20-Epi Analogues of 1,25-Dihydroxyvitamin D₃ Are Highly Potent Inducers of DRIP Coactivator Complex Binding to the Vitamin D₃ Receptor*

(Received for publication, February 19, 1999, and in revised form, March 31, 1999)

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1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) plays a major role in the stimulation of bone growth, mineralization, and intestinal calcium and phosphate absorption; it also acts as a general inhibitor of cellular proliferation. Several new, clinically relevant compounds dissociate anti-proliferative and calcemic activities of 1,25(OH)₂D₃, but the molecular basis for this has not been clearly elucidated. Here, we tested whether the potency of one class of compounds, 20-epi analogues, to induce myeloid cell differentiation, is because of direct molecular effects on vitamin D receptor (VDR). We report that two 20-epi analogues, MC1627 and MC1288, induced differentiation and transcription of p21WAF1/CIP1, a key VDR target gene involved in growth inhibition, at a concentration 100-fold lower than that of 1,25(OH)₂D₃. We compared this sensitivity to analogue effects on VDR interacting proteins: RXR, GRIP-1, and DRIP205, a subunit of the DRIP coactivator complex. Compared with the interaction of VDR with RXR or GRIP-1, the differentiation dose-response most closely correlated to the ligand-dependent recruitment of the DRIP coactivator complex to VDR and to the ability of the receptor to activate transcription in a cell-free system. These results provide compelling links between the efficiency of the 20-epi analogue in inducing VDR/DRIP interactions, transactivation in vitro, and its enhanced ability to induce cellular differentiation.

In the early part of this century, vitamin D₃ was discovered as a fat-soluble substance with antirachitic activity (1). It was subsequently found that vitamin D₃ must be metabolized to an active hormonal form before it can elicit its biological effects (2). In the kidney to its active form, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), several coactivators have been identified by biochemical techniques of the preinitiation complex (6, 7).

More recently, an intermediary class of proteins called coactivators was discovered that may facilitate the interaction between nuclear receptors and basal transcription machinery. To date, several coactivators have been identified by biochemical interaction or yeast two-hybrid assays (reviewed in Ref. 26). These identified coactivators include SRC1/NCoA-1, GRIP1/TFIP2/NCoA2, ACTR/p/CIP/AIB1/RAC3, and many others (8–10). These proteins are all related to one another, and contain a homologous leucine-rich sequence (LXXLL) that is required for their interaction with AF-2 domain of nuclear receptors in a ligand-dependent manner (11). Several coactivators have been shown to enhance nuclear receptor transactivation in transient transfection assays. Using an affinity column immobilizing the ligand binding domain (LBD) of VDR, we recently isolated a complex of at least 13 VDR interacting proteins (DRIPs) ranging in size from 30 to 250 kDa from Namalwa B-cell nuclear extracts (12, 13). These proteins selectively bind as a complex to VDR in a 1,25(OH)₂D₃-dependent manner. One of these proteins, DRIP205, interacts directly with the LBD in the presence of hormone and appears to anchor the rest of the subunits to the receptor. Furthermore, the DRIP complex strongly potentiates transcription mediated by VDR/RXR in a cell-free, ligand-dependent transcription assay on chromatin-assembled templates. These observations suggested that 1,25(OH)₂D₃ biological effects may be mediated through the capacity of VDR to recruit the DRIP coactivator complex in response to ligand.

A general effect of 1,25(OH)₂D₃ beyond maintaining calcium homeostasis is the inhibition of cellular proliferation (14). One mechanism for this is the induction of 1,25(OH)₂D₃-dependent gene expression (4). Transactivation by 1,25(OH)₂D₃ requires a carboxyl-terminal α-helical region, termed activation function-2 (AF-2), that forms part of the ligand-binding pocket (5). Upon binding to 1,25(OH)₂D₃, VDR dimerizes with the retinoid X receptor (RXR) to enable high affinity interactions with a specific vitamin D-responsive element (VDRE) located in 5′-flanking regions of target genes. Binding of VDR/RXR results in either the up-or down-regulation of transcription. Recent evidence has suggested that VDR exerts its transcriptional activity, at least partially, via direct interactions with TFIIB and TFIIF, proteins of the preinitiation complex (6, 7).

