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Mechanism of Action of Superactive Vitamin D Analogs Through Regulated Receptor Degradation

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Abstract We and others have previously shown that selected vitamin D analogs potentiate the vitamin D receptor (VDR) mediated transcription much more efficiently than the natural hormone itself. Here we show that the transcriptionally active 20-epi analogs, namely KH 1060 and MC 1288, protect VDR against degradation more efficiently than calcitriol at 10^{-10} M concentration (VDR $t_{1/2} > 48$ h, 17 h, and 10 h, respectively). The conformationally epi-like analog EB 1089 did not significantly alter the half-life of VDR (10.3 h), but retained the VDR levels over longer periods of time than calcitriol. The transcriptionally weak analog GS 1558, on the other hand, enhanced VDR degradation even more than what was observed with the unliganded receptor ($t_{1/2}$ 4.5 h and 5 h, respectively). Inhibition of proteasome activity by the inhibitor MG-132 resulted in a marked increase in the VDR levels in cells treated with the vehicle or GS 1558 (2.5-fold and 2.7-fold, respectively), more than twice the levels observed in the presence of calcitriol or EB 1089 (1.2-fold and 1.1-fold, respectively). MG-132 treatment did not increase the VDR levels in cells treated with KH 1060 or MC 1288. The electrophoretic mobility shift assay (EMSA) with nuclear extracts from MG-132-treated cells revealed formation of a high-molecular-weight RXRβ-VDR-VDRE complex, which also contained Sug1. In the presence of calcitriol, 34% of total VDR in its DNA binding state was present in this complex. The 20-epi analogs effectively prevented the formation of this complex, since, in this case, only 16% of total VDR was found in this complex. These results suggest that KH 1060 and MC 1288 induce a VDR conformation, which prevents binding of proteins mediating receptor degradation. As a result, the regulation of VDR degradation differs from that found with the calcitriol-VDR complex resulting in superactive transcriptional action of the analogs. J. Cell. Biochem. 76:548–558, 2000.

Key words: analog; calcitriol; degradation; proteasome; vitamin D receptor

1α,25-Dihydroxyvitamin D_3 (calcitriol), the biologically active form of vitamin D, regulates mineral homeostasis of the body by rapid stimulation of intestinal calcium and phosphate absorption and regulation of bone resorption via both genomic and nongenomic pathways [Norman et al., 1992]. Its genomic effects are mediated by the nuclear vitamin D receptor (VDR) [Baker et al., 1988] which, in its ligand binding state, promotes the formation of DNA-bound VDR-retinoid X receptor (RXR) heterodimers [Cheskis and Freedman, 1994]. The vitamin D response elements (VDREs) have been characterized from the promoter region of numerous genes and they can mediate either stimulation or repression of gene expression [Carlberg, 1995]. As suggested by crystal structures of the ligand binding domains of different steroid receptors, binding of the ligand induces conformational changes in this domain of the receptor [Wagner et al., 1995; Bourguet et al., 1995; Renaud et al., 1995; Parker and White, 1996; Wurtz et al., 1996]. These changes are thought to facilitate dimerization of VDR with RXR

Abbreviations used: calcitriol, 1α,25 dihydroxyvitamin D_3; GS 1558, 23-thia-aro-1α25-dihydroxyvitamin D_3; KH 1060, 20-epi-22-oxa-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; EB 1089, 1α,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3; MC 1288, 20-epi-1α,25-dihydroxyvitamin D_3; AF-2, activation function 2; DMEM, Dulbecco's modified Eagle's medium; DRIP, vitamin D receptor interacting proteins; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; RIP140, receptor interacting protein; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator-1; VDR, vitamin D receptor; VDRE, vitamin D response element.

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[Cheskis et al., 1995], to expose the activation function (AF-2) domain of the VDR for the binding of co-modulators [Masuyama et al., 1997], and to introduce a compact, protease resistant structure in the receptor [Wiese et al., 1992; Peleg et al., 1995; van den Bemd et al., 1996; Väisänen et al., 1997].

In spite of many advantageous properties of calcitriol on cellular proliferation and differentiation, its medical use is limited by the hypercalcemic effects of high doses needed to achieve a therapeutical value. As a consequence, numerous non-calcemic calcitriol analogs with high differentiation inducing and proliferation inhibiting properties have been recently developed [Bouillon et al., 1995]. With respect to transactivation, the most potent analogs developed thus far are those having a 20-epi orientation in their side chain [Binderup et al., 1991; Dilworth et al., 1994; Nayeri et al., 1996; Ryhänen et al., 1996, 1998]. The high transactivation potential of the 20-epi analogs may be due to reduced catabolism of the analogs [Dilworth et al., 1994] or to their increased affinity toward VDR [Dilworth et al., 1994; Nayeri et al., 1996]. The latter is not, however, a universally accepted feature for the 20-epi analogs, since in several studies essentially no differences in the binding affinities of calcitriol and the 20-epi analogs toward VDR have been found [Binderup et al., 1991; van den Bemd et al., 1995]. The 20-epi analog-VDR complexes are clearly more resistant against partial protease digestion than the calcitriol-VDR complexes suggesting that in vitro the conformational change induced by the 20-epi analogs is different from that induced by calcitriol [Peleg et al., 1995; Liu et al., 1997; Väisänen et al., 1997, 1999].

