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Induction of p18^{INK4c} and Its Predominant Association with CDK4 and CDK6 during Myogenic Differentiation

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Terminal cell differentiation involves permanent withdrawal from the cell division cycle. The inhibitors of cyclin-dependent kinases (CDKs) are potential molecules functioning to couple cell cycle arrest and cell differentiation. In murine C2C12 myoblast cells, G1 CDK enzymes (CDK2, CDK4, and CDK6) associate with four CDK inhibitors: p18^{INK4c}, p19^{INK4d}, p21, and p27^{Kip1}. During induced myogenesis, p21 and its associated CDK proteins underwent an initial increase followed by a decrease as cells became terminally differentiated. The level of p27 protein gradually increased, but the amount of total associated CDK proteins remained unchanged. p19 protein decreased gradually during differentiation, as did its associated CDK4 protein. In contrast, p18 protein increased 50-fold, from negligible levels in proliferating myoblasts to clearly detectable levels within 8–12 h of myogenic induction. This initial rise was followed by a precipitous increase between 12 and 24 h postinduction, with p18 protein finally accumulating to its highest level in terminally differentiated cells. Induction of p18 correlated with increased and sequential complex formation—first increasing association with CDK6 and then with CDK4 over the course of myogenic differentiation. All of the CDK6 and half of the CDK4 were complexed with p18 in terminally differentiated C2C12 cells as well as in adult mouse muscle tissue. Finally, kinase activity of CDK2 and CDK4 decreases as C2C12 cells differentiate, whereas the CDK6 kinase activity is low in both proliferating myoblasts and differentiated myotubes. Our results indicate that p18 may play a critical role in causing and/or maintaining permanent cell cycle arrest associated with mature muscle formation.

INTRODUCTION

Differentiation of many cell types involves the induction of lineage-specific gene expression and the withdrawal from the cell division cycle. Work over the past several years on myogenic differentiation using both animal model systems and cultured myoblasts has elucidated a hierarchical regulatory mechanism that controls skeletal myogenesis (Weintraub, 1993; Lassar *et al.*, 1994; Olson and Klein, 1994). After stimulation by an appropriate environmental signal, such as serum deprivation, members of the MyoD family of basic Helix–Loop–Helix (bHLH) transcription factors present in undifferentiated myoblasts initiate a cascade of events leading to the activation of other transcription factors necessary for the expression of muscle-specific genes. Coupled with this induction of muscle-specific gene expression is the permanent withdrawal of proliferating myoblasts from the cell cycle to become terminally differentiated myotubes. In contrast to the progress in understanding the mechanism that regulates the expression of muscle-specific genes, little is known on how the cell cycle arrest is initiated

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during muscle differentiation and maintained in terminal-differentiated myotubes.

The primary control of the eukaryotic cell cycle is provided by the sequential formation, activation, and subsequent inactivation of a series of structurally related Ser/Thr protein kinases, cyclin-dependent kinases (CDKs; recently reviewed, Hunter and Pines, 1994; Sherr, 1994). The enzymatic activity of a CDK is regulated at three different levels: cyclin binding and activation, subunit phosphorylation, and inhibition by a CDK inhibitor. In mammalian cells, there exists at least two distinct families of CDK inhibitors, represented by the two prototype CDK inhibitors *p21* and *p16* (Sherr and Roberts, 1995). *p21* (also known variously as *CIP1*, *WAF1*, *SDI1*, CAP20, *PIC1*, and *CDKN1*) was first identified in normal human fibroblasts as a component of quaternary cyclin D–CDK complexes that also contain proliferating cell nuclear antigen (PCNA; Xiong *et al.*, 1992). It is a potent inhibitor of most, if not all, cyclin–CDK enzymes (Gu *et al.*, 1993b; Harper *et al.*, 1993; Xiong *et al.*, 1993a). The *p21* family currently contains two additional related CDK inhibitor genes, $p27^{Kip1}$ (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994) and $p57^{Kip2}$ (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). The steady-state level of the *p21* mRNA is elevated during G1 cell cycle arrest by a wide range of cell growth-regulatory signals, including DNA damage, cellular senescence, negative cytokines, and cell differentiation (reviewed, Sherr and Roberts, 1995). Several transcription factors have been found to activate *p21* gene expression, including tumor suppressor p53 (El-Deiry *et al.*, 1993), vitamin D3 receptor (VDR; Liu *et al.*, 1996), signal transducers and activators of transcription (STAT; Chin *et al.*, 1996), and CCAAT/enhancer-binding protein α (C/EBPα; Timchenko *et al.*, 1996).

p16 represents the other family of CDK inhibitors that currently includes four isolated genes: *p15* (also known as *MTS2*, *INK4b*, and *p14*; Guan *et al.*, 1994; Hannon and Beach, 1994; Kamb *et al.*, 1994), *p16* (*INK4a* and *MTS1*; Serrano *et al.*, 1993), *p18* (*INK4c*; Guan *et al.*, 1994; Hirai *et al.*, 1995), and *p19* (*INK4d*; Chan *et al.*, 1995; Hirai *et al.*, 1995; Okuda *et al.*, 1995; Guan *et al.*, 1996). Members of the *p16* gene family are related in sequence and evolution, all encoding proteins composed of four repeated ankyrin motifs and containing an intron interrupting the coding sequence at the same position. Unlike p21/p27/p57, which bind to and inhibit the activity of a wide spectrum of CDK enzymes, the p16-family inhibitors specifically interact with two closely related CDK proteins, CDK4 and CDK6. Their substrate preference and the timing of the onset of their kinase activity strongly implicate both cyclin D–CDK4 and cyclin D–CDK6 as physiological kinases for the retinoblastoma susceptibility gene product pRB, the growth-suppressing activities of which are known to be down-regulated by cell cycle-dependent phosphorylation (recently reviewed, Sherr, 1994). The p16-family inhibitors suppress cell growth in a pRb-dependent manner (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Sherr and Roberts, 1995), suggesting a potential mechanism by which members of the p16 family inhibit cell growth: inhibiting the activity of CDK6 and CDK4 kinases, thereby preventing the phosphorylation of pRb and keeping pRb in its active growth-suppressing state.

The ability of CDK inhibitors to arrest cell growth and the induction of their expression in response to cell growth signals presents them as a group of ideal molecules that may link the cell cycle machinery to a variety of cell growth-regulatory pathways, including cell differentiation. Induction of a CDK inhibitor from either family during cell differentiation would be sufficient to arrest the cell cycle. Maintenance of high levels of a CDK inhibitor would arrest differentiated cells permanently. To explore the function of CDK inhibitors in cell differentiation, we analyzed the induction of CDK inhibitors and their interaction and regulation with target CDK proteins during myogenesis.

MATERIALS AND METHODS

Cell Lines and Cell Culture

C2C12 myoblasts (CRL 1772; American Type Culture Collection, Rockville, MD) were maintained in growth medium (Dulbecco's modified Eagle's medium with 4500 mg/l glucose [DMEM-H], 15% fetal bovine serum, and antibiotics). For myogenesis studies, C2C12 cells were plated on 150-mm tissue culture dishes at low density. Between 24 and 48 h after culturing in growth medium, C2C12 cells were induced to differentiate by switching to differentiation medium (DMEM-H supplied with 2% horse serum and 10 μg/ml bovine insulin; Life Technologies, Gaithersburg, MD). Myogenin protein was first detected 2 days postinduction. Myotubes became visible between 4 and 5 days postinduction.

