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Ligand modulates VDR-Ser/Thr protein phosphatase interaction and p70S6 kinase phosphorylation in a cell-context-dependent manner☆

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Abstract

We have recently shown that in colon cancer cells, Vitamin D receptor (VDR) interacts with the catalytic subunit of Ser/Thr protein phosphatases, PP1c and PP2Ac, and induces their enzymatic activity in a ligand-dependent manner. The VDR–PP1c and VDR–PP2Ac interactions were ligand independent in vivo, and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-mediated increase in VDR-associated phosphatase activity resulted in dephosphorylation and inactivation of p70S6 kinase in colon cancer cells. Here, we demonstrate that in myeloid leukemia cells, $1,25(OH)_2D_3$ treatment increased the Thr389 phosphorylation of p70S6 kinase. Accordingly, $1,25(OH)_2D_3$ decreased VDR-associated Ser/Thr protein phosphatase activity by dissociating VDR–PP1c and VDR–PP2Ac interactions. Further, $1,25(OH)_2D_3$ increased the association between VDR and Thr389 phosphorylated p70S6 kinase. Finally, by using non-secosteroidal VDR ligands, we demonstrate a separation between transactivation and p70S6 kinase phosphorylation activities of VDR and show pharmacologically that p70S6 kinase phosphorylation correlates with HL-60 cell differentiation.

Keywords: 1,25-Dihydroxyvitamin D_3; Vitamin D receptor; Ser/Thr protein phosphatase; p70S6 kinase; VDR; PP1c; PP2A; Caco-2 cells; HL-60 cells; THP-1 cells

1. Introduction

The biological actions of the active form of Vitamin D_3, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), are mediated through Vitamin D receptor (VDR), a sequence-specific ligand-dependent transcription factor, which belongs to the superfamily of nuclear receptors. $1,25(OH)_2D_3$ plays an important role in mineral ion homeostasis, cell proliferation, differentiation, and immunomodulation [1–4]. Recently, Bettoun et al. [5], provided an evidence of a cross-talk between VDR and Ser/Thr protein phosphatases, PP1c and PP2A, and showed that VDR interacts with PP1c/PP2Ac and p70S6 kinase ($p70^{S6k}$). VDR-associated with PP1c/PP2Ac in a ligand independent manner and $1,25(OH)_2D_3$ induced the VDR-associated phosphatase activity. The modulation of PP1c/PP2A activity by VDR resulted in ligand-dependent rapid and specific dephosphorylation at threonine 389 (Thr389) residue and inactivation of their substrate $p70^{S6k}$. Since $p70^{S6k}$ is essential for G1 to S phase transition, these results also provide one of the molecular pathways of $1,25(OH)_2D_3$-mediated G1 block in Caco-2 cells. 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]. Here, we demonstrate that in myeloid leukemia cells, $1,25(OH)_2D_3$ dissociated VDR–PP1c and VDR–PP2Ac interactions and resulted in decreased VDR-associated Ser/Thr phosphatase activity and increased Thr389 phosphorylation of $p70^{S6k}$. Further, by using non-secosteroidal VDR ligands, we show that the increased Thr389 phosphorylation of $p70^{S6k}$ is required for $1,25(OH)_2D_3$-mediated HL-60 cell differentiation. We propose that the diagonally opposite regulation of $p70^{S6k}$ phosphorylation by 1,25-dihydroxyvitamin D_3, via cell-context-dependent modulation of VDR-associated PP1c/PP2Ac activity, contributes to G1 block in colon

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cancer cells and macrophage/monocytic differentiation in myeloid leukemic cells.

2. Materials and methods

2.1. Cell culture

HeLa cells were maintained in DMEM supplemented with 10% FCS. THP-1 cells were maintained and passaged in RPMI medium supplemented with 10% FCS and β-mercaptoethanol (57 μM). HL-60 were maintained and passaged in Iscove's MEM supplemented with 20% FCS.

