2-METHYLENE-19-NOR-(20S)-1,25-DIHYDROXYVITAMIN D_3 POTENTLY STIMULATES GEN.

SPECIFIC DNA BINDING OF THE VITAMIN D RECEPTOR IN OSTEOBLASTS

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1 The abbreviations used are: 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; 2MD, 2-methylene-19-nor-(20S)-1,25-dihydroxyvitamin D3; DBP, vitamin D binding protein; VDR, vitamin D receptor; VDRE, vitamin D response element; ChIP, chromatin immunoprecipitation; RANKL, receptor activator of NF-κB ligand; RXR, retinoid X receptor; OPG, osteoprotegerin; Cyp24, 25 hydroxyvitamin D3-24 hydroxylase; OC, osteocalcin; OPN, osteopontin; HAT, histone acetyltransferase; SERM, selective estrogen receptor modulator; TRAP, tartrate-resistant acid phosphatase; BMD, bone mineral density; LBD, ligand-binding domain; ER, estrogen receptor;
ABSTRACT

2-Methylene-19-nor-(20S)-1,25-dihydroxyvitamin D₃ (2MD) is a highly potent analog of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) whose actions are mediated through the vitamin D receptor (VDR). In this report, we have replicated this increased potency of 2MD in vitro using osteoblastic cells, and explored its underlying molecular mechanism. 2MD stimulates the expression of several vitamin D-sensitive genes including 25-hydroxyvitamin D₃-24 hydroxylase (Cyp24), osteopontin and receptor activator of NFκB ligand and suppresses osteoprotegerin at concentrations two logs lower than that for 1,25(OH)₂D₃. 2MD is also more potent in stimulating transfected chimeric reporter genes under either Cyp24 or the osteocalcin promoter control. Enhanced potency is retained regardless of medium serum content. Interestingly, the uptake of both 1,25(OH)₂D₃ and 2MD into cells is similar, as is their rapid association with the VDR. This indicates that comparable levels of occupied VDR do not elicit equivalent levels of transactivation. Using chromatin immunoprecipitation (ChIP), however, we observed a strong correlation between DNA-bound receptor and the level of induced transcription suggesting a 2MD-induced increase in affinity of the VDR for DNA. Additional studies using a mammalian two-hybrid system and ChIP indicate that 2MD is also more potent in promoting interaction with RXR and the coactivators SRC-1 and DRIP205. Finally, protease digestion studies revealed a unique VDR conformation in the presence of 2MD. These studies suggest that the molecular mechanism of 2MD potency is due to its ability to promote enhanced levels of specific DNA binding by the VDR and
could suggest possible explanations for the tissue- and gene-selective actions of 2MD.

INTRODUCTION

The physiological actions of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) are mediated by the vitamin D receptor (VDR) (1). This predominantly nuclear protein is a member of the steroid receptor gene family of transcription factors and functions like other nuclear receptors to regulate the expression of genes involved in a wide variety of cellular activities. The major role of 1,25(OH)$_2$D$_3$ is to maintain calcium and phosphorus homeostasis through its direct actions on gene expression in the intestine, kidney and bone (2). This hormone also regulates proliferation, differentiation, activity and survival of a number of cell types, many of which are not involved in mineral homeostasis (3). The biological relevance of these latter actions remains to be determined, however, given the largely skeletal phenoptype associated with null mutations of the VDR gene in both humans and in mice (4,5). Ligand association with the VDR leads to rapid activation and subsequent accumulation of the receptor on vitamin D response elements (VDREs) located within the promoter regions of specific genes (1). VDRE binding is facilitated by the retinoid X receptor (RXR) which functions both as a heterodimeric DNA binding partner as well as a participant in transactivation (6). The role of RXR in VDR-mediated gene repression is unclear and appears to be somewhat controversial (7). Regardless, these initial molecular actions of 1,25(OH)$_2$D$_3$ and its receptor are essential to the subsequent alteration in the expression of genes
involved in a variety of highly pleiotropic cellular responses.

A number of steroid hormone analogs, including those for 1,25(OH)₂D₃ have been synthesized and characterized (8). With respect to vitamin D₃ analogs, many of these are highly potent activators of gene transcription despite the fact that their affinities for VDR are not different from that of the native hormone. While enhanced potency can arise in vivo as a result of an analog’s pharmacokinetic properties, recent studies in vitro have suggested that at least for some analogs increased potency may be due to their ability to induce unique VDR conformations that favor enhanced interaction with either RXR or coactivators such as SRC-1 or DRIP205 (9-11). Interestingly, while altered receptor conformations are supported through proteolytic digestion studies in vitro, they are not supported by three-dimensional structural analyses (12,13). Other 1,25(OH)₂D₃ analogs display disparate actions, exerting highly potent effects on cellular proliferation and differentiation or to suppress parathyroid hormone gene expression, yet demonstrating a restricted ability to induce calcium mobilization from bone or calcium uptake from the gut (14,15). Since increased serum calcium promotes soft tissue degeneration and other equally detrimental effects, compounds with a low hypercalcemic potential are highly desirable therapeutically. The underlying mechanism(s) for this selectivity is not known, but is likely to be complex given the diverse molecular mechanisms that drive bone resorption, intestinal absorption, and renal reabsorption of calcium.

2-Methylene-19-nor-(20S)-1,25-dihydroxyvitamin D₃ (2MD) is a highly
potent $1,25(OH)_2D_3$ analog that exhibits tissue-selective as well as gene-selective actions \textit{in vitro} and \textit{in vivo} (16,17). This compound binds to the VDR with a $K_d$ that is similar to that for $1,25(OH)_2D_3$, although its interaction with serum vitamin D binding protein (DBP) is exceedingly weak (16 and DeLuca, H.F., unpublished). \textit{In vivo} studies suggest that this analog appears to localize selectively to bone relative to intestine (16).

2MD strongly induces calcium resorption from bone in vivo, an activity that is likely due to its ability to induce receptor activator of NF-$\kappa$B ligand (RANKL) from supportive stromal cells and osteoblasts. RANKL is a key regulator of osteoclast formation, activity and survival (18,19), and is potently induced by both $1,25(OH)_2D_3$ and 2MD in cultured cells (17). Interestingly, treatment of ovariectomized adult rats with low doses of 2MD leads to a dramatic increase in bone mineral density (BMD) in aged, ovariectomized rats (17). While the mechanism of this dramatic anabolic activity of 2MD is not yet understood, \textit{in vitro} studies suggest that 2MD exhibits an exceptionally potent and unique ability to enhance VDR-mediated osteoblast activity that leads to increased mineralization (17). In this manuscript, we explore the underlying molecular basis for the increased potency of 2MD using osteoblast cell models. We find that $1,25(OH)_2D_3$ and 2MD bind equivalently to the VDR under serum-free conditions. Despite this, localization of the receptor to VDREs lying adjacent to $1,25(OH)_2D_3$-inducible promoters in intact cells occurs at concentrations of 2MD that are much lower than that for $1,25(OH)_2D_3$. VDR DNA-binding correlates directly with the ability of the two ligands to activate transcription. A 2MD-mediated increase in VDR DNA affinity may
result from enhanced VDR interaction with RXR and with coactivators such as SRC-1 and DRIP205, both of which could enhance the stability of the VDR on DNA.

