Examination of the Potential Functional Role of Conserved Cysteine Residues in the Hormone Binding Domain of the Human 1,25-Dihydroxyvitamin D$_3$ Receptor*

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The significance of conserved cysteines at positions 288, 337, and 369 in the hormone binding domain of the human vitamin D receptor was evaluated by individual site-directed mutagenesis to glycine. Neither nuclear localization nor heterodimerization with retinoid X receptors in binding to the vitamin D-responsive element was appreciably affected by altering these cysteines, but vitamin D hormone (1,25-(OH)$_2$D$_3$) activated transcriptions was moderately attenuated in C337G and very mildly in C369G. The degree of impairment of ligand binding at physiologic temperatures correlated with the requirement for increased concentrations of 1,25-(OH)$_2$D$_3$ ligand to maximally stimulate transcriptional activity in co-transfected COS-7 cells. Thus cysteine 288 and, to a lesser extent, cysteine 337 are important for high affinity hormone binding to the vitamin D receptor, which ultimately leads to ligand-dependent transcriptional activation.

The 1,25-dihydroxyvitamin D$_3$ receptor (VDR)$^1$ is a nuclear protein that mediates many of the biological actions of the 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) hormone, such as regulating calcium/phosphorus metabolism and cellular differentiation (1). The VDR belongs to the steroid/retinoid/thyroid hormone receptor superfamily and, as with other members of this receptor family, consists of a highly conserved domain, which contains two zinc finger motifs required for DNA binding, and a carboxyl-terminal hormone binding domain (HBD) responsible for the specific, high affinity binding of 1,25-(OH)$_2$D$_3$, the active form of vitamin D (2). The binding of ligand presumably initiates a conformational change in the VDR protein whereupon the hormone-receptor complex binds to distinct sequences of nucleotides, termed vitamin D-responsive elements (VDREs), which are upstream of target genes and thereby modulates transcription (3). VDRE sequences have been identified in the promoter regions of the human (4) and rat (5) osteocalcin genes as well as in the mouse osteopontin gene (6). Generally, VDREs consist of imperfect direct repeat of six nucleotide bases, GGGTGA, separated by a three-base pair spacer. Gel mobility shift analysis using these VDREs has revealed that an additional nuclear factor, the family of retinoid X receptors (RXRs), forms heterodimers with the VDR (7–9) and facilitates its binding to DNA. VDR-RXR heterodimerization on VDREs is postulated to play an essential role in transcriptional modulation of target genes through the VDR. Mutational analysis has revealed that a conserved region between residues 244 and 263 (10, 11), as well as the fourth and ninth heptad repeats (12) in the HBD of human VDR, are essential for heterodimerization on the VDRE. We have also reported that the region between amino acids 403 and 427 in the HBD of hVDR may be involved in transcriptional regulation (12). Therefore, the HBD of hVDR must be considered as a multifunctional domain important not only for binding to the 1,25-(OH)$_2$D$_3$ ligand, but also for forming a heterodimer with RXR and likely for interacting with the transcriptional machinery. These functions are closely related, since the 1,25-(OH)$_2$D$_3$ hormone enhances the formation of VDR heterodimers with RXR on the VDRE (9, 13) and co-expression of RXRs enhances ligand-dependent transactivation mediated by the VDR (9).

Cysteine residues are known to play a vital role in the formation and maintenance of protein conformation. Eight cysteine residues in the DNA binding domain are absolutely conserved among the steroid/retinoid/thyroid hormone receptors and form two zinc fingers which are involved in binding to the cognate-responsive elements of these receptors (14). Furthermore, in keeping with an important structural role for cysteines, several cysteinyl residues in the HBD of steroid hormone receptors have been proposed to be involved in ligand binding. Previous studies revealed that cysteine residues in the glucocorticoid receptor (GR) (15–20) and estrogen receptor (ER) (21–24) play important roles in high affinity ligand binding. In the HBD of VDR, cysteines at positions 288, 337, and 369 are conserved in the human (25), rat (26), and avian (27, 28) receptors. Little information is available on the importance of HBD cysteines in the mechanism of VDR action, although in an early biochemical experiment, Coty et al. (29) showed that treatment of hormone-occupied avian VDR with mercurial reagents causes a dissociation of the 1,25-(OH)$_2$D$_3$ ligand. This result suggested that VDR amino acids with sulfhydryl-containing side chains may play a crucial role in ligand binding,
perhaps by maintaining the proper conformation of the hormone binding pocket. Detailed involvement of specific cysteines in this and other functions of VDR remains to be elucidated. In the present study, we constructed several site-specific mutant hVDRs to examine the precise roles of each of these conserved cysteine residues in the HBD of hVDR.

