expected to disrupt the hydrophobic contact between helix H5 and helix H12 and would probably prevent formation of the hydrophobic cleft needed for coactivator binding. Our data clearly demonstrate that the I268T mutation inhibits coactivator binding at low concentrations of 1,25(OH)2D3. In fact, the amount of I268T mutant VDR to RXR is defective. However, it cannot be ruled out that additional factors may exist that contributed to these findings.

An interesting finding in this case of HVDRR is the fact that the patient does not have alopecia. Alopecia is found in many, but not all, cases of HVDRR reported to date (2). Molecular analysis of HVDRR cases provides new insights into the association of VDR defects with alopecia. Alopecia is found in all HVDRR patients who have premature termination codons that truncate the VDR and in all patients who have DBD mutations (2). Alopecia is also found in HVDRR patients who have mutations in the VDR LBD that prevent VDR from binding to RXR (11, 13, 26). In cases in which alopecia was not associated with HVDRR, the molecular cause of the disease was due to mutations within the LBD. Three patients without alopecia had mutations in contact points for the ligand. R274, the contact point for the 1-hydroxyl group, was mutated to R274L (10); H305, the contact point for the 25-hydroxyl group, was mutated to H305Q (12); and W286, which contacts the conjugated triene connecting the A and C rings, was mutated to W286R (27). A fourth patient without alopecia had an I314S mutation. I314 does not directly contact the ligand, but the mutation changed the ligand binding affinity of the receptor and interfered modestly with RXR heterodimerization (11).
Acknowledgments

Received January 23, 2004. Accepted August 6, 2004. Address all correspondence and requests for reprints to: Dr. Peter J. Malloy, Division of Endocrinology, Gerontology and Metabolism, Stanford University School of Medicine, Stanford University Medical Center, Room 50225, Stanford, California 94305-5103. E-mail: malloy@cmgm.stanford.edu.

This work was supported by National Institutes of Health Grants DK-42482 (to D.F.) and DK-50583 (to S.P.).

References


25. Nakajima S, Hsieh JC, MacDonald PN, Galligan MA, Haussler CA, Whitfield GK, Haussler MR 1994 The C-terminal region of the vitamin D receptor is essential to form a complex with a receptor auxiliary factor required for high affinity binding to the vitamin D-responsive element. Mol Endocrinol 8:159–172


Endocrinoiy is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.