MATERIALS AND METHODS

Reagents- 1,25(OH)$_2$D$_3$ was obtained from Solvay (da Weesp, The Netherlands). 2MD was provided by Deltanoid Pharmaceuticals, Inc. (Madison, WI). Tritiated 1,25(OH)$_2$D$_3$ (166 Ci/mmol), L-[$^{35}$S]-methionine (1175 Ci/mmol) and [$^{32}$P]-dCTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Tritiated 2MD (174 Ci/mmol) was prepared from an appropriate precursor synthesized by (Grzywacz, Scizinski and DeLuca, unpublished) and provided by Deltanoids Pharmaceuticals, Inc. Dexamethasone was obtained from Sigma Chemical Co. (St. Louis, MO). Alpha-Modified Eagles medium (α-MEM) and Dulbecco’s Modified Eagles medium (DMEM) were purchased from Life Technologies (Grand Island, NY). Oligonucleotide primers were obtained from IDT (Coralville, IA). Anti-VDR (H-81), RXR (ΔN197), and SRC-1 (M-341) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-RNA polymerase II antibodies (8WG16) were obtained COVANCE, Berkeley Antibody Company (Richmond, CA). Lipofectamine Plus was obtained from Invitrogen (Carlsbad, CA). BioGel HTP was obtained from BioRad (Hercules, CA).

Plasmids- The human osteocalcin (OC) promoter reporter plasmid phOC-3900 luc, the human Cyp24 reporter plasmid phCyp24-luc (ph24OHase-luc) and pGal4(5x)-luc were
previously reported (20,21). pcDNA-hVDR, phVDR-VP16 and pCH110-βgal have been also previously described (20, 22). pcDNA-hVDR(R417A/R420A) was prepared from pcDNA-hVDR using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The Gal4 (1-147) DNA-binding domain (DBD) fusion vectors pM-SRC-1NR (residues 621-765) and pM-GRIP-NR (residues 629-760) have also previously described (22,23). Gal4 (1-147) DBD fusion vector pBIND (Promega, Madison, WI) was used to create pBIND-mRXRα (residues 292-467) and pBIND-hDRIP205NR (residues 527-970). pET-hVDR comprised of the full-length VDR containing a C-terminal 6xHis tag was constructed from the pET-29b vector obtained from Novagen (Darmstadt, Germany). pGEX-SRC-1NR (residues 621-765), pGEX-GRIP-NR (residues 629-760), pGEX-mRXRα (residues 203-467) and pGEX-DRIP205-NR (residues 527-970) were constructed using the pGEX parent plasmid obtained from Amersham (Piscataway, NJ).

**Cell Culture-** MC3T3-E1 cells and ST2 cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum (FBS) from BioWhittaker (Walkerville, MD). Vitamin D₃ ligands were added in ethanol (1% maximum final concentration).

**Osteoclast Formation-** Spleen cells were obtained from six-week-old C57B6 mice (Harlan-Sprague Dawley, Indianapolis, IN), treated with lysis buffer (155mM NH₄Cl, 12mM NaHCO₃, 1mM EDTA) and prepared as previously described (17). Coculture
assays were carried out by plating ST2 cells (5x10^4/well) together with freshly isolated spleen cells (1x10^6/well) in 48-well plates. Cells were cultured in phenol red-free α-MEM with 10% charcoal dextran-treated FBS in the presence or absence of indicated compounds and the medium replaced on day 4 and day 7. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity on day 8-10 as previously described (24). Osteoclast number was determined by counting the total number of multinucleated (>3 nuclei), TRAP-positive cells/ well.

Transfection Analysis- MC3T3-E1 cells were seeded into 24-well plates at a density of 5x10^4 cells/well and transfected 16-24 hours later using Lipofectamine Plus as described (23). Individual wells received 300 ng of DNA comprised of 250 ng of phOC-3900 luc or phCyp24-luc plasmid and 50 ng pCH110-β-gal. In the two-hybrid system, cells received 250 ng of pGal4(5x)-luc, 50 ng of pVDR-VP16, 50 ng of either pBIND-mRXRα, pM-SRC-1NR, pM-GRIP-NR, or pBIND-hDRIP205NR and 50 ng pCH110-β-gal. After transfection, the cells were normally cultured in medium supplemented with 10% FBS for 24 hr with or without the indicated ligands. Cells were harvested 24 hr after stimulation and lysates assayed for luciferase and β-galactosidase (β-gal) activities using standard methods. In some experiments, cells were transfected as above and treated with the indicated ligands in the absence of FBS for periods ranging from 15 min to 8 hr. The medium was then changed to ligand-free medium containing 10% FBS. The cells were harvested 24 hr post-transfection and analyzed as above.
Luciferase units were normalized in all cases to β-gal activity.

RNA Isolation and Analysis- MC3T3-E1 or ST2 cells were plated in 100mm dishes in α-MEM supplemented with 10% FBS at densities of 5x10^5/ml and treated for up to 24 hrs with dexamethasone (10^{-7} M) without or with the indicated concentration of either 1,25(OH)_2 D_3 or 2MD. Total RNA was isolated using the Triazol reagent from MRC (Cincinnati, OH), resolved using denaturing gel electrophoresis and transferred to Hybond-N+ membranes obtained from Amersham. Double-stranded cDNA fragments encoding mouse RANKL (+270 to +940), mouse OPG (+346 to +1206), mouse Cyp24 (+435 to +707), mouse OPN (full-length cDNA) or mouse beta-actin (+806 to +1155) were labeled with [32P]-dCTP (NEN) using a Megaprime labeling kit (Amersham) and used as probes. Radioactive images were analyzed with a phosphorimager. Total RNA was also reverse transcribed using the SuperScript II RNase H Reverse transcriptase kit from Invitrogen (Carlsbad, CA) and subjected to PCR analysis using standard methods (see also ChIP method).