Northern Blot Analysis

For the multiple-tissue Northern analysis, 2 μg of poly(A)$^+$ RNA was isolated from different mouse tissues, resolved on a 1.2% agarose gel, and transferred to a nylon membrane (Clontech, Palo Alto, CA). Northern hybridizations were carried out according to the manufacturer's instruction. The *p18* probe used for Northern hybridization was a 1.1 kb DNA fragment containing the mouse *p18* cDNA isolated from a mouse skeletal muscle cDNA library using the coding region of human *p18* cDNA as a probe (Guan *et al.*, 1994). The β-actin probe (Clontech), used as a control for mRNA loading, is a 2 kb human cDNA probe that strongly cross-hybridizes with mouse β-actin mRNA. For the myogenin Northern analysis, the probe was a 1.4 kb mouse myogenin cDNA fragment kindly provided by Dr. W. Wright (Wright *et al.*, 1989). Approximately equal amounts of total RNA were loaded into each lane as determined by ethidium bromide staining, and hybridization was standardized with a rat GAPDH cDNA control probe (our unpublished results).

Antibodies and Immunochemistry Procedures

Procedures for immunoprecipitation, immunoblotting, and several antibodies used in this study have been described previously (Xiong *et al.*, 1993b; Jenkins and Xiong, 1995). After determining protein

concentration by Bradford assay, lysates containing equal amount of protein derived from different time points during myogenic induction or different tissues were used for immunoprecipitation or immunoblotting. For all coupled immunoprecipitation and immunoblotting (IP-Western) experiments presented in this study, after immunoblotting with the first antibody (e.g., anti-p21) blots were stripped and reprobed with different antibodies (e.g., anti-p18) to verify that consistent myogenesis patterns were obtained in each separate experiment.

All antibodies used in this study were rabbit polyclonal and were affinity-purified unless indicated otherwise. Most, but not all, antibodies raised against human peptides cross-react with corresponding mouse proteins with similar affinity. In the case in which anti-human peptide antibodies do not cross-react with mouse counterparts efficiently (CDK4 and p19), an antibody was raised against a synthetic peptide corresponding to the mouse protein. The specificity of all newly generated antibodies in cross-reacting with all other known family members (anti-p15, -p16, -p18, -p19, -CDK4, and -CDK6) was established by immunoprecipitating in vitro translated, ^{35}S-labeled proteins. To generate anti-peptide antibodies, a cysteine residue was added to the N-terminus of each synthetic peptide to covalently couple the peptide to activated keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL), which was then used to immunize rabbits. The sequence of synthetic peptides used in generating individual antibodies is underlined (underlined sequence corresponds to the C-terminal region of each protein): amino acid residue 117–130 of mouse p15 (CHRDIARYLHAATGD, nonaffinity antiserum; Quelle *et al.*, 1995); residue 155–168 of human p18 (CHRDIARYLHAATGD; Guan *et al.*, 1994); residue 153–166 of mouse p19 (CNLMDILQGHMMIPM, nonaffinity antiserum; Chan *et al.*, 1995; Hirai *et al.*, 1995); residue 290–303 of mouse CDK4 (CNLMDILQGHMMIPM; Matsushime *et al.*, 1992); residue 317–326 of human CDK6 (CSQNTSELNTA; Meyerson *et al.*, 1992); the nonaffinity anti-CDK2 peptide antibody (residue 287–298, CQDVTKPVPHLRL) has been described previously (Xiong *et al.*, 1993b; Zhang *et al.*, 1993). The nonaffinity anti-p16 antibody raised against a fusion of GST and full-length human p16 protein (Serrano *et al.*, 1993) cross-reacts efficiently with both mouse and human p15. A previously described nonaffinity anti-p18 antibody raised against full-length human p18 protein (Guan *et al.*, 1994) reacted with denatured p18 protein with higher sensitivity than the anti-p18 peptide antibody and, therefore, was used in immunoblotting. Anti-human p21 (sc-397), anti-human p27 (sc-528), anti-human CDK6 (sc-177), and anti-rat myogenin (sc-576) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

In Vitro Translation

[^{35}S]Met-labeled INK4 proteins were prepared in vitro with T7 or T3 RNA polymerase using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI). The cDNAs for mouse *p15* and *p16* (Quelle *et al.*, 1995), *p18* (E. Westphal and Y.X., our unpublished results), *p19* (Chan *et al.*, 1995), mouse CDK4 (Matsushime *et al.*, 1992), and human CDK6 (Meyerson *et al.*, 1992) were each subcloned into plasmids under either T7 or T3 RNA promoter. Each in vitro translation reaction was then immunoprecipitated with antisera raised against four p16 family members, or mouse CDK4 and human CDK6, to determine their immunogenic specificity.

Immunodepletion Procedures

Total cellular lysate was prepared from mouse skeletal muscle tissue using the same NP-40 lysis buffer containing (in mM): 50 Tris-HCl, pH 7.5, 150 NaCl, 0.5% NP-40, 50 NaF, 1 dithiothreitol (DTT), 1 Na_3VO_3, 1 phenylmethylsulfonyl fluoride, and 1× protease inhibitors (25 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM benzamidine, and 10 μg/ml trypsin inhibitor) as were used for C2C12 cell lysis. For each immunodepletion, 50 μl of protein A–agarose beads was coated with 50 μl of crude anti-p18 peptide antibody in 300 μl of NP-40 lysis buffer and mixed at 4°C for 1 h. Beads were pelleted by centrifuging for 3 min at 3000 rpm and 4°C and washed three times using 1 ml of lysis buffer. The protein A–agarose beads coated with anti-p18 peptide antibody were mixed with 3 mg of skeletal muscle tissue lysate for 2 h at 4°C. The mixture was then centrifuged for 3 min at 3000 rpm and 4°C. The supernatant was transferred to a new microcentrifuge tube with a fresh aliquot of anti-p18 antibody-coated beads for a second round of immunodepletion. After the three consecutive immunodepletions, the supernatant was requantitated and 500 μg of p18-depleted and nondepleted control lysates was used for each immunoprecipitation with peptide antibodies for p18, CDK2, CDK4, or CDK6.

Kinase Procedures

Protein lysate was prepared from proliferating and differentiating C2C12 cells in NP-40 lysis buffer and precipitated with anti-peptide antibodies for CDK2, CDK4, or CDK6 for 2 h at 4°C. Both anti-CDK4 and anti-CDK6 antibodies were affinity-purified; the anti-CDK2 antibody is capable of supporting kinase activity without requiring affinity purification. Protein A–agarose beads were added and incubated for 1 h at 4°C to precipitate immunoglobulin. The beads were washed two times with lysis buffer and one time with kinase buffer (50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, and 5 μM ATP). The washed beads were resuspended in 30 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP and 2 μl of a 2 mg/ml GST–pRb substrate (a fusion protein of GST and the C-terminal 137 amino acid residues of pRB) and incubated 30 min at 30°C. The reaction was stopped by adding 20 μl of 2× sodium dodecyl sulfate (SDS) loading dye, and 20 μl of sample was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 12.5% gel. The gel was stained with Coomassie blue to visualize immunoglobulin and GST–pRb protein to verify equal recovery of each individual immunoprecipitation and kinase reaction. The gel was dried and exposed for 2 h on a phosphorimage plate. The kinase activity of immunoprecipitated CDK6 is very low in both proliferating and differentiated C2C12 cells when assayed in vitro using GST–pRb (or histone H1) as a substrate. To rule out potential interference of kinase activity by the anti-CDK6 antibody, the same kinase assays were repeated in human peripheral T lymphocytes and proliferating Jurkat (acute T cell leukemia) cells. We have also included two different affinity-purified anti-CDK6 antibodies, one raised against a synthetic peptide corresponding to 10 amino acid residues (Meyerson and Harlow, 1994) and 21 amino acid residues (sc-177; Santa Cruz Biotechnology) at the C-terminal region of human CDK6. In both the T lymphocytes and the Jurkat cells, all three antibodies yielded CDK6 kinase activity that readily phosphorylated the GST–pRb fusion protein, yet a very low level of kinase activity was recovered from C2C12 cells.

