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1α,25 dihydroxi-vitamin D_3 modulates CDK4 and CDK6 expression and localization

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ABSTRACT

We recently reported that the vitamin D receptor (VDR) and p38 MAPK participate in pro-differentiation events triggered by 1α,25(OH)$_2$-vitamin D_3 [1,25D] in skeletal muscle cells. Specifically, our studies demonstrated that 1,25D promotes G0/G1 arrest of cells inducing cyclin D3 and cyclin dependent kinases inhibitors (CKIs) p21$^{Waf1/Cip1}$ and p27^{Kip1} expression in a VDR and p38 MAPK dependent manner. In this work we present data indicating that cyclin-dependent kinases (CDKs) 4 and 6 also play a role in the mechanism by which 1,25D stimulates myogenesis. To investigate VDR involvement in hormone regulation of CDKs 4 and 6, we significantly reduced its expression by the use of a shRNA against mouse VDR, generating the skeletal muscle cell line C2C12-VDR. Investigation of changes in cellular cycle regulating proteins by immunoblotting showed that the VDR is involved in the 1,25D −induced CDKs 4 and 6 protein levels at 6 h of hormone treatment. CDK4 levels remains high during S phase peak and G0/G1 arrest while CDK6 expression decreases at 12 h and increases again al 24 h. The up-regulation of CDKs 4 and 6 by 1,25D (6 h) was abolished in C2C12 cells pre-treated with the ERK1/2 inhibitor, UO126. Moreover, CDKs 4 and 6 expression induced by the hormone nor was detected when α and β isoforms of p38 MAPK were inhibited by compound SB203580. Confocal images show that there is not co-localization between VDR and CDKs at 6 h of hormone treatment, however CDK4 and VDR co-localizates in nucleus after 12 h of 1,25D exposure. Of relevance, at this time 1,25D promotes CDK6 localization in a peri-nuclear ring. Our data demonstrate that the VDR, ERK1/2 and p38 MAPK are involved in the control of CDKs 4 and 6 by 1,25D in skeletal muscle cells sustaining the operation of a VDR and MAPKs −dependent mechanism in hormone modulation of myogenesis.

1. Introduction

1α,25(OH)$_2$-vitamin D_3 [1,25D] is a steroid hormone with pleiotropic actions in several tissues [13,21]. The vitamin D receptor (VDR) is member of steroid hormone receptor superfamily expressed in numerous organs [1]. It was demonstrated the VDR expression in myoblasts and myotubes [18,23] and its participation in 1,25D dependent intracellular signaling pathways in skeletal muscle (reviewed in Ref. [2]. We previously reported that 1,25D modulates proliferation and differentiation in skeletal muscle cells [3,8]. Specifically, we evidenced that hormone stimulation prompts a peak of S phase (at 12 h of treatment) followed by a pro-differentiative arrest in G0/G1 phase (at 24 h), events which were dependent on VDR expression and p38 MAPK activation [8].

Cyclin dependent kinases (CDKs) are a family of serine/threonine kinases that actively participate in the regulation of cellular cycle, binding to cyclins and modulating pRb phosphorilation [16]. In G1 and start of S phases, CDKs expression controls the beginning of DNA replication [20]. Specially, CDKs 4 and 6 are essential to G1/S progression, by binding to cyclins D [14]. Although pRb dephosphorylates during the differentiation process, CDKs 4 and 6 may remain expressed [11]. Kato and collaborators reported that CDKs 4 and 6 associate with CKIs p21$^{Waf1/Cip1}$ y p27^{Kip1} [10] and it was evidenced the p18^{INK4c} association with these CDKs [7]. 1,25D or its analogs actions on CDKs 4 and 6 were only reported in cancer cells [15] and, until now, CDKs 4 and 6 regulations by the hormone in muscle cells has not been investigated.

In view that there is not information about 1,25D actions on CDK4 and 6 in skeletal muscle cells, the aims of this work was to

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investigate CDKs 4 and 6 expression and localization during different phases of the cellular cycle in the skeletal muscle cell line C2C12 and determinate the role of VDR, ERK1/2 and p38 MAPK in these events.

