Differential Interaction of 1α,25-Dihydroxyvitamin D₃ Analogues and Their 20-epi Homologues with the Vitamin D Receptor*

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A recent focus of structure-function studies of synthetic ligands for the vitamin D receptor (VDR) concerns the chiral center at carbon 20 of the steroid side chain; 20-epi analogues are 100–10,000 times more potent transcriptionally than the natural hormone 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃). We have compared the binding properties of three pairs of analogues either with a natural (N) or 20-epi (E) orientation. In intact cells, 45–60% of VDR were essential for 1α-directed mutagenesis revealed that residues 421 and 422 are required for 1α,25-(OH)₂D₃-induced transcriptionally than the natural hormone 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃, but not for binding of 20-epi analogues. Site-directed mutagenesis revealed that residues 421 and 422 were essential for 1α,25-(OH)₂D₃-induced conformational changes, high affinity of 1α,25-(OH)₂D₃ for VDR, and transcriptional activity, but not for binding of its 20-epi analogue. In contrast, deletion of residues 396–427 abolished binding of 1α,25-(OH)₂D₃, but binding of its 20-epi analogue was still detectable. The results suggest that the ligand-binding domain of VDR has multiple and different contact sites for the two families of side chain-modified ligands, resulting in VDR-ligand complexes with different half-lives and transcriptional activities.

The action of the vitamin D receptor (VDR), like that of other nuclear receptors, is dependent primarily on interaction with its biologically active ligand, 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃). The binding of ligand to its nuclear receptor leads to conformational changes in the receptor (4–6), promotes self-dimerization and heterodimerization with the retinoid X receptor (RXR) (7–11), and enhances binding to DNA and transcriptional activities (12–15). It is generally accepted that the transcriptional activities of nuclear receptors are directly correlated with their affinity for their respective ligands (16–18). There are, however, several exceptions to this rule. For example, the effective dose required to reach 50% saturation (ED₅₀) of the estrogen receptor by its ligand is several orders of magnitude greater than the ED₅₀ for maximal transcriptional activity (19). A possible explanation for this is that ligand-activated estrogen receptor acts in a cooperative manner with unoccupied estrogen receptor molecules during interaction with its DNA response element or with the transcriptional apparatus, whereas other steroid hormone receptors do not.

Another exception is the progesterone antagonist RU486; this synthetic ligand binds tightly to the progesterone receptor, but does not induce transcriptional activity (20). The explanation for this discrepancy is that the analogue/antagonist interacts with the progesterone receptor at a different site from progestosterone, thus creating a unique conformational change in the receptor and preventing its normal action (21).

Recently, we began to analyze the mechanism of action of analogues of 1α,25-(OH)₂D₃ that regulate receptor-mediated transcription more effectively than the natural hormone, although their affinity for VDR is not greater (6). For example, a concentration of 10⁻¹¹ M is required to induce 50% of maximal DNA binding and transcriptional activities of VDR by the 20-epi analogue MC 1288 (1-E; see Fig. 1), but this concentration of 1-E is 200-fold lower than the ED₅₀ of 2 × 10⁻⁹ M for saturation of VDR binding sites in equilibrium (6, 22). These results suggest that enhanced activation of VDR occurs after binding of the analogue to VDR, but before induction of transcription. Because analogue 1-E induces a unique conformational change in VDR in vitro and enhances dimerization of VDR with RXR in vivo (6), we speculated that the conformation of 20-epi analogue-activated VDR promotes better binding to DNA by stabilizing the VDR heterodimer. The conformational differences between 1α,25-(OH)₂D₃/VDR and 20-epi analogue-VDR complexes are probably due to differences in the sites where ligands contact the receptor, as are the differences in the interaction of progesterone and RU486 with the progesterone receptor (21). To test this hypothesis, it is necessary to map the ligand-binding domain of VDR, to identify the amino acids that are required for the binding of 1α,25-(OH)₂D₃ to it, and to determine whether the same amino acids are also required for binding of the 20-epi analogues (E-analogues). In the study presented here, we identified the amino acids required for interaction of 1α,25-(OH)₂D₃ at the C-terminal region of VDR. We also showed that the same amino acids were required for binding of 1α,25-(OH)₂D₃ and analogues with a natural orientation of the side chain (N-analogues). However, all of the
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E-analogues examined had different binding requirements at this region, and these differences in binding requirements were associated with increased stability of the ligand-receptor complexes.

