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Phosphorylation of pRb by cyclin D kinase is necessary for development of cardiac hypertrophy

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Abstract. *Objectives*: A number of stimuli induce cardiac hypertrophy and may lead to cardiomyopathy and heart failure. It is believed that cardiomyocytes withdraw from the cell cycle shortly after birth and become terminally differentiated. However, cell cycle regulatory proteins take part in the development of hypertrophy, and it is important to elucidate the mechanisms of how these proteins are involved in the hypertrophic response in cardiomyocytes. *Materials and methods, and Results*: In the present study, by immunohistochemistry with a phosphorylation-specific antibody, we found that cyclin D-cdk4/6-phosphorylated retinoblastoma protein (pRb) during hypertrophy and expression of an unphosphorylatable pRb mutant impaired hypertrophic growth in cardiomyocytes. Transcription factor E2F was activated by hypertrophic elicitors but activation was impaired by pharmacological inhibition of cyclin D-cdk4/6. Inhibition of cyclin E-cdk2 complex only partly impaired E2F activity and did not prevent hypertrophic growth, but diminished endoreplication during hypertrophy. *Conclusions*: These results indicate that cyclin D-cdk4/6-dependent phosphorylation of pRb and activation of E2F is necessary for hypertrophic growth in cardiomyocytes, whereas cyclin E-cdk2 kinase is not necessary for hypertrophy but regulates endoreplication in these cells. The data support the notion that hypertrophic growth of cardiomyocytes involves a partial progression through the G_1 phase of the cell cycle.

INTRODUCTION

The cell cycle is highly conserved and regulated in all eukaryotic cells, and expression of several cell cycle regulatory proteins is altered in various heart diseases, including cardiomyocyte hypertrophy. Many of the signalling molecules implicated in hypertrophy are proto-oncogenes, molecules essential for regulating the cell cycle (Reiss *et al*. 1996; Sadoshima & Izumo 1997; Poolman & Brooks 1998; Tamamori *et al*. 1998). Cardiomyocytes stop dividing shortly after birth and

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withdraw from the cell cycle (Poolman & Brooks 1998; Busk & Hinrichsen 2003). Instead, postnatal cardiomyocytes grow by hypertrophy (Zak 1974; Ueno *et al*. 1988; Busk & Hinrichsen 2003; Busk *et al*. 2005). Mammalian cardiomyocytes retain only limited cell cycle activity during adult life (Clubb & Bishop 1984; Rumyantsev 1991b; Li *et al*. 1996; Busk & Hinrichsen 2003). However, regeneration of injured adult myocardium has been documented in newt (Oberpriller *et al*. 1988; Busk & Hinrichsen 2003) and zebrafish (Poss *et al*. 2002; Busk & Hinrichsen 2003), and in one particular mouse strain (Leferovich *et al*. 2001).

The hypertrophic response is triggered by various stimuli, like left ventricular wall stress, growth factors, cytokines and hormones (Chien & Grace 1999; Busk & Hinrichsen 2003). The increased myocyte size is accompanied by an increase in RNA and protein content (Chien *et al*. 1999), along with re-expression of foetal genes. *In vitro*, cardiomyocyte hypertrophy can also be induced by various mitogens like serum (Lubic *et al*. 1994; Busk & Hinrichsen 2003) and angiotensin II (AII; Aceto & Baker 1990; Busk & Hinrichsen 2003). These stimuli activate multiple second-messenger systems and induce activity of various immediate-early genes, such as *c-fos* and *c-jun* (Simpson 1989; Bogoyevitch *et al*. 1995; Sadoshima & Izumo 1997; Busk & Hinrichsen 2003), genes that are essential components of the mitotic machinery. The same hypertrophic stimuli also induce expression of many cell cycle regulatory proteins in cardiomyocytes (Reiss *et al*. 1996; Sadoshima *et al*. 1997; Li *et al*. 1998; Poolman & Brooks 1998; Tamamori *et al*. 1998; Nozato *et al*. 2000; Busk *et al*. 2002; Busk & Hinrichsen 2003). It is thus becoming clear that hypertrophic and proliferative stimuli share certain intracellular signal pathways.

Progression through the four phases of the cell cycle is tightly regulated by the sequential expression, activation, inactivation and degradation of specific cell cycle regulatory proteins. Many of the 'decisions' whether a cell should divide or not are taken in G_1 phase, before DNA synthesis in S phase (Bartek & Lukas 2001; Busk & Hinrichsen 2003). As hypertrophy does not require DNA synthesis, events in G_1 phase are particularly interesting for cardiac hypertrophy. A key regulator of passage from G_1 phase into S phase is the E2F transcription factor (Nevins *et al*. 1997; Dyson 1998; Busk & Hinrichsen 2003). E2F is essential for cell cycle progression and regulation by activating necessary genes. E2F is regulated by the Rb family of proteins that bind to E2F and prevent E2F from activating transcription. E2F is released from this inhibitory complex by phosphorylation of the Rb protein (Nevins *et al*. 1997; Dyson 1998; Busk & Hinrichsen 2003). The molecules responsible for this phosphorylation are the cyclin-dependent kinase (cdk) complexes (Li & Brooks 1999; Busk & Hinrichsen 2003). In G_1 progression, these complexes include cyclins D1–D3 associated with cdk4/6 kinases and cyclin E associated with cdk2 kinase (Reed 1996; Busk & Hinrichsen 2003). The cyclin D-cdk4/6 kinase complex phosphorylates the Rb protein, thereby making it possible for E2F to activate transcription of various genes, including the cyclin E gene (Ohtani *et al*. 1995; Busk & Hinrichsen 2003). Cyclin E is the regulatory subunit for cdk2 kinase, and the cyclin E-cdk2 complex further phosphorylates the Rb protein, thereby releasing E2F from its inhibitory complex with retinoblastoma protein (pRb). This event controls entry into S phase and DNA synthesis (Ohtsubo *et al*. 1995; Busk & Hinrichsen 2003). The cyclin-cdk complexes are regulated in a variety of ways, including by cdk inhibitors (Sherr & Roberts 1999; Bartek & Lukas 2001; Busk & Hinrichsen 2003).