2. Materials and methods

1α,25-dihydroxyvitamin D_3 was from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Natocor (Villa Carlos Paz, Argentina). Free-phenol red Dulbecco's modified Eagle's medium (DMEM) was from US Biological Inc. (Massachusetts, MA, U.S.A). Anti-CDK4, anti-CDK6 and anti-tubulin antibodies were from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-VDR, anti-P-p38 MAPK, anti-p38 MAPK and anti-P-ERK1/2 antibodies and anti-mouse and anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) was from General Electric (Oklahoma, USA). The C2C12 wild type (WT) cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). SB203580 and UO126 are from TOCRIS Bioscience (Bristol, United Kingdom). Puromycin was from Invitrogen (Invivogen, San Diego, CA). Lentivirus particles containing a pLKO.1 vector with the information to express a shRNA against VDR were provided by Dr. V. Gonzalez Pardo, Universidad Nacional del Sur.

2.1. Lentivirus infection and selection

To generate a stable (long-term) knockdown of VDR gene expression in the C2C12 WT cell line, these cells were infected with lentivirus particles containing a pLKO.1 vector with the information to express a shRNA against mouse VDR. This plasmid also has a gene encoding puromycin resistance, thereby addition of puromycin allowed us to select cells stably expressing shRNA against VDR (C2C12-VDR). Detailed protocol was previously reported [4].

2.2. Cell culture and synchronization

The mouse skeletal myoblastic C2C12 WT and C2C12-VDR cell lines were seeded with DMEM supplemented with 10% FBS and 2% antibiotic-1% antimycotic solution, with or without puromycin (2 μg/ml), when corresponding. The cells were cultured at 37 °C under a humidified atmosphere of 95% air/5% CO_2. Subconfluent cells were rendered quiescent by placing in DMEM containing 1% FBS for 24 h (harvested) before steroid hormone treatments.

2.3. Electrophoresis and western blotting

Following treatment, the cells were lysed, the clarified lysates resolved by one-dimensional SDS-PAGE and then transferred to Immobilon P membranes as previously described [5]. The membranes were then probed with anti-VDR, anti-CDK4 or CDK6, anti-P-p38 MAPK and anti-P-ERK1/2 antibodies. After incubation with peroxidase-conjugated secondary antibody membranes were visualized by chemiluminiscence. The antibodies were then stripped and the membranes were reprobed with anti-tubulin or anti-p38α antibodies to account for equal loading. Autoradiograms were scanned with a Hewlett Packard densitometer to quantitate bands signals by the ImageJ software program.

