Inhibition of Retinoblastoma Protein Phosphorylation by Myogenesis-induced Changes in the Subunit Composition of the Cyclin-dependent Kinase 4 Complex

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Abstract
The retinoblastoma protein (Rb) is essential for the maintenance of the postmitotic state in terminally differentiated myocytes. Upon C2C12 myogenesis, the level of the cyclin-dependent kinase 4 (CDK4) protein does not change, but its Rb kinase activity is down-regulated markedly. Here, we show that the reduction in CDK4 activity results from (a) the irreversible induction and association of the p21 CDK inhibitor with the CDK4 complex and (b) a decline in overall D-type cyclin expression. Immunoprecipitation-coupled immunoblot analyses demonstrated that myocyte differentiation produces alterations in the subunit interactions within the CDK4 complex, including a diminished interaction with cyclin D1 and enhanced interactions with cyclin D3 and p21. The significance of the p21 interaction with CDK4 was indicated by the ability of anti-p21 antibodies to specifically immunodeplete a Rb kinase inhibitory activity that was bound to the CDK4 complex in myotubes. Furthermore, the restimulation of myotubes with serum did not lead to the re-activation of CDK4 or disrupt the CDK4-p21 interaction. Despite the increase in cyclin D3 expression during myogenesis, quantitative immunoblot analyses revealed that the combined levels of cyclin D1 and D3 declined during this process that CDK4 is expressed at much higher levels than either of these cyclin subunits in postmitotic myotubes. These results suggest that the myogenesis-induced up-regulation of p21 and down-regulation of the total D-type cyclin expression contribute to the inhibition of the CDK4 Rb kinase activity, leading to conditions that favor the accumulation of the hypophosphorylated Rb and growth arrest upon terminal differentiation.

Introduction
During skeletal muscle terminal differentiation, the postmitotic state is established prior to the expression of the contractile phenotype (1). Rb appears to be an important regulator of the cell cycle withdrawal during myogenesis. Myogenic differentiation is associated with an increase in the steady-state level of Rb mRNA (2) and the accumulation of the hypophosphorylated (active) form of the Rb protein (3, 4). Myocytes derived from a Rb−/− mouse can differentiate into multinucleated myotubes, but unlike wild-type cells, they remain capable of cell cycle reentry upon mitogen re-stimulation (5). In undifferentiated cells, Rb is hypophosphorylated in the quiescent state and in the early G1 phase of the cell cycle, where it blocks cell cycle progression because of the inactivation of the E2F transcription factor (6). The phosphorylation of Rb is initiated during mid- to late G1, and it becomes fully phosphorylated prior to G1-S transition. Subsequently, it is dephosphorylated during mitosis. CDK4 and CDK6 are thought to be the predominant Rb kinases in vivo (7). The cyclin A-CDK2 and cyclin E-CDK2 complexes can also phosphorylate Rb in vitro and promote Rb hyperphosphorylation in transfected cells (8). CDK2 activation by cyclin E occurs following the initial phosphorylation of Rb, and the activation of CDK2 by cyclin A occurs at even later time points. In contrast, activation of cyclin D-CDK complexes occur in the mid-G1 phase (9, 10), the same phase of the cell cycle in which myoblasts commit to terminal differentiation (11–13).

Although Rb becomes dephosphorylated during myocyte differentiation, the predominant Rb kinases, CDK4 and CDK6, are constitutively expressed (14–16). It has also been reported that cyclin D3 is up-regulated 20-fold during L6 cell myogenic differentiation, but no cyclin D3-associated Rb kinase activity could be detected in lysates prepared from the differentiated myotube cultures (15). Thus, the mechanisms leading to Rb dephosphorylation upon myogenesis are not obvious, and they likely involve complex changes in the subunit compositions of the CDK4 and CDK6 complexes. Recently, cyclin kinase inhibitors were identified that bind to the CDK complexes and function as negative regulators of cell growth. The general CDK inhibitor p21 (also referred to as sdi1, mda-6 CAP20, CIP1, and WAF1) is induced markedly upon skeletal muscle differentiation (1, 16–18). p21 functions to inhibit CDK2 activity in postmitotic myotubes (18), which presumably contributes to the establishment of the postmitotic state, and its expression also serves to promote myocyte survival in low mitogen differentiation medium (19). Here we examined the regulation of the CDK4 kinase