* This work was supported in part by National Institutes of Health Grant DK54540 (to L. P. F.) and CA08748 (Core Center Grant) to Sloan-Kettering Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by the Endocrine Research Training Program (DK07313).

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The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; AF-2, activation function-2; RXR, retinoid X receptor; VDRE, vitamin D-responsive element; LBD, ligand binding domain; DRIP, VDR interacting protein; DBP, vitamin D binding protein; OCT, 1,25-dihydroxy-22-oxa-vitamin D₃; FACS, fluorescence-activated cell sorter; BSA, bovine serum albumin; GST, glutathione S-transferase.
homeostasis is that of general inhibitor of cell proliferation. The antiproliferative effect of 1,25(OH)2D3 was first observed in cultured malignant melanoma cells (14). It was also found that 1,25(OH)2D3 induced the differentiation of immature mouse myeloid leukemia cells (15). Anti-tumor studies of 1,25(OH)2D3 have also been extended to in vivo systems, where 1,25(OH)2D3 prolonged the survival time of mice inoculated with myeloid leukemia cells (16). Since then, 1,25(OH)2D3 has been found to inhibit the growth of a number of primary and cultured tumor cell types, including breast, prostate, colon, and lymphomas. Although these findings suggest new therapeutic possibilities for 1,25(OH)2D3 in cancer, deleterious side effects such as hypercalcemia and soft tissue calcification prevent the parent 1,25(OH)2D3 being used as a therapeutic agent. Therefore, a great deal of effort has been made to develop new vitamin D analogues to dissociate antiproliferative and calce- 
mic activities. Ideal compounds should have potent effects in regulating cell growth and differentiation at concentrations well below those that cause side effects such as increased calcium absorption and bone mineralization. To date, several classes of vitamin D analogues with high antiproliferative and low calcemic activity have been identified, but the molecular mechanisms of their facilitated actions are poorly understood. Among them, 20-epi analogues represent a particularly noteworthy group. They are characterized by an inverted stereochemistry at carbon 20 in the side chain (Fig. 1). Their antiproliferative potency is several orders of magnitude higher than 1,25(OH)2D3, but their calcemic activity is comparable with 1,25(OH)2D3. The characterization of most 20-epi analogues has revealed that their affinity for VDR is similar to that of 1,25(OH)2D3 (17). In addition, the presence of vitamin D binding protein (DBP) in culture has little effect on the biological activity of 1,25(OH)2D3 or a 20-epi analogue, suggesting that the difference in cellular uptake of these ligand mediated by DBP is unlikely a major factor for the augmented activity of 20-epi compounds (18). It has been suggested that conformational effects on the VDR-LBD after analogue binding dictates their potent antiproliferative effects. In electrophoretic mobility shift assays using nuclear extracts from ligand treated cells, 20-epi analogue treatment had significantly stronger effects on VDR/RXR-DNA complex formation than 1,25(OH)2D3; however, this difference was limited to lower concentrations of ligands (19). Furthermore, the difference between 1,25(OH)2D3 and 20-epi compounds disappeared when using recombinant VDR and RXR proteins alone (20). Taken together, these observations suggest that additional factors present in nuclear extracts play critical roles in 20-epi analogue-induced transac- 
tivation. Thus, it is conceivable that 20-epi analogues may induce the binding of key coactivators at lower concentrations than 1,25(OH)2D3, ultimately leading to enhanced biological activity.

