

Cyclin D3-associated Kinase Activity Is Regulated by p27^{kip1} in BALB/c 3T3 Cells

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We report that cyclin D3/cdk4 kinase activity is regulated by p27^{kip1} in BALB/c 3T3 cells. The association of p27^{kip1} was found to result in inhibition of cyclin D3 activity as measured by immune complex kinase assays utilizing cyclin D3-specific antibodies. The ternary p27^{kip1}/cyclin D3/cdk4 complexes do exhibit kinase activity when measured in immune complex kinase assays utilizing p27^{kip1}-specific antibodies. The association of p27^{kip1} with cyclin D3 was highest in quiescent cells and declined upon mitogenic stimulation, concomitantly with declines in the total level of p27^{kip1} protein. The decline in this association could be elicited by PDGF treatment alone; this was not sufficient, however, for activation of cyclin D3 activity, which also required the presence of factors in platelet-poor plasma in the culturing medium. Unlike cyclin D3 activity, which was detected only in growing cells, p27^{kip1} kinase activity was present throughout the cell cycle. Since we found that the p27^{kip1} activity was dependent on cyclin D3 and cdk4, we compared the substrate specificity of the active ternary complex containing p27^{kip1} and the active cyclin D3 lacking p27^{kip1} by tryptic phosphopeptide mapping of GST-Rb phosphorylated *in vitro* and also by comparing the relative phosphorylation activity toward a panel of peptide substrates. We found that ternary p27^{kip1}/cyclin D3/cdk4 complexes exhibited a different specificity than the active binary cyclin D3/cdk4 complexes, suggesting that p27^{kip1} has the capacity to both inhibit cyclin D/cdk4 activity as well as to modulate cyclin D3/cdk4 activity by altering its substrate preference.

INTRODUCTION

Two families of proteins play major roles in the regulation of the cell cycle (Roberts, 1993; Morgan, 1995; Sherr, 1996; Wuarin and Nurse, 1996). One family, the cdk, exerts control on downstream processes by phosphorylating selected proteins, such as Rb family members, on serine and threonine residues (Morgan, 1995). The other family consists of specialized regulatory proteins, cyclins, that bind to cdk molecules and modulate their activity to phosphorylate appropriate target proteins (Swenson *et al.*, 1986; Hagan *et al.*, 1988; Booher *et al.*, 1989; Draetta *et al.*, 1989; Hadwiger *et al.*, 1989; Moreno *et al.*, 1989). The catalytic activity of monomeric cdk subunits is almost undetectable, and

the pathway to its formation requires, as a first step, the binding of a cyclin subunit (Solomon *et al.*, 1990). While the resulting binary cyclin/cdk complex is functionally competent, its activity is still subject to both positive and negative control. Maximal activity is attained only after phosphorylation of a conserved threonine residue covering the ATP-binding pocket on the catalytic subunit (Ducommun *et al.*, 1991; Gould *et al.*, 1991). This phosphorylation is performed by a constitutive cellular kinase activity, cdk-activating kinase (CAK), itself a cyclin/cdk complex (Poon *et al.*, 1993; Solomon *et al.*, 1993; Fisher and Morgan, 1994). In addition, cdk activity is also subject to negative control by two mechanisms: binding of inhibitory subunits and phosphorylation on an amino-terminal tyrosine residue (Picard *et al.*, 1989; Pondaven *et al.*, 1990). The cdk-inhibitory proteins are collectively called CKIs

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(Elledge and Harper, 1994; Sherr and Roberts, 1995), of which seven have been identified to date.

