A Cyclin D2–Rb Pathway Regulates Cardiac Myocyte Size and RNA Polymerase III After Biomechanical Stress in Adult Myocardium

Ekaterini Angelis, Alejandro Garcia, Shing S. Chan, Katja Schenke-Layland, Shuxen Ren, Sarah J. Goodfellow, Maria C. Jordan, Kenneth P. Roos, Robert J. White, W. Robb MacLellan

Abstract—Normally, cell cycle progression is tightly coupled to the accumulation of cell mass; however, the mechanisms whereby proliferation and cell growth are linked are poorly understood. We have identified cyclin (Cyc)D2, a G1 cyclin implicated in mediating S phase entry, as a potential regulator of hypertrophic growth in adult post mitotic myocardium. To examine the role of CycD2 and its downstream targets, we subjected CycD2-null mice to mechanical stress. Hypertrophic growth in response to transverse aortic constriction was attenuated in CycD2-null compared with wild-type mice. Blocking the increase in CycD2 in response to hypertrophic agonists prevented phosphorylation of CycD2-target Rb (retinoblastoma gene product) in vitro, and mice deficient for Rb had potentiated hypertrophic growth. Hypertrophic growth requires new protein synthesis and transcription of tRNA genes by RNA polymerase (pol) III, which increases with hypertrophic signals. This load-induced increase in RNA pol III activity is augmented in Rb-deficient hearts. Rb binds and represses Brf-1 and TATA box binding protein (TBP), subunits of RNA pol III–specific transcription factor B, in adult myocardium under basal conditions. However, this association is disrupted in response to transverse aortic constriction. RNA pol III activity is unchanged in CycD2−/− myocardium after transverse aortic constriction, and there is no dissociation of TBP from Rb. These investigations identify an essential role for the CycD2-Rb pathway as a governor of cardiac myocyte enlargement in response to biomechanical stress and, more fundamentally, as a regulator of the load-induced activation of RNA pol III. (Circ Res. 2008;102:1222-1229.)

Key Words: hypertrophy ■ genetically altered mice ■ animal models of human disease ■ cell signaling/signal transduction

During development, cell cycle progression is closely coupled to the accumulation of cell mass to maintain a uniform cell size. It has been suggested that proteins involved in cell cycle progression control both processes, thereby linking proliferation and cell growth.1 In the fetal heart, the proliferative activity of cardiac myocytes is accompanied by increases in cell mass, thereby maintaining myocyte size. However, in adult cardiac myocytes cell growth becomes uncoupled from proliferation after growth stimuli, resulting in hypertrophic growth.2 The molecular mechanisms by which proliferation and cell growth are coupled in myocytes are poorly understood; however, cell cycle regulatory proteins typically associated with G1 exit have also been implicated in regulating cardiac myocyte cell growth.3–5 Cyclin (Cyc)D2-Cdk4 in particular has been associated with cardiac hypertrophy, although the downstream effectors have not been identified.6,7 Previous studies have indicated that inhibiting G1 cyclin/Cdk activity in adult, postmitotic, cardiac myocytes can attenuate hypertrophic growth.8,9 Consistent with this finding, we recently reported that Myc-induced hypertrophic growth was dependent on the presence of CycD2, similar to what has been reported for Myc-induced cell cycle reentry but was independent of Cdk2 activity.10

The association between CycD and Cdk4 represents the rate-limiting step in a complex cascade that leads to the phosphorylation of a number of proteins, including the retinoblastoma gene product, or Rb.8,11–13 When phosphorylated Rb becomes inactive, its association with a family of transcription factors known as E2Fs is disrupted, thereby allowing them to activate transcription.14 Previous studies have determined that Rb phosphorylation by CycD-Cdk4 is essential for cell cycle progression, largely by regulating the transcriptional activities of these E2Fs.15,16 However, whether this signaling pathway is also important in cardiac hypertrophy is unknown. Among the
known hypertrophic signaling cascades, a direct mechanistic link to Rb is perhaps best suggested for its interaction and regulation of the RNA polymerases.17,18

Therefore, to understand the role of CycD2 and Rb proteins during cardiac hypertrophy, and to determine the mechanism by which these cell cycle regulators modulate cardiac hypertrophy in vivo, we used mouse models deficient in these proteins. Mice in which CycD2 has been genetically ablated are viable and display normal cardiac anatomy and function.19 Although normal at baseline, CycD2-deficient mice exhibited attenuated hypertrophy in response to pressure overload. To determine the importance of Rb as a downstream target, we used mice with a cardiac-specific deletion of Rb. These mice have normal heart size at baseline,20 but hypertrophic growth in response to mechanical or pharmacological stress was increased significantly in Rb-null mice. Furthermore, activity of RNA polymerase (pol) III was increased in Rb-deficient hearts subjected to hemodynamic stress, whereas this transverse aortic constriction (TAC)-induced increased activity was not seen in CycD2−/− myocardium. Thus, we propose that CycD and Rb work in an interdependent manner to control myocyte size in postmitotic myocardium after mechanical stress. Our data identify CycD2, through its phosphorylation target Rb, as a pivotal regulator of RNA pol III activation in myocardial hypertrophy.