We demonstrate here that the binding of two 20-epi ana- 
logues, MC1288 and MC1627, result in the induction of mye- 
loid cell differentiation and the transcriptional induction of p21Waf1/Cip1, a key VDR target gene involved in growth inhibi- 
tion and differentiation (21), at picomolar doses. Compared with the interaction of VDR with RXR or coactivators such as GRIP-1, this dose-response most closely correlates to the ligand- 
dependent recruitment of the DRIP coactivator complex to 
VDR and the ability of the receptor to activate transcription in 
vitro. These results provide compelling links between the effi- 
ciency of the 20-epi analogues in inducing VDR/DRIP interac- 
tions, VDR-RXR transactivation in vitro, and their enhanced 
ability to induce cellular differentiation.

MATERIALS AND METHODS

Ligands—1,25(OH)2D3 and 20-epi analogues (MC1627, MC1288, and MC1292) were kindly provided by Leo Pharmaceutical Products Ltd. (Denmark). 1,25-dihydroxy-22-oxa-vitamin D3 (OCT) was generously provided by Chugai Pharmaceuticals (Japan).

Cell Culture and Nuclear Extract Preparation—Namalwa cells (ATCC) were cultured in 4-liter spinner flasks and maintained in RPMI medium supplemented with 5% fetal bovine serum, 5% calf serum, and 300 µg/ml glucose. Nuclear extracts were prepared by the method described by Dignam et al. (22). U937 cells (ATCC) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 300 µg/ml glucose.

FACS Analysis—Early log-phase growing U937 cells were treated with tested ligands ranging from 10−9 to 10−13 M or ethanol for 72 h. Approximately 2 × 106 cells were harvested, followed by washing twice with ice-cold phosphate-buffered saline containing 1% bovine serum albumin (BSA). Cells pellets were then resuspended in 250 µl of phos- 
phate-buffered saline containing 1% BSA. 2 µl of fluorescein isothio- 
cyanate-conjugated CD14 and 2 µl of phycoerythrin-conjugated CD11b (Caltag) were added to 75 µl of each sample; an isotype-matched anti- 
body was incubated with 75 µl of each sample as a control for nonspecific 
binding. Cells were incubated in the dark on ice for 45 min. After 
washing twice, cells were resuspended in 0.5 ml of phosphate-buffered saline containing 1% BSA. Stained cells were analyzed by a FACS (Becton Dickinson).

Transient Transfection—Approximately 107 U937 cells were col- 
cected, washed twice and resuspended in 400 µl of RPMI 1640. Cells 
were then transiently cotransfected with 10 µg of WWP-Luc (p21), 2 µg of pCMV-human RXR (pOTCO) by electropora- 
tion in a 4-mm cuvette. Transfected cells were pulsed together, evenly 
distributed to 100-nm plates, and treated with tested ligands ranging from 10−8 to 10−12 M or ethanol for 24 h. Treated cells were harvested and lysed. Cellular extracts were assayed for luciferase activity by dilution in cell culture lysis reagent (Promega) and measurement in 100 µl of luciferase assay reagent (Promega) in a lumino- 

Gust Fusion Protein Pull-down Assay—For GST-VDR LBD and DRIP complex interactions, 40 µg of GST-VDR LBD-(110–427) fusion protein immobi- 
ized on beads were incubated with 10−8 M tested ligands in 

Gst-binding buffer (20 mM Tris-HCl (pH 7.9), 180 mM KCl, 0.2 mM 
EDTA, 0.05% Nonidet P-40, 0.5 µg of carrier plasmid (pOTCO) by electropora- 
tion in a 4-mm cuvette. Transfected cells were pulsed together, evenly 
distributed to 100-nm plates, and treated with tested ligands ranging from 10−8 to 10−12 M or ethanol for 24 h. Treated cells were harvested and lysed. Cellular extracts were assayed for luciferase activity by dilution in cell culture lysis reagent (Promega) and measurement in 100 µl of luciferase assay reagent (Promega) in a lumino-
immobilized on beads were incubated with GST-binding buffer containing 3.5 mg/ml BSA at 4 °C for 2 h. GST fusion proteins were then incubated with purified recombinant VDR in the presence of tested ligands ranging from $10^{-2}$ to $10^{-12}$ M at 4 °C for 2 h. After washing three times, samples were resolved by SDS-polyacrylamide gel electrophoresis, followed by transferring on polyvinylidene difluoride membrane and detected for VDR binding by Western blotting.