Both primary sequence homology and cdk target specificity indicate CKIs fall into two groups, the Kip/Cip family and the Ink4 family. Unlike the members of the Ink4 family, which are able to inhibit only cdk4/cdk6 activity, the members of the Kip/Cip family are able to exhibit inhibitory activity with all cdk complexes (Xiong *et al.*, 1993; Harper *et al.*, 1995; Matsuoka *et al.*, 1995). This latter group is currently composed of three members: p21^{Cip1} (Harper *et al.*, 1993; Xiong *et al.*, 1993), p27^{kip1} (Hengst *et al.*, 1994; Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994), and p57 (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). The earliest known inhibitors, p21^{Cip1} and p27^{kip1}, are also the most extensively studied and have been linked to growth arrest brought about through a number of different environmental signals. Conditions that promote DNA damage or otherwise cause an induction of p53 have almost invariably been demonstrated to result in the induction of p21^{Cip1} (el-Deiry *et al.*, 1993; Dulic *et al.*, 1994), whereas conditions such as high cell density, TGF- β growth inhibition, and serum withdrawal have been associated with induction of p27^{kip1} (Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994; Agrawal *et al.*, 1995). Sequence comparisons between p27^{kip1} and p21^{Cip1} revealed a region of identity in the N-terminal region of each protein that contains the motif responsible for its inhibitory activity (Nakanishi *et al.*, 1995). In addition to direct inhibition of catalytic activity, association of p27^{kip1} with either cdk2 or cdk4 complexes can block their CAK-mediated phosphorylation (Matsuoka *et al.*, 1994). Overexpression of p27^{kip1} was found to cause G₁ arrest (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994), and antisense inhibition of p27^{kip1} expression prevented cell cycle exit in response to mitogen depletion (Coats *et al.*, 1996; Rivard *et al.*, 1996), consistent with its proposed role as a negative regulator of cell cycle progression *in vivo*. Unlike p21^{Cip1}, p27^{kip1} protein levels either remain relatively constant in cycling cells or are high in quiescent cells and decreased in growing cells (Nourse *et al.*, 1994; Toyoshima and Hunter, 1994; Agrawal *et al.*, 1995). Thus, in some cell types, p27^{kip1} is an essential component of the pathway that connects mitogenic signals to the cell cycle at the restriction point and may be the primary CKI protein responsible for producing an inhibitory threshold for activation of cyclin/cdk complexes in quiescent cells.

It is widely agreed that cyclin D1 plays an important role in sequestering p27^{kip1} during the G₁ phase, allowing cyclin E/cdk2 to become active and thus permitting entry into S phase (Polyak *et al.*, 1994a). Although cdk activities are observed during G₁, the role of p27^{kip1} inhibition of cyclin D/cdk4 activity in cell cycle regulation remains to be clarified. Recently published data have led to several different conclusions

concerning the effect of p27^{kip1} on D type cyclin activity including: 1) p27^{kip1}-associated cyclin D/cdk4 is active (Reynisdottir and Massague, 1997), 2) p27^{kip1}-associated cyclin D complex is not active (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994; LaBaer *et al.*, 1997), and 3) p27^{kip1} must be removed before the cyclin D/cdk can be activated by CAK (Kato *et al.*, 1997). Moreover, it has recently been demonstrated that while p27^{kip1} readily inhibits cyclin/cdk2 activity, it requires multiple p27^{kip1} molecules to inhibit an active cyclin D/cdk4 complex. In contrast to its roles as an inhibitor of cdk, p27^{kip1}-immune complexes isolated from MANCA cells, a human Burkitts lymphoma cell line, were reported to contain an Rb kinase activity (Soos *et al.*, 1996). The p27^{kip1}-specific activity was incapable of phosphorylating histone H1 and was removed by depletion of cdk6, characteristics suggesting that it was a cyclin D-dependent activity. Furthermore, p27^{kip1} complexes prepared from primary mouse keratinocytes stimulated to undergo differentiation contained an Rb kinase activity that was dependent on cyclin D3/cdk4 (Hauser *et al.*, 1997). Clearly, the mechanisms whereby cyclin D/cdk complexes are activated and the role that p27^{kip1} may play on cyclin D/cdk activity, such as to inhibit this activity or to bring about a Rb kinase in association with cyclin D/cdk4/p27^{kip1} complexes, has not been elucidated.