The difference in the conformation of VDR may affect the binding of co-modulators to the surface of the receptor and ultimately result in enhanced transcription. Recently, the 20-epi analogs were shown to facilitate the binding of VDR interacting proteins (DRIPs) to the receptor and induce VDR-mediated transactivation more efficiently than calcitriol [Yang and Freedman, 1999]. Further, steroid receptor coactivator-1 (SRC-1) and receptor interacting protein (RIP140) both bind to the AF-2 domain of VDR and augment VDR-mediated transactivation [Masuyama et al., 1997]. This domain is also responsible for the interaction of VDR with Sug1, the mediator of proteasomal degradation of VDR [vom Baur et al., 1996; Masuyama et al., 1997; Masuyama and MacDonald, 1998].

The 26S proteasome is a large multiprotein complex, which degrades short-lived proteins related to metabolic regulation and cell-cycle progression. It is formed from the catalytic 20S core, which is activated by binding of two 19S regulatory complexes [Pickart, 1997; Tanaka, 1998]. Mammalian 19S regulatory complex consists of 14 non-ATPase subunits and at least six ATPase subunits, one of which is Sug1 (p45) [Tanaka, 1998]. Of the steroid hormone receptors, VDR [Masuyama and MacDonald, 1998], estrogen receptor (ER) [Nawaz et al., 1999], and progesterone receptor (PR) [Syvälä et al., 1998] have been found to be degraded by the proteasome. For ER and PR, ubiquitination of the receptor has been reported, but proteins can also be degraded in the proteasome without ubiquitination [Murakami et al., 1992]. It has been postulated that VDR is directed to the proteasome by Sug1 [Masuyama and MacDonald, 1998] but no evidence indicating VDR ubiquitination has been published.

We studied the ability of calcitriol and its synthetic analogs GS 1558, KH 1060, EB 1089, and MC 1288 to protect the VDR against degradation in a human osteoblast-like MG-63 osteosarcoma cell line. We have previously shown that GS 1558 has a weaker transactivation potential than calcitriol [Väisänen et al., 1999], whereas the 20-epi analogs KH 1060 [Ryhänen et al., 1996] and MC 1288 [Ryhänen et al., 1996; Väisänen et al., 1999] as well as the conformationally 20-epi-like analog EB 1089 [Mahonen et al., 1996; Väisänen et al., 1999] are superactive in VDR-mediated transcription. In this study, we show that these analogs increase the half-life of the receptor and protect the receptor against degradation over longer periods of time than calcitriol. We also show evidence indicating that this increase may result from conformational differences of the 20-epi analog-VDR complexes and differential binding of proteins participating in the degradation of VDR.

MATERIALS AND METHODS

Vitamin D_3 Compounds and Antibodies

The C-terminal antibody for VDR was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The VDR antibodies directed against the DNA-binding domain (VD2F12) [Dame et al., 1986] and against the N-terminal sequence (αhVDR-p103) [Tuohimaa et al., 1992]

have been previously described. The antibodies for RXRβ (recognizing both β1 and β2 forms), RXRβ1, and RXRβ2 were purchased from Santa Cruz Biotechnology, Inc. The Sug1 antibody 2SU-1B8 was a kind gift from Dr. Pierre Chambon. The secondary antibody used in Western immunoblotting was alkaline phosphatase conjugate of anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO).

Calcitriol and its synthetic analogs 23-thia-aro-1α,25-dihydroxyvitamin D_3 (GS 1558), 20-epi-22-oxa-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3 (KH 1060), 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3 (EB 1089), and 20-epi-1α,25-dihydroxyvitamin D_3 (MC 1288) were a kind gift from Dr. Lise Binderup, Leo Pharmaceuticals, Ltd, Denmark. Figure 1 shows the structures of calcitriol and the side-chain modifications of the analogs.

Cell Culture and Nuclear Extracts

Human MG-63 osteoblastic sarcoma cells and monkey CV-1 kidney cells (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin at 37°C in a humidified (95% air:5% CO_2) incubator. The medium was changed to DMEM + 2% FCS for 24 h before starting the treatments. Cells were harvested at the indicated time points and nuclear extracts were prepared as previously described [Hurst et al., 1990] without the heat denaturation step. Protein concentration was measured by Bio-Rad protein Assay Dye (Bio-Rad Laboratories, Hercules, CA). Nuclear extracts were aliquoted and stored at −70°C.

Western Immunoblotting

Nuclear extracts (20 μg) were boiled for 5 min in SDS-sample buffer. The samples were loaded onto a 4%/12% SDS-polyacrylamide gel and run for 2–3 h at a constant voltage (280 V). The proteins were electrotransferred onto PVDF-membranes. The membranes were blocked with TBS-Tween + 2% milk powder for 30 min at room temperature. The primary antibody was added in TBS-Tween + 2% milk powder and the reaction was carried out at +4°C overnight. The filters were washed five times with TBS-Tween and the secondary antibody was added for the next 1 h at room temperature. After washing the filters, the protein pattern was developed with nitro blue tetrazolium and bromo-chloro-indolyl-phosphate (Sigma-Aldrich).

Electrophoretic Mobility Shift Assay

Nuclear proteins (10 μg) were incubated on ice for 10 min with 2 μg poly(dI-dC) in 20 mM Hepes, pH 7.6, 4.2% glycerol (by vol), 70 mM NaCl, 2.3 mM $MgCl_2$, 2.0 mM EDTA, 2.2 mM dithiothreitol, 80 μM phenylmethylsulphonyl fluoride, 0.3 μg/ml trasylol, and 0.3 μg/ml leupeptin. An end-labelled human osteocalcin gene VDRE (ACCGGGTGAACGGGGGCA, upper strand) was added and the incubation was continued at room temperature for 20 min. Protein-bound DNA was separated from the free probe on a 7% polyacrylamide gel run in 0.25 × TBE (1 × TBE: 0.1 M Tris/borate, pH 8.3, 2 mM EDTA) and the gels were dried and visualized by autoradiography. In the antibody experiments, protein extracts were incubated at +4°C for 90 min with antibodies before addition of carrier-DNA and the labelled probe.

