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CYCLIN D1 AND CDK4 INDUCE HYPERTROPHY THROUGH PHOSPHORYLATION OF RNA POLYMERASE II C-TERMINAL DOMAIN IN NEONATAL RAT CARDIOMYOCYTES

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Abstract
We have previously reported that hypertrophic stimuli induce the expression of cyclinD1 and CDK4 in cardiomyocytes, and that overexpression of cyclin D1 and CDK4 causes hypertrophic change. Recently, it is reported that pTEFb (CDK9 and cyclinT) is involved in cardiac hypertrophy through phosphorylation of the RNA polymerase II C-terminal domain (pol II CTD), promoting transcript elongation by Pol II. We assessed the hypothesis that cyclinD1/CDK4 induce hypertrophy through phosphorylation of pol II CTD in cultured neonatal rat cardiomyocytes. In this study, we examined whether the overexpression of cyclinD1/CDK4 induces hypertrophy by using [^{3}H]leucine incorpolation assay. CyclinD1/CDK4 overexpression increased the rate of protein synthesis by 1.55 fold, compared to those in LacZ-overexpressed cells. Further, we assayed the rate of RNA synthesis by [^{3}H]uridine incorpolation. Results showed that CyclinD1/CDK4 cells increased the rate of RNA synthesis compared to the control cells (1.57 fold). Finally, we examined whether phophorylation of pol II CTD is involved in cyclinD1/CDK4-induced hypertrophy. DRB, a chemical antagonist of pol II CTD phosphorylation, decreased the rate of incorporation of [^{3}H]leucine and uridine in cyclinD1/CDK4 cells. These data altogether suggest that cyclinD1/CDK4 activates Pol II transcription through inducing CTD phosphorylation and is involved in cardiac hypertrophy.

Key words: hypertrophy, cell cycle, RNA polymerase II CTD, DRB, pTEFb

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Abbreviations: DRB: nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, pol II: RNA polymarase II, CTD: C-terminal domain, pTEFb: positive transcription elongation factor-b

Introduction

Cardiac hypertrophy is one of the serious complications which increase mortality due to cardiovascular diseases and it is characterized by increased cardiomyocyte protein synthesis, increased cell volume.

Terminal differentiated cardiomyocytes after birth withdraw from the cell cycle and grow by

hypertrophy. D-type cyclins and its kinase partner CDK4 phosphorylate the retinoblastoma (Rb) family proteins, and initiate the cell cycle progression in proliferating cells[1]. Therefore, the cyclin D1/CDK4 complex functions as a growth factor sensor that links extracellular signals to the cell cycle machinery. On the other hand, cyclin D1 is also expressed in terminally differentiated cells such as neurons[2], and cardiac cells[3]. It has been reported that the cyclin D/CDK4 complex promotes an enlargement of the cell size and protein synthesis in post-mitotic cells of Drosophila[4,5]. Consistent with this, knockout studies of cyclin D/CDK4 have showed that deregulation of growth was more often observed than the specific defects in the cell cycle. We have previously reported that hypertrophic stimuli induce the expression of cyclinD1 and CDK4 in cardiomyocytes, and that overexpression of cyclin D1 and CDK4 causes hypertrophic change[6-8]. However, it is unclear what molecule is the target of cylinD1/CDK4 in cardiac hypertrophy.

pTEFb is a key regulator of the process controlling the processivity of RNA polymerase II and possesses a kinase activity that can phosphorylate the carboxy-terminal domain of the largest subunit of RNA polymerase II. Recently, it is reported that pTEFb (CDK9 and cyclinT) is involved in cardiac hypertrophy through phosphorylation of the RNA polymerase II C-terminal domain (pol II CTD), promoting transcript elongation by pol II[9]. We assessed the hypothesis that cyclinD1/CDK4 induce hypertrophy through phosphorylation of pol II CTD in cultured neonatal rat cardiomyocytes. Here we showed that cyclinD1/CKD4 induced-hypertrophy would be involved in the increase of mRNA and protein synthesis through phosphorylation of pol II CTD.

Materials and Methods

Materials

Endothelin-1 (ET-1) was purchased from PEPTIDE INSTITUTE, INC. The nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Sigma) was dissolved in dimethylsulfoxide (DMSO) and then in culture medium to the desired final concentration in 0.1% (vol : vol) DMSO. [^{3}H]uridine and [^{3}H]leucine were obtained from NEN and Amersham Pharmacia Biotech, respectively. Rabbit antibodies against the carboxyl-terminal domain (CTD) of RNA polymarse II (C-21, sc-900, Santa Cruz Biotechnology) and cyclinD1 (Ab-3, Calbiochem) purchased. Other chemicals used were reagents grade.