EXPERIMENTAL PROCEDURES

Reagents—Synthetic oligonucleotides were prepared by the Macro-molecular Synthesis and Analysis Facility of the M. D. Anderson Cancer Center. [³²S]Methionine and 1α,25-(OH)₂[26,27-³²H]D₃ were obtained from Amer sham Corp. A coupled transcription/translation kit and a site-directed mutagenesis kit were obtained from Promega. The analogues used in this study were a generous gift from Dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark). The structural formulas for and abbreviations of these ligands are shown in Fig. 1.

Site-directed Mutagenesis—Single-stranded DNA templates were prepared from Escherichia coli JM109 cells bearing the plasmid pALTER (Altered Sites in vitro mutagenesis system, Promega) with an insert encoding the wild-type human VDR cDNA, a tetracycline resistance gene, and a mutated ampicillin resistance gene. A VDR primer with the desired mutation and an oligonucleotide containing repair sequences for the mutation in the ampicillin resistance gene were annealed to the single-stranded DNA, and then synthesized by complementary strand was catalyzed by T4 DNA polymerase. Synthetic plasmids were transformed into E. coli strain BMH171-18 (mutS) and isolated from ampicillin-resistant colonies. Plasmid DNA was transformed into JM109 for a second screening, and then DNA was isolated from individual colonies and screened for mutations by DNA sequencing through the mutated site. Subsequently, the DNA coding for a mutant VDR was subcloned into the plasmid pGEM-4 for in vitro studies with synthetic receptor or into a eukaryotic expression vector for functional studies in intact cells.

Cell Culture and Transfections—Rat osteosarcoma ROS 17/2.8 cells and monkey kidney CV-1 cells were plated in 35-mm dishes at a density of 3 × 10⁵/dish. ROS 17/2.8 cells were transfected with 2 μg of plasmid oVDRE containing the vitamin D-responsive element (VDRE) from the human osteocalcin gene (GGTGACTCACCGG/GTGAAACCGGGCGCAT) (23). This response element was attached to the thymidine kinase promoter/growth hormone fusion gene. Monkey kidney CV-1 cells were transfected with the osteocalcin VDRE/reporter fusion gene (4 μg/dish) and the recombinant human VDR expression vector (2 μg/dish). Monkey kidney COS-1 cells were plated in 150-mm dishes at a density of 6 × 10⁶/dish and transfected with recombinant human VDR plasmids (20 μg/dish).

All transfections were performed by the DEAE-dextran method (6). Medium samples for measurements of growth hormone were collected 2 days after transfection. Growth hormone production from the reporter gene was measured by a radioimmunoassay as described by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

Ligand Binding Assays—To assess the relative affinity of 1α,25-(OH)₂D₃ and the E-analogues for wild-type and mutant VDRs in vitro, whole-cell homogenates from COS-1 cells transfected with VDR expression plasmids were prepared in KTED buffer (0.3 M KCl, 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 1 mM dithiothreitol) as described previously (6). The homogenates were then aliquoted into tubes containing 0.2 pmol of 1α,25-(OH)₂[³²H]D₃, and increasing concentrations of nonradioactive ligand. For Scatchard analysis of 1α,25-(OH)₂D₃ binding to wild-type and mutant VDRs, the homogenates were incubated with increasing concentrations of 1α,25-(OH)₂[³²H]D₃ (0.05–5 nM). The mixtures were incubated on ice for 3–4 h, and then the free ligand was separated from bound by hydroxyapatite (24). The bound ligand was released from the hydroxyapatite by ethanol extraction, and the radioactivity was measured by scintillation counting. For the competition assays, the results were plotted as the inverse value of percent maximal binding against competitor concentration by the method of Weckels and Norman (24).