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3 The abbreviations used are: Rb, retinoblastoma protein; CDK, cyclin-dependent kinase; GST, glutathione S-transferase.
activity during myogenesis. We found that despite the constitutive expression of the CDK4 protein, its kinase activity was reduced markedly during C2C12 myocyte differentiation. Our data suggest that myogenesis-induced changes in the subunit composition of the CDK4 complex contribute to this inhibition of CDK4 kinase activity. These changes result from the up-regulation of the p21 CDK inhibitor and from a decrease in the combined levels of the D-type cyclins during terminal differentiation.

Results

Changes in Rb Phosphorylation and CDK4 Rb Kinase Activity upon Myocyte Differentiation. Immunoblotting analyses were performed to monitor changes in Rb phosphorylation during C2C12 differentiation under the cell culture conditions employed for these studies (Fig. 1A). As reported previously (3, 4), both the rapidly migrating hyperphosphorylated forms and the slowly migrating hyperphosphorylated forms of Rb are present in replicating myoblast lysates. However, the hypophosphorylated form predominates in lysates prepared from myotube cultures and in lysates prepared from postmitotic myotube cultures that were restimulated with the mitogen-rich growth media. We next examined the levels of D-type cyclins and their associated kinases, CDK4 and CDK6, which are the predominant Rb kinases in vivo. CDK4 and CDK6 protein levels remained constant during myocyte differentiation (Fig. 1B). Cyclin D1 protein levels decreased, whereas cyclin D3 protein levels increased 3–4-fold. Cyclin D2 was not detectable in the C2C12 myotube or myoblast lysates, but it has been reported that cyclin D2 protein levels decline during L6 myocyte differentiation (15). The changes in D1 and D3 cyclin levels are consistent with previously reported changes in their mRNA and protein levels during myocyte differentiation (14–16). Serum restimulation of differentiated myotubes had no effects on the levels of CDK4 or CDK6 but appeared to partially reverse the myogenesis-induced changes in cyclin D1 and cyclin D3 levels. Because the myotube cultures used for these experiments comprised greater than 90% of the nuclei in multinucleated cells, these data suggest that the myogenesis-induced changes in the expression cyclins D1 and D3 are reversible, and they are consistent with the notion that the mitogenic signal transduction pathway is intact in postmitotic myotubes (18, 20, 21). Because the CDK6 band was of low intensity in the immunoblot (Fig. 1B) and CDK6 immunoprecipitates displayed a very low Rb kinase activity under our assay conditions (not shown), we focused on CDK4 and its associated proteins in subsequent experiments.

To investigate the mechanisms that may contribute to the observed changes in Rb phosphorylation during C2C12 myogenesis, the in vitro Rb kinase activities of CDK4, cyclin D1, and cyclin D3 immunoprecipitates were determined using a GST-Rb fusion protein as substrate. Relatively high levels of Rb kinase activities were detected in all immunoprecipitates from myoblast cell lysates, whereas these Rb kinase activities were considerably lower in cell lysates prepared from myotubes and myotubes restimulated with serum (Fig. 2). Similarly, a reduction in cyclin D3 and CDK4-associated Rb kinase activity during L6 myocyte differentiation has also been observed (15). This reduction of in vitro Rb kinase activity is consistent with the observed changes in Rb phosphorylation states during myo-
Fig. 2. Down-regulation of Rb kinase activity during myogenic differentiation. Anti-CDK4, cyclin D1, and cyclin D3 immunoprecipitates from lysates of myoblasts (Mb; □), myotubes (Mt; ▼), or myotubes restimulated with serum (SSMt; ▼) were collected on protein A plus G beads. The Rb kinase activities of these protein complexes were measured using a GST-Rb fusion protein as substrate (see "Materials and Methods"). Kinase assays were performed simultaneously, and the intensities of the autoradiography bands can be compared directly. The average values from two independent experiments are shown in the histogram.