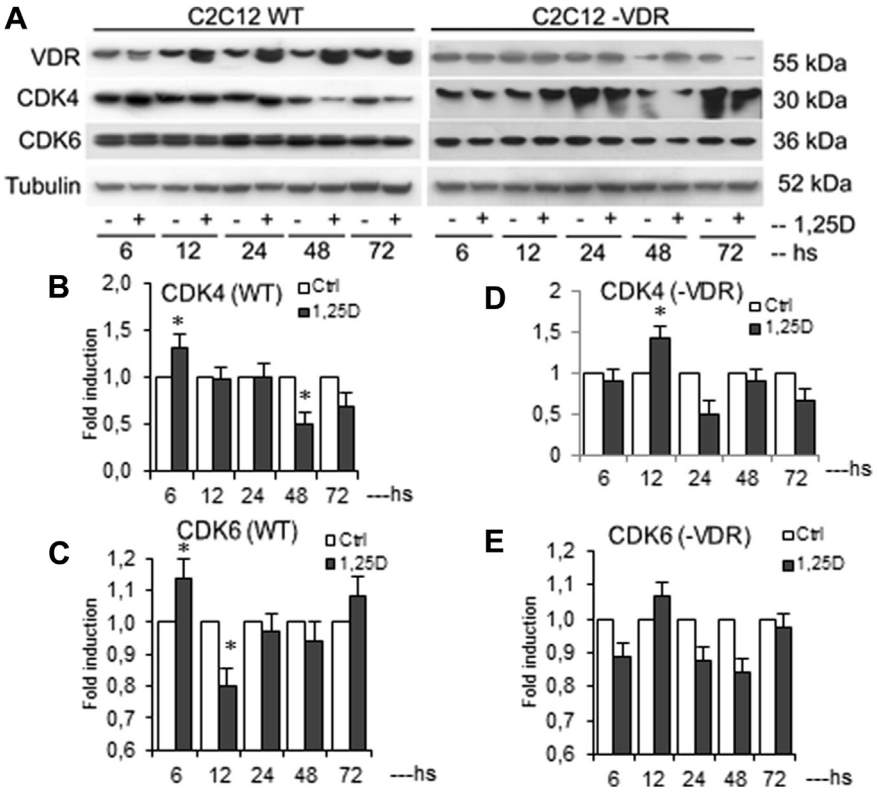

Fig. 1. 1,25D increase CDKs 4 and 6 protein levels in a VDR dependent manner at 6 h of hormone treatment. C2C12 WT and C2C12-VDR were stimulated with 1,25D or its vehicle (isopropanol 0.001%) for different times (6–72 h). Western blot analyses were carried out using anti-VDR, anti-CDK4, and anti-CDK6 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 (left: C2C12 WT, right: C2C12-VDR). (B–E) Quantifications by scanning volumetric densitometry of blots from the 3 experiments showing mean ± SD of CDKs protein levels. *$p < 0.05$ respect to the corresponding control.

2.4. Immunocytochemistry

C2C12 cells grown onto glass coverslips were fixed in methanol (at −20 °C), process which also permeabilized the cells. Non-specific sites were blocked with 5% BSA in PBS for 1 h. Samples were then incubated with the appropriate primary antibody prepared in 2% BSA in PBS (1 h, room temperature). After washing with PBS, the samples were incubated with secondary Cy5 goat anti-rabbit IgG or Cy3 goat anti-mouse conjugated antibodies (1 h, room temperature). The samples were examined using a Leica confocal laser microscope.

2.5. Statistical evaluation

The statistical significance of the results was evaluated by Student's t-test [19].

3. Results and discussion

3.1. 1,25D modulates CDKs 4 and 6 expression in C2C12 cells in a VDR dependent manner

C2C12 WT and C2C12-VDR were stimulated with 1,25D or its vehicle (isopropanol 0.001%) for different times (6–72 h). In C2C12 WT, western blot analyses evidenced that 1,25D increases VDR expression at all times assayed. The hormone was unable of up-regulate VDR protein levels in cells C2C12-VDR, which were transfected with shRNA against the receptor (Fig. 1A and Supplemental Fig. 1). The hormone induces CDKs 4 and 6 protein levels at 6 h of treatment. CDK4 protein levels returned to basal levels at 12 and 24 h of hormone incubation and further decreased between 48 and 72 h, while CDK6 diminish at 12 h and increase again at 24 h, not showing significant changes at 48 and 72 h. In cells with knockdown of VDR gene expression (C2C12-VDR) the hormone was unable to induced CDKs 4 and 6 increases at 6 h of treatment, however it was observed an augment of CDKs at 12 h of hormone incubation and a decrease of CDKs levels at 24 h, perhaps related with the role of VDR in cell cycle modulation previously reported [8]. This result shows that VDR is required in 1,25D dependent CDKs expression at 6 h (Fig. 1A,D and E). Hormone effects on CDKs protein levels are also dose dependent, been 1 nM of 1,25D which induces highest CDKs increases (data not shown). Until now, there are not reports about role of CDK4 and CDK6 in normal cells stimulated with vitamin D_3 or derivates. In leukemic cells it was demonstrated that a 1,25D analog prompts an arrest in G1 accompanied of CDKs 4 and 6 decreases [22]. Other report shows that the 1,25D analog EB1089 acts as a proliferation inhibitor where CDKs 4 and 6 levels diminish in NCI–H929 cells [15]. In this work, we evidenced that physiological concentrations (1 nM) of 1,25D increases CDKs 4 and 6 protein levels at 6 h of treatment, according to the S phase of cellular cycle previously observed [8]. This effect was only observed at 6 h while 12 and 24 h showed no changes in CDK4 levels by the hormone. The fall of CDK4 at 48 and 72 h agree with the start of differentiation process. CDK6 augmented at 6 h of 1,25D treatment and decreased its levels at 12 h of hormone stimulation, similarly to CDK4 and showed no significant changes at 24, 48 and 72 h. Of relevance, in cells lacking VDR the hormone was not able to up regulate CDKs expression at 6 h and CDK4 and 6 increases observed at 12 h was not statistically significant. Our results show that this receptor is required to enhance CDK4 and CDK6 protein levels during proliferation. This result suggests that CDKs 4 and 6 are part of the