Cell culture

Cardiomyocytes from 1- or 2-day post-natal Sprague-Dawley rats were isolated by a Percoll gradient centrifugation as described previously[10,11]. Cardiomyocytes were cultured in minimal essential medium (MEM) containing 5% calf serum (CS) in humidified air with 5% carbon dioxide at 37°C for 24 h, and then in serum-free medium for another 24 h. The purity of cardiomyocytes was determined by immunostaining for cardiac cell-specific sarcomeric actin. Only cultures that contained over 95% cells positive for sarcomeric actin were employed in this study.

Adenoviruses

Adenoviruses encoding cyclin D1 (Ad-D1)[12] were generous gifts from Dr. J.H. Albrecht. To prepare Ad-CDK4, a cDNA fragment encoding CDK4 was subcloned into the SwaI site of the cosmid pAx-CAwt, and recombinant adenoviruses were generated by homologous recombination in 293 cells using the Adenovirus Expression Vector Kit (Takara). Titers of viruses were determined by an indirect immunofluorescent assay using anti-72K serum[13]. Cardiomyocytes were infected by adenoviruses at a multiplicity of infection (MOI) of 20, in serum-free MEM and incubated for 1 h with brief agitation every 15 min. After infection, the medium was replaced by culture medium as indicated. The efficiency of viral gene transfer is $>$95% under the conditions used.

Northern blot analysis

Northern blot analysis and preparation of the probes were performed as previously described[14].

Western blotting

Whole cell extracts were prepared as described[15]. The extracts from equal number of cells (4×10^6) were separated on an 6% SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore). The membrane was then incubated with primary antibodies as indicated. The reacted bands were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies using the ECL detection system (Amersham Pharmacia Biotech).

Measurement of mRNA and protein synthesis

The rate of RNA and protein synthesis were determined by incorporation of [^{3}H]uridine and [^{3}H]leucine as described[6]. Briefly, cardiomyocytes (2×10^5 cells) on a 12-well plate were infected with adenoviruses for 44 h. At the end of this incubation, 0.5 μCi [^{3}H]uridine or [^{3}H]leucine were added into 1 ml of culture medium and the incubation was continued for another 4 h. The cells were then rinsed three times with ice-cold phosphate-buffered saline (PBS), and treated with 5% trichloroacetic acid (TCA) on ice for 20 min. After washing twice with ice-cold 5% TCA, cells were lysed in 0.5N NaOH. An aliquot of the TCA-insoluble materials was neutralized and its radioactivity was measured by a liquid scintillation counter.

Results

ET-1 induced cyclinD1 expression level of mRNA and protein

ET-1 functions as an autocrine/paracrine factor in the development of cardiac hypertrophy.

As shown in Figs. 1A and 1B, both cyclinD1 mRNA and protein was induced in ET-1-treated cardiomyocytes. Induction of cyclinD1 mRNA and protein was peaked at 6-12 hours and 12-18 hours after ET-1 (0.1 μM) treatment, respectively. These results indicate that cyclinD1 are rapidly upregulated by treatment of cardiac myocytes with ET-1 (Fig. 1).

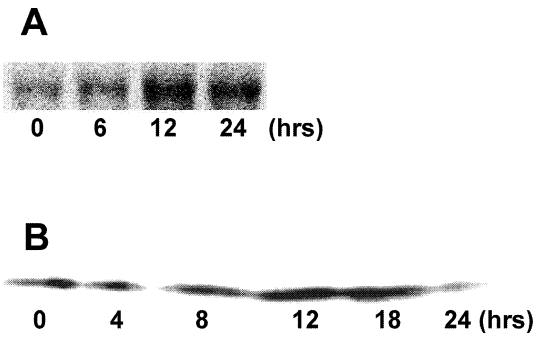

Fig. 1. Time course of mRNA and protein level of cyclinD1 induced by ET-1 in cardiomyocytes
Neonatal rat cardiomyocytes were exposed to ET-1 for indicated times. Northern blot hybridization (10 μg of total RNA/lane) was performed using 32P-labeled mouse cyclin D1 cDNA probes (Fig. 1A). Western blot was performed using antibody to cyclinD1 as in Materials and methods (Fig. 1B).

