0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(9):2309–2319
Copyright © 2005 by The Endocrine Society
doi: 10.1210/me.2004-0382

Calmodulin-Dependent Kinase IV Stimulates Vitamin D Receptor-Mediated Transcription

Tara I. Ellison, Diane R. Dowd, and Paul N. MacDonald

Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] promotes intestinal absorption of calcium primarily by binding to the vitamin D receptor (VDR) and regulating gene expression. 1,25-$(OH)_2D_3$ also exerts rapid actions at the cell membrane that include increasing intracellular calcium levels and activating protein kinase cascades. To explore potential cross talk between calcium signaling elicited by the nongenomic actions of 1,25-$(OH)_2D_3$ and the genomic pathway mediated by VDR, we examined the effects of activated Ca^{2+}/calmodulin-dependent kinases (CaMKs) on 1,25-$(OH)_2D_3$/VDR-mediated transcription. Expression of a constitutively active form of CaMKIV dramatically stimulated 1,25-$(OH)_2D_3$-activated reporter gene expression in COS-7, HeLa, and ROS17/2.8 cell lines. Metabolic labeling studies indicated that CaMKIV increased VDR phosphorylation levels. In addition, CaMKIV increased the independent transcription activity of the VDR coactivator SRC (steroid receptor coactivator) 1, and promoted ligand-dependent interaction between VDR and SRC coactivator proteins in mammalian two-hybrid studies. The functional consequences of this multifaceted mechanism of CaMKIV action were revealed by reporter gene studies, which showed that CaMKIV and select SRC coactivators synergistically enhanced VDR-mediated transcription. These studies support a model in which CaMKIV signaling stimulates VDR-mediated transcription by increasing phosphorylation levels of VDR and enhancing autonomous SRC activity, resulting in higher 1,25-$(OH)_2D_3$-dependent interaction between VDR and SRC coactivators. (*Molecular Endocrinology* 19: 2309–2319, 2005)

THE VITAMIN D RECEPTOR (VDR) is a member of the nuclear receptor family of transcription factors. 1,25-$(OH)_2D_3$, the most biologically active form of vitamin D, is the endogenous ligand for the VDR. Ligand-bound VDR heterodimerizes with the retinoid X receptor (RXR) and binds to DNA response elements (VDREs) in the promoter regions of 1,25-$(OH)_2D_3$-regulated target genes to alter transcription (1). Nuclear receptor coactivator proteins, such as steroid receptor coactivators (SRCs) (2), NCoA-62 (3–5), and mediator complex (6) stimulate transcription by recruiting RNA polymerase II, general transcriptional machinery, chromatin remodeling factors and other associated factors to the promoter template.

The major physiological role of VDR is to promote calcium absorption in the intestine and maintain calcium homeostasis in the body (1). Despite the essential role VDR plays in regulating calcium homeostasis, interplays between intracellular calcium levels and VDR transcriptional activity have not been studied extensively. Intracellular calcium levels regulate a variety of distinct cellular signaling pathways including ligand-gated ion channels, cell cycle progression, apoptosis, and transcriptional processes (7–9). Calmodulin (CaM) is a calcium sensor and an effector protein that functions in many of these calcium-dependent processes. Calcium binding induces a conformational change in CaM resulting in the activation of a variety of protein kinases, phosphatases, ion channels, receptors, and NO synthases (10). The kinases activated by CaM include myosin light chain kinase and the multifunctional Ca^{2+}/calmodulin-dependent kinases I, II, and IV (CaMKI, II, and IV). CaMKI and II are ubiquitously expressed in mammalian cells, whereas CaMKIV expression is more restricted. The brain, T lymphocytes, testes, ovary, adrenal glands, skin, and bone marrow contain high levels of CaMKIV (11–13). The intracellular localization of the CaM kinases also differs, with CaMKI and II primarily residing in the cytoplasm and CaMKIV residing primarily in the nucleus (14–16). CaMKIV consists of a catalytic domain that contains the active site, and an autoinhibitory domain that regulates the activity of the kinase (17). Ca^{2+}/CaM activates CaMKIV by binding to its regulatory region (11), thereby revealing its catalytic domain. Ca^{2+}/CaM kinase kinase phosphorylates CaMKIV, which induces full activity of CaMKIV and allows it to act independently of Ca^{2+}/CaM (17). Phosphorylated CaMKIV activates a variety of proteins, including cAMP response element binding protein (CREB), serum response factor, activating transcription factor 1, and CREB binding protein (10).

First Published Online May 26, 2005

Abbreviations: CaM, Calmodulin; CaMK, calcium/CaM-dependent kinase; CBS, calf bovine serum; CREB, cAMP response element binding protein; DBD, DNA binding domain; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; LBD, ligand binding domain; RID, receptor interaction domain; ROR, retinoid-related orphan receptor; RXR, retinoid X receptor; SRC, steroid receptor coactivator; VDR, vitamin D receptor; VDREs, vitamin D response elements; VP16, virion protein 16.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

Two studies suggest a potential role for CaMKIV in nuclear receptor-mediated transcription. Kane and Means (18) demonstrated that CaMKIV augments the constitutive activity of the retinoid-related orphan receptors (RORs) and other orphan nuclear receptors. A second study indicated that thyroid hormone receptor activity is modestly activated by CaMKIV, but this effect requires RORα (19). A direct role for CaMKIV in ligand-activated nuclear receptor-mediated transcription and specifically, in 1,25-$(OH)_2D_3$/VDR-activated transcription, has not been addressed. The potential for CaM/CaMK involvement in vitamin D signaling is supported by the well-known capacity of 1,25-$(OH)_2D_3$ to elicit rapid, membrane-initiated calcium transients in numerous cell types (20–22). However, there is scant evidence linking the 1,25-$(OH)_2D_3$-induced calcium transients to VDR transcriptional activity. Because VDR transactivation is a target for a variety of kinases including casein kinase II (23), protein kinase C (24), and the MAPK pathway (25), and because 1,25-$(OH)_2D_3$-induced intracellular calcium levels could activate CaMKIV, we tested the hypothesis that CaMKIV regulates the activity of VDR. Here, we provide new evidence that active CaMKIV stimulates 1,25-$(OH)_2D_3$- and VDR-mediated transcription by phosphorylating VDR, increasing the activity of SRC1, and by promoting the interaction between VDR and SRC coactivator proteins. These data suggest a potential intersection between two critical 1,25-$(OH)_2D_3$ signaling pathways, namely the well-established genomic mechanism mediated through the VDR and the emerging nongenomic pathways involving membrane-initiated stimulation of second messenger pathways, kinase cascades, and opening of ion channels.

RESULTS

CaMKIV Selectively Enhances 1,25-$(OH)_2D_3$/VDR-Mediated Transcription

We initially explored the effect of CaMKIV on VDR-mediated transcription using the $(VDRE)^4$-TATA-Luc reporter gene in HeLa cells. Full-length VDR was expressed in the presence or absence of a constitutively active form of CaMKIV containing amino acids 1–313 of the rat sequence ($CaMKIV_{1-313}$) (26). In the absence of $CaMKIV_{1-313}$, 1,25-$(OH)_2D_3$ activated reporter gene expression 16-fold. When $CaMKIV_{1-313}$ was ectopically expressed, 1,25-$(OH)_2D_3$-stimulated luciferase activity was induced 55-fold, a 4-fold activation compared with vector control (Fig. 1A). This increase in luciferase activity was dependent upon the 1,25-$(OH)_2D_3$ ligand, as expression of the reporter gene was unaffected by $CaMKIV_{1-313}$ alone. Binding of 1,25-$(OH)_2D_3$ by the VDR ligand binding domain (LBD) causes a conformation change, creating an interaction surface for RXR and a variety of comodulatory proteins. To determine whether this domain also mediates VDR activation by $CaMKIV_{1-313}$, the VDR LBD (amino acids 93–427) was expressed as a fusion protein with a heterologous gal4 DNA binding domain (DBD). The gal4-VDR fusion protein activated a gal4-dependent reporter gene $[(gal4)^5$-TATA-Luc] 9-fold in the presence of 1,25-$(OH)_2D_3$ (Fig. 1A). Coexpression of $CaMKIV_{1-313}$ enhanced 1,25-$(OH)_2D_3$ activation of the reporter construct 63-fold, seven times the activation observed in the absence of $CaMKIV_{1-313}$. Thus, the LBD of VDR was sufficient to mediate the effects of $CaMKIV_{1-313}$ on 1,25-$(OH)_2D_3$-activated reporter gene expression in this system.