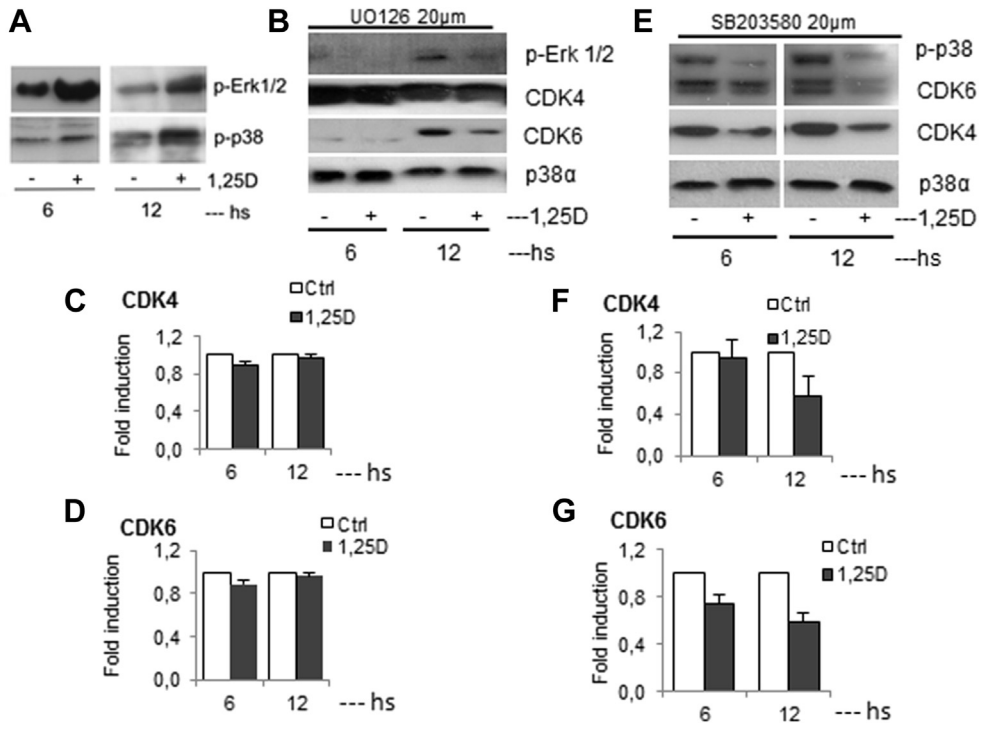

Fig. 2. ERK1/2 participates in 1,25D dependent CDKs induction. Isoforms α and β of p38 MAPK are also involved in CDKs modulation by the hormone. (A) C2C12 WT cells were stimulated with 1 nM 1,25D or its vehicle (0.001% isopropanol) for 6 and 12 h (B–G) C2C12 WT cells were preincubated with 20 µM UO126 or SB-203580 and then treated with 1 nM 1,25D or its vehicle (0.001% isopropanol) for 6 and 12 h. Western blot analysis were carried out using anti-P-ERK1/2 and anti-P-p38 MAPK, which recognizes only activated ERK1/2 and p38 MAPK respectively. The blotted membranes were reprobed with anti-CDK4 and anti CDK6 antibodies. In order to ensure equal loading of gels it was used anti p38 MAPK α antibody. (A, B and E) Representative immunoblots from three independent experiments. (C, D F and G) Quantifications by scanning volumetric densitometry of blots from the 3 experiments showing mean ± SD of cyclin D3 protein levels. *$p < 0.05$ respect to the corresponding control.

mechanism by which the hormone regulates cellular proliferation.