The physiological role of p27^{kip1}, in most cases, has been linked to inhibition of cyclin E/cdk2, and the association of p27^{kip1} with cyclin D/cdk4 is believed to be required only to lower the inhibitory threshold for cyclin E/cdk2 activation. This idea is strongly supported by numerous studies but does not exclude the possibility that a ternary cyclin D/cdk4/p27^{kip1} complex may be physiologically distinct from a binary complex lacking p27^{kip1}. Since it has been previously demonstrated that cyclin D3/cdk4 activity also appears during G₁ traverse in BALB/c 3T3 fibroblasts (Dong *et al.*, 1998) and that cyclin D3 is bound to p27^{kip1} complexes in quiescent cells (Agrawal *et al.*, 1996), we have examined the possibility that cyclin D3 activity may be subject to inhibition by p27^{kip1} in this cell line. Our results indicate that p27^{kip1} is removed from cyclin D3 complexes after mitogenic stimulation with PDGF, which may be necessary but is not sufficient for cyclin D3/cdk4 activation. In addition, we have found that the association of p27^{kip1} with cyclin D3 in quiescent cells also gives rise to a kinase activity that may be detected in p27^{kip1}-immune complexes. By comparing the phosphorylation specificity of ternary p27^{kip1}/cyclin D3/cdk4 complexes and binary complexes lacking p27^{kip1}, we demonstrate that the addition of p27^{kip1} results in an alteration of substrate specificity.

MATERIALS AND METHODS

Cell Culture

BALB/c 3T3 fibroblasts (clone A31) were maintained in an incubator with a humidified atmosphere (5% CO₂, 95% air) at 37°C. Experimental cultures were grown to confluence in 100-mm Petri dishes using DMEM supplemented with 10% calf serum (CS), 4 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Quiescent cultures were stimulated 2–3 d after density-arrest by the addition of fresh medium (DMEM) containing 10 ng/ml PDGF-BB, and 10% CS (Agrawal *et al.*, 1995; Winston *et al.*, 1996).

Reagents, Recombinant Plasmids, and Antibodies

Biological buffers, detergents, and chemicals (except for those indicated below) were from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Cell culture medium, antibiotics, and protein A-agarose beads were from Life Technologies (Gaithersburg, MD), and PDGF-BB was from BioSource (Camarillo, CA). Nitrocellulose paper and reagents for SDS-PAGE were from Bio-Rad (Hercules, CA). Rabbit anti-mouse HRP, ECL reagents, and radioisotopes were from Amersham (Arlington Heights, IL). Autoradiographic film was from Eastman Kodak (Rochester, NY). A plasmid encoding GST-Rb 379–928 was obtained from Doug Cress (H. Lee Moffitt Cancer Center and Research Institute). The cyclin D1 (72–13G) and D3 (C-16) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The cdk4 antibody was raised against C-terminal polypeptide of mouse cdk4. The cyclin E antibody was raised against C-terminal polypeptide of mouse cyclin E. The p27^{kip1} antibody was raised against His6–kip fusion protein (Agrawal *et al.*, 1996).

Preparation of Mouse Primary Fibroblasts (MPFs)

p27^{+/-} mice were the gift of Dr. Andrew Koff (Memorial Sloan-Kettering Cancer Center, New York, NY) (Kiyokawa *et al.*, 1996). Further intercross of the p27^{+/-} mice produced both p27^{-/-} and p27^{+/-} offspring. To prepare primary dermal fibroblasts, the bottom layer of skin was obtained from newborn mice, transferred to fresh sterile PBS containing 0.1% trypsin, and stirred in a small beaker for 30 min at room temperature. The PBS containing the subcutaneous tissue was filtrated through three layers of sterile abrasive cloth. The cells in the filtrate were grown in DMEM supplemented with 10% CS.

Preparation of Cell Extracts

Cultures were rinsed twice in ice-cold PBS, harvested by scraping, and collected by centrifugation at 12,000 × g for 6 s. The pellets were resuspended in lysis buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% Glycerol, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 2.5 µg/ml leupeptin, and 1 mM DTT), vortexed vigorously, and incubated on ice for 30 min. After centrifugation at 14,000 × g for 3 min, the supernatant was transferred into fresh tubes, and then stored at -70°C.