Relative Levels of CDK4, Cyclin D1, Cyclin D3, and p21. Because of differences in antibody efficiency, direct comparisons between cell cycle protein levels cannot be made based solely on the data presented in Fig. 1B. However, relative levels of cell cycle proteins could be estimated by comparing immunoblot band intensities after they were calibrated relative to the band intensities of known amounts of recombinant proteins that were simultaneously processed during the immunoblot procedure (Fig. 3). On the basis of these analyses, the calculated levels of CDK4 and p21 in myotube lysates are 20 and 31 fmol/μg of cell lysate, respectively. In contrast, the levels of cyclin D1 and cyclin D3 are significantly lower in myotube lysates: 1 and 3 fmol/μg of cell lysate, respectively. These estimates of cyclin and CDK4 levels in myotubes indicate that the majority of CDK4 is not complexed with cyclins in myotubes, and that the total amount of p21 greatly exceeds the levels of the active CDK4/cyclin complexes in these cells. In contrast, the levels of cyclin D1 in myoblast lysates are 10 fmol/μg of cell lysate (not shown). Thus, the large decline in cyclin D1 levels is only partially compensated by the increase in cyclin D3 expression, and this situation is likely to contribute to the low levels of CDK4 activity that are observed in myotube lysates (Fig. 2).

Fig. 3. Estimates of p21, CDK4, cyclin D1, and cyclin D3 levels in myotube lysates. Immunoblot analyses were performed simultaneously with specific antibodies on 40 μg of myotube lysate and on the indicated quantities of recombinant GST fusion protein. Band intensities were determined by scanning densitometry and plotted. The quantity of the specific protein in the myotube lysate was estimated from its band intensity (C) relative to the band intensities of known quantities of recombinant protein (●).

Myogenesis-induced Changes in CDK4-associated Proteins. CDK4-associated proteins were analyzed by co-immunoprecipitation using lysates prepared from [35S]methionine-labeled cultures of myoblasts and myotubes to further investigate the mechanism of the CDK4 Rb kinase inhibition (Fig. 4). Immunoprecipitation of C2C12 myoblast lysates with anti-CDK4 antibodies revealed predominant bands that appear to correspond to p32CDK4 and p32cyclinD2, which comigrate in SDS-PAGE, and p35cyclinD1. Several lower-intensity bands were also detected that had apparent molecular weights of M, 16,000, 21,000, 26,000, and 27,000 (Fig. 4, Lane 1). The immunoprecipitates of the corresponding myotube lysates displayed a reduction in the relative intensity of the putative p35cyclinD1 band and an increase in the intensities of the M, 21,000, 26,000, and 27,000 bands (Fig. 4, Lane 2). The specificity of the immunoprecipitated bands was demonstrated by their competition with an excess of immunogenic CDK4.
peptide (Fig. 4, Lanes 3 and 4). The myogenesis-inducible M, 21,000 and 27,000 proteins in the CDK4 immunoprecipitates had electrophoretic mobilities similar to that of the cyclin kinase inhibitors p21 and p27 (Kip1). Thus, immunoprecipitation analyses were also performed with anti-p21 (Fig. 4, Lanes 5–8) and anti-p27 (Fig. 4, Lanes 9–12) antibodies. These analyses revealed similar sets of 35S-labeled bands corresponding to p21 or p27 as well as their associated proteins, including p32CDK4, p32cyclinD3 and p35cyclinD1.

