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Structurally and Functionally Important Amino Acids of the Agonistic Conformation of the Human Vitamin D Receptor

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ABSTRACT

The crystal structures of the ligand binding domain of human vitamin D receptor (VDR) complexed with its natural ligand or the superagonists MC1288 or KH1060 have recently been reported. The crystallized ligand binding domain (LBD) of VDR, however, differs from the full-length VDR with respect to deletion of 50 amino acids between its helices 2 and 3. In this study, we investigated structurally and functionally important amino acid interactions within the ligand binding pocket of the full-length VDR in the presence of several synthetic vitamin D_3 analogs. We used site-directed mutagenesis scanning combined with limited proteolytic digestion, electrophoretic mobility shift assay, and reporter gene assay and correlated the findings with the crystal structures of truncated VDR LBD. Our results suggest that structurally different agonists have distinct ligand-receptor interactions and that the amino acid residues H229, D232, E269, F279, and Y295 are critical for the agonistic conformation of the VDR. Our biological data, which were obtained with the full-length VDR, fit well with the crystal structure of the truncated VDR LBD and suggest that removal of the insertion domain between helices 2 and 3 of the receptor does not markedly influence the functionality of the VDR.

The biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (calcitriol), regulates several important physiological and biochemical functions in mammals, such as calcium and phosphorus homeostasis as well as cellular growth, differentiation, and apoptosis (DeLuca et al., 1990; Walters, 1992). Binding of calcitriol to its receptor (VDR) induces a conformational change in the receptor and promotes formation of a heterodimer with retinoid X receptor (RXR) as well as binding of other transcriptional cofactors to the receptor. The VDR-RXR heterodimer attaches to a specific segment of DNA, the vitamin D response element (VDRE), which is located in the promoter regions of calcitriol target genes (Carlberg, 1995, 1996; Toell et al., 2000).

The VDR belongs to the nuclear receptor superfamily. Many members of this superfamily have conserved amino acid sequences in the C terminus of the receptor (Wurtz et al., 1996; Haussler et al., 1998). VDR, however, makes an exception in that it has a long, unordered loop structure between helices 2 and 3. The LBDs of several nuclear receptors have already been successfully crystallized (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995; Brzozowski et al., 1997; Williams and Sigler, 1998; Pike et al., 1999; Gampe et al., 2000; Egea et al., 2000; Clayton et al., 2001; Watkins et al., 2001), and a clear homology can be observed in their three-dimensional structures because they share a common helical sandwich structure with three layers of α-helices surrounding the hydrophobic ligand binding pocket. When the crystal structure of truncated VDR LBD was solved by Rochel et al. (2000), it could be observed that VDR does not make an exception to the structural principles of related receptors. However, the fact that the crystallized VDR lacks 50 amino acids directed us to investigate how the crystal structure agrees with data obtained from biological studies of full-length VDR.

Limited proteolytic digestion has been shown to be an important method for studying the conformation of nuclear receptors (Peleg et al., 1995; Nayeri and Carlberg, 1997). In a recently published article, Herdick et al. (2000) suggest that the three fragments resulting from the limited proteolytic digestion of VDR represent functionally different receptor conformations. These conformations are agonistic (c1LPD), antagonistic (c2LPD), and nonagonistic (c3LPD) (reviewed by Carlberg et al., 2001). With wild-type VDR, the main conformation is normally c1LPD, but c3LPD is also weakly visible with most ligands (see Fig. 3).

In this work, we have used site-directed mutagenesis scanning combined with limited proteolytic digestion to investi-

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ABBREVIATIONS: VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; LBD, ligand binding domain; EMSA, electrophoretic mobility shift assay; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; FCS, fetal calf serum; LPD, limited protease digestion.

gate which amino acid residues are important for the action of full-length VDR in the presence of calcitriol and 15 different vitamin D_3 analogs (Fig. 1). In addition, the effects of point mutations were studied in vitro by electrophoretic mobility shift assay (EMSA) and in vivo by transfecting COS-7 African green monkey kidney cells with the wild-type or mutated VDRs. The results of biological assays were compared with the crystal structure of truncated VDR LBD by molecular modeling to find out if the same amino acid residues have critical role for stable VDR conformation in the full-length and truncated VDR. Finally, molecular modeling was used to further study the differences in the results with calcitriol and its analogs.

We report here that the amino acid residues H229, D232, E269, F279, and Y295 are critical for the agonistic conformation of the full-length VDR. In addition, our results suggest that different amino acid residues within the ligand binding cavity of the VDR are important for binding of structurally different ligands and/or for the stable receptor conformation induced by these ligands.