To assess receptor occupancy by the ligand as well as the dissociation rate of the ligand in vivo, monolayers of VDR-transfected COS-1 cells were washed three times in PBS and incubated for 1 h with ligand in serum-free medium. Then the medium was discarded, the cells were washed three times in PBS, and fresh medium was added. At various times, the medium was discarded again, and the cells were washed three times in cold PBS, scraped into 10 ml of PBS, centrifuged, resuspended in KTED buffer, and homogenized. Aliquots (0.2 ml) of the homogenates were incubated on ice for 3–4 h with 0.2 pmol of 1α,25-(OH)₂[³²H]D₃ with or without a 100-fold excess of unlabelled ligand. To assess the number of unoccupied VDR sites, the free ligand was separated from the bound by hydroxyapatite as described above.

To assess exchange of unlabelled 1α,25-(OH)₂D₃ or its analogues with 1α,25-(OH)₂[³²H]D₃, monolayers of VDR-transfected COS-1 cells were washed three times in PBS and incubated for 1 h with ligand in serum-free medium. Then the medium was discarded, and the cells were washed three times in cold PBS, scraped into 10 ml of PBS, centrifuged, resuspended in KTED buffer, and homogenized. Aliquots (0.2 ml) of the homogenates were incubated at 30 °C with 0.2 pmol of 1α,25-(OH)₂[³²H]D₃ for various times and then transferred to ice for an additional 3 h. The free radioactive ligand was separated from the bound by hydroxyapatite as described above. Exchange was assessed by comparing the amount of 1α,25-(OH)₂[³²H]D₃ bound to unoccupied VDR and the amount of 1α,25-(OH)₂[³²H]D₃ bound to in vivo bound VDR at each time point.

Ligand-induced Sensitivity to Proteases—Synthetic wild-type and mutant human VDRs labeled with [³⁵S]methionine (1000 Ci/nmol) were prepared by in vitro coupled transcription/translation in reticulocyte lysates (Promega) with the human VDR cDNA inserted into the pGEM-4 plasmid. The receptor preparations were incubated with the indicated concentrations of 1α,25-(OH)₂D₃ or analogues for 10 min at room temperature. Then 0–25 μg/ml trypsin (Calbiochem) was added, and the mixtures were incubated for another 10 min. The digestion products were analyzed by 12% dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were dried and autoradiographed.

RESULTS

Role of Chemistry and Stereochemistry of the Side Chain in Regulation of VDR Transcription by Analogues of 1α,25-(OH)₂D₃—A recent study from our laboratories showed that the E-analogue 1-E had a 200-fold greater transcriptional activity than the VDR than did 1α,25-(OH)₂D₃ (6). Another E-analogue with a modified chemistry of the side chain, 3-E, also had enhanced transcriptional activity. These results suggested that side chains with 20-epi configuration facilitated receptor action. To further study the role of the chemistry and stereochemistry of the side chain in VDR actions, we examined the transcriptional activity of three E-analogues and their N-
analogue homologues (Table I). The transcriptional activity of these ligands was examined in ROS 17/2.8 cells transfected with a fusion gene containing the osteocalcin VDRE fused to the thymidine kinase promoter/growth hormone reporter gene. The cells were treated with the ligand for 1 h in serum-free medium to allow ligand uptake and saturation of cellular VDR ligand-binding sites in the absence of serum-binding proteins. The results of this assay (Table I) showed that extension of the 1α,25-(OH)₂D₃ side chain (as in 2-N) enhanced transcriptional activity by 50-fold, but the addition of oxygen at position 22 (as in 3-N) to this chemically modified side chain decreased transcriptional activity by 2-fold relative to 2-N. The 20-epi stereoisomer of each of these side chains further enhanced their transcriptional activities. The E-analogue 2-E had the greatest transcriptional activity, up to 5000-fold greater than that of 1α,25-(OH)₂D₃. Therefore, we concluded that the 20-epi configuration enhanced the transcriptional activity of these ligands whether or not their side chains were chemically modified.