3.2. ERK1/2 mediates hormone-dependent stimulation of CDKs and this event was also dependent on p38 MAPK isoforms α and β activation

ERK1/2 are members of MAPKs directly involved in skeletal muscle proliferation [6]. Malumbres and Barbacid reported the involvement of ERK1/2 in CDKs expression in tumor cells [14]. In C2C12 wild type cells, 1,25D induces ERK1/2 phosphorylation at 6 and 12 h of treatment (Fig. 2A). To evaluate the role of ERK1/2 in CDKs increase by the hormone, we inhibited ERK1/2 using the pharmacological compound UO126 (20 μM). Hormone-dependent CDKs induction observed at 6 h was abolished when ERK1/2 were inhibited, showing that these MAPKs participate in CDKs 4 and 6 -dependent 1,25D modulation (Fig. 2B–D). This result evidenced ERK1/2 involvement in CDK4 and CDK6 expression during cell proliferation, as was descript in other cellular types [14]. Activation of α and β isoforms of p38 MAPK promotes mostly differentiation; while γ isoforms is involved in proliferation (reviewed [12]. P38 MAPK α and β isoforms are implicated in the pro-differentiative arrest of C2C12 cells in G0/G1 phase [8]. In C2C12 wild type cells, p38 MAPK was activated by 1,25D at 6 and 12 h of treatment (Fig. 2A) and its α and β isoforms inhibited by compound SB203580 (20 μM) (Fig. 2E). When p38 MAPK activity was abolished by SB203580, CDKs 4 and 6 protein levels at 6 h of hormone treatment did not increase (Fig. 2E–G) showing that this MAPK pathway is also involved in 1,25D effect. There is not information of our knowledge about p38 MAPK involvement in CDK 4 and 6 protein expressions.

3.3. CDK4 and CDK6 levels induced by 1,25D at 6 h of treatment are also evidenced by immunocitochemistry

Data obtained by confocal images of immunocytochemical assays corroborate results acquired by western blot. Images show the augment in CDK4 and CDK6 protein levels (evidenced by the increase in red amount) and VDR expression (evidenced by the increase in blue amount), in C2C12 wild type cells treated for 6 h with 1,25D. Of relevance, these images reveal the nuclear localization of CDK4 and nuclear and perinuclear localization of CDK6 (Fig. 3).

