Vitamin D Receptors from Patients with Resistance to 1,25-Dihydroxyvitamin D_3: Point Mutations Confer Reduced Transactivation in Response to Ligand and Impaired Interaction with the Retinoid X Receptor Heterodimeric Partner

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Hereditary hypocalcemic vitamin D-resistant rickets is attributable to defects in the nuclear receptor for 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. Two novel point mutations (I314S and R391C) identified in the hormone-binding domain of the human vitamin D receptor (VDR) from patients with hereditary hypocalcemic vitamin D-resistant rickets confer the receptor with sharply reduced 1,25-$(OH)_2D_3$-dependent transactivation. These natural mutations, especially R391C, also lead to a second specific consequence, namely impaired heterodimeric interaction with retinoid X receptor (RXR). While the transactivation ability of the I314S mutant can be largely restored by providing excess 1,25-$(OH)_2D_3$, R391C activity is more effectively restored with exogenous RXR. These observations are reflected also in the clinical course of each patient: the patient bearing the I314S mutation showed a nearly complete cure with pharmacological doses of a vitamin D derivative, whereas the patient bearing R391C responded only partially to such therapy. Further tests with patient fibroblasts and transfected cells show that the activity of the I314S VDR mutant is augmented somewhat by added RXR, while transactivation by the R391C mutant is best corrected by RXR in the presence of excess hormone. Thus, the effects of hormone *vs.* RXR in bolstering these mutant VDRs, such that they mediate efficient transactivation, are not entirely separable. The unique properties of these genetically altered receptors establish a new subclass of natural human VDR mutants that illustrate, *in vivo*, the importance of both 1,25-$(OH)_2D_3$ binding and heterodimerization with RXR in VDR action. (Molecular Endocrinology 10: 1617–1631, 1996)

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INTRODUCTION

The human vitamin D receptor (hVDR) is a member of the superfamily of nuclear receptors that includes the receptors for steroid, retinoid, and thyroid hormones, as well as other orphan receptors whose natural ligands are unknown (1–3). These receptors all possess a well defined domain for DNA binding and a broader, less well defined domain for hormone binding. The hVDR mediates the known nuclear actions of the hor-

monal form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], by binding to an enhancer-like sequence in DNA, termed the vitamin D responsive element (VDRE), and modulating the transcription of target genes, presumably by interacting in some way with the transcriptional machinery (reviewed in Refs. 4 and 5).

Transcriptional activation by hVDR appears to require a heterodimeric interaction with another nuclear receptor, most likely one of the retinoid X receptors (RXRs) (see Ref. 6 and citations therein). This is analogous to the action of the thyroid hormone receptor (TR), the retinoic acid receptor (RAR), and peroxisome proliferator-activated receptor, which have also been reported to bind as heterodimers to responsive elements consisting of direct repeats (reviewed in Ref. 7). This is in contrast to the classic steroid hormone (*i.e.* estrogen, glucocorticoid, progesterone, androgen, etc.) receptors, which bind as homodimers to indirect (inverted) repeats (1–3).

A clinical syndrome, hereditary hypocalcemic vitamin D resistant rickets, exists in which the hVDR of the affected patient is impaired in its ability to activate transcription in response to the presence of the 1,25-$(OH)_2D_3$ ligand. A number of such patients have now been studied, and the hVDR defects can be classified into two groups. The largest group consists of point mutations in the conserved zinc finger region that reduce or abolish the affinity of the receptor for the DNA response element (8–12). A second, smaller group of patients contains point mutations that either introduce a premature stop codon or, in one case, create a missense mutation in the hormone-binding domain of the receptor such that the affected hVDR is no longer capable of binding to physiological levels of 1,25-$(OH)_2D_3$ (12–15).

The present study describes two novel missense mutations in the hormone-binding domain of hVDR from patients with hereditary hypocalcemic vitamin D-resistant rickets. Both of these mutations confer defects in 1,25-$(OH)_2D_3$-dependent transactivation. Further tests revealed that the predominant effect of one of these mutations is an impairment in the heterodimeric interaction of hVDR with RXR on the VDRE. The other mutation also influences heterodimerization, but this effect only partially explains the phenotype of this mutant receptor, which displays another, as yet undefined, defect related to hormone responsiveness.

RESULTS

Two Patients with Hereditary Resistance to Vitamin D

Patient 1, a 2-yr-old female when first seen, presented with the classic symptoms of rickets, combined with elevated levels of circulating 1,25-$(OH)_2D_3$, and was treated with 1 mg/day of vitamin D until age 18. After an uneventful pregnancy, she returned for treatment at age 20 with hypocalcemia along with elevated circulating levels of 1,25-$(OH)_2D_3$. Treatment with 50 μg/day of 25-hydroxyvitamin D elicited a very satisfactory response in serum calcium. A follow-up study (16) appeared to show normal binding of 1,25-$(OH)_2D_3$ in patient fibroblasts, but the normal induction of the vitamin D 24-hydroxylase enzyme seen in control cells after administration of 1,25-$(OH)_2D_3$ was lacking.

Patient 2, also a young (21-month-old) female when first seen, was diagnosed with rickets, displaying a very small stature, impaired dentition and alopecia, along with elevated circulating 1,25-$(OH)_2D_3$. This patient was also treated with high-dose vitamin D in the form of the 1,25-$(OH)_2D_3$ analog, dihydrotachysterol, which promoted some normalization of serum calcium and phosphate levels, although at 4.5 yr of age she still presented symptoms of mild osteopenia, distortion of upper teeth, sparseness of hair, and low percentile height.

Detection of 1,25-$(OH)_2D_3$ Binding Activity and Immunoreactive Protein in Cultures from Biopsy

Fibroblasts from both patients were tested for binding of the 1,25-$(OH)_2D_3$ ligand and for immunoreactive hVDR. Figure 1A shows the results of hormone-binding assays performed with duplicate fibroblast cytosols at 4 C. Included in this assay were normal fibroblasts, as well as cells from a patient with a known hormone-binding defect, R274L (14, 17). These results must be considered as semiquantitative because of the difficulty of assaying the low amounts of endogenous hVDR present in fibroblast lysates. Patient 1 cells possess specific 1,25-$(OH)_2D_3$-binding activity that is essentially normal, as observed previously (16), while patient 2 cells have activity that is slightly lower, but greatly above the levels seen with the R274L mutant receptor. A second experiment performed on separate duplicate cytosols (data not shown) yielded nearly identical results. Western immunoblots using the 9A7γ anti-VDR antibody (*insets below* Fig. 1A) showed an immunoreactive protein in each cell sample at approximately 48 kDa, corresponding to the predicted size of hVDR (18).

Endogenous hVDR Cannot Activate Transcription Normally in Transfected Patient Fibroblasts

The presence of apparently normal 1,25-$(OH)_2D_3$-binding activity and immunoassayable hVDR in both patient fibroblasts (Fig. 1A) called into question whether the VDR was in fact the site of defect, thereby raising the possibility of a postreceptor defect (16). We therefore first determined whether endogenous patient VDRs were capable of transactivation using a GH-linked reporter plasmid containing four copies of the rat osteocalcin VDRE (see *Materials and Methods*). When normal fibroblasts were transfected with the reporter plasmid, it can be seen (Fig. 1B, *light bars*) that addition of 1,25-$(OH)_2D_3$ elicited an 11-fold in-

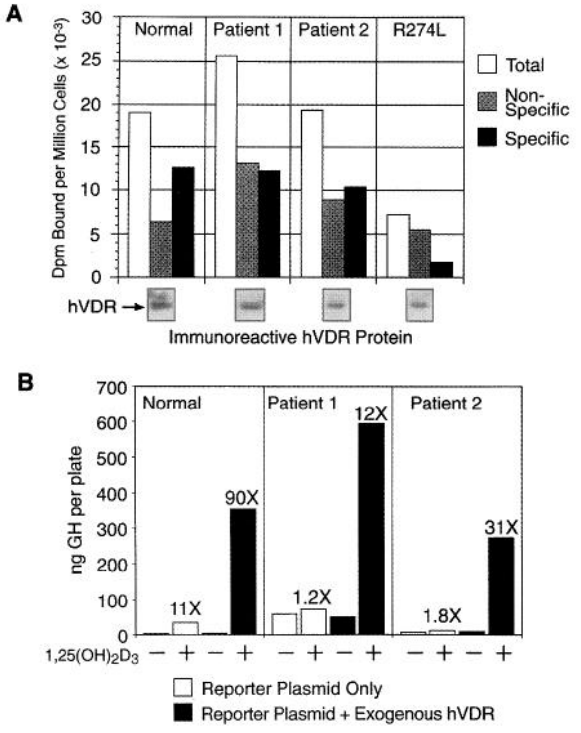

Fig. 1. Preliminary Characterization of hVDR in Fibroblasts from Two Patients with Hereditary Resistance to Vitamin D

