Actions of $1,25(OH)_2$-vitamin D_3 on the cellular cycle depend on VDR and p38 MAPK in skeletal muscle cells

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Abstract

Previously, we have reported that $1,25(OH)_2$-vitamin D_3 (1,25D) activates p38 MAPK (p38) in a vitamin D receptor (VDR)-dependent manner in proliferative C2C12 myoblast cells. It was also demonstrated that 1,25D promotes muscle cell proliferation and differentiation. However, we did not study these hormone actions in depth. In this study we have investigated whether the VDR and p38 participate in the signaling mechanism triggered by 1,25D. In C2C12 cells, the VDR was knocked down by a shRNA, and p38 was specifically inhibited using SB-203580. Results from cell cycle studies indicated that hormone stimulation prompts a peak of S-phase followed by an arrest in the G0/G1-phase, events which were dependent on VDR and p38. Moreover, 1,25D increases the expression of cyclin D3 and the cyclin-dependent kinase inhibitors, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, while cyclin D1 protein levels did not change during G0/G1 arrest. In all these events, p38 and VDR were required. At the same time, a 1,25D-dependent acute increase in myogenin expression was observed, indicating that the G0/G1 arrest of cells is a pro-differentiative event. Immunocytochemical assays revealed co-localization of VDR and cyclin D3, promoted by 1,25D in a p38-dependent manner. When cyclin D3 expression was silenced, VDR and myogenin levels were downregulated, indicating that cyclin D3 was required for 1,25D-induced VDR expression and the concomitant entrance into the differentiation process. In conclusion, the VDR and p38 are involved in control of the cellular cycle by 1,25D in skeletal muscle cells, providing key information on the mechanisms underlying hormone regulation of myogenesis.

Key Words

- vitamin D
- signal transduction
- muscle
- cell cycle

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Introduction

Recruitment of cells in G0/G1 stage is a pre- and pro-differentiation arrest of skeletal muscle myoblasts, necessary to the subsequent development of myotubes (Mercer *et al*. 2005). For cell cycle progression, the cellular exit from G1-phase and entry into S-phase require formation and activation of cyclin–cyclin-dependent kinases (CDKs) complexes. The CDK inhibitors (CKI), $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, can block both the cyclin/CDK activity and subsequent phosphorylation of pRb family proteins, resulting in G1-phase arrest (Chellappan *et al*. 1998). Specifically, in C2C12 skeletal muscle cells, the expression of most cyclins is downregulated at the onset of differentiation, as cells arrest in the G0/G1-phase of the cell cycle (Jahn *et al*. 1994, Wang & Nadal-Ginard 1995). However, cyclin D3 is paradoxically upregulated during muscle differentiation, showing to be a key player in

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myogenesis (Kiess *et al*. 1995, Rao & Kohtz 1995). It is relevant that cyclin D3 is involved in the permanent withdrawal of C2C12 myoblasts from the cell cycle (Cenciarelli *et al*. 1999) and an increase in its expression in proliferating myoblasts causes early myogenesis (Gurung & Parnaik 2012). The role of cyclin D1 in the C2C12 cell cycle is opposite to that of cyclin D3, i.e. cyclin D1 is highly expressed in proliferating cells and its expression is markedly reduced after 1 day on differentiation medium (Clemente *et al*. 2005). With respect to CKIs, $p21^{Waf1/Cip1}$ plays an important role inhibiting the progression of the cellular cycle in C2C12 cells (Davidovic *et al*. 2013), and an active role for $p27^{Kip1}$ at an early stage of differentiation has been established (Messina *et al*. 2005).

P38 MAPK (p38), a key signaling pathway involved in skeletal muscle differentiation, is a member of the MAPKs super family (Zetser *et al*. 1999). Inhibition of p38 activity abrogates myoblast differentiation and fusion (Lluís *et al*. 2005). Of the four isoforms of the p38 family described, it was reported that p38 α, β, and γ are all required for myogenic differentiation in C2C12 cells (Wang *et al*. 2008). The inhibitor SB-203580 has been successfully used to study the functional roles of p38 α and β because it selectively inhibits these isoforms without affecting the γ and δ isoforms (Kumar *et al*. 1997). Studies using this inhibitor implicated p38 α and β in the promotion of skeletal muscle differentiation *in vitro* (Cuenda & Cohen 1999). P38 facilitates withdrawal of myoblasts from the cell cycle, allowing the beginning of differentiation (Lee *et al*. 2002). Moreover, terminal muscle cell differentiation is dependent on a functional p38 MAPK pathway and the expression of CKIs, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, is affected in C2C12 cells lacking MKK3, the direct kinase activator of p38 (Cabane *et al*. 2003). Several novel p38 MAPK-target genes, including *CCDN3*, are required for myogenin expression. It is relevant that cyclin D3 expression in memory B cells has also been shown to be dependent on p38 (Ertesvag *et al*. 2007). In C2C12 cells, cyclin D3 is regulated by p38 β and it is required for myogenic differentiation (Wang *et al*. 2008).

The steroid hormone $1,25(OH)_2$-vitamin D_3 (1,25D) is involved in myogenesis events and its biological actions on skeletal muscle cells have been a subject of interest for more than 20 years. The effects of 1,25D vary according to the type of skeletal muscle cells, stage of proliferation or differentiation, and culture (serum) condition employed. We have previously obtained data indicating that 1,25D modulates proliferation and differentiation of C2C12 cells (Buitrago *et al*. 2012). However, there are no reports about the effects of 1,25D on cellular cycle phases, cyclins, and CKIs in skeletal muscle cells, nor on the role of the vitamin D receptor (VDR) and p38 in hormone-dependent events. 1,25D exerts most of its biological actions through VDR, which acts as a transcription factor (MacDonald *et al*. 2001). VDR bound to 1,25D and retinoid X receptor interacts with vitamin D-response elements (VDREs) in the promoters of vitamin D target genes (Aslam *et al*. 1999). In this regard, it is known that the VDR interacts with co-repressors as part of the transcriptional mechanisms by which myoblast arrest is induced (Campbell 2014). However, it is uncertain whether the binding of an agonist to the VDR allows RXR to bind the co-repressors. There are several proposals regarding the assembly of molecular machines during the cell cycle, involving both transcriptional and post-translational control of the dynamics of biological systems (Jensen *et al*. 2006). Recently, Jian *et al*. (2005) have reported that cyclin D3 interacts with VDR and regulates its transcriptional activity. This interaction was strengthened by treatment with 1,25D. However, the relationship between VDR and cyclin D3 was not studied in depth. In this work, we show for first time, to our knowledge, that silencing of cyclin D3 affects VDR and myogenin expression induced by a physiological dose of 1,25D and p38 inhibition conditions co-localization of VDR with cyclin D3 in skeletal muscle cells. Moreover, we explore the involvement of VDR in the action of 1,25D on the cellular cycle and evaluate the contribution of p38 in hormone-triggered pro-differentiative events in skeletal muscle cells.