**MATERIALS AND METHODS**

Preparation of Wild-type and Mutant VDRs—The hVDR expression vector, pSG5/hVDR (30), was utilized to create point-mutant hVDR plasmids by in vitro site-directed mutagenesis (31). Three cysteine residues in the HBD of hVDR at positions 288, 337, and 369, which are conserved among species, were replaced by glycines (designated C288G, C337G, and C369G, respectively). COS-7 monkey kidney cells (5 × 10^6 cells/150-mm culture dish) were transfected with 50 μg of each plasmid/plate by the calcium-phosphate DNA co-precipitation method, as described previously (32), and cultured in Dulbecco’s modiﬁed Eagle’s medium (DMEM) (Life Technologies, Inc.) with 10% dextran-coated charcoal-stripped fetal bovine serum (Gemini Bioproducts, Calabasas, CA) for 48 h. The cells were then scraped, washed three times with phosphate-buffered saline (136 mM NaCl, 26 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2), and resuspended in KETD-0.3 buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 300 mM KCl, 1% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15 μM aprotinin, 100 μg/ml leupeptin, 1 μg/ml pepstatin A). After sonication, samples were centrifuged at 215,000 × g for 30 min at 2°C. The supernatant was collected, divided into small aliquots, and stored at −70°C. Western blotting of VDR was performed with anti-VDR monoclonal antibody, 9A7γ, as described previously (33). Protein concentrations were determined according to the method of Bradford (34).

**Ligand Binding Assays**—COS-7 cell extracts containing wild-type or mutant VDR proteins were incubated with the indicated concentrations of 1,25-(OH)2D3 (Amersham Corp.) for 10 h at 4°C or for 2 h at 23°C along with 20 μg of rat liver nuclear extract in the presence or absence of a 400–600-fold molar excess of unlabeled 1,25-(OH)2D3. Addition of rat liver nuclear extract, which alone does not possess ligand binding activity, to samples containing overexpressed VDR is required for efficient high affinity, saturable 1,25-(OH)2D3 hormone binding (35). Bound and free ligand were separated with dextran-coated charcoal for analysis of hormone binding (36).

**Cellular Distribution of VDR**—COS-7 cells transfected with wild-type or mutant VDR plasmids were incubated in the absence or presence of 10−8 M 1,25-(OH)2D3 for 12 h at 37°C. The nuclear versus cytosolic partitioning of VDR was determined as described previously (33).

**Nuclear Uptake Assay**—Assessment of the nuclear uptake of 1,25-(OH)2D3 by COS-7 cells was performed essentially as described previously (37) with slight modifications. COS-7 cells overexpressing hVDRs were harvested with 0.025% trypsin, 0.02% EDTA and washed twice with DMEM (Life Technologies, Inc.) with 10% dextran-coated charcoal-stripped fetal bovine serum. Cells were then resuspended in DMEM containing 1% fetal bovine serum at a concentration of 2.5 × 10^6 cells/ml and incubated with labeled 1,25-(OH)2D3 at 37°C for 3 h (shaking 100 rpm). Nuclear fractions were obtained as described elsewhere (33), and the radioactivity was measured by liquid scintillation counting. Specific binding was calculated by subtracting nonspecific binding from total binding.