$CaMKIV_{1-313}$ also activated VDR-mediated transcription in COS-7 cells, which express low levels of endogenous VDR (less than 500 transcripts per cell) (Fig. 1B). Here, $CaMKIV_{1-313}$ enhanced 1,25-$(OH)_2D_3$-activated transcription 6-fold compared with vector control. $CaMKIV_{1-313}$ exerted minimal effects on the $(VDRE)^4$-TATA-Luc reporter in the absence of VDR. Residual activation was likely due to low levels of endogenous VDR in this system. In addition, full-length VDR activation due to 1,25-$(OH)_2D_3$ or $CaMKIV_{1-313}$ was not apparent using a reporter lacking vitamin D response elements $[(gal4)^5$-TATA-Luc, Fig. 1B]. These data show that $CaMKIV_{1-313}$ selectively augments 1,25-$(OH)_2D_3$-mediated transcription in a VDR- and VDRE-dependent manner.

$CaMKIV_{1-313}$ activated VDR in a dose-dependent manner (Fig. 1C). In the presence of 1,25-$(OH)_2D_3$, 100 ng of SG5-$CaMKIV_{1-313}$ activated the VDRE-reporter 4-fold compared with vector control. A maximal activation of approximately 14-fold was observed at 1000 ng of SG5-$CaMKIV_{1-313}$ expression plasmid. However, amounts of $CaMKIV_{1-313}$ greater than this exhibited lower efficacy, probably the result of squelching effects from the large quantity of plasmid. SG5-$CaMKIV_{1-313}$ had little effect in the absence of 1,25-$(OH)_2D_3$ (*open bars* in Fig. 1C). The $CaMKIV_{1-313}$ activation of VDR also depended upon the concentration of 1,25-$(OH)_2D_3$ (Fig. 1D). $CaMKIV_{1-313}$ augmented VDRE-dependent transcription 6-fold at ligand concentrations as low as 10^{-9} M 1,25-$(OH)_2D_3$, and the response dramatically increased to 13-fold and 17-fold at 10^{-8} M and 10^{-7} M 1,25-$(OH)_2D_3$, respectively. Taken together, these data establish a potent and selective effect of constitutively active CaMKIV on 1,25-$(OH)_2D_3$- and VDR-induced promoter activity.

CaMKIV and CaMKII Impact 1,25-$(OH)_2D_3$-Activated Transcription from Synthetic and Natural Promoters in a Cell-Selective Manner

CaMKIV belongs to a three-member family of multifunctional Ca^{2+}/CaM-dependent kinases, the best-characterized member being CaMKII. Because CaMKII and CaMKIV exhibit some overlapping substrate specificity, we tested whether CaMKII would similarly affect VDR-mediated transcription. Surprisingly, whereas $CaMKIV_{1-313}$ expression in COS-7 cells increased 1,25-$(OH)_2D_3$-activated transcription 8-fold, expression of constitutively active CaMKII (amino ac-

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

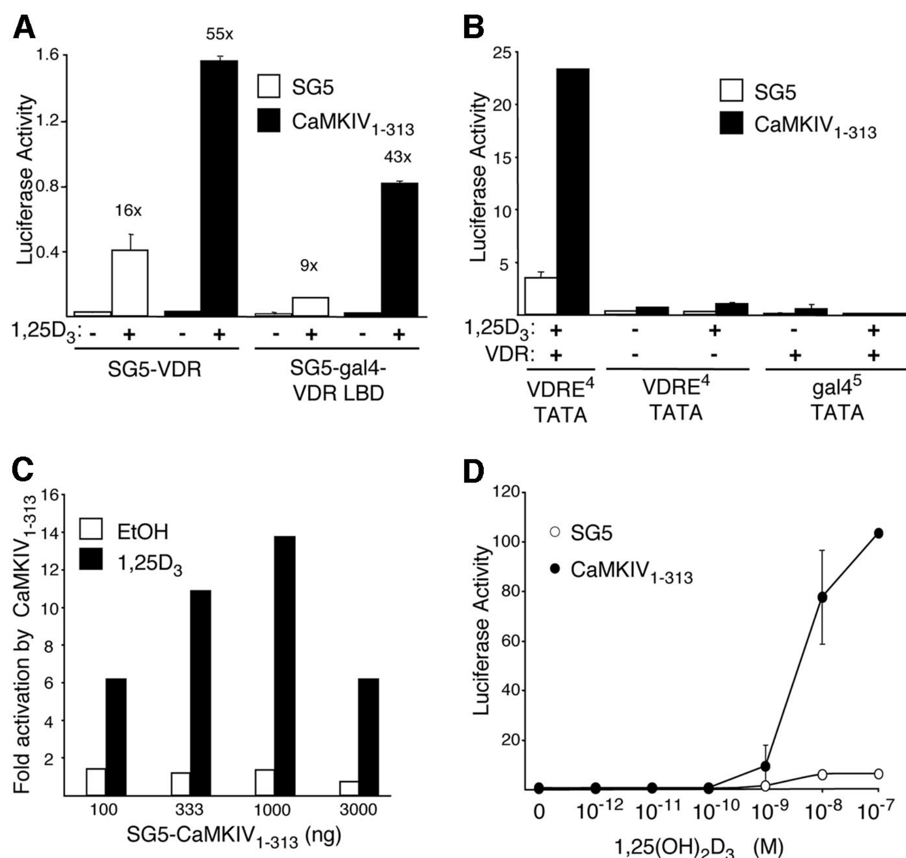

Fig. 1. CaMKIV Selectively Enhances 1,25-$(OH)_2D_3$/VDR-Mediated Transcription

A, Constitutively active CaMKIV activates both the full-length VDR and the VDR LBD fused to a heterologous gal4 DBD. HeLa cells were transfected with 1 μg of $(VDRE)^4$-TATA-Luc, 3 ng of SG5-hVDR, and 1 μg of SG5 or SG5-$CaMKIV_{1–313}$ per well, or with 1 μg of $(gal4)^5$-TATA-Luc, 30 ng SG5-gal4-VDR, and 1 μg of SG5 or SG5-$CaMKIV_{1–313}$ per well, as described in *Materials and Methods*. After a 24-h treatment with ethanol vehicle or 10^{-8} M 1,25-$(OH)_2D_3$, cell extracts were prepared and analyzed for luciferase expression. Data are graphed as the mean of duplicate samples ± SD. B, $CaMKIV_{1–313}$ produces little effect in the absence of VDR or vitamin D response elements. COS-7 cells were transfected with 3 ng of SG5-hVDR, 1 μg of SG5 or SG5-$CaMKIV_{1–313}$, and 1 μg of $(VDRE)^4$-TATA-Luc or $(gal4)^5$-TATA-Luc. Cells were treated and extracts prepared as described in part A. C, $CaMKIV_{1–313}$ activates VDR-mediated transcription in a dose-dependent manner. Fold activation by $CaMKIV_{1–313}$ was calculated as the luciferase activity obtained in the presence of SG5-$CAMKIV_{1–313}$ divided by the luciferase activity observed with the SG5 vector control. COS-7 cells were transfected with 1 μg of $(VDRE)^4$-TATA-Luc, 3 ng of SG5-VDR and increasing amounts of SG5-$CaMKIV_{1–313}$ or SG5 vector control, treated with ethanol or 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h, and analyzed as described in part A. D, $CaMKIV_{1–313}$ activates the VDRE reporter in a 1,25-$(OH)_2D_3$ concentration-dependent manner. COS-7 cells were transfected with 1 μg of $(VDRE)^4$-TATA-Luc, 3 ng of SG5-VDR and 300 ng of SG5-$CaMKIV_{1–313}$ or vector control, treated with the indicated concentrations of ethanol or 1,25-$(OH)_2D_3$ for 24 h, and analyzed as described in part A.

ids 1–290) produced minimal effects on 1,25-$(OH)_2D_3$-activated transcription in this VDR/VDRE-driven reporter gene system (Fig. 2A). However, when these studies were expanded to ROS 17/2.8 rat osteosarcoma cells, a 1,25-$(OH)_2D_3$ target cell line that expresses more than 20,000 copies of VDR (27), both $CaMKIV_{1–313}$ and $CaMKII_{1–290}$ strongly induced 1,25-$(OH)_2D_3$-activated reporter gene expression mediated by endogenous VDR (Fig. 2B). Therefore, both $CaMKIV_{1–313}$ and $CaMKII_{1–290}$ augment VDR-mediated transcription, although the magnitude of the $CaMKII_{1–290}$ effect varies by cell type.