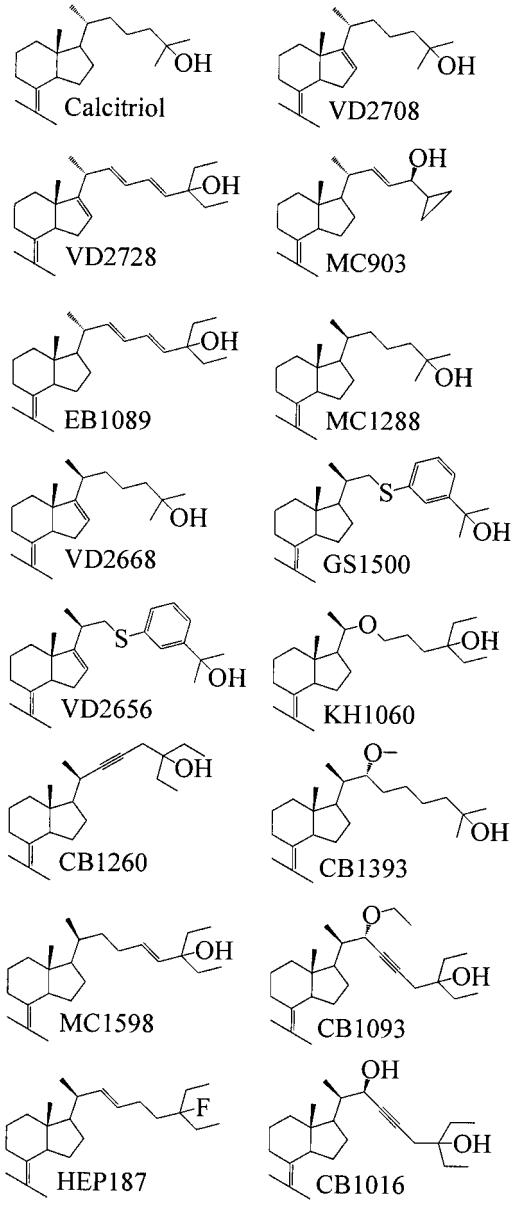

Fig. 1. CD-ring and side-chain structures of calcitriol and the analogs used in this study.

Materials and Methods

Materials. The QuikChange site-directed mutagenesis kit was provided by Stratagene (Cambridge, UK). Calcitriol and its synthetic analogs (Fig. 1) were a generous gift from Leo Pharmaceutical Products Ltd. (Ballerup, Denmark). L-[^{35}S]methionine (specific activity, >1000 Ci/mmol) was provided by Amersham International (Little Chalfont, Buckinghamshire, UK). Trypsin (EC 3.4.21.4) was from Sigma-Aldrich (St. Louis, MO). African green monkey kidney cells (COS-7) were obtained from the American Type Culture Collection (Manassas, VA). Promega (Madison, WI) provided TNT-coupled wheat germ extract system. Luciferase activity assay kit was from Bio-Orbit (Helsinki, Finland). DOTAP lipofection reagent was from ROTH (Karlsruhe, Germany). Vectors pSP65, pSG5, and pCMVβ were provided by Promega. The pXP-1 vector was from Dr. S. K. Nordeen (University of Colorado Health Sciences Center, Denver, CO) (Nordeen, 1988).

Site-Directed Mutagenesis. Amino acid residues for site-directed mutagenesis scanning were chosen based on the crystal structure of the truncated VDR LBD (Protein Data Bank entry 1DB1). The cDNA of VDR was subcloned into expression vector pSP65 and used as a template for site-directed mutagenesis. Thirty-three substitutions (H139A, T142A, Y143A, Y147A, R158A, S225A, H229A, D232A, V234A, S235A, Y236A, S237A, K240A, S265A, S266A, E269A, R274A, S275A, E277A, S278A, F279A, C288A, Y293A, Y295A, D299A, H305A, Q317A, H330A, N394A, E396A, H397A, Q400A, and Y401A) and one deletion (T415stop) was produced according to the manufacturer's instructions (see Fig. 7B). The cDNAs of the mutated VDRs were completely sequenced to ensure that no additional base changes were produced. It was also ensured by L-[^{35}S]methionine-labeled in vitro translation and SDS-PAGE that the mutated cDNAs translated properly into protein (Fig. 2).

Limited Protease Digestion Assay. Aliquots (5 μl) of mixtures containing the in vitro-translated, L-[^{35}S]methionine-labeled wild-type or mutated VDRs in a final volume of 20 μl were preincubated with 1 μM of calcitriol or its synthetic analogs for 30 min at 22°C. The samples were then exposed to limited proteolytic digestion using 25 μg/ml trypsin for 10 min at 22°C. The reaction was stopped by adding SDS-loading buffer and boiling for 5 min. The digestion products were separated by 12% SDS-polyacrylamide gel electrophoresis and the gels were dried and autoradiographed. The autoradiographs were scanned and the intensities of the LBD fragments were measured using the NIH Image program (ver. 1.62; http://rsb.info.nih.gov/nih-image/).

