We provide evidence of a cross-talk between nuclear receptor and Ser/Thr protein phosphatases and show that vitamin D receptor (VDR) interacts with the catalytic subunit of protein phosphatases, PP1c and PP2Ac, and induces their enzymatic activity in a ligand-dependent manner. PP1c specifically interacts with VDR but not retinoic acid receptor α and retinoid X receptor α in yeast. Although VDR-PP1c and VDR-PP2Ac interaction is ligand-independent in vivo, 1α,25-dihydroxy-vitamin D3 induces VDR-associated phosphatase activity. Further, VDR modulation of PP1c/PP2Ac activity results in a rapid and specific dephosphorylation and inactivation of their substrate, p70 S6 kinase (p70S6k). Finally, we demonstrate that the endogenous VDR, PP1c or PP2Ac, and p70S6k are present in a ternary complex in vivo, and the interaction of p70S6k with the VDR-PP complex is modulated by the phosphorylation state of the kinase. Since p70S6k is essential for G1-S transition, our results provide a molecular basis of 1α,25-dihydroxy-vitamin D3-induced G1 block in colon cancer cells.

Vitamin D receptor (VDR), a sequence-specific ligand-dependent transcription factor belonging to the family of nuclear receptors, mediates biological actions of 1α,25-dihydroxy-vitamin D3 (1,25(OH)2D3). VDR heterodimerizes with retinoid X receptor (RXR), and at the molecular level VDR-RXR heterodimers induce gene expression via interaction with vitamin D response elements present in the promoter regions of responsive genes (1). This mode of action is known as the "genomic action" of VDR. However, 1,25(OH)2D3 also induces gene expression by a mechanism distinct from its classical mode of action. For example, 1,25(OH)2D3-induced expression of monocyte differentiation markers CD14 and CD11b in THP-1 cells requires phosphatidylinositol 3-kinase (PI 3-kinase) via ligand-dependent interaction of VDR with the regulatory (p85) subunit of PI 3-kinase (2). Similarly estrogen receptor interacts with the p85 regulatory subunit of the PI 3-kinase where estrogen receptor-PI 3-kinase interaction leads to the activation of protein kinase B/AKT and endothelial nitric-oxide synthase (3). It thus appears that cross-talk between nuclear receptors and other signal transduction pathways can lead to either induction of gene expression in a nuclear receptor-responsive element-independent manner or to extranuclear/non-genomic induction of enzymatic activities that are physiologically important, for example, in explaining the vasoprotective effects of estrogen (3). Further, nuclear receptor ligands (dexamethasone, triiodothyronine, and retinoic acid) also induce a rapid dephosphorylation of c-Jun N-terminal kinase independently of their transcriptional activation (4). Therefore, nuclear receptors appear to have a functional role both inside and outside the nucleus. Ser/Thr phosphatases are implicated in the regulation of a wide variety of cellular functions, namely metabolism, transcription, translation, development, cell growth, and differentiation (5). There are two major and structurally related families of Ser/Thr phosphatases, termed PP1 and PP2A. PP1c and PP2Ac are the catalytic subunits of PP1 and PP2A, respectively, that associate with various regulatory and target subunits, thereby generating distinct holoenzymes with unique localizations, specificities, and cellular functions (6).

