A Central Dinucleotide within Vitamin D Response Elements Modulates DNA Binding and Transactivation by the Vitamin D Receptor in Cellular Response to Natural and Synthetic Ligands*

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There is considerable divergence in the sequences of steroid receptor response elements, including the vitamin D response elements (VDREs). Two major VDRE-containing and thus 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃)-regulated genes are the two non-collagenous, osteoblast-derived bone matrix proteins osteocalcin and osteopontin. We observed a stronger induction of osteopontin than osteocalcin mRNA expression by 1,25-(OH)₂D₃. Subsequently, we have shown that vitamin D receptor/retinoid X receptor α (VDR/RXRα) heterodimers bind more tightly to the osteopontin VDRE than to the osteocalcin VDRE. Studies using point mutants revealed that the internal dinucleotide at positions 3 and 4 of the proximal steroid half-element are most important for modulating the strength of receptor binding. In addition, studies with VDRE-driven luciferase reporter gene constructs revealed that the central dinucleotide influences the transactivation potential of VDR/RXRα with the same order of magnitude as that observed in the DNA binding studies. The synthetic vitamin D analog KH1060 is a more potent stimulator of transcription and inducer of VDRE binding of VDR/RXR in the presence of nuclear factors isolated from ROS 17/2.8 osteoblast-like cells than the natural ligand 1,25-(OH)₂D₃. Interestingly, however, KH1060 is comparable or even less potent than 1,25-(OH)₂D₃ in stimulating VDRE binding of in vitro synthesized VDR/RXRα. Thus, the extent of 1,25-(OH)₂D₃- and KH1060-dependent binding of VDR/RXRα is specified by a central dinucleotide in the VDRE, and the ligand-induced effects on DNA binding are in part controlled by the cellular context of nuclear proteins.

The classic role of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) includes regulating the expression of genes involved in calcium and bone metabolism (1). In addition, the hormone is an important mediator of cell growth and differentiation (2, 3). In a large number of 1,25-(OH)₂D₃-dependent target genes, distinct vitamin D response elements (VDREs) have been identified that are composed of two hexameric half-sites separated by three base pairs (DR3-type VDRE). The vitamin D receptor (VDR/retinoid X receptor (RXR) complex binds with defined polarity to these VDREs with the RXR occupying the distal half-site and the VDR the proximal half-site (4–7).

The spacing of the half-sites plays an important role in specifying the type of receptor pair that interacts with hormone response elements (7). There is considerable divergence in the nucleotide sequence of the distal and proximal half-site, which is attributable at least in part to redundancy in the consensus recognition motifs of the VDR and the RXR (8). The sequences of steroid hormone half-elements also influence the relative affinities for different steroid hormone receptors and thus may support receptor-specific gene activation by discriminating between different receptors (7). In addition, specific nucleotides in the VDRE appear to influence the conformation of bound VDR/RXR heterodimers, reflected by a VDRE-specific alteration in epitope accessibility (6). Furthermore, minor changes in the nucleotide sequence of half-elements can change a negative VDRE that suppresses transcription into a positive VDRE, which transactivates in a 1,25-(OH)₂D₃-dependent manner (9). The impact of minor changes in nucleotide sequences on receptor DNA binding is not restricted to VDR/VDRE interactions, as evidenced by half-element point mutations that have been shown to alter the affinity (10) or identity of the cognate receptor (11).

Two major 1,25-(OH)₂D₃-regulated genes are the two non-collagenous, osteoblast-derived bone matrix proteins osteocalcin and osteopontin. These genes have sequence variations in their VDREs (Refs. 12–14 and Table I). To gain insight into the biological relevance of nucleotide variation in naturally occurring VDREs for the action of 1,25-(OH)₂D₃ and its potent analog KH1060 (15–18), we examined the binding and activity of the osteocalcin and osteopontin VDREs. The main finding of our study is that nucleotide differences between osteopontin and osteocalcin VDREs affect DNA binding and transactivation in response to 1,25-(OH)₂D₃ and its potent analog, KH1060. Our findings show that the primary sequence of VDREs may influence the biological activities of natural and synthetic ligands that bind to the VDR. Finally, the strong biological potency of the analog KH1060 is only reflected by DNA binding of VDR/RXR heterodimers in the environment of nuclear proteins and not by the binding of in vitro synthesized VDR and RXRα, underscoring the importance of a cellular context for ligand-induced DNA binding.
E X P E R I M E N T A L P R O C E D U R E S