Materials and methods

Materials

1α,25-dihydroxyvitamin D_3 was purchased from Sigma Chemical Co. Fetal bovine serum (FBS) was from Natocor (Villa Carlos Paz, Argentina). Free-phenol red DMEM was from US Biological, Inc. (Salem, MA, USA). Anti-cyclin D3 (#2936), anti cyclin D1 (#2926), anti-$p21^{Waf1/Cip1}$ (#2946), and anti-$p27^{Kip1}$ (#2552) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-myogenin (sc-12732), anti-VDR (sc-1008) and anti-tubulin antibodies (sc-73242), HRP-conjugated secondary antibodies (anti-rabbit, sc-2004, and anti-mouse, sc-2005), and siRNA against cyclin D3 mRNA (sc-35137) were purchased from Santa Cruz Biotechnology. Alexa Fluor 488 (green) goat anti-rabbit IgG (A-11008) and Alexa fluor 647 (red) goat anti-mouse-conjugated antibodies (A-21236) are obtained from Molecular Probes (Eugene,

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OR, USA). The inhibitor SB-203580 was from TOCRIS (Tocris Bioscience, Bristol, UK). The Super Signal CL-HRP substrate system for ECL was from Perkin Elmer (Boston, MA, USA). The WT C2C12 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Puromycin was from Invitrogen. The plasmid to knock down the VDR was pLKO.1, clone ID TRCN0000027101 (Open Biosystems, Huntsville, AL, USA). Lentivirus particles containing a pLKO.1 vector with the sequences encoding a shRNA against VDR were kindly provided by Dr V Gonzalez Pardo, Universidad Nacional del Sur (Bahia Blanca, Argentina).

Lentivirus infection and selection

To generate a stable (long-term) knockdown of *VDR* gene expression in the C2C12 cell line, these cells were infected with lentivirus particles containing a pLKO.1 vector with the sequences encoding a shRNA against mouse VDR. This plasmid also has a gene encoding puromycin resistance, thereby addition of this antibiotic allowed us to select cells stably expressing shRNA against VDR (C2C12-VDR). We have described the protocol in a previous publication (Buitrago *et al*. 2013).

Cell culture and synchronization

The mouse skeletal myoblasts C2C12 WT and C2C12 (-VDR) grown in low-glucose DMEM supplemented with 10% FBS and 2% antibiotics (penicillin and streptomycin), 1% antimycotic (nistatine) solution with or without puromycin (2 μg/ml), were employed. The cells were not allowed to exceed 70% of confluence during passages and were discarded after ten passages (30 days), to minimize depletion of myoblasts in culture. We performed all the experiments independently at different passages (between the first and tenth passages). The cells were seeded at an appropriate density (100 000 cells/cm^2) in Petri dishes (100 mm diameter) with growth medium and cultured at 37 °C under a humidified atmosphere of 95% air/5% CO_2. Under these conditions, myoblasts divide within the first 48 h. The subconfluent cells were rendered quiescent by placing them in DMEM containing 2% FBS for 16 h (starved). The cells, cultured for 2 days (proliferative stage) and starved as indicated, were used for the treatments described in the Results section.

Cell cycle analysis by flow cytometry

C2C12 WT and C2C12 (-VDR) cells were dispersed by trypsinization, washed twice with PBS, and then resuspended in 1 ml PBS. After 24 h fixation in absolute ethanol, cells were suspended in PBS containing 5% Tween detergent. The fixed and permeabilized cells were pelleted and re-suspended with 500 μl of RNase-propidium iodide mix (BD Biosciences, San Diego, CA, USA) for 30 min. The fluorescence of DNA was measured using a flow cytometer (Becton–Dickinson FACS Calibur) and the cell distribution in different phases of the cellular cycle was analyzed by computer software (CELLQuest PRO, Becton–Dickinson, San Jose, CA, USA).

Electrophoresis and western blotting

Following treatment, the cells were lysed, the clarified lysates were resolved by one-dimensional SDS–PAGE and then transferred to Immobilon P membranes as previously described (Morelli *et al*. 2001). Standard samples were included in the first lane of all gels to make between-blot comparisons possible (data not shown). The membranes were then probed with specific primary antibodies. After incubation with peroxidase-conjugated secondary antibodies, the membranes were visualized by ECL captured by chemiluminescence film. The antibodies bound to proteins were then stripped and the membranes were reprobed with anti-tubulin or anti-p38 α antibodies to compensate for differences in gel loading. The films were scanned by a HP densitometer to quantify bands signals by the ImageJ software program.

Immunocytochemistry and confocal microscopy

C2C12 cells were grown on glass coverslips and after treatments were immediately washed in cold PBS solution and fixed in methanol (at −20 °C) for 20 min, a process which also permeabilized the cells. Non-specific sites were blocked with 5% BSA in PBS for 1 h. The samples were then incubated overnight at 4 °C with the appropriate primary antibody (anti-VDR or anti-cyclin D3 antibodies) prepared in 2% BSA in PBS. After washing with PBS, the samples were incubated with secondary anti-rabbit or anti-mouse fluorophore-conjugated antibodies (1 h, room temperature). The samples were examined using a Leica confocal laser microscope.

Silencing of cyclin D3 by transfection of siRNA

Six-well tissue culture plates with 2×10^5 cells/well starved in antibiotic-free medium supplemented with 2% FBS were used for transfection. After 6 h, the transfection mixture was removed and replaced with a normal growth medium. The cells were incubated for an additional 18 h

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until used for treatments. The protocol followed is in accordance with manufacturer's instructions (Santa Cruz Biotechnology).

Statistical analysis

The results were expressed as mean ± S.D. The differences between means values were analyzed using Student's *t*-test (Snedecor & Cochran 1967). The differences were considered statistically significant when $P \leq 0.05$.

Results

Cell cycle progression of C2C12 muscle cells after serum stimulation

We considered it necessary to characterize the time course of the cell cycle in C2C12 WT and C2C12 (-VDR) cells which had not, to our knowledge, been previously reported, following the procedure described for vascular smooth muscle cells (Nakano *et al*. 2005). To that end, all cells were deprived of FBS for 16 h to synchronize the cultures at the time in which 85% of cells were growth-arrested in the G0/G1-phase (data not shown). Then, the arrested cells were stimulated using 10% FBS for different times (6, 12, 18, and 24 h) and analyzed by flow cytometry. Figure 1 shows that in both C2C12 WT and C2C12 (-VDR) lines, approximately 90% of cells were in the G0/G1-phase after 6 h of serum stimulus, the progression of cell cycle from G1- to S-phase then takes place and a S-phase peak was observed at 12 h. After completion of the S-phase, a G2/M-phase increase is observed at 18 h. One round of the cell cycle seemed to be completed at 24 h, when most of the cells were again in G0/G1-phase, diminishing the percentage of cells in S-phase. This cell cycle pattern was similar in C2C12 WT and C2C12 (-VDR) cells.

Hormone stimulation of C2C12 muscle cells prompts a VDR-dependent S-phase peak followed by an arrest in the G0/G1-phase: the activation of p38 MAPK is required in 1,25D-induced G0/G1 arrest

We evaluated the role of the VDR in the distribution of myoblasts in the different phases of the cell cycle in the C2C12 WT and C2C12 (-VDR) lines treated with 1 nM 1,25D for 6, 12, and 24 h. As explained in the Materials and methods section, subconfluent cells were rendered quiescent by placing them in DMEM containing 2% FBS for 16 h (starved). Flow cytometry analyses were then carried out as in Fig. 1. In C2C12 WT cells, it was evidenced that the hormone promotes an increase in the S-phase peak at 12 h. This was followed by a significant recruitment of cells in the G0/G1-phase at 24 h. In C2C12 (-VDR) myoblasts, where the VDR is not significantly expressed, the percentages of cells in the S-phase at 12 h were lower than in WT cells and remained unchanged at 24 h. Moreover, the number of cells arrested in the G0/G1-phase at 24 h was significantly smaller than in the C2C12 WT line expressing the VDR (Fig. 2A). These results for the first time, to our knowledge, indicate the involvement of the VDR in modulation of skeletal muscle cell cycle by the hormone. It is noteworthy that these data indicate that the VDR is necessary to induce cell cycle arrest, which is essential for myogenic differentiation.