Effect of Ligand Structure on Its Dissociation Rate from VDR—In our previous study (6), we confirmed the earlier report (Binderep et al. (22)) that the increased growth inhibitory and differentiating activities of the E-analogues were not associated with a greater affinity for VDR. However, another aspect of ligand binding was not tested in these studies: the stability of the ligand-receptor complexes. This is an important aspect of analogue action because, in our experiments, cellular VDR binding sites were first saturated with the ligand under equilibrium conditions, but then the ligand was removed. Therefore, we tested the transcriptional activity of the VDR-ligand complexes under nonequilibrium conditions. Similar conditions may develop naturally during prolonged incubation of cell cultures with the ligand or in vivo. This may not be the case when the ligand is removed by catabolism. Therefore, we tested the possibility that the enhanced transcriptional activity of the E-analogue may in part be due to a decrease in the rate of dissociation from the VDR complexes. VDR-transfected COS-1 cells were incubated with 10⁻⁸ M 1α,25-(OH)₂D₃ or its analogues for 1 h; the excess ligand was then removed; and the rate of appearance of unoccupied binding sites was measured over the next 3 h (Fig. 2A). We found that unoccupied binding sites appeared at a rapid rate when cellular VDR was first saturated with 1α,25-(OH)₂D₃ or with the N-analogue: in 3 h, only 35% of the VDR binding sites remained occupied with 1α,25-(OH)₂D₃, and 50% of the VDR binding sites remained occupied with the analogues 2-N and 3-N. We also concluded that the chemistry of these analogues’ side chains did not have a significant effect on the stability of the ligand-receptor complexes. On the other hand, 20-epi configuration of these side chains significantly slowed down the replenishment of unoccupied binding sites. After 3 h of incubation, 80% of the VDR binding sites remained occupied by the E-analogues 1-E and 3-E, and all of the binding sites remained occupied by the most active 20-epi analogue, 2-E. Therefore, we conclude that ligands with a natural orientation of the side chain dissociate rapidly from VDR, whereas ligands with the 20-epi side chain either dissociate very slowly from VDR or induce irreversible inactivation of VDR ligand-binding sites by an unknown mechanism.

To determine whether the increase in the number of unoccupied binding sites in intact cells was due to dissociation of ligand from VDR or due to reactivation of inactive VDR, we performed an in vitro exchange assay. Again, VDR-transfected COS-1 cells were incubated with a 10⁻⁸ M concentration of each ligand for 1 h in serum-free medium; the ligands were removed; and the cells were washed three times and homogenized. The homogenates were incubated at 30 °C with 1α,25-(OH)₂[³H]D₃, and exchange of unlabelled and radioactive ligands was measured at various times. The results (Fig. 2B) show that the exchange rate of receptor-bound unlabelled 1α,25-(OH)₂D₃ with 1α,25-(OH)₂[³H]D₃ in vitro was very rapid in the first 15 min of incubation: 55% of the receptor-bound unlabelled ligand was exchanged for the radioactive ligand. On the other hand, 45% of the receptor binding sites did not bind the radioactive ligand even after 1 h of incubation. A similar pattern of rapid exchange of ligand from a high percentage of the occupied binding sites was seen with the two N-analogues. However, rapid exchange of 1-E occurred with only 20% of the occupied VDR binding sites, and no detectable rapid exchange occurred with VDR binding sites occupied with the analogue 2-E. Only 3-E had an exchange rate similar to that of its N-analogue homologue (3-N). So, although the dissociation rate of this analogue in the cells was very slow, its exchange rate in vitro was not affected by the orientation of the side chain. It is possible that the presence of the oxygen atom at position 22 controls the orientation of the side chain does not affect the complex process of exchange that requires the simultaneous movement of 3-E out of the receptor binding sites.

Mapping Ligand Binding Activity in the C-terminal Region of VDR—The results presented above, together with our earlier observations of differences in the conformation of VDR complexed with 1α,25-(OH)₂D₃ and the E-analogues (6), support the hypothesis that the mode of interaction of the 20-epi analogues with VDR is different from that of 1α,25-(OH)₂D₃ and the N-analogues. One possible explanation for these differences is that the contact points of the 20-epi analogues with the ligand-binding domain are different from the contact points of ligands with natural side chain stereochemistry. To test this hypothesis, we mapped the ligand-binding domain of VDR by deletions, testing the binding activities of the mutants to 1α,25-(OH)₂D₃ and the analogues. The mutation strategy was based on earlier studies by McDonnell et al. (25) and Nakajima et al. (7), who identified a C-terminal sequence between amino acids 390 and 427 as essential for ligand binding activity. To further map this region, we prepared deletion mutants by inserting stop codons at the positions shown in Fig. 3A. These deletion mutants were first examined for protein expression in COS-1 cells and then for binding and transcriptional activities. Analysis by Western blotting showed that all the mutants in Fig. 3