Overexpression of E2F-1 in adult cardiomyocytes drives a significant number of cells from G_1 phase into S phase of the cell cycle (Agah *et al*. 1997; von Harsdorf *et al*. 1999). Growing evidence indicates that several hypertrophic stimuli up-regulate the activities of G_1 cyclins, such as cyclins D1, D2, D3 and A, and associated cdks (cdk4/6 or cdk2) in cardiac myocytes *in vitro* and *in vivo* (Sadoshima & Izumo 1997; Poolman & Brooks 1998; Tamamori *et al*. 1998; Busk *et al*. 2002; Busk & Hinrichsen 2003), while expression of cdk inhibitors is down-regulated

(Li & Brooks 1997; Busk & Hinrichsen 2003). These data strongly suggest that G_1 cell cycle regulatory proteins are important factors during the hypertrophic response in cardiomyocytes.

In the present work, we have used inhibitor and expression studies to access the role cell cycle proteins have on hypertrophic development in the myocyte cultures. Results show that phosphorylation of pRb by cyclin D-cdk4/6 is necessary for hypertrophic growth and that this pathway leads to partial activation of the E2F transcription factor. Cyclin E-cdk2, which also phosphorylates pRb, had little impact on hypertrophy but prevented endoreplication observed in hypertrophic cardiomyocytes.

METHODS

Cardiomyocyte cultures

Protocols used in this investigation conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, reviser 1996).

Newborn, ventricular myocytes were prepared from 1- to 5-day-old neonatal Wistar rats (University of Copenhagen, Copenhagen, Denmark) as previously described (Busk *et al*. 2003).

Cells were plated at a density of 5×10^4 cells/cm^2 in ventricular myocyte medium (VMM) [Eagle's minimal essential medium supplemented with 100 nM insulin, 656 mg/L creatine, 396 mg/L carnitine, 626 mg/L taurine, 0.5 g/L bovine serum albumin, 0.1% foetal calf serum (Gibco, Paisley, UK), 50 μg/mL streptomycin (Gibco) and 50 U/mL penicillin (Gibco)] in culture dishes, pre-coated in PBS with 8% foetal calf serum, for 5 h at 37 °C. Cultures were tested for presence of non-myocytes by staining with a myocyte-specific anti-α-actin antibody (Sigma, St. Louis, MO, USA) and tetramethylrhodamine isothiocyanate (TRITC)-labelled phalloidin.

Inhibitors

Roscovitine (10 μM) (Calbiochem, San Diego, CA, USA) or differentiation inducing factor 1 (30 μM) (DIF-1; Affiniti Research, Exeter, UK) was used to inhibit cdk2 kinase and cyclin D, respectively.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Bartkova *et al*. 2003) with antibodies (Sigma) to sarcomeric α-actin (clone 5C5), sarcomeric tropomyosin (clone CH1), α-smooth muscle actin (clone 1A4), von Willebrand factor and serine 795-phosphorylated pRb (clone RB-10), on the myocyte cultures fixed with paraformaldehyde. pRb phosphorylation was visualized using Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA).

Morphology assay

The morphology assay was performed as described previously (Busk *et al*. 2002). Briefly, cells were plated in pre-coated chamber slides and after 3 days of incubation they were fixed in 4% paraformaldehyde, and actin was stained with TRITC-labelled phalloidin. The preparations were viewed by confocal laser microscopy (LSM510, Zeiss, Switzerland) and cell size was analysed using Metamorph software (Zeiss) and expressed in arbitrary units.

Protein synthesis

Myocytes were plated in pre-coated wells in VMM. Medium was changed to VMM with 30 μg/mL L-glutamine 2 days after plating. Cells were treated with roscovitine or DIF-1 on day 5

and ^{3}H-phenylalanine (25 Ci/mmol; Amersham/Pharmacia, Buckinghamshire, UK) was added to a final concentration of 0.5 μCi/mL in the medium. After 72 h, medium was removed and protein was precipitated with trichloroacetic acid (Higgins 1994). Precipitated protein was solubilized in 1% sodium dodecyl sulfate and incorporated radioactivity was measured by counting in a scintillation analyser using a ^{3}H program (Tri-carb 2900TR, Packard Bioscience, Meriden, CT, USA).

DNA synthesis

Myocytes were plated in pre-coated wells in VMM. Medium was changed to VMM with 30 μg/mL L-glutamine 2 days after plating. Cells were treated with roscovitine or DIF-1 on day 4 and ^{3}H-thymidine (25 Ci/mmol; Amersham/Pharmacia) was added to a final concentration of 0.5 μCi/mL in the medium. After 24 h, medium was removed and DNA was precipitated with trichloroacetic acid (Higgins 1994). Precipitated DNA was solubilized in 0.1 M NaOH; 1% sodium dodecyl sulfate and incorporated radioactivity was measured by counting using a scintillation analyser on a ^{3}H program (Tri-carb 2900TR, Packard Bioscience).

Recombinant adenovirus

Replication-deficient adenovirus expressing mock from the cytomegalovirus (CMV) promoter was used as described previously (Busk *et al*. 2002). The plasmid pECEΔcdk-HA contains the pRb gene (RbΔcdk) that cannot be phosphorylated by cyclin-dependent kinases due to mutations in the putative phosphorylation sites (Lukas *et al*. 1997). RbΔcdk was excised from the vector by digestion with Bsu36I, blunt ended with Klenow fragment, and digested with *Hin*dIII. The fragment was inserted into pAdTrack-CMV (Stratagene, La Jolla, CA, USA) and was digested with *Hin*dIII and *Eco*RV. Replication-deficient adenovirus expressing RbΔcdk from the CMV promoter was made by homologous recombination of pAdTrack-CMV containing RbΔcdk with pAdEasy-1, in *Escherichia coli* BJ5183 (He *et al*. 1998). A gene encoding mutated cdk2 (DNcdk2), which is dominant negative (van den Heuvel & Harlow 1993), was excised from the vector by digestion with *Bam*HI and inserted into pAdTrack-CMV cut with *Bgl*II. The viral vector was multiplied and packed by transfection into HEK293 cells (He *et al*. 1998). Titres were determined by infection of HEK293 cells and counting of green fluorescent protein (GFP)-stained cells as described previously (Busk *et al*. 2002). Cardiomyocytes were infected by adding adenovirus to a multiplicity of infection of 10. All cells in infected cultures expressed GFP and did therefore also express the gene of interest.