P38 activation is required for the withdrawal of myoblasts from the cell cycle that precedes muscle differentiation (Lee *et al*. 2002). Thereby, in order to establish if p38 activation is a requisite in the G0/G1-phase arrest promoted by 1,25D in C2C12 cells, we investigated changes in cellular phases by flow cytometry in presence of the p38 inhibitor SB-203580 (10 μM). Figure 2B shows that the 1,25D-induced G0/G1-phase arrest of cells is abrogated when p38 is inhibited, indicating that the hormone employs the p38 pathway to promote the onset of muscle differentiation.

The VDR and p38 MAPK are required in 1,25D-induced cyclin D3 expression in C2C12 muscle cells

It is well established that the expression of cyclin D1 rapidly declines in myoblasts induced to differentiate, arrested in G0/G1-phase, whereas cyclin D3 is induced at the transcriptional and post-translational levels (Kiess *et al*. 1995). We studied cyclin D3 and cyclin D1 expression in C2C12 cells stimulated by 1,25D. Figure 3A and B shows that the hormone increases cyclin D3 protein levels at 24 h of treatment, whereas no significant changes were observed in cyclin D1 in C2C12 WT cells. The increment in cyclin D3 induced by 1,25D was abolished in C2C12 (-VDR) cells, indicating that the VDR mediates hormone upregulation of cyclin D3 protein levels. No changes in the expression of the two cyclins were detectable at 18 h of hormone treatment.

The involvement of p38 activity in cyclin D3 expression was also investigated. Our data reveal that 1,25D activates p38 at 24 h and this effect is successfully abrogated by the use of 10 μM of SB-203580. It is of relevance that the results show that SB-203580 abolishes the expression of cyclin D3 induced by the hormone at 24 h, providing evidence that it is dependent on p38 activation (Fig. 3C and D).

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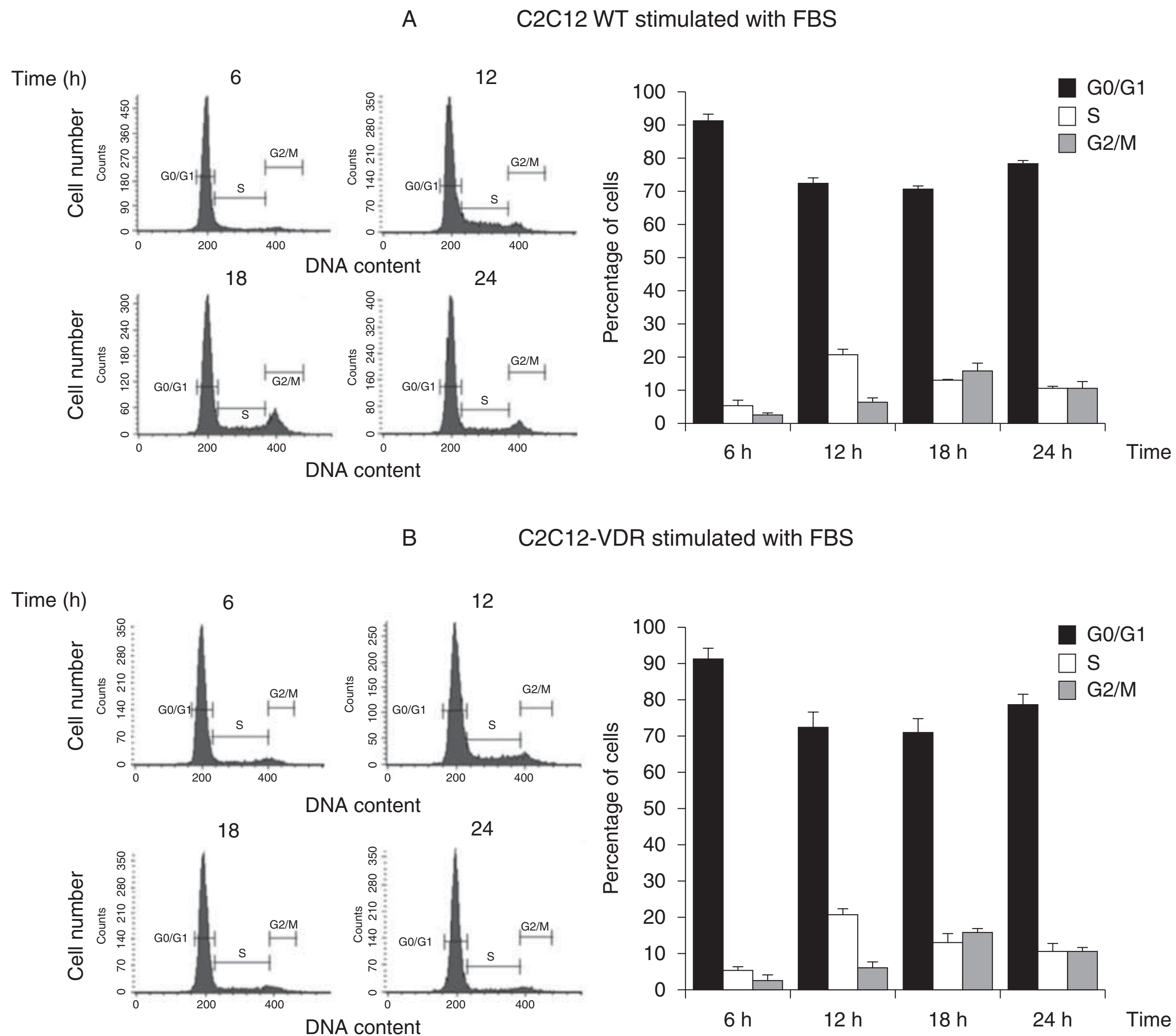

Figure 1
Cell cycle progression of C2C12 WT and C2C12-VDR muscle cells after serum stimulation. C2C12 WT cells and C2C12 cells lacking VDR expression (C2C12-VDR) were starved for 16 h and thereafter stimulated with 10% FBS for 6, 12, 18, and 24 h. At each time indicated, cells were trypsinized, stained with propidium iodide, and DNA contents were measured by flow cytometry. C2C12 WT (A) and C2C12-VDR cells (B): (left panels) representative DNA histograms and (right panels) quantifications of DNA histograms showing percentages of cells in G0/G1-, S- and G2/M-phases.

Induction of CKIs $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ by 1,25D occurs in a VDR- and p38 MAPK-dependent manner in C2C12 muscle cells

$P21^{waf1/Cip1}$ and $p27^{kip1}$ are CKIs that are overexpressed when C2C12 myoblasts withdraw from the cell cycle and commit to muscle fiber differentiation (Andres & Walsh 1996, Franklin & Xiong 1996). The involvement of the VDR in the expression of both CKIs by 1,25D was investigated. Figure 4A, B, and C shows that the hormone markedly increases CKIs $p21^{waf1/Cip1}$ and $p27^{kip1}$ levels in C2C12 WT cells at 24 h of stimulation, the time corresponding to the G0/G1-phase arrest, and increase in cyclin D3 observed previously. As expected, the effects of the steroid on both CKIs were not evidenced when VDR expression was suppressed in C2C12 (-VDR) cells.