A, Binding of 1,25-$(OH)_2[^3H]D_3$ in fibroblast cytosol preparations, obtained from a patient without abnormalities in calcium metabolism (Normal), patients 1 and 2 from this study, and a fourth patient with a previously described receptor that is defective in hormone binding (R274L). Lysate preparation and incubation with hormone were performed as described in *Materials and Methods*. *Bars* represent the average of binding obtained from lysates of two separate plates, with each result normalized per million cells. The presence of immunoreactive hVDR was demonstrated by Western blotting (see *insets below* panel A); lanes contain extracts from two separate plates and volumes loaded were normalized for protein content as determined using a BCA kit from Pierce (Rockford, IL). B, Transactivation mediated by endogenous receptor in fibroblasts cells from patients 1 and 2 compared with normal controls. The first two (*open*) bars in each section represent cultures that were transfected with a plasmid containing four copies of a vitamin D responsive element upstream of a human GH reporter gene using an electroporation protocol (see *Materials and Methods*). The last two (*solid*) bars in each section represent transactivation in cells that received the reporter plasmid and a second plasmid (pSG5hVDR) that drives the expression of exogenous, normal hVDR. Cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$ (+) or ethanol vehicle (−) as indicated. Values represent nanograms of human GH secreted per 60-mm culture dish during the 72-h incubation period.

crease in reporter gene activity. In contrast, cells from patient 1 showed a 1.2-fold, and cells from patient 2 a 1.8-fold response to the same (10^{-8} M) dose of 1,25-$(OH)_2D_3$, indicating that vitamin D-responsive transactivation is severely compromised in both patients.

Patient 1 cells appear to show a higher level of basal transcription, suggesting possible constitutive activity. Later tests indicated, however, that cells from patient 1 regularly transfect with a higher efficiency than control or patient 2 cells, as monitored by a β-galactosidase vector included as an internal standard. Thus, when normalized to efficiency of transfection, basal transcription levels in patient 1 fibroblasts are similar to those of normal and patient 2 fibroblasts (see Fig. 6C, below).

Next, normal hVDR was provided to replicate cultures to test whether this would restore responsiveness to 1,25-$(OH)_2D_3$ (Fig. 1B, *dark bars*). In normal fibroblasts, the presence of this exogenous hVDR boosted 1,25-$(OH)_2D_3$-dependent transactivation to almost 90-fold over controls without hormone treatment. Exogenous hVDR also elicited the appearance of a robust (12–31X) transactivational response to 1,25-$(OH)_2D_3$ in cultures from both patients, implicating the hVDR itself, and not some postreceptor step, as the site of the lesion.

Cloning of Patient hVDR cDNAs

A portion of the hVDR mRNA containing the entire protein-coding region was amplified from patient poly(A)$^+$RNA using RT-PCR. Sequencing from two independent RT-PCR reactions for each patient cell sample revealed, in addition to several apparent PCR artifacts, a consistent site of mutation for each patient. For patient 1, a T to G conversion altered codon 314 from Ile to Ser, while for patient 2, codon 391 was changed from Arg to Cys by a C to T mutation. The occurrence of these mutations was reconfirmed by amplification of total patient genomic DNA using primers specific for exons 7, 8, and 9 (see *Materials and Methods*). The I314S and R391C mutations were found in exons 8 and 9, respectively (this portion of the gene for hVDR has been partially described in Refs. 13 and 19). Thus, three separate clones were obtained by PCR from patient 1 cells, all containing the mutant Ser-314 codon, with no evidence of the wild type Ile codon; similarly, all three clones from patient 2 hVDR contained a Cys in lieu of the wild type Arg at codon 391. We therefore tentatively concluded that both patients represent homozygotes for the corresponding mutant gene. To circumvent the PCR artifacts, the I314S and R391C mutations were separately introduced by site-directed mutagenesis into a wild type hVDR cDNA (see *Materials and Methods*). Experiments utilizing the artificially created mutant cDNAs will be referred to using the nomenclature I314S or R391C, whereas experiments testing the endogenous mutant receptor in patient fibroblast cultures will be designated as patient 1 or patient 2, respectively.

Functional Testing of Mutated hVDRs in Transfected COS-7 Cells

The I314S and R391C mutant hVDRs were expressed by transfecting VDR-deficient COS-7 cells. Because

both mutations occur in the C-terminal hormone-binding domain of hVDR, we first prepared cytosols of transfected cells for saturation binding tests using 1,25-$(OH)_2[^3H]D_3$. The results of these assays performed at 4 C (Fig. 2) indicated that the two mutant receptors bound ligand with an affinity in the normal range [dissociation constant (K_d) ~0.1 nM], although both determinations for the I314S mutant hVDR were slightly higher than normal controls (average apparent K_d = 0.175 nM). Thus, the binding data obtained with artificially generated mutations (Fig. 2) correspond well with fibroblast cytosol binding results (Fig. 1A) and indicate that both mutant hVDRs bind the ligand nearly normally, at least at 4 C, *in vitro*.

The I314S and R391C mutations also reside in a general region of the receptor that has been proposed to mediate dimerization by other members of the nuclear receptor superfamily (see Refs. 20 and 21 and *Discussion*). Since hVDR has been shown to heterodimerize with RXRs on the VDRE of target genes (6), we tested for defects in complex formation with RXR on the rat osteocalcin VDRE (Fig. 3). When excess receptors are present, *in vitro*, heterodimer association with this VDRE does not absolutely require the 1,25-$(OH)_2D_3$ ligand (6, 22), allowing us initially to monitor these protein-protein-DNA interactions in the absence of hormone. To obtain a quantitative assessment of complex formation, constant volumes of cytosol from hVDR-transfected cells were combined with increasing amounts of hRXRα (Fig. 3, panels A and B). Mutant hVDRs were compared with controls from parallel cultures transfected with wild type hVDR. Under these conditions, the amount of RXR required to achieve maximal complex formation on the VDRE was similar when wild type and the I314S mutant were compared (Fig. 3A), although there is an indication in the presence of the two lowest amounts of hRXRα that slightly more RXR might be needed to heterodimerize with the I314S mutant. Three additional experiments confirmed that a slightly greater amount of RXR (~2- to 3-fold) is needed for I314S VDR to efficiently heterodimerize on the VDRE, and a plot of the fraction of VDRE probe bound *vs.* the quantity of RXR added is pictured in Fig. 3C as the most representative of four total experiments. In contrast to the modest attenuation of heterodimerization seen with I314S, the R391C mutant hVDR repeatedly required nearly 1 full order of magnitude more RXR to achieve complex formation comparable to that of wild type hVDR (Fig. 3, B and D). These results, which were normalized to hVDR expression in each cytosol sample (see *insets below* Fig. 3, A and B, and legend), are a strong indication that the R391C mutant, and possibly also the I314S hVDR, are impaired in their ability to heterodimerize with RXR.

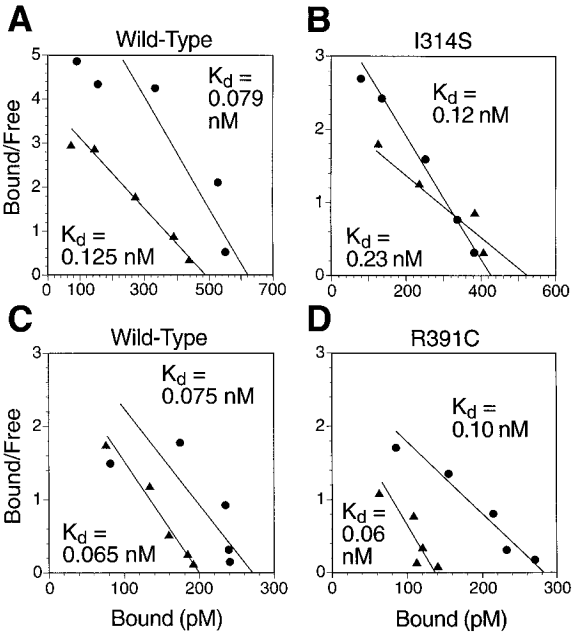

Fig. 2. Saturation Binding Assays of I314S and R391C hVDRs at 4 C

Lysates from transfected COS-7 cells (20–50 μg hVDR expression plasmid per 5 million cells in 150-mm plates) were tested for binding of radiolabeled 1,25-$(OH)_2D_3$ as described in *Materials and Methods*. Scatchard plots of two independent determinations (▲ *vs.* ●) are shown for mutant hVDRs I314S (panel B) and R391C (panel D). For each determination, a wild type control was also tested under identical conditions (compare matching symbols in panel A to those in panel B; likewise, panel C controls are paired with results in panel D).