The studies described thus far used heterologous reporter constructs containing minimal vitamin D response elements. To extend these studies to a more natural VDR target system, a reporter gene construct containing the native 482-bp promoter of the rat 24-hydroxylase gene was introduced into ROS 17/2.8 rat osteosarcoma cells. Expression of constitutively active CaMKIV in this context activated the 24-hydroxylase promoter 3.5-fold, and $CaMKII_{1–290}$ activated the promoter 5.5-fold in the presence of 1,25-$(OH)_2D_3$ (Fig. 2C). Neither kinase altered unliganded transcriptional activity from the 24-hydroxylase promoter. Thus, $CaMKIV_{1–313}$ and $CaMKII_{1–290}$ activate both a synthetic and a natural promoter construct in the native cellular context of a VDR target cell.

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

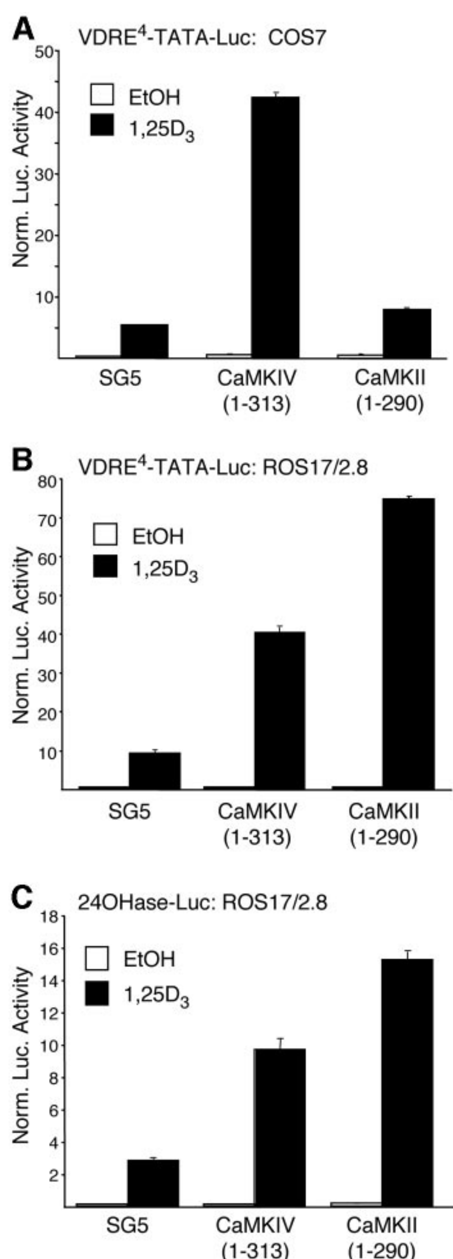

Fig. 2. CaMKIV and CaMKII Impact 1,25-$(OH)_2D_3$-Activated Transcription from Synthetic and Natural Promoters in a Cell-Selective Manner

A, COS-7 cells were transfected with 680 ng of $(VDRE)^4$-TATA-Luc, 200 ng of phRG-TK, 3 ng of SG5-VDR expression plasmid, and 1 μg of either SG5, SG5-CaMKIV$_{1–313}$, or SG5-CaMKII$_{1–290}$ as described in *Materials and Methods*. Luciferase activity was measured 24 h after treatment with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle. Data are graphed as the mean of duplicate measurements ± SD. B, ROS17/2.8 osteosarcoma cells were transfected with 680 ng of $(VDRE)^4$-TATA-Luc, 200 ng of phRG-TK, and 1 μg of either SG5, SG5-CaMKIV$_{1–313}$, or SG5-CaMKII$_{1–290}$. Cells were treated and extracts prepared as described in part A. C, ROS17/2.8 osteosarcoma cells were transfected with 680 ng of the rat24OHase-Luc reporter, 200 ng phRG-TK, and 1 μg of either SG5, SG5-CaMKIV$_{1–313}$, or SG5-CaMKII$_{1–290}$. Cells were treated and extracts prepared as described in part A.

CaMKIV$_{1–313}$ Increases Phosphorylation of VDR

To address potential mechanisms underlying the observed increase in VDR/1,25-$(OH)_2D_3$-activated transcription, we focused on CaMKIV$_{1–313}$ and the COS-7 cell system because CaMKIV$_{1–313}$ displayed the widest cell activity profile and because COS-7 cells express minimal levels of endogenous VDR. One potential mechanism may involve direct or indirect phosphorylation of VDR by CaMKIV$_{1–313}$. Sequence analysis of VDR revealed a putative CaMKIV consensus phosphorylation site at serine residue 172 (^{167}*P*S*R*PN*S*RHT*P*^{176}). The consensus CaMKIV site is (Hyd-x-R-x-x-S/T-x-x-x-Hyd) in which Hyd refers to any hydrophobic amino acid, R is arginine, S/T is a serine or threonine at the phosphorylation site, and x refers to any amino acid. VDR contains a proline residue in each of the hydrophobic positions, and an arginine at amino acid 169. To explore the importance of the VDR serine 172, site-directed mutagenesis was used to change this serine to an alanine. As shown in Fig. 3A, this mutation had no effect on 1,25-$(OH)_2D_3$ responsiveness of VDR because 1,25-$(OH)_2D_3$ induced an 8-fold activation of the $(VDRE)^4$-TATA-Luc reporter compared with vehicle treatment in both the wild-type VDR and S172A VDR-transfected cells. The wild-type and S172A VDR also responded similarly to CaMKIV$_{1–313}$ expression, as both were activated approximately 10- to 11-fold in the presence of 1,25-$(OH)_2D_3$. Western blot showed equivalent expression of the wild-type and S172A mutant VDR (data not shown). These data suggest that S172 is not required for 1,25-$(OH)_2D_3$ responsiveness and that S172 is not involved in CaMKIV$_{1–313}$-mediated activation of VDR-dependent gene expression.

To test whether other amino acids are targeted by CaMKIV, ^{32}P-labeling studies were used to examine overall phosphorylation of VDR. COS-7 cells expressing Flag-tagged VDR were labeled with ^{32}P-orthophosphate in the absence and presence of CaMKIV$_{1–313}$, and cell extracts were subjected to immunoprecipitation with an anti-Flag antibody. Expression of CaMKIV$_{1–313}$ increased the phosphorylation level of VDR 4-fold (Fig. 3B), both in the absence of 1,25-$(OH)_2D_3$ treatment (compare lane 3 with 1) and the presence of 1,25-$(OH)_2D_3$ treatment (compare lane 4 with 2). However, this increase in VDR phosphorylation was accompanied by a 2-fold increase in VDR expression as assessed by Western immunoblots of crude protein samples. When normalized to VDR protein levels, CaMKIV$_{1–313}$ expression enhanced both unliganded and liganded VDR phosphorylation, by 2.2- and 1.8-fold, respectively.