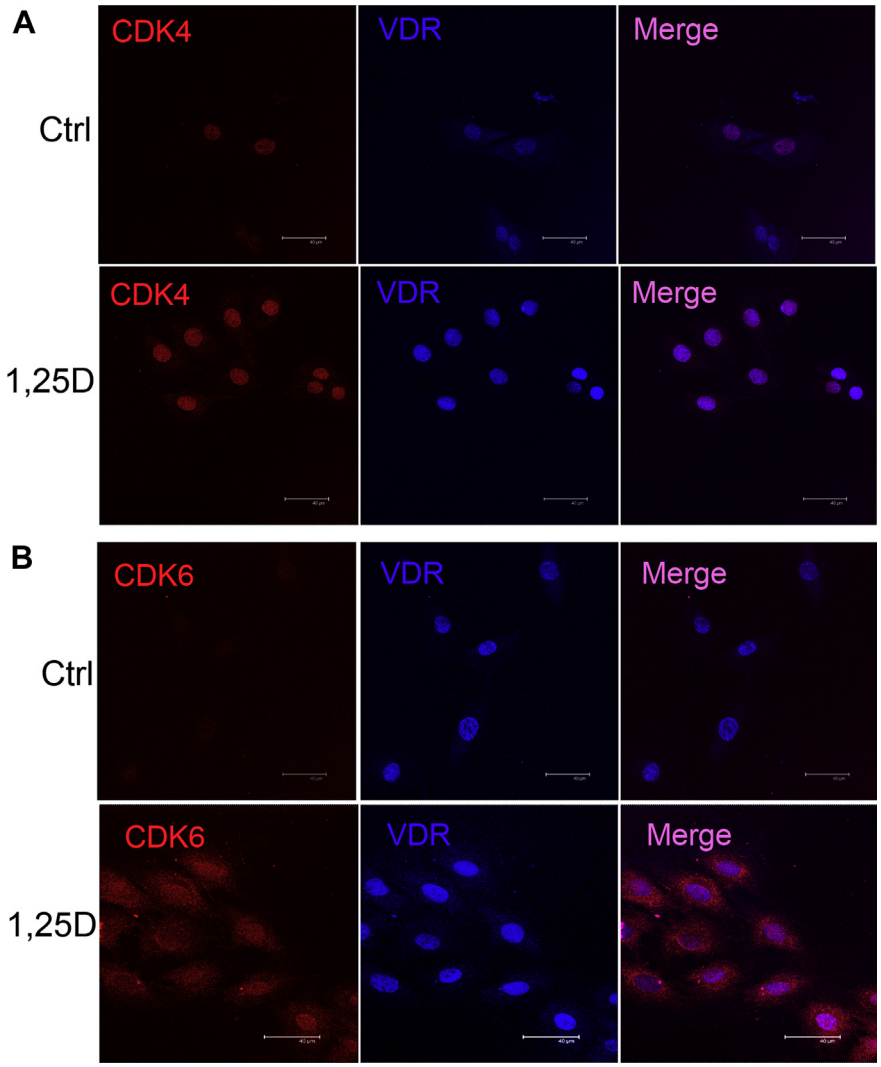

Fig. 3. 1,25D treatment for 6 h increases CDKs 4 and 6 levels. C2C12 WT cells grown over coverslips were stimulated with 1 nM 1,25D or its vehicle during 6 h. Confocal fluorescence digital images of C2C12 WT cells incubated with vehicle or 1,25D and double labeled with anti-VDR antibody (blue) and anti-CDK4 (red, panel A) or anti-CDK6 antibodies (red, panel B) are shown. White bars in images represent 40 μm. The right side of each panel shows merged microphotograghs. Representative images of different fields of all coverslips analyzed are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