Taking into account previous results indicating the involvement of p38 in cell growth modulation (Kim *et al*. 2002, Miura *et al*. 2005), the involvment of p38 in the effects of 1,25D on CKIs $p21^{waf1/Cip1}$ and $p27^{kip1}$ expression was evaluated. The use of SB-203580 proved that activation of p38 is required for the increase of $p21^{waf1/Cip1}$ and $p27^{kip1}$ levels in response to the hormone (Fig. 4D and E). The VDR and p38-dependent rise of

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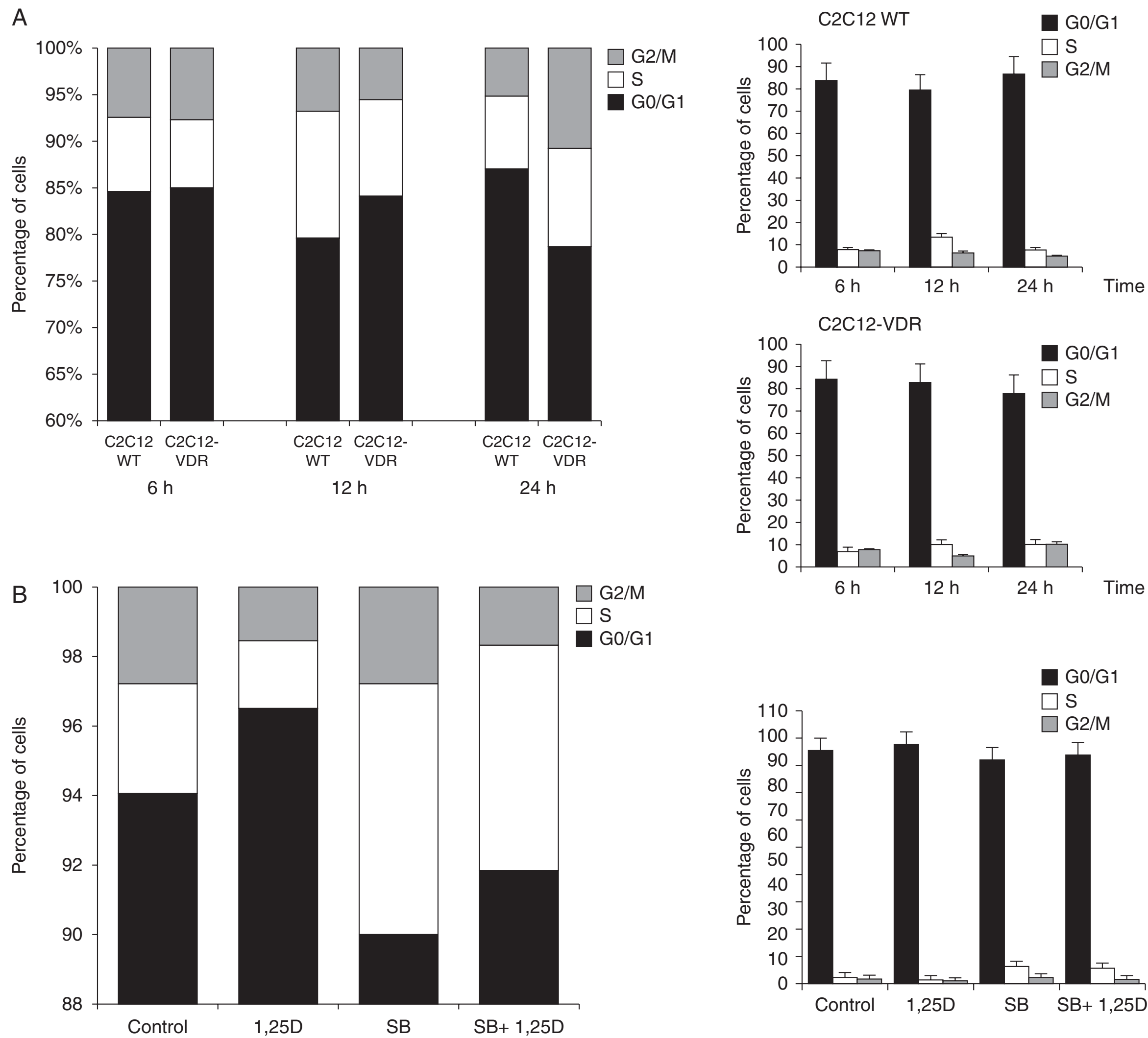

Figure 2

1,25D treatment promotes redistribution of cells in the C2C12 cellular cycle in a VDR- and p38-dependent manner. (A) C2C12 WT and C2C12-VDR cells were deprived of serum for 16 h and then treated with 1 nM 1,25D for 6, 12, and 24 h. At each time indicated, cells were trypsinized, stained with propidium iodide, and DNA contents were measured by flow cytometry, in three independent experiments. The panel shows the quantification of the DNA histograms with percentages of WT and -VDR cells in G0/G1-, S- and G2/M-phases in graphs with combined bars (left) and independent bars of each cellular line to show s.d. (right). (B) C2C12 WT cells were deprived of serum for 16 h, preincubated with 10 μM of p38 inhibitor SB-203580, and then treated with 1 nM 1,25D for 24 h. The cells were trypsinized, stained with propidium iodide, and DNA contents were measured by flow cytometry, in three independent experiments. The panel shows the quantification of the DNA histograms with percentages of WT cells in G0/G1-, S-, and G2/M-phases in graphs with combined bars (left) and independent bars (right).

$p21^{waf1/Cip1}$ and $p27^{kip1}$ at G0/G1-phase arrest of cells further supports the proposal that 1,25D induces muscle cell differentiation via the VDR and also involves p38.

The VDR and p38 MAPK are involved in 1,25D-promoted myogenin expression in C2C12 muscle cells

It is well established that myogenin expression is indispensable as the first step in the differentiation of myoblasts, which is followed by their withdrawal from the cell cycle (Andres & Walsh 1996). Since 1,25D promotes G0/G1-phase arrest of C2C12 cells, changes in myogenin protein levels were then investigated. Figure 5A and B shows that 1,25D induces a marked increase in myogenin expression at 24 h, which is still evident at 72 h, in C2C12 WT cells. Noteworthy, myogenin levels are undetectable in C2C12-VDR cells at all times studied, indicating that the VDR is required for the hormone-dependent synthesis of