EMSA. Aliquots (4 μl) of mixtures containing the in vitro-translated, nonlabeled wild-type or mutated VDRs and RXR were preincubated with 10 nM of calcitriol or its analogs in a final volume of 20 μl for 20 min at 22°C in a buffer containing 2 μg of poly(dI-dC) in 20 mM HEPES, pH 7.6, 4.2% glycerol (v/v), 70 mM NaCl, 2.3 mM $MgCl_2$, 2.0 mM EDTA, 2.2 mM dithiothreitol, 80 μM phenylmethylsulfonyl fluoride, 0.3 μg/ml aprotinin (Trasylol), and 0.3 μg/ml leupeptin. A ^{32}P-labeled double-stranded VDRE from the human osteocalcin gene promoter (5′-CCGGGTGAACGGGGGCA-3′) was then

Fig. 2. The full-length in vitro translation products of the wild-type and selected mutated VDRs.

added and the incubations were continued for additional 30 min at 22°C (underlined nucleotides indicate the binding site of VDR-RXR heterodimer; RXR binds to one site and VDR to the other). The samples were loaded onto 5% polyacrylamide gel and electrophoresed in 0.025 M Tris-borate, pH 8.3, and 0.5 mM EDTA. All experiments were done at least in triplicate. The gels were dried and analyzed by filmless autoradiographic analysis (FLA3000; Fuji, Tokyo, Japan).

Cell Culture. African green monkey kidney cells (COS-7) were maintained in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin in a humidified 95% air/5% CO_2 incubator. All experiments were performed in a medium containing 2% charcoal-treated FCS to eliminate endogenous steroid hormones. Calcitriol or its analogs were dissolved in ethanol before adding to the cultures and the control cultures were treated with 0.1% ethanol.

Transfection of COS-7 Cells. To study the effect of selected point mutations on the transactivation efficiency of calcitriol and its selected analogs (MC903, MC1288, HEP187, and KH1060), the COS-7 cells were transfected with full-length wild-type or mutated VDRs subcloned into expression vector pSG5. VDRE containing reporter plasmid pXP-1/hOC-910 was constructed by cloning a 910-base-pair promoter fragment, which was amplified from the human osteocalcin gene (nucleotides −881 to + 29) by polymerase chain reaction. Wild-type VDR or the mutated VDR pXP-1/hOC and the control plasmid pCMVβ were introduced into COS-7 cells using a DOTAP lipofection reagent using manufacturer's instructions. Twenty-four hours after transfections, the medium was replaced by fresh medium containing 2% charcoal-treated FCS and 1 nM calcitriol or its analogs, and the cells were incubated for 30 h. The cells were lysed and the luciferase activity was measured. The luciferase activities were normalized with respect to β-galactosidase activity. All experiments were done at least in triplicate.

Molecular Modeling. The X-ray crystal structure of the truncated VDR LBD complexed with calcitriol (Protein Data Bank entry 1DB1) was obtained from the Protein Data Bank (Berman et al., 2000; http://www.rcsb.org/pdb/). Crystallographic water (164 molecules) was included in the VDR-ligand complexes. Protein modeling and calculations were done using the SYBYL program version 6.5 (St. Louis, MO) running on a O_2 R5000 workstation (SGI, Mountain View, CA). The analogs VD2708, VD2728, MC903, EB1089, MC1228, VD2668, GS1500, VD2656, KH1060, CB1260, CB1393, MC1598, CB1093, HEP187, and CB1016 were placed in the ligand binding pocket of the VDR using X-ray coordinates of the complex. This was done by least-squares-fitting the A, B, and D rings of the analogs on calcitriol of the crystal structure. Carbon atoms 2, 5, 8, 12, 14, and 17 were used in the fitting procedure. Because 25-OH of calcitriol is important for binding (Bouillon et al., 1995; Rochel et al., 2000), the OH-groups of the side chains of the analogs were placed near this position within the receptor. Energy minimizations of the VDR-ligand complexes were done using Tripos force field and conjugate gradient optimization methods with a convergence criterion requiring a minimum energy change of 0.001 kcal/mol. In the minimizations, the coordinates of the analogs, the protein, and the water molecules within 8.0 Å of the analog atoms were allowed to move. The minimized complexes were visually checked. If there were bad contacts in the structures or several alternative ways to place the analog side chain in the binding site, additional minimizations were carried out using different starting geometries.