Materials and Methods

Animal Studies

Cardiac-restricted Rh-deficient mice (CRh−/−) have been described.20 CycD2-deficient mice were a kind gift from Dr P. Sicinski.19 For TAC, a fixed pressure overload was obtained by surgically constricting the transverse aorta, as described.21 Genotypes of mice were determined by PCR as described.10,20 All animals were handled and maintained in accordance with institutional guidelines and the NIH Guide for the Care and Use of Laboratory Animals.

Isolation of Cardiac Myocytes and Analysis

Neonatal rat ventricular myocytes (NRVMs) were prepared as described.10,20 Myocyte dimensions were determined and volumes were calculated using a computerized morphometric system.21 Details for viral propagation22 are available in the online data supplement at http://circres.ahajournals.org. For small interfering (si)RNA studies, NRVMs were transfected with 125 nmol/L CycD2 siRNA or nonspecific siRNA (Qiagen) with Lipofectamine 2000 (Invitrogen) according to the specifications of the manufacturer.

Histology and Immunostaining

For histology, hearts were either frozen in OCT compound or fixed overnight in 4% paraformaldehyde buffered with PBS and routinely processed. Details of immunostaining protocols and antibodies are available in the online data supplement.

RNA and Protein Analysis

Total RNA was extracted (Tri Reagent, Sigma) according to the instructions of the manufacturer. Northern blot and ribonucleic protein assays were conducted as previously described.20,23 Real-time quantitative PCR was conducted using the ABI PRISM 7700 Sequence Detection System; TaqMan (Applied Biosystems, Foster City, Calif). Primer sequences for all genes analyzed in the present study have been previously reported or are available on request.20,24 Western blotting and immunoprecipitations were performed as we have described,10,25 and details of the antibodies used are available in the online data supplement.

RNA Pol III Assays

RT-PCR analysis of ARPP P0 mRNA and primary tRNA5′ or tRNA17 transcripts was carried out as previously described.26,27 Gels were scanned and quantitated by a UVP image analysis system (Adobe PHOTOSHOP 4.0, Adobe Systems, Mountain View, Calif).

Statistical Analysis

All data are presented as means±SEM. Results were compared by 1-way ANOVA and Fisher’s protected least significant difference or Tukey’s multiple comparison post tests, using significance at a probability value of <0.05.

Results

CycD2-Null Mice Display Attenuated Hypertrophy in Response to Pressure Overload

Our previous work had shown that the hypertrophic effects of Myc were mediated through a CycD2-dependent pathway.10 In accordance with previous studies,4 CycD2 protein expression increases both in vitro and in vivo in response to hypertrophic stimuli (Figure I in the online data supplement). To determine whether the importance of CycD2 was specific to Myc-induced hypertrophy or whether it was more generally important in regulating hypertrophic growth, we subjected CycD2-null mice to pressure overload for 14 days. Homozygous CycD2-deficient mice are viable and appear normal but are sterile.19 Although not described in the original report, CycD2−/− mice had a 22.5% reduction in body weight compared with wild-type littermates at 12 weeks (supplemental Table I; 23.5±1.6 g [CycD2−/−] versus 28.8±0.9 g [CycD2+/+]; P<0.05); however, the heart-to-body weight ratios (HW/BW) were similar at baseline (4.35±0.14 mg/g [CycD2−/−] versus 4.40±0.16 mg/g [CycD2+/+]; P=NS). As expected, TAC increased CycD2 mRNA and protein levels in wild-type but not CycD2-deficient mice (Figure 1A and 1B). CycD2 mRNA increased 75% in CycD2-null mice compared with wild-type mice subjected to TAC, as measured by real-time PCR analyses (1.00±0.18 versus 1.75±0.19; P<0.05). Similarly, CycD2 protein expression increased 73% in CycD2−/− mice after TAC, as measured by Western blotting (1.62±0.36 versus 2.80±0.40; P<0.05). There was a 64% reduction in the hypertrophic response in CycD2-null mice compared with wild-type mice subjected to TAC (P<0.02). Two weeks of TAC induced a 25% increase in the HW/BW in CycD2 wild-type mice (Figure 1C; HW/BW: 4.35±0.14 versus 5.45±0.29 mg/g; P<0.005). In contrast, CycD2-null mice displayed an attenuated hypertrophic response with only a 9% increase in HW/BW (Figure 1C; HW/BW: 4.40±0.16 versus 4.81±0.14 mg/g; P<0.05). Fetal cardiac genes including atrial and B-type natriuretic peptides, as well as α-skeletal actin, were upregulated in response to TAC in both ventricles from mice of both genotypes despite the differences in myocardial mass, suggesting that not all aspects of the hypertrophic response were impaired (Figure 1D).