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**TABLE I**

**Binding of 20-epi Analogues of 1α,25-(OH)₂D₃ to VDR**

| Analogue          | Transcription (ED₅₀)  |
|-------------------|----------------------|
| 1α,25-(OH)₂D₃     | (3.0 ± 1.2) x 10⁻⁹   |
| 1-E               | (10.0 ± 0.5) x 10⁻¹² |
| 2-N               | (6.0 ± 0.6) x 10⁻¹¹  |
| 2-E               | (6.0 ± 3.1) x 10⁻¹³  |
| 3-N               | (1.0 ± 0.5) x 10⁻¹¹  |
| 3-E               | (2.0 ± 1.1) x 10⁻¹¹  |

ROS 17/2.8 cells were transfected by the DEAE-dextran method (6) with a thymidine kinase growth hormone fusion gene containing the osteocalcin VDRE. Immediately after transfection, the ligands (10⁻⁷ to 10⁻¹³ M) were added to serum-free medium for 1 h and then removed. The cells were washed twice in PBS, and then Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum was added. Forty-eight hours after transfection, the culture medium was collected, and growth hormone levels were determined with a radioimmunoassay. The effective dose required to reach 50% of maximal transcriptional activity (ED₅₀ ± S.E.) was calculated by plotting -fold induction of reporter gene expression against ligand concentration from three transfection experiments. Maximal -fold induction of transcription varied between experiments (6- to 15-fold), but maximal transcriptional activity was the same for 1α,25-(OH)₂D₃ (which was included in each transfection experiment) and the analogues in individual experiments.

The results of this assay (Table I) showed that extension of the 1α,25-(OH)₂D₃ side chain (as in 2-N) enhanced transcriptional activity by 50-fold, but the addition of oxygen at position 22 (as in 3-N) to this chemically modified side chain decreased transcriptional activity by 2-fold relative to 2-N. The 20-epi stereoisomer of each of these side chains further enhanced their transcriptional activities. The E-analogue 2-E had the greatest transcriptional activity, up to 5000-fold greater than that of 1α,25-(OH)₂D₃. Therefore, we concluded that the 20-epi configuration enhanced the transcriptional activity of these ligands whether or not their side chains were chemically modified.
expressed similar levels of immunoreactive VDR in COS-1 cells (data not shown). Fig. 3C shows that the transcriptional activity of VDR was abolished by deleting the C-terminal residues 420–427. This deletion did not abolish the binding activity of 1α,25-(OH)₂D₃ to VDR (Fig. 3B), but did decrease it by 50–70%. Further deletion of residues 410–420 had no marked effect on binding of 1α,25-(OH)₂D₃ to VDR, but deleting residues 403–410 induced an additional dramatic decrease, leaving only residual binding activity. This residual binding activity was completely abolished by deleting residues 396–403. From these mapping experiments, we concluded that at the C-terminal region, the amino acids important for binding of 1α,25-(OH)₂D₃ were located between residues 420 and 427 and between residues 396 and 410. Therefore, some of the amino acids required for binding of 1α,25-(OH)₂D₃ overlap with amino acids that are essential for transcriptional activity of VDR.