Immunological Assays

Immunoprecipitation was performed using 40 µg of total proteins from cell extracts. Specific antisera were incubated with protein A-agarose beads in 250 µl of the lysis buffer described above at 4°C for 2 h while shaking. The antibody-linked beads were washed once with lysis buffer, mixed with appropriate cell extracts, and incubated for 2 h at 4°C. For subsequent immunoblotting analysis, complexes bound to protein A-agarose were washed twice with lysis buffer, resuspended in 15 µl of SDS-PAGE loading buffer, heated to 95°C for 5 min, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The blots were incubated with the primary antibody for 1 h followed by a 1-h incubation with a

secondary antibody. The bound antibodies were visualized using the ECL detection system (Amersham) according to the manufacturer's instructions.

In Vitro Kinase Assay Using Purified Fusion Proteins

Specific antiserum (2 µl) and 30 µl of 25% protein A-agarose beads were added to 250 µl of lysis buffer, and the mixture was rocked for 2 h at 4°C, followed by the addition of 40 µg of total protein from cell extracts and an additional 2 h of mixing. Immune complexes were collected by centrifugation at 14,000 × g for 1 min and washed twice with lysis buffer, once with 2 × kinase reaction buffer (50 mM HEPES [pH 7.5], 10 mM MgCl₂, 5 mM MnCl₂, 10 mM DTT), resuspended in 8 µl of 1 × kinase reaction buffer containing 10 µCi [³²P]-ATP, 10 µM cold ATP, and 1 µg of GST-pRb. The mixture was incubated at 30°C for 30 min, resuspended in 10 µl of SDS-PAGE loading buffer, heated to 95°C for 5 min, and separated on an 11% SDS-polyacrylamide gel. Phosphorylated proteins were visualized by exposure to Kodak XAR-5 x-ray film for 30 min to 10 h and quantitated by exposure on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In Vitro Kinase Assay with Synthetic Peptide Substrates

For in vitro kinase assay using synthetic peptides, substrate peptides derived from the pRb sequence and previously demonstrated to reflect cdk4 phosphorylation sites were synthesized and purified by HPLC at Genemed Synthesis (South San Francisco, CA). The nomenclature utilized in this report corresponds to that previously reported (Kitagawa *et al.*, 1996).

Immune complexes were obtained as described above and resuspended in 8 µl of 1 × kinase reaction buffer containing 10 µCi [³²P]-ATP, 10 µM cold ATP, and 1 µg of synthetic peptide. The mixture was incubated at 30°C for 30 min, added with 10 µl of 2 × loading buffer, heated to 95°C for 5 min, and separated on a 16.5% T/6% C tricine-SDS-polyacrylamide gel. Tricine-SDS-PAGE was carried out essentially as described (Schagger *et al.*, 1990). A three-layer gel (stacking, 4%; spacing, 10%; and separating, 16.5%) was constructed, and phosphorylated protein was loaded and electrophoresed at 140 V until the tracking dye was within 3 cm of the bottom of the gel. The dye line was cut, and the remainder of the gel was fixed in a buffer containing 10% methanol and 10% acetic acid for 30 min. Phosphorylated protein was visualized by exposure to Kodak XAR-5 x-ray film in a Life Technologies magnetic film cassette for 2–24 h and quantitated by exposure on a PhosphorImager (Molecular Dynamics).

Tryptic Phosphopeptide Mapping

To examine the phosphorylation specificity of various immune complexes, GST-Rb was phosphorylated as described above in vitro. After ³²P-labeled samples were separated on an 11% SDS-polyacrylamide gel, the gel was dried, and the protein bands were cut out from individual lanes using a single-edge razor blade. The pieces of gel were placed in a microcentrifuge tube and incubated in 400 µl freshly prepared 50 mM ammonium bicarbonate, pH 7.4, for 5 min at room temperature. After removal of the paper backing, the pieces of gel were ground until they could pass through a disposable tip of a 200-µl adjustable pipette. To elute radioactive proteins from gel, 40 µl β-mercaptoethanol and 8 µl 10% SDS were added to the gel pieces, followed by boiling for 3 min and incubating for 90 min at room temperature on a shaker. After centrifugation, the supernatant was transferred to a new tube. Protein was recovered by adding 20 µg boiled RNase A and 250 µl ice-cold 100% trichloroacetic acid to the supernatant, followed by incubating the mixture for 1 h on ice. The precipitates were washed twice with 96% ice-cold ethanol, air-dried, resuspended in 50 µl of 50 mM ammonium bicarbonate,

pH 7.9, and digested for 10 h with 10 μ g trypsin (Boehringer Mannheim, Mannheim, Germany).

