Essential roles for $G_1$ cyclin-dependent kinase activity in development of cardiomyocyte hypertrophy

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Essential roles for $G_1$ cyclin-dependent kinase activity in development of cardiomyocyte hypertrophy. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2036–H2040, 1998.—Although cardiomyocytes undergo terminal differentiation soon after birth, irreversibly withdrawing from the cell cycle, growth stimulation induces cell hypertrophy. Such growth stimulation is also responsible for the upregulation of $G_1$ cyclins and cyclin-dependent kinase (CDK) activity in proliferating cells. We sought to determine whether $G_1$ CDK activity is involved in the hypertrophy of rat neonatal cardiomyocytes in culture. We show that serum stimulation promoted the $G_1$ CDK activity without induction of DNA synthesis in cardiomyocytes. Furthermore, overexpression of CDK inhibitors p16$^{NK4a}$ and p21$^{CIP1/WAF1}$ by use of the adenovirus vector effectively prevented cell enlargement and depressed serum-induced protein synthesis and expression of skeletal $\alpha$-actin and atrial natriuretic factor, genetic markers of cardiac hypertrophy. These results suggest that the $G_1$ CDK activity promoted by serum stimulation is required for the induction of cardiomyocyte hypertrophy and provide novel evidence for understanding the regulation of cardiac hypertrophy by cell cycle regulators.

in proliferating cells, during the $G_1$ phase of the cell cycle, proliferative stimuli lead to the sequential activation of $G_1$ cyclins and associated cyclin-dependent kinases (CDKs), including D-type cyclins (D1, D2, and D3) complexed to cdk4 and cdk6 and cyclin E bound to cdk2 (21, 26). In addition to regulation by cyclin binding, CDK activity can also be regulated by interactions with the members of two families of CDK inhibitors: the p16$^{NK4a}$ family proteins, which specifically inhibit the activity of cdk4/cdk6, and the p21$^{CIP1/WAF1}$/p27$^{KIP1}$ proteins, which inhibit the activities of cdk2 and cdk4/cdk6 (15, 22). $G_1$ CDKs are thought to target various cellular substrates, such as the retinoblastoma (Rb) gene product (pRb) family, phosphorylation of which is required for cell cycle progression (21, 26).

Cardiomyocytes undergo terminal differentiation soon after birth, irreversibly withdrawing from the cell cycle. Because of their inability to proliferate, postnatal cardiomyocytes grow by hypertrophy (25, 27). In adults, cardiac hypertrophy occurs in response to various stresses, such as increased afterload and ischemia, or as a mechanism to compensate for heart dysfunction. Elucidation of the mechanisms underlying cardiac hypertrophy is an important issue in cardiology, because severe heart dysfunction and arrhythmias may occur as a result of hypertrophy. Cardiac hypertrophy, which is accompanied by the upregulation of protein synthesis and expression of fetal isoforms of sarcomeric proteins, such as skeletal $\alpha$-actin (SK-A) and $\beta$-myosin heavy chain, is thought to be mediated by autocrine/paracrine mechanisms involving various growth and neurohumoral factors (6, 19). Also, atrial natriuretic factor (ANF) mRNA is considered to be a marker of cardiac hypertrophy (2). Serum (11, 12), basic fibroblast growth factor (16), insulin-like growth factors (7), endothelins (8), and ANG II (1) have been reported to induce cardiomyocyte hypertrophy in vitro. These stimuli activate multiple second messenger systems and induce various immediate-early genes, such as c-fos and c-jun, in cardiomyocytes (3, 18, 23). In view of similar responses being also observed in response to mitogenic stimuli in various cell types, it appears likely that hypertrophic and mitogenic stimuli share certain intracellular responses. Furthermore, recent experiments have indicated that serum stimulation upregulates the activities of $G_1$ cyclins and associated CDKs in terminally differentiated cardiomyocytes, similar to the observation in proliferating cells (17). We thus investigated whether $G_1$ CDK activity is involved in the process of hypertrophy in cultured rat neonatal cardiomyocytes.