Rb Regulates Hypertrophic Growth In Vitro

Although several CycD2 phosphorylation targets are present in adult cardiac muscle, Rb is the primary target of CycD-Cdk4 kinase complexes and has been implicated, at least indirectly, in regulating protein synthesis.7 Thus, we sought to determine whether phosphorylation of Rb in response to hypertrophic agonists was dependent on CycD2. To knock down CycD2 expression, we transfected NRVMs with either nonspecific siRNA or a specific siRNA to CycD2. As shown...
in Figure 2A, siRNA transfection was able to induce a 98% decrease in CycD2 protein expression in NRVMs compared with nonspecific siRNA treatment (1.37 ± 0.29 versus 0.03 ± 0.01; P < 0.05). To determine whether depleting CycD2 impaired Rb phosphorylation in response to hypertrophic agonists, NRVMs transfected with nonspecific or CycD2 siRNA and stimulated with phenylephrine (PE) were immunostained with an antibody specific for phosphorylated serine 780 on Rb (pRbS780), a target of CycD-Cdk4. As expected, infection of NRVMs with PE and measured protein accumulation and myocyte size regulating hypertrophic growth, we stimulated NRVMs with

**Figure 1.** CycD2<sup>−/−</sup> mice display attenuated hypertrophy in response to pressure overload. A, Representative ribonucleic protection assay and quantification of CycD2 mRNA by real-time PCR normalized to GAPDH (n = 4 per group). *P < 0.05 CycD2 mRNA in CycD2<sup>−/−</sup> after TAC compared with CycD2<sup>+/+</sup> sham. B, Western blotting on total ventricular lysates after 2 weeks of TAC from the indicated genotypes and conditions. Protein quantification revealed an increase in CycD2 protein expression in CycD2<sup>+/−</sup> hearts after TAC. Cardiac actin (α-CaA) was used to normalize total protein levels (n = 4 for each group). *P < 0.05 for CycD2<sup>−/−</sup> TAC as compared with sham. C, Two weeks of TAC induced a significant increase in the HW/BW (mg/g) in CycD2<sup>−/−</sup> but not CycD2<sup>+/−</sup> mice (n = 5 per group). *P < 0.005 for CycD2<sup>−/−</sup> TAC compared with CycD2<sup>+/−</sup> sham. An increase in the HW/BW in CycD2<sup>+/−</sup> mice in response to TAC was not observed. D, Northern blot on total ventricular RNA. Expressions of atrial (ANP) and B-type (BNP) natriuretic peptides and skeletal actin (α-SkaA) and 18S ribosomal RNA (18S rRNA) as a loading control are shown.

**Pressure Overload Results in Enhanced Hypertrophy in Adult Rb-Deficient Cardiac Myocytes In Vivo**

To determine the role of Rb in regulating the hypertrophic response in adult cardiac myocytes in vivo, we subjected cardiac-restricted Rb-deficient (CRb<sup>L/L</sup>) mice we had previously created to TAC. These mice are phenotypically and biochemically normal at baseline. Hearts from Rb-deficient CRb<sup>L/L</sup> mice developed a 39.3% increase in HW/BW (mg/g) after 1 week of TAC (Figure 3A; P < 0.05). Likewise, at 2 weeks (Figure 3A), CRb<sup>L/L</sup> mice developed a 39.3% increase in HW/BW (mg/g) after 2 weeks of TAC as compared with sham. C, Two weeks of TAC induced a significant increase in the HW/BW (mg/g) in CycD2<sup>−/−</sup> but not CycD2<sup>+/−</sup> mice (n = 5 per group). *P < 0.005 for CycD2<sup>−/−</sup> TAC compared with CycD2<sup>+/−</sup> sham. An increase in the HW/BW in CycD2<sup>+/−</sup> mice in response to TAC was not observed. D, Northern blot on total ventricular RNA. Expressions of atrial (ANP) and B-type (BNP) natriuretic peptides and skeletal actin (α-SkaA) and 18S ribosomal RNA (18S rRNA) as a loading control are shown.

Pressure Overload Results in Enhanced Hypertrophy in Adult Rb-Deficient Cardiac Myocytes In Vivo