Transactivation in the Presence of Exogenous RXR

The observation that supplying excess RXR can promote complex formation, especially by the R391C mutant, suggested that transactivation by these mutants could also be restored by this means. Accordingly, transactivation assays were performed in the presence of increasing amounts of plasmid pSG5-RXRβ, which mediates the expression of mouse RXRβ (mRXRβ). Previous results have shown that the rat osteocalcin VDRE responds equally to hRXRα and mRXRβ in both heterodimerization and transactivation assays (6, 22). As can be seen from the *insets below* Fig. 4A, the amounts of immunoassayable mRXRβ increase with the quantity of expression plasmid added. Boosting RXR levels did not result in an appearance of transactivation in cultures receiving no hVDR, nor did it appear to have any significant effect on normal hVDR (Fig. 4A). The I314S mutant displayed a very modest enhancement of transcription when RXR was increased (Fig. 4A). However, the R391C mutant showed a marked increase in transcriptional activity in the presence of excess RXR, approaching wild type levels when 0.5–1 μg of RXR expression plasmid was added (Fig. 4A). These results correlate well with those of gel mobility shift experiments (Fig. 3) and appear to explain the phenotype of the R391C mutant. However, the slight defect in RXR heterodimerization (Figs. 3, A and C, and 4A) cannot alone

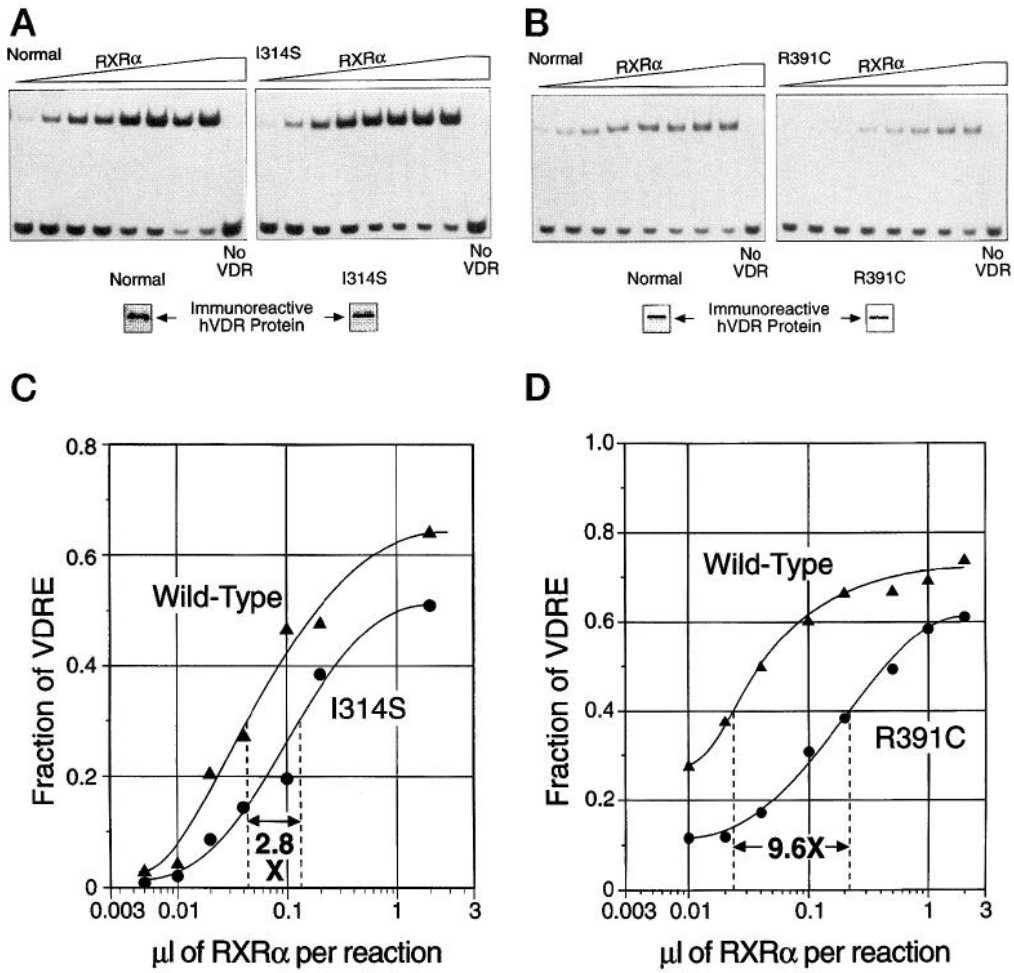

Fig. 3. Heterodimer-DNA Association in the Presence of Increasing Amounts of RXR

A labeled rat osteocalcin VDRE oligonucleotide (denoted "CT5"; see *Materials and Methods*) was mixed with lysates from COS-7 cells expressing either I314S (panel A) or R391C (panel B) hVDR mutants, transfected as described in the legend to Fig. 2 and subjected to gel mobility shift analysis as described in *Materials and Methods*. Control experiments with lysates containing equivalent amounts (see *insets below*) of wild type hVDR are included in each panel. The first lane of each gel contains a 1:200 dilution of baculovirus-expressed hRXRα. The next seven lanes contain 2 μl of, in sequence, 1:100, 1:50, 1:20, 1:10, 1:4, 1:2, and undiluted RXRα. The last lane of each gel also received undiluted RXRα, but contained lysate lacking expressed hVDR. In panels C and D, results for wild type hVDR (▲) or mutant hVDRs (●) from representative experiments are displayed by plotting the fraction of probe bound by the VDR-RXRα heterodimer *vs.* the amount of RXRα added (expressed in microliters, computed by dividing the 2 μl total added by the dilution factor). The excess amount of RXRα required to give equivalent binding by each mutant was estimated by the extent of rightward shift of the curve; this value was typically 2- to 3-fold for the I314S mutant and between 9- and 10-fold for the R391C mutant (both curves shown were corrected for expression of each mutant, which was 104% that of wild type for I314S and 74% for R391C in these particular experiments).

account for the severe transactivation impairment of the I314S mutant.

Pharmacological Doses of 1,25-$(OH)_2D_3$ Restore Some Transactivation Ability to Both Mutants

In spite of normal saturation binding at 4 C (Figs. 1A and 2), the question still remained whether the I314S and R391C mutant hVDRs might respond normally to elevated doses of hormone at physiological temperatures. Chief among the reasons for this uncertainty were the patient case histories, which indicated that patient 1 responded well to high dose 25-hydroxyvitamin D_3, while patient 2 responded only partially to similar treatment with another active vitamin D analog. We therefore tested transactivation by mutant and wild type hVDRs in the presence of up to 1 μM 1,25-$(OH)_2D_3$ (physiological concentrations are ~100 pM). The results of this analysis (Fig. 4B) indicated that, indeed, transactivation by the I314S mutant was effectively rescued in the presence of 1 μM hormone. The R391C mutant also displayed a significant recovery of activity, albeit incomplete, consistent with the partial response seen in the original patient. Thus, in contrast to the normal hormone binding seen in cell

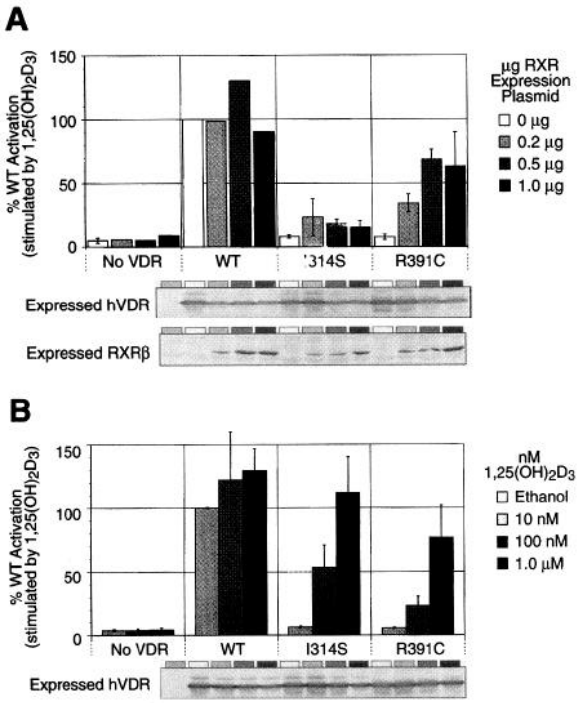

Fig. 4. Transactivation Can Be Restored to Varying Degrees by Boosting the Levels of Either RXR or 1,25-$(OH)_2D_3$