CaMKIV$_{1–313}$ Increases the Intrinsic Transactivation of SRC1

In addition to up-regulating phosphorylation of VDR, it is possible that CaMKIV$_{1–313}$ phosphorylates other proteins involved in activation of VDR-mediated tran-

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

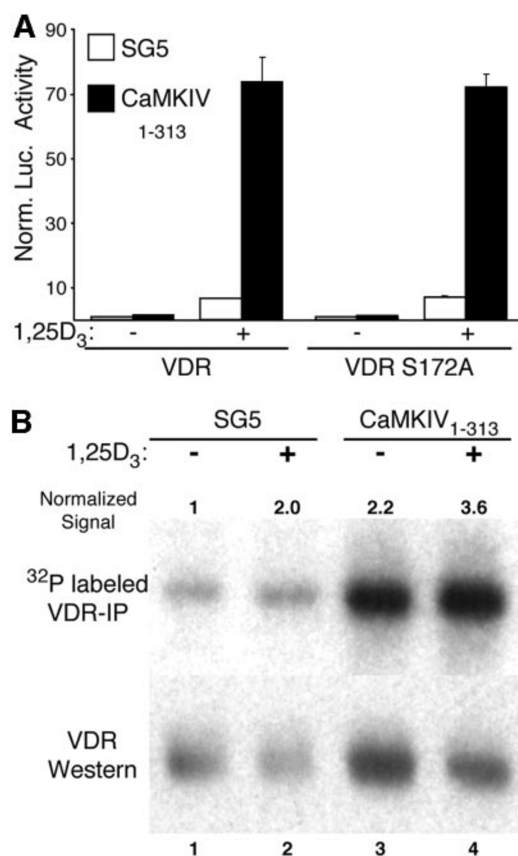

Fig. 3. CaMKIV$_{1-313}$ Increases Phosphorylation of VDR

A, VDR does not require serine 172 for activation by CaMKIV$_{1-313}$. COS-7 cells were transfected with 680 ng of (VDRE)^4-TATA-Luc reporter, 200 ng of phRG-TK, 3 ng of SG5-VDR (WT or S172A), and 500 ng of SG5-CaMKIV$_{1-313}$ or SG5 vector control. Luciferase activity was measured 24 h after treatment with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle. Data are graphed as the average of duplicate samples ± SD. B, Phosphorylation of VDR was examined using metabolic labeling with ^{32}P-orthophosphate. COS-7 cells were transfected with 500 ng of CMV-Flag-VDR and 1 μg of either SG5-CaMKIV$_{1-313}$ or SG5. Cells were incubated with [^{32}P]-orthophosphate and 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle in phosphate-free media for 2 h. Cells were harvested and Flag-VDR immunoprecipitated. The autoradiogram in the *upper row* shows immunoprecipitated ^{32}P-labeled VDR and the lower row is a Western blot of total VDR protein levels. Normalized signal states the quantitated intensity of ^{32}P-labeled VDR divided by intensity of the VDR Western for each lane, relative to the ratio of the bands in lane 1. The data shown are representative of three independent experiments.

scription. SRC1, a coactivator of several nuclear receptors, is phosphorylated at multiple sites by the MAPK family member Erk-2 (28). This phosphorylation augments the intrinsic transactivation activity of SRC1 and its coactivation of the progesterone receptor (29). To determine whether CaMKIV$_{1-313}$ impacts the intrinsic transactivation potential of SRC1, full-length SRC1 was expressed as a fusion protein with the DBD (amino acids 1–147) of the heterologous gal4 transcription factor in COS-7 cells (Fig. 4). The gal4-SRC1 fusion exhibited constitutive activity compared with the gal4 DBD alone, whereas the gal4-SRC1 receptor interaction domain (RID), containing only the LXXLL motifs, lacked this activity (*open bars* in Fig. 4). Coexpression of CaMKIV$_{1-313}$ increased gal4-SRC1 activity 6.8-fold compared with vector control, whereas CaMKIV$_{1-313}$ did not affect the activity of the gal4-SRC1 RID that lacks activation domains. In this system, CaMKIV$_{1-313}$ did not affect the activity of gal4-VP16 (virion protein 16), an unrelated transactivator with an acidic activation domain (data not shown). In addition, CaMKII$_{1-290}$ failed to augment transactivation of gal4-SRC1 and gal4-SRC1 RID, an expected outcome given the selective effect of CaMKII$_{1-290}$ on VDR-mediated transcription in ROS17/2.8 cells but not COS-7 cells. Taken together, these data show that CaMKIV$_{1-313}$ selectively augments the intrinsic transactivation of SRC1 in COS-7 cells.

CaMKIV$_{1-313}$ Increases VDR Interaction with the SRC Family of Coactivators in a Mammalian Two-Hybrid System

The extent of VDR interaction with its heterodimer partner RXR and with the SRC coactivators determines, in part, the degree of VDR transcriptional activity. Thus, the mammalian two-hybrid assay was used to determine whether phosphorylation events by CaMKIV$_{1-313}$ enhanced these interactions. In this assay, the VDR LBD was expressed as a VP16 activation domain fusion protein, whereas the RXRγ LBD and the LXXLL-containing RIDs of the coactivators were fused to the gal4 DBD. Protein-protein interactions were monitored using the $(gal4)^5$-TATA-luciferase reporter. As illustrated in Fig. 5 (*left panel*), CaMKIV$_{1-313}$ did not dramatically alter the interaction between VDR and

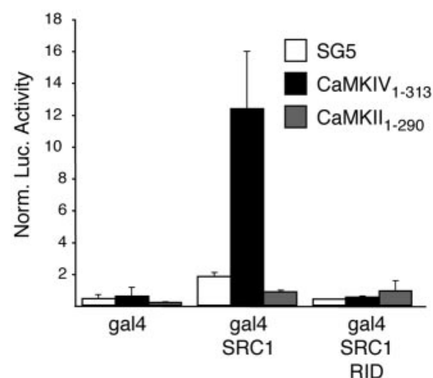

Fig. 4. CaMKIV$_{1-313}$ Increases the Intrinsic Transactivation of SRC1

COS-7 cells were transfected with 680 ng of $(gal4)^5$-TATA-Luc reporter, 200 ng of phRG-TK, 150 ng of SG5-gal4, SG5-gal4-SRC1, or SG5-gal4-SRC1 RID, and 300 ng of SG5-CaMKIV$_{1-313}$, SG5-CaMKII$_{1-290}$, or SG5 control. Luciferase activity was measured 42 h after transfection, and data are graphed as the average of triplicate samples ± SD.

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

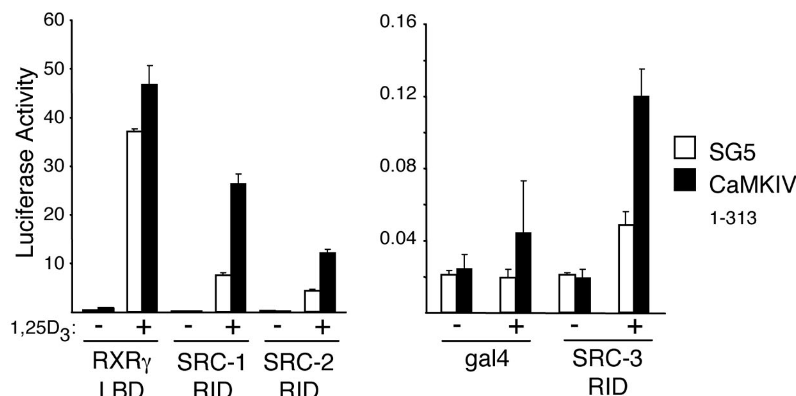

Fig. 5. $CaMKIV_{1–313}$ Increases Interaction of VDR with the RIDs of the SRC Family of Coactivators in a Mammalian Two-Hybrid System

COS-7 cells were transfected with 680 ng of $(gal4)^5$-TATA-Luc reporter, 150 ng of VP16-VDR, and 100 ng of SG5-gal4, SG5-gal4-RXRγ LBD, SG5-gal4-SRC1 RID, SG5-gal4-SRC2 RID, or SG5-gal4-SRC3 RID and 500 ng of SG5-$CaMKIV_{1–313}$ or SG5. Luciferase activity was measured 24 h after treatment with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle. Data are graphed as the average of duplicate samples SD.