Results

The studied ligands were able to stabilize the LBD of wild-type VDR against limited proteolytic digestion (Fig. 3). Substituting separately the amino acid residues H139, T142, Y147, R158, S225, V234, K240, S265, S266, E277, C288, Y293, Q317, H330, N394, and E396 with alanine affected the ligand-dependent stabilization of the VDR LBD only marginally or not at all (data not shown). This suggests that these amino acid residues are not important for ligand binding and the stable VDR LBD conformation with the studied ligands. In contrast, the substitutions R274A, F279A, Y295A, and the deletion of helix 12 caused formation of an unstable receptor with all ligands; with the substitution H397A, only the superagonist KH1060 was able to stabilize the agonistic LBD conformation c1LPD (Fig. 4). This conformation, however, was not biologically active, because KH1060 failed to induce transcription with H397A-substituted VDR, indicating that ligand binding does not necessarily result in functionally correct conformation. These results indicate that these amino acid residues are collectively important for the stable agonistic VDR conformation induced by the different ligands.

As expected, deletion of helix 12 resulted in a disappearance of the ligand-dependent c1LPD fragment with all ligands, confirming that helix 12 is a functional part of the c1LPD conformation (Fig. 4). Interestingly, the substitutions H305A and H397A, as well as deletion of helix 12, caused ligand treatment to induce a strong c3LBD conformation with most ligands (Fig. 4).

With some point mutations, the ligands stabilized the mutated VDR LBDs differently (Fig. 4, Table 1). In this group, the amino acid residues H229, D232, and E269 are noteworthy, because substitution of them by alanine completely prevented or strongly decreased the ligand-dependent stabilization of VDR LBD with most ligands. Comparing the stabilities with respect to ligand treatment, it was observed that the analogs VD2708, MC1288, KH1060, and MC1598 were able to stabilize the receptor more often than the other ligands. On the other hand, most of the substitutions could prevent the analogs EB1089, VD2656, CB1260, HEP187, and CB1016 from stabilizing the receptor.

Based on the results of site-directed mutagenesis scanning, we selected a series of mutated VDRs and analogs for a more detailed investigation. The amino acid residues H305 and H397 were chosen because they caused ligand binding to induce VDR conformation that is not usual under the conditions used. In addition, the amino acid residues H229, D232, E269, R274, F279, and Y295 were chosen because they seemed to be important for the stable agonistic conformation of VDR. These eight mutated VDRs were studied with calcitriol and the analogs MC903, MC1288, KH1060, and HEP187 by EMSA to determine the degree of heterodimerization with RXR. Regardless of the ligand used, the substitutions R274A,

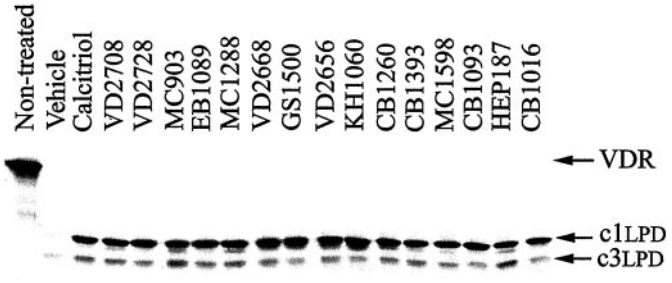

Fig. 3. Stability of the wild-type VDR against limited proteolytic digestion with trypsin. L-[^{35}S]methionine-labeled VDRs were preincubated with 1 μM calcitriol or analogs at 22°C before exposing it to limited proteolytic digestion with trypsin (25 μg/ml). The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