COS-7 cultures maintained at 37 C were transfected with the indicated hVDR vector (0.3 μg of WT or 0.75 μg of mutant hVDR plasmid) along with 5 μg of a 1,25-$(OH)_2D_3$-responsive reporter gene construct. Activation of transcription was assessed by measuring secretion of human GH, and fold stimulation by wild type receptor in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ over ethanol-treated control was set at "100%". Levels of expression of each hVDR, as monitored by Western immunoblotting, are shown *below* each panel. A, Transactivation in the presence of increasing amounts of expressed mouse RXRβ (see legend at *right* for amounts of expression plasmid added). Fold stimulations over ethanol-treated control are expressed as a percentage of stimulation by wild-type hVDR in the absence of added RXRβ expression vector. Mouse RXRβ was used in preference to hRXRα in these experiments because of the availability of monoclonal antibody specific for this isoform of RXR (see immunoblots, *bottom* of panel A). B, Transactivation in the presence of increasing concentrations of 1,25-$(OH)_2D_3$. Fold stimulations over ethanol-treated control are expressed as a percentage of stimulation by wild-type hVDR in the presence of 10^{-8} M 1,25-$(OH)_2D_3$. Doses tested are shown at *right*.

lysates at 4 C (Fig. 2), both mutant hVDRs appeared to be significantly impaired in their ability to mediate hormone-dependent transcriptional activation in intact cells at 37 C, a defect that can be partially overcome at very high doses of 1,25-$(OH)_2D_3$.

Interdependence of VDR Ligand Binding and Heterodimerization with RXR

The relationship between ligand binding and interaction with RXR was investigated for both VDR mutations in three independent experimental formats.

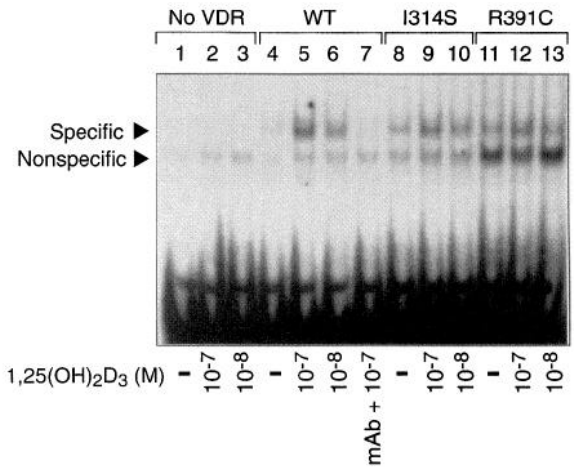

Fig. 5. Hormone-Dependent Gel Mobility Shift

Incubation of lysates with labeled VDRE oligonucleotide and electrophoresis were similar to those described in *Materials and Methods* and the legend to Fig. 3 except that amounts of lysate were reduced to visualize hormone dependence. Incubation mixtures received the indicated dose of 1,25-$(OH)_2D_3$ (10^{-7} or 10^{-8} M; *dashes* indicate mixtures that received ethanol vehicle rather than 1,25-$(OH)_2D_3$). Lysates were from COS-7 cells transfected with 100 ng of hVDR expression vectors as indicated at the *top* of figure. Lanes 1–7 (No VDR and Normal hVDR) received 1 μl lysate. Lanes 8–10 (I314S) received 2 μl lysate, while lanes 11–13 (R391C) received 4 μl lysate to facilitate detection of these weaker complexes. Lane 7 (WT) received one μl of 9A7γ monoclonal anti-VDR antibody. *Arrowheads* indicate the migration of the specific complex (inhibited by anti-VDR antibody) and a second, faster-migrating, nonspecific complex.

First, the I314S and R391C mutant VDRs were tested for their ability to form heterodimeric complexes on the rat osteocalcin VDRE as in Fig. 3, but this time utilizing endogenous RXRs in the presence of increasing amounts of the 1,25-$(OH)_2D_3$ ligand. To visualize hormone dependence of complex formation, the amounts of transfected VDR plasmid must be drastically reduced (see legend to Fig. 5) to produce a more limiting amount of expressed receptor, and the exposure times of mobility shift gels correspondingly increased. As shown in Fig. 5, under these conditions two shifted bands are seen in reactions containing lysates from transfected COS-7 cells, but only one of these bands contains VDR, as evidenced by its absence in lysates from mock-transfected cells (lanes 1–3) or in lysates treated with anti-VDR antibody (lane 7). This specific complex shows a marked increase when lysates containing normal hVDR are treated with ligand (compare lane 4 to lanes 5 and 6). The I314S and R391C mutant VDRs, on the other hand, show a blunted response to hormone (compare lane 8 to lanes 9 and 10 and lane 11 to 12 and 13). Further, a larger quantity of lysate was added for the I314S and R391C mutants as compared with the normal VDR (see figure legend; also note the corresponding increase in the intensity of the nonspecific band). The fact that 4 times

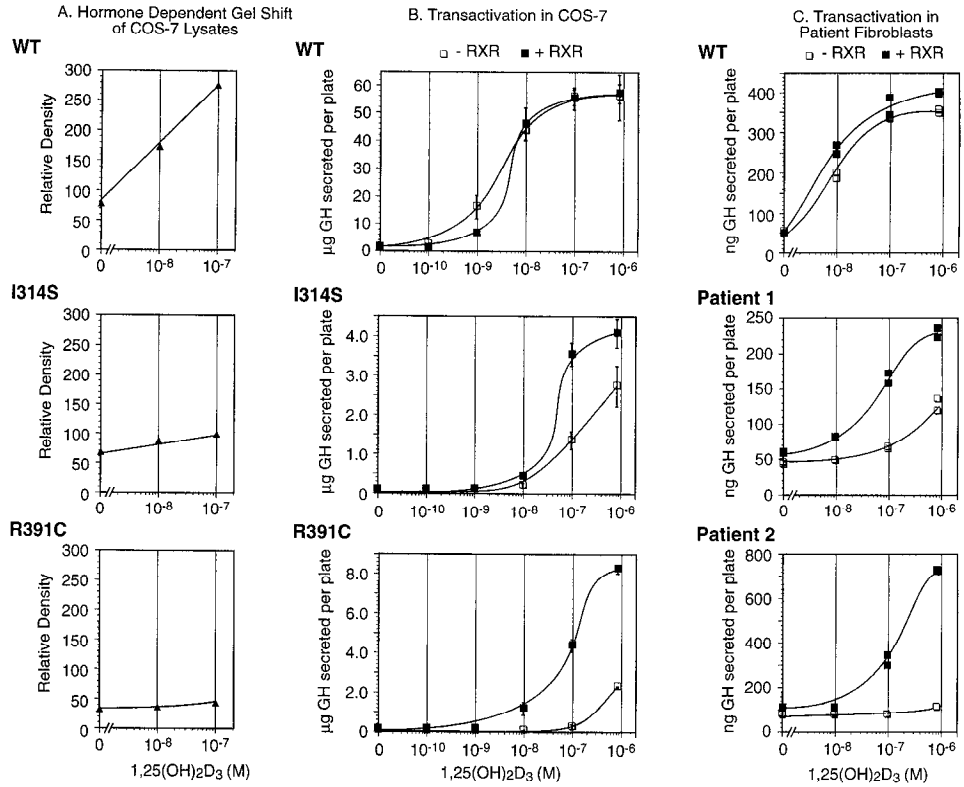

Fig. 6. Dose Response of Mutant VDRs

Column A displays densitometry results from the experiment shown in Fig. 5. The relative intensities of the specific bands from Fig. 5, lanes 4–6 (Normal), lanes 8–10 (I314S), and lanes 11–13 (R391C) were determined from digitized scanned images as described in *Materials and Methods*. Column B displays dose-response curves generated from COS-7 cells transfected with a vitamin D-responsive reporter construct and hVDR expression vectors with or without an expression vector for mRXRβ. Triplicate plates were incubated with the indicated dose of 1,25-$(OH)_2D_3$ for 28 h (WT hVDR), 24 h (I314S), or 19 h (R391C), and secreted GH was measured as indicated in *Materials and Methods*, then normalized to VDR expression as monitored by Western immunoblotting. Column C shows similar dose-response curves generated in patient fibroblast cultures. Individual determinations are from duplicate culture plates and are normalized to transfection efficiency as monitored by assays of β-galactosidase activity (see *Materials and Methods*).

as much of the R391C lysate still produced a weaker shifted band than that of wild type hVDR confirms the results of Fig. 3, which indicated a heterodimerization defect in VDR. In addition, both mutant VDRs appear to be unable to achieve optimal complex formation with RXR on the VDRE even in the presence of elevated levels of hormone. It should also be noted that these results were obtained at 25 C (ambient), which is customary for gel mobility shift procedures, suggesting that ligand responsiveness is impaired at this temperature.