Plasmid construction

Annealed oligonucleotides (TAG, Copenhagen, Denmark) containing the E2F promoter site (Muller *et al*. 1997) were cloned into the vector pLuc-MCS (Stratagene) by digesting the vector with *Hin*dIII and *Xho*I. The fragment containing the E2F promoter and the luciferase gene were excised by digesting the plasmid with *Bam*HI, blunt ended with Klenow fragment and digested with *Spe*I. This fragment was cloned into pAAV-LacZ (Stratagene), digested with *Eco*RV and *Spe*I. The luciferase gene was removed by digesting the plasmid with *Sac*I and *Eco*RV. Enhanced green fluorescent protein (EGFP) was excised by digesting pEGFP-N1 (Clontech, Palo Alto, CA, USA) with *Not*I, blunt ended with Klenow fragment and digested with *Sac*I, and the fragment was inserted instead of the luciferase gene. The fragment containing the E2F promoter site and the EGFP gene was excised from the plasmid by digesting with *Xmn*I and *Not*I. The fragment was ligated into the Shuttle vector (Stratagene AdEasy Adenoviral Vector System), digested with *Not*I and followed by treatment with calf intestine alkaline phosphatase.

E2F activity assay

Cardiomyocytes were plated in pre-coated 6-well plates in VMM. Using polyethyleneimine (Sigma), transient transfection was carried out with 1 μg E2F plasmid and 1 μg β-galactosidase plasmid/well as previously described (Busk *et al*. 2003). Medium was replaced the next day before treating the cells with roscovitine or DIF-1 and stimulated with serum. After an additional 3 days, the cells were harvested in 150 μL 1× Cell Culture Lysis Reagent (Promega, Madison, WI, USA) and were treated as described previously.

Luciferase and β-galactosidase assay

Luciferase and β-galactosidase assays were performed as previously described (Busk *et al*. 2003).

Endoreplication assay

Cells were plated in pre-coated chamber slides in VMM. After incubation, they were fixed in 4% paraformaldehyde and actin was stained with TRITC-labelled phalloidin (Sigma) (Busk *et al*. 2002) and Sytox Green nucleic acid stain (Molecular Probes, Eugene, OR, USA). Preparations were viewed by confocal laser microscopy (LSM510, Zeiss) and a number of nuclei per cell were counted.

Statistical analysis

Statistical comparison was performed by two-sided Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Two days after isolation of the myocyte culture, the cell preparation was analysed for possible contamination with other cell types. Immunohistochemical analysis showed that nearly all cells were positive for the cardiomyocyte markers sarcomeric α-actin (Fig. 1; Beltrami *et al*. 2003) and sarcomeric tropomyosin (von Harsdorf *et al*. 1999). Cells were also stained with antibodies against α-smooth muscle actin and von Willebrand factor, in order to detect non-myocytes. Very few cells were positive for smooth muscle actin but it stains fibroblasts and smooth muscle cells too, and for the endothelial cell marker von Willebrand factor (Beltrami *et al*. 2003) (data not shown).

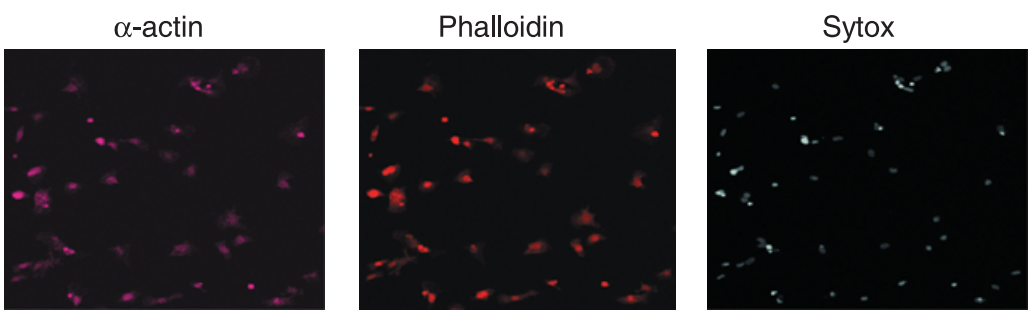

Figure 1. Immunohistochemical analysis of isolated cardiomyocytes. Cardiomyocytes were isolated from heart ventricles of newborn rats as described in the Materials and Methods section. They were plated in 0.1% serum medium and incubated for 2 days before staining with antibodies against sarcomeric α-actin (actin), non-specific staining of actin with phalloidin and DNA stained with Sytox Green nucleic acid.

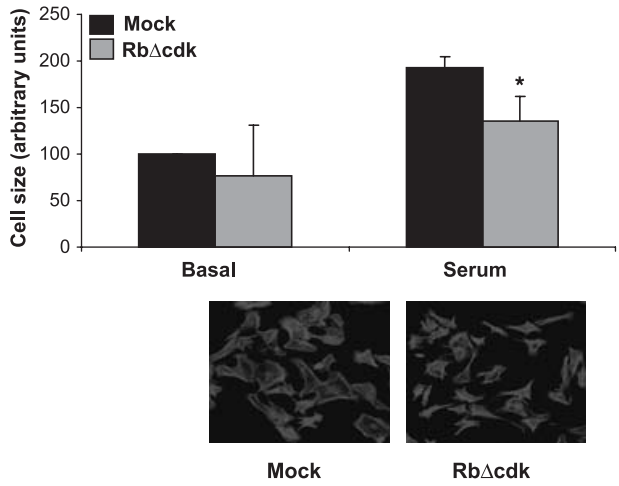

Figure 2. Constitutively active retinoblastoma protein (pRb) inhibited serum induced hypertrophy. Cardiomyocytes were infected with a viral vector expressing mock, or constitutively active pRb (RbΔcdk) (m.o.i. = 10) and were incubated for three days in 5% serum. Size of the cells (arbitrary units) under each condition was determined as described in the Material and Methods section. Error bars denote SD. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments. A minimum of 50 cells was measured.

Inhibition of pRb phosphorylation inhibits hypertrophic growth

pRb phosphorylation is a current molecular definition of a restriction point in the cell cycle (Planas-Silva & Weinberg 1997) and the cyclin D-cdk4/6 kinase complex plays a key role as growth sensor by phosphorylating pRb in the early stages of G_1. To investigate the effect of elimination of pRb phosphorylation on hypertrophic growth, constitutively active pRb, and RbΔcdk, was expressed in cardiomyocytes. They were infected with recombinant adenovirus expressing RbΔcdk protein or a mock virus expressing GFP, and we induced hypertrophy in the myocyte culture by stimulating the cells by addition of 5% serum. Serum is a frequently used inducer of hypertrophy and the size of the cardiomyocytes was subsequently determined as a measure for hypertrophic growth (Fig. 2). This experiment showed that RbΔcdk expression inhibited hypertrophic growth significantly ($P < 0.05$, Student's *t*-test) compared to the control cells.