RESULTS

Tissue-specific Expression of p18

The expression of *p18* in human tissues exhibits remarkably distinct tissue specificity (Guan *et al.*, 1996), prompting us to investigate the potential function of *p18* in initiating and/or maintaining cell cycle arrest caused by cell differentiation. The expression of *p18* mRNA varies significantly among different human tissues, with the highest levels observed in human skeletal and cardiac muscle cells (Guan *et al.*, 1996). We determined whether the expression of *p18* mRNA exhibits similar tissue specificity in the mouse using eight different adult mouse tissues (Figure 1A). Under high-stringency conditions for hybridization, the

probe derived from mouse *p18* cDNA sequence detected two discrete bands of sizes 2.4 and 1.2 kb. The relative intensity of these two bands seems to vary in different tissues. For example, whereas the 1.2 kb transcript is the predominant one in heart, lung, skeletal muscle, and kidney, it is expressed at a lower level than the 2.4 kb transcript in spleen and testis. The nature of these two *p18* transcripts and their potential regulatory function are currently under investigation. Low *p18* expression was observed in brain and liver. The pattern of *p18* gene expression seems to be similar between human and mouse in four tissues: high levels in skeletal muscle and heart, and low levels in brain and liver (Figure 1A; Guan *et al.*, 1996). There is, however, a distinct difference in *p18* expression between the two species in spleen, lung, and testis. The significance of species differences in the steady-state level of *p18* mRNA is not clear at present.

Consistent with the tissue-specific expression of *p18* mRNA, there was abundant p18 protein in mouse testis and kidney, moderate levels in skeletal muscle and heart, and low levels in liver and brain as determined by coupled immunoprecipitation and immunoblotting (IP-Western; Figure 1B). The level of p18 protein among different tissues correlates well with the level of p18-associated CDK4 and CDK6 (Figure 1B). In the testis, skeletal muscle, heart, and kidney, where p18 is expressed at high levels, CDK 4 and CDK6 are associated in p18 immunocomplexes. In both the brain and the liver, where p18 protein is low or undetectable, there were significantly lower levels of CDK4 protein present in anti-p18 immunoprecipitates.

These observations provided in vivo evidence potentially linking *p18* gene expression and terminal differentiation of several cell types, including the skeletal muscle lineage. Using established mouse C2C12 myoblasts that can be induced to undergo myogenic differentiation in vitro, and IP-Western technique, we have conducted a detailed analysis on the induction of p18 and other recently identified CDK-inhibitory proteins and the complex formations that these CDK inhibitors have with their target CDK proteins during myogenesis. IP-Western experiments determine not only the level of a specific polypeptide in vivo, but also the level of its association with other cellular protein(s). This is by far the most direct measurement for the regulation of CDKs by their inhibitors, because Northern analysis may not detect post-transcriptional regulation, and assaying kinase activity does not distinguish which specific inhibitor(s) is inhibiting the kinase because of the existence of multiple inhibitors in the same cell.

p21 Protein and Its Association with CDK Proteins during Myogenesis

Differentiation of skeletal myoblasts into contractile myotubes has been best studied in vitro using mouse muscle-derived cell lines like C2C12 that can be maintained as continuously proliferating myoblasts when cultured in growth factor-rich medium (e.g., 15% fetal bovine serum; see MATERIALS AND METHODS). After switching to a low-serum differentiation medium (containing 2% horse serum), C2C12 cells are induced to undergo myogenic differentiation that is associated with cell cycle withdrawal, expression of mature muscle-specific genes and, ultimately, fusion of myoblasts into multinucleated myotubes (Yaffe and Saxel, 1977; McMahon *et al.*, 1994). Myogenic differentiation of C2C12 cells was monitored by examining morphological changes including formation of multinucleated myotubes (our unpublished results)

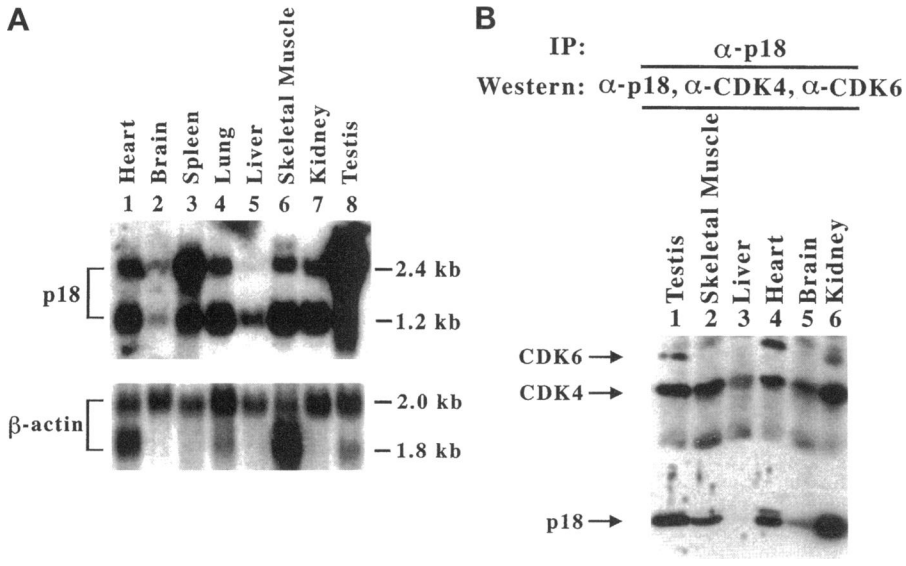

Figure 1. Expression of *p18* and *β-actin* mRNA and p18 protein in different murine tissues. (A) Poly(A)$^+$ RNA (2 μg) from eight different mouse tissues, as indicated at the top of each lane, were hybridized with a 1.1-kb mouse *p18* cDNA probe or a 2-kb human *β-actin* probe. (B) Total cell lysates were prepared from six adult mouse tissues, as indicated at the top of each lane. In all, 500 μg of each tissue lysate was immunoprecipitated with anti-p18 peptide antibody and resolved on a 15% SDS-polyacrylamide gel. After SDS-PAGE, proteins were transferred to nitrocellulose filters and immunoblotted with anti-p18, anti-CDK4, and anti-CDK6 antibodies.

and expression of the muscle-specific bHLH gene *myogenin* (Figure 2) by both Northern and Western blot analysis. The expression pattern for myogenin was typical for normal myogenesis.