RXRγ. However, expression of $CaMKIV_{1–313}$ increased the interaction between VDR and the LXXLL-containing domains of SRC1 and SRC2 by 3.5-fold and 3-fold, respectively. This enhanced interaction with SRC1 and SRC2 was dependent upon 1,25-$(OH)_2D_3$ because in the absence of ligand, VDR did not interact with SRC1 or SRC2, regardless of the expression of $CaMKIV_{1–313}$. Little interaction was observed between VDR and the RID of SRC3 (Fig. 5, *right panel*; note the expanded y-axis). $CaMKIV_{1–313}$ also increased this weak interaction 2- to 3-fold. In contrast, $CaMKIV_{1–313}$ did not affect the low basal interaction of VDR with the gal4 control protein. These data indicate that $CaMKIV_{1–313}$ increases interaction of VDR with the SRC family of coactivators, likely yielding enhanced transcriptional activity of VDR.

$CaMKIV_{1–313}$ Synergizes with SRC1 and SRC2 to Activate VDR-Mediated Transcription

We have shown that $CaMKIV_{1–313}$ increases VDR phosphorylation and enhances both SRC1 intrinsic transactivation and VDR/coactivator interactions. Figure 6 illustrates the functional consequences of these interplays between $CaMKIV_{1–313}$ and the SRC proteins on VDR-activated reporter gene expression. In the absence of coactivator (SG5 control), $CaMKIV_{1–313}$ augmented the 1,25-$(OH)_2D_3$-response of a VDRE-containing reporter 4.5-fold. SRC1 increased the 1,25-$(OH)_2D_3$-response 1.5-fold, and coexpression of SRC1 and $CaMKIV_{1–313}$ produced a 7.3-fold synergistic level of VDR-mediated transcription compared with vector control. Similarly, SRC2 increased the activity of ligand-activated VDR 2.2-fold, and coexpression of SRC2 and $CaMKIV_{1–313}$ activated liganded VDR 8-fold compared with vector alone, confirming the synergy between $CaMKIV_{1–313}$ and SRC2. In contrast, $CaMKIV_{1–313}$ and NCoA-62, an unrelated coactivator that lacks LXXLL motifs, activated VDR-mediated transcription in an additive manner. NCoA-62 increased the 1,25-$(OH)_2D_3$-response of VDR 1.2-fold, and together with $CaMKIV_{1–313}$ the two proteins produced a 3.2-fold increase in activation. Unsurprisingly, given the weak interaction observed in the mammalian two-hybrid assay, SRC3 produced minimal coactivation of VDR-mediated transcription regardless of $CaMKIV_{1–313}$ expression. Cumulatively, these studies suggest that the increased activity of VDR in the presence of $CaMKIV_{1–313}$ stems from increased VDR interaction with SRC1 and SRC2, whereas SRC3 and

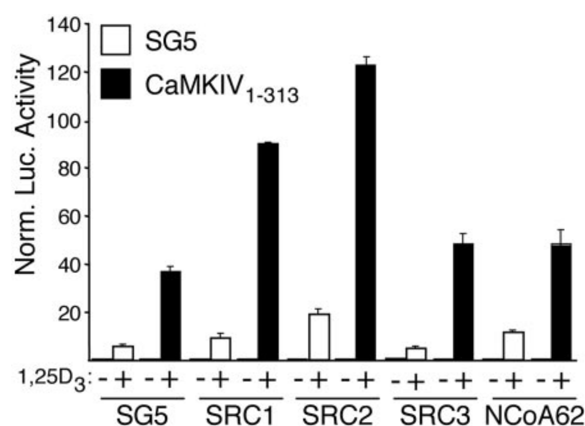

Fig. 6. $CaMKIV_{1–313}$ Synergizes with SRC1 and SRC2 to Activate VDR-Mediated Transcription

COS-7 cells were transfected with 680 ng $(VDRE)^4$-TATA-Luc reporter, 200 ng of phRG-TK, 3 ng SG5-VDR, and 25 ng of SG5-SRC1, SG5-SRC2, SG5-SRC3, SG5-NCoA62, or SG5 vector in the presence of 300 ng of SG5-$CaMKIV_{1–313}$ or SG5 vector control. After a 24-h treatment with ethanol or 10^{-8} M 1,25-$(OH)_2D_3$, cell extracts were prepared and analyzed for luciferase expression. Data are graphed as the mean of normalized duplicate samples ± SD.

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

NCoA-62 do not play an important role in the mechanism of $CaMKIV_{1-313}$ action in COS-7 cells.

DISCUSSION

$1,25\text{-}(OH)_2D_3$ exerts its biological effects through a multifaceted set of mechanisms. The genomic pathway involves $1,25\text{-}(OH)_2D_3$ binding to VDR, VDR heterodimerization with the RXR, and heterodimer binding to specific DNA response elements in the promoters of $1,25\text{-}(OH)_2D_3$ target genes, which leads to altered rates of transcription of selected target genes (1). A second established pathway involves membrane-initiated, nongenomic effects of $1,25\text{-}(OH)_2D_3$ (reviewed in Refs. 30 and 31). Indeed, numerous intracellular signaling pathways are rapidly activated (within seconds to minutes) by $1,25\text{-}(OH)_2D_3$, including fluctuations in intracellular calcium levels (21), alterations in calcium channel activity (32), protein kinase C activation and translocation (33, 34), and activation of the MAPK pathway (35). The molecular mechanisms governing the rapid cellular responses to $1,25\text{-}(OH)_2D_3$ are unresolved. Some reports suggest a VDR-independent pathway (36) that uses a novel membrane-associated receptor for $1,25\text{-}(OH)_2D_3$ (37). In contrast, recent data implicate a requirement for functional VDR in the nongenomic response of cells to $1,25\text{-}(OH)_2D_3$ (22, 38). Importantly, the potential for cross talk between the genomic and nongenomic pathways of $1,25\text{-}(OH)_2D_3$ action is becoming increasingly evident (25, 39). In this manuscript, we provide new evidence for potential cross talk between the genomic and rapid response pathways by showing that mediators of intracellular calcium signaling potentiate $1,25\text{-}(OH)_2D_3$-activated genomic responses mediated by the VDR.

Elevated intracellular calcium activates a number of intracellular signaling pathways and kinase cascades. Thus, to focus on Ca^{2+}/CaMKs as downstream mediators of calcium signaling, constitutively active forms of these kinases were used. We showed that $CaMKIV_{1-313}$ potently activates $1,25\text{-}(OH)_2D_3$/VDR-mediated transcription from synthetic and natural promoter-reporter genes. This effect is selective for VDR-activated reporter gene expression as $CaMKIV_{1-313}$ did not affect basal expression. Thus, it appears that $CaMKIV_{1-313}$ acts upon select components of nuclear receptor-mediated transcription rather than components of the general transcription machinery. A likely target is VDR itself, but mutation of a putative CaMKIV consensus motif within the VDR LBD did not alter VDR activation by $CaMKIV_{1-313}$ (Fig. 3A). This suggests that the activation of VDR occurs indirectly, perhaps through phosphorylation by another kinase or by phosphorylation of an intermediate protein. Alternatively, previous studies show that both CaMKIV and CaMKII also phosphorylate nonconsensus sequences (40, 41), so the possibility exists that $CaMKIV_{1-313}$ targets another site on VDR. Consistent with this possibility, whole cell labeling in COS-7 cells shows that $CaMKIV_{1-313}$ expression augments VDR steady-state phosphorylation approximately 2-fold, relative to the total amount of VDR protein, and also increases VDR protein levels (Fig. 3B). Therefore, whereas precise amino acid target identification on VDR will require more intensive research effort, our studies suggest that phosphorylation of VDR by CaMKIV plays a role in regulating VDR protein levels and transactivation.