**Gel Mobility Shift Assay**—Gel mobility shift assays were performed in the absence of 1,25-(OH)2D3 ligand as described previously (38). Briefly, cellular extracts from COS-7 cells overexpressing wild-type or mutant hVDR were incubated with 0.5 ng of 32P-labeled rat osteocalcin VDRE (5’-AGCTGACTGGTGATGAGGACATTACA-3’) in the presence of RXRs or RXRβ for 30 min at 22°C. The reaction mixtures were loaded onto 4% non-denaturating polyacrylamide gels in 0.25 × TBE (11 mM Tris, 11 mM borate, 0.5 mM EDTA). Gels were run at 10 mA for 70 min, dried, and subjected to autoradiography.

**Transcriptional Activation Assay**—Four copies of the CT4 oligonucleotide, which contains the rat osteocalcin VDRE, were subcloned into the upstream region of the viral thymidine kinase promoter–growth hormone reporter gene construct (Nichols Institute, San Juan Capistrano, CA) creating (CT4)4-TKGH (5). COS-7 cells (5 × 10^5 cells/60-mm culture plate) were co-transfected with 5 μg of CT4-TKGH and wild-type or mutant hVDR plasmids and then incubated with a final concentration of 10−10 to 10−6 M 1,25-(OH)2D3 or ethanol vehicle for 12 h at 37°C. Growth hormone concentrations in the culture medium were determined by radioimmunoassay (Nichols Institute).

**RESULTS**

Expression of Wild-type and Mutant hVDRs—Cysteine residues in the HBD of hVDR at positions 288, 337, and 369, which are conserved in VDRs from various species, were individually replaced with glycine by site-directed mutagenesis (designated C288G, C337G, and C369G, respectively). The resulting mutant hVDR proteins are depicted schematically in the context of the hormone binding domain. B, wild-type and mutant hVDRs were expressed in COS-7 cells, and cell extracts were prepared as described under “Materials and Methods.” These lysates were subjected to immunoblotting with a specific monoclonal antibody to VDR (9A7γ). Molecular weight standards are shown in the first and last lanes.

**Heterodimerization on the VDRE**—Because the replaced cysteines are near heptad repeat regions of hVDR (Fig. 1A) known to be required for dimerization with RXR, we first performed gel mobility shift assays with cellular extracts from transfected COS-7 cells overexpressing wild-type or mutant hVDRs in order to compare the abilities of the receptors to form heterodimers with RXR on the VDRE. The amount of COS-7 cellular extract utilized in these VDRE binding assays was adjusted based on the level of hVDR expression as determined by gel mobility shift assays. The mutations introduced do not appear to dramatically affect the expression or stability of the hVDR protein in COS-7 cells.
C337G and C369G were slightly diminished in their ability to form shifted complexes in relation to the native hVDR (see lanes 3 and 4 or 8 and 9, Fig. 2). This analysis utilizing enriched preparations of RXRα and RXRβ revealed that all mutant hVDRExamined formed qualitatively normal heterocomplexes with the RXRs, with only minor quantitative attenuation of C337G and C369G, the latter effect best detected by careful examination of the relative amounts of unshifted probe (Fig. 2). Extracts of COS-7 cells transfected with a control vector lacking the hVDR cDNA, even in the presence of RXRs (Fig. 2, lanes 5 and 10), did not form a complex on the VDRE. This observation, plus the fact that shifted complexes could be inhibited by VDR-specific monoclonal antibody 9A7 (data not shown), argues that the retarded bands are indeed comprised of complexes of hVDR. Finally, identical conclusions regarding the mutant hVDRExpressed in COS-7 cells were reached when heterodimerization was assessed in combination with human RXRα (lanes 1-5) or mouse RXRβ (lanes 6-10). RXRα and RXRβ were expressed and partially purified as described elsewhere (9).