To determine the role of Rb in regulating the hypertrophic response in adult cardiac myocytes in vivo, we subjected cardiac-restricted Rb-deficient (CRb<sup>L/L</sup>) mice we had previously created to TAC. These mice are phenotypically and biochemically normal at baseline. Hearts from Rb-deficient CRb<sup>L/L</sup> mice were significantly larger than those from control CRb<sup>+/+</sup> mice after being subjected to 1 week of TAC (supplemental Table II; HW/BW: 6.40 ± 0.34 versus 5.25 ± 0.25 mg/g; P < 0.05). Likewise, at 2 weeks (Figure 3A), CRb<sup>L/L</sup> mice developed a 39.3% increase in HW/BW (mg/g) for sham-operated mice; (P < 0.05), whereas Rb-deficient CRb<sup>−/−</sup> mice demonstrated a 59.4% increase (HW/BW: 6.44 ± 0.5 versus 4.21 ± 0.16 mg/g for sham-operated mice; P < 0.001). This enhancement of myocardial mass in CRb<sup>L/L</sup> mice compared with CRb<sup>+/+</sup> animals after 2 weeks of banding was also significant (Figure 3A; P < 0.05). Although the role of Rb in mediating cell cycle exit in striated muscle is controversial, increased myocyte proliferation cannot explain the increase in cardiac mass that we observed because no evidence of myocyte cell cycle reentry was seen either by 5-bromodeoxyuridine incorporation (Figure 3B) or Ki-67 expression (not shown). In contrast, myocyte fiber width was significantly increased in CRb<sup>L/L</sup> mice subjected to TAC when compared with sham-operated CRb<sup>L/L</sup> or CRb<sup>+/+</sup> mice.
Figure 2. CycD2 phosphorylates and inactivates Rb, which regulates protein synthesis in vitro. NRVMs were transfected with either non-specific siRNA (NS siRNA) or specific CycD2 siRNA and treated with 100 mmol/L PE for 24 hours. A, Western blot analysis reveals a 98% knockdown of CycD2 protein expression in CycD2 siRNA-transfected NRVMs (n=3 per condition; P<0.05). B, a and b, Immunostaining of NRVMs treated with nonspecific siRNA revealed robust staining of CycD2 and phospho-Rb S780 (pRb S780) in response to PE. B, c and d, CycD2 siRNA–transfected cells revealed diminished expression of both CycD2 and pRb S780 in response to PE. Scale bar=50 μm. C and D, NRVMs cultured in serum-free media for 48 hours and infected with the indicated virus. NRVM cultures were then stimulated with 100 μmol/L PE for 24 hours. C, Relative levels of Rb expression were determined by Western blotting. 3H-phenylalanine incorporation was quantified and normalized to unstimulated AdLacZ cultures. The pooled results of 3 experiments are presented. *P<0.01 vs unstimulated AdLacZ myocytes and P<0.001 vs AdRb-infected cultures.

Figure 3. Rb-deficient mice display an exaggerated hypertrophic response in vivo. Control (CRb+/+) or Rb-deficient (CRb−/−) mice underwent sham or TAC surgery and were followed for 2 weeks (n=6 for each group). A, Heart weights (mg) were normalized to body weight (g). *P<0.05 for CRb−/− TAC vs CRb+/+ sham and CRb−/− sham, **P=0.005 for CRb−/− TAC vs CRb+/+ TAC, and P<0.0001 for CRb−/− sham and CRb−/− sham. B, Percentage of 5-bromodeoxyuridine–positive (BrdU+ve) nuclei from sham and TAC mice were quantified (n=6 per group; P=NS). C and D, Fiber width of wheat germ agglutinin–stained hearts from sham and TAC mice were quantified (n=5 in each group). D, Representative wheat germ agglutinin–stained myocardial sections from CRb+/+ (a and b) or CRb−/− (c and d) subjected to sham (a and c) or TAC (b and d) surgery.
Expression of E2Fs and E2F Target Genes Is Unchanged in Rb-Deficient Myocardium

Although Rb associates with many proteins, classically it binds and inhibits members of the E2F family of transcription factors. Because E2Fs have been implicated in regulating cardiac hypertrophy, at least in vitro,29 we examined the expression of E2F family members in CRb+/+ or CRbL/L mice at baseline and after TAC with real-time PCR. E2F-1 mRNA increased 3-fold in CRb+/+ hearts in response to TAC compared with baseline (Figure 4A; 1.00±0.08 versus 3.05±0.83; P<0.05); however, there were no differences in expression between CRb+/+ and CRbL/L mice (Figure 4A). Interestingly, no significant change was noted with TAC in the expression of E2F-3, as determined by semiquantitative PCR analysis, which was the E2F family member previously associated with hypertrophic growth in cultured NRVMs (Figure 4A).29 No change in either E2F-4 or -5 expression was seen in response to TAC. Because E2F transcriptional activity could be altered in the absence of Rb, even if expression levels were not, we examined the levels of a panel of E2F-1-dependent target genes.24 Semiquantitative PCR revealed no significant differences in the expression of any of these E2F-target genes in CRb+/+ and CRbL/L myocardium after TAC (Figure 4B). Thus, differences in E2F expression or activity are unlikely to account for the enhanced hypertrophic response in Rb-null myocardium.