Overexpression of cyclinD1/CDK4 induces hypertrophy in cardiac myocytes

We examined whether the overexpression of cyclinD1/CDK4 induces hypertrophy by using [^{3}H] leucine incorpolation assay. CyclinD1/CDK4 overexpression increased the rate of protein synthesis by 1.55 fold, compared to those in LacZ-overexpressed cells as previously reported before (Fig. 3A)[8]. Further, we assayed the rate of RNA synthesis by [^{3}H]uridine incorpolation. Results showed that CyclinD1/CDK4 cells increased the rate of RNA synthesis compared to the control cells (1.57 fold) (Fig. 3B). These results show that the overexpression of cyclinD1/CDK4 induces hypertrophy in cardiac myocytes.

DRB inhibited the phosphorylation of pol II CTD induced with overexpression of cylcinD1 and CDK4

We hypothesized that cyclinD1/CKD4 induced-hypertrophy would be involved in the upreguration of mRNA and protein synthesis through activation of pTEFb.

Phosphorylation of Pol II CTD through activation of pTEFb induces the activation of RNA pol II and it causes hypertrophy through upreguration of RNA and protein synthesis[9]. To examine phosphorylation of pol II CTD after ET-1 treatment and overexpression of cyclinD1/CDK4 in cardiac myocytes, we performed western blotting with antibody that recognizes both hyperphosphorylated and hypophosphorylated pol II (IIo and IIa, respectively).

DRB was used as specifically inhibitor of pTEFb. Hyperphosphorylation of pol II were induced 15 min after ET-1 treatment and it was inhibited 2 hours preincubation with 50 μM DRB as previously reported9). The overexpression of CyclinD1/CDK4 using adenovirous vector also induced phosphorylation of Pol II CTD and it was inhibited with DRB (Fig. 2).

These results show that cyclinD1/CKD4 induced-hypertrophy would be involved in the phosphorylation of pol II CTD.

RNA and protein synthesis upregulated by the overexpression of cyclinD and CDK4 in cardiac myocytes were inhibited with DRB

As shown in Fig. 2, the overexpression of cyclinD1/CDK4 induced phosphorylation of pol II CTD. However, it is unclear whether the overexpression of cyclinD1/CDK4 induce the increase of RNA and protein synthesis through activation of pol II. To evaluate the synthesis of RNA and protein, the analysis of the incorporation of [^{3}H] uridine and [^{3}H]leucine were performed. Cardiomyocytes were infected with Ad-cyclinD1/Ad-CDK4. At 48 h after infection, cells were harvested. Fig. 3 revealed that the cyclin D1/CDK4 overexpressed-cells significantly increased the rate of RNA and protein synthesis compared to the control LacZ cells. DRB induced significant inhibition of cyclinD1/CDK4-induced RNA and protein synthesis in cardiac myocytes (Fig. 3).

In conclusion, these results show that cyclinD1/CKD4 induced-hypertrophy would be involved in the increase of RNA and protein synthesis through phosphorylation of pol II CTD.

Discussion

In this study, we showed that ET-1 induced cyclinD1 expression level of mRNA and protein and overexpression of cyclinD1/CDK4 induces hypertrophy in cardiac myocytes. Moreover, DRB inhibited RNA and protein synthesis and the phosphorylation of pol II CTD induced with overexpression of cylcinD1 and CDK4. These results demonstrate that cyclinD1/CKD4 induced-hypertrophy is involved in the increase of RNA and protein synthesis through pTEFb mediated-pol II CTD phosphorylation.

These results suggest the possibility that the inhibitor of CDK4 and pTEFb such as flavopiridol,

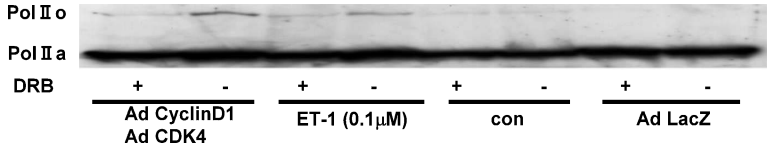

Fig. 2. DRB inhibited the phosphorylation of pol II induced with ET-1 and the overexpression of cylcinD1 and CDK4
Cardiac myocytes were serum-starved for 24 h, stimulated for 15 min with ET-1 (0.1 μM) or the vehicle with and without preincubation of 50 μM DRB for 2 h, and analyzed by Western blotting with antibody to the pol II CTD as in Materials and methods. Cells were infected with adenoviruses shown.