**In Vitro Transcription Assay**—Transcription assays were performed as described previously (23) with the following modifications: 20 μl of each receptor incubation mix including 180 ng of recombinant VDR and RXR plus tested ligands ranging from $10^{-8}$ to $10^{-12}$ M or ethanol was incubated on ice for 45 min. The volume of receptor-ligand mix was 3 μl instead of the 10 μl used in previous assays (23), and the volume of nuclear extract was increased to 15 μl from 8 μl so that the final reaction volume was 25 μl, as described previously.

**RESULTS**

**Effects of Analogues on Myeloid Cell Differentiation**—To examine how vitamin D analogues might influence VDR transcriptional activation, we first sought those analogues that had very potent effects on a biological process which could then be correlated to a transcriptional response. To do so, we chose to examine vitamin D$_3$-induced myeloid cell differentiation, whereby myelomonoblastic cell lines, such as U937, induce to differentiate to monocyte/macrophages upon exposure to 1,25(OH)$_2$D$_3$ (24). The degree of differentiation can be quantitated by FACS by assaying the expression of macrophage-specific markers on the cell surface, such as CD11b and CD14.

When U937 cells were treated for 72 h with 1,25(OH)$_2$D$_3$ at concentrations ranging from $10^{-13}$ to $10^{-9}$ M, FACS analyses indicated that concentrations of $10^{-10}$ M 1,25(OH)$_2$D$_3$ were sufficient to induce differentiation while concentrations above $10^{-9}$ M had no greater effect (Fig. 2, A and B). In contrast, when cells were treated with two related analogues of vitamin D belonging to the 20-epi group, MC1288 and MC1627, detectable CD11b and CD14 levels were apparent at $10^{-12}$ M, fully two orders of magnitude less than 1,25(OH)$_2$D$_3$ (Fig. 2, C and D). Maximal CD14 and CD11b expression was detected with MC1627 at $10^{-11}$ M; at this concentration, no effect on U937
differentiation was measurable with 1,25(OH)$_2$D$_3$.

We next asked if the effects of these potent analogues on myeloid differentiation correlated to VDR-mediated activation of transcription of a relevant target gene. Previously, we identified the p21/Waf1/Cip1 gene among a set of several primary response genes that are transcriptional targets of VDR following addition of 1,25(OH)$_2$D$_3$ to U937 cells (21). As we observed before, treatment of U937 cells by 1,25(OH)$_2$D$_3$ resulted in a 5- to 10-fold induction of p21 transcription at 10$^{-9}$ M (Fig. 3). In contrast, significantly lower amounts of MC1627 and MC1288 were required to elicit strong p21 induction. A 6-fold induction was apparent at 10$^{-12}$ M of MC1288, and MC1627 and peak inductions of 15- to 20-fold were measured at 10$^{-10}$ M with MC1288 and MC1627, respectively (Fig. 3). No induction of p21 transcription was discernible at 10$^{-11}$ M, 1,25(OH)$_2$D$_3$. The hormone and analogue effects on p21 transcription correlated remarkably closely to the differentiation responsiveness of U937 cells. Thus, the dose-response of the 20-epi analogues on myeloid cell differentiation mirrors p21 transcriptional activation, further linking differentiation and the expression of this cell cycle inhibitor (21).