A series of immunoprecipitation-coupled immunoblotting experiments were performed to confirm the identities of CDK4-associated proteins and more accurately assess the myogenesis-induced changes to their relative levels (Fig. 5). Consistent with data from the simple immunoblot analyses (Fig. 1), the immunoprecipitation-coupled immunoblots revealed no change in the total level of CDK4, a decrease in the level of cyclin D1, and increases in the level of cyclin D3. This analysis also revealed an increase in p21 expression. Analysis of CDK4 immunoprecipitates demonstrated enhanced associations of CDK4 with cyclin D3 (8-fold) and with p21 (10-fold) during myogenesis, whereas the level of CDK4-associated cyclin D1 decreased by a factor of 4 (Fig. 5; compare Lanes 6 and 7). These results were confirmed by using anti-CDK4 antibodies to immunoblot the anti-p21, anti-cyclin D1, and anti-cyclin D3 immunoprecipitates (Fig. 5, top row). Finally, these analyses did not detect cyclin D1 in cyclin D3 immunoprecipitates, and vice versa.

The immunoprecipitation-coupled immunoblot strategy was also employed to examine the interaction between p27 and CDK4 (Fig. 6). p27 was detected in CDK4 immunoprecipitates and CDK4 was detected in p27 immunoprecipitates. Upon myogenesis, the levels of p27 increased approximately 2-fold in the CDK4 immuno-precipitates.

The induced expression of p21 in myoblasts is not reversed by serum restimulation (17, 18), which is in marked contrast to the expression patterns of the D-type cyclins (Fig. 1). To evaluate whether the myogenesis-induced associations of CDK4 with p21 and p27 are a permanent feature of myocyte differentiation, myoblast cultures were restimulated with high mitogen growth media, and lysates from these cultures were subjected to immunoprecipitation-coupled immunoblot analysis. As shown in Fig. 7, the levels of CDK4-associated p21 and p27 in serum-restimulated myoblasts remained as high as that in myoblasts maintained in differentiation media. These data suggest that the sustained association between CDK4 and p21 or p27 is likely to contribute to the repression of CDK4 Rb kinase activity in myoblasts after serum stimulation, despite the reinduction of cyclin D1 expression.

**p21-mediated Inhibition of the CDK4 Rb Kinase Activity.** Cell lysate mixing experiments were performed to test for a myogenesis-induced CDK4 inhibitory activity. Some of the CDK inhibitors are stable to heat treatment, and lysates boiled to inactivate the kinases are useful in mixing experiments to assay for these inhibitory activities (18, 22–25). For these experiments, active CDK4 complex was immunoprecipitated from myoblast lysates and incubated with or without heat-treated cell lysates for 30 min at room temperature prior to the Rb kinase assays (Fig. 8A). The inclusion of heat-treated lysates from myoblast and serum-restimulated myoblast cultures in the Rb kinase reaction markedly reduced the activity of the CDK4 complex, but heat-treated myoblast lysates had little or no effect on CDK4 kinase activity.
Fig. 5. Immunoprecipitation coupled with immunoblotting analyses of CDK4-associated proteins. Cell lysates prepared from myoblasts (Mb) and myotubes (Mt) were immunoprecipitated with antibodies to the unrelated protein Gax (control, Lanes 1 and 2), anti-p21 (Lanes 3-5), anti-CDK4 (Lanes 6 and 7), anti-cyclin D1 (Lanes 8 and 9), and anti-cyclin D3 (Lanes 10 and 11). The immunoprecipitated protein complexes were separated on SDS-PAGE gels and subjected to immunoblot analyses with CDK4, cyclin D1, cyclin D3, and p21 antibodies (Immunoblot). In Lane 5, the anti-p21 was preincubated with its immunogenic peptide prior to immunoprecipitation. Anti-p21 immunoblot analysis of cyclin D1 and cyclin D3 immunoprecipitates is not presented, because the immunoglobulin proteins migrate at the same position in this assay.