2.2. Phosphatase assay

THP-1 or HL-60 cells (10^6 cells/ml) were incubated in phenol red-free, high glucose DMEM supplemented with 10% charcoal-stripped FCS for 24 h and treated with DMSO, 1,25$(OH)_2D_3$ (10^{-8} M), or rapamycin (20 μM) for 20 min. Cells were centrifuged (1000 rpm) at 4 °C for 5 min, washed once with cold MC buffer (20 mM Tris–HCl pH 7.5, 0.5 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 1 mM PMSF, and 1X mammalian cocktail of protease inhibitors (Sigma, St. Louis, MO)), centrifuged (1000 rpm) at 4 °C for 5 min and resuspended in MC buffer (3 ml) and subjected to three freeze-thaw cycles. Cell extracts were passed through a 21 mm-gauge needle and debris was removed by centrifugation (4000 rpm) for 10 min. Protein concentration was measured using BCA reagent (Pierce, Rockford, IL). The phosphatase activity was assayed as described previously [5].

2.3. Analysis of $p70^{S6k}$ phosphorylation

Cells were treated with vehicle, 1,25$(OH)_2D_3$ (10^{-8} M), LG190155 (10^{-7} M) or LG190178 (10^{-8}) for 20 min and analysis of $p70^{S6k}$ phosphorylation was performed on 1 mg of cytoplasmic extract as described previously [5].

2.4. Cell culture and transfection

HeLa cells passaged in phenol red-free, 10% charcoal-stripped DMEM were plated at 5000 cells per well in a 96-well plate. The next day, cells were transfected using 0.5 μl of Fugene (Roche Diagnostic Corporation, Indianapolis, IN), 100 ng of luciferase reporter pFR-Luc (Stratagene La Jolla, CA) and 10 ng each of pGal4-RXRα-LBD and pVP16–VDR–LBD expression vectors per well. Cells were treated with vehicle, 1,25$(OH)_2D_3$ (10^{-8} M), LG190155 (10^{-7} M) or LG190178 (10^{-8}) 24 h post-transfection and luciferase activity was quantitated the next day using Steady-Glo luciferase detection reagent (Promega, Madison, WI). Data were analyzed using GraphPad's Prizm analysis software.

2.5. FACS assay

HL-60 cells (5×10^4 cell/ml) were plated and stimulated with vehicle, 1,25$(OH)_2D_3$ (10^{-8} M), LG190155 (10^{-7} M) or LG190178 (10^{-8} M) in growth medium with charcoal-treated FCS in 24-well plate and incubated at 37 °C for 5 days. Cells were spun at 400 × *g* for 5 min, washed once in PBS containing 0.1% BSA, resuspended in 20 μl of PE-conjugated anti-CD14 antibody (Becton Dickinson, San Jose, CA), dark-incubated for 15 min, washed once again with PBS, 0.1% BSA and fixed in 400 ml of Cytofix buffer (BD Biosciences, San Jose, CA).

3. Results and discussion

In Caco-2 human colon carcinoma cells, 1,25$(OH)_2D_3$ upregulated VDR-associated Ser/Thr protein phosphatase activity, thereby leading to a rapid dephosphorylation of $p70^{S6k}$ at Thr389 residue. This dephosphorylation may contribute to Vitamin D_3-mediated G1–S arrest observed in these cells [5] To test whether similar mechanism participates in 1,25$(OH)_2D_3$-induced differentiation of myelocytic cells, VDR-associated phosphatase activity was assayed in