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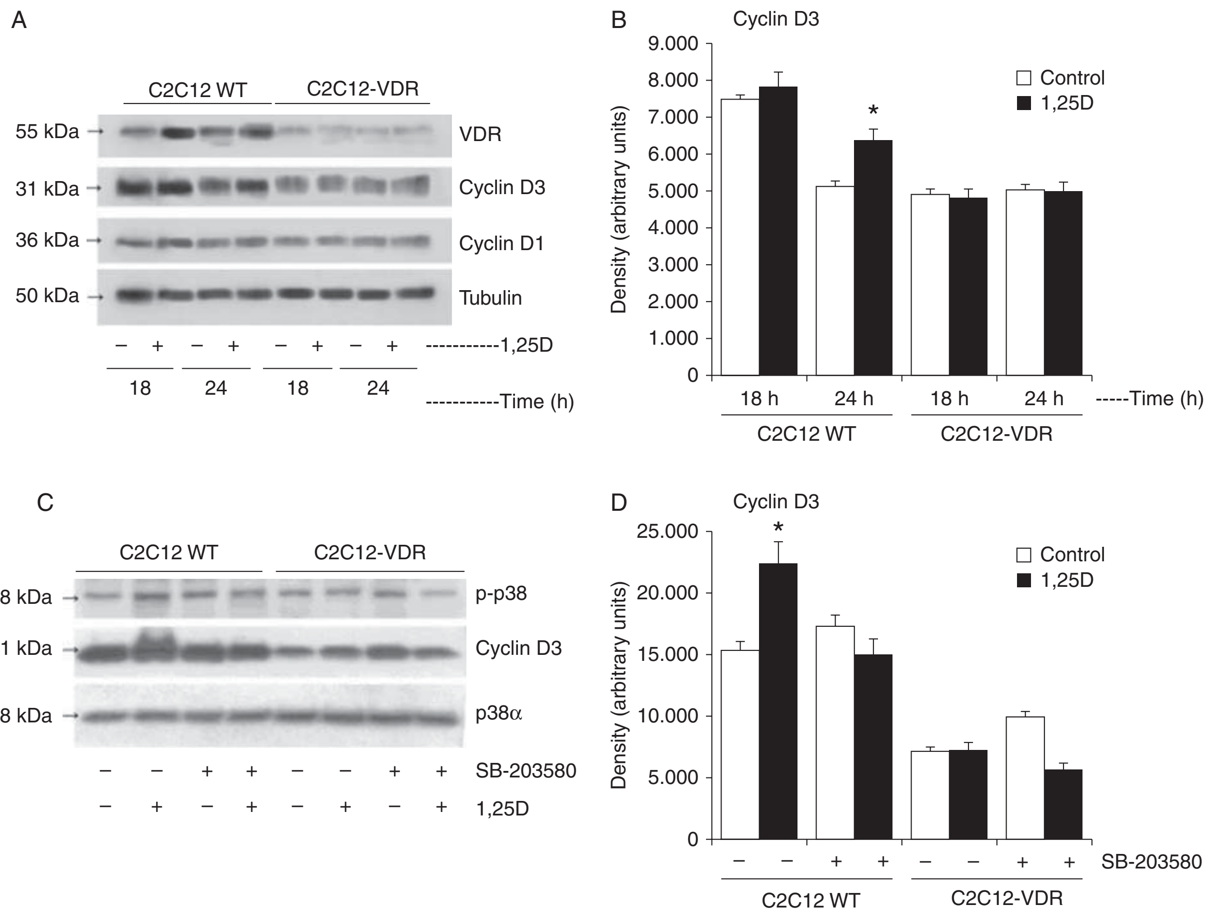

Figure 3

The VDR and p38 MAPK participate in the increase in cyclin D3 expression in response to the hormone in muscle cells. C2C12 WT and C2C12-VDR cells were treated with 1 nM 1,25D or vehicle (0.001% isopropanol) for 18 and 24 h. Western blotting analysis was carried out using anti-VDR, anti-cyclin D3, and anti-cyclin D1 antibodies. The blotted membranes were reprobed with anti-tubulin antibody in order to ensure equal loading of gels. (A) Representative immunoblots from three independent experiments. (B) Quantifications by scanning volumetric densitometry of blots from the three experiments showing mean ± s.d. of cyclin D3 protein levels. *$P<0.05$ with respect to the corresponding control. (C and D) C2C12 WT and C2C12-VDR cells were preincubated with 10 μM SB-203580 and then treated with 1 nM 1,25D or vehicle (0.001% isopropanol) for 24 h. Western blotting analysis was carried out using anti-P-p38 MAPK, which recognizes only activated p38, and anti-cyclin D3. The blotted membranes were reprobed with anti-p38 α antibody in order to ensure equal loading of gels. (C) Representative immunoblots from three independent experiments. (D) Quantifications by scanning volumetric densitometry of blots from the three experiments showing mean ± s.d. of cyclin D3 protein levels. *$P<0.05$ with respect to the corresponding control.

this early differentiation marker protein. Moreover, it was also evidenced that myogenin expression triggered by 1,25D is dependent on p38 activation (Fig. 5C and D).

VDR co-localizes with cyclin D3 in 1,25D-stimulated C2C12 muscle cells only when p38 MAPK is activated

As described previously, we showed that VDR expression is required for 1,25D-dependent upregulation of cyclin D3 in C2C12 cells. The interaction of cyclin D3 with VDR has been previously reported only by Jian *et al.* (2005) using another cell type. In this work, confocal immunocytochemistry was used to examine the intracellular localization of VDR and cyclin D3 in C2C12 cells committed to differentiation. In WT cells stimulated with 1,25D for 24 h, confocal images confirm that the hormone induces VDR expression and increases cyclin D3 protein levels (Fig. 6D and E vs A and B). There was no significant co-localization of VDR and cyclin D3 under basal conditions (Fig. 6C). 1,25D treatment promotes nuclear co-localization of VDR with cyclin D3 and also a re-distribution of VDR, observable in the plasma membrane region (Fig. 6F). Interestingly it was observed that WT C2C12 cells treated only with SB-203580 displayed significantly diminished VDR and cyclin D3-related fluorescence, indicating

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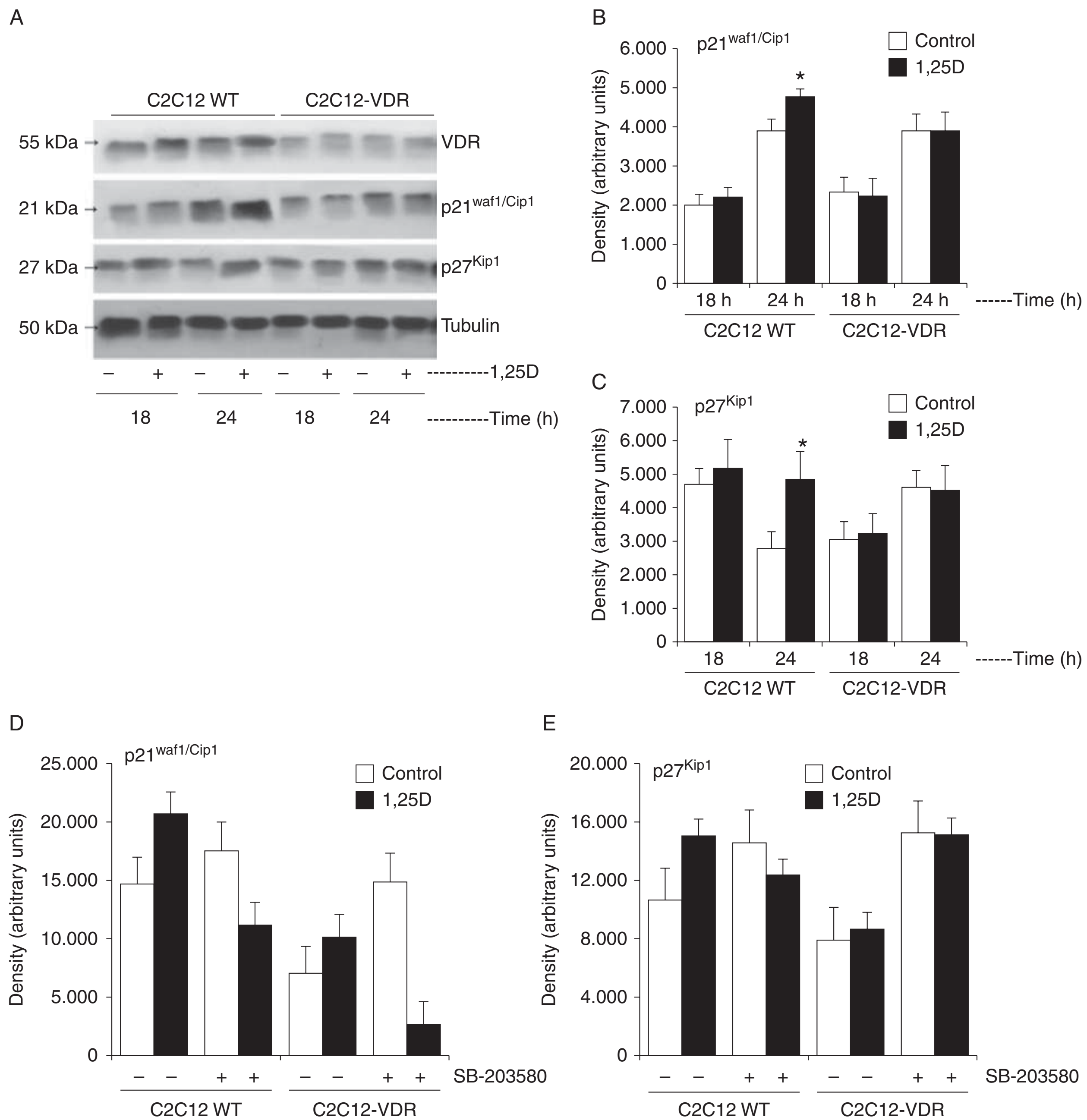