Reagents—1,25-(OH)2D3 and KH1060 were a gift from L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. a-Minimal essential medium was from Sigma. VDRE-encoding oligonucleotides, fetal bovine serum, penicillin, streptomycin, and L-glutamine were purchased from Invitrogen. The coupled in vitro transcription and translation rabbit reticulocyte lysate system (TNT lysate assay), Nhel, BglII, Tfx-50, the pG3 control and promoter plasmids, and the luciferase assay reagent were from Promega, Madison, WI. The rat osteocalcin and osteopontin cDNA probes were generously provided by Dr. M. Noda (West Point, PA). The rat osteocalcin and osteopontin cDNA control and promoter plasmids, and the luciferase assay reagent were from Promega, Madison, WI. The rat osteocalcin and osteopontin cDNA probes. The Northern blots presented in panels A–C were quantitated by densitometric scanning, and the absorbance ratios of osteocalcin mRNA or osteopontin mRNA to that of glyceraldehyde-3-phosphate dehydrogenase mRNA are expressed (D).

EXPERIMENTAL PROCEDURES

Reagents—1,25-(OH)2D3 and KH1060 were a gift from L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. a-Minimal essential medium was from Sigma. VDRE-encoding oligonucleotides, fetal bovine serum, penicillin, streptomycin, and L-glutamine were purchased from Invitrogen. The coupled in vitro transcription and translation rabbit reticulocyte lysate system (TNT lysate assay), Nhel, BglII, Tfx-50, the pG3 control and promoter plasmids, and the luciferase assay reagent were from Promega, Madison, WI. The rat osteocalcin and osteopontin cDNA probes were generously provided by Dr. M. Noda (West Point, PA).

Cells—The osteoblast-like osteosarcoma cell line ROS 17/2.8 was cultured in α-minimal essential medium supplemented with 2 mm L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% p-glucose, and 10% fetal bovine serum. When the cells reached subconfluence, the medium was replaced by α-minimal essential medium containing 2% charcoal-treated fetal bovine serum. After 24 h of culture, ligand incubations or transfections were performed for the indicated period of time.

RNA Extraction and Northern Blot Analyses—ROS 17/2.8 cells were cultured as described above, and 24 h after the addition of 1,25-(OH)2D3 RNA was isolated. RNA isolation was performed according to the method of Chomczynski and Sacchi (19). Electrophoresis of total RNA (30 μg) through a formaldehyde gel and Northern blotting (GeneScreen filters) were performed according to the method described by Davis et al. (20). The cDNA probes were previously (5) and the rat osteocalcin fragment, a 1.3-kb rat osteopontin fragment, and a 0.8-kb human glyceraldehyde-3-phosphate dehydrogenase fragment.

In Vitro Synthesis of VDR and RXRa and Preparation of Nuclear Extracts—Human recombinant VDR and RXRa were in vitro synthesized with the Promega Tnt lysate assay according to the instructions of the manufacturer using cDNA encoding for human VDR (in pGem4; a gift from M. R. Haussler, University of Arizona, Tucson, AZ) and RXRa (in pSG5; a gift from P. Chambron, INSERM, Strasbourg, France). For the preparation of nuclear extracts, ROS 17/2.8 rat osteoblast-like cells were incubated for 1 h with 1,25-(OH)2D3 or KH1060. Next, nuclear extracts were prepared in 20 mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol, and 0.2 mM EDTA according to the method described previously (6).

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed as described earlier (6). In brief, 10 μl of a mixture of in vitro synthesized VDR and RXRa was treated for 15 min at 37 °C with a ligand (1,25-(OH)2D3 or KH1060), or 10 μl of diluted nuclear extract (5 μg of protein) was incubated for 15 min at room temperature with 10 μl of 32P-labeled oligonucleotides (10 fmol). The oligonucleotides used in this study are presented in Table I. The protein-DNA complexes formed were separated on a 5% polyacrylamide gel (acrylamide/bisacrylamide, 80:1) in 0.5× TBE buffer (1× TBE is 50 mM Tris, 50 mM boric acid, and 1 mM EDTA). In the competition experiments, 32P-labeled oligonucleotides and different concentrations of unlabeled oligonucleotides were mixed and subsequently incubated with nuclear extract. The VDR ligand-responsive complex was visualized by exposure to Fuji RX medical x-ray film. All electrophoretic mobility shift assays were performed with excess probe, but for reasons of clarity in most figures only the shifted bands are shown.