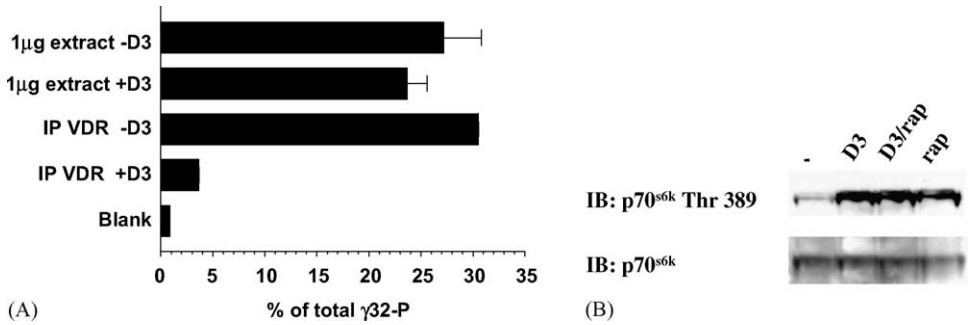

Fig. 1. Ligand inhibits VDR-associated Ser/Thr protein phosphatase activity in THP-1 cells. (A). Immunoprecipitations were performed on 1,25$(OH)_2D_3$-treated (IP VDR + D_3) or untreated (IP VDR − D_3) THP-1 cell extracts with VDR antibodies, and the immunecomplexes were assayed for phosphatase activity [5]. The amount of phosphatase activity present in 1 μg of control (−D_3) and treated (+D_3) THP-1 cells is also shown. (B). Ligand-dependent Thr389 phosphorylation of $p70^{S6k}$. Immunoblot analysis were performed on 1,25$(OH)_2D_3$, rapamycin (rap), or 1,25$(OH)_2D_3$ + rap treated (20 min) or vehicle treated (−) THP-1 cytoplasmic extracts using anti-$p70^{S6k}$ Thr389 and anti-$p70^{S6k}$ antibodies.

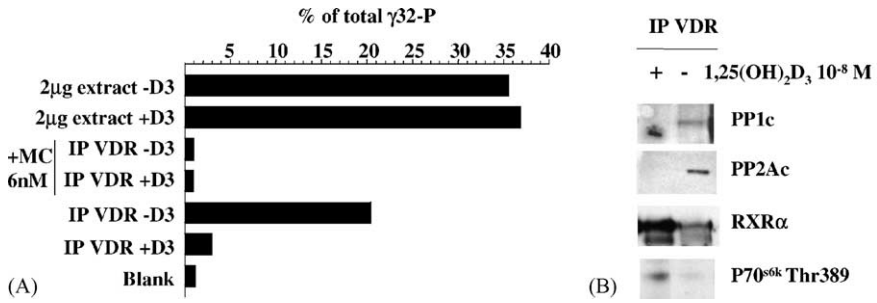

Fig. 2. Ligand disrupts VDR–PP1c/PP2Ac interactions. (A) Ligand inhibits VDR-associated Ser/Thr phosphatase activity in HL-60 cells. Immunoprecipitations were performed on $1,25(OH)_2D_3$-treated or untreated HL-60 cell extracts and assayed for phosphatase activity as shown in Fig. 1A. Incubation of immunecomplexes with microcystin (MC) abrogated the VDR-associated phosphatase activity. (B). Immunoprecipitations were performed on treated (+) or untreated (−) HL-60 cell extracts with VDR antibodies and analyzed by immunoblotting with antibodies to PP1c, PP2Ac, RXRα, and $p70^{S6k}$.

control or treated THP-1 cells. As shown in Fig. 1A, phosphatase activity could be immunoprecipitated from THP-1 cells using anti-VDR antibodies. Interestingly, 20 min treatment of the cells with $1,25(OH)_2D_3$ (10^{-8} M) resulted in an 88% reduction in VDR-associated phosphatase activity, whereas total phosphatase activity remained unaffected (Fig. 1A). These results are in sharp contrast with our findings in Caco-2 cells, where $1,25(OH)_2D_3$ treatment resulted in an increase in VDR-associated phosphatase activity [5]. In agreement with our previous observation that VDR-associated phosphatases control the Thr389 phosphorylation of $p70^{S6k}$, the phosphorylation of $p70^{S6k}$ at Thr389 was rapidly increased upon $1,25(OH)_2D_3$ treatment of THP-1 cells under conditions where the total amount of $p70^{S6k}$ protein was unaffected by treatment (Fig. 1B). Rapamycin, a known inhibitor of FRAP/mTOR, an upstream