In this report, we provide evidence of a cross-talk between VDR and Ser/Thr phosphatases and show that VDR interacts with PP1c in a ligand-dependent manner in yeast. Although the association of VDR and PP1c/PP2Ac is ligand-independent in vitro and in vivo in mammalian cells, 1,25(OH)2D3 induces the Ser/Thr phosphatase enzymatic activity of these phosphatases. This induction leads to a rapid and specific dephosphorylation at the Thr-389 residue of p70 S6 kinase (p70S6k), resulting in inactivation of this kinase. By co-immunoprecipitation, we also demonstrate the presence of a ternary complex containing VDR-PP-p70S6k where PP represents PP1c or PP2Ac. Using rapamycin, which specifically prevents Thr-389 phosphorylation, we show that the Thr-389-dephosphorylated p70S6k does not efficiently interact with the VDR-PP complex, suggesting that p70S6k phosphorylation state modulates its interaction with the VDR-PP complex. Since p70S6k is critical for G1-to-S phase transition, our observations provide a molecular basis of VDR ligand-induced G1 block in colon/epithelial cancer cells.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—the bait plasmid was generated by introducing a PCR-amplified VDR-LBD fragment (amino acids 89–427) in-frame with Gal4 DNA binding domain into EcoRI-BamHI sites of the plasmid pGBK7T7 (CLONTECH, Palo Alto, CA). Transformation of yeast was carried out by using the Yeastmaker transformation kit (CLONTECH). A colony from pGBK7T7-transformed yeast strain P669-2A was grown for 18 h in 50 ml of SD/Trp+ medium at 30 °C with shaking at 270 rpm. After a growth of 0.8 at A600, cells were pelleted (1000 × g, 5 min) and resuspended in 5 ml of the medium to give a final concentration of more than 1 × 109 cells/ml. The entire pGBK7T7-VDR-transfected P669-2A culture was combined with 1 ml of Y187 cells pretransformed with a mouse embryo cDNA library in pGADT7 expression vector (CLONTECH) in a 2-liter sterile flask. YPDA/kan (50 mg/
was added to the flask and incubated for 24 h at 30 °C with swirling (50 rpm) for mating. The mixture was centrifuged (1000 × g, 10 min), and the cells were resuspended in YPD/kan (10 ml). Double transformants were screened on medium lacking tryptophan, leucine, and histidine containing 25 mM 9-amino-1,2,4-triazole in the presence of 1.25(OH)2D3 (1 μM) galectoprotein was tested for galactosidase activity (7).

GST Pull-down Assays—PP1c deletion mutants Δ1PP1c (amino acids 105–323), Δ2PP1c (amino acids 177–323), and Δ3PP1c (amino acids 1–200) were prepared by cloning corresponding PCR amplification products into EcoRI-XbaI sites of pCDNA3 (Invitrogen). For protein expression, pGEX3X-VDR-LBD-transformed DH5α cells (25 ml) were grown overnight (18 h) at 30 °C with shaking. Proteins were collected (12,000 rpm for 10 min) at 4 °C in cold PBS, resuspended in 5 ml of extraction buffer (PBS, 5 mM EDTA, 1 mM DTT with protease inhibitors), and sonicated three times for 10 s with 15-s intervals. Following centrifugation to remove cell debris, bacterial extract was incubated (2 h) at 4 °C with a 50% slurry of glutathione-Sepharose beads (250 μl) (Amersham Biosciences). For GST pull-down assays, 15 μl of glutathione bead-bound GST-VDR-LBD or GST were mixed with 5 μl of in vitro transcribed and translated [35S]methionine-labeled wild type or mutant PP1c. Reactions were subsequently incubated in 1 ml of 75 mM KCl, 20 mM NaCl, 20 mM Hepes, pH 7.0, 1 mM EDTA, 0.1% Triton-X, 10% glycerol for 2 h at 4 °C in the presence or absence of 1,25(OH)2D3 (10−7 M). Beads were washed, resuspended in 2× protein loading dye (Invitrogen), denatured for 10 min at 70 °C, subjected to SDS-PAGE (12%), and analyzed by autoradiography.