When the results of the hormone-dependent gel mobility shift experiment (Fig. 5) are corrected for the amount of lysate used and plotted *vs.* dose of hormone (Fig. 6A), the dose-response pattern of each mutant hVDR is clearly distinct from that of wild type VDR. Thus, wild type hVDR (*top panel*, Fig. 6A) gives a strong, nearly linear response to 10^{-8} and 10^{-7} M doses of ligand, the I314S hVDR (*middle panel*, Fig. 6A) shows a much weaker, yet still measurable response at these doses, while the R391C mutant (*bottom panel*, Fig. 6A) requires the maximal 10^{-7} M dose for even a miniscule response. Of course, as demonstrated earlier (Fig. 3, B and D), there exists a fundamental defect in the ability of the R391C mutant to interact with RXR.

Second, to probe 1,25-$(OH)_2D_3$ and RXR interactions with hVDR simultaneously over a range of concentrations at 37 C, COS-7 cells were transfected with the indicated hVDR vector, the vitamin D-responsive reporter gene, and either carrier DNA or a third vector expressing mRXRβ, and transactivation was monitored by measuring GH secretion after incubation with varying doses of ligand (Fig. 6B). Consistent with the previous results shown in Fig. 4, the *top panel* of Fig. 6B illustrates that transactivation by normal hVDR is not increased significantly by supplying exogenous RXR. In contrast, as also seen previously, the R391C mutant (*bottom panel*, Fig. 6B) reveals a restoration of transactivation ability in the presence of exogenous RXR, particularly at the higher 1,25-$(OH)_2D_3$ doses. Although the absolute levels of GH secretion in the case of R391C (*bottom panel*, Fig. 6B) are less than those seen with the normal hVDR (*top panel*, Fig. 6B),

this can be partly explained by the shorter incubation time (19 h *vs.* 28 h); further, the fold effect of 10^{-6} M 1,25-$(OH)_2D_3$ on GH secretion is actually larger with the R391C mutant than with normal hVDR (67-fold *vs.* 47-fold).

Surprisingly, even though supplying exogenous RXR did not seem to have much effect on the I314S mutant at 10^{-8} M 1,25-$(OH)_2D_3$ (Fig. 4A), at higher doses of ligand the transactivation ability of this mutant seems to recover more completely when RXR is supplied (*middle panel*, Fig. 6B). This effect is most noticeable at 10^{-7} M 1,25-$(OH)_2D_3$.

Third, the results with artificially created I314S and R391C mutant hVDRs are mirrored in dose-response curves obtained from patient 1 and patient 2 fibroblasts, respectively (Fig. 6C). Although the absolute and fold effects of 1,25-$(OH)_2D_3$ administration on GH secretion are lower in fibroblast cultures, presumably due to a combination of lower receptor number and lower transfection efficiencies in these primary cultures, a similar pattern can be seen for each cell type when compared with the corresponding transfected COS-7 cultures. Thus, normal human fibroblasts, like COS-7 cells transfected with normal hVDR, show little enhancement of transactivation when exogenous RXR is provided (compare top panels of Fig. 6, B and C). In contrast, patient 2 fibroblasts, like the R391C-transfected COS-7 cells, show a high degree of dependence on exogenous RXR for activity (*bottom panels* of Fig. 6, B and C), while both patient 1 fibroblasts and I314S-transfected COS-7 cells (*middle panels*, Fig. 6, B and C) show a lesser, but still quite apparent, improvement of activity in the presence of excess RXR.

The consistency between the results obtained with the artificial mutants (Fig. 6, A and B) with those results from actual patient fibroblasts (Fig. 6C), argues strongly that the I314S and R391C mutations are sufficient to explain the phenotype of patients 1 and 2, respectively. There is a slight discrepancy in the effects of certain doses, with the transfected COS-7 cells generally responding somewhat better to the same dose (compare Fig. 6B and 6C at the 10^{-7} M dose). This finding could possibly be due to larger amounts of expressed VDRs in COS-7 cells or to some other feature of these cells compared with fibroblasts, such as a decreased ability to catabolize the ligand. Nevertheless, the data in Fig. 6 from experiments performed at 25–37 C reveal a close interdependence between hVDR binding of sterol ligand and heterodimerization with RXR.

Cellular Uptake of 1,25-$(OH)_2D_3$ Measured in Intact Cells at 37 C

The disparity between the saturation binding assays in cell lysates at 4 C (Fig. 2) and the transactivation results seen in Fig. 4B and Fig. 6 suggested that the two mutations might render the respective receptors less responsive to ligand. To test this possibility, replicate samples of intact transfected COS-7 cells

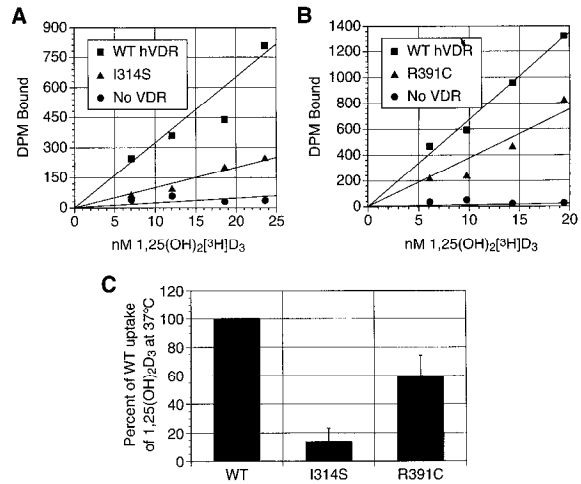

Fig. 7. Hormone Uptake in Intact Cells Measured at 37 C

COS-7 cells transfected with hVDR expression vectors were incubated with the indicated concentrations of 1,25-$(OH)_2[^3H]D_3$ for 2 h at 37 C, then processed as described in *Materials and Methods* to determine ligand uptake into the cells. Nominal concentrations of hormone were 5, 10, 15, and 20 nM, but actual concentrations were calculated from scintillation counting of total count tubes. Small variations in the amount of receptor present in transfected cells were monitored by Western immunoblotting, and results were corrected for those variations. A, COS-7 cells (4 million per 150-mm plate) were transfected with 1 μg wild type hVDR expression vector, 5 μg I314S vector, or carrier DNA. Expression of I314S was 108% that of wild type receptor by densitometric scanning of Western immunoblots. B, Cells were transfected with 10 μg wild type receptor vector, 20 μg of vector for R391C, or carrier DNA. Each plate also received 30 μg of a vector producing mouse RXRβ. Expression of R391C was 110% that of wild type hVDR, and expression of RXRβ was similar in all three lysates. C, A composite of independent determinations of cellular uptake ± SD by I314S (n = 2) and R391C (n = 3) at the highest ligand dose (nominally 20 nM).

were incubated at 37 C with increasing concentrations of 1,25-$(OH)_2[^3H]D_3$. Cells were washed at 4 C and then assayed for retention of intracellular labeled hormone. Although saturation of binding was not attained (Fig. 7, A and B), the results of several independent experiments (summarized in Fig. 7C), normalized to hVDR expression, clearly indicated that both mutants do not retain hormone normally at 37 C in the context of an intact cell. The I314S mutant was especially affected, with uptake of 1,25-$(OH)_2[^3H]D_3$ only 14% of that seen with wild type, while the R391C displayed 59% of wild type binding. Therefore, in spite of essentially normal results obtained at 4 C, a defect in the uptake or retention of ligand is suggested at physiological temperatures for both mutant receptors, an observation consistent with the requirement for supraphysiological doses of 1,25-$(OH)_2D_3$ to achieve significant levels of transactivation (Fig. 4B and Fig. 6).