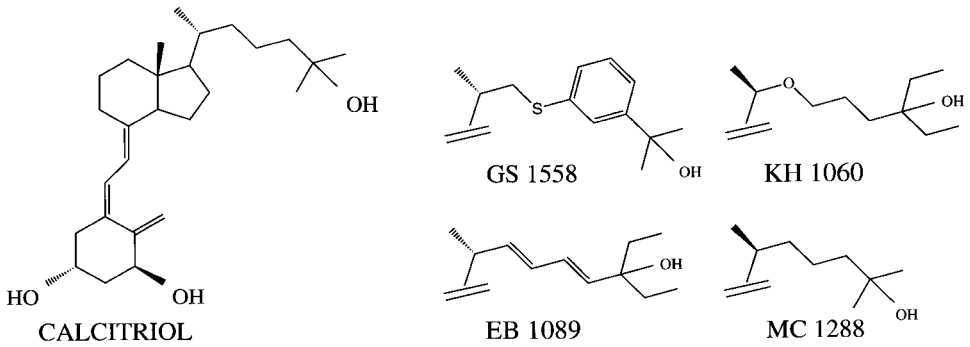


Fig. 1. Structures of calcitriol and the side-chain modifications of the analogs GS 1558, KH 1060, EB 1089, and MC 1288.

Statistical Analysis

Statistical analysis was performed by Student's *t*-test for two independent variables using a software package (Stat-Works™, Cricket Software Inc., PA).

RESULTS

Effects of Calcitriol and the Analogs on VDR Protein Levels

During the 24-h incubation, calcitriol upregulated VDR protein levels 2.5-fold compared with the vehicle treatment (Fig. 2A). The analogs KH 1060, EB 1089, and MC 1288 were even more potent in this respect inducing VDR levels, which were 1.9-fold, 1.3-fold, and 1.6-fold higher than those observed with calcitriol, respectively, whereas the GS 1558-induced levels were lower (−47%) than the calcitriol-induced levels (Fig. 2B).

Calcitriol and the Analogs KH 1060, EB 1089, and MC 1288 Protect VDR Against Degradation

The effects of calcitriol and the analogs on VDR degradation were studied in cycloheximide-treated MG-63 cells for 48 h (Fig. 3A). Without the ligand, 20% of the initial VDR level was detected at 31 h and 10% at 48 h. The analogs KH 1060, EB 1089, and MC 1288 protected VDR against proteolysis better than calcitriol throughout the 48-h experiment, whereas GS 1558 caused a more rapid degradation than calcitriol. After the 48-h incubation, the VDR levels from calcitriol-treated cells decreased almost to the control level, whereas the levels from cells treated with KH 1060, EB 1089, and MC 1288 stayed elevated (67%, 40%, and 38% from the initial level, respectively; Fig. 3A,B). We also performed these experiments with 10^{-8} M concentration of the vitamin D compounds and found that the differences observed with the 10^{-10} M concentration were then lost (data not shown).

Calcitriol doubled the VDR half-life from 5 h (vehicle treatment) to 10 h (Fig. 3C). In the presence of the analog EB 1089, the VDR half-life was also about 10 h. On the other hand, treatment with MC 1288 caused a statistically significant increase in the VDR half-life ($t_{1/2}$ = 17 h) and, in KH 1060-treated cells, the VDR levels did not decline to 50% level at all during the 48-h treatment. In the presence of the transactivationally weak analog GS 1558, the VDR half-life was only 4.5 h.