CaMKIV signaling increases VDR phosphorylation, and it also modulates VDR activity via the SRC family of proteins. SRC proteins are transcriptional coactivators of VDR and other nuclear receptors (2). They associate with VDR in a $1,25\text{-}(OH)_2D_3$-dependent manner (42, 43) and remodel chromatin at $1,25\text{-}(OH)_2D_3$-responsive promoters by acetylating histones. Targeting SRC proteins to promoter regions independent of nuclear receptors is sufficient for transactivation, as evidenced by their intrinsic transactivation capacity when fused to heterologous DNA binding domains (for example, gal4 DBD) (44). In this study, we found that $CaMKIV_{1-313}$ augments the intrinsic transactivation of SRC1. Importantly, this activation occurs independently of VDR and $1,25\text{-}(OH)_2D_3$. Therefore, these studies suggest a role for SRC phosphorylation by CaMKIV in augmenting VDR-mediated transcription.

We hypothesized that $CaMKIV_{1-313}$ promotes the ligand-dependent interaction between VDR and the SRC family of coactivators because the VDR LBD, which also recruits coactivators, was sufficient to mediate the enhanced $1,25\text{-}(OH)_2D_3$-activated transcription by $CaMKIV_{1-313}$ (Fig. 1A). Indeed, $CaMKIV_{1-313}$ increases interaction of VDR with the SRC proteins without affecting interactions between VDR and its heterodimer partner $RXR\gamma$ (Fig. 5). Processes that promote VDR-SRC interaction are expected to enhance the transactivation potential of the VDR, an effect that is illustrated by the synergistic activation of a $1,25\text{-}(OH)_2D_3$-responsive reporter gene by $CaMKIV_{1-313}$ and SRC1 and SRC2 (Fig. 6). $CaMKIV_{1-313}$ minimally affected the coactivator activity of SRC3, perhaps because VDR/SRC3 interaction and SRC3 coactivator activity is weak in this system. Functional synergy was not evident between $CaMKIV_{1-313}$ and NCoA62/SKIP, an AF2-independent nuclear receptor coactivator that is unrelated to the SRCs and that coactivates VDR through distinct mechanisms (3). Thus, the effects of $CaMKIV_{1-313}$ on VDR-activated transcription are restricted to select coactivators. Importantly, because $CaMKIV_{1-313}$ fails to change VDR interaction with its heterodimer partner $RXR\gamma$ (Fig. 5), it is unlikely that the increased interactions observed for VDR with SRC1 and SRC2 are caused by a general effect such as increased expression of VDR.

In addition to the activation of $1,25\text{-}(OH)_2D_3$/VDR-mediated transcription by $CaMKIV_{1-313}$, we also observed a striking increase by constitutively active CaMKII in ROS17/2.8 cells that was not apparent in

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

COS-7 cells (Fig. 2). Other studies have shown that CaMKIV and CaMKII mediate calcium-dependent activation of transcription factors such as CREB, although the activation varies greatly depending on cell type and which CRE-containing promoters are studied (45). Several possibilities exist to explain the differential effects of CaMKII in COS-7 and ROS17/2.8 cells. Species-related differences may play a role because COS-7 cell experiments used human VDR and experiments in ROS17/2.8 cells used rat VDR. Despite high sequence homology between human and rat VDRs, it is possible that $CaMKII_{1-290}$ phosphorylates residues within rat VDR that are not present in the human variant. Alternatively, coactivator expression profiles differ greatly between cell types, and we show that CaM kinases selectively target discrete coactivators in VDR-mediated transcription (Fig. 6). Thus, the cohort of coactivator proteins expressed in a particular cell may determine the degree to which that cell responds to CaMKIV or CaMKII in augmenting vitamin D responsiveness. Ultimately, the physiologic effects of CaMKIV and CaMKII depend on which cell types coexpress VDR with one or more of the CaM kinases. CaMKIV has a restricted cell expression pattern, limiting the number of tissues in which it can influence VDR-activated transcription. In contrast, CaMKII and VDR are coexpressed in a variety of cell types, providing CaMKII ample opportunity to regulate VDR activity in a physiologic setting.

A previous study demonstrated that constitutively active orphan nuclear receptors including RORs and COUP-TFI (chicken ovalbumin upstream promoter-transcription factor 1) are activated by $CaMKIV_{1-313}$ in keratinocyte cell lines (18). The authors suggest that the effect of $CaMKIV_{1-313}$ is selective for the constitutively active orphan receptors because $CaMKIV_{1-313}$ does not affect thyroid hormone receptor or estrogen receptor-mediated transcription. Importantly, our studies clearly extend a role for $CaMKIV_{1-313}$ to liganded nuclear receptors, namely 1,25-$(OH)_2D_3$-activated transcriptional processes mediated by the VDR. In addition, our data provide strong evidence that $CaMKII_{1-290}$ influences the actions of VDR in a cell-selective manner. Activation by $CaMKIV_{1-313}$ and $CaMKII_{1-290}$ was striking and selective for VDR-activated processes compared with the general transcriptional processes studied here. Importantly, this is the first indication that CaMKIV signaling results in phosphorylation of a nuclear receptor, although this may occur indirectly. Our studies also implicate novel actions of CaMKIV on the SRC family of proteins, resulting in increased interactions with VDR and augmented intrinsic transactivation. SRC1 contains several putative consensus sites for CaMKIV and CaMKII, possibly providing the opportunity for the CaM kinases to phosphorylate these residues.

In conclusion, we have shown that $CaMKIV_{1-313}$ augments 1,25-$(OH)_2D_3$/VDR-mediated transcription through multiple mechanisms, including promoting phosphorylation of VDR, increasing VDR protein expression, stimulating the intrinsic transactivation of SRC1 and increasing the interaction of VDR with SRC1 and SRC2. These multiple effects culminate in synergistic enhancement of VDR-mediated transcription by CaMKIV and SRC proteins. Further studies will be necessary to demonstrate the roles that calcium and the CaMKs play in the genomic actions of the VDR. However, our studies suggest the potential for convergence of calcium signaling cascades elicited by 1,25-$(OH)_2D_3$ with the genomic 1,25-$(OH)_2D_3$-activated processes mediated by the VDR because nongenomic pathways dramatically enhance intracellular calcium levels in a variety of 1,25-$(OH)_2D_3$ target cells. Cross talk between the two pathways may provide an important means to augment 1,25-$(OH)_2D_3$ action in target cells.

MATERIALS AND METHODS

Plasmid Constructs

SG5-gal4-VDR was constructed as described (46). The LBD of RXRγ (amino acids 209–463) was amplified using the PCR primers 5′-GCGCGAATTCGTGCAAGAAGAAAGACAG-3′ and 5′-GCGCGGATCCCCCAGGGGTCATCCTGGG-3′, and cloned into the SG5 vector (Stratagene, La Jolla, CA) containing amino acids 1–147 of the gal4 protein. VP16-VDR was constructed by digesting the VDR LBD from SG5-gal4-VDR using *Eco*RI/*Bam*HI restriction digest sites and cloning the fragment into pVP16 (CLONTECH, Palo Alto, CA). SG5-$CaMKII_{1-290}$ was constructed by inserting the 1.5-kb *Eco*RI fragment of pCDNA-CaMKII into SG5 (Stratagene).

$(gal4)^5$-TATA-Luc contains five copies of the gal4 response element upstream of the TATA box from the E1b promoter, cloned into pGL3 (Promega, Madison, WI). $(VDRE)^4$-TATA-Luc was created by cloning the VDRE (4)-TATA fragment (*Nde*I/*Bam*HI) from $(VDRE)^4$-TATA-GH (47) into *Sma*I and *Bg*/II sites of pGL3 basic (Promega).

The rat 1,25-$(OH)_2D_3$ 24-hydroxylase promoter (−369 to +113 relative to the transcriptional start site) was cloned by PCR using ROS 17/2.8 genomic DNA. The forward and reverse primers used for PCR were 5′-AGCGCTCGAGTGTC-CTCAGGGACCTTGC-3′ and 5′-CGATAAGCTTTGGCAGC-TATGGGGAGAG-3′, respectively. The PCR product was cloned into pGL3 basic (Promega), and the final construct was confirmed by sequencing.

