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Selective Gene Regulation by Vitamin D Receptor *via* Protein Kinase A Activation in Mouse Osteoblastic Cells

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Vitamin D receptor (VDR) is a member of nuclear receptor family of transcription factors and mediates the molecular actions of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).[1,2] The binding of $1,25(OH)_2D_3$ to its receptor VDR translocates the liganded receptor from cytoplasm into the nucleus and induces heterodimerization of VDR with its partner, the retinoid X receptor (RXR), on a specific DNA sequences termed vitamin D response element (VDRE).[3] VDR-ligand interaction is a major regulatory point in the regulation of calcium, bone development and mineralization and phosphorus homeostasis.[4]

Although most nuclear receptors are believed to function in response to their cognate ligands, a few of them have the capacity to recognize and bind regulatory sequences of target genes in the absence of a ligand and thus control transcriptional activity of the target genes.[5-7] Two related corepressors, silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR) have been identified in yeast two-hybrid assays based on their affinity to unliganded nuclear receptors.[8] In a subsequent report, unliganded thyroid receptor was shown to associate with repressive NCoR/HDAC3 complex and keep the local histones from coactivators harboring histone acetyltransferase (HAT) activity.[9]

VDR level is regulated by various stimuli *via* several signaling pathways. Although the basal level of VDR protein is minimally maintained by proteasome-mediated degradationin most cells, binding of $1,25(OH)_2D_3$ to VDR induces structural alterations and this conformational changes stabilize and significantly increase VDR proteinlevel while transcriptional autoregulation also accounts for part of the effect.[10] Protein kinase A (PKA) activators such as forskolin (FSK) and parathyroid hormone (PTH) are known as strong inducers of VDR in the absence of $1,25(OH)_2D_3$.[11,12] However, the effects of VDR upregulation by PKA activators in unliganded state on target genes have not been investigated.

In this study, to examine the effect of VDR upregulation by the activation of PKA, we chose MC3T3-E1 mouse osteoblastic cells as a model system and attempted to analyze transcriptional activity of two VDR target genes, 25-hydroxyvitamin D 24-hydroxylase (Cyp24) and osteopontin (Opn). In response to $1,25(OH)_2D_3$, VDR level was dramatically upregulated in a time-dependent manner (Fig. 1(a)). Activation of PKA phosphorylates a transcription factor

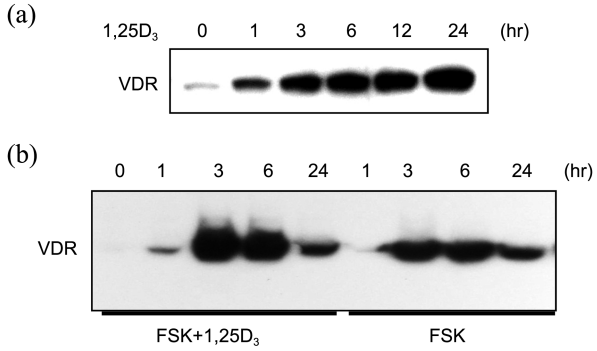

Figure 1. Upregulation of VDR by $1,25(OH)_2D_3$or a PKA activator FSK. (a) MC3T3-E1 cells were treated with $1,25(OH)_2D_3$ (10^{-7} M) for indicated periods and then subjected to Western blot analysis using monoclonal antibody 9A7 to VDR. (b) MC3T3-E1 cells were treated with FSK (10^{-6} M) or combination with $1,25(OH)_2D_3$ (10^{-7} M) for indicated periods and then subjected to Western blot analysis using monoclonal antibody 9A7 to VDR. $1,25D_3$ stands for $1,25(OH)_2D_3$.

cAMP response element-binding (CREB), which then binds to specific DNA sequences in gene's promoter and increases transcriptional output.[13] However, this signal also stimulates the production of inducible cAMP early repressor (ICER), an endogenous inhibitor of CREB-mediated transcription, generating bell-shaped transcriptional induction of downstream target genes.[14] As expected, a PKA activator FSK increased VDR level but exhibited maximal level of induction in 3-6 hrs of treatment (Fig. 1(b)). Combined treatment with both $1,25(OH)_2D_3$ and FSK significantly increased the VDR amount in an additive manner.