**Comparison of the Binding Requirements of the E- and N-analogues**—To determine whether the binding requirements of the E-analogues were different from those of the N-analogues, we performed binding assays in which wild-type or mutant VDRs were incubated with 1α,25-(OH)₂[³H]D₃ and increasing concentration of unlabeled ligand. The data were plotted as the inverse value of percent maximal binding against competitor concentration. The slopes of the linear plots reflect the affinity of each ligand for the 1α,25-(OH)₂D₃-binding site in wild-type and mutant VDRs. Fig. 3D shows that the slope of the competition plot of 1α,25-(OH)₂D₃ was significantly reduced by deleting residues 420–427, suggesting that the affinity of 1α,25-(OH)₂D₃ for this mutant was reduced. There was no significant reduction in the slope of the competition plot of 1α,25-(OH)₂D₃ using VDR that had an additional deletion of residues 410–420, suggesting that this deletion did not introduce another change in the affinity of 1α,25-(OH)₂D₃ for the receptor. To confirm the change in affinity of these deletion mutants for 1α,25-(OH)₂D₃, we performed saturation assays. Scatchard analysis of these assays in one experiment revealed that the equilibrium dissociation constants (Kᵢ) for 1α,25-(OH)₂D₃ binding to wild-type VDR, to deletion mutant 420/TGA, and to deletion mutant 410/TAA were 0.9, 4.5, and 3.75 nM, respectively. In another experiment, the Kᵢ values were 1.2 nM (wild-type VDR), 7.8 nM (mutant 420/TGA), and 7.5 nM (mutant 410/TAA).

The competition assays with the E-analogue 1-E showed that deleting residues 420–427 had no effect on the ability of 1-E to compete for the binding of 1α,25-(OH)₂[³H]D₃ to VDR, but deleting residues 410–420 induced a small but consistent reduction in the ability of 1-E to compete for the binding of 1α,25-(OH)₂[³H]D₃ to VDR. Therefore, we concluded that the
last seven amino acids of VDR were important for the binding of 1α,25-(OH)2D3, but not for 1-E, and that residues 410–420 seem to be more important for the binding of the E-analogue than for the binding of 1α,25-(OH)2D3.

Because the most significant difference in the binding requirements of 1α,25-(OH)2D3 and the E-analogue was within residues 420–427, we wished to determine if this difference was indeed due to the stereochemistry of the side chain and not due to its chemistry. Therefore, we repeated the competition assays with the six ligands shown in Fig. 1, using wild-type VDR and the VDR deletion mutant 420/TGA. Fig. 4 shows that the ability of 1α,25-(OH)2D3 and the N-analogues to compete for the binding of 1α,25-(OH)2D3 to VDR was significantly reduced by removal of residues 420–427. However, this deletion had either little or no effect on the ability of the E-analogues to compete for the binding of 1α,25-(OH)2D3 to VDR. The results of these experiments strongly suggest that the binding requirements of the E-analogues are upstream from...
the eight C-terminal amino acids of VDR, although we do not know which other residues are required for binding of these compounds.

To determine which residues between positions 420 and 427 were important for binding of 1α,25-(OH)₂D₃, we mapped this region by point mutations (Fig. 5A). The glutamic acid residue at position 420 was replaced with alanine (E420A). The two hydrophobic residues (valine at position 421 and phenylalanine at position 422) were mutated simultaneously to methionine and alanine, respectively (V421M/F422A), and the glutamic acid residue at position 425 was replaced with glutamine (E425Q). Each of these mutants was tested for its transcriptional activity in response to 1α,25-(OH)₂D₃ and its binding to 1α,25-(OH)₂[3H]D₃ by one-point binding analysis and by competition assays. Fig. 5A shows that amino acids 420–422 are located within a cluster of hydrophobic residues. A comparison with other steroid hormone receptors showed that this cluster overlaps a conserved transcriptional activation function 2 domain (TAF-2) (12, 13). As expected, these residues are also important for transcriptional activity of VDR (Fig. 5B). Mutating the glutamic acid residue at position 420 reduced the transcriptional activity of VDR, and the double mutation of amino acids 421 and 422 completely abolished it. The latter mutant did not have detectable transcriptional activity in response to the E-analogues (data not shown). In contrast, mutating the glutamic acid residue at position 425, which is located outside this cluster, had no effect on transcriptional activity.