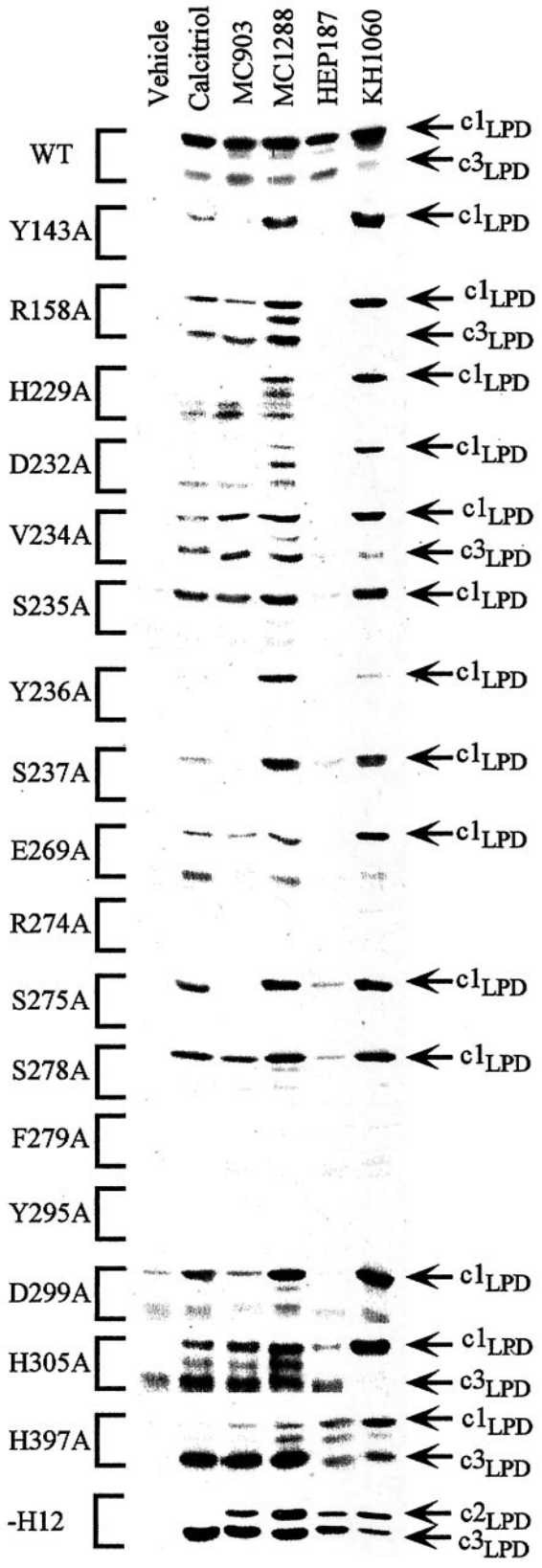

Fig. 4. Stability of the wild-type and selected mutated VDRs against limited proteolytic digestion with trypsin. L-[^{35}S]methionine-labeled VDRs were preincubated with 1 μM calcitriol or analogs at 22°C before exposing them to limited proteolytic digestion with trypsin (25 μg/ml). The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

F279A, and H397A completely prevented VDR from heterodimerizing and the substitutions H229A, D232A, E269A, and Y295A clearly influenced negatively the heterodimerization. In contrast, the substitution H305A showed activity comparable with wild-type receptor (Fig. 5A).

All vitamin D_3 compounds used induced luciferase activity when studied with the wild-type VDR (Fig. 5B). When studied with the mutated VDRs, significant differences in ligand action were observed. The substitutions H229A, D232A, R274A, F279A, Y295A, and H397A caused formation of an inactive receptor. The E269A-substituted receptor was inactive with calcitriol and MC903 but showed about 50% activity of the wild-type receptor when treated with MC1288, HEP187, or KH1060. After the substitution H305A, the VDR was fully active when treated with calcitriol, MC1288, or KH1060 and showed about 50% activity with MC903 and HEP187.

Molecular modeling revealed that it was possible with the studied ligands to position the hydroxyl group of the side chain into a position where it makes contacts with H305 and H397. A representative figure of two modeled ligands is shown in Fig. 7A. The compounds with a longer or more rigid side chain than that of calcitriol (e.g., HEP187, see Figs. 1 and 7A) were adapted to the available space within the receptor by bending their side chains. This is in agreement with recently published data (Yamamoto et al., 2000; Tocchini-Valentini et al., 2001). The A-ring of the ligands was always similarly positioned in the ligand binding pocket and the CD-ring showed only a slight variation in its positioning. In contrast, the different lengths and volumes of the side chains of the different ligands caused a significant variation in their positioning.

Discussion

Our results suggest that, with respect to the agonistic action of full-length VDR, the most important amino acids among the studied residues are H229, D232, E269, R274, F279, Y295, and H397. The importance of amino acids R274 and H397 is especially obvious, because they are the contact amino acid residues that anchor calcitriol into the ligand binding pocket of the truncated receptor (Rochel et al., 2000). In our studies, the substitutions R274A and H397A indeed rendered the full-length receptor to a labile conformation (Fig. 4) that was biologically inactive (Fig. 5). Because formation of a stable VDR LBD conformation indicates that ligand binding has occurred (Peleg et al., 1995; van den Bemd et al., 1996; Nayeri and Carlberg, 1997; Väisänen et al., 1998) and because substitution of these amino acid residues with alanine destabilized the receptor structure with all studied analogs, we suggest that these two amino acid residues are common contact sites for agonistic ligands.

Although R274 (contact site of 1α-OH) and H397 (contact site of 25-OH) are very important for the binding of vitamin D_3 compounds to full-length VDR, the rest of the reported contact sites in VDR seem to be less important. The substitution of Y143, S237, S278, and H305 by alanine did not completely destabilize the full-length receptor with all ligands, suggesting that these amino acid residues are not as important for the studied analogs as they are for calcitriol (Table 1). Our results thus suggest that the stable agonistic receptor conformation requires that the ligand must be at-

tached via its 1α-OH and side chain OH-groups to helices H5 and H11, respectively. When these contact sites are occupied, the ligand will be stabilized within the ligand binding pocket by several minor interactions that depend on the structure and flexibility of the ligand side chain and thus may differ between different ligands. Calcitriol and many of its analogs are very flexible compared with other steroid/nuclear hormones because of the open B-ring structure (Bouillon et al., 1995; Okamura et al., 1995). Therefore, they can change their conformation to fit into the ligand binding pocket and to reach the potential ligand contact sites. This flexibility probably makes it possible for the different ligands to have a distinct number of weaker interactions with the receptor because straight and bent side chains can interact with a distinct set of amino acid residues, as Tocchini-Valentini et al. (2001) have recently demonstrated. A similar conclusion can be drawn from our site-directed mutagenesis scanning results; they suggest that different amino acid residues are important for the agonistic c1LPD conformation with different ligands (Table 1).