MATERIALS AND METHODS

Production of recombinant adenoviruses. The replicate-deficient adenoviruses were prepared as described previously (14, 24). Briefly, rat p21$^{CIP1/WAF1}$ (Axp21) or human p16$^{NK4}$ (Axp16) cDNA was inserted into the cassette-cosmid vector (pAdEx1w) containing E1- and E3-deleted adenovirus sequences with the CAG (chicken $\beta$-actin promoter + cytomegalovirus enhancer) promoter and rabbit $\beta$-globin poly(A) signal sequences. The recombinant viruses were obtained by in vitro recombination in 293 cells. After separation of cell debris by centrifugation, the supernatant-containing virus particles were stored at $-80^\circ$C until use. The titer of each viral stock was determined by plaque assay in 293 cells, and the titers consistently ranged between $10^9$ and $10^{11}$ plaque-forming units/ml. Adenovirus without coding sequences (Ax1w1) was used as a control.

Cell culture and infection. Cardiomyocytes from 1- or 2-day postnatal Sprague-Dawley rats were isolated, subjected to Percoll gradient centrifugation, and cultured in vitro, as described previously (20); we routinely obtained cultures in which $>$95% of the cells were cardiomyocytes, as assessed by immunostaining with the mouse monoclonal antisarcomeric actin antibody (Dakopatts). Neonatal rat cardiomyocytes in culture were incubated in MEM (Flow Laboratories) with 5%...
FCS (Flow Laboratories) for 24 h. On the 2nd day, cardiomyocytes were infected with recombinant adenoviruses (50 plaque-forming units/cell) and further incubated with MEM without FCS for 48 h. Infected cells were analyzed after treatment with 10% FCS at 37°C for various periods. Nonmyocytes were prepared as controls, as described previously (4).

Bromodeoxyuridine incorporation. Cardiomyocytes/nonmyocytes that entered the S phase in response to serum stimulation were counted after incubation with bromodeoxyuridine (BrdU, 10 µmol/l) in the presence or absence of 10% FCS for 48 h at 37°C. To distinguish myocytes from nonmyocytes, cultures were first labeled with antisarcomeric actin antibody and then with 5 µg/ml of mouse monoclonal anti-BrdU antibody (Chemicon International). The distribution of labeled nuclei was visualized using an alkaline phosphatase-conjugated secondary antibody in conjunction with an ABC kit (Vector). The BrdU-labeling index is expressed as the number of BrdU-positive nuclei per 100 cells, with the total number of BrdU-labeled cells being the product of the labeling index and the mean cell number.

Northern blot analysis. Northern blot analysis and preparation of the probes were performed as previously described (2, 8).

Assay of cdk4 kinase. The nuclear extract of cardiomyocytes was immunoprecipitated with 1 µg of cdk4 polyclonal antibody (C-22, Santa Cruz Biotechnology), then the immunocomplexes were tested for cdk4 kinase activity using the glutathione S-transferase (GST)-Rb fusion protein as the substrate by a method described previously (13). In a control experiment, 1 µg of rat IgG was used for immunoprecipitation.

Evaluation of cardiac hypertrophy. To measure cell surface area, cells were fixed with 10% buffered Formalin after incubation in the presence or absence of 10% FCS for 48 h, then labeled with sarcomeric actin antibody. The mean surface area of 50 sarcomeric actin-positive cells was calculated (8).

To determine the level of protein synthesis, the cardiomyocytes (2 × 10⁶ cells) were incubated in the presence or absence of 10% FCS for 24 h. At 4 h before the end of the incubation period, 1 µCi of [³H]leucine was added, and the level of protein synthesis was determined from the extent of [³H]leucine incorporation (7). Statistical evaluation of the results of BrdU incorporation, [³H]leucine incorporation, and mean cell surface area was performed using Scheffé’s one-way ANOVA.