Cell Uptake Assays- MC3T3-E1 cells (1x10^6/ml) were seeded into 24-well plates and cultured overnight in normal FBS-supplemented α-MEM. The medium was then replaced with medium containing the indicated concentration of FBS and either 0.1 nM 1,25(OH)_2 D_3 (166 Ci/mmol) or 2MD (169 Ci/mmol) for periods ranging from 5 min to 2 hr. Following removal of the medium, cells were washed three times with PBS,
dissolved in scintillation fluid and evaluated for ligand uptake using a Packard Tricarb 2900TR Scintillation Spectrometer.

**Hormone-bindings Analysis** - MC3T3-E1 cells (1x10^6/ml) were seeded into 100mm plates and cultured overnight in normal FBS-supplemented α-MEM. The medium was then replaced with FBS-free medium, and 1,25(OH)₂D₃ (166 Ci/mmol) or 2MD (174 Ci/mmol) was added with or without a 100-fold molar excess of radioinert ligand for an additional 30 min. Following incubation, cells were washed, lysed in 50 mM Tris-HCl, pH 7.4, 0.3 M KCl, and 5mM DTT containing 0.5% NP-40, and the soluble fraction subjected to hydroxylapatite (HAP) assay to assess total and non-specific 1,25(OH)₂D₃- or 2MD-binding as previously described (25). The difference between total binding and nonspecific binding represents specific VDR binding. Total cellular uptake of both ligands in these experiments was also assessed as described above.

**GST Pulldown Assays** - pET-VDR was transformed into BL21(DE3) codon Plus RIL cells (Stratagene, USA). Transformed cells were cultured with 100 uM IPTG at 23°C for 6 hr and soluble VDR protein purified to homogeneity from lysates using sequential Ni-NTA and SP-Sepharose column chromatography. Lysates from pGST-RXRα-, pGST-SRC-1NR-, pGST-GRIP-NR- and pGST-DRIP205-NR-transformed E. coli were incubated with glutathione-Sepharose 4B for 2 hrs at 4 C, and then washed extensively to prepare an immobilized GST fusion reagent of mRXRα, hSRC-1, hGRIP
or hDRIP205. Purified VDR and immobilized GST fusion proteins were incubated with
10^{-8} to 10^{-12} M 1,25(OH)_2D_3 or 2MD in GST-binding buffer (20mM Tris-HCl, pH
7.9, 180mM KCl, 0.2mM EDTA, 0.05% Nonidet P-40, 0.5mM PMSF, 1mM DTT)
containing 1 mg/ml BSA for 30 min at RT. After 5 washes, dissolved samples were
resolved by SDS-PAGE, transferred to PVDF membranes and subjected to Western blot
analysis as previously described (20) using the anti-VDR monoclonal antibody 9A7 (26).
Blots were visualized using the ECL method.

Chromatin Immunoprecipitation (ChIP) Assays- Chromatin immunoprecipitation was
performed as described previously (27,28). Briefly, MC3T3-E1 cells were cultured in
FBS-free α-MEM and treated with 1,25(OH)_2D_3 or 2MD for the times or
concentrations indicated. Following a wash with PBS, cells were subjected to cross-
 linking with 1% formaldehyde. Cells were extracted in 5mM Pipes pH 8.0, 85mM KCl,
0.5% NP-40 and then in 1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1. Chromatin
pellets were sonicated to an average of 300-500bp fragments of DNA, centrifuged and
then diluted into ChIP buffer (16.7mM Tris-HCl pH 8.1, 150mM NaCl, 0.01% SDS,
1.1% Triton X-100, 1.2mM EDTA). Immunoprecipitations were performed overnight at
4 C with the indicated antibodies and then collected following a 1 hr incubation with
salmon sperm DNA- and BSA-pretreated Zysorbin (Zymed, San Francisco, CA).
Precipitates were then washed sequentially and the cross-links reversed with a 6 hr
incubation at 65°C in 1% SDS and 0.1M NaHCO_3. DNA fragments were purified using
Qiagen QIAquick Spin Kits (Valencia, CA) and subjected to PCR techniques using primers designed to amplify fragments of murine Cyp24 promoter region (-236 to -51) and murine OPN promoter region (-854 to -658). All PCR analyses for each primer set were carried out in a predetermined linear range of DNA amplification. PCR products were resolved on 2% agarose gels and visualized using ethidium bromide staining.

**Protease Digestion Studies**- The plasmids pcDNA-hVDR or pcDNA-hVDR(R417A/R420A) were utilized to prepare in vitro-translated, [35S]-methionine labeled hVDR or hVDR(R417A/R420A) using the TNT/T7 Coupled Reticulocyte Lysate System from Promega (Madison, WI). Appropriate aliquots were treated with the indicated concentrations of 1,25(OH)2D3 or 2MD for 10 min and then digested with trypsin (0.12 ug/sample) at RT for the indicated time periods as described (9). Digestion was terminated with denaturing buffer, whereupon the samples were resolved by SDS-PAGE. Dried gels were subjected to autoradiography.

**RESULTS**

**2MD is a 1,25(OH)2D3-Like Superagonist in Cell Culture**- Previous studies revealed that 2MD is a highly potent compound that exhibits both tissue- and gene-selectivity in vivo (16). Several of these characteristics have also been demonstrated in cell culture (17). For example, 2MD is at least 100-fold more active than 1,25(OH)2D3 in inducing a collection of vitamin D-sensitive genes in the mouse osteoblastic cell line ST2,
including Cyp24, OPN, and RANKL, and in suppressing the expression of OPG (Fig. 1a). Separate additional experiments indicated that while peak activity of 1,25(OH)\(_2\)D\(_3\) and 2MD was reached at 10\(^{-7}\) M and 10\(^{-9}\) M, respectively, there was no increase in the efficacy of 2MD over that of 1,25(OH)\(_2\)D\(_3\). Importantly, this increased potency of 2MD over 1,25(OH)\(_2\)D\(_3\) extends to its ability to stimulate osteoclast formation in cocultures of ST2 cells and mouse osteoclast precursors (Fig. 1b), an effect due to increased expression of RANKL and decreased expression of OPG (19). These observations verify that 2MD is a highly potent 1,25(OH)\(_2\)D\(_3\) agonist in cultured bone cells. This is an intriguing finding in view of the fact that the affinity of the VDR for 2MD (K\(_d\)) is not different from that of the native hormone (16).

Transcriptional Models Recapitulate 2MD Potency- To establish a functional model for evaluating the potency of 2MD, we introduced vitamin D-sensitive Cyp24 and OC promoter-reporter gene plasmids into pre-osteoblastic ST2 or MC3T3-E1 cells by transient transfection and assessed dose-dependent transcriptional activity in response to 2MD or 1,25(OH)\(_2\)D\(_3\). As can be seen in Fig. 2, both 1,25(OH)\(_2\)D\(_3\) and 2MD dose-dependently induced transcriptional activation of each reporter gene in MC3T3-E1 cells; similar results were obtained in ST2 cells (data not shown). The transcriptional potency of 2MD was at least two logs greater than for 1,25(OH)\(_2\)D\(_3\), however, reflecting that observed for endogenous gene expression. These results suggest that the enhanced
potency of 2MD is transcriptional in nature and provide a model for determining the underlying molecular basis for this increased potency.

