Putative helices 3 and 5 of the human vitamin D_3 receptor are important for the binding of calcitriol

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Abstract We have introduced eleven point mutations into the human vitamin D receptor by site-directed mutagenesis in order to identify some of the amino acid residues that are important for ligand binding. The amino acid residues Ser225, His229, Asp232, Val234, Ser235, Tyr236, Ser237, Lys240, Ile242, Lys246 (helix 3), and Ser275 (helix 5) of the human vitamin D receptor were substituted by alanine. We report here that His229, Asp232, and Ser237 have an important role in the binding of calcitriol. In addition, the amino acid residues Tyr236 and Ser275 also seem to participate in the ligand binding process.

Key words: Vitamin D receptor; Ligand binding; Site-directed mutagenesis; Conformation

1. Introduction

Human vitamin D receptor (hVDR) belongs to the superfamily of steroid receptors. These receptors are nuclear transcription factors that are known to have direct genomic effects. They regulate gene expression in response to binding of their specific ligands. The binding changes the conformation of the receptors which enables transactivation. This can result in either activation or repression of gene transcription [1–4].

Crystal structures of the unliganded ligand-binding domain (LBD) of human retinoid X receptor-α (hRXRα), and the liganded LBDs of human retinoic acid receptor-γ (hRARγ), rat thyroid hormone receptor-α (rTRα), human estrogen receptor (hER), and human progesterone receptor (hPR) have recently been determined [5–9]. The crystal cultures of the liganded hRARγ, rTRα, hER, and hPR have revealed that in all these cases the specific ligands have multiple distinct contact sites within their receptors. All contact sites are positioned in the ligand binding pocket, which is located between helices 5, 6, 11, and 3. Several mutational studies and molecular modeling studies have also indicated that in steroid receptors helices 3, 5, and 11 are important for ligand binding [10–14]. Furthermore, Klaholz et al. have recently studied the binding of 9-*cis* retinoic acid and the synthetic ligand BMS961 to hRARγ by X-ray crystallography and reported that interactions occurred between those ligands and helices 3 and 5 among other segments of the hRARγ [15].

Based on this information we have produced 11 distinct mutated clones of hVDR by site-directed mutagenesis. Ten mutated amino acid residues are located in the putative helix 3 of the vitamin D receptor and one in the putative helix 5. The abilities of 1α,25-dihydroxy[26,27-*methyl*-^{3}H]cholecalciferol and its synthetic analog [26,27-^{14}C2]-MC1288 to bind to the in vitro translated wild-type and mutated hVDRs were measured and the results were compared with each other to determine whether the mutations influenced ligand binding. We also used partial proteolytic digestion of in vitro translated hVDR to gain an insight into the ligand-induced conformations of the wild-type and mutated receptors. Finally, molecular modeling was used to find out the putative positions of the mutated amino acid residues with respect to their interactions with the ligands.

2. Materials and methods

2.1. Site-directed mutagenesis

To find the contact sites of calcitriol in the hVDR, the prospective amino acid residues were chosen for site-directed mutagenesis by comparing their hydropathy indexes [16] and the amino acid sequences of the hVDR with the known 3-D structures of hRARγ, rTRα, hER, and hPR [6–9]. The mutated amino acid residues of hVDR and the known contact sites of hRARγ, hER, hPR, and rTRα are shown in Fig. 1. The cDNA of hVDR was subcloned into expression vector pSP65 and used as a template for site-directed mutagenesis by QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK). Eleven clones of mutated hVDR were produced by changing the amino acid residues Ser225, His229, Asp232, Val234, Ser235, Tyr236, Ser237, Lys240, Ile242, Lys246, and Ser275 separately into alanines as described by the manufacturer. The vectors incorporating the desired mutations were then transformed into *Epicurian coli* XL1-Blue supercompetent cells. The cDNAs of the clones were purified by Qiagen Plasmid Kit (Qiagen, Valencia, CA, USA) and sequenced completely to ensure that no other base changes were produced.