Peptides were separated on cellulose TLC plates (Merck, Darmstadt, Germany) using electrophoresis in formic acid, glacial acetic acid, and deionized water (50:156:1794/2 l) as first dimension and chromatography in butanol, pyridine, glacial acetic acid, and deionized water (75:50:15:60/200 ml) as second dimension. The plates were allowed to dry in a fume hood overnight, and the phosphopeptides were visualized by exposure to a phosphoimaging screen.

RESULTS

Cyclin D3 Activity Is Accompanied by a Reduced Association with p27^{kip1}

It has been demonstrated previously that cyclin D3/cdk4 kinase activity appears after density-arrested BALB/c 3T3 cultures are stimulated to undergo cell cycle traverse, 12–18 h after mitogenic stimulation (Dong *et al.*, 1998). We determined whether this activity was present in continuously cycling cells and its fate upon density-dependent growth inhibition (Figure 1A). It is clear from these data that cyclin D3 kinase activity is present in growing cells and down-regulated upon growth inhibition and then reappears after mitogenic stimulation. The cyclin D3 protein level, however, did not fluctuate in a proportional manner and appeared to remain relatively constant throughout the course of these growth transitions (Figure 1B). This observation suggested the cyclin D3-associated activity was, therefore, controlled at a level other than cyclin accumulation. Although it has been established that the level of cdk4 protein also remains constant in this cell line, the levels of p27^{kip1} are known to increase during density-dependent growth arrest (Figure 1C and Agrawal *et al.*, 1995, 1996), suggesting that the association of p27^{kip1} with cyclin D3/cdk4 may have contributed to the loss of activity observed in Figure 1A.

We also analyzed the level of cyclin D3 protein and its associated cdk and CKI subunits more closely in populations synchronized by density arrest and then mitogenically stimulated. As with the experiment shown in Figure 1, synchronized populations did not exhibit a significant change in the level of cyclin D3 protein during cell cycle traverse (Figure 2A), although the level of cyclin D3 activity was significantly elevated between 12 and 18 h (Figure 2B). This pattern is in striking contrast to that observed for cyclin D1 kinase activity, which increases concomitantly with an increase in cyclin D1 protein during serum-dependent cell cycle traverse (Agrawal *et al.*, 1996; Winston *et al.*, 1996). To examine the possibility that cyclin D3 activity was dependent on cdk4, the catalytic subunit responsible for cyclin D1 activity in murine fibroblasts, we prepared cell extracts that were depleted of cdk4 (Figure 2C). We then compared the mock depleted and cdk4-depleted extracts for the presence of cdk4