During differentiation, the level of p21 CDK inhibitor protein steadily increased 10-fold to reach a peak 24 h after induction of myogenesis. This was followed by a continuous decrease to basal levels similar to those seen in proliferating cells by day 4 of differentiation (Figure 3A–C, lanes 1–9). Consistent with this pattern of p21 protein expression, the levels of p21-associated CDK2, CDK4, and CDK6 (Figure 3A–C, respectively, lanes 1–9) exhibited the same pattern of increasing during the first day followed by a continuous decrease as cells became terminally differentiated. The more intense band of CDK4 compared with p21 in anti-p21 immunocomplexes may be attributable to the fact that the CDK4 antibody is much more sensitive than the p21 antibody in immunoblotting. Reciprocal experiments to determine the level of p21 protein present in CDK complexes (Figure 3, lanes 10–18) gave rise to essentially the same results: a transient increase of p21 associated with CDK4 and CDK2 (at a lower level) peaking 24 h after differentiation followed by a decrease to nearly undetectable levels. This oscillation in the level of p21-associated CDK2 and CDK4 is apparently attributable to the change of p21 but not CDK proteins, because the level of all three CDK proteins (CDK2, CDK4, and CDK6) remained essentially unchanged during differentiation (Figure 3, lanes 10–18). In the reciprocal experiment, we were unable to detect p21 in anti-CDK6 immunocomplex (Figure 3C, lanes 10–18). This may reflect the fact that only a small amount of CDK6 seems to be complexed with p21 (Figure 3C, lanes 1–9).

In addition to these three previously characterized CDK proteins, we also detected a CDK2-related, 39-kDa polypeptide (p39) whose level, like CDK2, also

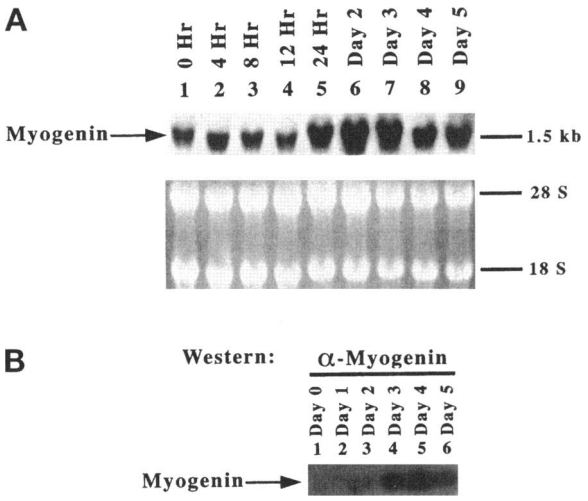

Figure 2. *Myogenin* gene expression during C2C12 cell differentiation. (A) Total RNA was prepared from C2C12 cells cultured in growth medium (lane 1) or in differentiation medium for different lengths of time (lanes 2–9), as indicated at the top of each lane. For each, 10 μg of the RNA samples were resolved on a 1% agarose gel. Approximately equal amounts of RNA were loaded as determined by ethidium bromide staining (bottom panel of Figure 2A), and hybridization was standardized with a rat GAPDH control probe (our unpublished results). Resolved RNA samples were transferred to a nitrocellulose filter, and the blot was hybridized with a probe derived from full-length mouse *myogenin* cDNA (Wright *et al.*, 1989). (B) Total cell lysates were prepared from C2C12 myoblasts cultured in growth medium (lane 1) or in differentiation medium for different lengths of time (lanes 2–6), as indicated at the top of each lane. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with an antibody to rat myogenin.

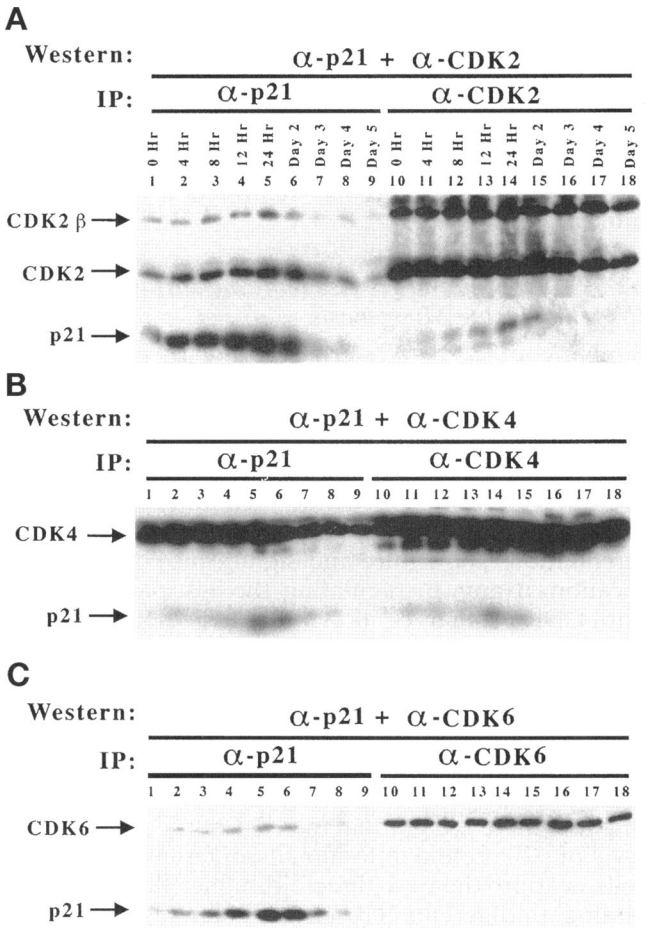

Figure 3. Expression of p21 and association of p21 with CDK proteins during myogenic differentiation of C2C12 cells. Lysates were prepared from C2C12 cells cultured in growth medium (lanes 1 and 10) or in differentiation medium for different lengths of time (lanes 2–9 and 11–18), as indicated at the top of each lane. Cell lysates were immunoprecipitated with antibodies to p21 (left half, all panels) and CDK2 (right half, panel A), CDK4 (right half, panel B), and CDK6 (right half, panel C) as indicated. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with antibodies to p21 and three CDK proteins as indicated.

remained unchanged and whose association with p21 similarly underwent an increase followed by a decrease during differentiation. Judging from its molecular weight, antigenicity, and specific association with both p21 and p27 (see below), but not p18 or p19, p39 most likely corresponds to the isoform of CDK2, CDK2β, which was recently identified in rat cells (Kotani *et al.*, 1995).

$p27^{Kip1}$ Protein and Its Association with CDK Proteins during Myogenesis

Similar coupled IP-Western experiments were carried out to determine the level of p27 protein and the association of p27 with various CDK proteins during myogenesis of C2C12 cells (Figure 4). After denaturing SDS-PAGE, p27 protein was resolved as a doublet. We have observed repeatedly a continued accumulation of the slower-migrating form of p27 during differentiation. The nature and significance of this modification, if any, in regulating the association of p27 with CDK proteins or its stability have not been studied. Estimated by comparing the intensity of CDK4 bands present in proliferating anti-p27 and anti-CDK4 immunoprecipitations (Figure 4B, lanes 1 and 10, respectively), p27 complexed with a significant amount (nearly one-half) of CDK4, the most highly expressed CDK in proliferating C2C12 cells. After myogenic induction, there is a gradual increase of p27 protein. The level of p27 protein in terminally differentiated C2C12 cells is approximately threefold higher than in proliferating, undifferentiated cells. A potential mechanism to account for the increase of p27 protein in G0-arrested C2C12 cells may be provided by the recent finding that p27 is degraded by the ubiquitin–proteasome pathway the activity of which is considerably lower in quiescent cells than in proliferating cells (Pagano *et al.*, 1995). Despite the increase in the level of p27 protein during differentiation, the levels of p27/CDK complexes (CDK2, CDK4, CDK6, and $p39^{CDK2\beta}$) in reciprocal experiments remained essentially unchanged for up to 5 days of differentiation (Figure 4).