pRb is phosphorylated by cyclin D-cdk4/6 during the hypertrophic response

To investigate whether cdk4/6 kinase phosphorylates the pRb, we measured phosphorylation of pRb on serine 795 by immunohistochemistry. Serine 795 is selectively phosphorylated by cyclin D-dependent kinases (Kitagawa *et al*. 1996; Grafstrom *et al*. 1999). Phosphorylation of this site has been shown to reflect activity of cyclin D-cdk4/6 kinase complex (Bartkova *et al*. 2003) and appears to be a critical event necessary for inactivation of pRb (Connell-Crowley *et al*. 1997). Stimulation of the myocytes lead to a significant ($P < 0.05$, Student's *t*-test) increase in pRb phosphorylation compared to the control cells (Fig. 3a). As expected, this was impaired by DIF-1, which inhibits cyclin D-cdk4/6 (Fig. 3b). In contrast, roscovitine did not inhibit pRb phosphorylation on serine 795. Roscovitine is a chemical cdk inhibitor that mainly inhibits cdk2 activity. Roscovitine can inhibit other cdk complexes, but roscovitine IC_{50} values for cdk4/6 are ≈100 and 1000 times higher than for cdk2, respectively (Penuelas *et al*. 2003). In summary, these results indicated that hypertrophic growth induces phosphorylation of pRb by activity of cyclin D-cdk4/6 kinase.

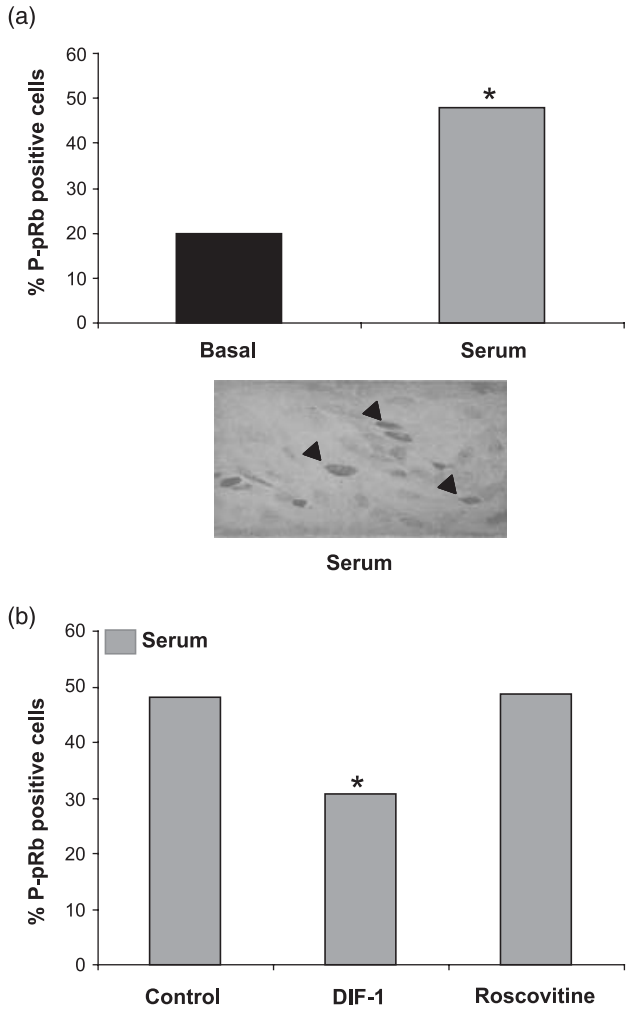

Figure 3. Serum induces retinoblastoma protein (pRb) phosphorylation in neonatal cardiomyocytes. Cells were stimulated with 5% serum (a) and were incubated for 2 days with 30 μM differentiation inducing factor 1 (DIF-1) or 10 μM roscovitine (b). Fraction of phosphorylated pRb-positive cells was counted. *indicates $P < 0.05$, Student's *t*-test. Data represent the result of three independent experiments. A minimum of 100 cells was counted.

Cyclin E-cdk2 kinase complex is not essential for hypertrophic growth

Both cdk4/6 and cdk2 kinase activities are induced during hypertrophic growth in cardiomyocytes (Sadoshima & Izumo 1997; Poolman & Brooks 1998; Tamamori *et al*. 1998; Busk *et al*. 2002; Busk & Hinrichsen 2003), and cyclin D-cdk4/6 is involved in cardiac hypertrophy *in vivo* and *in vitro* (Busk *et al*. 2002). To investigate the role of cyclin E-cdk2 kinase complex during hypertrophic growth, we induced hypertrophy in the myocyte culture by stimulating the cells with 5% serum and also with 0.5 μM AII (Fig. 4). In addition to serum, AII is a frequently used inducer of hypertrophy. We treated serum- or AII-induced cardiomyocytes with the cdk2 inhibitor roscovitine, and the size of cells was subsequently determined as a measure for hypertrophic growth. Roscovitine treatment had no significant effect on the size of the cells when treating them with serum compared to the control cells ($P < 0.05$, Student's *t*-test) (Fig. 4b). This indicated

