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A Novel Mutation in Helix 12 of the Vitamin D Receptor Impairs Coactivator Interaction and Causes Hereditary 1,25-Dihydroxyvitamin D-Resistant Rickets without Alopecia

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Hereditary vitamin D-resistant rickets (HVDRR) is a genetic disorder most often caused by mutations in the vitamin D receptor (VDR). The patient in this study exhibited the typical clinical features of HVDRR with early onset rickets, hypocalcemia, secondary hyperparathyroidism, and elevated serum concentrations of alkaline phosphatase and 1,25-dihydroxyvitamin D [1,25-$(OH)_2D_3$]. The patient did not have alopecia. Assays of the VDR showed a normal high affinity low capacity binding site for [^{3}H]1,25-$(OH)_2D_3$ in extracts from the patient's fibroblasts. However, the cells were resistant to 1,25-dihydroxyvitamin D action as demonstrated by the failure of the patient's cultured fibroblasts to induce the 24-hydroxylase gene when treated with either high doses of 1,25-$(OH)_2D_3$ or vitamin D analogs. A novel point mutation was identified in helix H12 in the ligand-binding domain of the VDR that changed a highly conserved glutamic acid at amino acid 420 to lysine (E420K). The patient was homozygous for the mutation. The E420K mutant receptor recreated by site-directed mutagenesis exhibited many normal properties including ligand binding, heterodimerization with the retinoid X receptor, and binding to vitamin D response elements. However, the mutant VDR was unable to elicit 1,25-$(OH)_2D_3$-dependent transactivation. Subsequent studies demonstrated that the mutant VDR had a marked impairment in binding steroid receptor coactivator 1 (SRC-1) and DRIP205, a subunit of the vitamin D receptor-interacting protein (DRIP) coactivator complex. Taken together, our data indicate that the mutation in helix H12 alters the coactivator binding site preventing coactivator binding and transactivation. In conclusion, we have identified the first case of a naturally occurring mutation in the VDR (E420K) that disrupts coactivator binding to the VDR and causes HVDRR. (*Molecular Endocrinology* 16: 2538–2546, 2002)

THE BIOLOGICAL ACTIONS of 1,25-dihydroxyvitamin D [1,25-$(OH)_2D_3$], 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). Like other steroid receptors, the VDR binds specifically to its cognate ligand, 1,25-$(OH)_2D_3$, triggering a series of molecular events leading to the activation of vitamin D-responsive genes. Regulation of specific gene transcription by the VDR requires binding as a heterodimer with the retinoid X receptor (RXR) to vitamin D response elements (VDREs) in the promoter region of target genes.

Abbreviations: AF-2, Activation function-2 domain; DBD, DNA binding domain; DRIP, vitamin D receptor-interacting protein; E420K, a highly conserved glutamic acid at amino acid 420 to lysine; GST, glutathione-*S*-transferse; HVDRR, hereditary vitamin D-resistant rickets; LBD, ligand binding domain; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D; RFLP, restriction fragment length polymorphism; RXR, retinoid X receptor; SRC, steroid receptor coactivator; VDR, vitamin D receptor; VDRE, vitamin D response elements.

Members of the steroid/nuclear receptor superfamily share a modular structure comprising an N-terminal transactivation domain of variable length, a DNA binding domain (DBD) that enables interaction with hormone response elements in promoter regions of target genes, and a C-terminal ligand binding domain (LBD). In addition, a highly conserved region at the carboxy terminus of the LBD, defined as the activation function-2 domain (AF-2), regulates transcription. Crystallographic studies of the VDR bound to 1,25-$(OH)_2D_3$ have shown that the LBD consists of 12 α-helices and 3 β-sheets (4). α-Helices H1-H11 form the ligand binding pocket, whereas ligand binding causes helix H12 to undergo a major reorientation locking the ligand inside the LBD.

Initiation of gene transcription also involves the recruitment of coactivator proteins, a family of closely related p160 proteins including steroid receptor coactivator 1 (SRC1)/nuclear coactivator 1 (NcoA1), transcriptional intermediary factor 2 (TIF2)/glucocorticoid receptor interacting protein 1 (GRIP1)/NcoA2/SRC2, and p300/CBP cointegrator-associated protein (pCIP)/receptor-associated coactivator 3 (RAC3)/acti-

vator of thyroid and retinoic acid receptors (ACTR)/amplified in breast cancer 1 (AIB1)/SRC3 (5). These coactivator proteins act as bridging factors linking the nuclear receptors to the preinitiation complexes and RNA polymerase II. They associate with the VDR in a ligand-dependent manner and enhance transactivation. Studies have shown that a conserved LXXLL motif in the nuclear receptor-interacting domains of the coactivators forms a short α-helix that binds in a hydrophobic groove on the nuclear receptor surface. Helices H3 and H4 on one side and helix H12 on the other form the hydrophobic groove (4).