*Enhanced Potency of 2MD is Not Due to Serum*- Ligand-induced transcriptional responses in cells are influenced by cell culture conditions and can be significantly affected by serum. Serum also contains DBP, an avid binder of and presumed primary although not exclusive carrier in the blood for most vitamin D metabolites (24). In contrast to 1,25(OH)2D3, 2MD binds only weakly to DBP, an effect that could lead to an increase in its functional concentration relative to 1,25(OH)2D3. We explored this possibility by transfecting the OC promoter into MC3T3-E1 cells and then treating the cells with increasing concentrations of 1,25(OH)2D3 or 2MD for 1 hr in serum-free medium. The medium was then replaced with ligand-free medium supplemented with 10% FBS and transcriptional activity assessed approximately 24 hrs later. The results indicate that 2MD continues to exhibit an approximately two-log increase in potency (Fig. 3a), revealing that its superagonist activity is not due to serum. Similar results were obtained with the hCyp24 promoter (data not shown). Interestingly, the level of transcription induced under serum-free conditions in 1 hr or less exposure to functionally equivalent levels of 1,25(OH)2D3 (10−7 M) and 2MD (10−9 M) is at least 50% of that achieved following continual treatment with the two ligands for 24 hr in the presence of serum (Fig. 3b). Irrespective of this, the data suggest that the potency of 2MD is not derived from its lack of interaction with serum components.
**Cellular Uptake of 1,25(OH)₂D₃ and 2MD Does Not Determine Potency**- We next tested the possibility that 2MD might enter cells more readily than 1,25(OH)₂D₃ and thus saturate intracellular VDR at much lower medium ligand concentrations. Cells were incubated with 0.1 nM tritiated 1,25(OH)₂D₃ or 2MD (a concentration which leads to transcriptional activation with 2MD but not with 1,25(OH)₂D₃) under various serum conditions for periods ranging from 0 to 15 min, then washed, and the cellular content of ligand determined by scintillation counting. As seen in Fig. 4a, incubation of cells in the absence of FBS leads to a rapid and equivalent accumulation of both ligands within the cells. Surprisingly, this accumulation peaked within 10 min (Fig. 4a), and then decreased to a steady state nadir by 15 to 30 min and remained relatively stable for the next several hr (data not shown). Interestingly, increasing concentrations of FBS in these experiments significantly reduced the uptake of both ligands into the cells (Figs. 4a and 4b). This effect was most evident for 1,25(OH)₂D₃ at the highest level of serum added (5-fold), although the difference does not seem able to account for the 2 log increase in potency. Despite this, the observation that virtually identical levels of both 1,25(OH)₂D₃ and 2MD accumulate in cells in the absence of serum indicates that the increased potency of 2MD is not due to altered cellular uptake.

**Binding of 1,25(OH)₂D₃ and 2MD to the VDR is Similar In Intact Cells**- Based upon the above findings, we tested an alternative possibility that VDR might exhibit an
increase in affinity for 2MD relative to 1,25(OH)_{2}D_{3} in intact cells. MC3T3-E1 cells were incubated in the absence of serum for 30 min (37 C) with increasing concentrations (0.05, 0.1, and 0.5 nM) of either 1,25(OH)_{2}D_{3} or 2MD in the absence or presence of 200-fold molar excess of each ligand. Total and VDR-bound ligands were then assessed. As observed in Fig. 5, neither total cellular uptake (Fig. 5a) nor VDR binding (Fig. 5b) was substantially different between 1,25(OH)_{2}D_{3} and 2MD at the concentrations tested. Although this experiment does not represent a complete saturation curve for VDR, approximately 5500 VDR mol/cell were detected at the 0.5 nM ligand concentration, roughly 60% of the MC3T3-E1 cell’s complement of saturable VDR (Pike, J.W., unpublished). These results indicate that when measured in intact cells, the relative affinities of the VDR for 2MD and 1,25(OH)_{2}D_{3} are probably not substantially different from that observed in broken-cell preparations and thus do not provide an explanation for the increased potency of 2MD. Rather, they indicate that this characteristic of 2MD resides in its enhanced ability to stimulate VDR-mediated transcription.

**2MD Potently Stimulates Binding of VDR to Active Promoters In Intact Cells** - Two possible mechanisms might account for the increased transcriptional potential of 2MD: 1) increased VDR affinity for DNA via some undefined mechanism or 2) increased transcriptional activity of individual receptor molecules. We therefore utilized chromatin immunoprecipitation (ChIP) assays to examine the relative abilities of the two ligands to
stimulate VDR binding to the VDREs located in OPN and Cyp24 promoters in intact MC3T3-E1 cells. The degree to which the OPN or Cyp24 DNA promoter fragment can be detected by PCR is directly related to the association of the VDR with these promoters. A dose response curve for VDR localization to the OPN promoter at 2 hr is observed in Fig. 6. Clearly, although the overall appearance of the VDR on this promoter is similar, the ligand concentrations required for maximal binding are quite different: while maximal binding is achieved with 0.1 nM 2MD, at least 10 nM is required for 1,25(OH)2D3. Not surprisingly, this accumulation of VDR on the promoters was also associated with recruitment of RNA pol II (Fig. 6). To ensure that the kinetics of VDR binding was not different, we treated cells with 0.1 nM 1,25(OH)2D3 or 2MD and assessed DNA localization on both the OPN and Cyp24 promoter every 15 min up to 135 min. While VDR localized to both promoters following treatment with 2MD (maximal at 45 min), VDR binding was undetectable with 1,25(OH)2D3 at any of the time points evaluated (data not shown). In contrast, treatment of cells with functionally equivalent concentrations of the two ligands (1 nM 2MD and 100 nM 1,25(OH)2D3) led to qualitatively and quantitatively similar results (Fig. 6 and data not shown). These studies indicate that the increased potency of 2MD is likely due to enhanced VDR localization on promoter DNA, although whether this is the result of increased VDR DNA-binding or increased VDR complex stability cannot be distinguished by this experiment.