To analyze transcriptional regulation of VDR, MC3T3-E1 cells were treated with several inducers of VDR level for 24 hrs.12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C (PKC) activator, was previously shown to functionally cooperate with $1,25(OH)_2D_3$ to increase the level of human VDR.[15] However, TPA alone did not seem to regulate the levels of VDR (Fig. 2(a)). FSK was the most powerful inducer of VDR transcription whereas $1,25(OH)_2D_3$ and TPA marginally increased VDR transcript (Fig. 2(a)). $1,25(OH)_2D_3$ appears to decrease FSK-induced VDR level. Cyp24 is induced only in the presence of $1,25(OH)_2D_3$ but the induction was further consolidated by FSK and TPA as previously reported.[15] PTH is a polypeptide hormone secret-

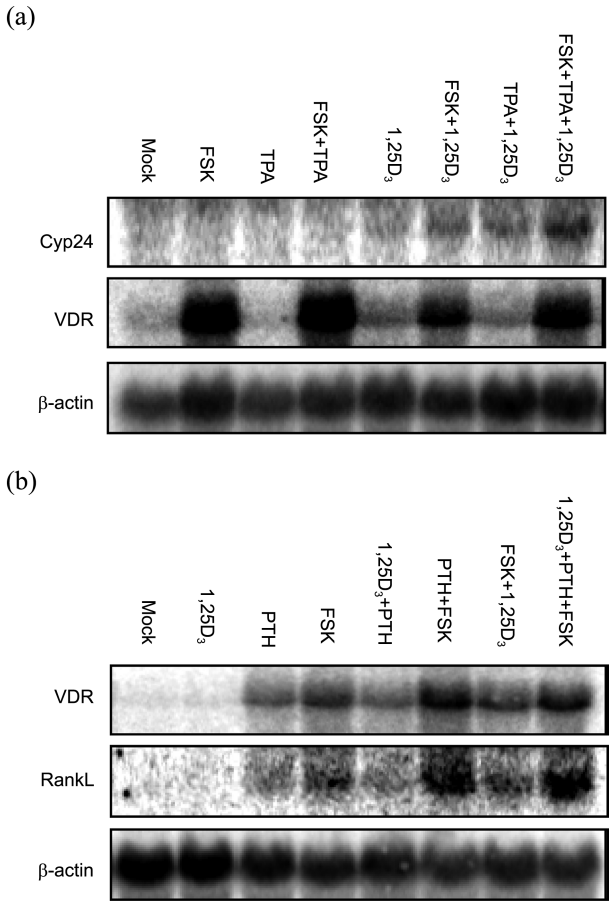

Figure 2. Transcriptional regulation of VDR by $1,25(OH)_2D_3$, FSK, TPA or combinations. (a) MC3T3-E1 cells were treated with $1,25(OH)_2D_3$ (10^{-7} M), FSK (10^{-6} M) and/or TPA (100 ng/mL) for 24 hrs and 20 g of total RNA was subjected to Northern blot analysis. (b) Primary mouse osteoblasts were treated with $1,25(OH)_2D_3$ (10^{-7} M), FSK (10^{-6} M) and/or another PKA activator PTH for 24 hrs and 20 g of total RNA was subjected to Northern blot analysis.