Nuclear Translocation of Expressed Receptors—Steroid/retinoid/thyroid hormone receptors are localized in the nucleus of all mammalian cells. In the case of VDR, like the retinoid and thyroid hormone receptors, the unoccupied receptor is already predominantly localized in the nucleus (39, 40). Although the "hinge" region between the DNA binding and HBD of many nuclear receptors has been proposed to be responsible for nuclear translocation (14), it is possible that conformational changes resulting from the introduction of point mutations in the hormone binding domain may attenuate nuclear translocation and therefore diminish DNA binding and transcriptional activity of mutant hVDRExamined. This observation, plus the fact that shifted complexes could be inhibited by VDR-specific monoclonal antibody 9A7 (data not shown), argues that the retarded bands are indeed comprised of complexes of hVDR. Finally, identical conclusions regarding the mutant hVDRExpressed in COS-7 cells were reached when heterodimerization was assessed in combination with human RXRα (lanes 1-5) or mouse RXRβ (lanes 6-10). RXRα and RXRβ were expressed and partially purified as described elsewhere (9).

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VDR immunoblotting utilizing equivalent amounts of total protein of nuclear (N) and cytosolic (C) fractions of COS-7 cells transfected with the indicated receptor and incubated in the absence or presence of 1,25-(OH)2D3. As expected, VDR is enriched in the nuclear fraction and the subcellular partitioning is not markedly affected by treatment with the hormonal ligand. Most relevant, all of the mutant hVDRExhibit a similar apparent N/C ratio when compared with the wild-type receptor as determined by quantitative densitometric scanning of these immunoblots (data not shown). In this experiment, we noted that C337G was either expressed in lower amounts or was more unstable (especially in the absence of ligand) during the fractionation procedure than wild-type receptor and the other two hVDR mutants, but nuclear localization was still evident. These results strongly suggest that mutations at cysteine 288, 337, or 369 do not significantly alter the ability of the receptor to translocate into the nucleus.

1,25-(OH)2D3 Ligand Binding at 4 °C, in Vitro—Considering that mercurial reagents had been found to dissociate 1,25-(OH)2D3 from VDR (29), we next examined the ligand binding activity of the cysteine-mutant hVDRExamining an assay performed in vitro. Extracts from transfected COS-7 cells were incubated at 4 °C for 10 h with 1,25-(OH)2D3 at concentrations of 0.23, 0.45, 0.90, 1.35, and 1.8 nM. Scatchard analyses of saturable, specific hormone binding by wild-type and mutant hVDRExamined yielded evidence for a single class of high affinity binding sites in each case (data not shown). A summary of the average results (±S.E.) from four separate experiments is as follows. The dissociation constant of C288G (293 ± 40 pm) was 3.4 times higher than the wild-type Kd of 86 ± 15 pm (p < 0.005). In contrast, C337G (Kd = 35 ± 7 pm) demonstrated approximately one-half of the dissociation constant of the wild-type (p < 0.02), while C369G (Kd = 77 ± 2 pm) was essentially equivalent to the wild-type receptor with respect to this parameter. Thus, only mutation of cysteine 288 appears to significantly diminish the affinity of hVDR for the 1,25-(OH)2D3 ligand, at least as assessed by assay of binding at 4 °C, in vitro.

Ligand-Dependent Transcriptional Activation by 10 nm 1,25-(OH)2D3—Even though alteration of the three cysteines in question did not dramatically affect ligand or DNA binding, in vitro, we proceeded to investigate 1,25-(OH)2D3-mediated transcriptional activation by wild-type or mutant hVDRExamined in COS-7 cells. Fig. 4A shows the effects of a slightly supraphysiologic concentration of 10-8 M 1,25-(OH)2D3 on VDRE-mediated transactivation. The hormone generated a 33-fold increase
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To amplify this analysis of transactivation, we performed a series of dose-response experiments in co-transfected COS-7 cells treated with 1,25(OH)2D3 concentrations ranging from 10−10 to 10−6 M. A representative result is pictured in Fig. 4B, showing that the C369G hVDR mediates transcriptional activation nearly as effectively as wild-type receptor, with only a slight defect apparent at the lower doses of 10−9 and 10−10 M 1,25(OH)2D3. In the case of C337G, the ligand response curve is appreciably shifted to the right, with normalization of transactivation only occurring at the relatively high level of 10−7 M 1,25(OH)2D3 and no significant effect at 10−9 M 1,25(OH)2D3 (Fig. 4B). C288G is clearly the most severely affected mutant, with a minuscule but statistically significant response at 10−7 M 1,25(OH)2D3 and the requirement for a concentration of 10−6 M 1,25(OH)2D3 to restore maximal transactivation. From the experiment depicted in Fig. 4B and two independent repeats, the following EC50 values (in nM 1,25(OH)2D3) were calculated: wild-type VDR = 1.0 ± 0.2; C337G = 1.7 ± 0.3; C327G = 4.3 ± 1.5; and C288G = 187 ± 23. These data provide indirect evidence for a mild hormone binding suppression in C369G and more substantial reductions in ligand binding affinities at 37°C for C337G and especially C288G. The observation that transactivation can be effectively rescued in all mutant hVDRs by increasing ligand concentrations argues against any of the three cysteines in question participating in subsequent interaction with the transcription machinery.