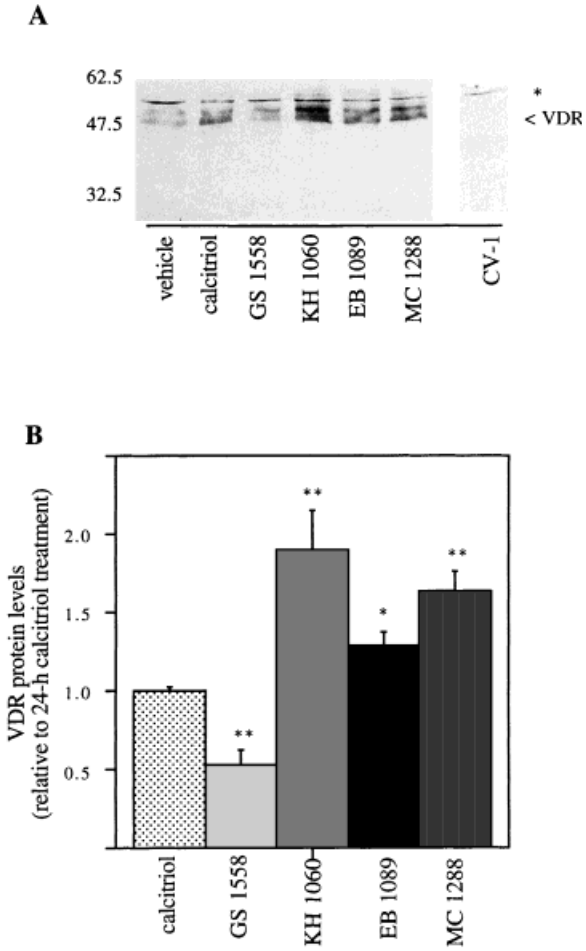
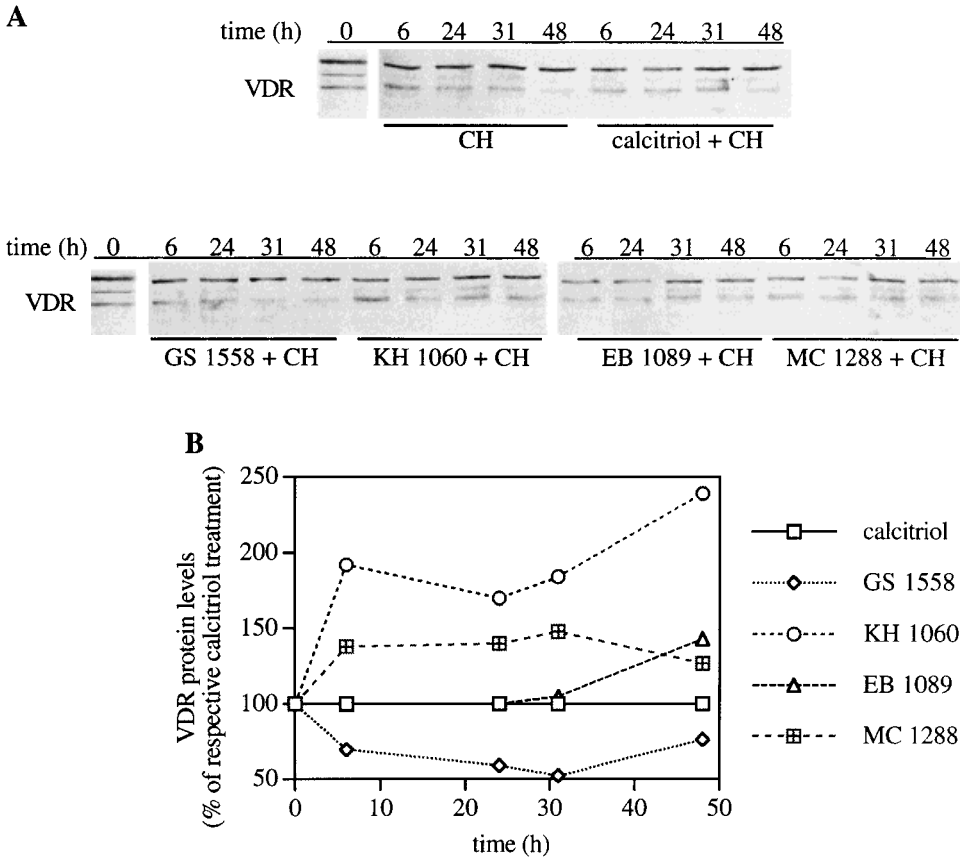


Fig. 2. VDR protein levels stimulated by calcitriol or its synthetic analogs GS 1558, KH 1060, EB 1089, and MC 1288. MG-63 cells were treated with a 10^{-10} M concentration of the vitamin D compounds for 24 h. Nuclear proteins were prepared as described in Materials and Methods and 20 μg samples of the extracts were resolved on 12% SDS-PAGE. **A:** Western immunoblot of MG-63 and CV-1 cell nuclear extracts using anti-VDR antibody raised against the C-terminus of the VDR. The asterisk denotes an unrelated band also present in CV-1 nuclear extracts deficient of VDR. Migration of molecular weight markers is indicated on the left. **B:** VDR levels from three (KH 1060, EB 1089, and MC 1288) or four (GS 1558) Western immunoblots are presented in relation to the VDR level obtained with calcitriol treatment. The results represent means ± SEM. Asterisks denote the statistical significance for the differences between calcitriol vs. analog treatment: $*P \leq 0.05$, $**P \leq 0.01$.

VDR Protein Levels After Inhibition of Proteasome Activity by MG-132

We used MG-132 to inhibit the proteasomal degradation of VDR and evaluated the VDR levels by Western immunoblotting (Fig. 4). Without MG-132, calcitriol and especially the analogs KH 1060, EB 1089, and MC 1288 clearly increased VDR levels already in 3 h (Fig. 4A).



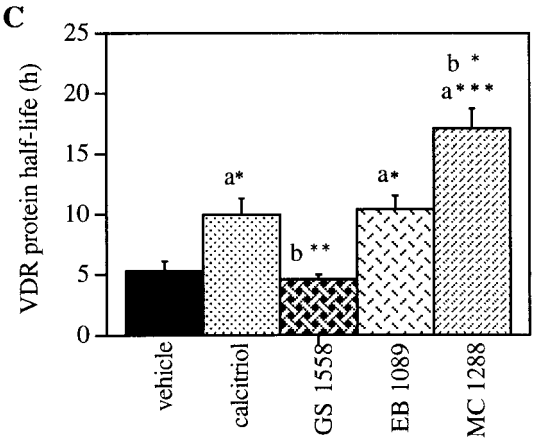


Fig. 3. Time-course of VDR degradation in the presence of calcitriol or the analogs GS 1558, KH 1060, EB 1089, and MC 1288. **A:** MG-63 cells were treated with the protein synthesis inhibitor cycloheximide (CH, 20 µg/ml medium) alone or together with calcitriol or the analogs (10^{-10} M) for the indicated periods of time. Nuclear extracts and Western immunoblotting were performed as described in Materials and Methods. **B:** VDR bands from Western immunoblots shown in A were scanned and the VDR protein levels from the analog-treated MG-63 cells are shown in relation to the VDR levels obtained with calcitriol-treated cells. **C:** Half-life of unliganded VDR or VDR in the presence of calcitriol, GS 1558, KH 1060, EB 1089, or MC 1288. MG-63 cells were treated as described in A. Nuclear proteins were prepared and the VDR protein levels were determined as described in Materials and Methods. The results represent means ± SEM of three to five experiments. Statistical significance for the difference between vehicle vs. ligand treatment (a) and between calcitriol vs. analog treatment (b). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. In the KH 1060-treated cells, the VDR protein did not decrease to 50% level during the 48 h incubation ($t_{1/2} > 48$ h).