Figure 4

The VDR is required in 1,25D-promoted increase in p21$^{waf1/Cip1}$ and p27 ^{Kip1} CKIs. Activation of p38 is also involved in the increased expression of the CKIs, p21$^{waf1/Cip1}$ and p27 ^{Kip1} in response to the hormone. (A, B, and C) C2C12 WT and C2C12-VDR cells were treated with 1 nM 1,25D or vehicle (0.001% isopropanol) for 18 and 24 h. Western blotting analysis of lysates was carried out using anti-VDR, anti-p21$^{waf1/Cip1}$, and anti-p27^{Kip1} antibodies. The blotted membranes were re-probed with anti-tubulin antibody in order to ensure equal loading of gels. (A) Representative immunoblots from three independent experiments. (B and C) Quantifications by scanning volumetric densitometry of blots from three independent experiments showing mean ± s.d. of p21$^{waf1/Cip1}$ and anti-p27^{Kip1} protein levels. *$P<0.05$ with respect to the corresponding control. (D and E) C2C12 WT and (-VDR) cells were pre incubated with 10 μM SB-203580 and then treated with 1 nM 1,25D or vehicle (0.001% isopropanol) for 24 h. Western blotting analysis was carried out using first anti-P-p38, which recognizes only activated p38, and anti-p21$^{waf1/Cip1}$ and anti-p27^{Kip1} antibodies (data not shown). The blotted membranes were re-probed with anti-p38α antibody in order to ensure equal loading of gels (data not shown). Immunoblots from four independent experiments were quantified by scanning volumetric densitometry showing mean ± s.d. of (C) p21$^{waf1/Cip1}$ protein levels and (D) of p27^{Kip1} protein levels. *$P<0.05$ with respect to the corresponding control.

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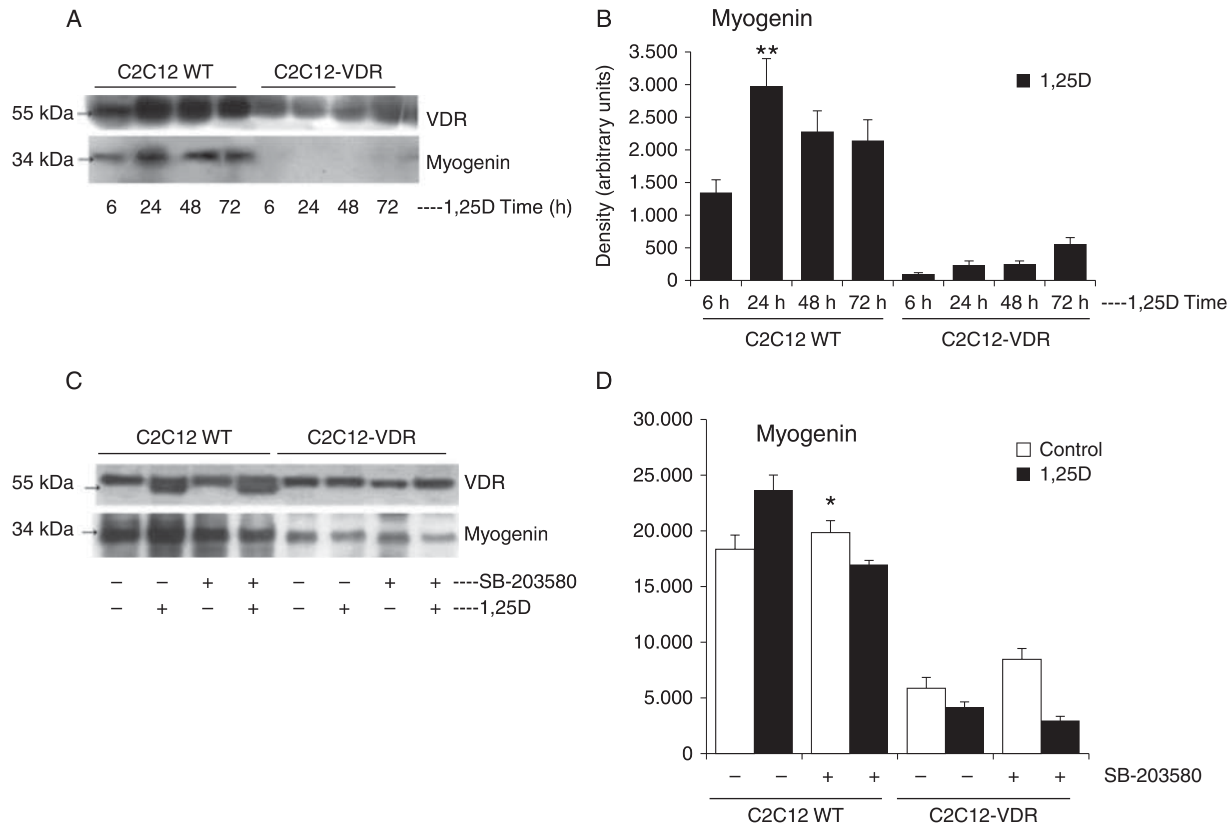