*Increased VDR DNA Binding May be Due to Enhanced Interaction with RXRα* - The
VDR is believed to bind to DNA as a ligand-induced RXR heterodimer (30,31). Thus, the possibility exists that binding of 2MD to the VDR enhances the receptors association with RXR leading in turn to VDRE DNA binding. To explore this hypothesis, we contrasted the ability of 1,25(OH)2D3 and 2MD to promote VDR interaction with RXRα using both an in vitro GST pull-down assay and a mammalian two-hybrid assay in MC3T3-E1 cells. GST-RXRα fusion protein was retained on glutathione-Sepharose beads and incubated with highly purified human VDR in the presence of increasing concentrations of either 1,25(OH)2D3 or 2MD. Following a 30 min incubation at RT, the beads were washed, and solubilized VDR resolved by SDS-PAGE and subjected to western blot analysis. Figure 7a reveals that while both 1,25(OH)2D3 and 2MD promote the association of VDR with RXR, the concentrations of the two ligands necessary for interaction are relatively high and do not appear to be different. In the mammalian two-hybrid system, MC3T3-E1 cells were cotransfected together with RXRα-Gal4 DBD, a full-length VDR-VP16 fusion construct and a luciferase reporter gene containing tandem Gal4 response elements and then incubated with increasing concentrations of either 2MD or 1,25(OH)2D3 for 1 hr. Fresh medium containing serum was then added and the cells were allowed to incubate for an additional 24 hr period. Figure 7b documents that while both ligands induce an interaction between VDR and RXR, 2MD is at least two logs more potent than 1,25(OH)2D3, a potency which appears to correspond to that seen with the transfected OC and Cyp24 gene promoters. Enhanced association of the VDR with RXR in the presence of 2MD is also supported in vivo by ChIP analysis.
Again, while 0.1 nM 1,25(OH)$_2$D$_3$ was ineffectual, the same concentration of 2MD strongly induced association of RXR as well as VDR to the Cyp24 gene promoter. These observations indicate that 2MD potently induces VDR/RXR interaction in intact cells but not in vitro, suggesting that the effects of 2MD require either additional factors or perhaps post-translational modifications of the receptor(s) that do not occur in vitro.

**Increased VDR DNA Binding May be Influenced by Interaction With Coactivator**—In order to examine the possibility that additional factors might affect VDR/RXR DNA binding or influence the stability of the complex, we assessed whether 2MD promoted association of the VDR with coregulators such as DRIP205 or the p160 coactivators such as SRC-1 and SRC-2/GRIP. The interaction between receptor and these coactivators is believed to occur via the AF-2 region of the receptor and LxxLL motifs located within coactivators (32). As in the RXR assessment, both the GST pull-down assay and the mammalian two-hybrid assay were employed. The results seen in Figure 8a indicate that as with GST-RXR/VDR interaction, the two ligands induce GST-SRC-1 and GST-DRIP205 interactions with the VDR in vitro with similar potency profiles. The results with GST-GRIP were identical (data not shown). In the two-hybrid system, however, 2MD again manifested a two-log increase in potency over 1,25(OH)$_2$D$_3$ in promoting VDR interaction with both SRC-1 (Fig. 8b) and DRIP205 (Fig. 8c) and similar findings were observed with GRIP/SRC-2 (data not shown). ChIP analysis supported this finding. Thus, while 0.1 nM 1,25(OH)$_2$D$_3$ was ineffectual, the same concentration of
2MD strongly induced association of not only VDR and RXR to the Cyp24 gene promoter, but SRC-1 as well (Fig. 8d). These results support the idea that 2MD promotes DNA binding though enhanced formation of a complex that involves not only RXR but LxxLL-containing p160 and DRIP205 coactivators as well.

2MD Induces a Unique VDR Conformation- Enhanced interaction of VDR with either RXR or SRC-1 seen in intact cells in response to 2MD is likely to be mediated through a unique receptor conformation. We therefore prepared in vitro translated full-length VDR and subjected it to limited proteolytic digestion using trypsin. Previous studies with the VDR (as well as other nuclear receptors) have revealed that ligand-specific cleavage products can be demonstrated following SDS-PAGE (9, 33). Figure 9a demonstrates that incubation of receptor with either 1,25(OH)₂D₃ or 2MD protects the VDR from complete degradation over time, and results in the production of two receptor fragments of 28 and 34 kDa (see arrows). These fragments are believed to comprise the carboxy terminal hormone-binding domain of the VDR. No difference in ligand potency was evident (data not shown). Treatment with 2MD, however, leads to the appearance of a unique third fragment of approximately 32 kDa (Fig. 9a, see arrows). This result indicates that the conformation of the VDR differs depending upon the nature of the activating ligand. We also examined the tryptic digestion pattern of a hVDR which contained mutations in helix 12 of VDR AF-2. These mutations do not alter hormone-binding activity, but completely abrogate its transcriptional capabilities (34). More importantly, proteolytic cleavage products can be demonstrated more readily with this VDR product
High concentrations of both 1,25(OH)2D3 and 2MD protect this mutant VDR from complete degradation (Fig. 9b). More importantly, while both the 34 and the 28 kDa bands are evident with the 1,25(OH)2D3-treated samples, it is the 28 and 32 kDa bands that are most apparent in the 2MD-treated samples. These data provides strong structural evidence that the VDR exhibits a conformation in the presence of 2MD that is different from that with 1,25(OH)2D3.

**DISCUSSION**

2MD is a 1,25(OH)2D3 analog in which the 20-carbon methyl group resides in the S rather than the normal R configuration and a methylene group is located at carbon 2 rather than carbon 19 (16). Like 1,25(OH)2D3, 2MD displays an affinity (Kd) for the VDR of ~1x10^{-10} M, although its affinity for serum DBP is weak. These characteristics are likely to influence its pharmacokinetic properties and thus its actions in vivo. Biologically, 2MD is an extremely potent analog with significant anabolic actions in bone both in vivo and in vitro (16,17). These observations suggest that 2MD may be both tissue- and cell-specific in its actions. In this report, we focused upon the molecular mechanisms that underlie the enhanced potency of 2MD. This potency is manifested in cell culture at the level of endogenous gene expression as well as transcription, and occurs despite the fact that both 2MD and 1,25(OH)2D3 bind equally well to the VDR. Indeed, this property is a characteristic of many chemically related classes of vitamin D analogs. We show herein using intact cell chromatin localization
and cofactor recruitment assays as well as intact cell protein interaction assays and *in vitro* assays that the potency of 2MD is due to its ability to induce a unique conformation within the VDR that is manifested by increased VDRE binding. This increased binding is likely the result of enhanced interaction with RXR but also with receptor comodulators such as the p160 coactivator SRC-1 and/or DRIP205. These findings provide a molecular explanation for the increased potency of 2MD and together with previous studies may explain the potency of other analogs of vitamin D that exhibit similar properties.