Microcystin Affinity and Phosphatase Activity—T47D and Caco-2 cells were cultured in phenol red-free, high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal calf serum. For microcystin chromatography, Caco-2 or T47D cells were treated with 1.25(OH)2D3 (10−7 M) for 30 min, washed with cold PBS, and scraped in MC buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM MgCl2, 2 mM DTT, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1× mammalian mixture of protease inhibitors (Sigma)). Cells were centrifuged (1000 × g) for 5 min, resuspended in MC buffer (3 ml), and 1× microcystinase were passed through a 21-gauge needle, and debris was removed by centrifugation (4000 rpm) for 10 min. Protein concentration was measured using BCA reagent (Pierce). The control sample was preincubated with 5 mM free microcystin for 1 h at 4 °C. Cell extract (1 mg of protein) was mixed with 30 μl of microcystin-Sepharose beads (50 mM slurry) (Upstate Biotechnology, Lake Placid, NY) pre-equilibrated with MC buffer and incubated overnight at 4 °C with shaking. Beads were washed with cold MC buffer containing 150 mM NaCl, resuspended in 10 μl of 2× protein loading dye, and electrophoresed on a 10% SDS-polyacrylamide gel together with aliquots from the unbound fraction (20 μg each). 1,25(OH)2D3 was present in all washes and buffer for the treated samples. For phosphatase activity, cell extracts (600 μg of protein) were incubated overnight at 4 °C with agarsose-conjugated rabbit anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and beads were washed four times with washing buffer (25 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij35) supplemented with 150 mM NaCl and 1,25(OH)2D3 (4 M) (Fig. 2A). Beads were resuspended in washing buffer, and aliquots (40 μl) were used for phosphatase activity assay or Western blotting. Ser/Thr phosphatase activity was measured using the Ser/Thr phosphatase assay kit (New England Biolabs, Beverly, MA).

Analysis of p70S6k Phosphorylation—Cells were treated with 1.25(OH)2D3 (10−8 M) or vehicle for 5, 15, or 30 min, rinsed once with cold PBS containing phosphatase inhibitor mixture I (Sigma), and scraped off in the same buffer. Cells were centrifuged at 450 × g for 5 min and resuspended in 5 volumes of hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT with protease and phosphatase inhibitors) for 5 min. IgE-p460 was added (0.6% final concentration), and cells were vortexed (10 s) and centrifuged (10,000 × g, 30 s). The supernatant was kept as the cytoplasmic fraction. Protein extracts (30 μg) were subjected to 10% SDS-PAGE and Western blotting with various p70S6k antibodies (Cell Signaling, Beverly, MA). Immunoprecipitation of VDR- or PP1c-bound p70S6k was performed on 1 mg of cytoplasmic extract as described for the phosphatase assay.

RESULTS AND DISCUSSION

To gain a better understanding of the mechanism of action of 1,25(OH)2D3, a yeast two-hybrid system was used to identify proteins that interacted with VDR in the presence of the ligand. The sequence of VDR harboring the D-E regions of the receptor (amino acids 89–427) was used to prepare the bait construct (pGBK77-VDR), and cDNAs encoding VDR-interacting proteins were isolated from a 17.5-day mouse embryo library. Upon characterization, most of the cDNA clones, whose protein products interacted with VDR in a ligand-dependent manner, were found to be mouse RXRa, β, and γ (Fig. 1A). However, only when pGBK77-VDR was expressed with pGADT7-PP1c in the presence but not in the absence of 1,25(OH)2D3 (Fig. 1, A and C). Ligand-dependent interaction of VDR-LBD with PP1c was also found to be dose-dependent (Fig. 1B), and the interaction was also observed with full-length VDR but not with RARα and RXRα (Fig. 1, C and D).

To further explore VDR-PP1c interaction, a GST-VDR-LBD fusion protein (containing amino acids 90–427) was purified from pGEX3X-VDR-LBD-transformed bacterial cells using glutathione-Sepharose beads. In vitro translated [35S]methionine-labeled PP1c was retained by the glutathione bead-bound GST-VDR-LBD. In contrast to the yeast two-hybrid results, in vitro interaction between GST-VDR-LBD and PP1c was found to be ligand-independent (Fig. 2A). No interaction was observed between PP1c and glutathione-bound GST protein. To identify the VDR interaction domain of PP1c, deletion mutants of PP1c were generated and assayed for interaction in GST pull-down experiments. Deletion of up to 177 amino acids in the N-terminal portion of PP1c did not prevent binding to VDR-LBD (Fig. 2A), used in the in vitro two-hybrid experiments. Deletion of 123 amino acids from the C terminus of PP1c significantly reduced but did not abolish its binding to VDR (Fig. 2B), suggesting that although the C terminus of PP1c provides the major interacting region, similar interactions were also observed with PP1c deletion mutants that contained the C terminus (Fig. 2B, lane 4).