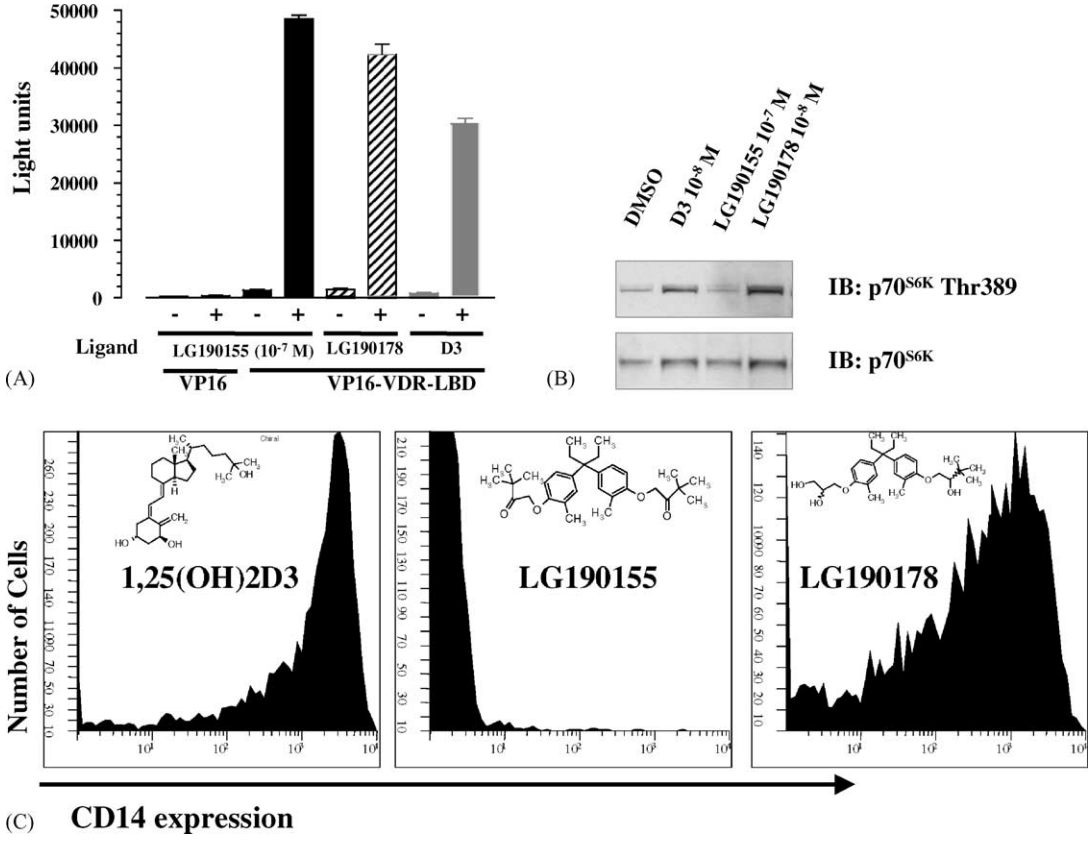

Fig. 3. $p70^{S6k}$ phosphorylation is required for HL-60 differentiation. (A) VDR ligands induce RXR-VDR heterodimerization. HeLa cells were transfected with pGal4-RXRα-LBD, pVP16-VDR-LBD, and pFR-Luc, and treated with vehicle or VDR ligands as indicated. The activation of the reporter is presented in arbitrary light units as mean + S.E. from experiments performed in triplicate. (B) LG 190155 does not induce $p70^{S6k}$ phosphorylation. Immunoblot analysis were performed on vehicle or ligand-treated (as indicated) HL-60 cell cytoplasmic extracts using $p70^{S6k}$ Thr389 and $p70^{S6k}$ antibodies. (C) LG 190155 does not induce HL-60 differentiation. HL-60 cells were treated with VDR ligands and cell differentiation was measured by quantitating CD14 expression by FACS analysis.

kinase of $p70^{S6k}$ also induced Thr389 phosphorylation, indicating that FRAP/mTOR is not involved in $p70^{S6k}$ Thr389 phosphorylation in THP-1 cells. Further, addition of rapamycin did not affect $p70^{S6k}$ phosphorylation induced by $1,25(OH)_2D_3$.