Neither Mutation Creates a Dominant Negative Phenotype

Because pedigree information was not available for either patient, it was not possible to establish definitively whether patients 1 and 2 were homo- or heterozygous for the described mutations. However, there was no indication of the wild type receptor in any of the above described amplification reactions (three independent reactions for each patient), either by sequencing or by restriction enzyme analysis. Nevertheless, the possibility existed that these mutations might create dominant negative receptors. Transactivation was therefore measured for each mutant receptor, both in the presence or absence of an equal quantity of wild type hVDR (Fig. 8). This experiment also included an artificial hVDR mutant (T box mutant: K91N/E92Q) with a confirmed dominant negative phenotype (23). As previously demonstrated (Fig. 1B), hVDR from patients 1 and 2 yielded greatly reduced 1,25-$(OH)_2D_3$-dependent transactivation (1.6- and 3.5-fold in this experiment) as compared with the 50-fold stimulation by wild type hVDR. When the amount of expression plasmid for each mutant hVDR was halved and mixed with an equal portion of plasmid for wild type hVDR, transactivation by the mixed receptor rose to very nearly half that seen with wild type (approximately a 20-fold stimulation in both cases). In contrast, the artificial T box mutant gave less than a 9-fold stimulation even when an equal portion of wild type hVDR was provided, confirming the ability of this mutant to suppress transactivation by wild-type hVDR. These data indicate that mutant hVDRs from neither patient 1 nor patient 2 appear capable of potent dominant negative activity in an experimentally created, heterozygotic context.

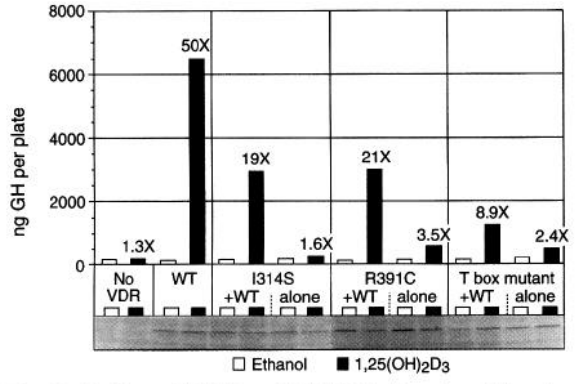

Fig. 8. Probing of I314S and R391C for Dominant Negative Activity

COS-7 cells were transfected with expression vectors for the indicated mutants or wild type hVDR plus the vitamin D-responsive $(CT4)_4$TKGH reporter gene construct (see *Materials and Methods*). Amounts of expression vector used were: 100 ng (if alone) or 50 ng (in combination with a mutant) of wild type hVDR vector; 250 ng (if alone) or 125 ng (in combination with wild type) of I314S vector; 1 μg (if alone) or 0.5 μg (in combination with wild type) of R391C vector; and 250 ng (if alone) or 125 ng (in combination with wild type) of the T box mutant. Values of GH secretion shown here were corrected for expression of each mutant relative to wild type hVDR as monitored by Western immunoblotting (see *bottom* of figure); the fold-stimulation by each mutant in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ was not affected by this calculation.

DISCUSSION

Two missense mutations localized to the hormone-binding domain of hVDR have been shown to display unique attributes compared with previously described natural hVDR mutants, which are mainly found in the DNA-binding zinc finger region (see Fig. 9, *bottom*). Both mutant hVDRs reported here display two apparent defects: a reduced ability to respond to the hormonal ligand and an impaired interaction with the RXR heterodimeric partner on the VDRE. This last observation places both mutations into a novel category of natural hVDR mutations.

Although gel mobility shift analysis quickly revealed the dimerization defect with the R391C mutant and suggested a minor defect in the I314S mutant as well, the ability of both mutant receptors to bind hormone normally at 4 C initially confounded a full assessment of the functional consequences of each mutation. However, the fact that both patients responded to pharmacological doses of vitamin D analogs led to further testing, including transactivation and gel mobility shift assays at varying concentrations of 1,25-$(OH)_2D_3$ and cellular uptake of ligand at 37 C, which together provided a more complete picture of the phenotype of each mutant VDR. The results of these experiments, summarized in Fig. 6, show a distinctive combination of impairments in ligand responsiveness and heterodimerization for each mutant. Thus, the I314S mutation generates a VDR with very poor response to ligand at physiological temperatures, a defect that appears to secondarily amplify a mild defect in heterodimerization with RXR. Conversely, the R391C mutation yields a VDR with markedly reduced ability to heterodimerize and a coexisting modest attenuation of response to 1,25-$(OH)_2D_3$. The ultimate consequences of both mutations are similar, with the occurrence of a fundamental block in 1,25-$(OH)_2D_3$-dependent heterodimerization of endogenous levels of hVDR and RXR at 37 C and resultant poor VDRE binding, *in vivo*. This conclusion is substantiated by the fact that neither I314S nor R391C generates a dominant negative hVDR species (Fig. 8). In our hands, to become a dominant negative VDR, the mutant receptor must still heterodimerize with RXR and exert its effects as an inactive heterodimer.

Whether our results can be interpreted as indicating temperature-sensitive hormone binding by either mutant is not clear. Indeed, a previous study of patient 1 fibroblasts (16) indicated that ligand binding approached the normal range after incubations at 37 C.

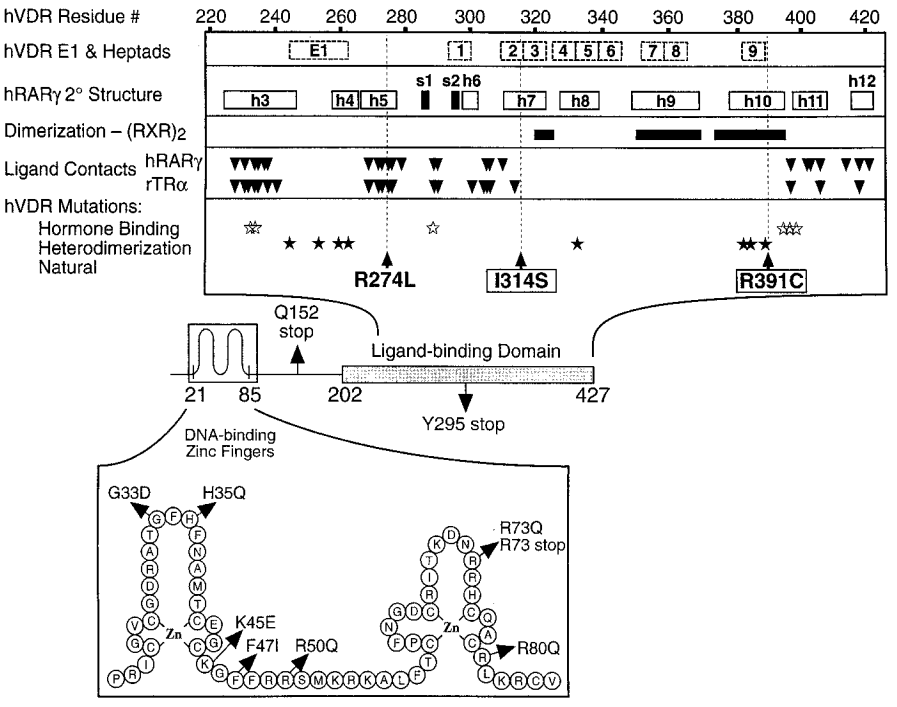

Fig. 9. Location of Known Natural hVDR Mutations Relative to Functional Domains

Note that the numbering scheme of Baker *et al.* (18) has been used for uniformity; an alternate numbering scheme beginning at the fourth codon (also methionine) has been used in many of the cited reports. The DNA and hormone-binding domains of hVDR have been enlarged (two *insets*) to allow for depiction of individual mutations in these regions (8–15). Regions of the hVDR hormone-binding domain corresponding to either the proposed heptad repeats (21) or to the α-helices/β-strands found in the crystal structure of hRARγ (28) are shown in the *upper inset* (top two registers). Regions that correspond to homodimer interfaces in the hRXRα crystal (20) are indicated by *solid boxes* in the third register. Positions corresponding to residues that participate in ligand binding by either hRARγ (28) or rTRα (27) are depicted by *arrowheads* in the fourth register. The *bottom* register displays the position of the two natural mutations described here, as well as the previously described R274L mutation that results in a hormone-binding defect (14, 17). Also shown are published artificial hVDR mutations from our laboratory which result in either hormone binding (☆) or heterodimerization (★) defects (22, 46, 52, 53).