Hereditary vitamin D-resistant rickets (HVDRR), also known as vitamin D-dependent rickets type II, is a rare genetic disorder caused by mutations in the gene encoding the VDR (2). Patients with HVDRR exhibit a constellation of features including early onset rickets, hypocalcemia, and secondary hyperparathyroidism. Patients with HVDRR have significantly elevated serum levels of the 1,25-$(OH)_2D_3$, and in most cases, total body alopecia. Consangunity is often a factor in the autosomal transmission of the disease. Since the first description of HVDRR in 1978, over 100 cases have been recorded, and a number of these have been analyzed at the biochemical and molecular level (2). Several abnormalities have been found in the VDR gene usually missense mutations and nonsense mutations but also including a partial gene deletion, and splice site mutations. Mutations in the DBD do not affect ligand binding but disrupt VDR-DNA interaction and transactivation (6–10). Mutations in the VDR LBD affect ligand binding by disrupting ligand contact points or interfere with RXR heterodimerization and result in partial or total hormone unresponsiveness (11–14). One patient with HVDRR has been described in which a mutation was not detected within the VDR (15). In this report, we describe a patient having a novel missense mutation in helix H12 in the VDR LBD that affects coactivator interaction and results in the syndrome of HVDRR without alopecia.

Case History

The patient is a Caucasian boy born at 42 wk gestation by cesarean section. Both parents are healthy with no known consanguinity, although one grandfather was adopted and no information about his birth family is known. At 9 months of age, the patient was noted to be small for age and slow in developing motor skills. Evaluation at 18 months found marked hypotonia, torticollis, thoracic kyphosis, and shoulder muscle weakness. There was no alopecia. Growth in length had fallen from the 90th percentile to below the 5th percentile and weight had fallen from the 95th percentile to the 25th percentile. The patient was then hospitalized for further evaluation of failure to thrive, hypotonia, and recurrent respiratory distress.

The patient was noted to have thickened wrists and ankles, a rachitic rosary, and some frontal bossing. Radiographic evaluation revealed demineralization of the ribs and spine on chest x-ray with upper lumbar and lower thoracic kyphosis. Cardiomegaly with increased pulmonary vasculature was also noted. Wrist and knee radiographs showed diffuse osteopenia with widening and fraying of the metaphysis and a fracture of the left ulna. A skeletal survey showed widespread osteopenia, metaphyseal changes, poor ossification of the epiphyses, and thirty poorly healing fractures. Laboratory evaluation revealed normal electrolytes, elevated alkaline phosphatase (3170, normal 115–460 IU/liter), normal phosphorus (3.8 mg/dl), and a markedly decreased total calcium (5.2, normal 9–10.5 mg/dl). Thyroid levels were normal, PTH was elevated (315, normal 10–55 ng/liter), and 25-hydroxyvitamin D level was normal (11.9, normal 2.2–33.7 ng/ml).

The patient was started on oral calcium and 1,25-$(OH)_2D_3$ replacement. His calcium levels ranged from 4.5–6 despite large doses of both and dramatically elevated serum 1,25-$(OH)_2D_3$ levels (400, normal 6–60 pg/ml). When ionized calcium levels dropped below 0.7 (normal 1–1.4 mmol/liter), he experienced laryngospasm with respiratory distress and full body tetany. Attacks occurred as frequently as every 7–10 d. The addition of magnesium and calcitonin did not change calcium levels or the frequency or severity of attacks.

At age 26 months, the patient was started on daily calcium infusions through a central indwelling catheter. Serum calcium levels were maintained in the 8–9 mg/dl range with ionized calcium between 1.0 and 1.2. Phosphorus levels have been normal with magnesium levels ranging 1.3–1.8. Serial radiographic evaluations have demonstrated dramatic remodeling of all features of rickets, with minimal residual bowing of the legs and left arm present at age 5 yr. All fractures healed quickly, and no additional fractures have occurred. After healing of the fractures a marked acceleration in growth was seen and gross motor development progressed quickly.

RESULTS

Fibroblasts from the patient were cultured from a forearm skin biopsy and analyzed for 1,25-$(OH)_2D_3$ responsiveness using 24-hydroxylase as the marker gene. As shown in Fig. 1, the 24-hydroxylase gene is highly induced by 10 nM 1,25-$(OH)_2D_3$ from a normal control cell. However, the patient's cells did not respond to 1,25-$(OH)_2D_3$ even when treated with 1000 nM of the hormone clearly demonstrating extreme resistance to 1,25-$(OH)_2D_3$. We have previously shown that analogs of 1,25-$(OH)_2D_3$ with increased activity compared with 1,25-$(OH)_2D_3$ can be potent activators of mutant VDRs (16). We tested two of these vitamin D analogs, MC1288 and Ro25–5318 for 24-hydroxylase induction but the analogs also failed to induce the 24-hydroxylase gene (Fig. 1).