Transactivation Assay—Oligonucleotides with BglII- and Nhel-compatible ends containing the rat osteocalcin (5′-CTACCTGCGACTCTGGGTGGAGAGACGACATTCTGAG-3′) or the OC/OP VDRE (5′-CTACCTGCGACTCTGGTTGATGGTCTACATTAGA-3′; VDREs) were used in bold; the underlined nucleotides are distinct from the corresponding nucleotides within the proximal rat osteocalcin VDRE half-sites and the multiple cloning site of the pGL3 promoter vector containing luciferase cDNA as the reporter gene. Sequences were confirmed by dyeexy sequencing using a 310 genetic analyzer (PerkinElmer Life Sciences). Cells (ROS 17/2.8 rat osteoblast-like cell line, MG-63 human osteoblast-like cell line, ECC-1 human endometrium carcinoma cell line, and MCF-7 human breast cancer cell line) cultured in 10 cm2 dishes were transfected with 5 μg of reporter plasmid/well using Tfx-50. The luciferase mRNA are expressed (D).
RESULTS

Vitamin D-regulated Expression of Osteocalcin and Osteopontin mRNA—Incubation for 24 h of ROS 17/2.8 osteoblasts with 1,25-(OH)₂D₃ resulted in a dose-dependent induction of both osteocalcin and osteopontin mRNA expression (Fig. 1). Fig. 1 clearly demonstrates that 1,25-(OH)₂D₃ more potently induces osteopontin mRNA than osteocalcin mRNA expression in ROS 17/2.8 osteoblast-like cells.

Ligand-dependent Binding of the VDR/RXR Heterodimer—Electrophoretic mobility shift assays revealed that the binding of in vitro synthesized VDR/RXR/H₉₂₅₁ to the VDREs (Table I) was 1,25-(OH)₂D₃ concentration-dependent. In the absence of 1,25-(OH)₂D₃, only minor VDR/RXR/H₉₂₅₁ binding was observed, and 1,25-(OH)₂D₃ enhanced the binding of in vitro synthesized recombinant VDR/RXR to the human and rat osteocalcin VDREs and the mouse osteopontin VDRE in a dose-dependent manner (Fig. 2). The importance of Fig. 2 is that the VDR/RXR heterodimer preferentially interacts with the osteopontin VDRE, exhibits intermediate binding to the human osteocalcin VDRE, and binds less strongly to the rat osteocalcin VDRE. The electrophoretic mobility shift assays were also performed with nuclear extracts of ROS 17/2.8 osteoblast-like cells, for which we have previously demonstrated with monoclonal antibodies that 1,25-(OH)₂D₃ induces VDR/RXR complexes (6). Also here, the intensity of the shifted complex was strongest for the osteopontin VDRE and lowest for the rat osteocalcin VDRE (Fig. 3A).