1,25(OH)2D3 Binding at Elevated Temperatures—Although ligand binding studies with cytosolic extracts at 0−4°C are traditional for steroid hormone receptors, the dissociation constants for the three cysteine mutant hVDRs obtained in this fashion (see above) are inconsistent with apparent ligand binding affinities inferred from the transcriptional dose-response patterns shown in Fig. 4B. For example, C288G exhibits only a 3.4-fold increase over wild-type in apparent Kd as assessed at 4°C, while the EC50 for 1,25(OH)2D3 action via this mutant is 187 times that of the wild-type receptor. To resolve this discrepancy, we more directly examined the ligand binding activities of wild-type and mutant hVDRs at 37°C, in vivo, by measuring the nuclear uptake of tritiated 1,25(OH)2D3 in intact COS-7 cells overexpressing the various hVDRs. Fig. 5A illustrates the specific binding of ligand in nuclear fractions of transfected COS-7 cells at five different concentrations of 1,25(OH)2D3. In studies such as the one pictured in Fig. 5A, we have found that saturation of wild-type hVDR even at low, endogenous receptor levels occurs only at relatively high concentrations of 1,25(OH)2D3 ligand, i.e. 5 nM (37). All three mutant hVDRs showed reduced nuclear uptake of the ligand in the intact COS-7 cells. Significantly, C288G demonstrated the lowest uptake, which was approximately 5% of wild-type levels; this finding correlates with the requirement for very high concentration of hormonal ligand (10−6 M) to stimulate receptor-mediated transactivation for this mutant (Fig. 4B). C337G, which paradoxically exhibited higher ligand binding activity, in vitro, at 4°C as compared with the wild-type receptor, but a shift to the right in the transcriptional activity dose-response curve with 1,25(OH)2D3 at 37°C (Fig. 4B), displayed markedly reduced nuclear uptake of 1,25(OH)2D3. Because nuclear partitioning of all mutant hVDR proteins appears normal (Fig. 3), these data suggest that 1,25(OH)2D3 ligand binding activity, in vivo, at 37°C is attenuated by the mutation at cysteine 337 and to a greater degree by altering cysteine 288. The results illustrated in Fig. 5A also reveal a small but significant suppression of nuclear uptake of ligand at 37°C for the C337G receptor. Again, this slight defect appears to be in concert with the 70% increase in EC50 for 1,25(OH)2D3-stimulated transcription with this mutant as noted in Fig. 4B and described in