We next sought to determine whether the lack of responsiveness was due to an alteration in 1,25-

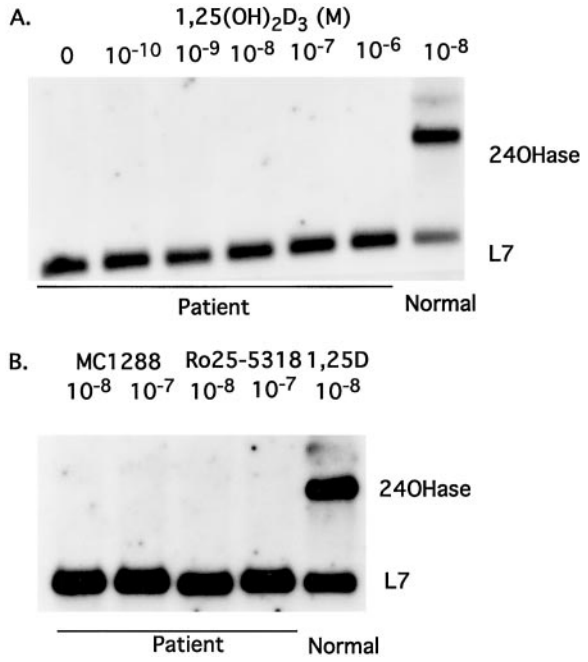

Fig. 1. Induction of 24-Hydroxylase mRNA in the Patient's Fibroblasts

Fibroblasts from the patient with HVDRR were treated with increasing doses of 1,25-$(OH)_2D_3$ (A) or the vitamin D analogs MC1288 and Ro25–5318 (B) for 6 h and then assayed for 24-hydroxylase mRNA by Northern blotting. The blot was incubated with ^{32}P-labeled 24-hydroxylase and L7 ribosomal protein cDNA probes. Normal fibroblasts treated with 10 nM 1,25-$(OH)_2D_3$ served as a control.

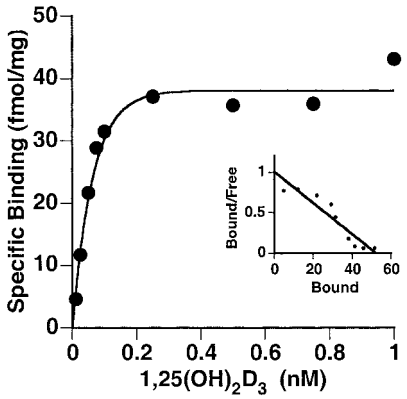

Fig. 2. Analysis of Specific [^{3}H]1,25-$(OH)_2D_3$ Binding in Extracts from the Patient's Fibroblasts

Cell extracts from the patient's fibroblasts were incubated with increasing concentrations of [^{3}H]1,25-$(OH)_2D_3$ in the presence and absence of 250-fold excess radioinert 1,25-$(OH)_2D_3$. After incubation at 4 C for 16 h, the bound and free hormone was separated by hydroxylapatite. The saturation curve and Scatchard analysis plot (*inset*) are shown.

$(OH)_2D_3$ binding to the VDR. As shown in Fig. 2, the patient's cells exhibited a high affinity (dissociation constant = $3.2 \pm 1.7 \times 10^{-11}$ M, n = 3) low capacity (maximum binding capacity = 46 ± 4 fmol/mg protein, n = 3) binding protein similar to values found in normal fibroblasts (2).

We determined the DNA sequence of the VDR cDNA from the patient's cells and identified a single C to A missense mutation that changed the codon for glutamic acid (CAA) to lysine (AAA) (Fig. 3, A and B). The mutation occurs at amino acid residue 420 (E420K) in helix H12 in the LBD (Fig. 3C). The mutation resulted in the loss of a *Taq*I site in exon 9. We used the *Taq*I enzyme to perform restriction fragment length polymorphism (RFLP) analysis and showed that the patient is homozygous for the mutation and his mother is a heterozygous carrier of the mutant allele (Fig. 4). The biological father's DNA was not available.

To analyze the properties of the E420K mutant VDR, we recreated the mutation in the VDR cDNA by site directed mutagenesis. As shown in Fig. 5, in transactivation assays in COS-7 cells, the mutant receptor was unable to activate gene transcription from the 24-hydroxylase reporter even when 1000 nM 1,25-$(OH)_2D_3$ was added. Similar results were found using an osteopontin VDRE reporter (data not shown). Western analysis showed equal expression of the WT and E420K mutant VDRs. These data show that the E420K mutation totally inhibits transactivation.

We then asked whether the mutation interfered with RXRα heterodimerization or with DNA binding using glutathione-*S*-transferse (GST)-pull down and gel shift assays. In the GST-pull down assay shown in Fig. 6, the E420K mutant VDR bound to GST-RXRα in a 1,25-$(OH)_2D_3$-dependent manner similar to the WT VDR. As shown in Fig. 7 in the gel shift assay, the WT VDR formed a more intense complex with the osteopontin VDRE when 1,25-$(OH)_2D_3$ was present. Addition of an anti-VDR antibody caused a supershift of the WT VDR-DNA complex. The E420K mutant VDR also formed a strong complex with the osteopontin VDRE when 1,25-$(OH)_2D_3$ was added. However, the antibody failed to supershift the E420K VDR-DNA complex. Because the mutation in the VDR occurs in the antibody epitope, this finding further demonstrates the specificity of the binding complex. These data demonstrate that the E420K mutation does not interfere with RXR heterodimerization or binding to DNA.