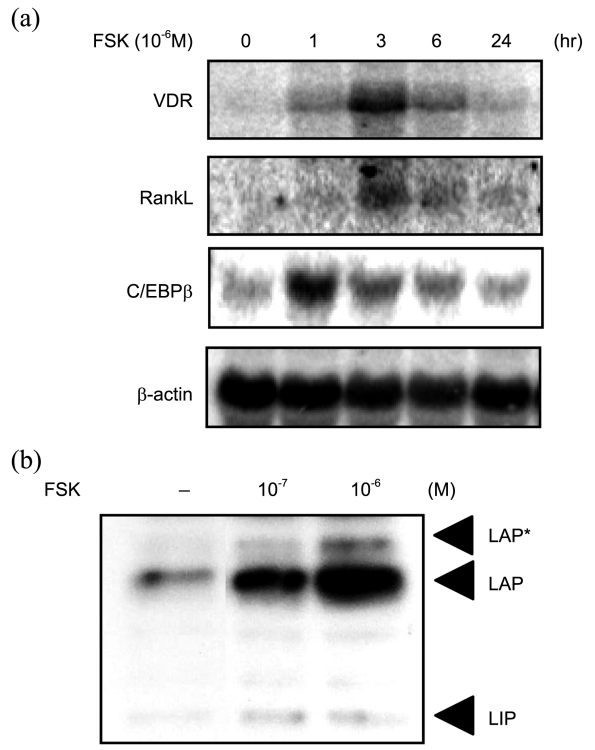

Figure 3. Induction of C/EBPβ by a PKA activator FSK (a) MC3T3-E1 cells were treated with FSK (10^{-6} M) for indicated periods and then subjected to Northern blot analysis. (b) MC3T3-E1 cells were treated with FSK (10^{-6} M) for 24 hrs and then subjected to Western blot analysis. C/EBPβ isoforms (40 kDa LAP*, 35 kDa LAP and 20 kDa LIP) are indicated by arrowheads.

ed by chief cells of the parathyroid glands and functions as a natural PKA activator.[16] Since MC3T3-E1 cells lack the expression of PTH receptor, we decided to use primary osteoblasts isolated from mouse cavalry, which are known to respond to PTH.[17] Results indicate that both PKA activators markedly increased VDR level while $1,25(OH)_2D_3$ does not seem to have clear effects in Northern blot analysis (Fig. 2(b)). RankL, a VDR target gene, followed the similar pattern to VDR, confirming the importance of VDR as a transcriptional regulator. These data demonstrate that the activation of PKA strongly increases VDR level mainly through the transcriptional regulation.

Upon the activation of PKA, several coactivators are recruited on the regulatory sequences of the VDR locus for the production of the transcript. One of the key coactivators required to be recruited is a transcription factor, the CCAAT enhancer-binding protein β (C/EBPβ). C/EBPβ produces several N-terminally truncated isoforms.[15] 35 kDa Liver-enriched activator protein (LAP) and 40 kDa LAP* function as transcriptional activators whereas 20 kDa protein liver-enriched inhibitory protein (LIP) is regarded as a functional antagonist of LAP and LAP*.[18] C/EBPβ induction was peaked in 1-3 hrs of FSK treatment in MC3T3-E1 cells, indicating that the C/EBPβ upregulation precedes FSK-stimulated VDR induction (Fig. 3(a)). In addition, the induction levels of activating isoforms LAP and LAP* by FSK are much higher than the changes in the amount of LIP (Fig. 3(b)). The results indicate that PKA activation exerts its effects on the increase of VDR level by the induction of activating isoforms of C/EBPβ.

Next, we explored the possibility that increased production of VDR by a PKA activator enables its binding to the target genes. In chromatin immunoprecipitation (ChIP) assays, strong VDR binding was observed on both Cyp24 and Opn promoters in response to $1,25(OH)_2D_3$ (Fig. 4(a)). Opn VDRE is composed of two identical half-sites separated by 3 base pair spacer region.[19] Due to symmetric nature of the sequences and the ability of VDR without its binding partner RXR to bind Opn VDRE in electrophoretic mobility shift assays (EMSA), it has been proposed that VDR homodimers may function on the Opn promoter.[20] Indeed, upon VDR induction by FSK, comparable VDR binding was observed on the Opn VDRE while its binding was not detectable on Cyp24 VDRE (Fig. 4(a)), This result indicates that simple upregulation of VDR even in the absence of its ligand $1,25(OH)_2D_3$ is sufficient to induce its measureable binding depending on the context of VDREs. We then