FIG. 1. Isolation of VDR-interacting proteins. A, β-galactosidase activity of clones expressing RXRα, β, γ, Gal4-AD or PP1c-Gal4-AD and Gal4-VDR-LBD in yeast in the presence (black bars) or absence (hatched bars) of 1,25(OH)2D3 (10−6 M). B, dose-responsive VDR-PP1c interaction. Yeast cells were cotransformed with pGBK77-VDR-LBD along with pGADT7-PP1c. Gal4-AD or PP1c specifically interacts with wild type VDR. Yeast cells transformed with pGBK77-T9-DV or pGBK77-RARα (3 and 4), or pGBK77-Gal4-AD (5 and 6) or pGADT7-PP1c (1, 3, and 4) or with empty pGADT7 vector (2, 4, and 6) were plated on Leu−Trp−His−plates in the presence or absence of 1,25(OH)2D3 for VDR, arylotinid acid (TTNPB) for RAR, and 9-cis-retinoic acid for RXR. D, β-Galactosidase assay of clones shown in C. Data are representative of three independent experiments.
**VDR-Ser/Thr Protein Phosphatase Interaction**

**Fig. 2. VDR-PP1c interaction in vitro and in vivo.**

A, wild type or N-terminal deletion mutants of PP1c radionabeled with \[^{35}S\]methionine were incubated with GST-VDR-LBD fusion protein bound to GST-Sepharose beads either in the presence (+) or absence (−) of 1,25(OH)\(_2\)D\(_3\). Incubation of the radiolabeled protein with GST alone served as a control. **The Input lane represents 20% of the total volume of the crude lysate used in each reaction.** Beads were washed, and bound proteins were eluted and analyzed by SDS-PAGE and subjected to fluorography for \[^{35}S\].

B, analysis of a C-terminal deletion of PP1c in the presence or absence of 1,25(OH)\(_2\)D\(_3\). The experiment was performed as described in A. C, VDR-phosphatase interaction in Caco-2 cells. 1,25(OH)\(_2\)D\(_3\)-treated (+) or untreated (−) Caco-2 cell extracts were incubated with microcystin. Beads were washed, and bound proteins were eluted and analyzed by SDS-PAGE and Western blotting with anti-VDR antibodies.

C and D, VDR-phosphatase interaction in Caco-2 cells. PP1c- or PP2Ac-attached proteins were eluted and analyzed by SDS-PAGE and Western blotting with anti-VDR antibodies. A VDR-PP1c interaction was observed in untransfected cells. 1,25(OH)\(_2\)D\(_3\)-treated (black bars) and ligand-dependent interaction (striped bar) VDR-associated Ser/Thr phosphatase activity. The amount of Ser/Thr phosphatase activity present in 5 μg of control and treated Caco-2 cell extract is also shown. Data are representative of three independent experiments performed in triplicate. B, ligand-independent association of VDR with PP1c and PP2Ac. Immunoprecipitations were performed from 1,25(OH)\(_2\)D\(_3\)-treated (+) or untreated (−) Caco-2 cell extracts with VDR antibodies. After extensive washing, the immune complexes were analyzed by SDS-PAGE and immunoblotting with PP1c, PP2Ac, RXRα, and p70\(^{S6}\)k antibodies. The relative intensity of the bands in arbitrary units is shown below the blot. Preincubation of Caco-2 cell extract with microcystin-Sepharose beads, PP1c- or PP2Ac-attached proteins were eluted and analyzed by SDS-PAGE and Western blotting with VDR, RXRα, and PP1c antibodies. VDR, RXRα, and PP1c proteins were analyzed in the total cell extract, unbound flow-through, and microcystin bead-bound fraction. Data are representative of three independent experiments.