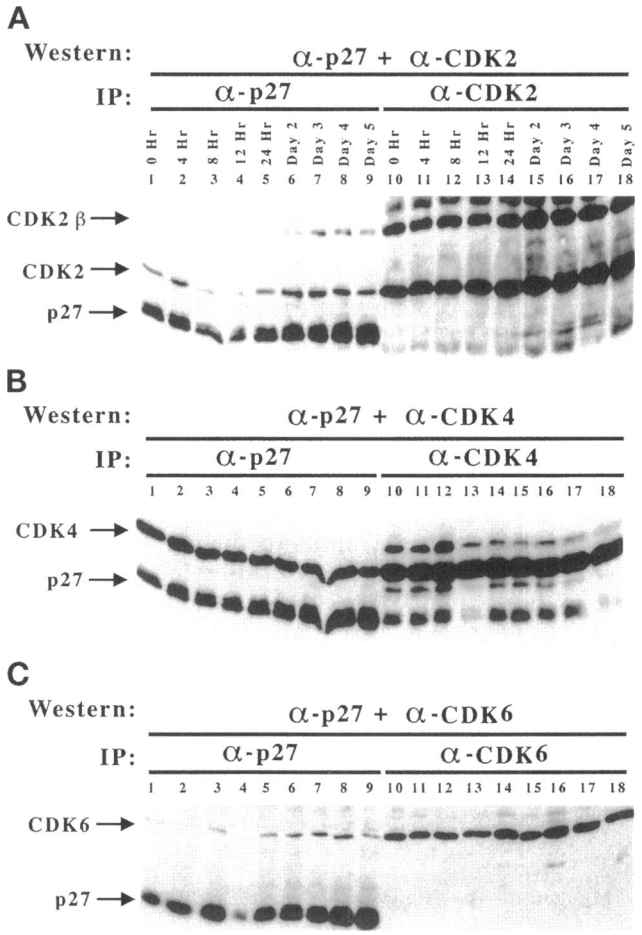

Figure 4. Expression of $p27^{Kip1}$ and association of $p27^{Kip1}$ with CDK proteins during myogenic differentiation of C2C12 cells. Lysates were prepared from C2C12 cells cultured in growth medium (lanes 1 and 10) or in differentiation medium for different lengths of time (lanes 2–9 and 11–18) as indicated at the top of each lane. Cell lysates were immunoprecipitated with antibodies to $p27^{Kip1}$ (left half, all panels) and CDK2 (right half, panel A), CDK4 (right half, panel B), and CDK6 (right half, panel C) as indicated. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with antibodies to $p27^{Kip1}$ and three CDK proteins as indicated.

Down-regulation of $p19^{INK4d}$ Protein during C2C12 Cell Differentiation

$p16^{INK4a}$ represents the other family of CDK inhibitors, which is distinct from the p21/p27 family, and currently includes four isolated genes: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. Members of the INK4 family are more closely related than members of the p21 family, and antibody raised against full-length individual INK4 proteins often cross-react with other members of the family. To determine accurately the expression of individual INK4 proteins and their association with CDK4 and CDK6 during C2C12 cell differentiation, we first characterized the antibodies for each INK4 protein. At least two different antibodies were raised for each INK4 protein, using either a full-length protein or a synthetic peptide as an antigen. Each antibody was used to immunoprecipitate all four mouse INK4 proteins that were in vitro translated and labeled with [^{35}S]Met (see MATERIALS AND METHODS). Of the four anti-INK4 antibodies that were chosen for this study, the anti-peptide antibodies for p15, p18, and p19 were each specific for their mouse protein and did not cross with other INK4 proteins. The anti-GST–p16 antiserum recognized not only p16 protein, but also mouse p15 (Figure 5A).

By Northern hybridization, coupled metabolic labeling and immunoprecipitation ([^{35}S]IP), and IP-Western, we detected neither p16 nor p15 gene expres-

sion in terminally differentiated muscle tissues and C2C12 cells (our unpublished results). p19 protein was expressed at relatively high levels in proliferating, undifferentiated C2C12 cells and complexed with a significant amount of CDK4 (Figure 5B, lane 1). After 4 days culturing in differentiation medium, p19 protein decreased to very low levels (Figure 5B, lanes 2–5). Consistently, p19-associated CDK4 also decreased as cells differentiated. Down-regulation of p19 during myogenesis seems to be consistent with its normal accumulation during S-phase and its nadir in G1-phase of the cell cycle (Hirai *et al.*, 1995; Okuda *et al.*, 1995). Therefore, these three INK4 proteins (p15, p16, and p19) do not seem to play a critical role in causing and maintaining cell cycle arrest during C2C12 cell differentiation.

Fifty-fold Induction of p18 during Myogenesis and Its Predominant Association with CDK4 and CDK6

Distinct from other CDK inhibitors, there is a striking increase of p18 protein during myogenesis. A very low level of p18 protein can be detected in proliferating C2C12 cells (overexposure of Figure 6; our unpublished results). p18 protein became clearly detectable within 8–12 h of myogenic induction and underwent a sharp increase between 12 and 24 h postinduction. p18 continued to accumulate >50-fold higher by day 5 of differentiation (Figure 6, A and B, lanes 1–9). Accompanying this induction is the continued increase of p18-associated CDK6 and CDK4. Consistent with the higher affinity of p18 for CDK6 than for CDK4 (Guan *et al.*, 1994), increased association of p18 with CDK6 preceded the increased association of p18 with CDK4, despite the fact that CDK4 is apparently expressed at a much higher level in C2C12 cells than CDK6. In fact, CDK4 first became detectable in p18 immunocomplexes between 24 h and 2 days after induction (overexposure of Figure 6B; our unpublished results), by which time nearly all CDK6 seemed to be complexed with p18 (Figure 6A). This raises an intriguing possibility of sequential inhibition of CDK enzymes during differentiation. Reciprocally, CDK6- and CDK4-associated p18 also continued to increase during differentiation, whereas the level of both CDK4 and CDK6 proteins remained relatively constant (Figure 6, A and B, lanes 10–18). Consistently, formation of CDK6–p18 complex preceded the formation of CDK4–p18 complex. In proliferating murine macrophages, *p18* mRNA is periodically expressed with a nadir in G1 and maximal synthesis during S-phase (Hirai *et al.*, 1995). This cell cycle oscillation profile excludes the possibility that the high p18 levels induced during myogenesis of C2C12 cells resulted from the enrichment of the G1 cell population and, additionally, supports a direct function for *p18* in controlling cell cycle arrest during myogenesis.