Treatment with MG-132 had the greatest influence on VDR levels in cells treated with the vehicle or GS 1558, where 2.5-fold and 2.7-fold increases, respectively, were observed in relation to treatment without MG-132. In cells treated with calcitriol or EB 1089, MG-132 upregulated VDR levels 1.2-fold and 1.1-fold, respectively. In KH 1060-treated cells, MG-132 had no effect on VDR levels. Interestingly, in MC 1288-treated cells, MG-132 treatment reduced VDR levels so that only 80% of VDR obtained without MG-132 was left (Fig. 4B).

Effects of MG-132 on VDR-VDRE Interactions

We used MG-132 to inhibit the proteasome activity in MG-63 human osteosarcoma cells and used nuclear proteins in electrophoretic mobility shift assays (EMSA) to detect VDR complexes bound to human osteocalcin gene VDRE (Figs. 5 and 6). Three complexes desig-

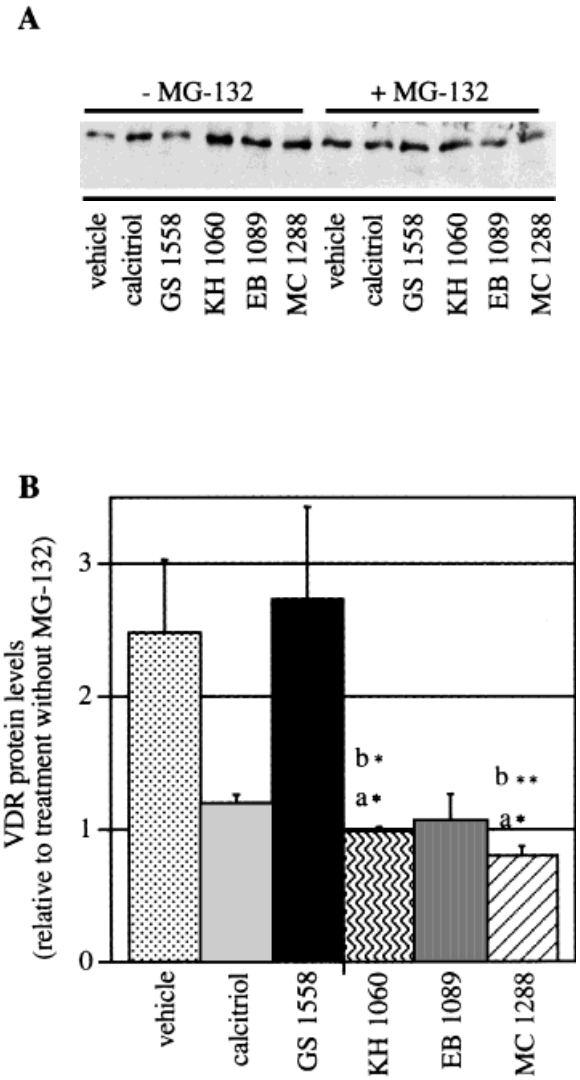


Fig. 4. Effects of the proteasome inhibitor MG-132 on VDR levels in cells treated with calcitriol or the analogs. **A:** MG-63 cells were treated with calcitriol, GS 1558, KH 1060, EB 1089, or MC 1288 (10^{-10} M) alone or in the presence of 40 μM MG-132 for 3 h. Nuclear extracts were prepared and Western immunoblots were performed. **B:** VDR protein levels from MG-132 plus calcitriol or analog-treated MG-63 cells were compared with the respective treatments without MG-132. The results represent means ± SEM of three experiments. Statistical significance for the differences between vehicle vs. ligand treatment (a) and between calcitriol vs. analog treatment (b). $*P \leq 0.05$, $**P \leq 0.01$.

nated as B2, B3, and B4 were detected with nuclear extracts from vehicle-treated MG-63 cells and the end-labelled osteocalcin gene VDRE (Fig. 5A). VDR antibodies directed against the C-terminus, the DNA-binding domain, and the N-terminus of the receptor all recognized the complexes, whereas the Sug1 antibody recognized complexes B2 and B4 but not complex B3 (Fig. 5A, left panel, Table I). The RXRβ antibody, which identifies both β1 and β2 receptor forms, recognized all these complexes, whereas the RXRβ1 antibody did not recognize complex B4 (Fig. 5B, right panel, Table I). Treatment of cells with MG-132 resulted in the formation of a new, slowly migrating complex B1, which was recognized by the VDR antibodies, the Sug1 antibody, and the RXRβ1 antibody (Figs. 5B and 6A, Table I). RXRβ2 was not found in complexes B1, B2, or B3. We conclude that VDR present in complex B1 represents, from the total VDR concentration, the portion which will be directed to degradation by the proteasome.

We also evaluated the percentage of VDR in complexes B1, B2, and B3 in MG-132-treated cells (Fig. 6B,C). In the presence of calcitriol, 34% of VDR was in complex B1. The respective portions in cells treated with GS 1558 or EB 1089 were 28% and 25%. When the analogs KH 1060 and MC 1288 were present, only 16% of VDR was in this complex formed by inhibition of proteasomal degradation, and when evaluated in relation to calcitriol treatment, the difference is statistically significant (Fig. 6C).