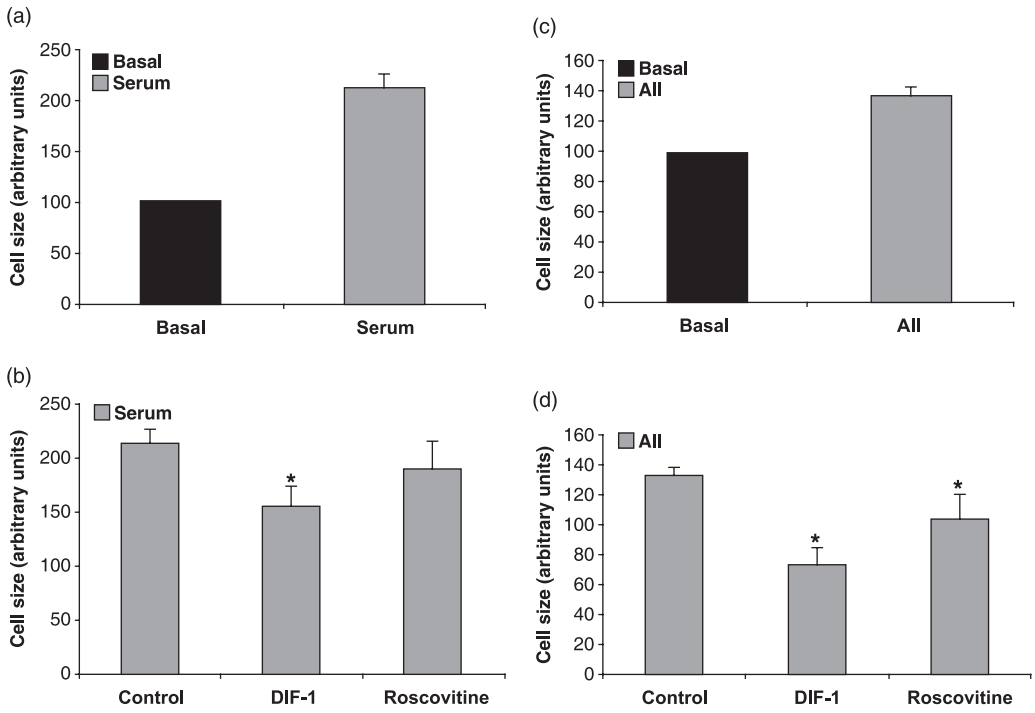

Figure 4. Inhibition of cyclin D impairs hypertrophic growth in neonatal cardiomyocytes. Average cell size in neonatal myocyte cultures after stimulation with 5% serum (a) or 0.5 μM angiotensin II (AII) (c) and were incubated for 3 days with 30 μM differentiation inducing factor 1 (DIF-1) or 10 μM roscovitine (b) (d). Size of the cells (arbitrary units) under each condition was determined as described in the Material and Methods section. Error bars denote SD. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments. A minimum of 50 cells was measured.

that cyclin E-cdk2 complex is not essential for hypertrophic growth of cardiomyocytes. As a control, we also treated the cardiomyocytes with DIF-1, resulting in expected inhibition of hypertrophic growth in the cardiomyocytes. However, AII treatment did result in a significant reduction in size when treated with roscovitine (Fig. 4d). To test whether this was the result of cdk2 inhibition, we expressed the inhibitory cdk2 mutant DNcdk2 in the cardiomyocytes. Inhibition of the cyclin E-cdk2 kinase complex with DNcdk2 had no effect on the size of hypertrophic cardiomyocytes (Fig. 5a), but inhibited DNA synthesis (Fig. 5b). This indicated that the discrepancy between roscovitine treatments in serum- and AII-stimulated cells is not due to inhibition of the cyclin E-cdk2 complex. In summary, inhibition of cyclin E-cdk2 kinase complex had no effect on the size of the cells. Because roscovitine inhibits entry into S phase, these data support the notion that cardiomyocytes can develop hypertrophy without passing into S phase.

Detection of induced protein synthesis is used as an indicator of hypertrophy. To verify hypertrophic growth observed, total protein synthesis in the cultures was measured by ^{3}H-phenylalanine incorporation. Serum- and AII-induced hypertrophy up-regulated protein synthesis in the cultures compared to the control cells (Fig. 6a–d). This induction of protein synthesis was down-regulated when inhibiting cyclin D-cdk4/6 with DIF-1 (Fig. 6a,c). However, protein synthesis was also dependent on cyclin E-cdk2, as ^{3}H-phenylalanine incorporation could be impaired by treating the cells with roscovitine (Fig. 6b,d). This result indicated that protein synthesis was not an optimal marker for hypertrophy when using serum or AII as hypertrophic elicitors in the cultures.

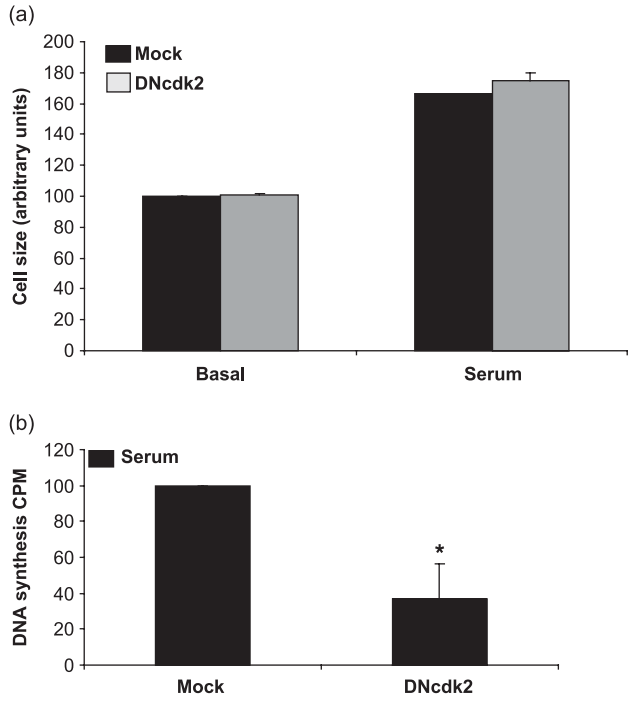

Figure 5. DNcdk2 expression does not impair hypertrophic growth, but inhibits DNA synthesis in neonatal cardiomyocytes. Average cell size in neonatal myocyte cultures infected with dominant-negative CDK2 (DNcdk2) or adenoviral vectors expressing mock (m.o.i. = 10) and incubated for 3 days in basal serum or with 5% serum (a). Size of the cells (arbitrary units) under each condition was determined as described in the Material and Methods section. Error bars denote SD. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments. A minimum of 50 cells was measured. (b) Cells were infected as in (a) and 1 day after infection DNA synthesis was measured by ^{3}H-thymidine incorporation over a 24-h period in cells treated with 5% serum. Data represent results of three independent experiments.

Hypertrophy is almost always associated with increased expression of brain natriuretic peptide and atrial natriuretic peptide in the heart, as well as with increased circulation levels of the two peptides in the blood (reviewed in Tremblay *et al*. 2002). Therefore, these peptides are often used as markers for the hypertrophic response in cardiomyocytes. As an additional control, we analysed levels of brain natriuretic peptide mRNA and found it to be induced in the cardiomyocyte cultures stimulated with serum or AII (data not shown).