Analysis of CDK and CDK Inhibitor Protein Complexes in Adult Mouse Muscle Tissues

To confirm the results obtained from cultured C2C12 cells induced to differentiate in vitro, we analyzed CDK and CDK inhibitor complexes in vivo using adult mouse tissues. Cell lysates were prepared from terminally differentiated mouse skeletal muscle tissue and immunoprecipitated with peptide antibodies specific to p18, p21, p27, CDK4, and CDK6. The five

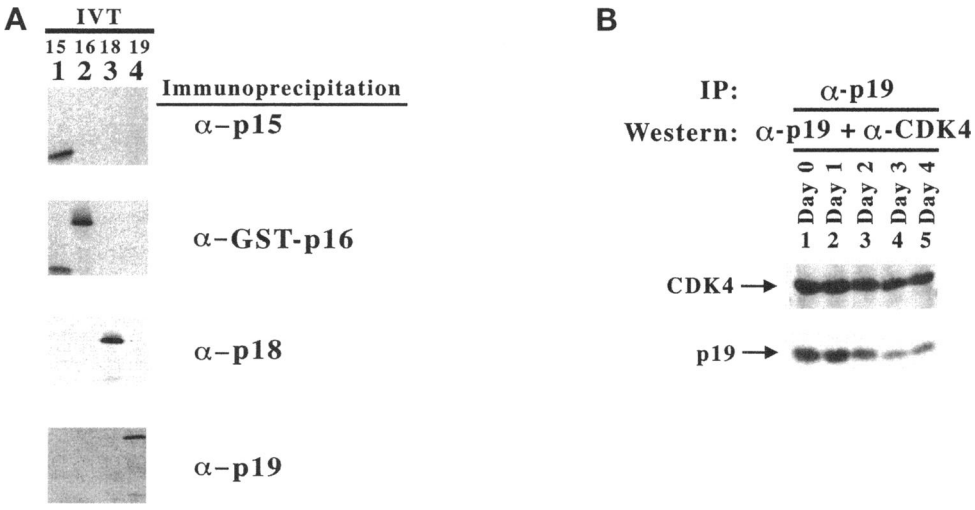

Figure 5. Characterization of anti-INK4 antisera and expression of $p19^{INK4d}$. (A) Plasmids containing cDNA for mouse *p15*, *p16*, *p18*, and *p19* were translated in vitro with [^{35}S]Met. Each translated product, as indicated at the top of each lane, was immunoprecipitated with antisera for p15, p16, p18, and p19 as indicated to the right of each panel. Immunoprecipitates were resolved on a 15% SDS-polyacrylamide gel. (B) Expression of and association of $p19^{INK4d}$ with CDK proteins during myogenic differentiation of C2C12 cells. Lysates were prepared from C2C12 cells cultured in growth medium (lane 1) or in differentiation medium for different lengths of time (lanes 2–5) as indicated at the top of each lane. Cell lysates were immunoprecipitated with antibodies to $p19^{INK4d}$. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with antibodies to $p19^{INK4d}$ and CDK4.

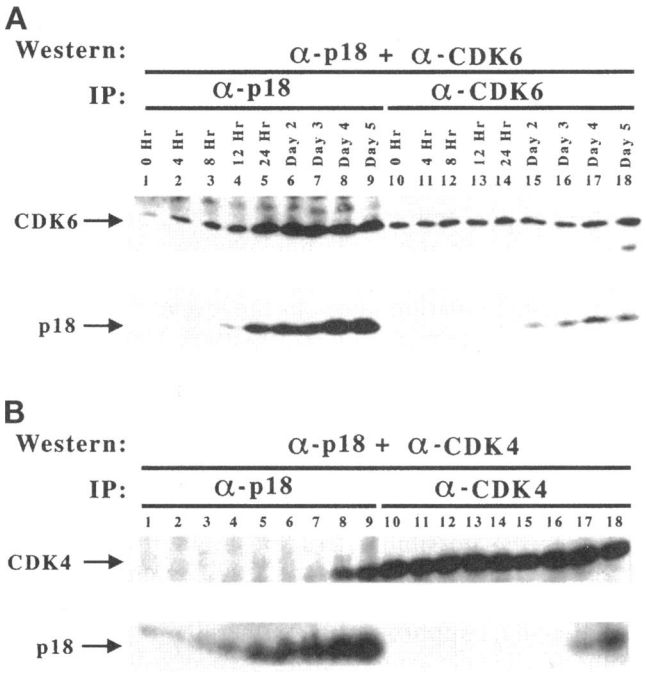

Figure 6. Expression of $p18^{INK4c}$ and association of $p18^{INK4c}$ with CDK proteins during myogenic differentiation of C2C12 cells. Lysates were prepared from C2C12 cells cultured in growth medium (lanes 1 and 10) or in differentiation medium for different lengths of time (lanes 2–9 and 11–18) as indicated at the top of each lane. Cell lysates were immunoprecipitated with antibodies to p18 (left half, all panels), CDK6 (right half, panel A), and CDK4 (right half, panel B) as indicated. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with antibodies to p18 and CDK4 or CDK6 proteins as indicated.

immunoprecipitates were resolved on the same SDS-polyacrylamide gel and immunoblotted with a mixture of the five antibodies to allow a direct comparison of the levels of the different CDK inhibitors complexed with the same CDK proteins (Figure 7A). As observed in differentiated C2C12 cells, no p21 protein was detected in anti-p21, anti-CDK4, or anti-CDK6 immunoprecipitates derived from adult mouse skeletal muscle cells. The p18 protein was expressed at a high level in adult mouse skeletal muscle. Most, if not all, CDK6 protein is complexed with p18 in differentiated adult mouse muscle cells (Figure 7, compare lanes 1 and 5). In contrast, neither p21 nor p27 was seen complexed with CDK6 as determined by reciprocal immunoprecipitations. Approximately equal amounts of CDK4 proteins were co-immunoprecipitated in p18 and p27 immunocomplexes, despite the apparent presence of higher p27 protein levels. Because p27 was already associated with nearly half of the CDK4 in both proliferating and differentiated myoblasts (Figure 4B, compare lanes 1, 9, and 10), it seems that as cells became terminally differentiated, p18 complexed with the major fraction of CDK4 that was not previously associated with p27.

To provide a more accurate assessment of how much CDK6 and CDK4 are associated with p18 in differentiated muscle cells, we carried out immunodepletion experiments using cellular lysate prepared from mouse skeletal muscle tissue (see MATERIALS AND METHODS). After three consecutive p18 immunodepletions, the supernatant samples were resolved by SDS-PAGE. Depletion of p18 had no effect on CDK2, with which p18 does not interact (Figure 7B, compare lanes 1 and 2). Immunoblotting with antibody specific to p18 indicated that all p18 protein was depleted from the lysate (Figure 7B, compare lanes 3 with 4 and lanes 7 with 8). Consistent with complete depletion of p18, no CDK4 (Figure 7B, lane 4) or CDK6 (Figure 7B, lane 8) was detected in anti-p18 immunoprecipitates derived from p18-depleted lysates. Recip-

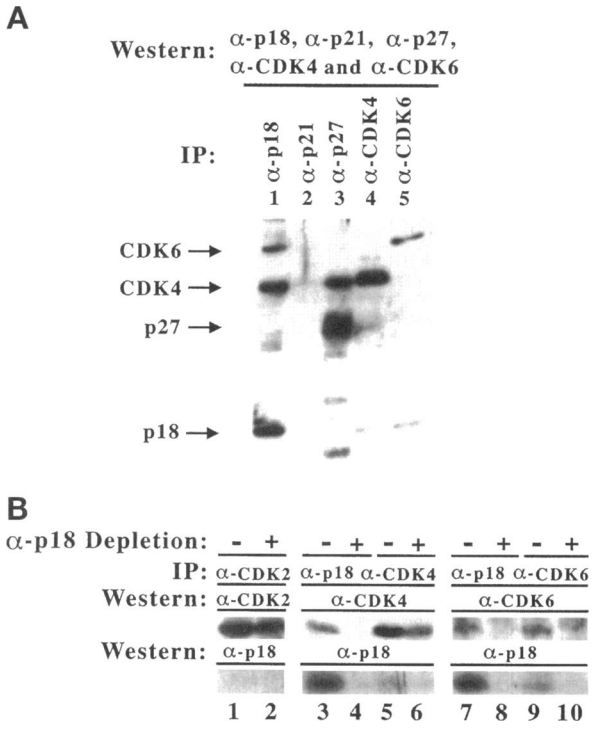

Figure 7. Expression and association of CDK and CDK inhibitor proteins in adult mouse muscle tissues. (A) Total cell lysate was prepared from adult mouse skeletal muscle tissue. Total cell lysate (800 μg) was precipitated with a specific antibody as indicated at the top of each lane. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with a mixture of five indicated antibodies. (B) Total cell lysate was prepared from adult mouse skeletal muscle tissue. Total cell lysate (3 mg) was immunodepleted three times with the peptide antibody for p18. Total cell lysates (500 μg) from anti-p18–depleted and nondepleted samples were each immunoprecipitated with peptide antibodies for p18, CDK2, CDK4, or CDK6 as indicated. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with antibodies to p18 and CDK2, CDK4, or CDK6 proteins as indicated.