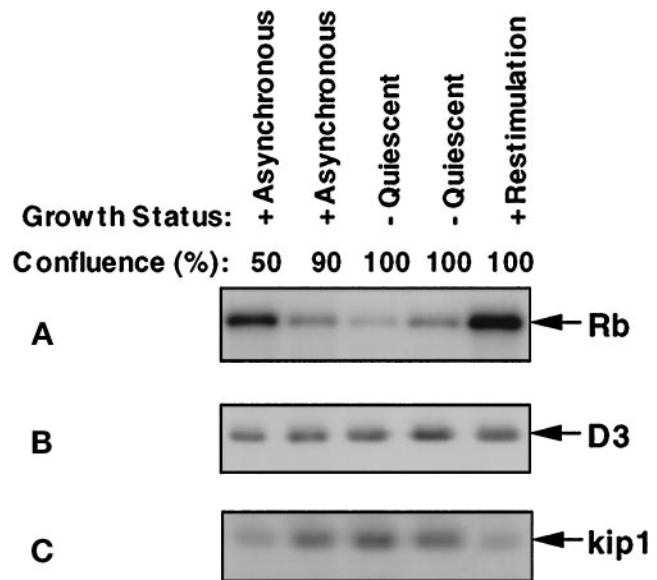


Figure 1. Cyclin D3-dependent kinase activities are inversely correlated with the level of p27^{kip1} in a cell cycle-dependent manner. To examine whether cyclin D3 activity was regulated during growth transitions, we utilized asynchronous cultures of BALB/c 3T3 cells in growth medium (DMEM) supplemented with 10% CS. Cultures were harvested at 24-h intervals during the growth cycle until density arrest was attained (lanes 1–4). After growth arrest, quiescent cells were mitogenically stimulated with growth medium supplemented with 10% CS + 10 ng/ml PDGF and harvested 18 h later (lane 5). (A) Portions of each cell extract, containing 40 μ g of protein, were subjected to immunoprecipitation with cyclin D3 antibody-linked beads. Immunoprecipitates were used to measure *in vitro* kinase activity using GST-Rb as a substrate. Phosphorylated GST-Rb was separated on 11% SDS-PAGE and visualized by autoradiography. Parallel samples of each cell extract, containing 40 μ g of protein, were subjected to Western blot analysis and probed with cyclin D3 antibodies (panel B) and p27^{kip1} antibodies (panel C). Proteins were detected using the ECL system as described in MATERIALS AND METHODS.

kinase activity and cyclin D3 kinase activity (Figure 2D). The data revealed that the cyclin D3 activity was quantitatively removed by immunodepletion of cdk4, demonstrating that the activity observed in Figure 2B reflected cyclin D3/cdk4 complexes.

It has recently been demonstrated, however, that ectopic expression of both cyclin D1 and cdk4 does not result in the formation of an active complex primarily as a result of a failure of the subunits to associate in the absence of serum factors (Cheng *et al.*, 1998). To examine the possibility that the lack of cyclin D3-associated activity was due to the absence of a catalytic subunit, the *in vivo* association of cyclin D3 with cdk4 was evaluated in extracts derived from quiescent cells and those mitogenically stimulated for various lengths of time. Figure 3A reveals that the amount of cdk4 protein remained constant throughout the course of the experiment. In addition, examination of cyclin D3-immune complexes for cdk4 protein revealed the

