Hereditary Vitamin D Resistant Rickets Caused by a Novel Mutation in the Vitamin D Receptor That Results in Decreased Affinity for Hormone and Cellular Hyporesponsiveness

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Abstract

Mutations in the vitamin D receptor (VDR) result in target organ resistance to 1α,25-dihydroxyvitamin D [1,25(OH)$_2$D$_3$], the active form of vitamin D, and cause hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR). We analyzed the VDR of a patient who exhibited three genetic diseases: HVDRR, congenital total lipodystrophy, and persistent müllerian duct syndrome. The patient was treated with extremely high dose calcitriol (12.5 μg/d) which normalized serum calcium and improved his rickets. Analysis of [^{3}H]1,25(OH)$_2$D$_3$ binding in the patient's cultured fibroblasts showed normal abundance of VDR with only a slight decrease in binding affinity compared to normal fibroblasts when measured at 0°C. The patient's fibroblasts demonstrated 1,25(OH)$_2$D$_3$-induction of 24-hydroxylase mRNA, but the effective dose was approximately fivefold higher than in control cells. Sequence analysis of the patient's VDR gene uncovered a single point mutation, H305Q. The recreated mutant VDR was transfected into COS-7 cells where it was 5 to 10-fold less responsive to 1,25(OH)$_2$D$_3$ in gene transactivation. The mutant VDR had an eightfold lower affinity for [^{3}H]1,25(OH)$_2$D$_3$ than the normal VDR when measured at 24°C. RFLP demonstrated that the patient was homozygous for the mutation while the parents were heterozygous. In conclusion, we describe a new ligand binding domain mutation in the VDR that causes HVDRR due to decreased affinity for 1,25(OH)$_2$D$_3$ which can be effectively treated with extremely high doses of hormone. (*J. Clin. Invest.* 1997. 99:297–304.) Key words: receptors • calcitriol • steroid • vitamin D–dependent rickets, type II • 1,25-dihydroxyvitamin D

Introduction

Vitamin D is essential for promoting calcium transport across the small intestine and for the normal development of bone. The hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] mediates these biological actions by binding to the vitamin D receptor (VDR),[1] a nuclear transcription factor that regulates gene expression. Hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR), also known as vitamin D–dependent rickets, type II, is an autosomal recessive disorder that has been shown to be caused by mutations in the VDR (1, 2). The disease is characterized by early onset rickets, hypocalcemia, secondary hyperparathyroidism, elevated 1,25(OH)$_2$D$_3$ levels, and resistance to 1,25(OH)$_2$D$_3$ treatment (3, 4). Earlier research assaying [^{3}H]1,25(OH)$_2$D$_3$ binding in skin fibroblasts from affected individuals demonstrated two types of binding defects. In some cases fibroblasts were devoid of [^{3}H]1,25(OH)$_2$D$_3$ binding (so-called receptor negative phenotype) and in other cases fibroblasts had normal [^{3}H]1,25(OH)$_2$D$_3$ binding (so-called receptor positive phenotype) (1–18). In the receptor positive cases the VDR was shown to exhibit a decreased binding affinity for DNA. The cloning and sequencing of the human VDR cDNA (19) has provided the basis to elucidate the molecular abnormalities in affected individuals. The identification of the specific mutations in the gene encoding the VDR (20–32) has demonstrated that heterogeneous genetic defects in the VDR cause HVDRR.

The VDR is a member of the steroid/thyroid/retinoid receptor gene superfamily of transcription factors and shares a common structural and functional organization with the members of the thyroid/retinoid subgroup of receptors that includes the retinoic acid receptor (RAR), the retinoid x receptor (RXR), and the thyroid receptor (TR) (33, 34). The structural organization of VDR includes a highly conserved amino-terminal DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD). In addition, the VDR must form a complex with RXR in order to transactivate 1,25(OH)$_2$D$_3$ responsive genes via vitamin D response elements (VDREs) although recent evidence suggests that other co-activating factors are also involved. The heterodimerization of VDR with RXR is thought to occur via a region of the LBD that contains a 9-heptad repeat, a common structural motif found in this subfamily of nuclear hormone receptors (35).

To date, a total of 13 mutations causing HVDRR have been characterized (20–26, 28–32). The HVDRR syndrome is inherited as a recessive trait that requires two mutant VDR alleles in order to be expressed. Heterozygotic individuals appear to be phenotypically normal. Most of the mutations have been found in the DBD, a location that does not affect the

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1. *Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; HVDRR, hereditary 1,25-dihydroxyvitamin D resistant rickets; LBD, ligand-binding domain; DBD, DNA-binding domain; OP, osteopontin; RXR, retinoid x receptor; TR, thyroid receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