According to the truncated VDR LBD crystal structure (Rochel et al., 2000), E269 (H5) is connected by hydrogen bonding to C337 (H8), R343 (coil between H8 and H9), and N394 (H10). Thus, this single amino acid residue connects four helices with each other suggesting that although E269 does not directly interact with calcitriol, its importance is in the stabilization of the interior of VDR structure. Our results support this suggestion, because substituting E269 with ala-

TABLE 1

Stability of the wild type and mutated VDR LBDs against limited proteolytic digestion with trypsin

(−)-Ligand treatment stabilizes the LBD by less than 20% of that of the wild-type VDR; (+)-ligand treatment stabilizes the LBD by more than 20% of that of the wild-type VDR. Locations of mutated amino acids within VDR are indicated as coil (C) or helix (H).

Location in VDR	Mutated VDRs	20-Normal Compounds					20-epi Compounds										
		Calcitriol	VD 2708	VD 2728	MC 903	EB 1089	MC 1288	VD 2668	GS1 500	VD 2656	KH1 060	CB1 260	CB 1393	MC1 598	CB1 093	HEP 187	CB 1016
C	Y143A	+	+	+	–	–	+	+	–	–	+	–	+	–	+	–	–
	R158A	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–
H3	H229A	–	+	–	–	–	+	–	–	–	+	–	–	+	–	–	–
	D232A	–	+	–	–	–	+	–	–	–	+	–	–	+	–	–	–
	V234A	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+
	S235A	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+
	Y236A	–	–	+	–	–	+	–	–	–	–	–	–	+	–	–	–
	S237A	–	+	–	–	–	+	–	–	–	+	–	–	+	+	–	–
H5	E269A	–	+	+	+	–	+	–	+	–	+	–	+	+	+	–	–
	S275A	+	+	+	–	+	+	–	+	–	+	–	+	+	+	+	–
	S278A	+	+	+	+	–	+	+	+	–	+	+	+	+	+	–	–
H6	D299A	+	+	+	–	–	+	–	+	–	+	–	+	+	+	–	–
C	H305A	+	–	+	+	+	–	–	+	+	+	–	–	+	–	–	–