The construction of SG5-VDR was described previously (24). CMV-Flag-VDR was constructed by ligating the 1.6-kb *Eco*RI/Klenow/*Bam*HI-digested fragment of SG5-VDR into Flag-CMV2 (Sigma, St. Louis, MO). This resulted in an in-frame fusion of VDR with an N-terminal FLAG epitope. SG5-gal4-SRC2 (RID) contains amino acids 595–780 encompassing the three nuclear receptor-interacting boxes of SRC2 fused to the gal4 DBD (amino acids 1–147) in the SG5-gal4 vector. SG5-gal4-SRC1 (RID) contains amino acids 595–840 fused to gal4 (amino acids 1–147) in the SG5-gal4 vector (4). Amino acids 588–774 of SRC3 composing the RID were amplified by PCR from SRC3 CMX-SRC3 (48) and cloned in-frame into the SG5-gal4 vector. SG5-gal4-SRC1 was constructed by cloning the SRC1 fragment from pCR-SRC1 (49) in-frame into SG5-gal4. SG5-SRC1 was constructed by inserting the SRC1 fragment from pCR-SRC1 into the SG5 vector (Stratagene), and SG5-SRC2/GRIP (glucocorticoid receptor-interacting protein) was described previously (50). SG5-SRC3 was constructed by inserting the SRC3 cDNA from CMX-SRC3 (48) into SG5 (Stratagene).

SG5-VDR (S172) was made using the GeneEditor Site Directed Mutagenesis System (Promega) and the primer 5′-

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

GTGTGTCTGGCGTTGGGCCTG-3′. Briefly, the primer was phosphorylated and annealed to the denatured SG5-VDR DNA template. The mixture was incubated for 1 h for mutant strand synthesis and ligation, and DNA was transformed into a mismatch repair-deficient *Escherichia coli* strain for DNA production. DNA was purified using the Wizard Plus SV Miniprep kit (Promega), transformed into DH5α *E. coli*, and plated for colony selection. Accuracy of the S172A SG5-VDR mutation and surrounding sequence was confirmed by sequencing.

Cell Culture and Transfections

All cells were grown in 5% CO_2 at 37 C. COS-7 cells were cultured in DMEM supplemented with 5% charcoal-stripped calf bovine serum (CBS) for 4 d before transfection. COS-7 cells were seeded for transfection in six-well plates at a density of 7×10^4 cells per well. HeLa cells were maintained in DMEM with 10% fetal bovine serum and seeded for transfection in six-well plates at a density of 7×10^4 cells per well. ROS 17/2.8 cells were maintained in DMEM with 5% charcoal-stripped CBS and seeded for transfection in six-well plates at a density of 8×10^4 cells per well. Cells were transfected by standard calcium phosphate precipitation procedures (27). The phRG-TK vector (Promega) was cotransfected for normalization of transfection efficiency, as indicated. After 18 h, cells were washed twice with PBS and media were replaced, then cells were treated with 1,25-$(OH)_2D_3$ or ethanol control, as indicated. After 24 h, cells were washed twice with PBS and lysed in 300 μl Passive Lysis Buffer (Promega) per well. Luciferase activity was determined from 20 μl samples of cell lysate using the Dual Luciferase Assay System (Promega) and a LMax luminometer (Molecular Devices, Sunnyvale, CA).

Whole Cell Labeling with ^{32}P Orthophosphate

COS-7 cells were seeded in 60-mm plates at a density of 1.8×10^5 cells per plate. Cells were grown for 24 h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 5% charcoal-stripped CBS, then transfected with CMV-Flag-VDR in the absence or presence of SG5-$CaMKIV_{1-313}$ by standard calcium phosphate precipitation techniques. After 18 h, the precipitate was removed by washing twice with PBS, pH 7.2 (PBS), followed by replenishment of the media. After 24 h, the cells were washed in sterile Tris-buffered saline pH 7.5 (TBS) and phosphate-free DMEM was added, supplemented with 2% CBS dialyzed against TBS. [^{32}P]-orthophosphate (0.06 mCi) was added to each plate in the absence or presence of 10 nM 1,25-$(OH)_2D_3$. The cells were labeled for 2 h, then washed twice with ice-cold TBS and harvested in 500 μl ice-cold lysis buffer [25 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, with 0.1% Nonidet P-40, 1 mM dithiothreitol, 50 mM NaF, 0.65 mM $NaVO_3$, and a protease inhibitor cocktail (Sigma, St. Louis, MO)]. The cells were lysed for 10 min with rocking at 4 C, scraped, and transferred to microfuge tubes. After a 10-min centrifugation at 4 C, the supernatant was transferred to a new tube, a crude sample was reserved, and 50 μl of 50% M2 affinity gel slurry (Sigma) was added. After a 2-h incubation at 4 C, the M2 beads were collected and washed three times with cold lysis buffer containing 0.1% Nonidet P-40. The washed pellet was solubilized in SDS-PAGE sample buffer and separated on 10% denaturing polyacrylamide gels. The gel was dried and exposed to X-OMAT-ARS film for 8 h. A second gel containing crude samples was transferred to polyvinylidene difluoride membrane and processed by standard Western blotting procedures using the 9A7 rat monoclonal antibody raised against VDR and chemiluminescent detection. No ^{32}P signal was observed from the exposure time used for Western blot. Quantitation of ^{32}P and Western signal was performed from film scans by the Gene-Genius imaging system (Syngene, Frederick, MD), using GeneSnap and GeneTools (Syngene) software for analysis.

Acknowledgments

We thank B. W. O'Malley (Baylor University) for the generous contribution of pCR3.1-SRC, and A. R. Means (Duke University) for SG5-$CaMKIV_{1-313}$ and pCDNA-$CaMKII_{1-290}$. We also thank D. C. Schultz for generous use of equipment, and A. L. M. Sutton for thoughtful discussion of the manuscript.

Received September 27, 2004. Accepted May 17, 2005.

Address all correspondence and requests for reprints to: Paul N. MacDonald, Ph.D., Department of Pharmacology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106. E-mail: pnm2@case.edu.

This work was supported by Grant RO1DK50348 and RO1DK53980 from The National Institute of Diabetes and Digestive and Kidney Diseases. T.I.E. is supported by an Institutional National Research Service Award from the National Institutes of General Medical Sciences (T32GM 08803). Portions of this work were presented at the 25th Annual Meeting of the American Society for Bone and Mineral Research, September 2003.

REFERENCES

1. Sutton ALM, MacDonald PN 2003 Vitamin D: more than a "bone-a-fide" hormone. Mol Endocrinol 17:777–791
2. Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270: 1354–1357
3. Zhang C, Dowd DR, Staal A, Gu C, Lian JB, Van Wijnen AJ, Stein GS, MacDonald PN 2003 NCoA62/SKIP is a nuclear matrix-associated coactivator that may couple vitamin D receptor-mediated transcription and RNA splicing. J Biol Chem 278:35325–35336
4. Zhang C, Baudino TA, Dowd DR, Tokumaru H, Wang W, MacDonald PN 2001 Ternary complexes and cooperative interplay between NCoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription. J Biol Chem 276:40614–40620
5. Baudino TA, Kraichely DM, Jefcoat Jr SC, Winchester SK, Partridge NC, MacDonald PN 1998 Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. J Biol Chem 273:16434–16441
6. Rachez C, Suldan Z, Ward J, Chang CP, Burakov D, Erdjument-Bromage H, Tempst P, Freedman LP 1998 A novel protein complex that interacts with the vitamin D_3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. Genes Dev 12:1787–1800
7. Berridge MJ, Bootman MD, Roderick HL 2003 Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4:517–529
8. Demaurex N, Distelhorst C 2003 Cell Biology: apoptosis—the calcium connection. Science 300:65–67
9. Chawla S, Hardingham GE, Quinn DR, Bading H 1998 CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science 281:1505–1509
10. Means AR 2000 Regulatory cascades involving calmodulin-dependent protein kinases. Mol Endocrinol 14:4–13