ligand-binding properties of the receptor and hence were referred to earlier as the receptor positive phenotype. These mutations cause changes that interfere with the high affinity binding of the VDR to DNA, although the exact mechanism has not yet been elucidated. When these defective receptors are co-expressed in CV-1 or COS-7 cells along with a VDRE-reporter plasmid, the mutant receptors are unable to activate the transcription of the reporter gene even in the presence of very high concentrations of hormone (23, 25, 29, 30, 32, 36). On the other hand, only four mutations have been identified in the LBD (21, 22, 26, 28, 30). Two of these mutations, identified in individuals with the receptor negative phenotype, were stop mutations which resulted in the premature termination of the protein coding sequence and deletion of portions of the LBD (21, 22, 28, 30). One of these stop mutations was shown to have a secondary affect on VDR transcription and/or message stability that resulted in absent or diminished mRNA (22). The first missense mutation identified in the LBD, R271L, was found in a patient who was unresponsive to vitamin D_3 therapy (28). Cultured fibroblasts from the patient failed to induce 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) when exposed to as much as 1 μM 1,25$(OH)_2D_3$. Interestingly, when this mutation was recreated and expressed in CV-1 cells, the mutant receptor was capable of transcriptional activation. However, the mutant receptor required 1,000-fold more hormone than the normal receptor in order to respond in these assays (28). The authors speculated that this mutation decreased the affinity of the receptor for 1,25$(OH)_2D_3$ by a factor of 1,000 although the ligand binding data were not presented. A C190Y mutation has also been identified in the VDR of a patient with HVDRR; however, details concerning hormone binding or functional properties of the mutant receptor were not described (26).

In this report, we update our recent findings on a child with HVDRR (37). We now describe the presence of a novel mutation (H305Q) in the VDR gene that results in a modest decrease in the affinity of the receptor for 1,25$(OH)_2D_3$ when measured at 0°C (approximately twofold) and a substantial decrease when measured at 24°C (approximately eightfold). The mutation leads to cellular resistance and decreased responsiveness to hormone. This type of mutation is unique in that it marks the first time that a patient with HVDRR has shown clinical improvement when treated with very high doses of 1,25$(OH)_2D_3$ which overcome the affinity defect and render the patient's cells responsive to hormone. The case is also unique in that the child has three rare recessive genetic diseases.

Methods

Culture conditions. Human skin fibroblasts and COS-7 cells were incubated at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% FBS and appropriate antibiotics.

[³H]1,25(OH)₂D₃ receptor binding assay. [³H]1,25$(OH)_2D_3$ binding was assayed by incubating cytosol with radiolabeled ligand in the absence or presence of a 250-fold excess of radioinert 1,25$(OH)_2D_3$ as previously described in reference 17. Hydroxylapatite was used to separate bound and free hormone. Protein concentration was determined according to Bradford's method (38).

Northern blot analyses. Fibroblasts were grown to confluence and then treated with 1,25$(OH)_2D_3$ for 6 h in media containing 1% FBS. RNA was prepared as described in reference 22. Total RNA (5 μg) was electrophoresed on 1% agarose gels and then transferred to nylon filters. RNA was immobilized on the filter by ultraviolet irradiation. The filter was then hybridized with the 24-hydroxylase and L7 ribosomal RNA probes labeled by the random primer method as described in reference 39. L7 has been shown in multiple experiments to be unaffected by 1,25$(OH)_2D_3$ treatment and therefore can serve as a control for loading and transfer.

PCR and DNA sequencing. Exons seven and eight were amplified from genomic DNA using the oligonucleotide primers and PCR conditions as described in reference 22 except that the amplification steps were reduced to 30 s each. The PCR product was cloned and sequenced using [α³⁵S]dATP and Sequenase Version 2.0 DNA sequencing kit (Amersham Corp., Arlington Heights, IL). The amino acid numbering system used in this paper is consistent with that previously described by Baker et al (19).

AlwNI RFLP. The PCR amplified products were digested with the restriction enzyme AlwNI at 37°C in the buffer supplied by the manufacturer (New England Biolabs Inc., Beverly, MA). Digestion products were resolved on 6% polyacrylamide gels and visualized by silver staining (Bio-Rad Laboratories, Richmond, CA) (22).

Site-directed mutagenesis and construction of VDR expression vectors. Site-directed mutagenesis of the wild-type VDR cDNA was done using the pALTER system (Promega Corp., Madison, WI) as described in reference 32. The mutagenic oligonucleotide used was 5′-AAGCCGGACAGAGCCTGGAGC (the mutated base is underlined). Plasmid DNA was isolated and sequenced to confirm the presence of the desired point mutation in the VDR cDNA. The mutated VDR cDNA was then subcloned into the mammalian expression vector pEUK-C1 (Clontech, Palo Alto, CA) (32).

VDR, VDRE-CAT cotransfection assay. COS-7 cells, in 60 mm plates, were transfected with plasmid DNA using LipofectAMINE (Gibco BRL, Gaithersburg, MD) as described in reference 32. A chloramphenicol acetyltransferase (CAT) ELISA (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to determine the amount of CAT protein produced in the assay (32). β-galactosidase activity was determined as described in reference 32.

In vitro production of normal and mutant VDR. The normal and H305Q mutant VDR cDNAs cloned in pBluescript KSII (Stratagene, San Diego, CA) were amplified by PCR using primers: 5′-GGGATCCATGGAGGCAATGGCGGCCAGCACTTCCCTGCCT and 5′-GAGGTCGACTAGTCAGGAGATCTCATT. The PCR products were digested with BamHI and XhoI and cloned into pCITE 3A (Novagen, Inc., Madison, WI). The normal and mutant proteins were produced by coupled in vitro transcription and translation of the VDR cDNA templates using the Single Tube Protein System 2 along with T7 RNA polymerase according to the manufacturer's instructions (Novagen, Inc.).