Fig. 4. Transcriptional activation by 1,25(OH)2D3 via wild-type and cysteine point mutant hVDRs. COS-7 cells were co-transfected with wild-type or mutant pSG5hVDR expression plasmids along with the (CT4)4-TKGH reporter construct. The cells were then incubated with 1,25(OH)2D3 or ethanol vehicle for 12 h, and growth hormone secretion into the media was assessed. A, transcriptional activation in the presence of 10−8 M 1,25(OH)2D3. The amounts of growth hormone secreted into the media were compared with the amount in cultures receiving wild-type hVDR + 1,25(OH)2D3, which was set at 100 arbitrary units. Means (±S.E.) from four separate experiments are depicted. Expression of mutant hVDR proteins in the cells used in this experiment was similar to that of wild-type hVDR as determined by immunoblotting (data not shown). B, dose-response curves. COS-7 cells were transfected with wild-type hVDR (■), C369G (○), C337G (▲), or C288G (▲) expression plasmids along with (CT4)4-TKGH reporter and then treated with various concentrations of 1,25(OH)2D3 as indicated. Each point represents the average of assays on duplicate plates of cells, and the data were normalized to the maximal transcriptional activation effect of 1,25(OH)2D3 with the wild-type receptor. Virtually identical dose-response curves were obtained in two repeats of the experiment shown (see “Results” for compilation of EC50 values). The typical maximal stimulation of transactivation by 1,25(OH)2D3 with the C337G and C288G mutant receptors was 15-fold, comparable with wild-type and C369G in these experiments, but less than the 33-fold effect reported for the experiment in A.

in the transcription of the reporter in the presence of wild-type hVDR. C337G demonstrated transcriptional activation by 1,25(OH)2D3 at levels approximately 60% of the wild-type hVDR, while the activity of C369G was not significantly different from the wild-type receptor. Surprisingly, C288G did not show detectable transcriptional activation (Fig. 4A) by the 1,25(OH)2D3 ligand, even though this mutant exhibited reduced but still relatively high binding affinity for the ligand under the conditions of the in vivo binding assay at 4°C.
Evidence for the involvement of cysteine residues in steroid hormone receptor-ligand binding was originally reported when it was observed that mercurial reagents which interfere with protein thiol residue interaction reduce ligand binding to the ER (21) and GR (15, 41). Coty et al. (29) also found that treatment of occupied avian VDR with mercurial reagents causes a dissociation of $125\text{-(OH)}_2\text{D}_3$. With active analogs utilized for affinity labeling of the receptor, such as dexamethasone 21-mesylate, Cys-656 of the rat GR (corresponding to Cys-644 of the mouse GR) was identified as a critical residue for ligand binding (16–18). Utilizing arsenite as a thiol bridging reagent, it has been demonstrated that Cys-656 and -661 of rat GR are important for hormone binding (20). In the case of ER, Harlow et al. (22) reported that both an estrogen agonist and an estrogen antagonist covalently bind to Cys-530 in the HBD of human ER.

The present data advance our understanding of the ligand binding domain of hVDR and provide insight into the potential role of the three conserved cysteine residues in this region. Cysteine 288 is clearly essential for normal high affinity hormone binding (Fig. 5, A and B) and stimulation of transcription at physiologic doses of $125\text{-(OH)}_2\text{D}_3$ ligand (Fig. 4, A and B), but is not required for heterodimeric association of hVDR with RXR on the VDRE (Fig. 2). Because significant transcriptional activation can be generated when cells expressing C288G hVDR are treated with the high dose of $10^{-6}\text{ M}$ $125\text{-(OH)}_2\text{D}_3$ (Fig. 4B), cysteine 288 does not seem to be as crucial for transactivation, per se, as are residues 403–427 (12). In contrast, cysteine 369 is not critical for high affinity hormone binding at $4\text{ °C}$, and mutation of this residue results in only minor suppression of $125\text{-(OH)}_2\text{D}_3$ nuclear uptake (Fig. 5A), ligand binding at $23\text{ °C}$ (Fig. 5B), and possibly of heterodimerization with RXRs (Fig. 2); these effects are manifest as a small but significant shift in the dose-response curve with respect to transactivation (Fig. 4B). Alteration of cysteine 337 to glycine elicits a paradoxical enhancement of ligand binding at $4\text{ °C}$ and a minor attenuation of RXR heterodimerization capacity (Fig. 2), but results in a significant diminution in hVDR transactivation function (Fig. 4, A and B), the latter finding most likely being explained by relatively ineffective $125\text{-(OH)}_2\text{D}_3$ ligand binding at 23–37 °C (Fig. 5, A and B). Strong evidence supporting this conclusion is provided by the fact that transactivation by C337G is restored to normal in the presence of $10^{-7}\text{ M}$ $125\text{-(OH)}_2\text{D}_3$ (Fig. 4B). Mutations such as C337G, therefore, appear to result in a temperature-dependent phenotype for as yet unexplained reasons and reveal a necessity for functional testing at or near physiological temperatures. This concept is further illustrated in the case of C288G. When cell extracts were assayed by traditional ligand binding, at $4\text{ °C}$, in vitro, the Cys-288 mutant exhibited approximately one-third of the binding affinity of the wild-type receptor. Yet this mutant VDR displayed only 5% of wild-type nuclear uptake of the hormonal ligand in vivo, at $37\text{ °C}$ (Fig. 5A). Although there are several possibilities to explain these results, including: i) instability and degradation of the receptor protein at physiologic temperatures, ii) attenuated nuclear translocation of the receptor, and iii) weaker binding to the ligand in vivo; the first two possibilities are not likely because nuclear fractions from COS-7 cells expressing the C288G mutant receptor contained a similar amount of intact hVDR protein compared with the cells expressing wild-type hVDR (Fig. 3). Thus, we again conclude that there is a temperature-sensitive defect, in this case in the hormone binding activity of the C288G mutant.