Induced E2F activity during the hypertrophic response

If cyclin D-cdk4/6-dependent phosphorylation of pRB and not cyclin E-cdk2-dependent phosphorylation of pRB is involved in hypertrophic growth, does this result in altered E2F activity? Because E2F is known to be involved in cell cycle progression in cardiac myocytes (Flink *et al*. 1998; von Harsdorf *et al*. 1999), we investigated activity of E2F during the hypertrophic response, using a construct consisting of a minimal promoter controlled by an E2F enhancer element ahead of a *luciferase* reporter gene. Cardiomyocytes were transiently transfected with the construct and a control plasmid with the CMV promoter ahead of a *β-galactosidase* reporter gene. After transfections, the cardiomyocyte cultures were treated with DIF-1 or roscovitine to inhibit cyclin D and cdk2, respectively. To induce the hypertrophic response, cells were stimulated with

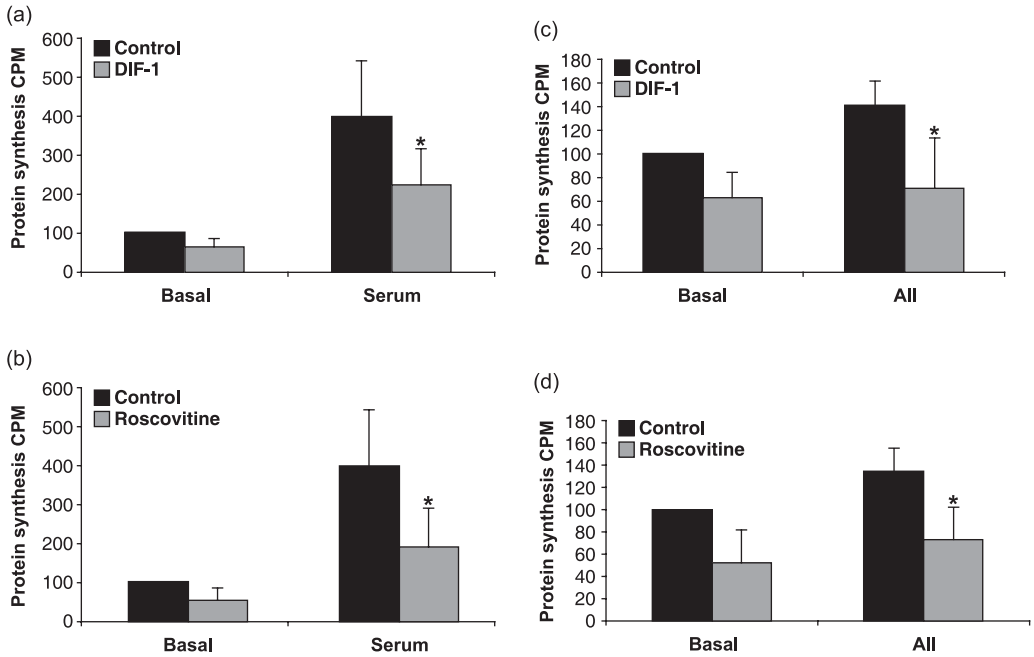

Figure 6. Serum or angiotensin II (AII) induced protein synthesis is inhibited by differentiation inducing factor 1 (DIF-1) or roscovitine in cardiomyocytes. Cells stimulated with 5% serum (a and b) or 0.5 μM AII (c and d) and incubated for 3 days with 30 μM DIF-1 or 10 μM roscovitine. Protein synthesis was measured by ^{3}H-phenylalanin incorporation in the stimulation period of 3 days. Error bars denote SD. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments.

serum. Low E2F promoter activity was detected in samples without serum and stimulation of the hypertrophic response induced E2F activity (Fig. 7). DIF-1 abolished the E2F activity entirely as expected as DIF-1 leads to inhibition of cyclin D-cdk4/6, and cyclin E-cdk2 activity depends on cyclin D-cdk4/6 (Lundberg & Weinberg 1998). Inhibition of cyclin E-cdk2 with roscovitine lowered E2F activity significantly ($P < 0.05$, Student's *t*-test), but there was still some activity-above background. This indicated that phosphorylation by cdk4/6 was sufficient to induce E2F-regulated transcription in hypertrophic cardiomyocytes. We also analysed expression of E2F1, E2F2 and E2F3 by Western blotting but found no regulation of protein levels in basal cells or cells stimulated with serum and treated with DIF-1 or roscovitine (data not shown). In summary, these data show that E2F activity was induced during the hypertrophic response of the cardiomyocytes. Transcription factor activity, and not protein expression, seems to be affected by cyclin D-cdk4/6 and cyclin E-cdk2 kinase activity.

Induced E2F activity up-regulates DNA synthesis

To test whether E2F activity observed during hypertrophic growth induced DNA synthesis in the cardiomyocytes, total DNA synthesis in the cultures was measured by ^{3}H-thymidine incorporation. Serum- or AII-induced hypertrophy up-regulated DNA synthesis in the cultures compared to the control cells (Fig. 8a–d). This induction was significantly ($P < 0.05$, Student's *t*-test) down-regulated by DIF-1 (Fig. 8a,c). The effect was also dependent on cyclin E-cdk2, as ^{3}H-thymidine incorporation could be impaired by treating the cells with roscovitine (Fig. 8b,d). In agreement with the literature, we found that 10 μM roscovitine inhibited DNA synthesis, which depends

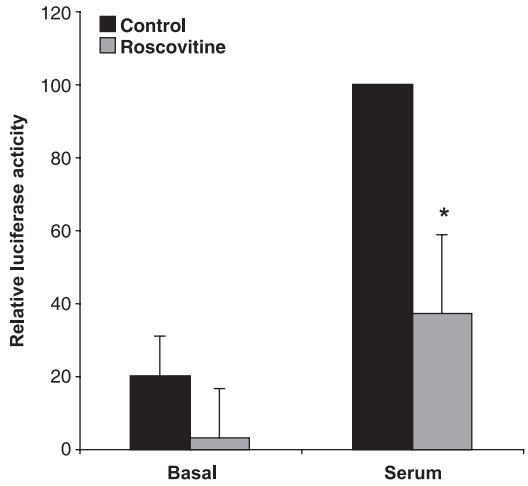

Figure 7. Inhibition of E2F activity in roscovitine treated cardiomyocytes. Cardiomyocytes were polyethyleneimine transfected with an E2F-luciferase reporter vector and a plasmid expressing β-galactosidase for 24 h as described in the Material and Methods section. Cells were stimulated with 5% serum and were further incubated for 3 days with 10 μM roscovitin. Error bars denote SD. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments.

on cdk2 activity, whereas cdk4/6-dependent phosphorylation of pRb was not inhibited by this concentration of roscovitine, and these results indicate that 10 μM roscovitine inhibits cdk2 but not cdk4/6 in cardiomyocytes. In addition, DNA synthesis inhibition is observed in basal cardiomyocytes treated with DIF-1 or roscovitine. Leaving cardiomyocytes to incorporate ^{3}H-thymidine for an additional 3 days did not absolutely abolish this activity, indicating continuing repair of DNA in the cardiomyocyte culture (data not shown). In summary, even though cardiomyocytes are known to preferentially stay in G_1 phase during the hypertrophic response, some myocytes do exceed the S-phase boundary and start DNA replication. This effect appears to involve both the cyclin D-cdk4/6 and the cyclin E-cdk2 kinase complex.