rocally, immunoblotting of anti-CDK4 and anti-CDK6 immunoprecipitates, derived from control or p18-depleted lysates, with antibody specific to CDK4 or CDK6 indicated that nearly half of CDK4 (Figure 7B, compare lanes 5 with 6) and all CDK6 (Figure 7B, compare lanes 9 with 10) had been depleted by the anti-p18 antibody.

Analysis of CDK Activity during Myogenesis

Finally, we determined the kinase activity of CDK2, CDK4, and CDK6 during C2C12 cell differentiation (Figure 8). Anti-CDK2, -CDK4, and -CDK6 immunoprecipitates derived from C2C12 cells at different time points after myogenic induction were assayed for pRb kinase activity. As cells differentiate, we have observed repeatedly a slightly initial increase of both CDK2 and CDK4 kinase activity followed by a continued decrease of both kinase activities. The significance of this initial increase of both kinase activities is not clear at present. A possible factor that may contribute to the initial increase of CDK2 and CDK4 kinase activity is p21, whose protein level and association with CDK2 and CDK4 underwent an initial increase after myogenic induction before declining (Figure 3, A and B). p21 interaction with CDK–cyclin complexes has been implicated in stabilizing their association and, therefore, potentially increasing the kinase activity of the CDK–cyclin complex (Zhang *et al.*, 1994; Harper *et al.*, 1995). During C2C12 cell differentiation, the level of CDK2 kinase activity decreased to a nearly undetectable level, whereas the level of total CDK2 protein remained essentially unchanged (Figures 3A and 4A). We do not know at present the mechanism responsible for the down-regulation of CDK2 kinase activity during the differentiation, because the association of CDK2 with p21 or p27 did not increase and because members of the INK4 family do not interact with CDK2. The decrease in CDK4 kinase activity is most likely attributable to the increased association of CDK4 with p18, the only CDK inhibitor that we have found to increase its association with any of the three G1 CDKs during C2C12 differentiation.

Immunoprecipitated by three different anti-CDK6 antibodies and assayed in vitro using the GST-pRb protein as a substrate, the CDK6 kinase activity is very low in both asynchronously proliferating C2C12 myoblasts and differentiated myotubes (Figure 8, right panel). Using the same antibodies, considerably higher CDK6 kinase activity was immunoprecipitated from both human peripheral T lymphocyte and proliferating Jurkat cells (our unpublished results). This rules out the possibility that the low level of immunoprecipitated CDK6 kinase activity in C2C12 cells results from interference by the anti-CDK6 antibodies. The low level of CDK6 kinase activity in proliferating cells may be the combination of use of asynchronized

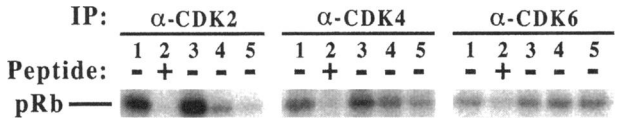

Figure 8. Kinase activity during myogenesis of C2C12 cells. Lysates were prepared from C2C12 cells cultured in growth medium (lanes 1 and 2) or in differentiation medium for 24 h (lane 3), 3 days (lane 4), or 5 days (lane 5). Cell lysates were immunoprecipitated with antibodies to CDK2 (left panel), CDK4 (middle panel), and CDK6 (right panel) as indicated. Competing peptide was preincubated with antisera before adding lysate from proliferating cells (lane 2) to show the specificity of the IP-kinase assay. The immunoprecipitates were assayed for kinase activity using a GST–pRb fusion protein as the substrate, resolved by SDS-PAGE, and exposed to a phosphorimage plate.

cells and higher levels of p19. Reciprocal decrease of p19 and increase of p18 during myogenesis raise the possibility that p18 may replace p19 as the CDK6 inhibitor as C2C12 cells differentiate.

DISCUSSION

A hallmark of terminal cell differentiation is the cessation of cell proliferation. Early cell fusion experiments between myocytes and nonmyogenic cells had suggested that differentiated muscle cells contain a dominant inhibitor(s) of cell cycle progression (Clegg and Hauschka, 1987). As negative cell cycle regulators, CDK inhibitors are ideal candidate proteins to initiate cell cycle arrest required for cell differentiation and/or prevent terminally differentiated cells from re-entering the division cycle. This notion is supported by the existence of a potentially large number of related CDK inhibitor genes whose expression exhibit distinct tissue specificity (Guan *et al.*, 1994, 1996; Lee *et al.*, 1995; Matsuoka *et al.*, 1995). Our studies in both in vitro cultured cells and adult tissues identified p18 as a major CDK4- and CDK6-associated inhibitor that is significantly induced during myogenesis. This provides the first example that correlates the differentiation of a particular cell lineage with not only induced expression of a specific CDK inhibitor but also increased association with and inhibition of its target CDK proteins.

Five lines of evidence support p18 as the major CDK inhibitor that plays a critical role in regulating G1 CDK4 and CDK6 during myogenic differentiation. First, there is a >50-fold induction of p18 protein during myogenesis in C2C12 cells (Figure 6), significantly higher than the threefold increase of p27, the only other CDK inhibitor that was found to accumulate continuously through the differentiation process. Second, *p18* mRNA normally accumulates during S-phase of the cell cycle in proliferating cells (Hirai *et al.*, 1995), arguing against the possibility that induction of p18 protein in G1-arrested myotubes is the consequence of enrichment of G1 cell population. Third,

accompanying its induction, p18 protein is increasingly complexed first with CDK6 and then with CDK4, from nearly undetectable levels in CDK4 and CDK6 immunocomplexes in proliferating cells to become the predominant CDK4- and CDK6-associated protein in terminally differentiated C2C12 cells. None of the other three CDK inhibitors expressed in C2C12 cells that we have examined—p19, p21, and p27—increased association with any of the three examined CDK proteins. Fourth, consistent with the results obtained in cultured C2C12 cells, p18 protein is expressed at a high level in skeletal muscle tissues (Figure 7) and complexes with all CDK6 and with the major portion of CDK4 that was not associated previously with p27. Finally, the pRb kinase activity of CDK4 was inhibited significantly in differentiated myotubes (Figure 8). p18 clearly plays an important role in inhibiting CDK4 kinase activity because it is the only CDK inhibitor that was found to increase its association with CDK4.