Fig. 6. CDK4 and p27 interactions in C2C12 myocytes. Anti-p27 immunoprecipitates from myoblast (Mb) and myotube (Mt) lysates were subjected to immunoblot analysis with p27 and CDK4 antibodies. Anti-CDK4 immunoprecipitates and immunoprecipitates with a control antibody were also probed with p27 antibody.

To determine whether p21 contributes to the heat-stable CDK4 inhibitory activity that is induced upon myogenesis, heat-treated lysates were first immunodepleted with antibodies raised against peptides specific to p21 and then tested for their CDK4 inhibitory activity (Fig. 8B). The treatment of the myotube lysates with anti-p21 antibodies either partially or completely depleted the CDK4 inhibitory activity that was present in the myotube lysates. The specificity of the p21 immunodepletion was demonstrated by the inclusion of excess p21 immunogenic peptide, which prevented the removal of the CDK4 inhibitory activity. Furthermore, the inclusion of the p21 immunogenic peptide alone had no effect on the CDK4 kinase activity, indicating that it does not interfere with the kinase assay. These data indicate that p21 is a potential inhibitor of the CDK4 activity in myotube lysates.

The analyses represented in Fig. 8, A and B, demonstrate that the p21 inhibitory activity increases upon myogenesis; however, these analyses cannot discern between either the possibility that the kinase inhibitory activity is actually bound to CDK4 in myotubes or the possibility that this activity is released from latent, non-CDK4 pools by the heat treatment. Thus, to test whether the myogenesis-induced Rb kinase inhibitory activity is bound to CDK4 in myotubes, the CDK4 protein complexes were immunoprecipitated from myoblast or myotube lysates, boiled in lysis buffer, and then analyzed for their ability to inhibit CDK4 activity. As shown in Fig. 8C, a heat-stable inhibitory activity was detected in CDK4 immunoprecipitates prepared from myotube lysates, but little if any was detected in myoblast lysates. Detection of this inhibitory activity in myotubes was abolished by preincubation with the CDK4 immunogenic peptide prior to immunoprecipitation. This CDK4-bound inhibitory activity was also removed by immunodepleting the myotube lysate with anti-p21 antibody prior to the CDK4 immunoprecipitation step.
CDK4 Regulation during Myogenesis

These data demonstrate that p21 is a significant component of the Rb kinase inhibitory activity that is bound to the CDK4 complex in myotubes.

Discussion

During myogenesis, the expression of CDK4, a major Rb kinase, does not change at the levels of mRNA and protein (Fig. 1; Refs. 14–16). However, the Rb kinase activities of CDK4, cyclin D1, or cyclin D3 immunoprecipitates are reduced significantly. Here, we investigated whether this reduction in Rb kinase activity could result from the differential interactions of CDK4 with CDK inhibitor and cyclin regulatory subunits upon myocyte differentiation.

The reduction in the CDK4 Rb kinase activity upon myogenesis correlated with changes in the subunit composition of CDK4 complex. Immunoprecipitation analyses of 35S-labeled myocyte lysates revealed four low molecular weight proteins, with apparent molecular weights of M, 16,000, 21,000, 26,000, and 27,000, which were specifically associated with the CDK4 complex (Fig. 4). Of these, the M, 21,000, 26,000, and 27,000 protein bands were of greater intensity in the myotube lysates compared with the myoblast lysates. Some cyclin kinase inhibitors are reported to have molecular weights in this range, and immunoprecipitation-coupled immunoblot experiments confirmed that p21 and p27 were associated with CDK4 in the myocyte lysates (Figs. 5 and 6). There was a small but reproducible increase in the interaction between p27 and CDK4 during myogenesis (approximately 2-fold), but the association between CDK4 and p21 increased more substantially (10-fold). In proliferating myoblasts, the low level of p21 (or p27) interaction with CDK4 may function to promote the association between the cyclins and CDK4, leading to the activation of the Rb kinase activity.