DISCUSSION

The hyperactive transcriptional actions of a number of new calcitriol analogs may be explained by properties of the analogs themselves such as their increased affinity toward VDR [Nayeri et al., 1996], their facilitated uptake by cells, their reduced catabolism, or metabolism possibly leading to physiologically active metabolites [Dilworth et al., 1997]. On the other hand, the analogs, by binding to VDR, induce different conformations in the receptor [Peleg et al., 1995; van den Bemd et al., 1996; Väisänen et al., 1997, 1999] and these conformations may have consequences on the subsequent events of transactivation. The conformation of the ligand-receptor complex affects the binding to RXR and VDREs [Cheskis et al., 1995] and, further, to other DRIPs through the differentially exposed AF-2 domain of the receptor [Rachez et al., 1999; Yang and Freedman, 1999]. Also Sug1, which mediates the proteasomal degradation of VDR, binds to this domain of the receptor [Masuyama et al., 1997; Masuyama and MacDonald, 1998].

In this study, we show that the transcriptionally superactive 20-epi analogs KH 1060 and MC 1288 protect VDR against degradation more

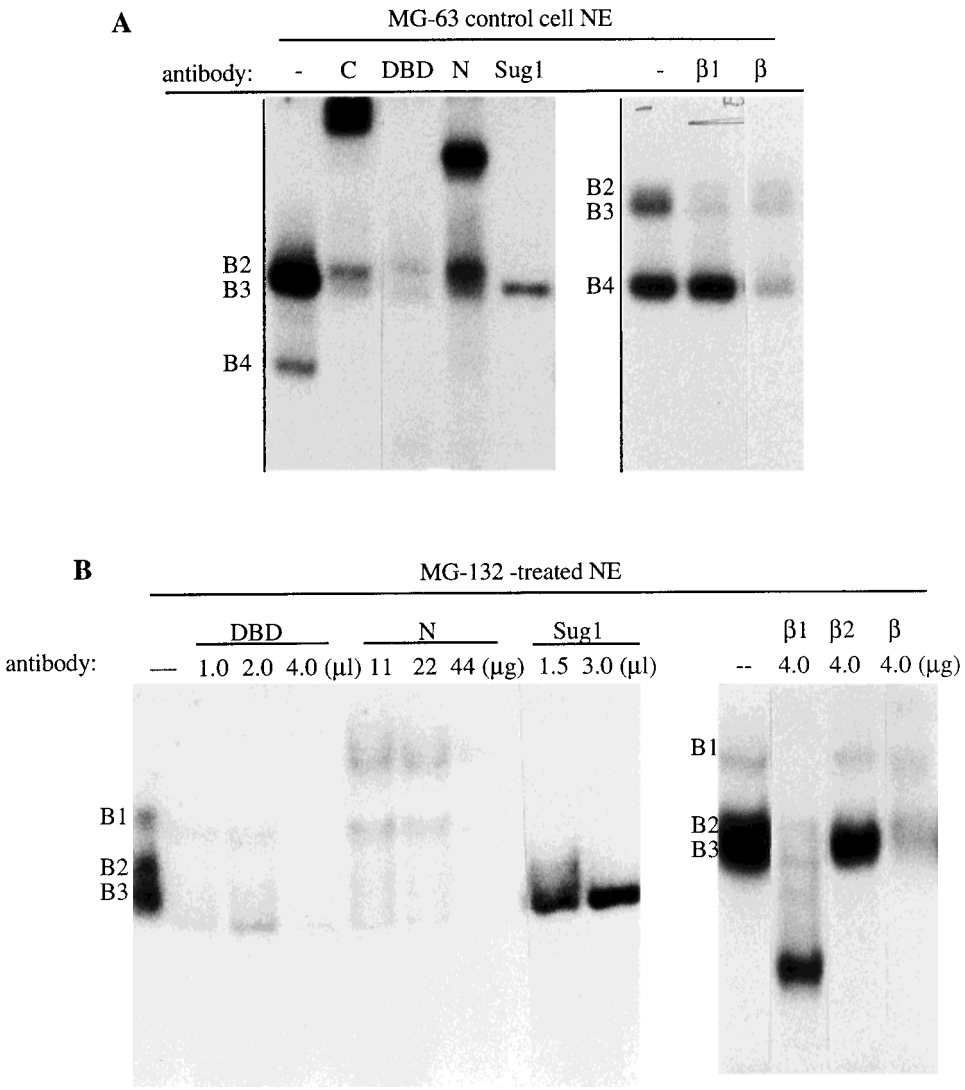
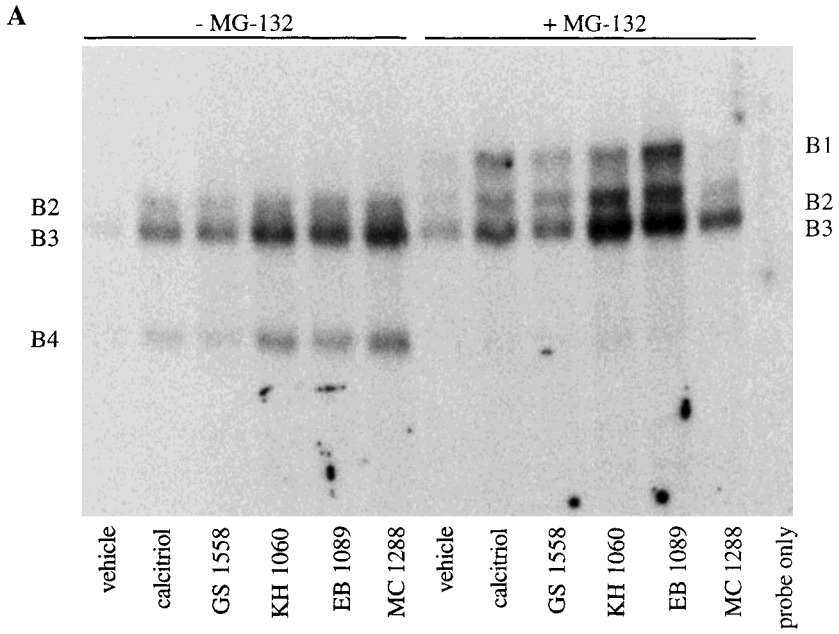