We next determined whether the mutation affected interaction with the coactivator proteins SRC-1 and DRIP205. As shown in Fig. 8, the WT VDR bound to GST-SRC-1 and to GST-DRIP205 in a 1,25-$(OH)_2D_3$-dependent manner. In contrast, the E420K mutant VDR failed to bind to either coactivator (Fig. 8). These data clearly demonstrate that the E420K mutation disrupts the formation of the coactivator binding site on the VDR.

The E420 residue has been postulated to form a salt bridge with a lysine residue in helix H5, K264 (4). We asked whether the loss of transactivation activity of the E420K mutation could be rescued by generating the double mutation K264E/E420K, potentially reestablishing the E to K salt bridge. We also tested whether a second double mutant K246E/E420K had

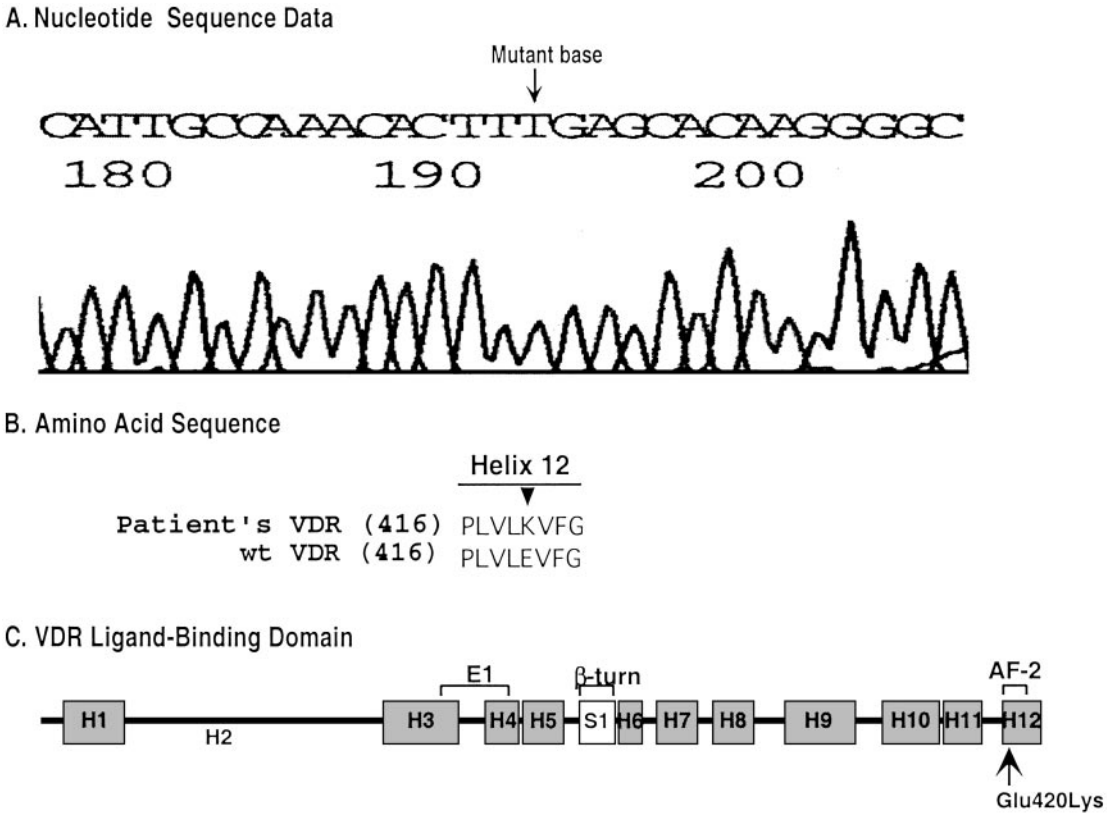

Fig. 3. Analysis of the VDR cDNA Sequence from the Patient

A, VDR cDNA was synthesized from total RNA using RT-PCR. PCR products were cloned and then sequenced. Shown is the sequence of the noncoding strand with the location of the C to A mutation indicated. B, Amino acid sequence of helix 12 of the WT and mutant VDR. C, Schematic representation of the VDR LBD and location of the mutation. α helices are shown as *rectangles with helix number inside*.

any effect on transactivation because K246 has been shown to be critical for coactivator binding (17). Mutations in both K246 and K264 have been previously shown to have no affect on ligand binding, DNA binding and heterodimerization with RXR; however, VDR transactivation was impaired (18, 19). As shown in Fig. 9, the transactivation capacity of the single mutations K246E and K264E were significantly reduced compared with the WT VDR. In contrast, the double mutants were totally inactive indicating that the reciprocal mutations at K246 or K264 to reestablish the postulated salt-bridge failed to rescue the E420K mutation.

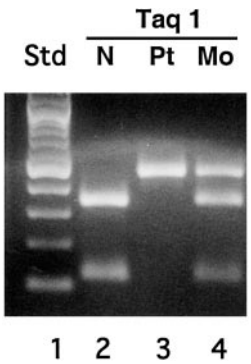

Fig. 4. Genotype of the Patient and his Parents Determined by RFLP

Exon 9 was amplified by PCR and digested with *Taq*I. The C to A base substitution eliminates a *Taq*I restriction site within the 500-bp amplified product. Digestion products were electrophoresed on 2% agarose gels and visualized with ethidium bromide stain. Lanes 1–4 symbols: Std, 100-bp marker; N, normal control; Pt, patient; Mo, mother.