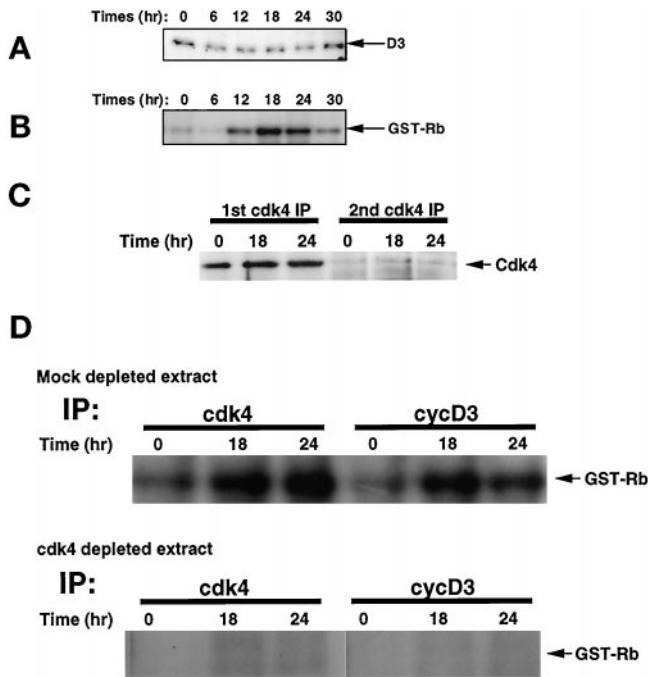


Figure 2. Cyclin D3 level and cyclin D3-dependent kinase activity throughout the cell cycle. Quiescent, density-arrested cells were mitogenically stimulated by the addition of fresh DMEM supplemented with 10% CS and 10 ng/ml of PDGF-BB. Cell cultures were harvested at the times indicated. (A) Portions of each cell extract containing 40 μ g of total proteins were subjected to Western blot analysis and probed with anti-cyclin D3 antibody. (B) Forty micrograms of protein from each cell extract were utilized for immunoprecipitation with cyclin D3 antibody-linked beads. Immunoprecipitates were used to measure *in vitro* kinase activity using GST-Rb as a substrate. Phosphorylated GST-Rb was separated on 11% SDS-PAGE and visualized by autoradiography. (C) Depletion of cdk4 was accomplished by immunoprecipitation of cdk4 from 40 μ g of protein derived from cell extracts prepared from quiescent cells ($t = 0$) or cells that were mitogenically stimulated for 18 or 24 h (indicated above individual lanes). The depleted extracts were subjected to a second immunoprecipitation with anti-cdk4, and the products of both rounds of immunoprecipitation were separated by SDS-PAGE and examined for the presence of cdk4 to determine the portion of cdk4 that was removed by this treatment. (D) Cyclin D3-associated and cdk4 kinase activities were determined in cdk4-depleted or mock-depleted extracts by incubating the immune complexes (indicated above individual lanes) with GST-Rb as a substrate. Products were separated by SDS-PAGE, and phosphorylated proteins were detected by autoradiography.

amount of cdk4 associated with cyclin D3 also remained proportional throughout the cell cycle (Figure 3B), indicating that the increased cyclin D3-associated activity was not the result of increased amount of binary complexes. Since the total level of p27^{kip1} was inversely proportional to the level of cyclin D3 activity, we also determined the amount of p27^{kip1} associated with cyclin D3. The results, shown in Figure 3, C and D, revealed that cyclin D3-associated p27^{kip1} declined upon mitogenic stimulation, reaching a minimum in the interval in which cyclin D3 activity ap-

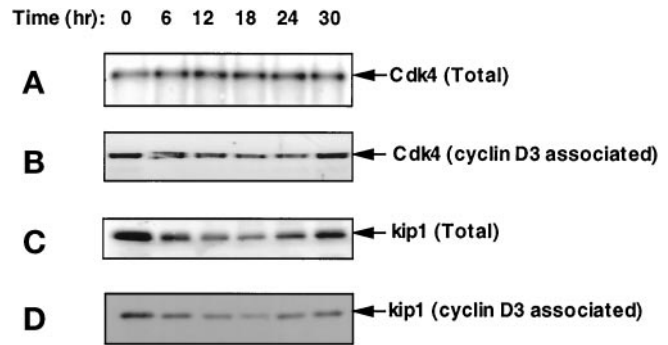


Figure 3. The level of total and cyclin D3-associated cdk4 does not fluctuate during cell cycle traverse, whereas the level of p27^{kip1} does. Quiescent, density-arrested cultures were mitogenically stimulated by the addition of fresh DMEM supplemented with 10% CS and 10 ng/ml of PDGF-BB and harvested at the times indicated. Cell extracts containing 40 μ g of total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with cdk4 antibody (panel A), and p27^{kip1} antibody (panel C). Parallel samples were subjected to immunoprecipitation with cyclin D3 antibody-linked beads. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with cdk4 antibody (panel B) or p27^{kip1} antibody (panel D), respectively.

peared. Thus, the amount of p27^{kip1} associated with cyclin D3 was inversely proportional to the cyclin D3 Rb kinase activity. Taken together, the data in Figures 1–3 support the hypothesis that p27^{kip1} plays a role in the regulation of the activity of cyclin D3/cdk4.

PDGF does not bring about cell cycle traverse of BALB/c-3T3 cells unless growth factors found in platelet-poor plasma (PPP) are also present; however, PDGF does bring about a reduction in the amount of p27^{kip1} (Agrawal *et al.*, 1996; Winston *et al.*, 1996). To determine whether PDGF was sufficient to cause a reduction in the amount of p27^{kip1} associated with cyclin D3, we examined cultures treated for 18 h with PDGF, 10% PPP, or PDGF + 10% PPP for the level of cdk4 and p27^{kip1}. All three treatments resulted in cultures that exhibited similar levels of total cdk4 protein and cyclin D3-associated cdk4 (Figure 4A), clearly indicating the absence of activity was not the result of a failure to assemble cyclin D3/cdk4 complexes. In addition, cultures stimulated with PDGF, either in the presence or in the absence of PPP, exhibited a reduced level of p27^{kip1} protein (Figure 4B). Consistent with this reduction, the portion of cyclin D3 protein that was not complexed to p27^{kip1}, as determined by Western blotting of cell extracts that had been immunodepleted of p27^{kip1} (Figure 4C), differed dramatically between quiescent and mitogenically stimulated cells. In extracts prepared from quiescent cells and cells treated with PPP alone, cyclin D3 was quantitatively removed by immunodepletion of p27^{kip1}, while similar levels of cyclin D3 were present in p27^{kip1}-immunodepleted extracts prepared from cells treated with