A series of myogenic events occurs in proliferating MM14 myoblasts when cells are deprived of basic fibroblast growth factor (FGF). Myoblasts begin to withdraw from the cell cycle within 2–3 h at a point in G1 that has yet to be well defined. This is followed by the expression of muscle-specific proteins within 6–7 h and initiation of cell fusion within 12–14 h (Clegg *et al.*, 1987). Although the differentiation of other lines of myoblasts may not be regulated identically to MM14, it is generally believed that cell cycle withdrawal normally precedes differentiation. The induction of p18 protein in an exponentially growing C2C12 myoblast population, as determined by immunoblotting analysis, became evident between 8 and 12 h after switching from growth medium to differentiation medium (Figure 6). This suggests that if p18 indeed causes cell cycle arrest, it might begin to do so at an early point of differentiation. Induction of p18 is before growth arrest of C2C12 cells as they differentiate, which occurs between 24 and 48 h postmyogenic induction, as measured by [^{3}H]thymidine incorporation (our unpublished results).

Equally important is the accumulation and maintenance of high p18 levels in C2C12 myotubes and adult mouse skeletal tissue, suggesting that p18 may also function in terminally differentiated cells to prevent them from re-entering the cell cycle. It is worthy to notice in this context the mechanistic difference between two families of CDK inhibitors in inhibiting CDK activity. The p21 family of inhibitors binds cyclin–CDK complexes, and inhibition over these complexes can be released by changing the ratio of inhibitor-to-cyclin/CDK (Zhang *et al.*, 1994; Harper *et al.*, 1995). Alternately, the p16 family of inhibitors forms binary inhibitor–CDK complexes (e.g., p18–CDK6) that, assembled in vitro, are highly stable and cannot be dissociated with excess amounts of cyclin D1 protein (Hirai *et al.*, 1995; Guan *et al.*, 1996). By forming stable binary inhibitor–CDK complexes that cannot be dissociated by the cyclins, cells may be able to arrest stably or even permanently.

During C2C12 cell differentiation, we observed similar induction of the steady-state level of *p21* mRNA to that of previous reports (Guo *et al.*, 1995; Halevy *et al.*, 1995; Missero *et al.*, 1995; Parker *et al.*, 1995; Li and Xiong, unpublished results). We interpret the initial increase of *p21* mRNA and protein during myogenesis as the result of the accumulation of the G1 cell population. *p21* mRNA is periodically expressed in proliferating cells with maximal synthesis in G1-phase and a nadir during S-phase (Li *et al.*, 1994; Noda *et al.*, 1994). Indeed, we find that cell cycle arrest of C2C12 cells also coincides with maximum p21 protein levels. Increased p21 protein levels at this time point may aid in the initial growth arrest of C2C12 cells. Alternately, the increase in p21 levels, and association with CDK2 and CDK4, may stabilize these CDK–cyclin complexes and cause the increase in kinase activity we observed (Figure 8; Zhang *et al.*, 1994; Harper *et al.*, 1995). A possible explanation for the difference between the level of *p21* mRNA and p21 protein in later stages of differentiation may come from the steady induction of p18 protein and its increasing association with CDK4 and CDK6. As the level of CDK proteins remained unchanged, increased p18–CDK4 and p18–CDK6 association may lead to the release of p21 protein from CDK4 and CDK6, causing a decrease of total p21 protein as the result of its short half-life (~30 min; Timchenko *et al.*, 1996; Jenkins and Xiong, unpublished results). Because of its decrease at later stages of C2C12 cell differentiation, its very minor amounts associated with CDKs in both terminally differentiated C2C12 cells (Figure 3) and adult mouse muscle tissues (Figure 7), and the fact that mice lacking p21 undergo normal development (Deng *et al.*, 1995), we suggest that although p21 could have a certain function in helping to establish the initial G1 arrest, it does not seem to have a critical role in maintaining a permanent arrest in terminally differentiated muscle cells. Similarly, because p27 both in proliferating and differentiated C2C12 cells (Figure 4B, lanes 1, 9, and 10) and in muscle tissue (Figure 7A) associates with similar amounts of CDK4 (approximately one-half of the total CDK4), our results for p27 do not indicate that it is the major inhibitor of G1 CDK activity during myogenesis. This conclusion is also consistent with the observation that mice lacking p27 undergo normal muscle development (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996).

The findings presented in this study raise several important issues concerning the mechanism involved in myogenesis and cell cycle control during differentiation in general. First, the level of the three major G1 CDK proteins—CDK2, CDK4, and CDK6—each re-

mains essentially unchanged during differentiation of C2C12 cells (Figures 3, 4, and 6, lanes 10–18). Similarly, cyclin E and cyclin D3 both are present at relatively high levels in differentiated, nondividing C2C12 cells (Jahn *et al.*, 1994; Guo *et al.*, 1995). Therefore, it seems that, instead of simply turning off the expression of CDK or cyclin genes, inhibition of CDK activity during differentiation and maintenance in terminally differentiated cells may be achieved by assembling the kinases into inhibitor-associated, catalytically inactive complexes. These observations pose a challenging question as to what function CDKs may have in terminally arrested, nondividing cells. Intriguingly, we noticed that the kinase activity of CDK6 did not decrease in differentiated myotubes as did CDK2 and CDK4 (Figure 8). This result, however, should be interpreted cautiously because we only detected very low level CDK6 kinase activity in C2C12 cells and are comparing asynchronously proliferating cells with a G1-arrested population. A more sensitive assay for CDK6 kinase activity and further characterization of CDK6 kinase activity during the cell cycle are needed to firmly establish this notion.

The second issue concerns the mechanism by which p18 arrests the cell cycle. Cell growth inhibition by *p18* (and *p16*) seems to dependent on the endogenous wild-type pRb function, suggesting that pRb is the major, if not the only, target of *p18* function in vivo (Guan *et al.*, 1994). pRb has been implicated in regulating and, in particular, maintaining cell cycle arrest in terminally differentiated myotubes (Braun *et al.*, 1992; Endo, 1992; Mymryk *et al.*, 1992; Cardoso *et al.*, 1993; Caruso *et al.*, 1993; Gu *et al.*, 1993a; Schneider *et al.*, 1994). Although the direct physical interaction between pRb and MyoD could provide, in principle, a molecular basis to maintain pRb in an active state (Gu *et al.*, 1993a), the alternative pathway that involves cyclin-dependent kinases and their inhibitory proteins seems more appealing. Hence, induction of p18 by growth factor deprivation may lead to the formation of p18–CDK6 and p18–CDK4 complexes. This would inhibit cyclin D–CDK4 and cyclin D–CDK6 kinase activity, thus resulting in the accumulation of hypophosphorylated, active growth-suppressing pRb in G1. Inhibition of CDK4 kinase activity may be the direct result of increased association of CDK4 with p18 in combination with inhibition of CDK4 by p27. Permanent arrest may also be maintained by the inactivation of CDK4 (and perhaps CDK6) by p18.

A final key issue concerns the regulation of *p18* gene expression, which is currently unknown. A simple and seemingly logical model can be envisioned in which expression of *p18* gene is activated by a MyoD-mediated transactivation pathway to initiate cell cycle arrest. However, we have observed induction of *p18* by growth factor deprivation in fibroblast cells that do not express myogenic determination factor MyoD (e.g., NIH3T3 and 10T1/2 cells; our unpublished results), arguing that the expression of *p18* gene is not exclusively regulated by MyoD, if at all. Consistent with this notion, *p18* was also highly expressed in several additional types of terminally differentiated cells including heart, spleen, lung, kidney, and testis (Figure 1), suggesting a broad role of *p18* in cell differentiation.

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