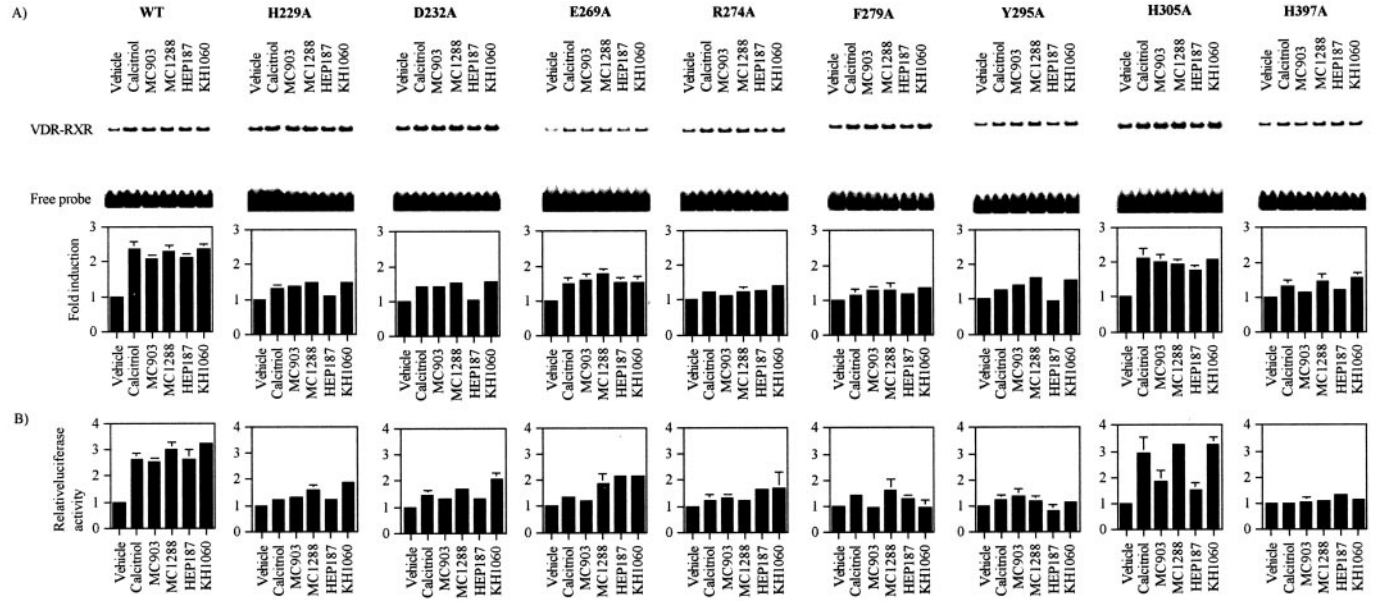

Fig. 5. Effects of selected point mutations on heterodimerization of VDR with RXR and on transactivation efficiency of VDR. A, the in vitro-translated, nonlabeled wild-type and mutated VDRs and RXR were preincubated with 10 nM of calcitriol or its selected synthetic analogs at 22°C. Then a ^{32}P-labeled double-stranded VDRE from the human osteocalcin gene promoter (5′-CCGGGTGAACGGGGGCA-3′) was added and the incubations were continued for additional 30 min at 22°C. The samples were loaded onto 5% polyacrylamide gel and electrophoresed in 0.025 M Tris-borate, pH 8.3, and 0.5 mM EDTA. The gels were dried and analyzed by filmless autoradiographic analysis. The fold induction was calculated by dividing the intensity of the ligand treated protein complex with that of the vehicle. B, wild-type VDR or the mutated VDR pXP-1/hOC and the control plasmid pCMVβ were introduced into COS-7 cells using a DOTAP lipofection reagent using manufacturers instructions. Twenty-four hours after transfections, the medium was replaced by a fresh medium containing 2% charcoal-treated FCS and 1 nM calcitriol or its analogs, and the cells were incubated for the next 30 h. The cells were lysed and the luciferase activity was measured. The luciferase activities were normalized with respect to β-galactosidase activity (n = 3–6).

nine decreased significantly the stability of full-length VDR LBD and biological activity of the receptor with all studied ligands.

In VDR crystal structure, F279 is located near H7 and it forms a strong hydrophobic interaction with W286 (β-sheet), thus stabilizing the receptor. This important stabilizing interaction was also detected in our studies with full-length VDR, whereas the substitution F279A strongly decreased the stability of the receptor. In addition, Swamy et al. (2000) reported recently that substitutions W286A and W286F completely abolish binding of calcitriol. We suggest that this dramatic decrease in the ligand binding affinity and stability is attributable to a breakdown of the hydrophobic interaction between W286 and F279 that destabilizes the receptor structure.

H229 (helix 3) and Y295 (helix 7) do not interact directly with the ligand in the VDR crystal structure. However, these amino acid residues are bound together by a hydrogen bond connecting helix 3 and the β-sheet stabilizing the receptor structure. This hydrogen bonding seems to have an important structural role also in full-length VDR, because replacing any of these two amino acid residues with alanine leads to a labile receptor protein that is biologically inactive. Similarly, D232 (helix 3) has a stabilizing role in the truncated VDR LBD; it forms three hydrogen bonds with R154 in the coil between helices 1 and 2, connecting these areas strongly together. Indeed, the substitution D232A destabilized the full-length receptor structure and caused formation of a nonfunctional receptor protein.

Removal of the entire H12 (including the AF-2 domain) does not break down the structure of VDR. Instead, because helix 12 has been removed and the conformation c1LPD is no longer possible, the receptor is forced to adopt antagonistic and nonagonistic conformations even with strongly agonistic ligands (Fig. 4, Fig. 6). Interestingly, the substitutions H305A and H397A also clearly shift the conformation of full-length VDR to the nonagonistic direction (Fig. 4). With respect to VDR crystal structure, the structural area including H12 and the amino acid residues mentioned above is relatively small and surrounds the side chain of the ligand (Fig. 7B). This suggests that it is an important functional part of the receptor that discriminates between agonistic, antagonistic, and nonagonistic functions of the VDR (Bourguet et al., 1995; Renaud et al., 1995; Brzozowski et al., 1997; Shiau et al., 1998; Pike et al., 1999).