DISCUSSION

The patient described here exhibited the classical clinical pattern of HVDRR. He initially presented with early onset rickets, secondary hyperparathyroidism, hypocalcemia, and elevated serum 1,25-$(OH)_2D_3$ levels. However, he did not have alopecia. The child did not respond to high dose calcitriol therapy. After we documented that his cultured fibroblasts exhibited complete resistance to calcitriol, he was eventually treated with daily iv calcium infusions that cured the hypocalcemia and the rickets subsequently resolved (20, 21). We identified a novel mutation in his VDR gene, E420K

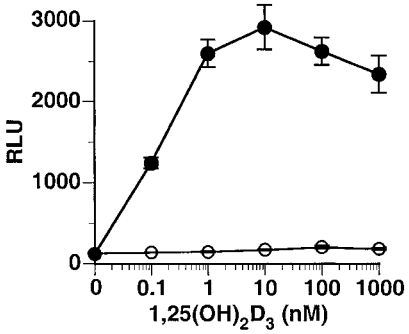

Fig. 5. Transactivation Activity of the E420K Mutant VDR by 1,25-$(OH)_2D_3$

COS-7 cells were transiently transfected with E420K mutant or WT VDR expression plasmids, a 24-hydroxylase promoter-luciferase reporter plasmid and a *Renilla* luciferase reporter plasmid that serves as an internal control. The COS-7 cells were treated with increasing dose of 1,25-$(OH)_2D_3$ as indicated. *Error bars* represent ± SD. Symbols are WT VDR (●) and E420K (○).

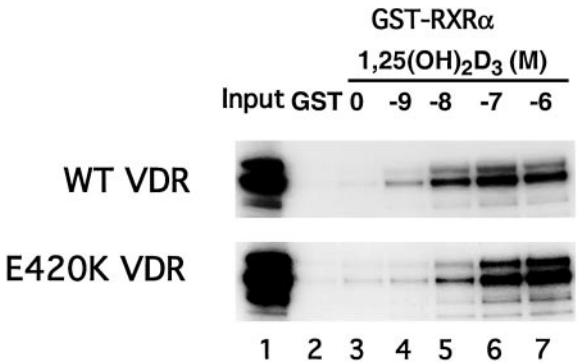

Fig. 6. The E420K Mutant VDR Binds to RXRα

In vitro [^{35}S]-labeled E420K mutant and WT VDRs were incubated with GST-RXRα bound to glutathione agarose beads and increasing concentrations of 1,25-$(OH)_2D_3$. After washing, the bound proteins were eluted and subjected to SDS-PAGE and autoradiography. A, WT VDR; B, E420K mutant VDR.

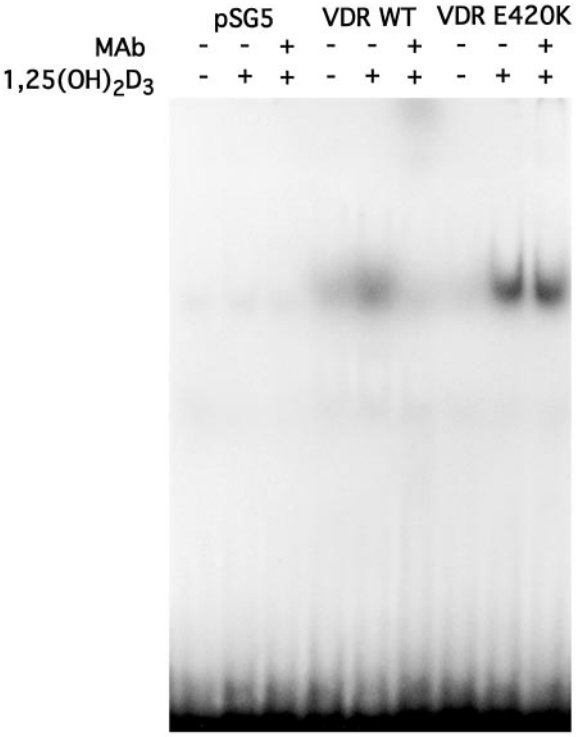

Fig. 7. The E420K Mutant VDR Binds to VDREs

COS-7 cells extracts containing E420K mutant and WT VDRs were incubated with ^{32}P-labeled osteopontin VDRE with and without 10 nM 1,25-$(OH)_2D_3$. The bound complexes were then resolved on nondenaturing gels. In some samples, a monoclonal antibody against the C-terminal region of the VDR was added to supershift the complex.

and demonstrated that this was the molecular basis for vitamin D resistance in this patient. The E420K mutation occurs in helix H12 in the LBD and does not affect ligand binding, heterodimerization with RXR, or DNA binding. However, the mutation disrupts binding to coactivators.