2.2. Binding of radioactive calcitriol and MC1288 to in vitro translated wild-type and mutated hVDRs

The wild-type and mutated hVDRs were prepared in vitro by the coupled wheat germ extract system with either wild-type or modified hVDR cDNA inserted into plasmid pSP65 as described by the manufacturer (Promega, Madison, WI, USA). The binding of radioactive calcitriol or MC1288 to wild-type and mutated hVDRs was assessed by one point analysis. Five-μl aliquots of mixtures containing in vitro translated unlabeled hVDRs (25 μg of total protein) in a final volume of 20 μl were treated for 30 min at 22°C with 0.5 nM 1α,25-dihydroxy[26,27-*methyl*-^{3}H]cholecalciferol (179 Ci/mmol, Amersham International, Buckinghamshire, UK) or 5 μM [26,27-^{14}C2]-MC1288 (a gift from Leo Pharmaceutical Products, Ballerup, Denmark) diluted with ethanol. Unbound radioactivity was removed by treatment with dextran-coated charcoal and the bound radioactivity was measured by scintillation counting. The experiment was repeated three times and the results were corrected for background. For Scatchard analysis, 5 μl aliquots of in vitro translated receptor proteins (25 μg of total protein) in a final volume of 20 μl were treated as above with

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Abbreviations: Calcitriol, 1α,25-dihydroxyvitamin D_3; hER, human estrogen receptor; LBD, ligand binding domain; MC1288, 20-epi-1α,25-dihydroxyvitamin D_3; hPR, human progesterone receptor; hRARγ, human retinoic acid receptor-γ; hRXRα, human retinoid X receptor-α; rTRα, rat thyroid hormone receptor-α; hVDR, human vitamin D receptor

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Table 1
Comparison of K_d values of the wild-type and mutated hVDRs

hVDRs	K_d of calcitriol (nM)[a]
Wild type	1.02 ± 0.02
Ser225Ala	1.12 ± 0.03
His229Ala	26.75 ± 0.13
Asp232Ala	29.54 ± 0.05
Val234Ala	1.46 ± 0.03
Ser235Ala	1.42 ± 0.03
Tyr236Ala	3.50 ± 0.08
Ser237Ala	23.29 ± 0.16
Lys240Ala	1.37 ± 0.03
Ile242Ala	1.22 ± 0.01
Lys246Ala	1.92 ± 0.03
Ser275Ala	5.29 ± 0.09

[a]Mean ± S.E.

0.005–5 nM 1α,25-dihydroxy[26,27-*methyl*-^{3}H]cholecalciferol. Statistical analysis was performed by the SPSS (6.1.1.) program.

2.3. Digestion of in vitro translated hVDRs

L-[^{35}S]Methionine (>1000 Ci/mmol, Amersham International, Buckinghamshire, UK) labeled wild-type and mutated hVDRs were prepared in vitro by the coupled wheat germ extract system as described above. Five-μl aliquots of mixtures containing the in vitro translated hVDRs in a final volume of 20 μl were pre-incubated with 1 μM of non-radioactive calcitriol or MC1288 (a gift from Leo Pharmaceutical Products, Ballerup, Denmark) diluted with ethanol for 30 min at 22°C before exposing them to partial proteolytic digestion by 25 μg/ml trypsin (EC 3.4.21.4) (Sigma, St. Louis, MO, USA) for 10 min at 22°C. The reaction was stopped by adding 4 μl of 6-fold SDS-loading buffer and boiling for 5 min. The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

2.4. Molecular modeling

The ligand binding domain of hRARγ complexed with all-*trans* retinoic acid (pdb entry 2lbd) was used to locate the mutated amino acid residues of hVDR and their putative positions with respect to the ligand binding pocket. With molecular modeling, helices 3 and 5 of hRARγ were replaced by helices 3 and 5 of hVDR. The figure was constructed with the program SETOR [17].

3. Results and discussion

The binding studies revealed that changing the amino acid residues Ser225, Val234, Ser235, Lys240, Ile242, and Lys246 separately into alanines did not markedly affect the ability of the radioactive calcitriol to bind to the mutated receptor compared with the wild-type hVDR (Fig. 2a). However, when either His229, Asp232, or Ser237 were replaced by alanine, the binding affinities decreased significantly indicating that these amino acid residues are important for ligand binding

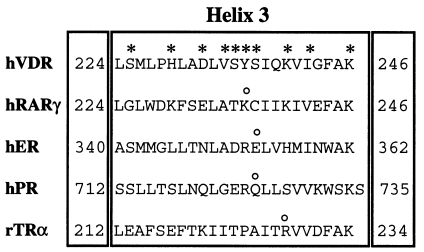

Fig. 1. Sequence alignments of helix 3 regions of hVDR, hRARγ, hER, hPR, and rTRα. (*) Target amino acid residues for site-directed mutagenesis; (◦) known contact amino acid residues in hRARγ, hER, hPR, and rTRα for ligand binding.