To verify that endogenous PP1c was physically associated with endogenous VDR, we purified PP1c-associated proteins using microcystin-Sepharose (an affinity resin for PP1c and PP2Ac) beads (8). Cell extracts obtained from control or 1,25(OH)\(_2\)D\(_3\) (10^{-7} M)-treated Caco-2 cells were incubated overnight at 4 °C with microcystin-Sepharose beads. Following extensive washing, microcystin bead-bound proteins were separated by SDS-PAGE and analyzed by Western blotting using anti-VDR antibodies. A VDR-PP1c interaction was observed in Caco-2 cells that was not dependent on the presence of the VDR ligand (Fig. 2C, a). Preincubation of Caco-2 extract with 0.5 nmol of free microcystin resulted in a loss of detectable VDR and PP1c, suggesting that VDR-PP1c association was specific (Fig. 2C, b). Further, microcystin chromatography of control and 1,25(OH)\(_2\)D\(_3\) (10^{-7} M)-treated Caco-2 and T47D cell extracts followed by Western blotting with anti-VDR antibodies showed VDR-PP1c interaction in Caco-2 but not T47D cells (Fig. 2D), suggesting that VDR-PP complex is cell context-dependent. Moreover, RXRα was not detected in the VDR-PP complex resolved on microcystin-Sepharose beads (Fig. 2D).

The presence of ligand-independent interaction between VDR and PP1c in GST and microcystin systems but ligand dependence in yeast prompted us to examine the possibility that 1,25(OH)\(_2\)D\(_3\) induced the enzymatic activity of VDR-bound Ser/Thr protein phosphatases. A ligand-mediated increase in phosphatase activity could explain the increase in VDR gene expression in yeast. Therefore, we measured VDR-associated Ser/Thr phosphatase activity. Anti-VDR antibody could pull down Ser/Thr protein phosphatase enzymatic activity from Caco-2 cell extracts (Fig. 3A). Further, 30-min treatment of cells with 1,25(OH)\(_2\)D\(_3\) (10^{-8} M) resulted in an enhancement of VDR-associated phosphatase activity (Fig. 3A). The specificity of VDR-associated Ser/Thr phosphatase activity was demonstrated by incubation of the VDR-IP reaction with microcystin (6 nM) that completely abolished the VDR-associated enzymatic activity (Fig. 3A). Microcystin, a cyanobacterial toxin, is a potent inhibitor of both PP2Ac and PP1c (9). Aliquots from the VDR-IP used to measure phosphatase activity were subjected to Western blot analysis to monitor differences in VDR-associated Ser/Thr phosphatases between control and 1,25(OH)\(_2\)D\(_3\)-treated samples. In immunoprecipitations, VDR antibody could pull down not only PP1c but also PP2Ac, and identical levels of PP1c and PP2Ac proteins were detected in control and treated cell extracts (Fig. 3B). These results demonstrate that the increased Ser/Thr phosphatase activity associated with VDR-IP derived from 1,25(OH)\(_2\)D\(_3\)-treated cells did not result from increased recruitment of Ser/Thr phosphatases to the receptor. In contrast, as expected, 1,25(OH)\(_2\)D\(_3\) treatment of Caco-2 cells resulted in increased immunoprecipitation of RXRα by anti-VDR antibodies (Fig. 3B). Taken together these
data suggest that 1,25(OH)_{2}D_{3} increases the enzymatic activity of VDR-associated Ser/Thr phosphatases in Caco-2 cells.

A number of cellular mechanisms dependent on Ser/Thr phosphorylation and relevant to 1,25(OH)_{2}D_{3} action could be affected by increased VDR-bound phosphatase activity. The effect of 1,25(OH)_{2}D_{3} on G_{1}-S phase transition is of particular interest since Ser/Thr phosphatases complex with a number of key regulators of cell cycle progression. PP2Ac is known to dephosphorylate and inactivate p70S6k. Anti-VDR antibody could pull down Thr-389-specific p70S6k antibodies showed dephosphorylation of p70S6k after 5, 15, and 30-min treatments of cells with 1,25(OH)_{2}D_{3} (Fig. 3C). In contrast, treatment with 1,25(OH)_{2}D_{3} did not change either the protein level of p70S6k or the level of phospho-p70S6k at Thr-389 at Thr-242/Ser-244 or Ser-411 positions (Fig. 3A). This inhibition resulted in a significant dissociation of p70S6k from VDR under conditions where Thr-389 phosphorylation of p70S6k was not secondary to reduced FRAP/mTOR activity.