**Effects of 20-Epi Analogues on VDR-Cofactor Interactions—** Because of the astonishingly potent effects the 20-epi compounds had on p21 induction and myeloid cell differentiation, we wished to determine whether their activity could be correlated with an enhancement of known VDR-protein interactions that are functionally important. Such interactions would include, for example, VDR-coactivator binding or ligand-stimulated dimerization. An obligatory cofactor for VDR transactivation is its heterodimeric partner, RXR. We previously reported that three analogues containing 16-ene-23-yne-D$_3$ substitutions conferred distinct rate and equilibrium constants for VDR-RXR heterodimerization and specific DNA binding to a VDRE relative to the natural 1,25(OH)$_2$D$_3$ ligand (25). Here, we have used a glutathione S-transferase (GST) fusion to RXR to perform in vitro pull-down assays with purified VDR in the presence of 1,25(OH)$_2$D$_3$ or the two 20-epi analogues. When compared with the natural ligand, MC1627 was able to stimulate VDR/RXR heterodimerization at a lower dose (10$^{-11}$ M), whereas MC1288 did not differ significantly from 1,25(OH)$_2$D$_3$ (Fig. 4). At 10$^{-10}$ M, a greater stimulation of heterodimerization was observed with both analogues than with 1,25(OH)$_2$D$_3$ (Fig. 4). Thus, although we cannot rule out a contribution of the 20-epi compounds on VDR-RXR heterodimerization, the effects of the analogues on dimerization per se do not appear to mirror the same dose-response as observed in the differentiation and p21 transactivation assays.

Recruitment of transcriptional coactivators by VDR is also regulated by VDR ligands. Such coactivators include SRC1/NCoA-1, GRIP1/TIF2/NCoA2, ACTR/p/CIP/AIB1/RAC3, P/CAP, and CBP/p300. Several of these coactivators (i.e. the p160 family) are structurally related and have been shown to possess histone acetyl transferase activity. To test the effect the 20-epi analogues had on the interaction between VDR and members of the p160 family, we assessed GRIP-1/VDR interactions as an example using a GST pull-down assay. As shown in Fig. 5, MC1627 but not MC1288 was more effective than 1,25(OH)$_2$D$_3$ at lower concentrations in inducing the GRIP/VDR interaction (Fig. 5). The concentrations required for inducing 50% of maximal VDR/GRIP-1 interaction were 5.9 × 10$^{-10}$, 4.7 × 10$^{-10}$, and 4 × 10$^{-11}$ for 1,25(OH)$_2$D$_3$, MC1288, and MC1627, respectively. The difference between 1,25(OH)$_2$D$_3$ and MC1627 is approximately 1 log (Fig. 5B and Table I) and does not correlate with the 2 log difference observed using U937 differentiation or p21 transactivation as a
readout. Therefore, it is not likely that the increased effect these analogues have on VDR activity is because of a direct role in the GRIP-1/VDR induction.

Effects of Analogues on VDR-DRIP Interactions—Because the 20-epi analogues did modulate GRIP-1/VDR binding somewhat, we considered whether they might affect binding between VDR and other coactivators. The DRIPs comprise a multiprotein coactivator complex of at least thirteen subunits that we recently discovered and have been characterizing that bind to VDR and other nuclear receptors in a strictly ligand-dependent manner (12, 13). The DRIP complex lacks histone acetyl transferase activity, and its individual subunits do not share any homology to other characterized coactivators (12). A single subunit, DRIP205, directly associates with VDR via its ligand-binding domain. This association is dependent on the AF-2 subdomain of the receptor and one of two LXXLL (NR) motifs in the central portion of DRIP205 (residues 630–635).

To see whether the 20-epi analogues affected the DRIP205/VDR interaction, we employed a GST fusion protein containing the nuclear receptor interaction domain of DRIP205 to pull-down purified VDR in the presence of 1,25(OH)2D3 or the 20-epi compounds at concentrations ranging from 10−12 to 10−6 M. The results shown in Fig. 6A illustrate that 1,25(OH)2D3 induced the association of VDR and DRIP205 in a dose-dependent manner, beginning at 10−10 M. MC1627 and MC1288 exhibited significantly more potent effects on VDR/DRIP205 interactions than the natural ligand. The minimum concentration required for inducing the VDR/DRIP205 association was 10−12 M, at least 100 times lower than that of 1,25(OH)2D3 (Fig. 6, A and B and Table I).