RESULTS

Lack of DNA synthesis in cultured cardiomyocytes. First, we evaluated the entry of quiescent rat primary neonatal cardiomyocytes into the S phase of the cell cycle by determining the extent of BrdU incorporation. Rat cardiac fibroblasts (nonmyocytes) were used as positive controls. As summarized in Table 1, stimulation of cardiomyocytes with 10% serum led to no marked increase in the number of BrdU-positive cells (~2% above background); i.e., cell proliferation was, in effect, halted, whereas a 49% increase (P < 0.01) was noted among the corresponding control nonmyocytes. These results indicate that under our experimental conditions the addition of serum cannot stimulate cardiomyocyte DNA synthesis. Although serum stimulation had no effect on cardiomyocyte proliferation, as evidenced by the lack of DNA synthesis, it did lead to cardiomyocyte hypertrophy, causing a twofold increase in cell size, as determined by cell surface area measurements, upregulation of protein synthesis, and induction of SK-A transcription (see below).

Activation of G1 CDKs in serum-stimulated cardiomyocytes. In agreement with the results of recent experiments (17), we detected upregulation of the expression of the mRNAs of cyclin D1 as well as cyclin A, which accumulates around S phase commencement in serum-stimulated cardiomyocytes. Although we detected cyclin E mRNA expression in unstimulated cardiomyocytes, it was not upregulated by serum stimulation (Fig. 1). Consistent with the upregulation of cyclin D1 mRNA expression, serum-stimulated cdk4 kinase activity was also increased (Fig. 2), as determined by kinase assays in which the GST-Rb fusion protein was used as the substrate. The samples immunoprecipitated with IgG did not phosphorylate GST-Rb (data not shown).

G1 CDK activity is involved in cardiomyocyte hypertrophy. To determine whether endogenous D-type CDK activity is directly involved in cardiomyocyte hypertrophy, the cells were infected with a recombinant adenovirus A xp16, encoding a specific inhibitor of D-type CDK activity, p16, and A xp21, encoding a broad CDK inhibitor, p21. A xp16 was used as the control virus. Infection of both CDK inhibitor-expressing viruses, but not of the control A xp1 w1 virus, inhibited the increase in the level of DNA synthesis in serum-stimulated nonmyocytes (Table 1). In addition, after infection of cardiomyocytes with A xp21, cdk4 kinase activity was effectively inhibited (Fig. 2), confirming that the proteins encoded by these viruses inhibit such activity. From cell surface area measurements (Fig. 3), it was determined that, with the exception of the control A xp1 w1 virus (data not shown), serum-stimulated cardiomyocytes treated with A xp21 or A xp16 showed a substantial reduction in the extent of hypertrophy in comparison with those not possessing A xp21 or A xp16. Morphologically, the cells treated with A xp21 or A xp16 alone were similar to the control cells. The results of measurements of [³H]leucine incorporation, indicating the rate of protein synthesis, are summarized in Fig. 4. The degree of protein synthesis stimulated by 10% serum was inhibited by conjuction with A xp21 or A xp21 in a dose-dependent manner. On the other hand, serum and serum with A xp1 w1 had significantly different effects on unstimu-
lated control cardiomyocytes, indicating no increase in the level of protein synthesis in the inhibitor-infected serum-stimulated cardiomyocytes. Finally, we asked whether p21 and p16 can inhibit expression of markers of cardiac hypertrophy, SK-A and ANF. Each virus effectively inhibited serum-induced SK-A and ANF mRNA expression, whereas the control virus (Ax1w1) did not (Fig. 5). These results indicate that p21 and p16 suppress the development of cardiomyocyte hypertrophy.