Figure 5

Myogenin expression is induced by 1,25D in muscle cells where VDR is expressed and p38 is activated. (A and B) C2C12 WT and C2C12-VDR cells were treated with 1 nM 1,25D for 6, 24, 48, and 72 h. Western blotting analysis was carried out using anti-VDR and anti-myogenin antibodies. The blotted membranes were reprobed with anti-tubulin antibody in order to ensure equal loading of gels (data not shown). Representative immunoblots from three independent experiments are shown (A) and quantifications by scanning volumetric densitometry of blots from three independent experiments showing averages ± S.D. of cyclin D3 protein levels. **$P<0.01$ with respect to the corresponding control. (C and D) C2C12 WT and C2C12-VDR cells were pre incubated with 10 μM of SB-203580 and then treated with 1 nM 1,25D or vehicle (0.001% isopropanol) for 24 h. Western blot analysis was carried out using first anti-P-p38, which recognizes only activated p38, and anti-VDR, and anti-myogenin antibodies. The blotted membranes were reprobed with anti-tubulin antibody in order to ensure equal loading of gels (data not shown). Representative immunoblots from three independent experiments are shown (C) and quantifications by scanning volumetric densitometry of blots from three independent experiments showing mean ± S.D. of cyclin D3 protein levels. *$P<0.05$ with respect to the corresponding control on (D).

that activation of p38 is important for achieving VDR and cyclin D3 expression (Fig. 6G and H). Although 1,25D stimulation together with p38 inhibitor treatment of C2C12 WT cells induced VDR expression, its distribution was different to that observed in cells treated only with the hormone (Fig. 6J vs D). 1,25D treatment after preincubation with SB-203580 did not promote the increase in cyclin D3 protein levels. Merged images supported the idea that p38 participates in the VDR–cyclin D3 interaction (Fig. 6L).

Silencing of cyclin D3 inhibits VDR expression and myogenin upregulation by 1,25D in C2C12 muscle cells

In view of the role of cyclin D3 in skeletal muscle and its relationship with VDR, it was investigated whether cyclin D3 participates in VDR and myogenin expression. We successfully performed the silencing of cyclin D3 using siRNA technology in WT C2C12 cells. Figure 7 shows that silencing of cyclin D3 resulted in diminished expression of VDR as well as totally abolishing the induction of myogenin by 1,25D.

Discussion

C2C12 is a murine highly myogenic myoblastic cell line that remains in the proliferative stage in a medium with 10–20% FBS, and when these myoblasts become confluent switching them into 2% horse serum medium, causes them to begin to elongate, become multinucleate, fuse, and finally differentiate into myotubes (Florini *et al*. 1991). We previously reported that 1,25D activates the MAPK family

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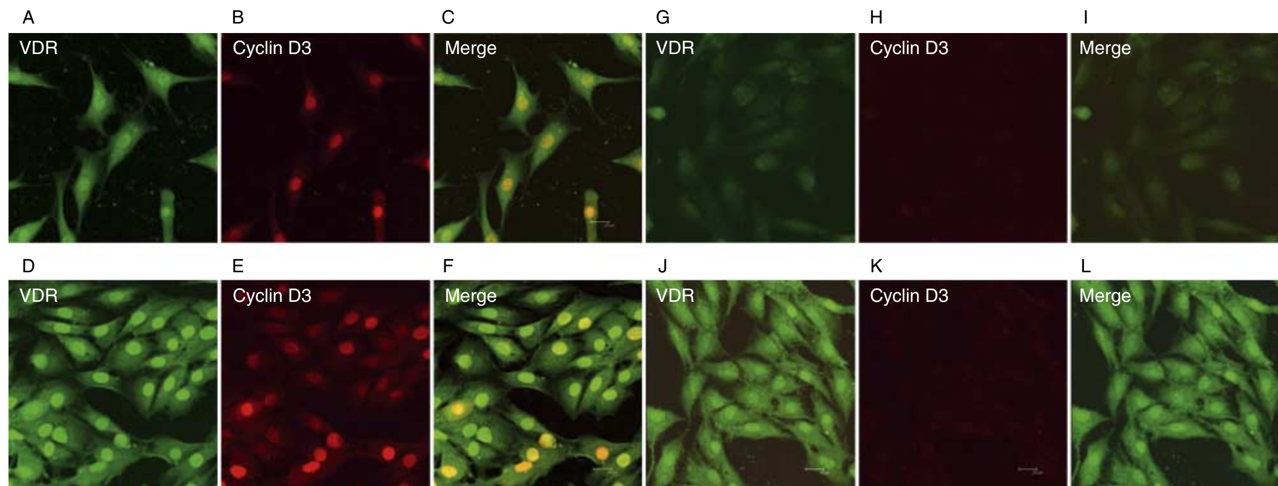

Figure 6
VDR co-localization with cyclin D3 is dependent on p38 activation. C2C12 WT cells grown on coverslips were stimulated with 1 nM 1,25D or vehicle during 24 h, in the presence or absence of SB-203580 (10 μM). Immunocytochemistry assays were performed as described in Methods. Confocal fluorescence digital images of C2C12 WT cells incubated with vehicle (panels A, B, and C), 1,25D (panels D, E, and F), SB-203580 (panels G, H, and I), SB-203580 and 1,25D (panels J, K, and L) and double labeled with anti-VDR antibody (green), and anti-cyclin D3 antibody (red) are shown. White bars in images represent 20 μm. The right side of each panel shows merged microphotograghs. Representative images of different fields of all coverslips analyzed are shown. A full colour version of this figure is available via http://dx.doi.org/10.1530/JME-14-0102.

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of proteins in cultured C2C12 myoblasts, thereby influencing the early expression of genes involved in cellular proliferation and differentiation (Ronda *et al*. 2007). It was observed that for C2C12 cells (grown in DMEM with 10% FBS) incubation with 100 nM 1,25D stimulated differentiation by inhibiting cell proliferation and modulating the expression of promyogenic growth factors and myostatin (Garcia *et al*. 2011). However, these results contradict an earlier report that described the downregulation of myogenin and myogenic transcription factor 5 (Myf5) mRNA in C2C12 myoblasts treated with 1,25D for 48 and 96 h (Endo *et al*. 2003). It is possible that differences in experimental designs and conditions (e.g. treatment times and concentrations) may account for these contradictory findings. Recently, it was observed that addition of 1,25D at supraphysiological concentrations (10^{-7} and 10^{-5} M) appeared to inhibit myotube formation and decreased mRNAs encoding key myogenic regulatory factors (Girgis *et al*. 2013, Ryan *et al*. 2013).

In this work, we obtained evidence that VDR expression in C2C12 cells did not affect the progress of the cell cycle under FBS stimulus. However, it was observed that 1 nM 1,25D promotes an increase in the S-phase (at 12 h) followed by a G0/G1-phase arrest (24 h) and that these events decrease in cells lacking VDR, showing a dual effect of 1,25D. In view of our previous results (Buitrago *et al*. 2012), it was expected that a cell proliferative state would be observed at 24 h. This may be explained by the fact that now the cells were deprived of serum for 16 h, instead of 2 h as reported previously. Okuno *et al*. (2012) have observed that stimulation of C2C12 cells with 1,25D in presence of 10% FBS for 72 h induced an increase in the percentage of cells in the G0/G1-phase in the absence of a proliferative stimulus from the hormone. The 1,25D inhibition of proliferation of myoblasts grown in DMEM

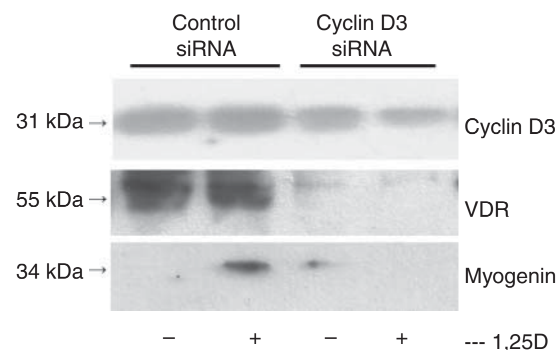

Figure 7
Silencing of cyclin D3 by a specific siRNA affects VDR and myogenin expression in muscle cells. C2C12 WT cells were transfected with 50 pmol cyclin D3 siRNA or control siRNA for 6 h according to the manufacturer's specifications. Transfected cells were incubated for an additional 18 h period in fresh medium until treatments were administered (as indicated previously). Western blot analyses were carried out with anti-cyclin D3, anti-VDR, and anti-myogenin antibodies. The blotted membranes were reprobed with anti-tubulin antibody in order to ensure equal loading of gels (data not shown). Representative immunoblots of blots from two independent experiments are shown.