We also tested two additional vitamin D compounds for their effects on VDR/DRIP205 interaction. MC1292, which, relative to other 20-epi compounds, induces a distinct conformation in the VDR-LBD, as indicated by protease digestion patterns,2 and confers a relatively low antiproliferative activity (only 4-fold higher than 1,25(OH)2D3) (17), here showed a similar VDR/DRIP binding pattern as 1,25(OH)2D3. Similarly, a chemically distinct vitamin D3 analogue, OCT, conferred weaker ability to induce the VDR/DRIP205 interaction than the 20-epi compounds and even 1,25(OH)2D3 (Fig. 6, A and B and Table I), suggesting that the biological effects of the 20-epi analogues may occur selectively through VDR/DRIP recruitment. Taking together all the data from Figs. 4–6, it appears that the most potent effects of the 20-epi analogues on VDR-cofactor interactions occur between the receptor and DRIP205. This observation suggests that the DRIP complex is the preferred VDR coactivator complex, at least in response to the 20-epi analogues.

Influence of 20-Epi Analogues on VDR Transactivation in Vitro—The VDR-DRIP205 results suggested that the ability of vitamin D analogues to induce VDR-DRIP binding might cor-

2 L. Binderup, personal communication.
relate with their effects on VDR-mediated transactivation, since we have previously found that the addition of the DRIP coactivator complex to a purified transcription system strongly potentiated VDR-RXR transactivation in response to 1,25(OH)_{2}D_{3} (12). Because the 20-epi compounds conferred the most potent effect on VDR-DRIP205 binding, and DRIP205 anchors the entire complex to VDR, we chose to focus our efforts on this class of vitamin D analogues. As expected, MC1288 and MC1627 were able to stimulate a stronger degree of recruitment of several subunits of the DRIP coactivator complex than 1,25(OH)_{2}D_{3} at 10^{-8} M (Fig. 7A). In particular, the binding to VDR of DRIP250, 240, 205, 100, and 97 were enhanced by the 20-epi compounds relative to 1,25(OH)_{2}D_{3}.

To directly link VDR/DRIP binding in response to analogue dose to transcriptional activity, we repeated the 20-epi analogue titration using a cell-free, VDR-RXR-responsive transcription assay. This allowed us to monitor VDR transcriptional enhancement as a consequence of analogue potency without the confounding effects that cellular uptake or metabolism of the compounds might have on the biological readout. With the natural ligand, activation by VDR-RXR above basal levels was weakly detectable at 10^{-9} M and reproducibly strong at 10^{-8} M (Fig. 7, B and C). As was the case with both the VDR/DRIP205 and VDR/DRIP complex interactions, the two 20-epi compounds showed a stronger transactivation by VDR-RXR in vitro at both of these concentrations; moreover, some analogue-stimulated transcription could be observed as low as 10^{-10} M (Fig. 7B). These results provide a strong correlation between the proactivity of the 20-epi analogues to recruit the DRIP complex and their ability to stimulate VDR-RXR transactivation in vitro at lower concentrations than 1,25(OH)_{2}D_{3}.

**FIG. 7.** A, vitamin D analogue-dependent interaction between the VDR LBD and the multisubunit DRIP coactivator complex from Namalwa B-cell nuclear extracts. Immobilized GST-VDR LBD was incubated with a Namalwa B-cell nuclear extract in the presence of ethanol (lane 1), 1,25(OH)_{2}D_{3} (lane 2), or MC1627 (lane 3), or MC1288 (lane 4), all at 10^{-8} M. Bound proteins were eluted by incubation with N-lauroylsarcosine. Eluted proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis and visualized by silver nitrate staining. B, vitamin D analogue-induced transactivation activity in a cell-free transcription assay. 50 ng of VDR-RXR in the presence of ethanol (lane 1) or tested ligands at indicated concentrations were used. A VDRE-linked E1B promoter G-less cassette template and a reference template (not shown) were included in each reaction. Transcription was initiated by the addition of nucleoside triphosphates and allowed to proceed for 45 min. Radiolabeled transcripts were recovered and separated on a 6% denaturing polyacrylamide gel. Data was quantitated with MacBas Version 2.5 program. C, quantitation of data shown in panel B. The results are normalized to a reference template and are expressed as fold-induction over the (-) ligand control set at a value of 1.0. Each point represents the mean of three independent experiments. 1,25(OH)_{2}D_{3}, MC1627, and MC1288 are denoted as (□), (○), and (○), respectively.