Although PP2Ac-p70S6k interaction is well documented (10–12), PP1c-p70S6k interaction has not been reported. As shown in Fig. 3D, p70S6k could be immunoprecipitated using anti-PP1c antibody, suggesting the presence of a PP1c-p70S6k signaling module. We next tested the possibility that VDR-PP1c or -PP2Ac complexes also contain p70S6k, thereby providing an opportunity for PP2Ac or PP1c to dephosphorylate and thus inactivate p70S6k. Anti-PP1c antibody could pull down Thr-389-phosphorylated p70S6k in the absence but not in the presence of the ligand (Fig. 3D). Further, p70S6k did not directly interact with VDR since in vitro translated p70S6k was not retained by GST-VDR-LBD bound to glutathione beads (data not shown). Dissociation of p70S6k from VDR under conditions where Thr-389 was mainly dephosphorylated (via activation of associated PP1c or PP2Ac in the complex) suggested that the interaction could be dependent upon the phosphorylation state of p70S6k. To support this hypothesis, Caco-2 cells were treated with rapamycin (20 nM, 45 min), and VDR immunoprecipitates were analyzed by using phospho-Thr-389-specific antibody. Since rapamycin preferentially inhibits FRAP/mTOR, the upstream kinase of p70S6k (16), as expected rapamycin treatment of Caco-2 cells inhibited phosphorylation of p70S6k at Thr-389 (Fig. 3D). This inhibition resulted in a significant dissociation of p70S6k from VDR, supporting the idea that phospho-Thr-389 preferentially interacted with the VDR-PP complex in the absence of the ligand (Fig. 3D). These results suggest that VDR may act as a platform to compartmentalize VDR-PP1c-p70S6k and VDR-PP2Ac-p70S6k complexes to the same intracellular location or bring them in close proximity so that ligand stimulation could result in induction of Ser/Thr phosphatase activity, which in turn may result in dephosphorylation and inactivation of p70S6k. Therefore, in addition to vitamin D-dependent induction of p21 gene expression (17), VDR ligand-mediated dephosphorylation and inactivation of p70S6k may also play a role in 1,25(OH)_{2}D_{3}-induced G_{1} arrest of colon cancer cells. Alternatively the VDR-PP1c/PP2Ac-p70S6k pathway may contribute to ligand-mediated inhibition of proliferation of epithelial cancer cells that do not show p21 up-regulation after 1,25(OH)_{2}D_{3} treatment (18).

Epidemiological studies show that vitamin D confers significant protection against colorectal cancer (19) and increased VDR expression is observed in cancerous lesions than normal colon (20, 21). Therefore, as shown in our model (Fig. 3E), it is tempting to speculate that in colon cancer cells, at least a part of the cytoplasmic complement of VDR is compartmentalized as VDR-PP1c/PP2Ac-p70S6k signaling module. Increased VDR levels coupled with low 1,25(OH)_{2}D_{3} may inhibit the activity of PP1c/PP2Ac in this complex, thus rendering the associated p70S6k inactive by virtue of its Thr-389 phosphorylation. This is evident from Fig. 3A since VDR-associated Ser/Thr phosphatase activity is drastically inhibited in the absence of the ligand. VDR ligand, upon binding to LBD, appears to impart a conformational change in PP1c/PP2Ac proteins, thus rendering them active in terms of their enzymatic activity (Fig. 3A), which is evident by decreased Thr-389 phosphorylation, inactivation, and dissociation of VDR-associated p70S6k (Fig. 3C, D). The final result of this sequence of events is cell cycle arrest at the G_{1}-S transition state. Accordingly, VDR ligands inhibited the proliferation of colon cancer cells in vitro and reduced tumorigenesis in vivo (22, 23). Finally, since down-regulation of PP2Ac activity is one of the key steps in cellular transformation (24, 25), our observation that 1,25(OH)_{2}D_{3} regulates Ser/Thr phosphatase activity might contribute to elucidating the role of VDR ligands in controlling growth and differentiation of normal and cancer cells.

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A Vitamin D Receptor-Ser/Thr Phosphatase-p70 S6 Kinase Complex and Modulation of Its Enzymatic Activities by the Ligand
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