Fig. 5. Identification by antibodies of the EMSA complexes formed with the osteocalcin gene VDRE and control MG-63 cell nuclear extract (**A**) or MG-63 cells treated with the proteasome inhibitor MG-132 (**B**). MG-63 cells were treated with the vehicle or 40 µM MG-132 for 3 h. Nuclear proteins (10 µg) were incubated together with the different antibodies for 90 min at +4°C. Carrier-DNA and the labelled osteocalcin gene VDRE were added and EMSA was performed as indicated in Materials and Methods. C, DBD, and N denote VDR antibodies directed against the C-terminal domain (4 µg), the DNA-binding domain (2 µl mouse ascites), and the N-terminal domain (22 µg IgG), respectively. Sug1: Sug1 antibody reaction, where 3 µl of 1:1 diluted ascites fluid was used. β1 and β: RXRβ1 and RXRβ antibodies (4 µg each), respectively. B1, B2, B3, and B4 denote the different complexes formed. Only the complexes are shown.

efficiently than calcitriol in human MG-63 cells. The half-life of VDR increased from 5 h (vehicle-treated cells) to 10 h found in the presence of 10^{-10} M calcitriol. In rat osteoblastic sarcoma cells, the unliganded VDR is degraded faster than in MG-63 cells with a half-life of 1.5 h [van den Bemd et al., 1996], or less than 4 h [Masuyama and MacDonald, 1998] and 10^{-8} M calcitriol clearly increases the half-life to 15 h and 8 h, respectively. Also in mouse fibroblasts calcitriol doubles the VDR half-life from 4 h to 8 h [Wiese et al., 1992].

In the presence of EB 1089, the half-life of VDR did not differ from that obtained with calcitriol treatment but, at the 48-h time point, 40% of the initial VDR level was left, whereas in the presence of calcitriol, the corresponding number was 13%. The most dramatic effect was obtained with KH 1060. In its presence the VDR protein levels did not decline to 50% level at all during the 48-h incubation and, at 24 h, 84% of the initial VDR level was still present. This result is in agreement with the previous result of van den Bemd et al. [1996], who fol-



B

Percentage of VDR present in complexes B1, B2, and B3 in MG-132 -treated MG-63 cells in the presence of calcitriol or different analogs.

	vehicle	calcitriol	GS 1558	KH 1060	EB 1089	MC 1288
B1	25.9±6.5	33.9±8.0	27.9±4.4	16.1±2.9	25.3±3.4	16.4±4.5
B2	26.8±2.3	26.8±3.6	28.3±2.9	33.6±2.3	29.1±3.7	27.6±1.5
B3	47.4±4.2	39.3±4.8	43.7±2.6	50.3±3.4	45.5±4.3	56.0±4.2

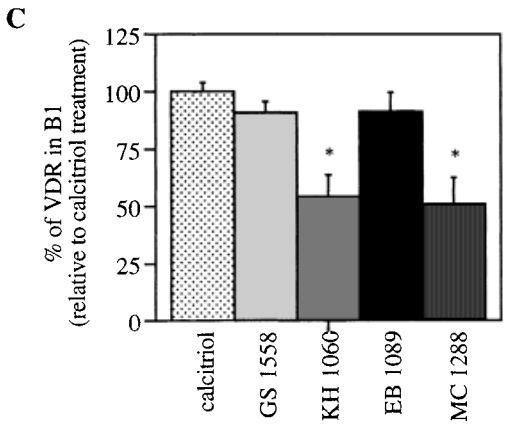


Fig. 6. Effect of the proteasome inhibitor MG-132 on VDR-VDRE complex formation. MG-63 cells were treated with the vehicle, calcitriol, GS 1558, KH 1060, EB 1089, or MC 1288 (10^{-10} M) alone or in the presence of 40 μM MG-132 for 3 h. Nuclear proteins (10 μg) were allowed to react with the end-labelled osteocalcin gene VDRE and EMSA was performed as described in Materials and Methods. **A:** EMSA complexes B1, B2, B3, and B4. The last lane represents the reaction without nuclear proteins. Only the complexes are shown. **B:** The table represents the percentage of VDR in the complexes B1, B2, and B3. The results represent means ± SEM of three experiments. **C:** Percentage of VDR in complex B1 in analog-treated cells shown in relation to the respective percentage in the calcitriol-treated cells. The results represent the means ± SEM from three different experiments. Statistical significance for the difference between MG-132 plus calcitriol and MG-132 plus analog treatment: $*P \leq 0.05$.

lowed VDR degradation in ROS 17/2.8 cells for 24 h and found that, at the 24-h time point, 80% of the initial VDR was left. Another analog, which effectively protected VDR against proteolysis, was MC 1288, which increased the VDR half-life to 17 h. Interestingly, the analog GS 1558 did not protect VDR at all but rather promoted its degradation even when compared with the unliganded receptor ($t_{1/2}$ 4.5 h and 5 h, respectively). This may explain our previous results that this analog is transcriptionally very weak [Väisänen et al., 1999]. Also, its binding affinity towards VDR is 30% less than that of calcitriol and the conformation induced in VDR is clearly different from that induced by calcitriol, as shown by partial trypsin digestion.