Endoreplication in hypertrophic cardiomyocytes

Binucleation of cardiomyocytes occurs shortly after birth in mice and rats, and is concurrent with withdrawal from the cell cycle (Brodsky *et al*. 1980; Rumyantsev 1991a). The molecular and cellular mechanisms responsible for regulating cardiomyocyte binucleation are largely unknown. Both endoreplication and hypertrophic growth involves activation of cell cycle machinery, induced protein synthesis and passage through G_1 phase. Because cyclin E-cdk2 complex is involved in endoreplication in various cell types, we wanted to investigate whether the same were true for cardiomyocytes. Thus, they were serum-stimulated to undergo hypertrophy with or without roscovitine treatment, and the percentage of binuclear cardiomyocytes was obtained by counting the number of nuclei per cell. Under basal conditions, no considerable endoreplication was observed (Fig. 9). However, serum stimulation resulted in significant induction of binucleation ($P < 0.05$, Student's *t*-test), and this was impaired by cdk2 inhibitor roscovitine, indicating that active cdk2 kinase is necessary for endoreplication. Although cyclin E may bind the cdc2 kinase (Aleem *et al*. 2005) and this complex could at least in theory be involved in endoreplication and S-phase transition (Kang & Koh 1997; Flink *et al*. 1998), roscovitine also inhibits cdc2 kinase (Canduri *et al*. 2004). This indicates that S-phase transition and DNA synthesis are inhibited by roscovitine and thus are not necessary for hypertrophic growth in cardiomyocytes.

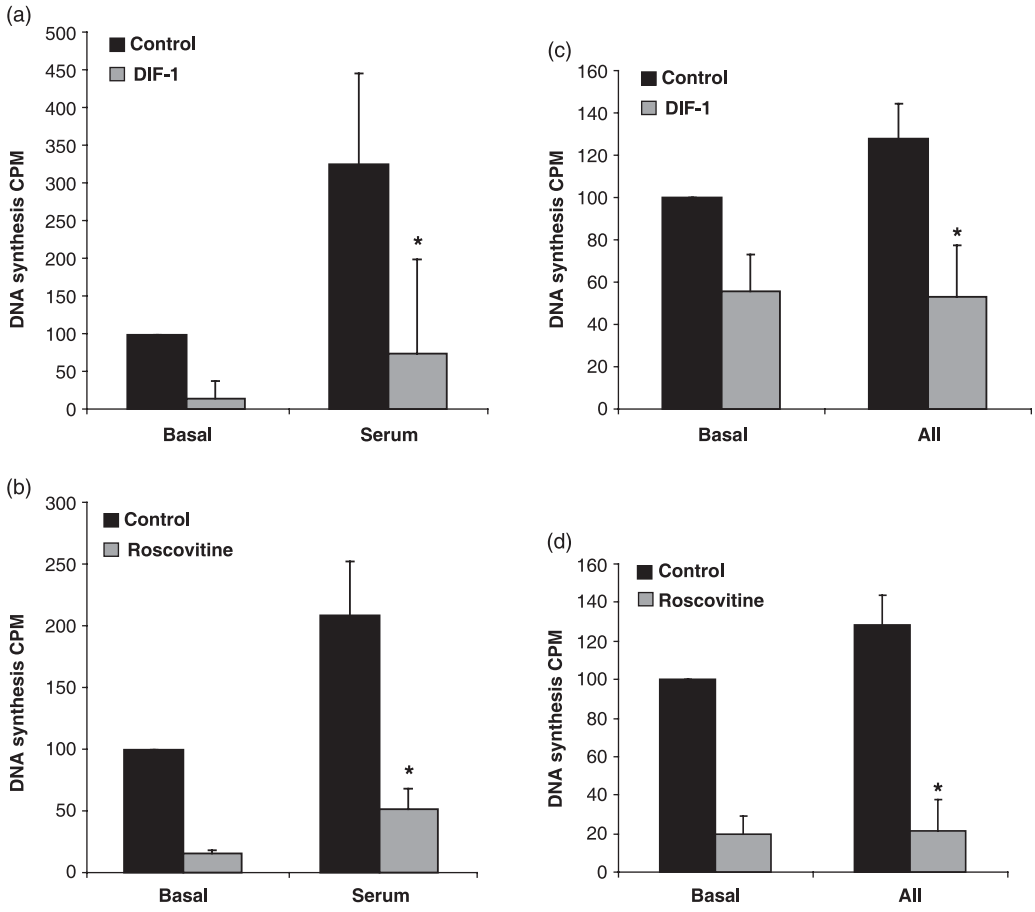

Figure 8. Differentiation inducing factor 1 (DIF-1) and roscovitine inhibits DNA synthesis in cardiomyocytes. Cells were stimulated with 5% serum (a and b) or 0.5 μM AII (c and d) and 30 μM DIF-1 or 10 μM roscovitine. After 1 day of incubation, DNA synthesis was measured by ^{3}H-thymidine incorporation over a 24-h period. Error bars denote SD. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments.

DISCUSSION

Hypertrophic and mitogenic stimuli share certain intracellular responses in various cell types, and both processes activate the cell cycle (Busk & Hinrichsen 2003). Hypertrophic growth in cardiomyocytes involves exit from G_0 and partial progression through the cell cycle. Over the past decade, a large number of studies have revealed the central role of the Rb pathway in regulation of G_1- to S-phase transition, and control of the cell cycle, by modulating activity of the E2F transcription factor (reviewed by Stevens & La Thangue 2003). Although regulation of cyclin-cdk activities is essential to cell size control in eukaryotes (Morgan 1997), and the E2F transcription factor is an important regulator of G_1 phase (Johnson *et al*. 1993; Duronio *et al*. 1995; Wu *et al*. 1996), their role in the hypertrophic response in cardiomyocytes is incompletely understood.