Reported modifications of 1,25(OH)$_2$D$_3$ on carbon 2 have been infrequent (36,37). Perhaps the most notable is 2β (3-hydroxypropyl)-1,25(OH)$_2$D$_3$ (ED-71), a compound which like 2MD also promotes bone formation *in vivo*, although its effects are much weaker (36). Modifications in other regions of the 1,25(OH)$_2$D$_3$ molecule, particularly the side-chain, are more common. Of considerable interest are those that contain altered stereochemistry at carbon 20. The (20S) or 20-epi analogs of the native hormone are numerous, and many of them display significantly increased potency despite an otherwise similar affinity for VDR (38). As with other analogs, the effects of this modification on receptor activity have been investigated rather extensively, although often with conflicting results. Earlier studies, for example, indicate that (20S) analogs enhance DNA binding by promoting VDR interaction with RXR *in vitro* (33). Additional results also suggested a hypothesis that (20S) analog-induced coactivator interactions can enhance VDR DNA-binding and/or alter transactivation due to
differences in receptor-ligand contacts within the LBD of the VDR. Freedman and colleagues were unable to confirm enhanced DNA binding and RXR interaction with VDR in vitro, but did demonstrate increased interaction with the coactivator DRIP205 (10). This interaction appeared to increase the extent of activity in a cell-free transcriptional assay, although this system utilized non-chromatin templates that are to a large degree independent of hormonal regulation. It is unclear at present whether either of the above observations is correct, but they do indicate the difficulties of relying exclusively on biochemical interaction assays in vitro. Accordingly, we focused upon VDR complex formation on specific chromatin DNA in intact cells using ChIP assays. We also evaluated the interaction of the VDR with its heterodimeric partner RXR as well as with potential facilitators of transactivation such as SRC-1 and DRIP205 using a mammalian two-hybrid system.

Despite the differences in potency between 1,25(OH)$_2$D$_3$ and 2MD both in the presence and absence of serum, the uptake of the two ligands into cells and their capacity to occupy significant levels of cellular VDR in the 0.05 nM to 0.5 nM range were very similar. Since only 2MD was capable of transcription at these concentrations, we conclude that 1,25(OH)$_2$D$_3$- and 2MD-ligated receptors differ in their apparent transcriptional capabilities. To explore this further, we assessed the ability of the two ligands to induce VDR binding to the OPN and Cyp24 promoters in cells using ChIP. This assay measures the relative amount of VDR bound to an endogenous gene promoter in intact cells in response to ligand activation. The results revealed a direct dose-dependent correlation between DNA-bound receptor content and transcriptional...
activation, with 2MD exhibiting enhanced potency relative to 1,25(OH)_{2}D_{3}. This correlation makes it unlikely that 2MD-activated receptors exhibit a “hyperactive” receptor state. Interestingly, when VDR ligand affinity and VDR DNA occupancy are both considered, it is not the apparent potency of 2MD but rather the “impotency” of 1,25(OH)_{2}D_{3} that underlies the differences between the two ligands. Presumably, this characteristic of the 1,25(OH)_{2}D_{3} ligand was evolutionarily advantageous.

Tryptic digestion of nuclear receptors has resulted in specific, ligand-dependent proteolytic patterns indicative of different receptor conformations (39,40). That these conformational differences exist with various ligands have been substantiated particularly well for the ER in X-ray diffractions studies (41,42). We observed such an altered pattern of digestion of the VDR, suggesting that 2MD promotes a receptor conformation different from that with 1,25(OH)_{2}D_{3}. This conformation was most evident when a form of the VDR that contained mutations in two of the residues responsible for the formation of the transactivation helix-12 was examined. These patterns did not reveal a difference in dose response between the two ligands, however, suggesting that potency differences are manifested only in the cell. Interestingly, the pattern of tryptic digestion was reminiscent of that obtained by Peleg and colleagues (9, 33) following VDR protection with 20-epi-1,25(OH)_{2}D_{3}. Indeed, in our hands the proteolytic digestion pattern of VDR with 20-epi-1,25(OH)_{2}D_{3} was the same as that with 2MD (data not shown). Thus, it seems likely that the altered VDR pattern seen in our studies with 2MD is due to the (20S) configuration in this analog and not to its 2-methylene substitution. Additional
studies using different proteases may be necessary to establish whether 2MD can promote
a conformation different from that with 20-epi-1,25(OH)₂D₃. Interestingly, the three
dimensional structure of the VDR LBD does not reveal differences in structure at the
atomic level when VDR is associated with 1,25(OH)₂D₃ or 20-epi-1,25(OH)₂D₃
(12,13). Our current crystallography studies of the VDR comparing 1,25(OH)₂D₃ with
2MD suggest similar conclusions (Vanhooke, J., manuscript in preparation). It is
possible that ligand-dependent differences will become apparent when the structure of
the full-length receptor or the VDR/RXR heterodimer is solved.

Differences in VDR structure in the presence of 2MD may be responsible for the
enhanced interactions of VDR with RXR as well as with the coactivators SRC-1 or
DRIP205 that are observed in the two-hybrid system. Since potency is not manifested in
vitro, it suggests that formation of a multi-protein DNA complex is essential for
increased DNA affinity, either as a result of increased DNA binding or as a function of
enhanced complex stability. Little is known of the processes that terminate nuclear
receptor-mediated transactivation or of the signals that initiate the event, although it is
known that receptor ubiquitination and 26S proteosome-mediated degradation are
involved (43,44). A key question is whether off signals are initiated though the loss of
receptor ligand or whether other signals are responsible for triggering transcriptional
termination. If ligand dissociation is integral to transcriptional termination, increased
stability could derive from a decreased off-rate of 2MD. This would, however,
necessitate a comparable decrease in the association rate in view of the similarity in K_d
between 1,25(OH)$_2$D$_3$ and 2MD. Such a possibility has been suggested recently for a progesterone receptor mutant (45). Regardless of the mechanism, it is clear that 2MD promotes enhanced affinity of the VDR for multiple components.

2MD is both tissue-as well as gene-selective, eliciting preferential activity in bone relative to the gut and stimulating bone formation that is not normally associated with 1,25(OH)$_2$D$_3$ or its analogs (16). Why bone is a focal point of 2MD action in vivo is unknown currently, although evidence suggests that its anabolic activity may be due to its ability to promote osteoblast mineralization in vitro (17). Many vitamin D analogs appear to exhibit characteristics of tissue and/or gene selectivity. Perhaps most notable is the inability of many analogs to provoke calcium homeostatic actions in the intestine, bone and kidney that lead to an elevation in serum calcium levels while simultaneously modulating cellular events such as proliferation and differentiation or regulating the expression of specific genes (46). The physiologic basis for this selectivity is not understood; it may be pharmacokinetic in some cases (47) and mechanistic in others (9,10,33). The mechanistic basis for selectivity could involve many aspect of VDR action. Many VDREs, for example, differ in structure from canonical direct repeats, and may well influence VDR binding and coactivator recruitment (48). This has not been proven, however. In addition, VDREs are often located immediately adjacent to binding sites for other transcription factors, suggesting the possibility of ligand-dependent selectivity for protein-protein interactions that could involve not only the adjacent transcription factors but coactivators or repressors as well (49,50). With respect to the comodulator class of transcription factors, many have been identified (51). They differ in
concentration from cell to cell, are often promoter-specific in their actions, and their individual activities are modulated through diverse signaling pathway (51,52). Thus, ligand-selective recruitment of these coregulators could also occur, providing additional mechanisms of cell- and gene-selectivity. Thus, while our findings focus primarily on the potency of 2MD, they may well provide avenues to explore additional interesting features of this analog’s actions.