**DISCUSSION**

The promising therapeutic potential of vitamin D_{3} has stimulated interest in developing similar vitamin D compounds for a variety of clinical applications. Although several classes of analogues with high antiproliferative but low calcemic activities have been identified, the mechanisms by which these analogues possess potent effects in regulating cell proliferation and differentiation are poorly understood. In particular, whether these compounds work indirectly via pharmacokinetic phenomena or through direct conformational changes on VDR is still a matter for debate. It is certainly reasonable to assume that either mechanism is not mutually exclusive, in that different classes of compounds act through either route.

20-epi analogues were identified initially as potent inhibitors of cell proliferation. Because previous characterization of this particular group has suggested that their intrinsic binding affinities for VDR and DBP do not differ from those of the natural ligand (17, 18), 20-epi compounds represent a class of compounds that might instead act by affecting VDR structure, which in turn would somehow lead to enhanced VDR function and biological activity. Previous studies using nuclear extracts prepared from ligand-treated cells indicated that 20-epi analogues at low concentrations have much stronger effects on VDR/RXR-DNA complex formation than 1,25(OH)_{2}D_{3} (19). However, these results could not be duplicated in a cell-free system with recombinant VDR and RXR alone (20). We performed pull-down assays with purified RXR and VDR to examine the ligand effect on VDR/RXR dimerization and found little or no difference between 1,25(OH)_{2}D_{3} and 20-epi analogues at low concentrations. 20-epi analogues are therefore unlikely to affect VDR-mediated transactivation through enhanced VDR/RXR dimerization; instead, these analogues appear to increase
the biological activity of VDR by inducing receptor interaction with coactivators present in nuclear extracts that are important for VDR-mediated transactivation, such as p160 family members, like GRIP-1, or, more potently, DRIP205, the subunit bridging the interaction of VDR with the multi-protein DRIP complex. The dose-response patterns of DRIP recruitment by VDR in response to MC1627 and MC1288 were remarkably similar to the induction of U937 cell differentiation and p21 transactivation at the same low concentrations of these compounds. That these cellular responses to the analogues are occurring through the ability of VDR to regulate transcription is closely correlated to the ability of the 20-epi analogues to induce VDR-RXR-dependent transactivation in a cell-free, in vitro transcription system at concentrations (i.e. 10^{-10} M) at least 100-fold lower than the minimum concentration required for 1,25(OH)_{2}D_3 induction (Fig. 7). To our knowledge, this is the first direct demonstration that vitamin D analogues can act directly at the level of gene transcription and provides the best evidence to date that some classes of analogues can influence biological readouts directly through VDR-dependent transactivation. In agreement with our results, the reported IC_{50} for inhibition of cell proliferation by the 20-epi analogues correlates with their ED_{50} for VDR/DRIP 205 interaction. For example, IC_{50} values for 1,25(OH)_{2}D_3, MC1288, and MC1292 are 1.4 × 10^{-8}, 2.8 × 10^{-10}, and 3.4 × 10^{-9}, respectively (17); and their ED_{50} values are 7 × 10^{-10}, 7 × 10^{-12}, and 2.2 × 10^{-10}, respectively (Table 1).