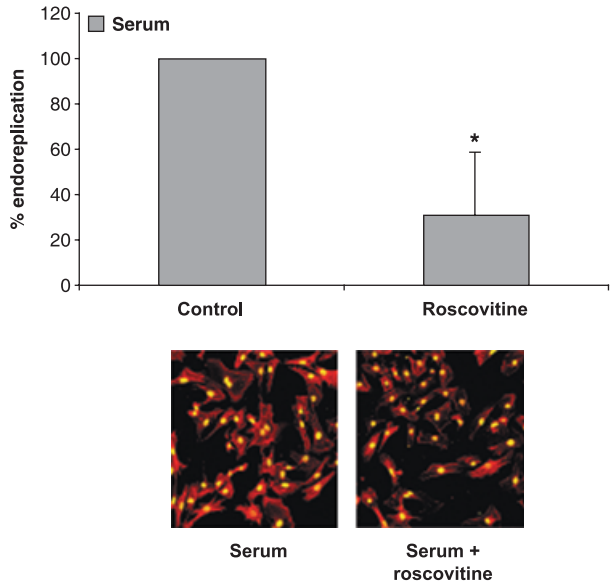

Figure 9. Roscovitine repressed endoreplication in hypertrophic neonatal cardiomyocytes. Cardiomyocytes stimulated with 5% serum were incubated with 10 μM roscovitine for 3 days followed by staining for actin with phalloidin and DNA with Sytox Green nucleic acid. Fraction of cells endoreplicating was counted. *indicates $P < 0.05$, Student's *t*-test. Data represent results of three independent experiments. A minimum of 100 cells was counted.

pRb phosphorylation by cyclin D-cdk4/6 kinase complex appears to be a key event necessary for inactivation of pRb in various cells. Despite the fundamental role cyclin D-cdk4/6 kinase complex plays as growth sensors, to date, only the Rb family and Smad proteins have been found to be substrate for this kinase complex (Matsuura *et al*. 2004). Cyclin D-cdk4/6 kinase complex is important for hypertrophic growth and the kinase activity is induced during the hypertrophic response in cardiomyocytes *in vivo* and *in vitro*. Inhibition of cyclin D inhibits cyclin D kinase activity and impairs their hypertrophic growth (Busk *et al*. 2002). This suggests that cyclin D-dependent kinase activity is necessary for development of hypertrophy in cardiomyocytes. We observed that expression of unphosphorylatable mutant of pRb, RbΔcdk, and inhibition of cyclin D-cdk4/6 impaired hypertrophic growth. Furthermore, stimuli that activated cyclin D-cdk4/6 induced phosphorylation of pRb on serine 795 and activity of the E2F transcription factor. This indicates that the pRb pathway is essential for hypertrophic growth of cardiomyocytes. This includes the phosphorylation of pRb by cyclin D-cdk4/6 kinase complex, followed by induction of E2F activity. Ectopic expression of RbΔcdk could also affect other pRb targets. However, pRb interactions with other proteins besides E2F and histone deacetylases are not that well established and the precise effect is therefore difficult to predict.

We found that inhibition of cyclin E-cdk2 kinase complex had no effect on hypertrophic growth here. Because cyclin E-cdk2 kinase complex is a key regulator of transition from G_1 to S phase and not involved in hypertrophic growth, the similarities shared by mitogenic stimuli and hypertrophic response are apparently only comparable in passage from G_0 to G_1 phase, and in the first part of G_1.

E2F protein expression is significantly down-regulated during myocyte development. Highest levels are found in foetal myocytes, but in adult ones E2F protein levels are undetectable (Brooks *et al*. 1998). In this study, hypertrophic stimuli induced E2F activity and this was either abolished or down-regulated when inhibiting cyclin D-cdk4/6 or the cyclin E-cdk2 kinase complex. This is consistent

with the observation that direct inhibition of E2F transcription factor, with a decoy oligonucleotide, inhibits hypertrophic growth of cardiomyocytes (Vara *et al*. 2003). Induced E2F activity is in agreement with the notion that hypertrophic cardiomyocytes re-enter the cell cycle from G_0 phase, and pass through G_1 phase. Earlier studies suggest that complete inactivation of pRb requires phosphorylation by both cyclin D-cdk4 and cyclin E-cdk2 (Lundberg & Weinberg 1998). Each kinase complex is activated at different points in G_1 phase. Cyclin D-cdk4 becomes active in mid- to late G_1 phase, and cyclin E-cdk2 in late G_1 phase is consistent with it contributing to pRb hyperphosphorylation. In this study, we found that only cdk4/6-dependent phosphorylation of pRb is required for participation of E2F in hypertrophy. This suggests that E2F transcription factor activity released following cyclin D-cdk4/6 phosphorylation, is activating genes necessary for hypertrophic growth in cardiomyocytes.

Mitogen stimulation leads to activation of cyclin D-cdk4/6 kinase complex in proliferating cells (Reed 1996), followed by induced E2F transcription factor activity and passage from G_1 to S phase. It is possible that mitogen-induced activation of cyclin D-cdk4/6 kinase complex in cardiomyocytes (Busk & Hinrichsen 2003) is responsible for previously reported S-phase passage and DNA synthesis (Yan *et al*. 1999; Matturri *et al*. 2002). We also observed DNA synthesis in hypertrophic cardiomyocyte cultures, indicating that cell cycle regulatory proteins, in at least some of the cardiomyocytes, are capable of driving the cells not only to re-enter the cell cycle, but also to exceed the G_1- to S-phase boundary and replicate DNA. Induced DNA synthesis in hypertrophic cardiomyocytes was impaired when either cyclin D or cdk2 was inhibited.

Knockout studies have shown that cyclin E and cdk2 are dispensable for mitosis in mice (Geng *et al*. 2003; Ortega *et al*. 2003). However, cyclin E is indispensable for endoreplication (Geng *et al*. 2003). Endoreplication arises as a consequence of nuclear mitotic division without cytoplasmic separation. Both endoreplication and hypertrophic growth involve induction of the cell cycle and passage through G_1 phase. As observed here, some hypertrophic cardiomyocytes are apparently able to pass further through the cell cycle, through G_1 phase and undergo endoreplication (Katzberg *et al*. 1977). This induced endoreplication can be impaired by inhibiting cyclin E-cdk2 kinase complex. This suggests that cyclin E-cdk2 kinase complex is necessary for endoreplication in cardiomyocytes. In spite of the molecular similarities between hypertrophic growth and endoreplication, activity of the cdk2 kinase is not a common feature of the two.

Scrutinizing G_1 kinase complexes provides valuable information on a possible approach for developing treatment for hypertrophy. In addition, with these data we are one step closer to elucidate the mechanisms necessary for getting cardiomyocytes to undergo mitosis and thereby proliferate. Normal cardiomyocytes have down-regulated levels of cell cycle regulatory proteins, but maintain the ability to re-express them during hypertrophy (Li *et al*. 1998). Because cardiomyocytes do not generally divide, this implies that cell cycle machinery is suppressed under normal conditions. This would in theory mean that by eliminating the suppressive mechanism, cardiomyocytes would be able to divide.

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