It is now clear from crystallographic studies of the VDR and other members of the steroid receptor superfamily that the LBDs of these receptors are composed of 11–13 α-helices forming a hydrophobic core that is occupied by the cognate ligand (4). The repositioning of helix H12 is a critical event that occurs as a consequence of ligand binding and is essential for transactivation. The repositioning of helix 12 involves both hydrophobic contacts and polar interactions and is critical for creating the correct surface interface for coactivator binding to the receptor (4). The polar interactions that stabilize helix H12 positioning involve a conserved salt-bridge between K264 in helix H4 and E420 in helix H12 and a hydrogen bond between S235 in helix H3 and T415 in helix H12 (4). The E420K mutation described here would be expected to disrupt the salt bridge formation and prevent the correct repositioning of helix H12 after binding the ligand. The substitution of the negatively charged glutamic acid (E420) with a positively charged lysine residue (K420) would prevent the polar interaction with the positively charged lysine (K264) salt bridge partner. The double mutant K264E/E420K would theoretically be expected to restore the salt bridge and therefore reconstitute transactivation activity. However, the K264E/E420K mutant was totally devoid of transactivation activity. Interestingly, the K264E mutation alone does exhibit some functional activity, which is surprising because the K264E mutation would also be expected to similarly disrupt the salt bridge. It is possible that, in the K264E mutant, the E420 forms a salt bridge at an alternative site with other positively charged residues thus preserving the formation of the hydrophobic cleft for coactivator binding.

In a computer generated model of the VDR LBD, it was suggested that a charge clamp is formed by E420 and K246, which allows coactivators to place its LxxLL

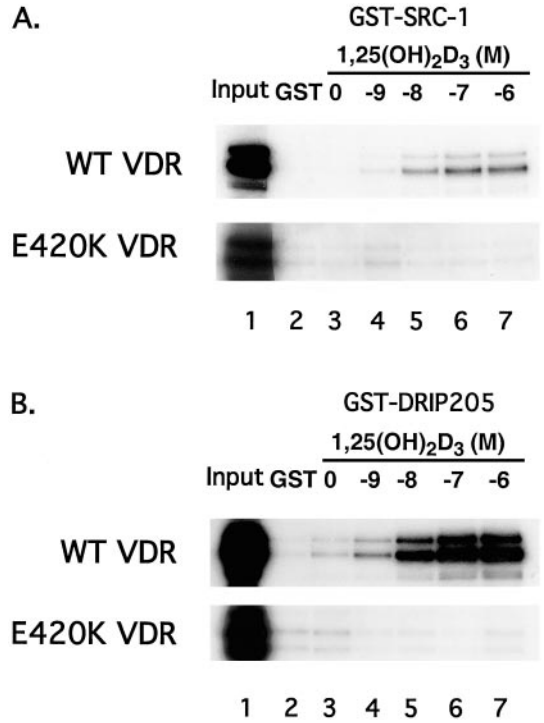

Fig. 8. The E420K Mutant VDR Is Unable to Bind to Coactivators

In vitro [^{35}S]-labeled E420K mutant and WT VDRs were incubated with GST-SRC-1 or GST-DRIP205 bound to glutathione agarose beads and increasing concentrations of 1,25-$(OH)_2D_3$. After washing, the bound proteins were eluted and subjected to SDS-PAGE and autoradiography. A, WT VDR and E420K mutant VDR bound to GST-SRC-1; B, WT VDR and E420K mutant VDR bound to GST-DRIP205.

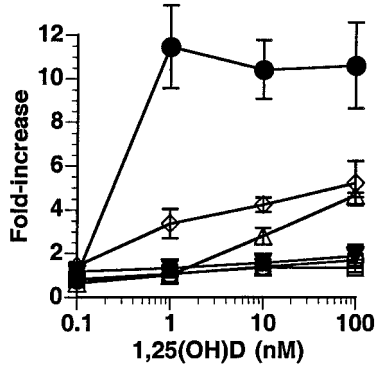

Fig. 9. Mutational Analysis of Conserved Lysine Residues

Mutant VDRs with mutations in the putative E420K salt bridge partner K264E and a neighboring lysine K246E as well as double reciprocal mutants K264E/E420K and K246E/E420K were assayed for 1,25-$(OH)_2D_3$ transactivation as described in Fig. 4. *Error bars* represent ± SD (n = 3). Symbols are WT VDR (●), K246E (△), K264E (◇), E420K (○E), K246E/E420K (□), and K264E/E420K (▼).

motif in the proper orientation and position (22). The K246 in helix 3 and E420 in helix 12 are thought to be indispensable for binding the LxxLL peptide (17, 23). We therefore examined whether the double mutant K246E/E420K could restore transactivation to the E420K mutant receptor. The K246E mutation alone does exhibit some functional activity, however, like the other double mutant, the K246E/E420Kmutant was totally devoid of transactivation activity. These data suggest a critical role for the correct positioning of E420 in transactivation. As we have shown here, the E420K mutation prevents VDR interaction with the coactivators SRC-1 and DRIP205 and results in the loss of transactivation. Our data clearly demonstrate that the E420K mutation disrupts coactivator binding and causes the hormone resistance seen in the patient. This case represents the first description of a naturally occurring mutation in the VDR that disrupts coactivator interaction and causes HVDRR.