Ligand Specificity for Binding Requires a Cellular Context—In comparison with 1,25-(OH)₂D₃ we also investigated the effect of the biologically very active 1,25-(OH)₂D₃ analog KH1060 (15–18) on VDR/RXR VDRE binding. Using in vitro synthesized VDR/RXR and the mouse osteopontin VDRE, KH1060 was only slightly more potent than 1,25-(OH)₂D₃. Unexpectedly, in view of its potent induction of osteocalcin production (15), KH1060 and 1,25-(OH)₂D₃ had a comparable stimulatory effect on VDR/RXR complex formation with 1,25-(OH)₂D₃. Interestingly, however, when nuclear extracts of 1,25-(OH)₂D₃- or KH1060-treated ROS 17/2.8 osteoblast-like cells were used, KH1060 induced DNA binding more strongly than 1,25-(OH)₂D₃ not only to the human osteocalcin
FIG. 3. Binding of nuclear proteins of ROS 17/2.8 osteoblast-like cells to hybrid rat osteocalcin and mouse osteopontin (MOP) VDREs. Electrophoretic mobility shift assays were performed with nuclear extracts from 1,25-(OH)_{2}D_{3} or KH1060-treated (10^{-10} or 10^{-8} M, 1 h) ROS 17/2.8 osteoblast-like cells. Nuclear extracts were incubated with 10 fmol of 32P-labeled oligonucleotides encoding for wild-type (A) and substitution mutation VDREs (B) (see Table I for VDRE sequences). The arrowheads indicate shifted complexes. In panel C, a computerized optical density scan of the shifted complex is shown. The absorbance value of the shifted ROC VDRE-VDR/RXR complex at 10^{-8} M 1,25-(OH)_{2}D_{3} was set to 1. Data represent the means of four independent experiments ± S.E.
VDRE (data not shown) and osteopontin but also to the rat osteocalcin VDRE (Fig. 3A). Thus, bone cell nuclear proteins are required for VDR/RXR binding to reflect physiological responsiveness to the hormone.

The VDR Binding Site of the Mouse Osteopontin Gene Confers High Affinity DNA Binding—To study in more detail the contribution of the various hexamer motifs in this differential preference for DNA binding, we introduced substitution mutations, replacing rat osteocalcin VDRE half-sites with osteopontin VDRE half-sites (Table I). With both nuclear extracts (Fig. 3B) and in vitro synthesized VDR and RXR (Fig. 4), replacement of the distal rat osteocalcin VDRE half-element with the corresponding osteopontin VDRE half-site (OP/OC) only slightly increased the intensity of the 1,25-(OH)_2D_3- and KH1060-induced shifted band. In contrast, substitution of the proximal half-site alone (OC/OP) or in combination with the distal half-site (OP/OP) led to a strong increase in DNA binding to levels comparable with that of the intact osteopontin VDRE. This was observed with both in vitro synthesized VDR/RXR and nuclear extracts and with 1,25-(OH)_2D_3 as well as KH1060 (Figs. 3 and 4). These findings clearly show that the proximal VDRE half-site, i.e. the VDR-binding site, has the largest impact on the extent of VDR/RXR binding to DNA.

Both Position and Sequencing of the VDRE Half-sites Are Critical Components—Using single substitution mutations, we further investigated which nucleotide(s) in the proximal half-site is/are significant in determining the observed differences in VDRE binding. The significance of the 3T and 4T nucleotides in the proximal half-site of the osteopontin VDRE was already noted (6, 21). Here, we show that the introduction of one (PM 3T, PM 4T) or both of these nucleotides (PM 3T4T) strongly enhanced 1,25-(OH)_2D_3-induced VDR/RXR VDRE binding. Additional substitution of 1G (PM 1G3T and PM 1G4T) had no clear supplementary effect (Fig. 5).

Sequence Variations within VDRE Half-sites Determine VDR/RXR Affinity for DNA Binding—We also studied whether the differences in binding to VDREs are reflected by the differences in affinity for these VDREs. Competition analysis using ^32P-labeled rat osteocalcin VDRE and increasing amounts (10–1,000 fmol) of unlabeled competitor oligonucleotides (rat osteo-
calcium or mouse osteopontin VDRE) showed that in vitro synthesized VDR/RXR displayed an increased affinity for mouse osteopontin VDRE (Fig. 6). We further investigated the impact of differences in nucleotide sequences on the affinity of nuclear extracts for the wild-type rat osteocalcin VDRE and the OC/OP VDRE. Competition assays revealed that nuclear proteins of ROS 17/2.8 osteoblast-like cells displayed an increased binding affinity for OC/OP VDRE (Fig. 7).