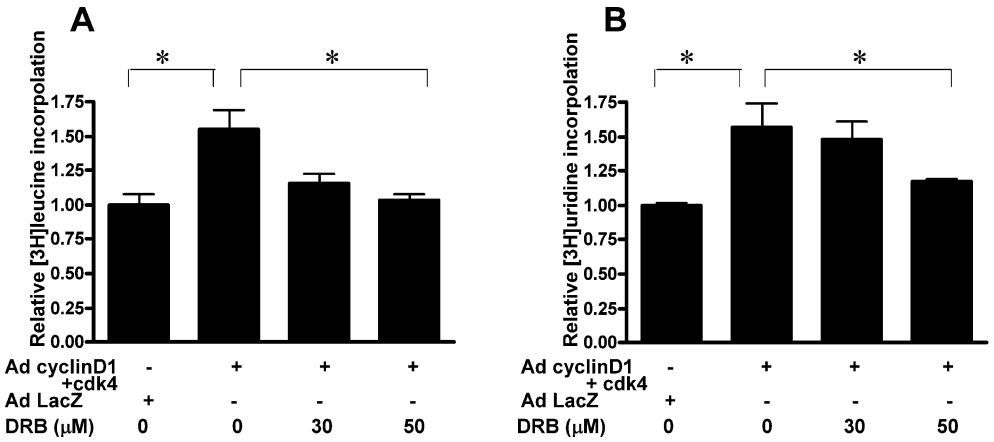

Fig. 3. Increase of RNA and protein synthesis was inhibited by DRB in the cyclin D1 and CDK4 overexpressed-cardiomyocytes
Cardiomyocytes were infected with Ad-cyclinD1/Ad-CDK4. At 48 h after infection, cell culture medium was replaced by [^{3}H]leucine (Fig. 3A) and [^{3}H]uridine (Fig. 3B)-containing medium and its incorporation was determined as in Materials and methods. Relative incorporation of [^{3}H]uridine and [^{3}H]leucine to that of LacZ-cells is shown. Each column represents the mean of four independent experiments with error bars of SEM. $^*P<0.05$.

DRB and seliciclib might be the candidate agents for the patient with cardiac hypertrophy[16]. Flavopiridol is a broad specificity CDK inhibitor with a distinct preference for CDK9. K_i values for CDK9/Cyclin T (3 nM) are approximately 10-fold lower than those for other CDKs (40-70 nM)[17]. Flavopiridol is currently in clinical trials as a CDK9 inhibitor for a number of cancers[18], with promising results for the treatment of chronic lymphocytic leukaemia[19]. The 2,6,9-trisubstituted purines were among the first low molecular weight inhibitors of CDKs[20]. One of these, (R)-roscovitine (CYC202, Seliciclib)[21] has now reached clinical phase 2 trials against non-small-cell lung cancer and nasopharyngeal cancer.

Not only Phosphorylation but also dephosphorylation of the Pol II CTD represents a critical regulatory checkpoint for transcription. Transcription initiation requires Fcp1/Scp1-mediated dephosphorylation of phospho-CTD[22]. Zheng H *et al.* isolated a novel phosphatase gene by large-scale sequencing analysis of a human fetal brain cDNA library. It contains a ubiquitin-like domain and a CTD phosphatase domain. Therefore, it was termed ubiquitin-like domain containing CTD phosphatase 1 (*UBLCP1*)[23]. These genes might be other targets for treatment of hypertrophy.

In Fig. 2, overexpression of cyclinD1/CDK4 induced phosphorylation of Pol II CTD. However, it is not clear whether cyclinD1/CDK4 directly phosphorylate pol II CTD or indirectly in cardiac myocytes and what is the upstream of cyclinD1/CDK4. It has been reported that p16 (INK4A) inhibited the CTD phosphorylation by TFIIH[24] and dominat negative Ras markedly inhibited phosphorylation of the pol II CTD[25]. These genes might be involved in the phosphorylation of pol II CTD with cyclinD1 and CDK4.

Cell cycle related-genes other than cyclinD1/CDK4 have been reported to be involved in hypertrophy. A cyclin D2-Rb pathway regulates cardiac myocyte size and RNA polymerase III after biomechanical stress in adult myocardium[26]. Inhibition of E2F abrogates the development of cardiac myocyte hypertrophy[27].

In conclusion, understanding more about the effect of cell cycle related-genes in cardiac myocytes could lead to new therapies for the

patients with hypertrophy.

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