The recent crystal structure elucidation of the HBD of human RXRα (42) appears to provide a prototype for this region in

**FIG. 5.** Specific binding of $125\text{-(OH)}_2\text{D}_3$ in intact cells at $37\text{ °C}$ and in cellular extracts at $23\text{ °C}$, in vitro. **A**, nuclear uptake of $125\text{-(OH)}_2\text{[H]}\text{D}_3$ by COS-7 cells expressing wild-type or mutant hVDRs. COS-7 cells expressing hVDRs were harvested, resuspended in culture medium containing 1% fetal bovine serum, and incubated with five concentrations of $125\text{-(OH)}_2\text{[H]}\text{D}_3$ in the presence or absence of a 600-fold molar excess of unlabeled $125\text{-(OH)}_2\text{D}_3$ at $37\text{ °C}$ for 2 h. **B**, specific binding of $125\text{-(OH)}_2\text{[H]}\text{D}_3$ to extracts of COS-7 cells transfected with hVDR and cysteine point mutants. Incubations were carried out for 2 h at $23\text{ °C}$ in the presence of $4.3\text{ nM}$ labeled ligand ± a 400-fold molar excess of radiolabeled $125\text{-(OH)}_2\text{D}_3$ to obtain specific binding. The binding shown is corrected for level of expression/degradation as determined by Western blotting.

The accompanying text. Therefore, the level of nuclear uptake of the $125\text{-(OH)}_2\text{D}_3$ ligand by COS-7 cells transfected with the various mutant hVDRs (Fig. 5A) correlates with the EC$_{50}$ values for $125\text{-(OH)}_2\text{D}_3$-enhanced transcriptional activity of these mutant receptors (Fig. 4B), thereby demonstrating that reduced hormone binding is the major source of attenuation in transcriptional activation exhibited by the C288G and C337G mutants.