Electrophoretic mobility shift assay (EMSA). Cell extracts of *Saccharomyces cerevisiae* CB023 expressing the human RXRα cDNA via the yeast expression vector pG5 (40) was used as the source of RXRα. For EMSA, 5 μl of in vitro translated VDR or 10 ng of partially purified VDR (Panvera Corp., Madison, WI) was first incubated at ambient temperature for 30 min with 10 μg yeast extract containing RXRα in 20 mM Tris, pH 7.9, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM DTT, 50 μg/ml poly dI–dC in the presence of 1–10 nM 1,25$(OH)_2D_3$ or ethanol vehicle control. After the initial incubation, complementary annealed oligonucleotides corresponding to the human osteopontin VDRE (OP-VDRE) nucleotide sequence (5′-CCTGTAAAGGGTCGTATGGTTCATCTCA) (41) were added and the incubation continued for an additional 20 min. Before annealing one of the oligonucleotides was labeled using [γ-³²P]-ATP (3,000 Ci/mmol, Amersham Corp.) and T4 polynucleotide kinase (New England Biolabs Inc.). The final volume of the binding reaction was 30 μl. In some samples the VDR mAb, 9A7γ, was added prior to the addition of labeled OP-VDRE or a 200-fold excess of unlabeled OP-VDRE was added. In other control samples, in vitro translated β-galactosidase or partially purified VDR (PanVera Corp.) was substituted for in vitro translated VDR. For EMSA, 20 μl of the binding mixture was applied to a 6% polyacrylamide gel

Table I. Family Clinical Data

	Patient	Sister	Mother	Father
Phenotype	HVDRR	HVDRR	Normal	Normal
Genotype	Homozygous	Homozygous	Heterozygous	Heterozygous
Treatment	Rocaltrol 12.5 µg/d	Dedrogyl 150 µg/d	—	—
Serum concentrations*				
Calcium (8.5–10.4 mg/dl)	8.6	9.8	10.2	10.1
$25(OH)_2D_3$ (7–60 ng/ml)	< 2.5	38	8	13
$1,25(OH)_2D_3$ (20–80 pg/ml)	1,491	2,021	120	110
PTH (3–40 pg/ml)	20	36	12	9
Osteocalcin (11–43 ng/ml)	35	51	31	37

*Normal ranges are given in parentheses.

and electrophoresed for 90 min at 85 V at ambient temperature in 0.5× Tris–borate EDTA buffer system (1× = 0.045 M Tris borate, 0.001 M EDTA). After electrophoresis, the gel was dried onto filter paper and exposed to Hyperfilm (Amersham Corp.) at −70°C before development.

Results

Case report. The patient described in this report, a Turkish boy born to first cousins, initially presented at two years of age with rickets and subsequently was diagnosed with HVDRR. As recently described by Van Maldergem et al. (37), the boy has three rare genetic diseases: HVDRR, total lipodystrophy, and persistent müllerian duct syndrome. In brief, the child exhibited the classical features of HVDRR, including severe rickets, hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and elevated circulating $1,25(OH)_2D_3$ levels. Interestingly, he was unresponsive to a one month treatment regimen with cholecalciferol (4,800 IU/d) followed by a trial with conventional doses of Rocaltrol (37).

Since the initial report, additional clinical data on this patient and other members of his immediate family have been obtained (Table I). The patient's sister also has been diagnosed with HVDRR. The patient is currently being treated with 12.5 µg Rocaltrol per d, while his sister is being treated with 150 µg of Dedrogyl [calcifidiol or $25(OH)D_3$] per d. The children both have responded to these treatments with restoration of eucalcemia, suppression of elevated PTH and healing of rickets (Table I). The $25(OH)D_3$ serum values of the patient and sister generally reflect the particular treatment regimen that they were receiving, i.e., the $25(OH)D_3$ level in the patient on Rocaltrol went to < 2.5 ng/ml while the sister's $25(OH)D_3$ levels on Dedrogyl were normal (38 ng/ml). During treatment, the serum $1,25(OH)_2D_3$ levels in each child have been maintained at concentrations 20- to 25-fold above the normal range. Since this therapy has only normalized the serum calcium, the lack of hypercalcemia in the presence of serum concentrations of $1,25(OH)_2D_3$ greater than 1,000 pg/ml, clearly demonstrated severe resistance to the action of the hormone (Table I).
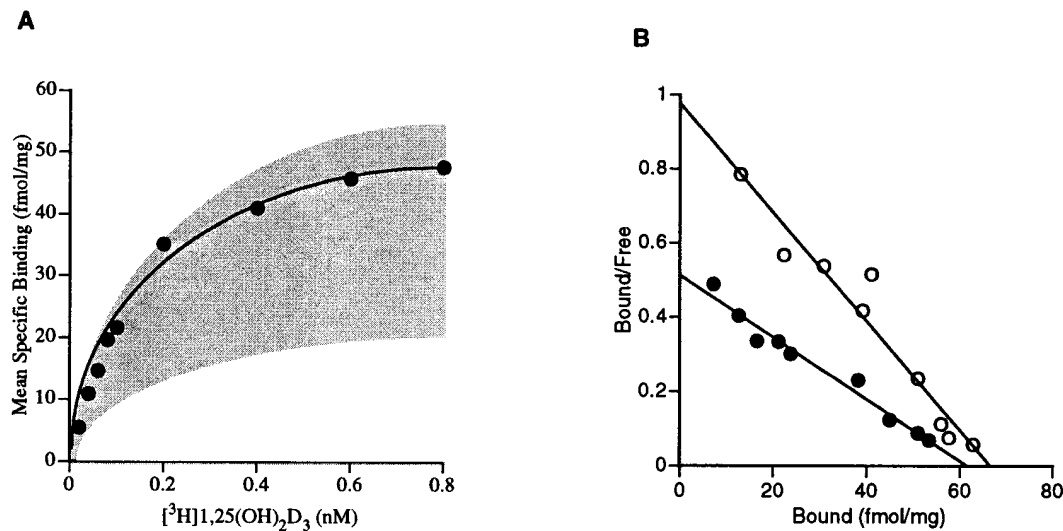