These mutants were also tested by one-point binding assay for binding of 1α,25-(OH)₂[3H]D₃ (Fig. 5B) and for changes in affinity for 1α,25-(OH)₂D₃ and its E-analogue by competition assays (Fig. 5C). In the one-point saturation analysis, there was some decrease in the binding of 1α,25-(OH)₂D₃ to the VDR mutants E420A and V421M/F422A, but no change in the binding activity of the VDR mutant E425Q. When we tested the effect of these mutations on the affinity of 1α,25-(OH)₂D₃ and the E-analogue 1-E, we found that only the double mutation at amino acids 421 and 422 significantly decreased the affinity of 1α,25-(OH)₂D₃ for VDR; the other point mutations had no such effect. However, the ability of the E-analogue 1-E to compete for binding of 1α,25-(OH)₂[3H]D₃ to these three mutants was the same as for wild-type VDR, suggesting that these mutations had no effect on the affinity of 1-E for VDR. Therefore, we concluded that amino acids downstream from residue 420 were completely unnecessary for binding of the E-analogues, although residues 421 and 422 were required for transcriptional activity of these ligands.

Effect of Mutations on Ligand-induced Changes in Receptor Conformation—Our recent studies have shown that binding of 1α,25-(OH)₂D₃ changes the sensitivity of VDR to protease digestion, producing a 34-kDa protease-resistant fragment (probably encompassing the entire ligand-binding domain (25)) and a 28-kDa fragment. The binding of the E-analogues also induced resistance to protease digestion, but with a different pattern: in addition to the 34- and 28-kDa protease-resistant fragments, there was a 32-kDa fragment. Therefore, the conformation of VDR-1α,25-(OH)₂D₃ complexes was clearly different from the conformation of the VDR-E-analogue complexes. To determine the relationship between ligand-induced receptor conformation and its action, we performed a protease sensitivity assay with receptor mutants (Fig. 6). Deleting residues 420–427 was sufficient to diminish 1α,25-(OH)₂D₃-induced resistance to protease digestion of synthetic VDR (Fig. 6A), al-
though this mutant receptor still had significant binding activity (Fig. 3). Incubation of the same receptor mutant with the E-analogue 1-E did induce a significant resistance to protease digestion. However, no 34-kDa protease-resistant fragment was produced; only the 32- and 28-kDa fragments were seen, and their intensity was significantly greater than with wild-type VDR. Therefore, residues 420–427 were clearly required for proper receptor folding whether or not ligand binding activity was detectable.

Because deletion of these amino acids diminished transcriptional activity, we presumed that the loss of amino acids that were necessary for proper folding of VDR in response to the ligand also caused a loss of ligand binding activity to be detectable.

The experiments described above strongly suggested that ligand-induced resistance to protease digestion does not necessarily reflect the affinity of VDR mutants for the ligand. However, it is reasonable to assume that as long as ligand-dependent resistance to protease digestion is detectable, VDR will still display ligand binding activity. Therefore, the protease sensitivity assay could be used to map the binding sites for the E-analogue 1-E in the deletion mutants that could not be examined by the competition assays because they had lost binding for 1α,25-(OH)2D3. Fig. 6 (A and B) shows that the analogue 1-E continued to induce resistance to protease diges-
tion even after deletion of amino acids 390–427 (390/TGA). The intensity of the protease-resistant fragment induced by deleting residues 390–427 was weaker than that of the fragment induced by deleting residues 396–427 (396/TAA), but so was the translation efficiency of the 390/TGA deletion mutant. To determine whether there was a real difference in ligand-induced resistance to protease digestion between these two mutants, we assessed the dose response to 1-E with the two mutants (Fig. 7) and found that they induced resistance to protease digestion in the same concentration range, which confirmed that the binding activity of 1-E depended not on residues between positions 390 and 396, but on residues farther upstream.

The results of the binding and competition assays are combined with the results of the protease sensitivity assays and summarized schematically in Fig. 8. From this illustration, we concluded that critical amino acids required for binding of 1α,25-(OH)₂D₃ were located between positions 420 and 427 and between positions 396 and 410. On the other hand, the critical amino acids required for binding of 1-E were between positions 410 and 420 and between positions 396 and 403, and the residual binding activity of this ligand depended on amino acids upstream from position 390. Therefore, the binding requirements of these two ligands, which differ only in the orientation of their side chains, were clearly different.