Pressure Overload Induces Enhanced RNA Pol III Activity in Rb-Null Hearts

RNA pol III activity is known to increase in cardiac myocytes subjected to hypertrophic signals; however, the mechanisms regulating this effect are poorly understood.30 Rb is known to negatively regulate RNA pol III activity,17 via binding to Brf-1 and TATA box binding protein (TBP), resulting in inhibition of the pol III–specific transcription factor complex TFIIIB.25,31 To clarify the relationship between Rb and these 2 main subunits of TFIIIB, in response to a hypertrophic stimulus, we performed immunoprecipitation studies using total ventricular extracts from wild-type mice exposed to TAC for 7 days. Expression of phosphorylated and total Rb, Brf-1, and TBP in sham and TAC ventricles are displayed in Figure 5A and 5B. Although total expression levels of these factors did not change with TAC, phosphorylation of Rb increased in hypertrophic ventricles (Figure 5B). Brf-1 and TBP associated with Rb in wild-type sham ventricles (Figure 5C), but phosphorylation of Rb in banded ventricles disrupted...
this interaction and Rb was no longer associated with Brf-1 and TBP in myocardial lysates from TAC ventricles (Figure 5C). To determine the functional significance of the interactions of Rb with these factors, we assayed RNA pol III activity in Rb-deficient hearts. Intron-specific primers were used to assay levels of RNA pol III–specific transcripts from tRNA Tyr or tRNA Leu genes on RNA extracted from ventricles of mice after sham or TAC surgery (Figure 5D and 5E). RNA pol III activity was similar in sham-operated CRb+/+ and CRb L/L myocardium. However, RNA pol III activity was increased in Rb-null hearts after TAC compared with CRb+/+ TAC animals (Figure 5D and 5E). When compared with pressure-overloaded CRb+/+ mice, inducible tRNA Tyr transcripts in CRb L/L animals were 40% higher (1.73 ± 0.17 versus 1.33 ± 0.24; P < 0.05), although tRNA Leu transcripts were not elevated significantly (1.52 ± 0.07 versus 1.22 ± 0.12; P = 0.12). These data are consistent with a model in which Rb is bound to TFIIB under baseline conditions, but this association is disrupted by hypertrophic stimuli leading to increased RNA pol III activity.

To determine whether the CycD2-Rb interaction that we have proposed has functional significance in hypertrophic myocardium regulating the RNA pol III machinery, we examined the interaction of TBP and Rb in ventricles of CycD2−/− mice (Figure 6A). Although the association of TBP and Rb is disrupted by hypertrophic stimuli in CycD2+/+ hearts as expected, TAC did not disrupt the interaction of TBP and Rb in ventricles of CycD2−/− mice (Figure 6A). Similarly, both tRNA Tyr and tRNA Leu transcripts were elevated in response to TAC in the CycD2−/− animal after TAC (Figure 6B and 6C; tRNA Tyr: 1.00 ± 0.08 versus 1.48 ± 0.07, P < 0.05; tRNA Leu: 1.00 ± 0.10 versus 1.54 ± 0.10, P < 0.05). However, neither tRNA Tyr nor tRNA Leu transcripts were altered in CycD2−/− mice after TAC.

**Discussion**

We have previously shown that CycD2 is critical for mediating Myc-induced hypertrophy.10 In the present study, we examined the role of CycD2 in mediating the hypertrophic response more generally and its possible downstream effectors. Ablation of CycD2 expression resulted in attenuated cardiac hypertrophy in response to pressure overload; conversely, the same mechanical stress led to an exaggerated hypertrophic response in an Rb-null background. This could be explained, at least in part, by the fact that the activity of RNA pol III, which is normally upregulated with hypertrophic stimuli, was further enhanced in the absence of Rb. However, RNA pol III activity remained unchanged in CycD2−/− mice after TAC as compared with wild-type counterparts. These data provide the first direct evidence for a role for the CycD-Rb pathway in regulating cardiac hypertrophy in vivo and provide a rational mechanistic link between the observation by many investigators of the upregulation of G1 cyclins and increased protein synthesis via activation of RNA polymerase family members.

Hypertrophy was not completely abolished in CycD2−/− mice, and fetal gene induction was unchanged, implying that CycD2 is not the only pathway involved in the development of hypertrophy. This conclusion is consistent with our previous data suggesting that Myc-independent hypertrophic pathways also exist.10 Thus, although CycD2 may be obligate for Myc-induced hypertrophic growth, separate pathways must regulate Myc induction of fetal genes. The CycD2-Rb pathway would be expected to have a selective effect on the regulation of the translational machinery, and therefore growth (increased protein synthesis) would be more affected than other transcriptional aspects of the hypertrophic response. Because we are proposing that the effects of CycD2-Rb are mediated through RNA pol III, the transcription of genes such as atrial or B-type natriuretic peptides that
studies in cell cycle inhibitors. This conclusion is consistent with cycle activators unable to overcome the block imposed by cardiomyocyte hypertrophy is attributable to levels of cell proliferation, whereas in postmitotic cells, CycD-Cdk4 caused accelerated cell division, whereas in proliferating cells, CycD-Cdk4 caused cell

are controlled by RNA pol II would not be expected to be effected.