Figure 1. Saturation analysis and Scatchard plot of specific $[^3H]1,25(OH)_2D_3$ binding in the patient's fibroblasts. Cytosol from fibroblasts of the patient and normal controls were incubated at 0°C with $[^3H]1,25(OH)_2D_3$ and then the bound and free hormone were separated by HAP. (*A*) Saturation plot of specific $[^3H]1,25(OH)_2D_3$ binding (shaded area represents the normal range from multiple previous experiments performed in our laboratory); (*B*) Scatchard plot of the binding data from a representative experiment. Symbols are index case (●), and a concurrently assayed normal (○).
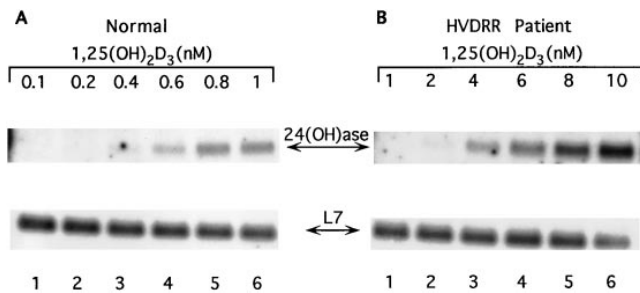
Figure 2. Northern blot analyses of the expression of the 24-hydroxylase mRNA in normal fibroblasts and fibroblasts from the index case with HVDRR. Fibroblasts were treated with the indicated dose of $1,25(OH)_2D_3$ for 6 h in media containing 1% FBS and then RNA was isolated. Northern blots were prepared and then hybridized with labeled 24-hydroxylase and L7 ribosomal protein cDNA probes. The concentration of $1,25(OH)_2D_3$ is shown above the blot. (*A*) Normal control; (*B*) index case. Expression of the L7 ribosomal protein mRNA served as a control. Lane numbers are at the bottom.

[^3H]1,25(OH)$_2$D$_3$-binding. Studies were conducted in fibroblasts cultured from family members to determine whether the cause of the HVDRR in this family was due to a mutation in the VDR. As depicted in Fig. 1, the cultured fibroblasts from the index case showed a normal complement of VDR (60 fmol/mg protein) as determined by [^3H]1,25(OH)$_2$D$_3$ binding assays. The values are at the high end of a range of five normal fibroblasts assayed in our laboratory. However, as compared to VDR from normal fibroblasts, a slight (approximately twofold) decrease in the affinity of the receptor for $1,25(OH)_2D_3$ was demonstrated (K_d $2.3\pm1.3 \times 10^{-10}$M for the patient, $n = 3$, versus K_d $0.52\pm0.18 \times 10^{-10}$M for the controls, $n = 5$). This modest difference in binding affinity was independently shown in our previous study (37) and demonstrates the mild nature of this defect that could be detected by ligand binding when performed at 0°C.

1,25(OH)$_2$D$_3$ response. To ascertain whether the fibroblasts from the patient were responsive to $1,25(OH)_2D_3$, the induction of 24-hydroxylase mRNA, a $1,25(OH)_2D_3$-responsive gene, was assayed by northern blot following treatment of cells with a series of increasing concentrations of hormone (0.1–10 nM $1,25(OH)_2D_3$) for 6 h. Exposure to $1,25(OH)_2D_3$ for 6 h induces normal cells to synthesize the 2.4 kb 24-hydrox-

ylase mRNA (42). As shown in Fig. 2, in normal fibroblasts the 24-hydroxylase mRNA is undetectable at 0.1 nM $1,25(OH)_2D_3$ (Fig. 2, *A*, lane *1*). However, when the concentration of the hormone is raised to 0.4 nM and higher, the abundance of the transcript increases in a $1,25(OH)_2D_3$ dose-dependent manner (Fig. 2, *A*, lanes *3–6*). In contrast, the 24-hydroxylase mRNA was not detected in the fibroblasts from the patient when the cells were treated with 1 nM $1,25(OH)_2D_3$ (Fig. 2, *B*, lane *1*). The 24-hydroxylase mRNA was detected when the concentration was raised to 4 nM $1,25(OH)_2D_3$ (Fig. 2, *B*, lane *3*). The cells then responded to the increasing amounts of $1,25(OH)_2D_3$ in a dose-dependent manner (Fig. 2, *B*, lane *3–6*). A trace amount of 24-hydroxylase mRNA was detected in the 2 nM $1,25(OH)_2D_3$ lane when the blot was exposed for a longer time. These data demonstrate that the patient's fibroblasts are hyporesponsive to $1,25(OH)_2D_3$ treatment. Approximately fivefold more hormone is required by the patient's fibroblasts to achieve the same level of response as normal fibroblasts. L7 mRNA levels served as control for loading. In other experiments, the affected sister was shown to have resistance to $1,25(OH)_2D_3$ similar to the index case whereas the mother had a normal response (data not shown).