FIG. 7. Dose response of E-analogue-induced sensitivity of C-terminal mutant VDRs to trypsin. The effect of 1-E dose on sensitivity of deletion mutants of VDR to trypsin digestion was assessed with ³⁵S-labeled synthetic VDR. Mutants 396/TAA and 390/TGA were incubated with increasing concentrations (10⁻⁶ to 10⁻⁸ M) of 1-E, digested with trypsin, and analyzed by gel electrophoresis and autoradiography as described in the legend to Fig. 6. un, untreated with trypsin.

DISCUSSION
This study is a direct extension of our previous work on the mechanism of action of analogues of 1α,25-(OH)₂D₃ (6). In the previous study, we demonstrated for the first time that the E-analogues can activate VDR differently from the natural
ligand and hypothesized that the E-analouges interacted with VDR at contact points not used by the natural hormone. We also speculated that the association at these alternative contact points facilitated dimerization with RXR, binding to DNA, and transcriptional activation of VDR. Here we confirmed a part of this hypothesis by comparing the binding properties and amino acid requirements for interaction of two groups of ligands with VDR: ligands with natural side chain orientation and 20-epi analogues. However, it is still necessary to investigate the relationships between specific binding requirements of the analogues and facilitated dimerization of VDR with RXR.

The ligand-binding domain of human VDR is a 37-kDa polypeptide encompassing two-thirds of the receptor molecule. Earlier studies have identified an upstream region between amino acids 114 and 166 (25) and a downstream region between amino acids 390 and 427 that are essential for binding of 1α,25-(OH)₂D₃ to VDR (7, 25). But these experiments did not define the exact residues that are required for ligand interaction with VDR or their role in other receptor functions. In our mapping experiments, we focused only on the C-terminal region of the ligand-binding domain of VDR and RXR (using the Genetics Computer Group program to predict secondary structures) in Fig. 9 clearly shows that helices 7, 10, and 11, which form the putative ligand-binding pocket B in RXR, aligned very well with similar secondary structures in VDR. Interestingly, our mutational analysis showed that the C-terminal structures homologous to helices 10 and 11 in RXR were also essential for binding of 1α,25-(OH)₂D₃ and its analogues to VDR. Furthermore, amino acids 396–410, which were important for binding of 1α,25-(OH)₂D₃, overlapped with a region essential for contact of estradiol, glucocorticoids, and androgens with their respective receptors (29–33). These striking similarities in the mapping of ligand binding activity strongly suggest that there is a certain rigidity of the ligand-binding pocket in different steroid hormone receptors and that the amino acids mapped in this study are probably contact sites for the ligands.

Does changing the stereochemistry of the side chain affect the requirement of ligands for the amino acids between positions 396 and 410? Our binding analysis showed that residues 403–410 were important for binding of 1α,25-(OH)₂D₃ and that residual binding activity was lost by deleting residues 396–403. Analysis of the binding requirement of the E-analogue 1-E by competition assays and by the protease sensitivity assay

![Graph showing binding requirements for 1,25D3 and 1-E](image-url)
strongly suggested that the residues required for binding of this analogue were between positions 396 and 403 and upstream from position 390. Therefore, from these data, it appears that binding of the E-analogue is shifted within the putative ligand-binding pocket into a region that overlaps with the ninth heptad repeat of the dimerization domain (7). We hypothesize that this shift in binding site also reflects a shift in the physical contact of the analogue in the ligand-binding pocket and away from the C terminus.

Another piece of evidence supporting a general shift in binding requirements of the E-analogues was seen in a region closer to the C terminus of VDR. The N-analogues and 1α,25-(OH)₂D₃ required residues 420–427 for interaction with VDR, whereas the E-analogues did not. The C-terminal region contains a cluster of hydrophobic amino acids termed TAF-2. This region is conserved in ligand-binding nuclear receptors (13), and its function is to modulate transcriptional activity. In VDR, these residues are also essential for maintenance of the appropriate function is to modulate transcriptional activity. In VDR, these TAF-2 residues so that they become available for protein-protein interactions. We hypothesize that in VDR, ligands with hydrophobic residues at the C-terminal region. However, we cannot exclude the possibility that amino acids inside the ligand-binding pocket may also be important. To test this hypothesis, it will be necessary to examine the effect of individual point mutations on the dissociation rate of ligands with natural side chain orientation and 20-epi analogues.

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