Also, our own tests of hormone binding in cytosols at 23 C failed to directly demonstrate a ligand-binding defect with either mutant (data not shown). However, the observation that the I314S and R391C mutant hVDRs cannot mediate normal uptake of ligand into transfected cells (Fig. 7) leaves the possibility open that some aspect of ligand binding or dissociation is affected by temperature in these mutations.

Recent studies on the role of ligand in transactivation by the nuclear receptors TR, RAR, and NGFI-B/Nurr1 (24–26), as well as our own studies with VDR (P. D. Thompson and M. R. Haussler, unpublished results), suggest an alternative explanation for the loss of hormone responsiveness in these mutants. It has been proposed that the role of ligand is to induce conformational changes in these receptors that not only promote heterodimerization with RXR but also potentiate transactivation by the resulting heterodimer. Thus, the defect in I314S or R391C hVDR might be not in hormone-binding, *per se*, but in the ability of these receptors to undergo the subsequent conformational changes that both promote the retention of ligand and mediate transactivation via productive contact with coactivators.

Recently, crystal structures have been reported for the hormone-binding domains of hRXRα (20), rat TRα (27), and hRARγ (28), providing important structure-function insights into the ligand-binding and dimerization activities of nuclear hormone receptors. The ligand-binding domain of hRXRα was obtained as an apohomodimer (20), while both rat TRα and hRARγ were crystallized as monomeric species bound to their respective ligands (27, 28). Despite the differences in receptor identity and the circumstances of crystallization, the general location of the structural elements (mainly α-helices) seems quite consistent among the three receptors, although the precise beginning and end of each element varies slightly (see Fig. 1 in Ref. 27).

An examination of the position of the I314S and R391C mutations relative to these structural elements is revealing (Fig. 9). Ile-314 lies within the seventh α-helix, a position near one of the dimer contacts in hRXRα, and even closer to a ligand contact in rat TRα. This location between ligand-binding contacts and the dimerization interface is consistent with the observed defects, both in heterodimerization and in responsiveness to ligand. A natural mutation has been found in

the human androgen receptor in a location (Met-786) almost exactly corresponding to Ile-314 in hVDR (29). This mutant receptor, from a patient with complete androgen insensitivity, retains hormone poorly in a whole cell assay, like I314S, but whether this altered receptor also has a minor dimerization defect has not been reported.

An interesting contrast is provided by the Arg-391 mutation, which occurs at a position near the C-terminal end of helix 10 and one residue outside of heptad 9 (Fig. 9). This location is well within the dimerization interface of hRXRα and only six residues removed in the primary sequence from a ligand contact in either rat TRα or hRARγ. Again, the location of the mutation relative to the structural elements is consistent with the phenotype of the cloned R391C mutant VDR, which shows a strong heterodimerization defect (Figs. 3, B and D, 5, and 6) and a more modest defect in ligand responsiveness (Figs. 4B, 6, and 7).

A previous report from our laboratory (22) had included *in vitro* tests of artificial hVDR mutants within heptad 9. The mutants K382E, M383G, Q385K, and, most significantly, L390G (immediately adjacent to Arg-391) each appeared to display primarily RXR heterodimerization defects, while ligand binding affinities at 4 C were less affected (22). Other reports involving mutagenesis of TR and estrogen receptor (30–34) implicate the helix 10/heptad 9 region in both homo- and heterodimerization. Taken together, these data suggest that the helix 10/heptad 9 region may be vital for the dimerization activities of nuclear receptors in general.

A natural arginine mutant (R429Q) has been recently reported in human TRβ (35), at a position exactly corresponding to Arg-391 in hVDR. This mutant shares relevant characteristics with R391C, including partial loss of dimerization as well as incomplete rescue by elevated levels of hormone (presumably at 37 C). In a study by a different laboratory (36), the same R429Q mutation in hTRβ was artificially created. Their results indicated that: 1) hormone binding by R429Q was normal at 4 C; and 2) R429Q did not display the dominant negative phenotype seen in many natural hTRβ mutations (36). This last observation can be explained by the reduced dimerization potential of R429Q, which may be required for dominant negative activity by hTRβ (30, 35, 36).

Mutations in the hVDR are assumed to be inherited as autosomal recessive traits (8). Our results suggest that both patients described here are either homozygous or have suffered a deletion of one allele, since no evidence was seen of the wild type allele in three independent PCR amplifications from each patient fibroblast culture. Our demonstration (Fig. 8) that neither mutant hVDR exhibits dominant negative character is also consistent with homo- or monozygosity at this locus. The I314S mutation shares many traits with dominant negative mutant hTRβs in that DNA binding and heterodimerization potential are largely preserved (30, 35). However, no dominant negative hTRβ mutation has yet been found in a location corresponding to I314S in the central portion of helix 7, a position that is outside the two clusters of natural hTRβ mutations (35). An explanation for these findings is not entirely clear at present. Moreover, given the lack of known natural hVDR dominant negative mutations, there may be a fundamental difference between VDR and TR in the ability of the unliganded receptor to suppress transactivation. Indeed, a corepressor that inhibits transactivation by binding the unliganded TR (and RAR) on DNA, but which does not appear to bind VDR, has been recently reported (37).

The ability of excess hormone to override the defect in both mutant hVDRs (see also Ref. 38) argues for a role of the 1,25-$(OH)_2D_3$ ligand in stabilizing the receptor in a configuration that can both form a complex with RXR on a VDRE and interact with the transcription machinery. Whether the loss of hormone responsiveness by these mutant VDRs is a direct result of impaired ligand binding, or of the ability of the receptor to respond conformationally after ligand binding, cannot be discerned with the present data. Even though important details concerning the transcriptional activation mechanism await further study, the present results provide a unique demonstration of the clinical significance of both ligand and RXR in transactivation by VDR.

MATERIALS AND METHODS

Patient Fibroblasts

Skin punch biopsies were performed on patients 1 and 2 to obtain viable fibroblasts for cell culture. Characterizations of patient 1 (16) and the R274L (14, 17) fibroblasts have been reported elsewhere. The biopsy sample for patient 2 was cultured essentially according to the method of Sly and Grubb (39). All fibroblast cultures were maintained in 50% DMEM and 50% Ham's F-12 medium (supplied by Life Technologies, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Calabasas, CA).

Initial Cloning of hVDR Sequences from Patient Fibroblasts

Poly(A)$^+$ RNA was obtained from 1–2 $\times$ 10^6 fibroblast cells using a FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA). RT-PCR was then performed on 1 μl of isolated poly(A)$^+$ RNA (total volume, 10–20 μl) using a GeneAmp RT kit (Perkin Elmer, Foster City, CA) and an Eppendorf thermocycler (Brinkmann, Westbury, NY). Primers were designed flanking either side of the hVDR cDNA coding region. The 5′-primer, corresponding to −42 to −21 in the hVDR cDNA, but containing an internal *Eco*RI site (*underlined*), was 5′-GCACCCCTGAATTCCACTTAC-3′, and the 3′-(antisense) primer, from +1333 to +1353 and containing an internal *Bam*HI site (also *underlined*), was 5′-CGCCAGCCCGGATCCTGGCACG-3′. The reverse transcriptase reaction (3′-primer only) was for 30 min at 42 C, followed by a brief denaturation and rapid cooling to 5 C. After addition of the second (5′) primer and *Taq* polymerase, 30 cycles of amplification were performed using an annealing temperature of 58 C and an extension temperature of 72 C. PCR products of approximately 1400 bp (predicted size 1395 bp) were di-

gested with *Eco*RI and *Bam*HI, then cloned into the pSG5 shuttle vector. Sequencing of the PCR products was performed using a Sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

Human genomic DNA was prepared from patient fibroblasts (1–2 × 10^6 cells) using a Qiagen DNA isolation kit. To confirm the I314S mutation in patient 1, a portion of the hVDR gene containing exons 7 and 8 was amplified using primers adapted from Malloy *et al.*, (40). The upstream primer (sense) had the sequence 5′-GCGAATTCCGTTACTGGTAACCTGACCTCTTC-3′, with an added *Eco*RI site (*underlined*) near its 5′-end. The downstream, antisense primer, was 5′-TGTCTAGAATACACCCCGCTCCCCAGTCCCTGAG-3′ with an added *Xba*I site. To confirm the R391C site in patient 2, a portion of exon 9 was amplified by using the complement of the antisense probe given above, with the sequence 5′-CCGAATTCCTCAGGGACTGGGGAGCGGGGTGTAT-3′ and an introduced *Eco*RI site (*underlined*). For the downstream antisense probe, the oligonucleotide 5′-CCTCTAGACCCACCCAGGCACCGCACAGGCTGTCCTAG-3′ was used (from −30 to −1 in the 3′-untranslated region of the hVDR cDNA; see Ref. 18), with an *Xba*I site near its 5′-end. The PCR reaction conditions were similar to those described above for RT-PCR, omitting the initial reverse transcriptase step. Products of each amplification reaction were digested with *Eco*RI and *Xba*I, then subcloned into the pTZ18U plasmid vector (U.S. Biochemical Corp.) for sequencing as above.