Our data that CycD is a critical factor in regulating cell size are seemingly at odds with existing data that CycDs are both necessary and sufficient for cardiac cell cycle progression. However, under normal physiological conditions, cell cycle progression is tightly coupled to the accumulation of cell mass (cell growth). Thus, even in situations where CycD2 induces proliferation of cardiac myocytes, it must also be stimulating cell growth because the resultant new myocytes were not smaller in size and CycD2 induced “hypertrophic” growth or increased myocyte size when cell cycle progression was inhibited. These authors concluded that cardiomyocyte hypertrophy is attributable to levels of cell cycle activators unable to overcome the block imposed by cell cycle inhibitors. This conclusion is consistent with studies in Drosophila, which revealed that in undifferentiated proliferating cells, CycD-Cdk4 caused accelerated cell division, whereas in postmitotic cells, CycD-Cdk4 caused cell

Figure 6. TBP does not dissociate from Rb and RNA pol III activity is not altered in CycD2−/− myocardium after TAC. A, TBP levels remain unchanged in both CycD2+/+ and CycD2−/− mice after TAC, as determined by Western blotting. A, Immunoprecipitation (IP) assays reveal that TBP does not dissociate from Rb in CycD2−/− ventricles after TAC. B, Representative blots of RNA pol III-dependent tRNA^Tyr and tRNA^Leu transcripts in CycD2+/+ mice (sham, n=4; TAC, n=5, respectively) are displayed; \( P<0.05 \) for both tRNA^Tyr and tRNA^Leu transcripts in CycD2−/− TAC vs CycD2−/− sham. No significant changes were noted in tRNA^Tyr and tRNA^Leu transcripts in CycD2−/− hearts.

enlargement. Thus, in the adult mouse heart, in which there is general agreement that CycDs are upregulated with hypertrophic stimuli, clearly the normal physiological consequence is not cell cycle reentry. This does not negate the observation that if expressed at high enough levels CycD2 can overcome the normal restraints to cell cycle reentry that are seen in adult cardiac myocytes. Our results with inducible Myc mice highlight this concept, in which high level activation of Myc induced both hypertrophic growth and cell cycle reentry, although only \( \approx 1\% \) of the myocytes reentered the cell cycle.

Pocket proteins, and Rb in particular, have a critical role in regulating cardiac cell cycle, but little evidence exists directly implicating Rb in cell size control in mammals, although several lines of evidence implicate it in this process. Classically, during cell cycle progression, Rb is inactivated in part by CycD-Cdk4 complexes freeing E2F transcription factors, which then drive cell cycle progression.

Although E2F-3 has been implicated in regulating the hypertrophic response, at least in vitro, we were not able to demonstrate differences in the expression or activity of E2F-1 or -3 in Rb-null mice. These data do not rule out a role for E2F family members in regulating cardiac growth but do question whether they are the primary downstream effectors of the CycD-Rb pathway in cardiac hypertrophy. Given that E2F-1 levels increase with TAC and a number of E2F target genes linked to the control of cell size control were also regulated with TAC, E2Fs may still play a key role in regulating cardiac hypertrophy through a separate pathway.

Interestingly, deletion of CycD2 did not effect the basal cardiac phenotype in CycD2−/− mice as compared with their wild-type counterparts, confirming that significant redundancy exists between CycD isoforms (D1, D2, and D3). However, minor differences were noted in CycD2 mice. Specifically, we noted a reduction in body weight in CycD2−/− animals as compared with wild-type mice, although the HW/BW was normal. Thus, it is likely that other family members can substitute for many but not all functions. Likewise, cardiac-specific deletion of Rb also displayed normal basal cardiac size as compared with CRb−/− mice. In both cases, this is consistent with the observation that basal RNA pol III activity was normal, although RNA pol III activity was augmented after mechanical load elicited in Rb-deficient mice. Rb normally represses pol III transcription by binding pol III transcription factor TFIIIB and sequestering it in an inactive complex. Our findings support a model in which both Brf-1 and TBP (pivotal TFIIIB subunits) become dissociated from Rb in response to a hypertrophic stimulus in vivo. Repression of tRNA and rRNA synthesis may explain the ability of Rb to inhibit protein synthesis and hence hypertrophic growth. Given the critical link between cell cycle progression and cell mass, it is not surprising that mechanisms exist that integrate these 2 modes of growth.

In summary, our results suggest a novel role for the CycD-Rb pathway in the regulation of hypertrophic growth in the adult heart. Future studies to better understand how this novel pathway interacts with other hypertrophic pathways to modulate the translational apparatus should provide insight into how proliferation and cell growth are linked.
Acknowledgments

We thank Dr P. Sicinski for CycD2-null mice and K. Hayakawa for technical assistance.

Sources of Funding

This work was supported by gifts from the Laubisch Fund (W.R.M. and K.P.R.); American Heart Association Established Investigator Award 0340087N (W.R.M.); and NIH grants P01 HL080111 and R01 HL70748 (to W.R.M.).

Disclosures

None.

References

A Cyclin D2–Rb Pathway Regulates Cardiac Myocyte Size and RNA Polymerase III After Biomechanical Stress in Adult Myocardium
Ekaterini Angelis, Alejandro Garcia, Shing S. Chan, Katja Schenke-Layland, Shuxen Ren, Sarah J. Goodfellow, Maria C. Jordan, Kenneth P. Roos, Robert J. White and W. Robb MacLellan

Circ Res. 2008;102:1222-1229; originally published online April 17, 2008; doi: 10.1161/CIRCRESAHA.107.163550

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/102/10/1222

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/04/17/CIRCRESAHA.107.163550.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL DATA:

MATERIALS AND METHODS

Animal studies.