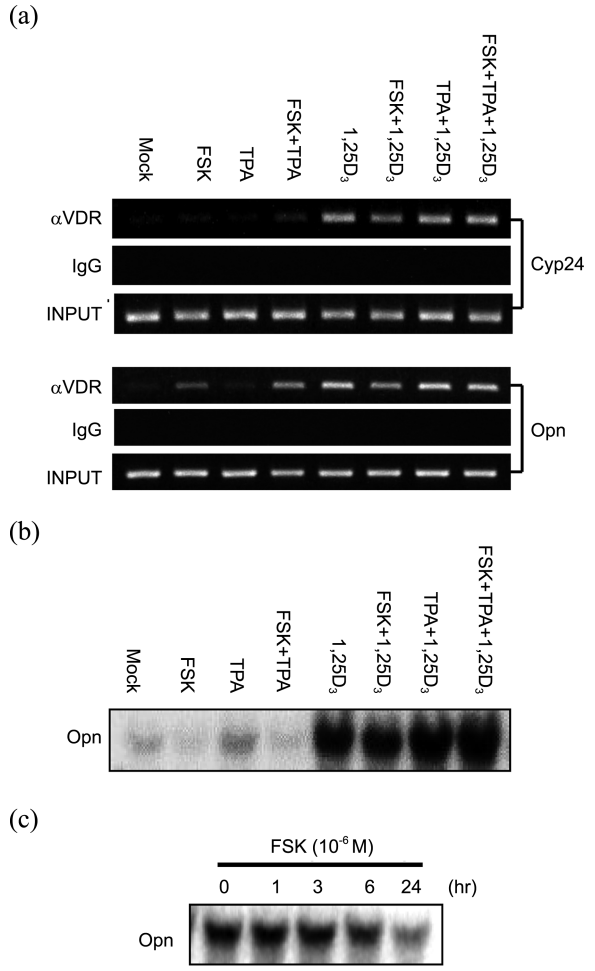

Figure 4. Suppression of Opn by FSK-stimulated upreguation of VDR (a) VDR recruitment on VDREs in the promoter regions of Cyp24 and Opn. MC3T3-E1 cells weretreated with indicated reagents for 6 hrs and then subjected to ChIP assays using a polyclonal antibody (H81) to VDR or rabbit IgG. (b) Regulation of Opn transcript by indicated reagents. The same blot used in Figure 2(a) was re-evaluated. Note that basal transcriptional activity was lowered by FSK. (c) Time-dependent downregulation of Opn transcript. The same blot used in Figure 3(a) was re-evaluated.

investigated the effects of FSK-induced VDR binding on the transcriptional activity of Opn. The Northern blot membrane used in Figure 2(a) was re-evaluated for the detection of Opn mRNA. Notably, the basal level of Opn transcript was significantly downregulated by the treatment with FSK whereas such an effect was not observed for Cyp24 (Fig. 2(a) and 4(b)). Moreover, this downregulation was further confirmed in time dependent manner (Fig. 4(c)), indicating that the induced VDR binding in the absence of $1,25(OH)_2D_3$ may function as a repressor. These data suggest that the conformational changes induced by $1,25(OH)_2D_3$ are prerequisite for the activating functions of VDR.

In summary, to better understand biological effects of VDR in its unliganded state, PKA activators were used in MC3T3-E1 osteoblastic cells and the VDR target genes, Opn and Cyp24, were analyzed. In contrast to the molecular actions of $1,25(OH)_2D_3$, PKA activators positively regulate the levels of VDR predominantly *via* transcriptional induction of the gene. We also showed that the VDR upregulation was preceded by the induction of C/EBPβ, one of the essential regulators for the transactivation of VDR, in both mRNA and protein levels. The increased VDR levels in the cells triggered its exclusive binding to the Opn promoter, which in turn repressed the transcriptional output of the gene in the absence of $1,25(OH)_2D_3$. Future studies will be required to elucidate the roles of other cofactors and epigenetic changes involved in the repression, part of which is already under way.

Experimental Section

Reagents. $1,25(OH)_2D_3$ was obtained from Solvay (da Weesp, The Netherlands). 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) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-VDR-9A7 was purchase from Abcam.

Cell Culture. MC3T3-E1 cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum (FBS) from BioWhittaker (Walkerville, MD). Primary calvarial osteoblasts from neonatal mice were also obtained as previously described[21] and cultured in α-MEM medium supplemented with 10% FBS from BioWhittaker (Walkerville, MD). All cells were plated at 80% confluence 24-72 hours before ligand treatment. $1,25(OH)_2D_3$ was added in ethanol (0.1% maximum final concentration).