A similar role for p21 has been described previously for the CDK2 complex (26). However, the large up-regulation of p21 during myogenesis will increase the stoichiometry of the p21 subunit within the CDK4 complex, leading to its inactivation. This hypothesis is supported by the demonstration that anti-p21 antibodies can largely immunodeplete the heat-stable CDK4 and CDK2 inhibitory activities that are present in myotube, but not myoblast, cell lysates (Fig. 8 A and B; Ref. 18). Furthermore, heat-inactivated CDK4 immunoprecipitates from myotubes possess a cyclin kinase inhibitory activity that can be immunodepleted by anti-p21 antibodies (Fig. 8 C). Collectively, these data support the hypothesis that the induction of p21 functions to inhibit the Rb kinase activity of (SC-397P) alone has no significant effect on CDK4 kinase activity. C, p21 is a component of CDK4-bound kinase inhibitory activity in myotubes. CDK4-associated proteins were immunoprecipitated from either myoblast (Mb) or myotube (Mt) lysates. These CDK4 immunoprecipitates were boiled in lysis buffer, and the supernatants were added to active CDK4 complex to test their ability to inhibit the Rb kinase activity. As a control, CDK4 immunogenic peptide was incubated with the CDK4 antibody prior to CDK4 immunoprecipitation. To measure the contribution of p21, myotube lysates were preincubated by immunoprecipitation with anti-p21 antibody before performing CDK4 immunoprecipitation. These experiments were repeated twice with different preparation of cell lysates, and similar results were obtained.
CDK4 upon myogenesis, which can lead to the accumulation of hypophosphorylated Rb in myotubes.

The experiments described herein also demonstrate that cyclin D1 is a predominant CDK4-associated cyclin in myoblast lysates, but the level of the cyclin D1-CDK4 complex declines upon myogenesis. Although the levels of cyclin D3 and cyclin D3/CDK4 complex increase during myogenesis, the total level of cyclin D3 in the myotube is not sufficient to form complexes with the majority of CDK4 molecules, and this situation is likely also to contribute to the decrease in CDK4 activity. The immunoprecipitation-coupled immunoblot analysis also did not detect cyclin D1 in cyclin D3 immunoprecipitates nor cyclin D3 in cyclin D1 immunoprecipitates, consistent with the notion that the CDK4 interactions with cyclin D1 and cyclin D3 are mutually exclusive. The cyclin D3-CDK4 complex can phosphorylate Rb 

in vitro (9), and thus, the decline in Rb kinase activity of the cyclin D3 immunoprecipitate upon myogenesis is likely due to the enhanced association of p21 with these complexes. It is paradoxical that cyclin D3 is induced upon myogenesis to become associated with an inactive CDK4 complex, and the significance of cyclin D3 induction during differentiation is not understood at present. One possibility is that cyclin D3, in association with another cyclin kinase partner, may function to phosphorylate myotube-specific proteins. Support for this speculation includes the finding that neurofilament proteins and tau are phosphorylated by CDK5 in postmitotic neurons, demonstrating that this class of kinases can also have differentiation-specific functions (27, 28). It has also been proposed that cyclin D3 functions to sequester p107 in myotubes, which serves to enhance the interaction between E2F and p130 (29).

The data presented here also demonstrate that the myogenesis-induced down-regulation of cyclin D1 and up-regulation of cyclin D3 are reversible in that the levels of these cyclins can be restored partially to their myoblast levels by restimulating cultures of postmitotic myotubes with serum. The reversibility of cyclin D1 and cdc2 mRNA (20) and of cyclin A and CKD2 protein (18) has also been noted in cultures of postmitotic myotubes. In contrast, the up-regulation of p21 during myogenesis is not reversed by serum restimulation (17, 18), and p21 remains bound to the CDK4 complex under these conditions (Fig. 7). Thus, the sustained interaction of p21 with CDK4 may be an important feature that contributes to the irreversibility of cell cycle withdrawal that is exhibited by skeletal muscle cells. Of course, this does not rule out the possibility of other regulatory mechanisms that may also contribute to irreversible CDK4 inactivation and cell cycle arrest. For example, tyrosine phosphorylation of CDK4 has been shown to be required for the G1 arrest in response to UV irradiation (30), but a potential role for CKD phosphorylation upon myogenesis has yet to be explored.