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with 10% FBS was in addition reported by Srikuea *et al.* (2012), who also demonstrated VDR expression in C2C12 cells. The authors used 20 nM of 1,25D for 48 h and under these conditions, they observed an increase in VDR expression and a significant decrease in the number of cells (Srikuea *et al.* 2012).

We also demonstrated that p38 participates in 1,25D-dependent G0/G1-phase arrest in C2C12 cells. In agreement with these data, it has been previously shown that p38 activation results in G1-phase arrest and differentiation (Puri *et al.* 2000). Moreover, our data indicate that VDR and p38 participate in hormone up-regulation of cyclin D3 protein levels. The relationship between cyclin D3 and VDR has been previously reported. Cyclin D3 interacts with VDR and, in this way, regulates its transcriptional activity, and ligand-activated VDR induces an accumulation of cyclin D3 in the nuclear region (Jian *et al.* 2005). In this study, it was shown that 1,25D-induced VDR expression was abolished when cyclin D3 was silenced by siRNA. Cyclin D3 is a target gene downstream of p38 in hepatic cells (Sun *et al.* 2013), and in C2C12 cells evidence has been obtained that silencing of specific p38 isoforms affects cyclin D3 expression (Wang *et al.* 2008). It is of relevance that evidence of the participation of p38 in cyclin D3 expression induced by 1,25D is reported for the first time, to our knowledge, in this work. In addition, we obtained evidence that 1,25D induces the co-localization between VDR and cyclin D3 in a p38-dependent manner. In C2C12 cells, an increase in cyclin D3 levels promotes myogenic differentiation (Gurung & Parnaik 2012) and cyclin D3 is also greatly induced at later stage differentiating cells (Kiess *et al.* 1995). In skeletal muscle cells, the differentiation process is accompanied by a decrease in cyclin D1 expression (Rao & Kohtz 1995). However, our results showed no significant changes in cyclin D1 levels in C2C12 cells stimulated with 1,25D. The fact that the hormone promotes a G0/G1-phase arrest of C2C12 myoblasts without changes in cyclin D1 protein would indicate that it does not require this cyclin in particular to carry out its actions.

Given that differentiation of skeletal muscle precursor cells is always accompanied by an increase in cyclin D3 expression, as we observed in WT C2C12 cells, we propose the hypothesis that the VDR- and p38-dependent arrest in the G0/G1-phase is a pro-differentiative event. 1,25D regulation of chick myoblast differentiation was described for the first time in our laboratory (Capiati *et al.* 1999). Later, it was demonstrated that the VDR is necessary for normal skeletal muscle development and the correct expression of myoregulatory transcription factors in mice (Endo *et al.* 2003). Moreover, we have previously shown that the hormone induces myosin heavy chain (MHC) and myogenin in the early stage of differentiation of C2C12 cells grown in low-serum medium (Buitrago *et al.* 2012). The presence of myogenin ensures MHC expression and modulates the subsequent myotube development, demonstrating that myogenin acts early in the determination of myoblast differentiation (Davie *et al.* 2007). In this work, 1,25D induced an acute and steady increase in myogenin expression in C2C12 WT cells, while myogenin protein levels remained undetectable in C2C12-VDR cells and when p38 was inhibited. Moreover, we obtained evidence that in C2C12 cells transfected with a siRNA against cyclin D3 mRNA, myogenin expression is abolished. This clearly shows that the beginning of the differentiation program induced by 1,25D in C2C12 cells requires the expression of the VDR and activation of p38. However, in cyclin D3-deficient C2.7 myoblasts, changes in myogenin expression were not found (De Luca *et al.* 2013). This may be explained by differences in experimental design (like the use of differentiation medium) or other pathways which are not regulated by 1,25D being involved in myogenin expression.

Both P21$^{waf1/Cip1}$ and p27^{Kip1} are CKIs; however, they have specific actions in myogenic differentiation (el-Deiry *et al.* 1994, Guo *et al.* 1994). The induction of p27^{Kip1} CKI is an early critical step of the N-cadherin-dependent signaling involved in myogenesis, showing an active role of p27^{Kip1} CKI in the decision of myoblasts to undergo differentiation (Messina *et al.* 2005). It is of relevance that when C2C12 myoblasts start expressing CKI, p21$^{waf1/Cip1}$ (Andres & Walsh 1996) and p27^{Kip1} (Franklin & Xiong 1996), they withdraw from the cell cycle and become determined to be differentiated into mature muscle cells. In this study, we showed that 1,25D increases protein levels of p21$^{waf1/Cip1}$ and p27^{Kip1} CKIs during G0/G1-phase arrest in a VDR- and p38-dependent manner. In accordance with these results, 1,25D has been shown to upregulate CKIs, p21$^{waf1/Cip1}$ and p27^{Kip1}, expression (Verlinden *et al.* 1998) and, more interestingly the *CKI p21$^{waf1/Cip1}$* gene was identified as a transcriptional target of VDR (Freedman 1999), with a functional VDRE being recognized in its promoter (Liu *et al.* 1996). In vascular smooth muscle cells, p38 mediates transcription of the *CKI p21$^{waf1/Cip1}$* gene (Moon *et al.* 2004). However, participation of p38 in 1,25D-induced CKI p21$^{waf1/Cip1}$ protein expression has not, to our knowledge, been reported previously. With respect to p27^{Kip1}, the VDR functions as the transactivation component of the VDR–Sp1 complex to trigger the expression of its gene

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(Cheng *et al.* 2006). Also, 1,25D-upregulation of CKI $p27^{Kip1}$ is blocked by a p38 inhibitor (Miura *et al.* 2005). Similarly to CKI $p21^{waf1/Cip1}$, this is the first report, to our knowledge, showing that 1,25D induces CKI $p27^{Kip1}$ with the involvment of p38.

Conclusions

We conclude that in the 1α,25-dihydroxyvitamin D_3-induced differentiation of C2C12 cells the VDR and p38 are significantly involved, leading to G0/G1 arrest of cells accompanied by cyclin D3-augmented expression and elevated levels of CKIs, $p21^{waf1/Cip1}$ and $p27^{kip1}$. Moreover, 1,25D promotes a p38-dependent co-localization of VDR and cyclin D3 and this cyclin is required in 1,25D-induced expression of VDR and myogenin. Our findings help to elucidate the mechanism by which the hormone contributes to the differentiation of myoblasts, which accounts for its effects on skeletal muscle growth and regeneration, revealing an important role of the VDR and p38 in the physiological actions of 1,25D on skeletal muscle.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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