Recreation of I314S and R391C Mutations in Wild Type hVDR by Site-Directed Mutagenesis

Due to the presence of PCR artifacts in the amplified patient cDNAs, which renders each cDNA unusable for functional testing, the point mutations that were identified in patients 1 and 2 were separately introduced into wild type hVDR by site-directed mutagenesis according to the method of Kunkel *et al.* (41) using the MutaGene kit (Bio-Rad Laboratories, Hercules, CA) as described previously (42). Each site-directed mutant was confirmed by sequencing.

Transfection of Cultured Cells and Transactivation Assays

Patient fibroblasts (4 × 10^6 cells per cuvette) were transfected with 40 μg of each plasmid using a Bio-Rad Gene Pulser (set at 200 V) with capacitance extender (set at 960 μFarads), then plated in 60 mm plates at 1 × 10^6 cells per plate. Cells were incubated for 72 h with or without hormone treatment (10^{-8} M) before preparation of cell lysates or harvesting of medium. For the experiment shown in Fig. 6, transfection efficiency was monitored by the inclusion of pCMVβ expression plasmid (Clontech Laboratories, Inc., Palo Alto, CA), followed by assay of β-galactosidase activity in cell lysates using a commercial kit (Promega Corp., Madison, WI).

COS-7 cells were maintained in DMEM (supplied by Life Technologies, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts). Cells were plated in culture dishes of various sizes and transfected with pSG5 expression vectors containing a cDNA encoding either mutant or wild type hVDR. The calcium phosphate coprecipitation technique was used without glycerol shock (43). Seven to 16 h posttransfection, cells were washed, then incubated for an additional 48 h with or without hormone treatment before preparation of cell lysates or harvesting of medium.

For transactivation assays, transfections included the $(CT4)_4$TKGH vector, which contains four copies of the rat osteocalcin VDRE [as oligonucleotide "CT4" (44)], inserted into a human GH reporter gene construct (44, 45). After transfection and washing, cells were treated for 48 h (COS-7) or 72 h (patient fibroblasts) with either 10 nM 1,25-$(OH)_2D_3$ or ethanol. Aliquots of medium were assayed for secreted human GH (hGH) by RIA using a commercial kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). Cells were then washed three times in ice-cold isotonic buffer and lysed in 125 mM Tris-HCl, pH 6.8, containing 2% SDS and 10% glycerol for Western immunoblot analysis (see below). Digitized computer images of each immunoblot were obtained using an Apple OneScanner with Ofoto software (Light Source Computer Images, Inc. and Apple Computer, Inc., Cupertino, CA), and the relative expression of hVDR proteins was determined from the scanned images using Scan Analysis software (Biosoft, Ferguson, MO) for normalization of results.

Ligand-Binding Assays

Patient fibroblasts (Fig. 1) or transfected COS-7 cells (Fig. 2) were harvested by scraping, and lysates were prepared as described by Nakajima *et al.* (22). Lysates were divided into small aliquots and stored at −70 C.

To test patient fibroblasts (Fig. 1A), 12.5-μl aliquots of radiolabeled hormone [1,25-$(OH)_2$-26-27[^{3}H]dimethyl-vitamin D_3, 165 Ci/mmol, Amersham Life Science, Arlington, IL], together with 6.25 μl of either unlabeled 10^{-5} M 1,25-$(OH)_2D_3$ or ethanol vehicle, were dried in 12 × 75-mm glass tubes, then redissolved in 2.5 μl ethanol and combined with 250 μl cell lysate for a final 1,25-$(OH)_2D_3$ concentration of 2.9 nM. Lysates were from duplicate plates of each fibroblast type, divided into four tubes each (two with ethanol vehicle, two with 10^{-5} M unlabeled hormone). After a 7- to 16-h incubation at 4 C, bound and free ligand were separated with DE-81 filters as described previously (46). Duplicate tubes receiving ^{3}H-labeled hormone and 6.25 μl ethanol vehicle gave estimates of total binding from each plate, while those tubes receiving labeled hormone combined with unlabeled competitor (~100-fold excess), provided a measure of nonspecific binding. The averages of two values from each plate were used to compute specific binding (total minus nonspecific), after which values from two plates of each cell type were averaged to obtain the values plotted in Fig. 1A.

To test transfected COS-7 cells (Fig. 2), 65 μl radiolabeled hormone were diluted to 18 Ci/mmol. Dilutions of this stock (10 μl) were combined in a series of 200-μl reactions, each containing 20–50 μl cell lysate and 30 μl rat liver nuclear extract (22), but varying in the concentrations of 1,25-$(OH)_2[^3H]D_3$ (ranging from 0.1 to 1.6 nM). After incubation at 4 C for 7–16 h, bound and free ligand were separated with a Dextran-coated charcoal suspension as described by Dokoh *et al.* (47). Aliquots of supernatant were removed for scintillation counting, and data were analyzed using a Scatchard analysis routine originally written in BASIC (48) and adapted for the Macintosh by Robert E. Williams (Xoma Corp., Santa Monica, CA).

For assessing cellular uptake at 37 C, transfected COS-7 cells were harvested by trypsinization, then resuspended at 3.5 × 10^6 cells/ml in DMEM containing 1% FBS. Aliquots of this suspension (200 μl) were incubated for 2 h at 37 C with shaking in the presence of radiolabeled hormone, which had been adjusted to a specific activity of 8 Ci/mmol, then diluted to yield final concentrations of 1,25-$(OH)_2D_3$ in the 5–20 nM range. Cells were washed twice at 4 C with Dulbecco's PBS containing 1% BSA followed by a wash with PBS alone. Cell pellets were lysed in 300 μl KETZ-0.3 buffer containing protease inhibitors as described by Nakajima *et al.* (22). After a 30-min incubation at 4 C, lysates were clarified by centrifugation at 14,000 × *g*, and 250-μl aliquots were removed for determination of ligand retention by scintillation counting. Portions of lysate were also taken for immunoblot analysis (see below).

Gel Mobility Shift Analysis of hVDR Mutants

A synthetic double-stranded oligonucleotide containing the rat osteocalcin VDRE, 5′-AGCTGCACTGGGTGAATGAGGACATTACA-3′, denoted CT5 (44), was labeled as described previously (22). RXR proteins overexpressed in the baculovirus-infected Sf9 cell system (6) were partially purified. Briefly, whole cell lysates were prepared by sonication as described (6), then subjected to chromatography on a Blue Dextran Sepharose column as detailed for similarly expressed hVDR (49) and stored at −70 C. Typically, 5–6 μg transfected COS-7 cell lysate (prepared as above) were incubated with ≤ 1 μl partially purified RXR in DNA-binding buffer [10 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 mg BSA, 1 mg poly(deoxyinosinic-deoxycytidylic acid)] for 15 min at 22 C and incubated with 0.5 ng of ^{32}P-labeled CT5 oligonucleotide for an additional 15 min. Electrophoresis and autoradiography conditions were as described previously (22).

Western Immunoblotting

Cultured cells were lysed directly in 2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 10% glycerol, and 40–100 μg of cellular protein were run on 10% SDS/polyacrylamide gels. Cell lysates prepared by other means were combined with one volume of a 2X form of the above buffer. Electrophoretic fractionation, blotting, and immunological detection by either 9A7γ anti-VDR or MOK 13.17 (50) anti-mRXRβ monoclonal antibodies were as described (6, 42). Protein bands on the developed blot were then subjected to densitometric quantification (see *Transactivation Assays* above).

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