Strength of VDR/RXR Binding to VDREs Determines Ligand-dependent Transactivation—Finally, to establish the functional consequences of differences in VDRE binding strength, we monitored ligand-dependent transactivation by the VDR using VDRE-driven luciferase reporter gene constructs that were transfected into ROS 17/2.8 osteoblast-like cells. ROS 17/2.8 osteoblast-like cells were transfected with a pGL3 luciferase reporter vector containing rat osteocalcin or OC/OP VDRE sequences. As shown in Table I, these VDREs differ only by 3 nucleotides in the proximal half-site. We found that the transactivation activity of 1,25-(OH)2D3 and KH1060 was enhanced 2-fold when the first, third, and fourth nucleotides of the proximal half-site of the rat osteocalcin VDRE were replaced by the corresponding nucleotides of the mouse osteopontin VDRE (Fig. 8). Similar findings were observed using other cell lines, i.e. MG-63 osteoblast-like cells, MCF-7 breast cancer cells, and ECC-1 endometrium cancer cells (data not shown). These data corroborate our initial observations on the more potent induction of osteopontin than of osteocalcin mRNA expression by 1,25-(OH)2D3.
In this study, we have shown that the biological activity of osteocalcin and osteopontin enhancer VDREs depends on specific nucleotides in the proximal half-sites, providing a mechanism to explain the differences in mRNA expression of these genes in osteoblastic cells. The key mutations we have tested involving receptor-dependent repression. An important observation in the present study is the difference in intensity of DNA-bound complexes between the three naturally occurring VDRE types tested. We show that the ligand-induced binding of VDR/RXR complexes to the rat osteocalcin VDRE was less pronounced compared with the binding to human osteocalcin and mouse osteopontin VDREs. This is in line with the previously described differences between these VDREs (22–25). We also show that the intensity of ligand-induced VDR/RXR binding to functional VDREs can mainly be attributed to the VDR half-site, in particular to the 3T and/or 4T. The impact on the extent of VDR/RXR DNA binding by minor changes in nucleotide sequences is also illustrated by the work of others (9, 26, 27). Ozono et al. (27) showed that a non-VDR binding accessory element within the rat 24-hydroxylase gene was converted to a VDR-binding site when the fourth nucleotide within its proximal half-site was substituted by adenine or thymidine. Koszewski et al. (9) demonstrated that two mutations in the proximal half-site of the avian parathyroid hormone (PTH) VDRE converted the negative activity of this VDRE into a positive one. The large impact of only small changes in nucleotide sequences on receptor-DNA binding is not restricted to VDR-VDRE interaction. For instance, within estrogen response elements a change of 1 base pair in the proximal half-site (the vitellogenin A2 estrogen response element) resulted in 3-fold lower estrogen receptor affinity (10), and the introduction of two mutations converted the vitellogenin A2 estrogen response element into a glucocorticoid responsive element (11). These reports and the present study demonstrate that only minor changes in the nucleotide sequences of nuclear hormone response elements can affect receptor-DNA binding and receptor conformation. This will, in its turn, have an impact on the recruitment of cofactors to the receptor-DNA complex. Altogether, nucleotide variations within response elements can have a major influence on the transcriptional activation of the target gene.

In addition, the present paper shows that the increased biological potency of the vitamin D analog KH1060 (15–18) is not reflected by an increased binding of in vitro synthesized VDR and RXRs to various VDREs. KH1060-induced binding of in vitro synthesized VDR/RXRs to the mouse osteopontin VDRE was slightly increased, whereas binding to human osteocalcin and rat osteocalcin VDREs was comparable with or even less than 1,25-(OH)2D3. Studies by Imai et al. (28) with
RO 24-2637 and RO 23-7553 also demonstrated a lack of parallelism between the potency of these analogs to induce the binding of recombinant human VDR and RXRα to the human osteocalcin VDRE and the ability of these analogs to activate a human osteocalcin VDRE-driven reporter gene. However, when we performed electrophoretic mobility shift assays with nuclear extracts of ROS 17/2.8 osteoblast-like cells, the biological potency of KH1060 was paralleled by an increased binding of nuclear factors to the different VDREs studied. These observations underline the absolute importance of the cellular context, i.e., the absence or presence of cellular cofactors for the interaction between VDR/RXR and VDRE and the observation that KH1060 induced VDR/RXR-DNA binding that reflects its increased biological potency (15–18). Therefore, this study implicates the significance of these nuclear cofactors for determining the extent of transcriptional activity. An important issue for following this is the identification of ligand-specific cofactors. In general, our findings are consistent with the concept that the dinucleotide motif may alter the conformation of VDR/RXR heterodimers, their affinity for DNA, and the intrinsic transactivation potential, which provides a framework for understanding the different biological responses of cells to 1,25-(OH)2D3 and its analogs.

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