A number of studies have examined the role of the AF-2 domain in detail using site directed mutagenesis (19, 24–28). In each case, mutations in E420 had no affect on ligand binding, heterodimerization with RXR or binding to VDREs. However, all E420 mutants had abrogated transactivation (24–26, 28). The E420 mutations were also shown to block SRC-1 or glucocorticoid receptor interacting protein-1 binding to the VDR (25, 28, 29). Interestingly, an E420A mutant VDR that does not bind to SRC-1 and is transcriptionally inactive was shown to bind the DRIP complex (27). Our results on the other hand, clearly show that the E420K mutation prevents the VDR from binding to DRIP205, the one member of the DRIP complex that binds directly to the VDR (30). From these data, it appears that the positively charged lysine in the 420 position would disrupt the charge clamp, creating an unfavorable binding site for the coactivators, whereas the uncharged alanine at 420 does not disrupt the coactivator binding site for the DRIP complex. Therefore, the findings indicate that both SRC-1 and DRIP205 must bind to the VDR in order for the VDR to become transcriptionally active. Although the SRC-1/p160 class of coactivators and the DRIP complex may have complementary activities because both coactivators are required for transactivation, the data suggest that some independent function is provided by each coactivator. Also, in one study, an E420A mutant VDR was shown to exhibit dominant negative activity in transactivation assays (24). However, our patient's mother is heterozygous for the E420K mutation and is phenotypically normal with no signs of vitamin D resistance suggesting that dominant negative activity is not manifested by the E420K mutant VDR *in vivo*.

An additional interesting aspect of this case of HVDRR is the fact that the patient does not have alopecia. Alopecia is found in almost all cases of HVDRR that have been reported to date (2). In those cases examined at the molecular level, alopecia has been associated with all patients having DBD mutations, premature stop mutations, and mutations that interfere with RXR heterodimerization. In those few HVDRR cases in which alopecia was not present, the molecular cause of HVDRR was the result of mutations

in the VDR LBD (R274L, I314S, and H305Q) (11–13). Two of these residues R274 and H305 are contact points for the 1-hydroxyl and 25-hydroxyl groups of 1,25-$(OH)_2D_3$, respectively (4). In all three cases, the mutations affected ligand binding. On the other hand, the patient described here has normal ligand binding but has a defect in coactivator binding. Despite being substantially resistant to high doses of hormone, he is free of alopecia. This case thus expands the spectrum of defects in the VDR that can be associated with HVDRR without alopecia. It is clear that the mechanism causing alopecia in HVDRR cases is complex and remains to be fully resolved.

In conclusion, we have identified a new mutation in the VDR LBD, E420K, that represents the molecular basis of HVDRR in this child. This is the first case demonstrating that a VDR mutation that interferes with coactivator binding can cause HVDRR.

MATERIALS AND METHODS

Cell Culture

Fibroblast cultures were established from skin biopsies and grown in DMEM containing 10% iron-supplemented calf serum (HyClone Laboratories, Inc., Logan, UT) and antibiotics as previously described (31). Cells were incubated at 37 C under a 5% CO_2 atmosphere.

1,25-$(OH)_2D_3$ Induction of 24-Hydroxylase mRNA

Cultured fibroblasts were grown to confluence and then treated with 1,25-$(OH)_2D_3$ for 6 h in medium containing 1% FBS. Total RNA was prepared using TRIZOL reagent (Life Technologies, Inc., Grand Island, NY) and 5 μg of RNA were electrophoresed on 1% agarose gels, transferred to nylon filters, and immobilized by UV cross-linking. The filters were hybridized with cDNA probes for 24-hydroxylase and L7 ribosomal protein. The probes were labeled with Redivue [α^{32}P]deoxy-CTP using the Rediprime DNA labeling system (Amersham Pharmacia Biotech, Arlington Heights, IL). L7 has been shown in multiple experiments to be unaffected by 1,25-$(OH)_2D_3$ treatment and therefore serves as a control for loading and transfer efficiency.

[^{3}H]1,25-$(OH)_2D_3$-Binding and Western Blotting

For ligand binding assays, cytosol from sonicated extracts of cultured fibroblasts prepared in KTEDM buffer [300 mM KCl, 10 mM Tris (pH 7.4), 1.5 mM EDTA, 5 mM dithiothreitol, and 10 mM sodium molybdate] and a mini complete protease inhibitor tablet (1 tablet/10 ml) (Roche Molecular Biochemicals, Indianapolis, IN) were incubated with [^{3}H]1,25-$(OH)_2D_3$ with or without 250-fold excess of radioinert 1,25-$(OH)_2D_3$ as previously described. Hydroxylapatite was used to separate bound and free hormone (32). Protein concentrations were determined by the Bradford method (33).

Gene Amplification and DNA Sequencing

The VDR cDNA was synthesized from total RNA using SuperScript Preamplification System (Life Technologies, Inc.) as previously described (14). PCR products were cloned into pCR2.1-TOPO (Invitrogen Corp., Carlsbad, CA) and sequenced. Fluorescent DNA sequencing was performed at the Stanford Protein and Nucleic Acid core facility.