In conclusion, we have demonstrated that the potency of 2MD is due to its ability to promote increased affinity of the VDR for specific DNA. Whether this increase is due to enhanced binding or increased stability of the transcriptional complex remains to be determined. Nevertheless, these features of 2MD action may provide hints as to how 1,25(OH)2D3 analogs such as 2MD might exert tissue- and gene-selective actions in vivo. Future studies focus on these biological properties of 2MD.

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REFERENCES


FIGURE LEGENDS

Fig. 1. 2MD is a highly potent regulator of endogenous gene expression in osteoblastic MC3T3-E1 cells. A, dose response modulation of Cyp24, OPN, RANKL, OPG and β-actin mRNA levels by 1,25(OH)2D3 and 2MD in MC3T3-E1 cells. Cells were treated in the presence of dexamethasone with the indicated concentrations of either 1,25(OH)2D3 or 2MD. Total RNA was isolated 36 hr later and analyzed by Northern blot analysis. Autoradiographic signals were quantitated using densitometry, normalized to β-actin levels and plotted as fold changes compared to untreated controls. These results are representative of several different experiments. B, osteoclast formation in cocultures of ST2 stromal cells and mouse spleen cells. Cells were cultured in the presence of dexamethasone and the indicated concentrations of either 1,25(OH)2D3 or 2MD for 10 days. Cells were fixed, stained for tartrate-resistance acid phosphatase, and quantitated.
by counting the total number of TRAP+, multi-nucleated (>3 nuclei) cells.

1,25(OH)₂D₃- (upper left panel) or 2MD- (upper right panel) induced osteoclasts photographed at 20x. Quantitation of osteoclast number as a function of ligand dose (lower panels). The results are the means ± SEM of triplicate wells for each condition. Similar results were obtained in three independent experiments.

Fig. 2. 2MD stimulates transcription of Cyp24 and OC promoter-dependent reporter genes with high potency in osteoblast cells. MC3T3-E1 cells were seeded into 24 well plates and transfected 24 hr later with pCH110-βgal and either phOC-3900-luc (A) or phCyp24-luc (B). Cells were treated with the indicated concentrations of either 1,25(OH)₂D₃ or 2MD in 10% serum-supplemented medium, and cell lysates assessed for β-gal and luciferase activities 24 hr later. Each point represents the average of quadruplicate analyses ± SEM normalized for β-gal activity. Similar results were obtained for three independent experiments.

Fig. 3. The potency of 2MD is not affected by serum content. A, MC3T3-E1 cells were transfected with phOC-3900-luc and pCH110-βGal and then treated with either 1,25(OH)₂D₃ (10⁻⁷ M) or 2MD (10⁻⁹ M) for 1 hr in the absence serum. Ligand-containing medium was then removed and replaced with normal serum-supplemented, ligand-free medium. Cells were lysed and β-gal and luciferase activities were assessed 24 hr later. Each point represents the average of quadruplicate analyses ± SEM
normalized for β-gal activity. Similar results were obtained for at least three independent experiments. B, MC3T3-E1 cells were transfected with phOC-3900 luc and pCH110-β-Gal and then treated with either 1,25(OH)_{2}D_{3} (10^{-7} \text{ M}) or 2MD (10^{-9} \text{ M}) for periods ranging from 0 to 8 hr in the absence serum. Ligand-containing medium was then removed and replaced with normal serum-supplemented ligand-free medium. All lysates were analyzed for β-gal and luciferase activity 24 hr following the original transfection. Control wells were transfected as above and received either vehicle, 1,25(OH)_{2}D_{3} (10^{-7} \text{ M}) or 2MD (10^{-9} \text{ M}) for 24 hr in the presence of normal serum-supplemented medium. The luciferase activities of quadruplicate wells were averaged, normalized for β-gal activity and plotted as fold-induction vs untreated controls ± SEM. Cells exposed to ligand for 24 hr were treated identically. These data are representative of at least two experiments.

Fig. 4. Uptake of 1,25(OH)_{2}D_{3} and 2MD into MC3T3-E1 cells is similar. A, time course of radioligand uptake. MC3T3-E1 cells were seeded into 24 well plates and incubated with 0.1 nM 1,25(OH)_{2}D_{3} (166 \text{ Ci/mmol}) or 2MD (174 \text{ Ci/mmol}) for 0 – 15 min in the presence of 0 (open or closed circles), 1 (open or closed squares), or 10% (open or closed triangles, 5 min time point only) serum-supplemented medium. At term, cells were washed 3x with PBS, dissolved in scintillation fluid, and evaluated for radiolabeled ligand content. B, FBS reduces the uptake of both 1,25(OH)_{2}D_{3} and 2MD. MC3T3-E1 cells were incubated with medium containing the indicated concentrations of
serum and either 0.1 nM 1,25(OH)₂D₃ (166 Ci/mmol) or 2MD (174 Ci/mmol) for 5 min. Cells were then treated as in (A). All assays were carried out in triplicate and the data represent the mean ± SEM. Similar results were obtained for at least three independent experiments.

Fig. 5. Specific binding of 1,25(OH)₂D₃ and 2MD to the VDR is similar in intact cells. A, total cellular uptake of 1,25(OH)₂D₃ or 2MD. Total cellular uptake of radiolabeled 1,25(OH)₂D₃ or 2MD corresponding to (B) below was determined as in Fig. 4. The 100 fold molar excess of unlabeled ligand did not alter radiolabeled ligand uptake (not depicted) indicating a non-saturable process. Each estimate represents the mean ± SEM for a triplicate assay. B, specific 1,25(OH)₂D₃- or 2MD-binding activity in intact cells. MC3T3E1 cells were seeded into 100mm plates and at confluency incubated with 0.05, 0.1 or 0.5 nM 1,25(OH)₂D₃ (166 Ci/mmol) or 2MD (174 Ci/mmol) in FBS-free medium with or without a 100-fold molar excess of radioinert ligand for 30 min. Following incubation, cells lysates were subjected to hydroxylapatite (HAP) assay to assess total and non-specific 1,25(OH)₂D₃- or 2MD-binding. Specific binding is depicted (nonspecific binding was less than 20% of total binding activity). Each point represents the mean ± SEM for triplicate assays. These data are representative of three similar experiments.