Western Blot Analysis. MC3T3-E1 cells were treated with either vehicle, forskolin or $1,25(OH)_2D_3$ for the indicated periods. Cells were washed twice with PBS and then dissolved directly on the plate in 10 mM Tris-HCl, pH 7.6, 0.3 M KCl, and 1% NP-40. After centrifugation for 10 min, lysates were evaluated for protein content and 60 μg subjected to SDS-PAGE. Proteins were transferred to Immunoblot PVDF membranes from BioRad (Hercules, CA) and subjected to western blot analysis using the anti-VDR monoclonal antibody 9A7 or the anti-C/EBPβ antibody (Santa Cruz).

RNA Isolation and Northern Blot Analysis. Cells were plated in 100 mm dishes in α-MEM supplemented with 10% FBS at densities of 5×10^5 cells/mL and treated for up to 24 hrs without or with the indicated concentrations of either vehicle, $1,25(OH)_2D_3$, forskolin, TPA, PTH or the combination. Total RNA was isolated using the TRI reagent (MRC, Cincinnati, OH). Radioactive DNA probes for Cyp24, VDR, C/EBPβ, RankL, Opn and actinwere prepared by a random primed DNA labeling kit (Roche Molecular Biosciences, Indianapolis, IN) and [α-^{32}P] dCTP (800 Ci/mmol; Perkin-Elmer Life Sciences, Norwalk, CT). 20 μg of total RNA was fractionated by electrophoresis on a 1% formaldehyde agarose gel, transferred to nitrocellulose membranes, and hybridized to the radiolabeled cDNA fragment (48% form-

Table 1. List of ChIP primers used

Gene	Forward primer	Reverse primer
Cyp24	GGTTATCTCCGGGGTGGAGT	AGTGGCCAATGAGCACGC
Opn	ACCACCTCTTCTGCTCTATATGGC	TTGACACTTGAACTATGCAGCCGC

amide, 10% dextran sulfate, 5× SSC, 1× Denhardt's solution, and 100 μg/mL salmon sperm DNA) at 42 °C overnight. The membranes were washed in 0.1% SDS plus 2× SSC for 15 min at room temperature and then in 0.1% SDS plus 0.1% × SSC for another 15 min at 60 C. The autoradiograms were prepared using Kodak BioMax film at −80 °C with intensifying screens.

Chromatin Immunoprecipitation (ChIP) Assays. Chromatin immunoprecipitation was performed as described previously (13, 28). Briefly, MC3T3-E1 cells or primary MOBs from mice were plated in α-MEM supplemented with 10% FBS 72 hrs prior to the experiment and then treated with or without $1,25(OH)_2D_3$ for the times and under the conditions indicated. Treated cells were washed several times with PBS and subjected to a cross-linking reaction with 1% formaldehyde. Cells were extracted sequentially in 5 mM Pipes pH 8.0, 85 mM KCl, 0.5% NP-40 and then in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, and the chromatin pellets sonicated to an average DNA size of 300-500 bp DNA (assessed by agarose gel electrophoresis) using a Fisher Model 100 Sonic Dismembranator at a power setting of 1. The sonicated extract was centrifuged and then diluted into ChIP buffer (16.7 mM Tris-HCl pH 8.1, 150 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA). Immunoprecipitations were performed overnight at 4 °C with the indicated antibodies and collected by centrifugation after 1 hr incubation with salmon sperm DNA- and BSA-pretreated Zysorbin (Zymed, San Francisco, CA). Precipitates were washed and cross-links reversed during an overnight incubation in 1% SDS and 0.1 M $NaHCO_3$ at 65 °C. DNA fragments were purified using Qiagen QIAquick PCR Purification Kits (Valencia, CA) and subjected to PCR using primers designed to amplify fragments of murine Cyp24 promoter (−234 to −51) region and murine Opn promoter (−854 to -658) region (Table 1). Analyses for each primer set were carried out at a pre-determined linear range of DNA amplification. PCR products were resolved on 2% agarose gels and visualized using ethidium bromide staining. DNA acquired prior to precipitation was used to assess the presence of genes following the ChIP procedure and designated "as input". PCR evaluation was performed using 10% of the input DNA. Densitometric quantitation was carried out using Kodak 1D Image Analysis (software version 3.5).

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