RFLP Analysis

Genomic DNA was isolated from whole blood using the QIAmp Blood kit (QIAGEN, Chatsworth, CA). Exon 9 of the VDR gene was amplified using the oligonucleotide primers VDR ApaU 5′-TAG GGG GTG CTG CCG TTG AGT GTC and VDR 1471L 5′-ACG GGT GAG GAG GGC TGC TGA GTA G. Reactions were performed in 1× PCR buffer (QIAGEN) containing 1.5 mM $MgCl_2$, 0.2 μM each deoxy-NTP, 10 pmol each primer, and 0.5 μg DNA. PCR was initiated by the addition of *Taq* DNA polymerase (QIAGEN). After an initial 5-min denaturation at 95 C, the samples were cycled at 95 C for 10 sec, 55 C for 10 sec, and 72 C for 30 sec. PCR products were digested with the restriction endonuclease *Taq*I at 55 C in buffer supplied by the manufacturer (New England Biolabs, Inc., Beverly, MA). The digestion products were analyzed on 2% agarose gels, stained with ethidium bromide, visualized by UV light, and photographed.

Site-Directed Mutagenesis and Plasmid Construction

Site-directed mutagenesis of the WT VDR cDNA in pSG5 was done using the Gene Editor system (Promega Corp., Madison, WI). The mutant oligonucleotide used was 5′-CTT GTG CTC AAA GTG TTT GGC. Clones were sequenced to confirm the presence of the point mutation.

Transactivation Assays

COS-7 cells were grown to 60–80% confluence in six-well tissue culture plates. Cells were transfected using Polyfect (QIAGEN) with 0.25 μg WT or mutant VDR expression plasmids and 0.5 μg of the rat 24-hydroxylase gene promoter linked to a luciferase reporter gene (34). The reporter plasmid contains −1399 to +76 nucleotides of the rat 24-hydroxylase promoter and contains two VDRE sequences at −262 to −238 and −154 to −134. A *Renilla* luciferase plasmid, pRL-null (0.01 μg) (Promega Corp.) that serves as an internal control for transfection efficiency was included in each transfection. After a 16 h transfection, the cells were incubated in DMEM containing 1% FBS with or without 1,25-$(OH)_2D_3$. Twenty-four hours after transfection, the cells were washed and prepared for dual luciferase assays according to the manufacturers' instruction (Promega Corp.). Luciferase activities were determined using a Turner Design luminometer (Turner Design, Sunnyvale, CA).

GST-Pull Down Assay

GST-RXRα, GST-SRC-1, and GST-DRIP205 fusion proteins were expressed in *Escherichia coli* BL21(DE3) after induction with 0.1 mM isopropyl-β-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 (1 tablet/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 mutant VDRs were labeled with [^{35}S]-methionine (Amersham Pharmacia Biotech) by *in vitro* transcription/translation using the TNT Quick-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 on a rotating mixer. Unbound proteins were removed by washing the beads three times with 0.5 ml of GST-binding buffer (50 mM Tris buffer, pH 7.5; containing 100 mM KCl; 10 mM $MgCl_2$; 0.3 mM dithio-

threitol; 0.1% Nonidet P-40, and 10% glycerol). [^{35}S]-Labeled VDRs and 1,25-$(OH)_2D_3$ were added to the beads, which were then incubated at 4 C for 1 h on a rotating mixer. The bead suspension was then transferred to spin columns (Amika Corp., Columbia, MD) and washed three times with GST-binding buffer. Bound proteins were eluted in 25 μl of 2$\times$ lithium dodecyl sulfate sample buffer (Invitrogen) then heated at 70 C for 10 min. The samples were electrophoresed on 10% NuPAGE gels in 3-(*N*-morpholino)propane sulfonic acid-sodium dodecyl sulfate running buffer (Invitrogen). Gels were fixed in 50% methanol, 10% acetic acid for 10 min, and then incubated in Amplify (Amersham Pharmacia Biotech) for 15 min. Gels were dried and exposed to Hyperfilm (Amersham Pharmacia Biotech) at −80 C. Nonspecific binding was determined using extracts containing GST alone.

DNA Binding Assay

DNA binding was assessed by gel mobility shift assays. The human osteopontin VDRE was end-labeled using [γ^{32}P]ATP and polynucleotide kinase. Cell extracts from COS-7 cells expressing WT and E420K VDRs were prepared by incubating the cell pellet in M-PER extraction buffer (Pierce Chemical Co.) containing 300 mM KCl and a protease inhibitor tablet (1 tablet/50 ml) (Roche) for 10 min at ambient temperature. Cell extracts were incubated with vehicle (ethanol) or 10 nM 1,25-$(OH)_2D_3$ in buffer containing 0.1 mg/ml poly(deoxyinosine: deoxycytidine) for 15 min at ambient temperature. The [^{32}P]-labeled osteopontin VDRE probe was then added for an additional 20 min. The final concentration of salt in the binding assay was 150 mM KCl (35). For supershift assays, a polyclonal antibody against the carboxy terminus of the VDR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and incubated for 30 min prior to the addition of the probe. The samples were then electrophoresed on 6% polyacrylamide gels (acrylamide:bis-acrylamide; 29:1) in 0.5$\times$ Tris-borate buffer at 180 V for 90 min at ambient temperature (36). The gel was then dried and subjected to autoradiography at −80 C.

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