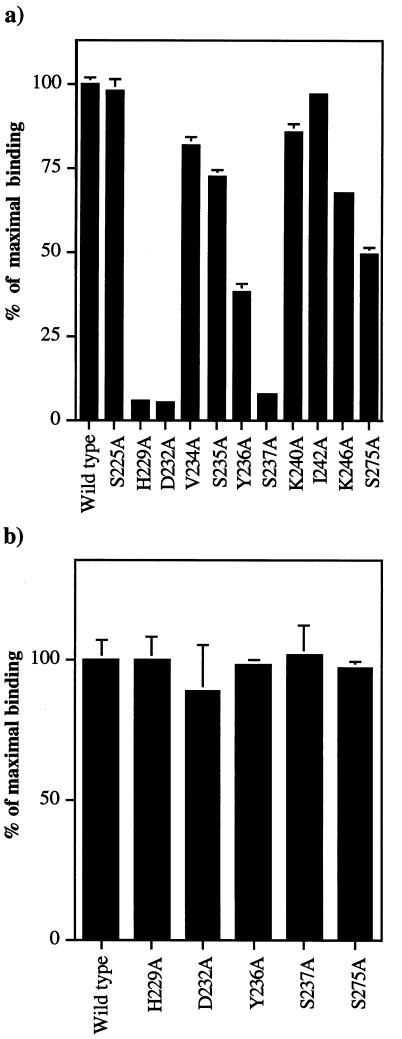

Fig. 2. Binding of radioactive calcitriol (a) and MC1288 (b) to in vitro translated wild-type and mutated hVDRs. Wild-type and mutated hVDRs were treated for 30 min at 22°C with 0.5 nM 1α,25-dihydroxy[26,27-*methyl*-^{3}H]cholecalciferol or [26,27-^{14}C2]-MC1288. Unbound radioactivity was removed by treatment with dextran-coated charcoal and the bound radioactivity was measured by scintillation counting. Means ± S.E. are presented.

(Table 1). Furthermore, the amino acid residues Tyr236 and Ser275 also seem to participate in the ligand binding process. These results suggest that the putative helix 3 of hVDR and, especially the central part of it, is an important structural element in the formation of the ligand binding pocket. In addition, the putative helix 5 of hVDR seems to have a role in the ligand binding.

We and others have previously reported that ligand binding changes the conformation of hVDR resulting in resistance against partial proteolytic digestion [12,13,18–20]. In the present study, we utilized this feature of the receptor to obtain information on the conformations of the mutated receptors. When the mutated receptors were exposed to partial proteolytic digestion, the substitutions Ser225Ala, Val234Ala, Ser235Ala, Lys240Ala, Ile242Ala, and Lys246Ala did not markedly change the protective effect of ligand binding against partial proteolysis compared with that of the wild-type hVDR (Fig. 3a). The substitution Ser275Ala decreased