Fig. 6. 2MD potently stimulates binding of VDR to active promoters in intact cells.
MC3T3-E1 cells were seeded into 100mm plates and treated with increasing concentrations of 1,25(OH)$_2$D$_3$ or 2MD in serum-free medium. After 15 min, the medium was changed to that containing 10% FBS. Two hr later, cells were harvested and subjected to ChIP analysis as described in Materials and Methods using anti-VDR, anti-RNA pol II, or in the absence of antibody. Isolated DNA was subjected to PCR (28 cycles) using primers designed to amplify a 196 bp fragment of the OPN promoter from –854 to –658 that contained the VDRE at –764 to –748. Upper panel: amplified ethidium bromide stained DNA fragments. Lower panel: densitometric quantitation of the PCR fragments. Precipitation was anti-VDR or anti-RNA pol II antibody concentration-dependent and similar results were obtained using the anti-VDR monoclonal antibody 9A7. Immunoprecipitation in the absence of antibody or in the presence of an irrelevant antibody was identical. PCR amplification of the coding region of either gene did not produce visible products. The results are typical of at least three experiments performed under similar conditions.

Fig. 7. 2MD is a potent inducer of VDR/RXR interaction. A, biochemical interaction between VDR and RXR in vitro. GST-RXR$\alpha$ immobilized on glutathione-Sepharose beads was incubated with purified hVDR (100ng) in the presence of increasing concentrations of 1,25(OH)$_2$D$_3$ or 2MD. Complexes were collected after 30 min, washed extensively, solubilized in denaturing buffer, and subjected to Western blot analysis using the anti-VDR 9A7 antibody. The data are representative of two experiments. B, 2MD induces interaction of VDR with RXR using mammalian two-
hybrid assay. MC3T3-E1 cells were transfected with phVDR-VP16, pBIND-mRXRα and pGal4(5x)-luc and treated and evaluated as described in Materials and Methods using a 1 hr ligand treatment in the absence of serum. Each point represents the mean ± SEM of quadruplicate transfections. The experiment is representative of several identical analyses as well as evaluations carried out in the presence of serum. C, 2MD recruits RXR to the OPN promoter in intact cells via ChIP analysis. MC3T3-E1 cells were treated with either vehicle, 1,25(OH)2D3 (0.1 nM) or 2MD (0.1 nM). After 45 min, cells were harvested and subjected to ChIP analysis using anti-VDR, anti-RXR or no antibodies. DNA input is indicated. Two different anti-VDR antibodies and two anti-RXR antibodies produced similar results. PCR analysis was performed for 28 cycles using primers designed to amplify a mouse Cyp24 promoter fragment from −236 to −51 with a VDRE at −163 to −148. These data are representative of at least three similar experiments.

Fig. 8. 2MD is a potent inducer of VDR/SRC-1 and VDR/DRIP205 interaction. A, biochemical interaction of VDR with either SRC-1 or DRIP205 in vitro. GST-SRC-1 or GST-DRIP205 immobilized on glutathione-Sepharose beads were incubated for 30 min with purified hVDR (100ng) in the presence of increasing concentrations of 1,25(OH)2D3 or 2MD. Complexes were collected after 30 min, washed extensively, dissolved in denaturing buffer, and subjected to Western blot analysis using the anti-VDR 9A7 antibody. These data are representative of several similar experiments. B
and C, 1,25(OH)2D3- or 2MD-induced interaction of VDR and either SRC-1 or DRIP205 using mammalian two-hybrid assay. MC3T3-E1 cells were transfected with pHVDR-VP16, pM-SRC-1NR or pBIND-hDRIP205NR and pGal4(5x)-luc and the cells treated and evaluated as described in the Materials and Methods using a 1 hr ligand treatment in the absence of serum. Each point represents the mean ± SEM of quadruplicate transfections. The data are representative of several similar experiments carried out either in the absence or in the presence of serum. B, SRC-1; C, DRIP205. D, 2MD recruits SRC-1 to the murine Cyp24 promoter in intact cells. MC3T3-E1 cells were treated with either vehicle, 1,25(OH)2D3 (0.1 nM) or 2MD (0.1 nM). Cells were harvested 45 min later and subjected to ChIP analysis using anti-VDR or anti-SRC-1 or in the absence of antibody. PCR analysis was performed for 28 cycles using primers designed to amplify a mouse Cyp24 promoter fragment from −236 to −51 with a VDRE at −163 to −148. These data are representative of several similar experiments.

Fig. 9. 2MD induces a unique VDR conformation. [35S]-methionine-labeled hVDR or mutant hVDR(R417A/R420A) was incubated with the indicated concentrations of either 1,25(OH)2D3 or 2MD for 10 min followed by treatment with trypsin (1 ug) for the indicated time (A) or 10 min (B). Digestions were terminated using denaturing buffer and the proteins resolved using SDS-PAGE and autoradiographed. Input VDR is indicated. Arrows denote receptor fragments in kDa. Upper panel, wildtype hVDR; lower panel, hVDR(R417A/R420A). The results are typical of several similar
experiments.
Figure 1A

- Fold Increase (RANK/β-actin)
- Percent Repression (OPG/β-actin)
- Fold Increase (OPN/β-actin)
- Fold Increase (CYP24/β-actin)

(Y-axis and x-axis specifications are shown for each plot.)
Figure 2

A

B

- NT
- 2MD
- 1,25(OH)₂D₃

hOC Promoter
RLU/β-gal (10⁶)

Ligand (log M)

hCyp24 Promoter
RLU/β-gal (10²)

0 2 4 6 8 10 12 14 16

- NT
- 2MD
- 1,25(OH)₂D₃

0 2 4 6 8

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Figure 3

A

B
Figure 4

A

![Graph showing ligand uptake over time.]

B

![Bar graph showing total uptake at different serum concentrations.]

1,25(OH)_{2}D_{3} and 2MD uptake comparison across varying serum concentrations.
Figure 5

A

B

Ligand Uptake (fmol/30min)

Specific Ligand Binding Activity (fmol/30min)

Ligand (nM)

Ligand (nM)
2-methylene-19-NOR-(20S)-1,25-dihydroxyvitamin D3 potently stimulates
gene-specific DNA binding of the vitamin D receptor in osteoblasts
Hironori Yamamoto, Nirupama K. Shevde, Anjali C. Warrier, Lori A. Plum, Hector F.
DeLuca and J. Wesley Pike

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