TABLE I. Identification by Antibodies of Complexes Formed on the Human Osteocalcin Gene VDRE[a]

	C	DBD	N	Sug1	β1	β2	β
B1	nd	+	+	+	+	−	?
B2	+	+	+	+	+	−	+
B3	+	+	+	−	+	−	+
B4	+	+	+	+	−	nd	+

[a]The cells were treated with the vehicle or 40 μM MG-132 for 3 h. C, DBD, and N denote VDR antibodies directed against the C-terminal domain, the DNA-binding domain, and the N-terminal domain, respectively. β1, β2, and β: RXRβ antibodies recognizing only the β1 form, only the β2 form, and both β1 and β2 forms, respectively. ? denotes poor recognition of B1 by the RXRβ antibody. nd, not determined.

The results from the MG-132-treated cells correlated well with the results from cycloheximide treatments. The effects of MG-132 on the VDR levels in cells treated with calcitriol and EB 1089 were equal and minimal when compared with the effects in cells treated with the vehicle or GS 1558. The incubation time with MG-132 was only 3 h and, during that time, the degradation of VDR in the presence of calcitriol or EB 1089, as shown by the cycloheximide treatment in Figure 3, was only minimal. On the other hand, the unliganded receptor and the GS 1558-VDR complex were rapidly degraded and, already at 3 h, the effect of MG-132 could be detected. In the presence of KH 1060, the MG-132 treatment had no effect on VDR levels. This may have resulted from the lack of VDR degradation in the KH 1060-treated cells, even without MG-132, during the 3-h incubation, as suggested by the cycloheximide treatment (Fig. 3). According to these results, it is also possible that the KH 1060-VDR complex is not degraded by the proteasome, but by some other mechanism. Different degradation mechanisms have also been suggested for the ER in the presence of estradiol or the antiestrogen RU 58668 [El Khissiin and Leclercq, 1999]. The most interesting finding from the MG-132 treatment was that it decreased the VDR levels in the MC 1288-treated cells (20% decrease in 3 h). We and others have previously speculated that MC 1288 may have entirely different binding requirements in VDR than calcitriol [Liu et al., 1997; Väisänen et al., 1998]. This may lead to a markedly different 3D-structure of the receptor-ligand complex possibly revealing new surface domains of the receptor for binding of co-modulators.

These speculations are further supported by the present EMSA results. The existence of VDR in three different complexes (B1, B2, and B3), one of which (B1) is formed as a result of the MG-132 treatment, gave us a possibility to determine the portion of VDR in the complex to be degraded by the proteasome. In the calcitriol-treated cells, 34% of total VDR in its DNA binding state was marked for degradation and the respective portions in GS 1558 and EB 1089-treated cells were somewhat less (28%

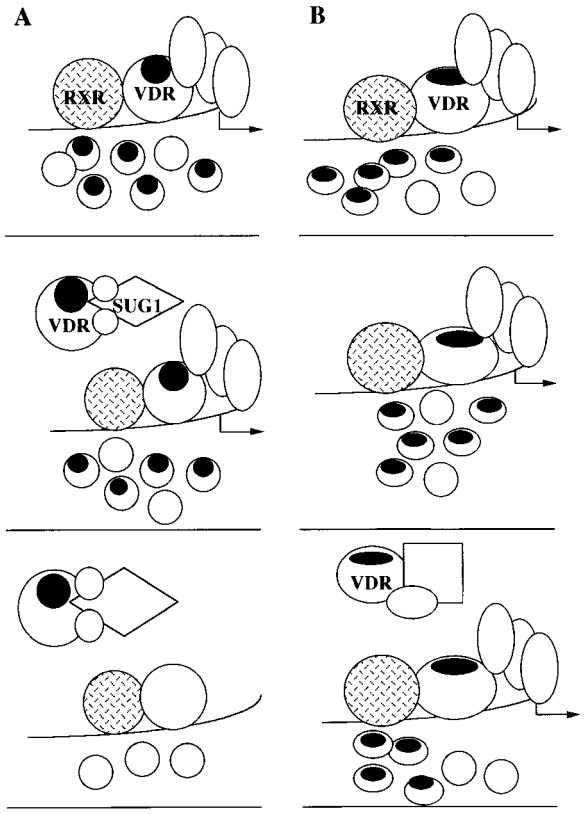


Fig. 7. The proposed mechanism of the superactive action of the calcitriol analogs. In its transcriptionally active form, VDR is heterodimerized with RXR and binds to the VDRE in the presence of either calcitriol (**A**) or the active analog (**B**). In the presence of calcitriol, VDR reacts with Sug1 and possibly other proteins needed for VDR degradation, is detached from the VDRE, and the ligand is metabolized. A new calcitriol-VDR complex binds to the VDRE and is eventually degraded. Finally, all calcitriol-VDR complexes are degraded, only unliganded VDR molecules are available, and transcription is turned off (A, bottom). The analog-VDR complex poorly interacts with Sug1 and possibly other proteins which deliver the ligand-VDR complexes from the VDRE to degradation (B, bottom). This occurs at a slower rate than the degradation of the calcitriol-VDR complexes. At low ligand concentration transcription in the calcitriol-treated cells is eventually turned off, whereas those cells treated with the superactive analogs still retain their ability to transactivate.

and 25%). The presence of KH 1060 and MC 1288 significantly altered the portions of VDR in the different complexes, and only 16% of the receptor was in complex B1.

In this paper, we show that the 20-epi analogs of calcitriol, KH 1060, and MC 1288, induce a VDR conformation, which apparently prevents the binding of proteins mediating the degradation of the receptor. This results in a longer-lasting existence of VDR active in transcription and may, in part, explain the high transactivational efficacy of these analogs (Fig. 7). These results also suggest that the conformationally 20-epi-like analog EB 1089 apparently protects the VDR like calcitriol but over longer periods of time.

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