Because of the striking differences between ligand binding kinetics with receptor extracts at $4\text{ °C}$ and in intact cells at $37\text{ °C}$ (Fig. 5A), we performed a final $125\text{-(OH)}_2\text{D}_3$ binding experiment with cellular extracts at the intermediate temperature of $23\text{ °C}$. Incubation of extracted VDR at this elevated temperature was found to elicit degradation (data not shown), so we were limited to the relatively short incubation time of 2 h to preserve the receptor. Under these conditions, saturation kinetics were not achieved, precluding the determination of K$_d$ values. However, specific binding levels at $23\text{ °C}$ for each mutant at a $125\text{-(OH)}_2\text{D}_3$ ligand concentration of $4.3\text{ nM}$ (Fig. 5B), corrected for receptor expression by normalizing the results to the signals from a Western blot performed after a 23 °C incubation (data not shown), strongly support the conclusion that C369G binds $125\text{-(OH)}_2\text{D}_3$ reasonably well at elevated temperatures while C337G and especially C288G hVDRs are defective in ligand binding.
nuclear receptors. The RXRα ligand binding domain consists of an antiparallel α-helical sandwich containing 11 α-helices surrounding two β-strands (42), and the proposed ligand binding pocket is a hydrophobic cavity bordered by helix 5, both β-strands, helix 7, the COOH-terminal portion of helix 10 and the NH2-terminal part of helix 11. That a similar ligand binding pocket may exist in the other members of the nuclear receptor superfamily is suggested not only by the homologies seen in this region (typically 20–30% across the superfamily), but also by mutagenesis studies with ER and GR. Katzenellenbogen et al. (43) have previously suggested that Cys-381 and Cys-530 lie at the “mouth” of a putative ligand binding pocket; these two residues in fact correspond to positions in human RXRα within helix 5 and the NH2-terminal portion of helix 11, respectively. The participation of the two β-strands and helix 7 in a generalized hormone binding site is confirmed by the findings of Chakraborti et al. (20), who implicate Cys-640 (1st β-strand), as well as Cys-656 and Cys-661 (both in helix 7), as being important for ligand binding by rat GR. In addition, a previous report describing the effect of an artifactual mutation in the cloned human ER from MCF-7 cells (44) indicates that Gly-400 raises the dose of estradiol-17β required for maximal transactivation by 10–100-fold; this residue is also located in the region of ER that corresponds to the second β-strand in RXRα. More recent site-directed mutagenesis of the mouse ER (45) points to the significance of Cys-742 (COOH-terminal portion of helix 10) in ligand-dependent transcriptional activation.

The location of all of these residues implicated in hormone-binding or hormone-dependent functions of the respective receptors seems in complete agreement with the proposed prototypical hydrophobic binding pocket.

The two mutants reported here for hVDR which have substantial effects on hormone binding and hormone-dependent transactivation, namely Cys-288 and Cys-337, occur in areas corresponding to the 1st β-strand in RXRα and in helix 8, respectively. Cys-288 would therefore take its place along with Cys-640 in rat GR and Gly-400 in human ER as confirming the general importance of the β-strand region in ligand binding. Furthermore, recently reported natural mutations of hVDR which display impaired hormone binding lie in helix 5 (Arg-274 (46) and helix 7 (Ile-314 (47)), both critical regions in RXR ligand association. In contrast, Cys-337 resides in an area of hVDR corresponding to helix 8, which places it outside the proposed ligand binding pocket. However, mutations at analogous positions in the human ER at Cys-447 (48) and in the mouse GR at Cys-671 (45) result in impaired ligand-induced transcriptional activation at physiological temperatures. Because these mutations have marked effects on hormone binding and transactivation, it is suggested that helix 8, which lies adjacent and parallel to helix 5 in the structure of RXRα, might somehow be important in stabilizing the conformation of the ligand binding cavity. Thus, the fact that RXR, ER, GR, and VDR represent widely diverse members of the nuclear receptor superfamily argues strongly that many features of the proposed ligand binding pocket may be shared across the nuclear receptor superfamily.

Covalent modification of specific residues in hVDR with ligands using affinity labeling techniques will be required to extend the present conclusions. Further studies of the type carried out in this report could involve altering Cys-288 and Cys-337 in hVDR to serine or alanine residues, since they may better preserve the size of the R-group and possibly the protein conformation. Ultimately, a physical examination of the molecular structure of the 1,25-(OH)2D3-occupied, hormone binding domain of VDR, such as through x-ray crystallography, will be necessary to elucidate the mechanism of 1,25-(OH)2D3 ligand binding and how this can influence the control of gene transcription.
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Role of Cysteine Residues in the Vitamin D Receptor

49.
Examination of the Potential Functional Role of Conserved Cysteine Residues in the 
Hormone Binding Domain of the Human 1,25-Dihydroxyvitamin D Receptor
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