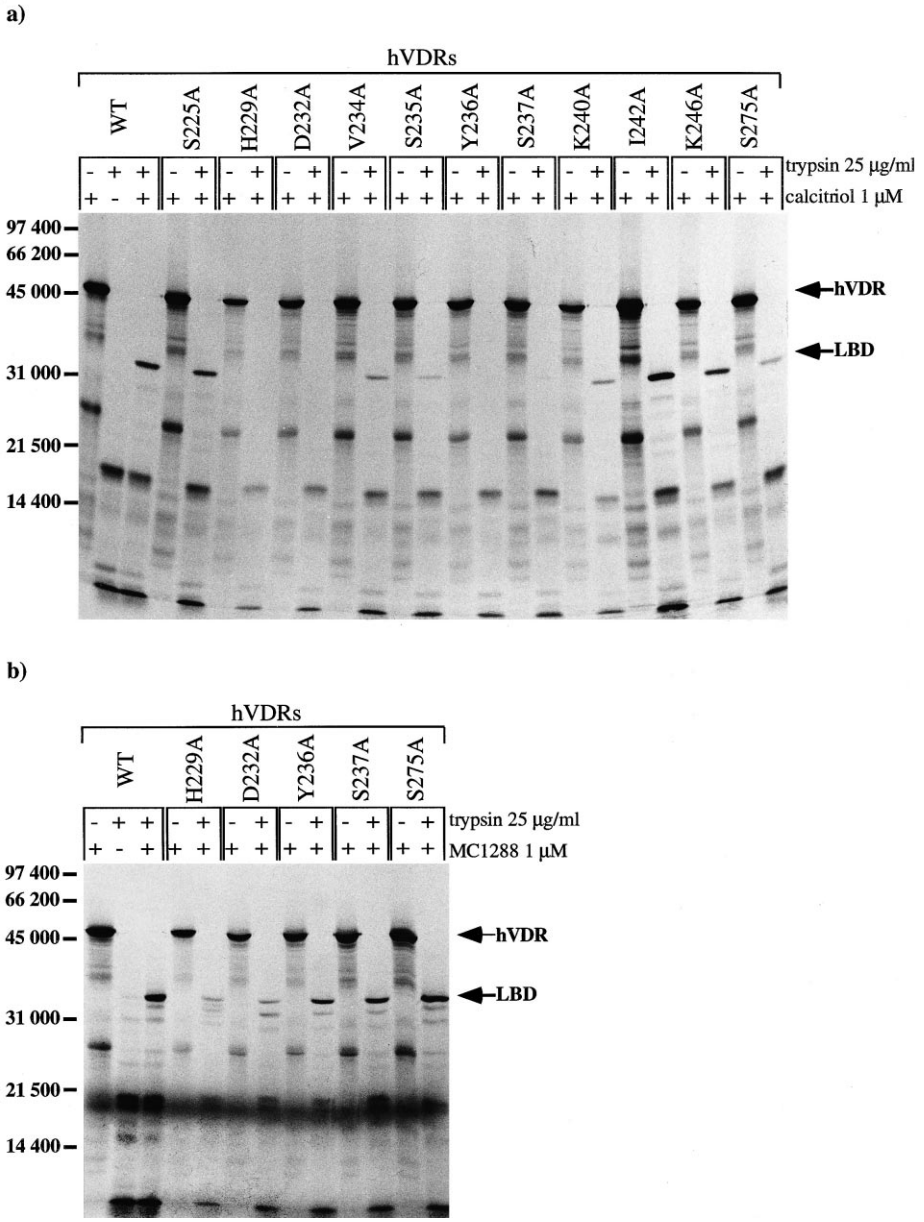

Fig. 3. Ligand-dependent protection of the LBDs of wild-type and mutated hVDRs against partial proteolytic digestion with trypsin. L-[^{35}S]Methionine-labeled wild-type and mutated hVDRs were pre-incubated with 1 μM calcitriol (a) or MC1288 (b) at 22°C before exposing them to partial proteolytic digestion with trypsin (25 μg/ml).

the ligand dependent protection, but the entire LBD of the hVDR was still visible in the gel, indicating that ligand binding had occurred and changed the conformation of the receptor. The substitutions His229Ala, Asp232Ala, Tyr236Ala, and Ser237Ala, however, resulted in complete degradation of the LBD in the presence of the ligand. This suggests that either no ligand binding had occurred or that the ligand induced conformational changes of the receptors were markedly different from those of the wild-type hVDR resulting in sensitivity to partial proteolytic digestion. Our study confirms previous reports on the occurrence of correlation between ligand binding and the resistance of hVDR to partial proteolysis [12,13,18–20]. Furthermore, our point mutation results confirm the molecular modeling results of Wurtz et al. on the importance of Ser237 and Ser275 for the binding of calcitriol [14]. Based on these data it is reasonable to suggest that the loss of ligand dependent protection with the indicated mutants resulted from decreased ability of the ligand to bind to the hVDR.

Molecular modeling supported these results. From Fig. 4 it can be observed that the amino acid residues His229, Tyr236, and Ser237 are positioned so that they can readily interact with the ligand. If the ligand is in a more bent conformation, like in the 6-s-*trans* conformation of calcitriol [21], the interaction may also occur with Asp232. Tyr236 and Ser275, in turn, are located close enough for potential interactions, but the substitutions Tyr236Ala and Ser275Ala only partially blocked the ligand binding (−62% and −51%, respectively). Thus these amino acid residues probably do not make direct interactions with the ligand, but their importance may be in the stabilization of the ligand-receptor complex. We suggest