Cardiac-restricted Rb-deficient mice (CRb<sup>L/L</sup>) have been described<sup>1</sup>. CRb<sup>L/L</sup> mice were mated and maintained on a FVB background homozygous for the αMHC-Cre transgene. Cyclin D2-deficient mice were a kind gift from Dr. P. Sicinski<sup>2</sup>. Cyclin D2 mice were maintained on a C57 background. Littermate controls were used throughout the study. Genotypes of mice were determined by PCR as described<sup>1,3</sup>.

For TAC, a fixed pressure overload was obtained by surgically constricting the transverse aorta, as described<sup>4</sup>. All surgical procedures (TAC) were performed on age-(8-10 weeks) and sex-matched mice. To induce hypertrophy pharmacologically, seven-day osmotic minipumps (Alzet, model 2001) loaded with 0.2 ml of isoproterenol (28 µg/ml per 25 g body weight) were implanted into the subcutaneous space of 3-month-old mice. M-mode echocardiography was performed as described<sup>4</sup>.

Isolation of cardiac myocytes and analysis.

Neonatal rat ventricular myocytes (NRVM) were prepared as described<sup>1,3</sup>. Myocyte dimensions were determined and volumes calculated using a computerized morphometric system<sup>4</sup>. For immunocytochemical studies, freshly cultured NRVM were placed in serum-free media for 48 hours, after which cells were treated with 100 µM phenylephrine (PE, Sigma) for 24 hours. An adenovirus vector expressing the wild-type human Rb, named AdRb, was generated using standard techniques. NRVMs were
treated with AdRb at multiplicity of infection of 25 pfu/cell\textsuperscript{5}. To estimate protein synthesis, myocytes were labeled with 5 µCi/ml of \([\textsuperscript{3}H]\)phenylalanine (Amersham Corp.) for 4-6 hours, after which cell precipitates were solubilized and counted.

**siRNA studies.**

NRVMs were transfected with 125nM of CycD2 siRNA or non-specific siRNA (Qiagen) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Two independent CycD2 siRNAs were used in equal amounts for all knock-down experiments. 48 hours after transfection NRVMs were treated with 100 µM phenylephrine (PE, Sigma) for an additional 24 hours, after which cells were immunostained or cell lysates subjected to Western blotting. The mouse CycD2 and non-specific (NS) siRNA oligonucleotide sequences are as follows: CycD2-1, 5'-CGAUGUGGAUUGUCUCAAAdTdT-3' and 5'-UUUGAGACAAUCCACAUCGdGdT-3'; CycD2-2, 5'-GCAAUGUUCCUAUUCAAdTdT-3' and 5'-UUGAAAUAGGAACAUUGCGdGdG-3'; NS, 5'-UUCUCGAACUGUCACGUdTdT-3' and 5'-ACGUGACACGUUCGGAGAAdTdT-3'.

**Histology and immunostaining.**

For histology, hearts were either frozen in OCT compound or fixed overnight in 4% paraformaldehyde buffered with PBS and routinely processed. If DNA synthesis was to be quantified, BrdU labeling was achieved by injecting 50 µg of BrdU per gram of body weight intraperitoneally. To assess rates of cell cycle reentry, fresh frozen sections were probed with antibodies to BrdU (Roche). Antibodies to myosin (MF20; Developmental Studies Hybridoma Bank, Univ. of Iowa) and wheat germ agglutinin
(Molecular Probes) were used according to the manufacturer’s recommendations. Secondary antibodies were purchased from Molecular Probes.

For cultured cells, NRVMs were fixed in 4% paraformaldehyde (Sigma) and permeabilized using 1% Triton-X 100 (Sigma). Immunostaining was performed as described\textsuperscript{3}. Images were acquired using a confocal TCS SP2 AOBS laser-scanning microscope system (Leica Microsystems Inc., Exton, PA, www.leica.com) and processed with Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA). Antibodies used include: Cyclin D2: mouse monoclonal, 1:200 (Neomarkers, Lab Vision); phospho-Rb\textsuperscript{S780}: rabbit polyclonal, 1:50 (Cell Signaling Technology).

**Western blotting and immunoprecipitations.**

Western blotting was performed as previously described\textsuperscript{3}. Antibodies against Cyclin D2, $\alpha$-cardiac actin and tubulin, Rb-G3-245, and phospo-Rb\textsuperscript{S795} were purchased from Neomarkers (Lab Vision), Sigma, BD Biosciences, and Cell Signaling Technologies, respectively. Protein expression was visualized using horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Biosciences, Sunnyvale, California). For immunoprecipitations (IP), 300-500 $\mu$g of tissue lysates were precleared with A/G agarose (Santa Cruz) and then incubated with 20 $\mu$g of anti-Brf-1, anti-TBP (Abcam), anti-Rb (BD Biosciences), or appropriate IgG control (Santa Cruz) and subjected to Western blot analysis\textsuperscript{6}.