In summary, the dephosphorylation of Rb is a critical event in myocyte terminal differentiation, yet the protein levels of the RB kinase do not change upon myogenesis. Here, we demonstrated a marked reduction in the RB kinase activity of CDK4 that correlates with changes in the proteins that are associated with the CDK4 complex in cell lysates. The decline in CDK4 activity appears to result from a decline in the combined levels of the D-type cyclins and from an irreversible increase in the stoichiometry of the p21 subunit. The decrease in CDK4 activity may be a mechanism that leads to a reduction in RB phosphorylation, leading to cell cycle withdrawal upon skeletal muscle differentiation.

Materials and Methods

Cell Culture. Mouse myoblast C2C12 cells were cultured in DMEM supplemented with 20% fetal bovine serum (growth medium). Myogenic differentiation was initiated by shifting subconfluent culture of C2C12 cells to differentiation medium (DMEM and 2% heat-inactivated horse serum). To prepare myotube cultures, myoblasts were cultured in differentiation medium for 2 days, then exposed to 10 μM cytosine β-d-arabinofuranoside for 48 h to eliminate undifferentiated myoblasts, and then switched back to differentiation medium for 2 more days. Treated under these conditions, the resulting myotube cultures typically displayed >90% of the nuclei to be present in multinucleated cells by visual examination. Serum restimulation of myotubes was performed by transferring myotube cultures to growth medium for 24 h.

Antibodies. Anti-cyclin D1 and anti-cyclin D3 monoclonal antibodies, anti-CDK4 COOH-terminus polyclonal antibody and corresponding immunogenic peptide, anti-p21 and anti-p27 (Kip1) COOH-terminus antibodies, and corresponding immunogenic peptides were all from Santa Cruz Biotechnology. Monoclonal anti-PCNA antibody was also from Santa Cruz Biotechnology. Anti-CDK4 antibody and corresponding immunogenic peptide were a gift from M. Meyerson (Massachusetts General Hospital Cancer Center, Boston, MA) (10). Anti-Rb monoclonal antibody was from PharMingen.

Immunoblotting. Cells were lysed in NP40 lysis buffer [0.5% NP40, 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin and aprotonin], and insoluble materials were cleared off by centrifugation at 12,000×g for 10 min. Cell lysates (40 μg) were separated on denaturing acrylamide gel and transferred to Immobilon-P membrane (Millipore). The membranes were blocked in 5% milk in PBS with 0.2% Tween 20 and incubated sequentially with the primary and secondary antibodies. Dilution of the primary antibodies was 1:100, as suggested by the manufacturer. The proteins were visualized using the Amersham enhanced chemiluminescence reagent.

To determine the relative levels of D-type cyclins, CDK4, and p21 in myotube lysates, we devised a semiquantitative immunoblot assay. In these experiments, a series dilution of recombinant GST fusions of CDK4, cyclin D1, cyclin D3, and p21 (Santa Cruz Biotechnology, Inc.), ranging from 1 to 100 ng, and 40 μg of myotube lysate were electrophoresed on the same gel and subjected to immunoblotting. Estimates of protein levels in cell lysates were obtained by comparing the immunoblot band intensity from cell lysates with that of known amounts of recombinant protein that were loaded onto adjacent lanes in the denaturing acrylamide gel. Band intensities were quantified on an Eagle-Eye densitometer (Stratagene). To avoid possible variation of results due to antibody availability, the experiments were repeated several times with different dilutions of antibodies (1–4 μg/ml), and similar results were obtained. Levels of the cell cycle proteins, expressed as fmol/μg of myotube lysate, were calculated using the molecular weights of GST fusion proteins.