Nucleotide sequence analysis. Since the $1,25(OH)_2D_3$ binding and functional response data indicated that the VDR from this patient was abnormal and northern blots demonstrated the VDR mRNA to be expressed normally and to be the correct size (data not shown), the nucleotide sequence of the VDR gene was examined for mutations in the exons encoding the VDR protein. The findings detailed above, suggesting a defect in the LBD, led us to focus on this region of the VDR. As shown in Fig. 3, a C to G missense mutation was identified in exon eight. This mutation replaces a histidine residue with a glutamine residue at position 305 (H305Q) in the VDR. Sequence analysis of the entire coding region of the VDR gene found no other mutations.

Family analyses by RFLP. To determine whether the sister, who also suffers from HVDRR, had the same mutation as the patient and whether other members of the family were carriers of this mutation, DNA samples from each available family member were obtained and analyzed for the mutation. Since the mutation generates an AlwN I restriction site, we developed an RFLP analysis to detect the mutation using this restriction endonuclease. PCR was used to amplify the region of DNA containing the mutation and these products were then
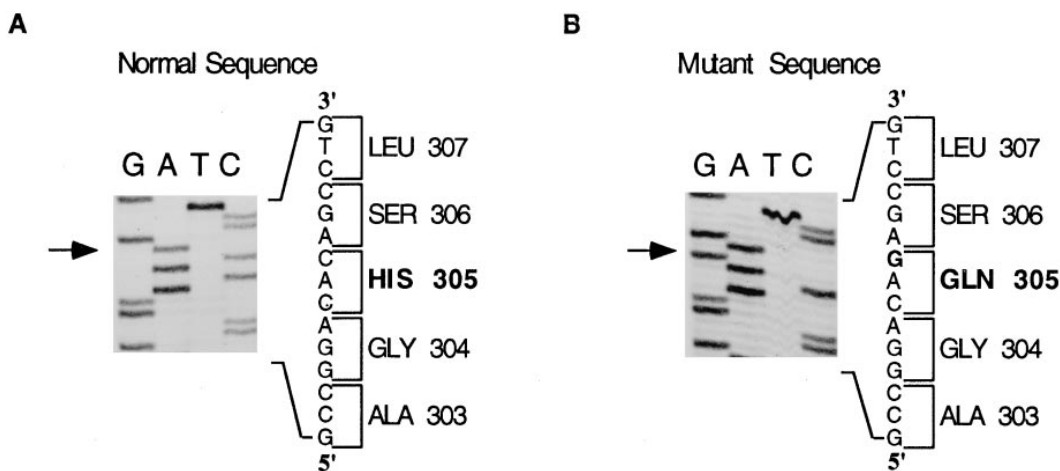

Figure 3. DNA sequence analysis of the VDR gene. Exon 8 DNA from the index case and normal fibroblasts was amplified by PCR and then sequenced. (*A*) Nucleotide sequence of normal VDR; (*B*) nucleotide sequence of the index case. The position of the single C to G missense mutation is indicated by the arrows and the altered amino acid in boldface type.
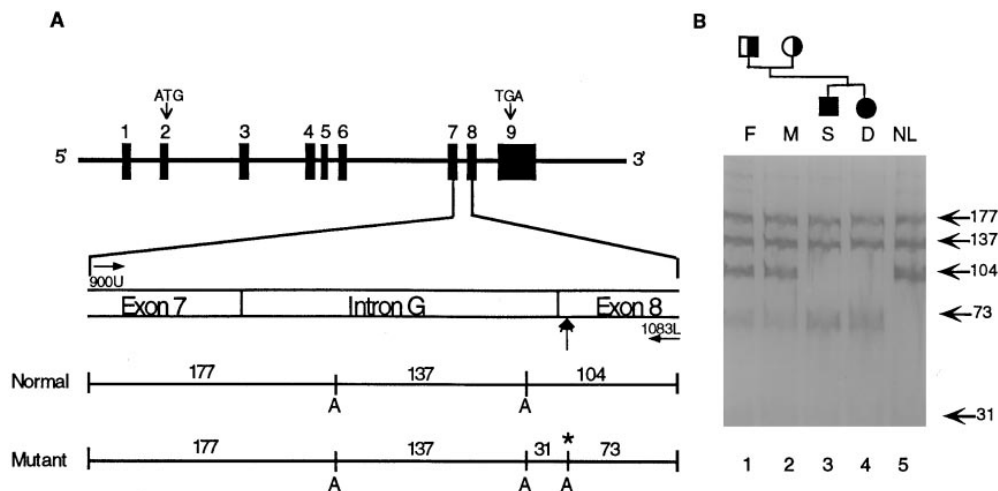
Figure 4. AlwN I RFLP. (*A*) Schematic representation of *AlwN* I digestion showing amplification from genomic DNA by oligonucleotide primers 900U and 1083L to generate a 418 bp fragment. The *AlwN* I (*A*) restriction sites for both normal (*top*) and mutant (*bottom*) alleles are shown. The site that is generated by the point mutation is indicated with an asterisk. (*B*) Polyacrylamide gel electrophoresis of PCR products after digestion with *AlwN* I in the father (*F*, lane *1*), mother (*M*, lane *2*), son (index case) with HVDRR (*S*, lane *3*), daughter with HVDRR (*D*), lane *4*), and a normal control