**DISCUSSION**

Consistent with the recent report by Sadoshima and co-workers (17), we showed that D-type CDK activity, which plays a key role in cell proliferation in various cell types, is promoted during hypertrophic processes of serum-stimulated cardiomyocytes in culture. To investigate the role of endogenous D-type CDK activity in the development of cardiac hypertrophy, we inhibited
the activity by using the p21- and p16-expressing viruses and demonstrated that these viruses inhibited a variety of markers of cardiomyocyte hypertrophy. These observations, together with the fact that D-type CDKs are common targets of p21 and p16, suggest that the D-type CDK activity is a prerequisite for cardiomyocyte hypertrophy and provide direct evidence of a new role for G1 CDKs in terminally differentiated cells in addition to their known critical functions in proliferating cells. A number of experiments demonstrated that multiple second messenger systems, such as the Ras-mitogen-activated protein kinase and the Janus protein-signaling transduction and activator of transcription pathways, are also activated in response to mitogenic stimuli in various cell types and play critical roles in development of cardiac hypertrophy. A number of experiments demonstrated that multiple second messenger systems, such as the Ras-mitogen-activated protein kinase and the Janus protein-signaling transduction and activator of transcription pathways, are also activated in response to mitogenic stimuli in various cell types and play critical roles in development of cardiac hypertrophy. Our results indicate that D-type CDK activity also contributes to the upregulation of protein synthesis for cardiomyocyte hypertrophy.

We used serum stimulation as an inducer of cardiomyocyte hypertrophy, because serum is a commonly used cell cycle activator in a variety of experiments. One may be concerned that the inhibition of hypertrophy seen in these experiments may be secondary to the inhibition of proliferation of the nonmyocytes in the culture. Thus we have performed the same experiments using cardiomyocytes pretreated with cytosine arabinose to prevent the proliferation of nonmyocytes (data not shown) and obtained data that p16 and p21 effectively suppressed the hypertrophy. These data indicate that the inhibition of hypertrophy is not secondary to effects on nonmyocytes and that p21 and p16 directly inhibit hypertrophy of cardiomyocytes. Furthermore, in our preliminary study, hypertrophy of cardiomyocytes induced by several other factors, such as ANG II and endothelin-1, which are known to play an important role in cardiac hypertrophy but not to cause fibroblast proliferation potently, was also inhibited by Axp21 or Axp16 (T. Nozato, M. Tamamori, and H. Ito, unpublished observations).

The mechanisms underlying stimulation of hypertrophy by D-type CDK activity remain unclear. One simple explanation is that E2F activity is also involved in the induction of hypertrophy. However, it appears unlikely that E2F activity contributes to the upregulation of SK-A and other sarcomeric genes that are activated during the hypertrophy process, since E2F sites have not been found within the promoter sequences of these genes. In fact, overexpression of E2F1 causes the opposite effect, i.e., suppression of the SK-A promoter activity in cardiomyocytes (9). Moreover, adenovirus E1A oncoprotein also suppresses SK-A transcription in a manner dependent on E1A domains required for binding to the Rb family members (10). Taken together, although the possibility of the Rb family-E2F pathways contributing to protein synthesis remains to be determined, these observations suggest that, unlike the control of cell proliferation, the Rb family-E2F pathway appears not to be the main pathway involved in the hypertrophic process.

Alternatively, D-type cyclins/cdk4 may directly activate unknown factors that bridge intracellular cascades of cardiac hypertrophy. Recent evidence suggests that D-type CDKs phosphorylate substrates other than the Rb family members, such as DMP1, an myb-like transcription factor (5). This suggests the interesting possibility that G1 CDKs target substrate(s) other than that involved in the Rb family-E2F pathway for promoting hypertrophic responses in cardiomyocytes. Further studies to define the target substrates for D-type CDK activity in relation to cardiac hypertrophy would clarify the differences between hypertrophic and proliferative processes and provide important insights into the pathogenesis of cardiac hypertrophy.

Despite recent advances in the therapy of heart failure, it is likely that the incidence of heart failure will continue to increase. The limitation of pharmacological therapy for heart failure is, in part, due to the inability of the myocardium to reproduce once injured by severe ischemia or inflammation of the heart. We have offered several new insights into the understanding of the cell cycle in cardiomyocytes that may ult-
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