Metabolic Labeling and Immunoprecipitation. Cells were labeled by incubating in DMEM, without methionine and cysteine, supplemented with dialyzed fetal bovine serum and 100 μCi/ml each of [35S]methionine and [35S]cysteine for 4 h at 37°C. Cell lysates were prepared as described above. The cell lysates were precleared once by incubation with nonspecific serum and protein A beads (Santa Cruz Biotechnology, Inc.). For immunoprecipitation, cleared lysates were incubated with 1 μg of the specific antibodies and protein A beads for 3 h at 4°C. Protein complexes collected on the beads were washed five times with lysis buffer and separated on 12% SDS-PAGE gel. The [35S]-labeled protein bands were visualized by fluorography. For peptide competition, the antibodies were first incubated with 2 μg of corresponding immunogenic peptides for 15 min at room temperature before performing the immunoprecipitation. For immunoprecipitation-coupled immunoblotting, immunoprecipitated protein complexes from 500 μg of cell lysates were eluted from the protein A beads by boiling in 1 × SDS sampling buffer (as in Ref. 3) and subjected to immunoblotting analysis.

In vitro RB Kinase and CDK4 Inhibition Assay. In vitro RB kinase assay was performed as described (9) by using as substrate a protein
covering the COOH terminus (605–921) of mouse Rb protein that was fused to GST (GST-Rb; Ref. 3). Briefly, cyclin kinase complexes were immunoprecipitated from cell lysates (200 μg) with the specific antibodies and collected on protein A beads. The beads were washed twice with kinase buffer [50 mM Tris (pH 8.0), 10 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin and aproti- nin] and incubated with 2 μg of GST-Rb fusion protein and 4 μCi [γ-32P]ATP in 50 μl of kinase buffer at 4°C. Reactions were terminated by the addition of 20 μl of 4 × SDS sample buffer and boiled for 5 min. Samples were separated by electrophoresis on polyacrylamide gel, and the phosphorylated proteins were visualized by autoradiography of the dried gels. For quantitative measurement of the CDK4 kinase activities, the intensity of each band on autoradiography films were scanned with an Eagle-Eye densitometer (Stratagene) and expressed in the histogram as relative kinase activity to that of CDK4 complex from myoblast lysates. The CDK2-associated histone H1 kinase activity was measured as described (18).

The GST-Rb fusion protein was purified from Escherichia coli extracts by batch chromatography using glutathione-Sepharose 4B beads (Phar- macia). GST-Rb was eluted from the beads by incubation in kinase buffer with 5 mM reduced glutathione at 4°C. Eluted proteins were visualized by staining with Coomassie blue following electrophoresis on a denaturing polyacrylamide gel. The concentration of GST-Rb fusion proteins was estimated by comparing the density of GST-Rb band to that of protein standards of known mass.

For CDK4 inhibition assays, cell lysates were boiled for 5 min to release the bound CDK4 inhibitors and cleared by brief centrifugation in an Eppendorf tube. These heat-treated cell lysates were then incubated with active CDK4 complexes immunoprecipitated from myoblast lysates for 30 min at room temperature. The protein complexes were washed with kinase buffer, and Rb kinase assay was performed as described above. For analysis of CDK inhibitory activity in CDK4 immunoprecipitates, cell lysates were incubated with anti-CDK4 antibody and protein A beads for 2 h at 4°C and washed three times with lysis buffer. The beads were boiled for 5 min in 0.5 ml of lysis buffer and centrifuged. The supernatants were collected and mixed with active CDK4 proteins complexes to assay their inhibitory activities as described above. For peptide competition, immunogenic CDK4 peptide (1 μg) was included in the CDK4 immunoprecipitation reaction. For p21 depletion, cell lysates were first immunoprecipitated with anti-p21 antibody prior to CDK4 immunoprecipitation.

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References