(*NL*, lane *5*). Fragment size (bp) is shown on the right. The 31 bp band runs as a faint band at the bottom of the gel and is better detected on the gels then the photograph in all lanes but the *NL*.

digested with *AlwN* I (Fig. 4, *A*). The restriction patterns generated from the digests were then analyzed by gel electrophoresis. As predicted from the restriction map (Fig. 4, *A*), a normal control exhibits three bands (177, 137 and 104 bps) due to two *AlwN* I sites in the region (Fig. 4, *B*, lane 5). The patient and his affected sibling, exhibit four bands (177, 137, 73 and 31 but not 104 bps) (Fig. 4, *B*, lanes *3* and *4*). This pattern is consistent with the presence of a third *AlwN* I site created by the mutation in this region (Fig. 4, *A*), and confirms their homozygosity for the mutation. The parents exhibit all five bands (Fig. 4, *B*, lanes *1* and *2*) which demonstrates that they are heterozygous for the mutation and carriers of the mutant allele.

Having determined the nature of the mutation in the VDR gene and established the autosomal recessive inheritance of the defective gene, the mutation was reconstructed in the VDR cDNA by site directed mutagenesis. The effects of this single base change in the VDR on hormone binding, transactivation potential, and ability to complex with RXR and form a heterodimer on a VDRE were examined.

[³H]1,25(OH)₂D₃-binding of recreated mutation. Scatchard analyses of [³H]1,25(OH)₂D₃-binding in COS-7 cells transfected with normal and mutant VDR cDNAs showed that the mutant VDR had an average K_d of 1.2×10^{-10} M which was slighty lower affinity than the normal VDR which had a K_d of 0.9×10^{-10} M when assayed at 0°C ($n = 2$). As shown in Fig. 5, when [³H]-1,25(OH)₂D₃ binding was performed at 24°C, the normal VDR exhibited a K_d of $1.1 \pm 0.3 \times 10^{-10}$ M which was similar to that observed at 0°C. In contrast, the mutant VDR showed a K_d of $8.7 \pm 3.1 \times 10^{-10}$ M at 24°C, approximately eightfold lower than the Kd for the normal VDR assayed at 24°C ($n = 3$).

Transactivation analysis of recreated mutation. Fig. 6 shows the results of transactivation experiments performed in COS-7 cells cotransfected with either the normal or mutant VDR cDNA expression vectors and a VDRE-CAT reporter plasmid. When the cells were transformed with the normal VDR cDNA, a 1,25(OH)₂D₃ dose-dependent (0.5–100 nM) rise in CAT protein was observed. A 3–3.5-fold increase was achieved with a definite response detected at 0.5 nM and a maximum re-

sponse approached at 10 nM 1,25(OH)₂D₃. A 1,25(OH)₂D₃ dose-dependent rise in CAT protein was also observed in the cells transformed with the mutant VDR cDNA. However, a 5–10 fold higher concentration of 1,25(OH)₂D₃ was required by the cells transformed with the mutant VDR to achieve the same effect observed in cells transformed with the normal gene. No response could be detected at 1 nM 1,25(OH)₂D₃. Interestingly, with higher concentrations of 1,25(OH)₂D₃, the
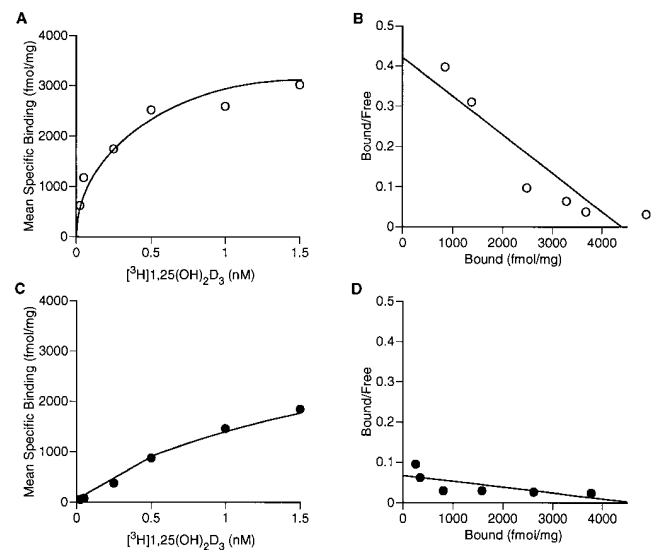

Figure 5. Saturation analysis and Scatchard plot of specific [³H]1,25(OH)₂D₃ binding of normal and mutant VDR expressed in COS-7 cells. Cytosol from COS-7 cells were incubated at 24°C with [³H]1,25(OH)₂D₃ and then the bound and free hormone were separated by HAP. (*A*) Saturation plot of specific [³H]1,25(OH)₂D₃ binding in the normal VDR; (*B*) a representative Scatchard plot of the binding data from normal VDR; (*C*) saturation plot of specific [³H]1,25(OH)₂D₃ binding in the mutant VDR; (*D*) a representative Scatchard plot of the binding data from mutant VDR. Symbols are mutant VDR (●), and normal VDR (○).
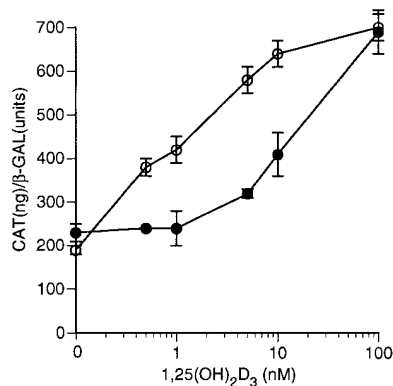
Figure 6. Transcriptional activation of a VDRE reporter plasmid by H305Q mutant VDR. COS-7 cells were cotransfected with the mutant or normal VDR cDNA and a VDRE-CAT reporter plasmid and cultured in 1% FBS. Cells were treated with increasing concentrations of hormone and assayed for $1,25(OH)_2D_3$-dependent transactivation of the CAT gene. Symbols represent normal (○) and H305Q mutant (●) receptors. The data represent the mean±SD of three experiments.