Among nine vitamin D analogues we tested, the strongest effects on VDR/DRIP205 interaction were limited to three 20-epi analogues. Although some ligands such as OCT possess potent antiproliferative effects, they show similar or even weaker effects on DRIP 205 binding to VDR as 1,25(OH)_{2}D_3, indicating that multiple mechanisms may account for the biological activities of different ligands. One possibility is that pharmacokinetic phenomena may lead to their altered activities. For example, the affinity of OCT for DBP is 580-fold lower than that of 1,25(OH)_{2}D_3 (28). Lower affinity for DBP may increase cellular uptake and thus activity in target cells. A second possibility is that the existence of multiple VDR coactivators may allow target cells to respond to different stimuli in a more specific manner. Besides the DRIP complex, other coactivators, such as SRC-1, TIF-2/GRIP-1, and AIB1/ACTR have also been found to interact with the VDR-LBD and enhance VDR-mediated transcription in transfection assays. For example, it has been reported that OCT selectively induces VDR to interact with TIF2 but not with SRC-1 or AIB1/ACTR (29).

How do different analogues confer differential effects on VDR activity? The most likely explanation is that distinct classes of analogues induce different LBD conformations, which in turn might be selective for particular kinds of coactivators. Several recent LBD crystal structures have established that upon ligand binding, the AF-2 helix (helix 12) undergoes a major reorientation in the context of the overall LBD structure, forming part of a charged clamp that accommodates the binding of coactivators (30, 31). Moreover, the crystal structure of the rat α1 thyroid hormone receptor LBD in complex with a thyroid hormone agonist indicates that the AF-2 domain contributes to hormone binding, suggesting a structural role for ligand in forming the active conformation of the LBD (32). In contrast, estrogen antagonists such as tamoxifen and raloxifene appear to alter the position of the AF-2 helix such that helix 12 occupies space in the LBD in a similar manner as has been described for SRC-1, thereby precluding coactivator binding (27, 33). Alterations in VDR structure in response to the 20-epi compounds might result in a conformation that preferentially accommodates DRIP205 (and therefore the entire DRIP complex) over other coactivators, such as SRC-1 or GRIP-1. Differential effects on VDR-LBD structure by analogues relative to 1,25(OH)_{2}D_3 is consistent with the observations that the binding of 1,25(OH)_{2}D_3 versus 20-epi analogues result in unique ligand-dependent VDR sensitivities to proteases. When liganded VDR was treated with trypsin or chymotrypsin, 20-epi analog-VDR complexes showed digestion patterns significantly different from the 1,25(OH)_{2}D_3-VDR complex, indicating the intrinsic alterations in LBD conformation induced by 1,25(OH)_{2}D_3 versus 20-epi analogues (19). Differential preferences for coactivators may not be surprising, given that GRIP-1 is structurally unrelated to DRIP205, except the requirement of one of two LXXLL motifs for interaction with the LBD (26). Moreover, while an intact AF-2 core is essential for VDR/DRIP binding, the details of these contacts must be different from that for VDR/SRC-1. The glutamate residue at position 420 of VDR is critical for SRC-1 binding but not for DRIP binding, reflecting subtle but perhaps key differences between the mode of interactions of these two coactivators with the LBD (13).

In summary, our findings suggest that the DRIP complex, and most likely other coactivators, play a central role in ligand-dependent, VDR-mediated transcription which in turn is reflected in key biological responses, such as anti-proliferation and differentiation. The strong correlation between VDR/DRIP interactions and cellular responses to vitamin D analogues might be valuable in developing high throughput methodologies for discovering new lead vitamin D compounds that possess highly potent anti-proliferative activities.

Acknowledgments—We thank Lise Binderup and Leo Pharmaceutical Products for 20-epi compounds, and Chugai Pharmaceuticals for OCT. We also thank C. Rachez, V. Bromleigh, and M. Stallcup for reagents, and G. Farner and C. Rachez for constructive comments during the preparation of the manuscript.

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doi: 10.1074/jbc.274.24.16838

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