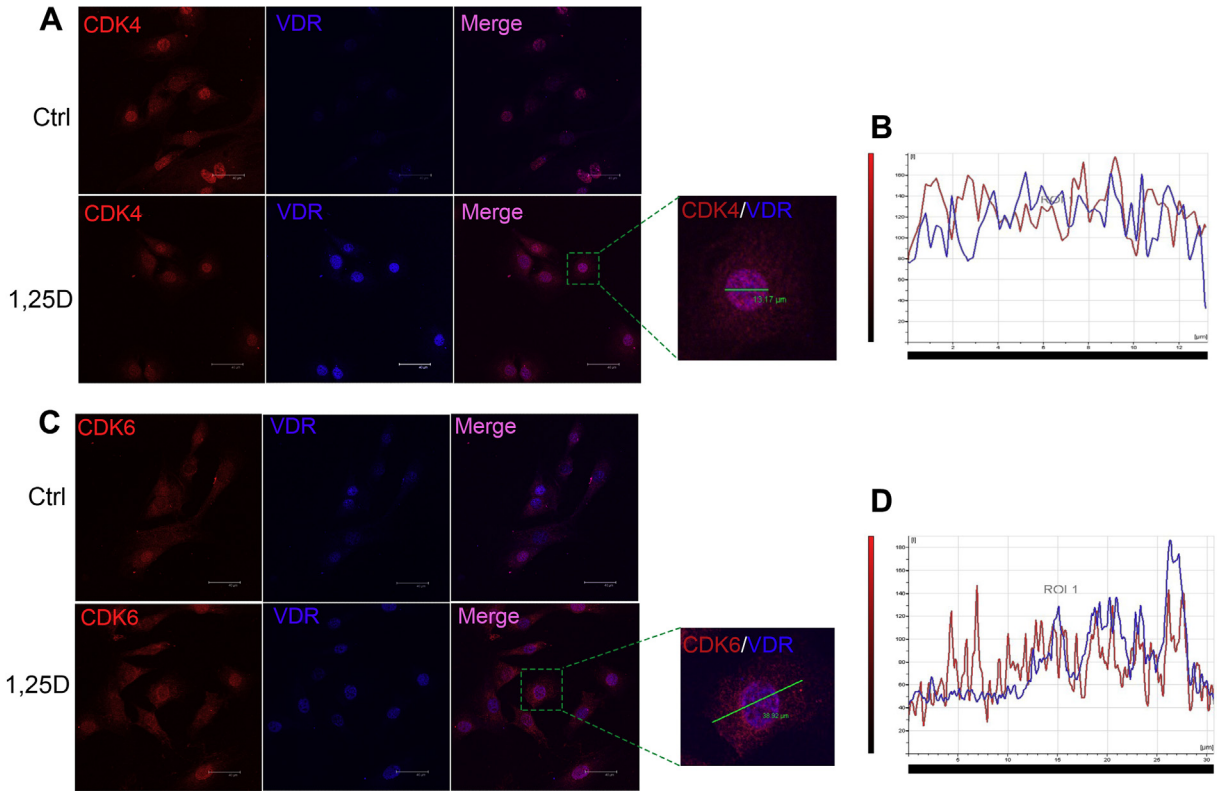

Fig. 4. Hormone stimulation for 12 h promotes nuclear VDR co-localization with CDK4 and increases CDK6 peri-nuclear ring accumulation. C2C12 WT cells grown over coverslips were stimulated with 1 nM 1,25D during 12 h. Confocal fluorescence digital images of C2C12 WT cells incubated with 1,25D and double labeled with anti-CDK4 (red) and anti-VDR antibody (blue) (A) and anti-VDR antibody (blue) and anti-CDK6 antibodies (red) (C) are shown. (B and D) Histograms of merged images showing quantifications of fluorescence intensity across a traced line (green) over the image. Representative images of different fields of all coverslips analyzed are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

None of CDKs co-localize with nuclear VDR at this hormone time treatment (Fig. 3A and B right panels).

3.4. *CDK4 co-localizates with VDR in nucleus after 12 h of 1,25D treatment, whereas CDK6 increase its perinuclear localization in response to the hormone*

When cells were stimulated with 1,25D for 12 h, confocal images show that CDK4 co-localizates with VDR in the inner of the nucleus. This is evidenced by the magenta color formed by fusion of red and blue color of merge images (Fig. 4A, right above) and by the corresponding histogram showing the intensity of both colors over a traced line (Fig. 4B). CDK6 localization in a perinuclear ring immediately outside of nucleus (evidenced with red color) is enhanced by 12 h of 1,25D treatment (Fig. 4C, right above). The corresponding histogram shows approximately 12 μm of the perinuclear ring with presence of red and absence of blue color, indicating no co-localization of CDK6 with VDR (Fig. 4D). We consider this unexpected result of relevance and a discovery that open doors to future investigations about the role of VDR in cellular proliferation. We speculate that hormone treatment (at this proliferation state) let the interaction between VDR and CDK4 to regulate gene expression, perhaps VDR expression similarly to the report of Jian and collaborators when described that cyclin D3 interacts with VDR to regulates its transcription activity and this event is counteracted by CDKs 4 and 6 over expression [9].

Altogether, results presented in this work show that VDR, ERK1/2 and p38 MAPK are implicated in 1,25D dependent induction of CDKs 4 and 6 protein levels at 6 h of treatment. 1,25D modulates CDKs intracellular localization and promotes CDK4 and VDR co-localization, perhaps to regulate the transcriptional activity of this hormone receptor.

Conflict of interest

None

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.083.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.083.

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