normal maximal CAT response could be achieved. These findings are consistent with the experiments performed in the patient's cultured fibroblasts which showed a diminished $1,25(OH)_2D_3$ response in the induction of 24-hydroxylase gene expression which could be overcome at high concentrations of $1,25(OH)_2D_3$. There are minor differences in the absolute concentration of $1,25(OH)_2D_3$ that the cells respond to, probably reflecting differences between the fibroblast (endogenous genes) and the COS cell (transfected VDR-reporter gene) systems. Overall, the results clearly indicate that the H305Q mutation in the VDR imparts a binding affinity defect with cellular hyporesponsiveness to $1,25(OH)_2D_3$ action that can be overcome at high doses of hormone.

Heterodimerization and VDRE-binding of mutant VDR. Since the location of the H305Q mutation resides in the area of the VDR that is near to and may be involved in heterodimer formation with RXR, the ability of the mutant receptor to heterodimerize with RXR and bind to the human osteopontin

Figure 7. Heterodimerization and DNA-binding activity of H305Q mutant VDR. Normal and H305Q mutant VDR produced by in vitro translation were evaluated for RXRα heterodimer formation and DNA-binding activity by GMSA using 6% polyacrylamide gels as described in Methods. Radiolabeled OP-VDRE sequences were used in the incubation mixture. The VDR-RXR DNA-binding activity is highlighted by the arrow. In vitro produced β-galactosidase (*β-gal*) and partially purified VDR (*pVDR*) served as controls. Lane numbers are at the bottom.

(OP) VDRE was examined by GMSA. Following in vitro transcription of the normal VDR and H305Q mutant VDR cDNAs, the receptor proteins were produced by in vitro translation using reticulocyte lysates. As shown in Fig. 7, a band shift was observed with the wild type VDR and the intensity of the band shift increased as the amount of hormone increased (Fig. 7, lanes *1–3*). The band shift was blocked by addition of anti-VDR mAb or by addition of excess unlabeled OP-VDRE (Fig. 7, lanes *4* and *5*). When the H305Q mutant VDR was tested, a normal band shift was observed which could also be blocked by the antibody or excess unlabeled probe (Fig. 7, lanes *6–10*). In the absence of RXR, neither the wild type VDR nor the H305Q mutant VDR bound to the OP-VDRE (data not shown). No band shift was observed with the β-galactosidase control (Fig. 7, lanes *11–13*) and a strong band shift was demonstrated using partially purified VDR which could be blocked by the anti-VDR mAb or by addition of excess unlabeled oligonucleotide (Fig. 7, lanes *14–16*). These data suggest that the H305Q mutation does not interfere with RXR dimerization or binding to the OP-VDRE.
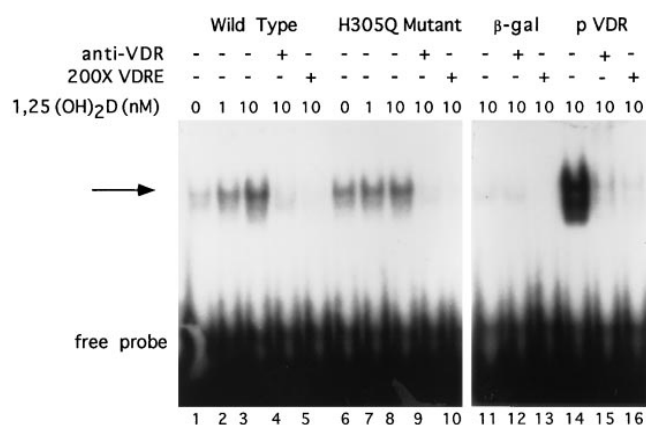
Discussion

In this study, we analyzed the biochemical and molecular properties of the VDR in a family with HVDRR. The index patient exhibited the classical features of the syndrome including early onset rickets, hypocalcemia, secondary hyperparathyroidism and elevated serum $1,25(OH)_2D_3$ levels. The child did not have alopecia, a finding often associated with this disease (4–6, 9, 10, 12, 13, 16, 17, 27, 43–46). Unlike other patients who required intravenous calcium infusions to treat the symptoms of HVDRR because they failed to improve with $1,25(OH)_2D_3$ therapy (46–49), the patient in this study responded to treatment with extremely high doses of Rocaltrol. During treatment, the patient's serum $1,25(OH)_2D_3$ levels rose to extraordinarily high values ($> 1,000$ pg/ml) which provided additional evidence of his resistant state. However, the treatment improved the rickets and restored the patient's calcium levels to normal. Similar results, including the healing of rickets, were observed in the patient's sister who is also affected with HVDRR.