Similar results, showing a decrease in VDR-associated phosphatase activity, were also observed in cytoplasmic extracts obtained from $1,25(OH)_2D_3$ (10^{-8} M) treated HL-60 cells and incubation of VDR immunoprecipitates with microcystin completely inhibited VDR-associated phosphatase activity, suggesting that microcystin sensitive phosphatases (PP1c and PP2Ac) were responsible for the VDR-associated phosphatase activity (Fig. 2A). Immunoblot analysis of VDR immunoprecipitates showed that in the absence of $1,25(OH)_2D_3$ both PP1c and PP2Ac interacted with VDR in HL-60 cells (Fig. 2B). Interestingly, the addition of $1,25(OH)_2D_3$ (10^{-8} M) resulted in a complete loss of VDR-associated PP1c or PP2Ac, under conditions where RXRα was readily recruited to VDR (Fig. 2B). Therefore, $1,25(OH)_2D_3$ treatment disrupts VDR–PP1c/PP2Ac interaction, resulting in a loss of VDR-associated phosphatase activity. Further, in contrast to results obtained in Caco-2 cells, $1,25(OH)_2D_3$ induced the association of VDR with the phosphorylated form of $p70^{S6k}$ in HL-60 cells (Fig. 2B).

We next examined whether the increased phosphorylation of $p70^{S6k}$ plays a role in HL-60 cell differentiation. This question was explored pharmacologically by treating HL-60 cells with $1,25(OH)_2D_3$ and two non-secosteroidal synthetic VDR ligands, LG190155 and LG190178 [7]. Since RXR-VDR is the functional unit of Vitamin D_3-mediated gene expression and RXR-VDR heterodimerization is stimulated by $1,25(OH)_2D_3$, we next examined these ligands for their ability to promote heterodimerization of VDR with RXR. In HeLa cells transfected with pGal4-RXRα-LBD and pVP16-VDR-LBD, $1,25(OH)_2D_3$ (10^{-8} M), LG190155 (10^{-7} M), and LG190178 (10^{-8} M) induced RXR-VDR heterodimerization in a ligand-dependent manner (Fig. 3A). However, in HL-60 cells, $1,25(OH)_2D_3$ (10^{-8} M) and LG190178 (10^{-8} M) but not LG190155 (10^{-7} M) induced $p70^{S6k}$ phosphorylation at Thr389 position (Fig. 3B). Interestingly, LG190155 (10^{-7} M), which did not induce $p70^{S6k}$ phosphorylation, also failed to differentiate HL-60 cells when analyzed for increased CD14 expression by FACS analysis (Fig. 3C). In contrast, a robust induction of CD14 expression and therefore differentiation of HL-60 cells into cells of monocyte/macrophage lineage was observed with $1,25(OH)_2D_3$ (10^{-8} M) and LG190178 (10^{-8} M) (Fig. 3C). These results show that Thr389 phosphorylation of $p70^{S6k}$ plays a role in HL-60 differentiation and also demonstrate that RXR-VDR heterodimerization/transactivation and $p70^{S6k}$ phosphorylation are pharmacologically separable activities of VDR. Vitamin D receptor modulators (VDRMs) that are potent in inducing phosphorylation of $p70^{S6k}$ may exhibit increased efficacy in differentiating leukemic cells and therefore, may show better therapeutic indices for the treatment of leukemias and myelodysplasia.

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