The analogs VD2708, MC1288, KH1060, and MC1598 seemed to stabilize the full-length VDR LBD against limited proteolytic digestion more often than the other ligands (Table 1). This suggests that these ligands were stabilized within the receptor better than the other ligands (e.g., by a larger number of interactions with the receptor). On the other hand, the analogs EB1089, VD2656, CB1260, HEP187, and CB1016 seemed to be less potent in this respect, suggesting that these ligands may not form as many interactions with the receptor as the ligands mentioned above. It is notable that of the weak analogs, all but EB1089 are 20-epi compounds. This suggests that even though both of the superagonists, MC1288 and KH1060, are 20-epi compounds, the 20-epi conformation does not necessarily make the analog a potent compound. The 20-epi structure obviously directs the ligand side chain to the direction that may be favorable for better interactions with the receptor. However, in addition, the high potency demands a flexible side chain, as MC1288 and KH1060 have. The double bonds in D-ring or double- and triple bonds in side chain as well as increase of the volume of the side chain may affect negatively to ligands possibilities to find the optimal position and the potency will be lost, as could be observed with analogs HEP187, CB1260, CB1016, and VD2656.

When the biological data obtained here and the crystal structure of the truncated VDR are compared, it can be concluded that the low biological activity of the R274A- and H397A-substituted VDRs results in decreased interactions between the ligands and the receptor. On the other hand, the

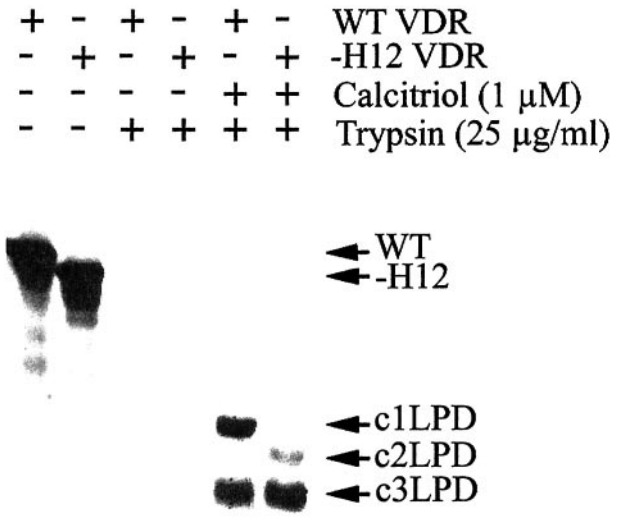

Fig. 6. Stability of the wild-type VDR and H12 VDR against limited proteolytic digestion with trypsin. L-[^{35}S]methionine-labeled VDRs were preincubated with 1 μM calcitriol at 22°C before exposing them to limited proteolytic digestion with trypsin (25 μg/ml). The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

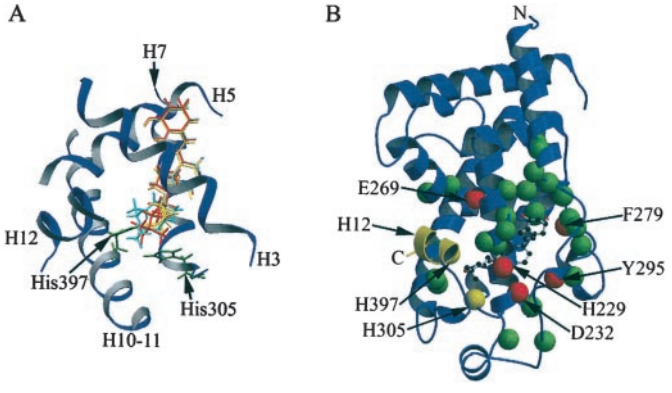

Fig. 7. Comparison of molecular models of selected ligands after energy minimization and location of point mutations in the VDR. A, modeled structures of MC903 (yellow) and HEP187 (light blue) within crystallized VDR LBD (Rochel et al., 2000). Calcitriol is in red. The helices lining the ligand binding pocket and amino acid residues that interact with 25-OH of calcitriol (green) are presented. B, location of the mutated amino acid residues within the VDR LBD. The coordinates of the truncated VDR LBD complexed with calcitriol (Protein Data Bank entry 1DB1) were used to position the mutated residues. The protein backbone of VDR LBD is shown in blue and the positions of the mutated amino acid residues are indicated with balls. The amino acid residues collectively important for the structurally different ligands are indicated in red. Helix 12 and the amino acid residues important for the agonistic ligand action are in yellow. The bound calcitriol is shown as a ball and stick model.

amino acid residues H229, D232, E269, F279, and Y295 are critical for the stable, agonistic conformation of the VDR. In addition, our results suggest that the contacts between the ligand and the receptor vary between structurally different ligands and that, in general, the potency of a given vitamin D_3 compound is related to the number of contacts.

As shown above, our biological data obtained with the full-length VDR can be explained well with the structural properties of the crystal structure of the truncated VDR LBD. This suggests that removal of the VDR loop between helices 2 and 3 does not markedly influence the functions studied here. However, the fact that this loop that makes VDR unique among the nuclear receptors has not disappeared during evolution suggests that functions connected to this loop still remain to be characterized.

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