Because of the clinical impression of HVDRR, fibroblasts cultures were established and used to determine whether the VDR from the child harbored a mutation. In cell extracts, $[^3H]1,25(OH)_2D_3$ binding was clearly detectable and the abundance of the receptor was in the high end of the normal range. However, a slight decrease in the affinity of the VDR for $1,25(OH)_2D_3$ could be discerned. To ascertain whether the patient's fibroblasts were resistant to $1,25(OH)_2D_3$, the induction of the 24-hydroxylase gene was assayed as a marker of $1,25(OH)_2D_3$ responsiveness. Interestingly, the cells did respond to the hormone but the concentration required was approximately 5–10-fold higher than the amount of hormone needed by normal cells to achieve the same response. The cells were clearly resistant to 1 nM $1,25(OH)_2D_3$ while the normal cells were responsive to this concentration. The binding and cell response findings suggested that the defect in the VDR was likely to be in the LBD and not in the DBD. Also, most cases with alopecia have mutations in the DBD.

The LBD of the VDR is located in the carboxyl half of the protein and encompasses ~ 300 amino acids. The crystal structures of the LBDs of the related receptors TR (50), RAR (51),

and RXR (52) have been determined and a common structural design for the LBD has emerged. The LBD consists of a hydrophobic core that is composed of 11–12 α-helices (H1–H12) and two to four short β-sheets. Extrapolating from this crystallographic data to the VDR, the mutation H305Q identified in this study would be found within this hydrophobic core. The H305Q mutation would most likely occur in the loop connecting two α-helices together (the loop connecting H6 to H7 in RXR and H7 to H8 in TR) and would appear to disrupt the normal contacts between the protein and the ligand or to destabilize the bound conformation. In either case, the net effect of the H305Q mutation could be envisioned to alter the binding affinity for ligand, thereby possibly decreasing the ability of the receptor to change to the active conformation. However, at higher hormone concentrations, we found that this affinity defect could be overcome and once fully occupied, the receptor can achieve full transcriptional activity.

A comparison of the amino acid sequences in the loop that connects the two α-helices together among members of the thyroid/retinoid subgroup reveals that H305, which is found only in the VDR, aligns to a glycine which is highly conserved at this location among the other receptors. Interestingly, in TR this glycine has been the site of at least four different mutations which leads to generalized resistance to thyroid hormone (53). The TR mutations are inherited as autosomal dominant negatives since only one of the alleles has been mutated. A natural dominant negative mutation has not yet been described in the VDR and in most cases the heterozygotic parents have been reported to have a normal phenotype. In this family, the parents appear to display a very mild form of hormone resistance, since their serum $1,25(OH)_2D_3$ levels are elevated above the normal range (Table I). The H305Q mutant receptor requires a higher hormone concentration to fulfill its role as a transactivator and maintain normal calcium homeostasis. We speculate that the mild hormone resistance in the heterozygous parents leads to increased production of $1,25(OH)_2D_3$ as a consequence of feedback regulation.

Two additional facts about this case are of substantial interest and warrant discussion. First, the presence of three genetic diseases: HVDRR, total lipodystrophy, and persistent mül–lerian duct syndrome, affecting a single individual is highly unusual and extremely unlikely to occur by chance alone. However, at this time we can not explain this statistically remote event. We now know that the HVDRR is the result of a point mutation in the VDR gene, and since the sister has the same genetic defect present in the index case without the association of the other two diseases, we have tentatively concluded that the HVDRR syndrome is inherited independently of the other two genetic defects. However, we have not ruled out that there may be a common connection among the defects in the index case which may involve the VDR.

A second area that requires comment concerns the apparent discrepancy between the mild resistance observed in the patient's cultured fibroblasts with the level of resistance seen in both children. While receiving high dose vitamin D therapy, the $1,25(OH)_2D_3$ serum levels were ∼ 25-fold higher than normal indicating a substantial resistance to the hormone. Ligand-binding could only detect an approximately twofold difference in affinity between the mutant and normal receptors at 0°C but the affinity defect was more pronounced (approximately eight-fold lower) when measured at elevated temperatures (24°C). The difference in hormone responsiveness between the cul-

tured mutant and normal fibroblasts (assayed at 37°C) was ∼ 5 to 10-fold. However, these estimates (up to 10-fold) of resistance are distinctly less than the 25-fold difference in circulating $1,25(OH)_2D_3$ levels found in the serum of the two children. We can not explain this discrepancy at this time but other defects that only become manifest in the in vivo setting rather than in isolated cultured fibroblasts may be at play. It is possible, for instance, within a cell-specific context that other tissues might exhibit more resistance than dermal fibroblasts. Another possibility is that pharmacokinetic factors may make the hormone less available in vivo. Resolution of this discrepancy between cultured cells and the in vivo setting would no doubt add to our understanding of the $1,25(OH)_2D_3$ action pathway.

The H305Q mutation reported here adds to the growing list of mutations found in the VDR and further defines the structural and functional organization of this protein. This mutation occurs in an amino acid that is not highly conserved among the thyroid/retinoid subgroup but is critical for the normal functioning of the VDR. In conclusion, we have identified a new mutation in the VDR gene that causes cellular hyporesponsiveness to $1,25(OH)_2D_3$ that can be overcome by increasing concentrations of hormone.

Acknowledgments

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