**RNA analyses.**

Total RNA was extracted from frozen tissue samples and cells by a modification of the acid-guanidinium-phenol-chloroform method (Tri Reagent, Sigma) as per
manufacturer’s instructions. Detection of specific mRNA via Northern blot analyses using digoxigenin (DIG)-labeled ANP, and α-skeletal actin probes (10 ng/mL) were conducted as previously described\(^7\) and carried out as per manufacturer’s protocol (Roche Applied Science). Ribonucleic protection assays (RPA) were completed in accordance with the manufacturer’s (BD Bioscience) specifications\(^1\).

For semi-quantitative PCR analysis, all primers were cycled in the linear range and resolved on 1% agarose gels. Real-time quantitative PCR was conducted using the ABI PRISM 7700 Sequence Detection System; Taqman (Applied Biosystems, Foster City, CA). PCR amplicons were detected by fluorescent detection of SYBR Green (QuantiTect SYBR Green PCR Kit, Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. Primers sequences for all genes analyzed in the present study have been previously reported or are available upon request\(^7,8\).

**RNA pol III assays.**

RT–PCR analysis of ARPP P0 mRNA and primary tRNA\(^\text{Ty}r\) or tRNA\(^\text{Leu}\) transcripts was carried out as described previously\(^9,10\). Reaction products were resolved on 1% agarose gels and visualized by ethidium bromide staining. Gels were scanned and quantitated by a UVP image analysis system (Adobe PHOTOSHOP 4.0, Adobe Systems, Mountain View, CA).
Table S1. Cardiac and body weights for Cyclin D2<sup>+/+</sup> and Cyclin D2<sup>-/-</sup> mice after 2 weeks of pressure overload.

<table>
<thead>
<tr>
<th></th>
<th>TAC</th>
<th>BW (g)</th>
<th>HW (mg)</th>
<th>HW:BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CycD2&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>-</td>
<td>28.80 ± 0.87</td>
<td>124.8 ± 1.6</td>
<td>4.35 ± 0.14</td>
</tr>
<tr>
<td>CycD2&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>+</td>
<td>27.77 ± 0.49</td>
<td>151.1 ± 7.6</td>
<td>5.45 ± 0.29</td>
</tr>
<tr>
<td>CycD2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>-</td>
<td>23.46 ± 1.56</td>
<td>83.5 ± 22.4</td>
<td>4.40 ± 0.16</td>
</tr>
<tr>
<td>CycD2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>+</td>
<td>23.80 ± 0.69</td>
<td>114.4 ± 4.5</td>
<td>4.81 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± SEM. BW, body weight; HW, heart weight; HW:BW, heart weight: body weight. CycD2<sup>+/+</sup>-Sham, n=5; CycD2<sup>+/+</sup>-TAC, n=5; CycD2<sup>-/-</sup>-Sham, n=5; CycD2<sup>-/-</sup>-TAC, n=9.
Table S2. Cardiac and body weights for CRb<sup>+/+</sup> and CRb<sub>L/L</sub> mice after 1 week of pressure overload.

<table>
<thead>
<tr>
<th></th>
<th>TAC</th>
<th>BW (g)</th>
<th>HW (mg)</th>
<th>HW:BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRb&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>-</td>
<td>23.50 ± 1.80</td>
<td>110.4 ± 13.7</td>
<td>4.64 ± 0.23</td>
</tr>
<tr>
<td>CRb&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>+</td>
<td>22.99 ± 1.45</td>
<td>120.7 ± 9.9</td>
<td>5.25 ± 0.25</td>
</tr>
<tr>
<td>CRb&lt;sub&gt;L/L&lt;/sub&gt;</td>
<td>-</td>
<td>22.91 ± 1.61</td>
<td>108.0 ± 5.2</td>
<td>4.75 ± 0.20</td>
</tr>
<tr>
<td>CRb&lt;sub&gt;L/L&lt;/sub&gt;</td>
<td>+</td>
<td>22.78 ± 1.25</td>
<td>145.8 ± 10.8</td>
<td>6.40 ± 0.34</td>
</tr>
</tbody>
</table>

Values are means ± SEM. BW, body weight; HW, heart; weight HW:BW, heart weight: body weight. n=5 for all groups.
REFERENCES


Figure S1. Cyclin D2 is upregulated during hypertrophy. To clarify the expression of CycD2 in response to hypertrophic stimuli, we performed immunostaining of neonatal rat ventricular myocytes (NRVM) cultured in serum-free conditions for 72 hours and then exposed them to 100 mM phenylephrine (PE) treatment for 24 hours. A; a-b, CycD2 protein levels are increased in NRVMs after exposure to PE. CycD2 (green), phalloidin (red), DAPI (nuclear stain, blue). Scale bar = 50μm. B, Representative RPAs performed on total RNA (4μg) from ventricles of mice after TAC. mRNA for CycD2, and GAPDH was examined. (n=4 per time point; * P< 0.01 compared to baseline levels).