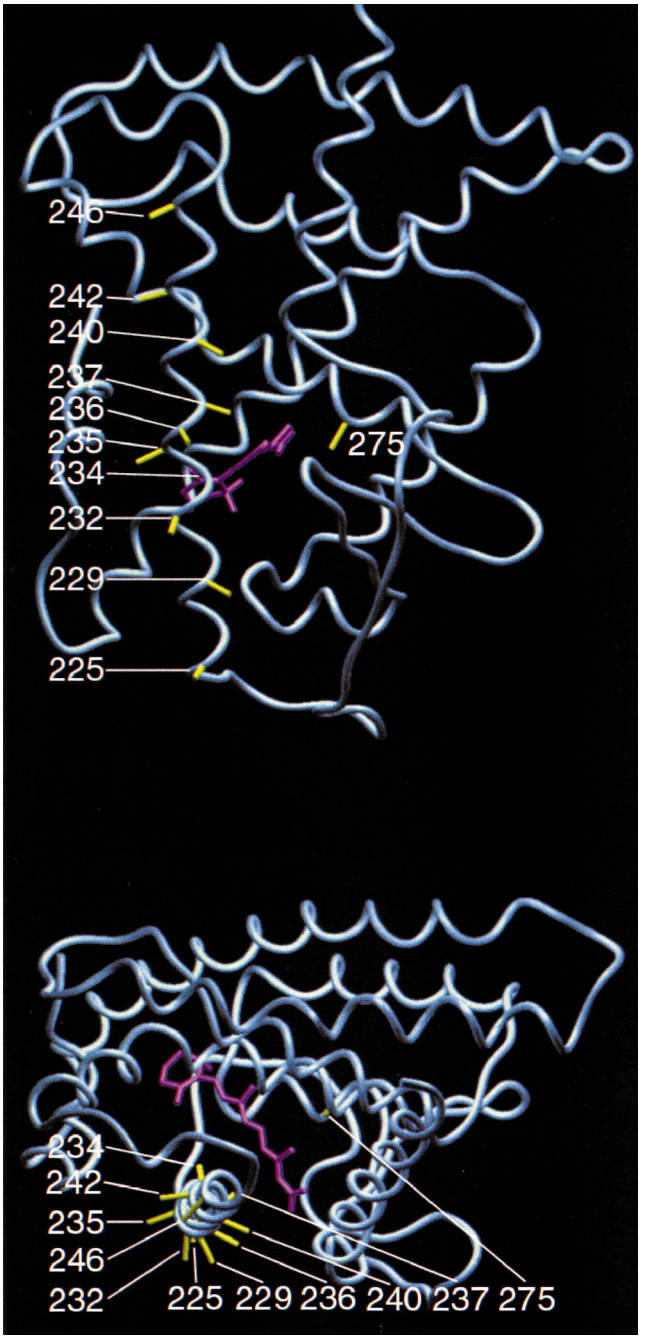

Fig. 4. Location of the mutated amino acid residues in the putative helices 3 and 5 of hVDR. The coordinates of the LBD of hRARγ complexed with all-*trans* retinoic acid (pdb entry 2lbd) were used to position the mutated residues of hVDR. The protein backbone of hRARγ is shown in cyan, the bound retinoic acid in red, and the positions of the mutated amino acid residues are in yellow. The numbers refer to the numbering of hVDR.

that calcitriol is positioned in the ligand binding pocket similarly to all-*trans* retinoic acid with its A-ring at the bottom of the ligand binding cavity.

To ensure that the decreased binding affinities of His229Ala, Asp232Ala, Tyr236Ala, Ser237Ala, and Ser275Ala substituted hVDRs are not due to a differential folding of the mutated receptors compared with the wild-type receptor we repeated the binding and conformational analyses of these hVDRs with the synthetic vitamin D analog MC1288. From Fig. 2b it can be seen that the point mutations did not markedly influence the binding of MC1288 to the mutated hVDRs compared with that of the wild-type receptor. After partial proteolytic digestion by trypsin the digestion patterns of Tyr236Ala, Ser237Ala, and Ser275Ala substituted hVDRs were similar with that of the wild-type receptor indicating that these point mutations had not influenced the folding of the receptor. The digestion pattern of His229Ala substituted receptor was slightly different from that of the wild-type receptor suggesting that this mutation had changed the folding of the receptor to some extent. In addition, even though the digestion pattern of the Asp232Ala substituted receptor was similar to that of the wild-type receptor, the intensity of the LBD was less than that of the wild-type receptor, suggesting that also this mutation had slightly changed the folding of the receptor. Therefore we cannot exclude the possibility that the decreased affinity of calcitriol to these receptors is due to different conformations of these receptors.

Our results also suggest that MC1288 and calcitriol have somewhat different binding sites in the putative helices 3 and 5 of hVDR. While Liu et al. have recently reported that MC1288 has different binding requirements in helices 11 and 12 of hVDR from that of calcitriol [12], it is interesting to notice that MC1288 may have different overall binding requirements compared with calcitriol, even though their only structural difference is the 20-epi bond.

We have demonstrated here that the putative helix 3 of hVDR and, especially, the central part of it, is important for the binding of calcitriol to its receptor. In addition, helix 5 seems to participate in the binding process. We suggest that the amino acid residues His229, Asp232, and Ser237 may act as contact sites between calcitriol and hVDR. We further suggest that Tyr236 and Ser275 may also have important roles in ligand binding, e.g. by stabilizing the ligand-receptor complex. To our knowledge this is the first report demonstrating specific contact sites of calcitriol in the putative helices 3 and 5 of hVDR.

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