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

11. Ohmstede CA, Jensen KF, Sahyoun NE 1989 Ca^{2+}/calmodulin-dependent protein kinase enriched in cerebellar granule cells. Identification of a novel neuronal calmodulin-dependent protein kinase. J Biol Chem 264: 5866–5875
12. Frangakis MV, Chatila T, Wood ER, Sahyoun N 1991 Expression of a neuronal Ca^{2+}/calmodulin-dependent protein kinase, CaM kinase-Gr, in rat thymus. J Biol Chem 266:17592–17596
13. Wang SL, Ribar TJ, Means AR 2001 Expression of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) messenger RNA during murine embryogenesis. Cell Growth Differ 12:351–361
14. Nairn AC, Greengard P 1987 Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase I from bovine brain. J Biol Chem 262:7273–7281
15. Ouimet CC, McGuinness TL, Greengard P 1984 Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. Proc Natl Acad Sci USA 81:5604–5608
16. Jensen KF, Ohmstede CA, Fisher RS, Sahyoun N 1991 Nuclear and axonal localization of Ca^{2+}/calmodulin-dependent protein kinase type GR in rat cerebellar cortex. Proc Natl Acad Sci USA 88:2850–2853
17. Tokumitsu H, Brickey DA, Glod J, Hidaka H, Sikela J, Soderling TR 1994 Activation mechanisms for Ca^{2+}/calmodulin-dependent protein kinase IV. Identification of a brain CaM-kinase IV kinase. J Biol Chem 269: 28640–28647
18. Kane CD, Means AR 2000 Activation of orphan receptor-mediated transcription by Ca^{2+}/calmodulin-dependent protein kinase IV. EMBO J 19:691–701
19. Kuno-Murata M, Koibuchi N, Fukuda H, Murata M, Chin WW 2000 Augmentation of thyroid hormone receptor-mediated transcription by Ca^{2+}/calmodulin-dependent protein kinase type IV. Endocrinology 141:2275–2278
20. Nemere I, Yoshimoto Y, Norman AW 1984 Calcium transport in perfused duodena from normal chicks: enhancement within fourteen minutes of exposure to 1,25-dihydroxyvitamin D_3. Endocrinology 115:1476–1483
21. Lieberherr M 1987 Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. J Biol Chem 262:13168–13173
22. Nguyen TM, Lieberherr M, Fritsch J, Guillozo H, Alvarez ML, Fitouri Z, Jehan F, Garabedian M 2004 The rapid effects of 1,25-dihydroxyvitamin D_3 require the vitamin D receptor and influence 24-hydroxylase activity: studies in human skin fibroblasts bearing vitamin D receptor mutations. J Biol Chem 279:7591–7597
23. Jurutka PW, Hsieh JC, Nakajima S, Haussler CA, Whitfield GK, Haussler MR 1996 Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. Proc Natl Acad Sci USA 93:3519–3524
24. Hsieh J-C, Jurutka PW, Galligan MA, Terpening CM, Haussler CA, Samuels DS, Shimizu Y, Shimizu N, Haussler MR 1991 Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. Proc Natl Acad Sci USA 88:9315–9319
25. Qi X, Pramanik R, Wang J, Schultz RM, Maitra RK, Han J, DeLuca HF, Chen G 2002 The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D_3-induced growth inhibition. J Biol Chem 277: 25884–25892
26. Chatila T, Anderson KA, Ho N, Means AR 1996 A unique phosphorylation-dependent mechanism for the activation of Ca^{2+}/calmodulin-dependent protein kinase type IV/GR. J Biol Chem 271:21542–21548
27. MacDonald PN, Dowd DR, Nakajima S, Galligan MA, Reeder MC, Haussler CA, Ozato K, Haussler MR 1993 Retinoid X receptors stimulate and 9-*cis* retinoic acid inhibits 1,25-dihydroxyvitamin D_3-activated expression of the rat osteocalcin gene. Mol Cell Biol 13:5907–5917
28. Rowan BG, Weigel NL, O'Malley BW 2000 Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. J Biol Chem 275:4475–4483
29. Rowan BG, Garrison N, Weigel NL, O'Malley BW 2000 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. Mol Cell Biol 20:8720–8730
30. de Boland AR, Nemere I 1992 Rapid actions of vitamin D compounds. J Cell Biochem 49:32–36
31. Norman AW, Okamura WH, Bishop JE, Henry HL 2002 Update on biological actions of 1α,25(OH)2-vitamin D_3 (rapid effects) and 24R,25(OH)2-vitamin D_3. Mol Cell Endocrinol 197:1–13
32. Caffrey JM, Farach-Carson MC 1989 Vitamin D3 metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. J Biol Chem 264: 20265–20274
33. Massheimer V, de Boland AR 1992 Modulation of 1,25-dihydroxyvitamin D_3-dependent Ca^{2+} uptake in skeletal muscle by protein kinase C. Biochem J 281:349–352
34. Bissonnette M, Tien XY, Niedziela SM, Hartmann SC, Frawley Jr BP, Roy HK, Sitrin MD, Perlman RL, Brasitus TA 1994 1,25(OH)2 vitamin D_3 activates PKC-α in Caco-2 cells: a mechanism to limit secosteroid-induced rise in [Ca^{2+}]i. Am J Physiol 267:G465–G475
35. Beno DW, Brady LM, Bissonnette M, Davis BH 1995 Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D_3-stimulated Egr induction. J Biol Chem 270:3642–3647
36. Wali RK, Kong J, Sitrin MD, Bissonnette M, Li YC 2003 Vitamin D receptor is not required for the rapid actions of 1,25-dihydroxyvitamin D_3 to increase intracellular calcium and activate protein kinase C in mouse osteoblasts. J Cell Biochem 88:794–801
37. Nemere I, Farach-Carson MC, Rohe B, Sterling TM, Norman AW, Boyan BD, Safford SE 2004 Ribozyme knockdown functionally links a $1,25(OH)_2D_3$ membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. Proc Natl Acad Sci USA 101:7392–7397
38. Zanello LP, Norman AW 2004 Rapid modulation of osteoblast ion channel responses by 1α,$25(OH)_2$-vitamin D_3 requires the presence of a functional vitamin D nuclear receptor. Proc Natl Acad Sci USA 101:1589–1594
39. Dwivedi PP, Hii CS, Ferrante A, Tan J, Der CJ, Omdahl JL, Morris HA, May BK 2002 Role of MAP kinases in the 1,25-dihydroxyvitamin D_3-induced transactivation of the rat cytochrome P450C24 (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. J Biol Chem 277: 29643–29653
40. Corcoran EE, Joseph JD, MacDonald JA, Kane CD, Haystead TAJ, Means AR 2003 Proteomic analysis of calcium/calmodulin-dependent protein kinase I and IV in vitro substrates reveals distinct catalytic preferences. J Biol Chem 278:10516–10522
41. Sun P, Enslen H, Myung PS, Maurer RA 1994 Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev 8:2527–2539
42. Masuyama H, Brownfield CM, St.-Arnaud R, MacDonald PN 1997 Evidence for ligand-dependent intramolecular folding of the AF-2 domain in vitamin D receptor-activated transcription and coactivator interaction. Mol Endocrinol 11:1507–1517
43. Kraichely DM, Collins Jr J, DeLisle RK, MacDonald PN 1999 The autonomous transactivation domain in helix H3 of the vitamin D receptor is required for transactivation

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025

and coactivator interaction. J Biol Chem 274: 14352–14358
44. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194–198
45. Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS 1994 Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol Cell Biol 14: 6107–6116
46. Kraichely DM, Nakai YD, MacDonald PN 1999 Identification of an autonomous transactivation domain in helix H3 of the vitamin D receptor. J Cell Biochem 75:82–92
47. Masuyama H, Jefcoat SC, MacDonald PN 1997 The N-terminal domain of transcription factor IIB is required for direct interaction with the vitamin D receptor and participates in vitamin D-mediated transcription. Mol Endocrinol 11:218–228
48. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM 1997 Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90:569–580
49. Smith CL, Onate SA, Tsai MJ, O'Malley BW 1996 CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. Proc Natl Acad Sci USA 93:8884–8888
50. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, Stallcup MR 1998 Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 12:302–313

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/mend/article/19/9/2309/2738007 by guest on 24 April 2025