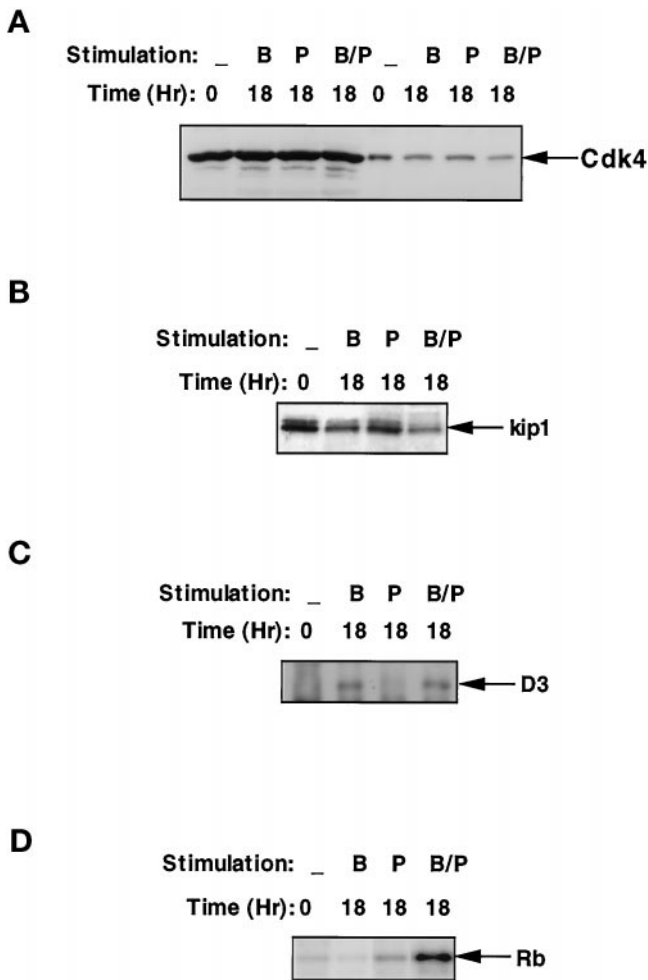


Figure 4. p27^{kip1} is not the only factor that can affect the activity of cyclin D3-dependent kinase. Density-arrested BALB/c 3T3 cultures were stimulated with DMEM supplemented with 10 ng/ml PDGF alone (lanes denoted with "B"), 10% PPP alone (lanes denoted with "P"), or 10 ng/ml PDGF and 10% PPP (lanes denoted with "B/P") for 18 h. Cell extracts were prepared from each culture, and portions containing 40 μ g of protein were utilized for all the following experiments: (A) Determination of the level of cdk4 protein. The total amount of cdk4 was determined with cell extracts (lanes 1–4), while the cyclin D3-bound cdk4 was determined by separating cyclin D3-immune complexes prepared from parallel samples (lanes 5–8). (B) Determination of the level of total p27^{kip1} protein. (C) Determination of the amount of cyclin D3 that was not complexed with p27^{kip1}. Cell extracts were first immunodepleted for p27^{kip1}, and the resulting supernatants were subjected to immunoprecipitation with cyclin D3 antibodies. The cyclin D3-immune complexes were separated by SDS-PAGE and immunoblotted with a cyclin D3 antibody. (D) Rb kinase activity associated with cyclin D3-immune complexes. Cyclin D3-immune complexes were prepared and utilized for *in vitro* kinase assays with a GST-Rb substrate. Phosphorylated proteins were separated on SDS-PAGE and visualized by autoradiography.

PDGF or with PDGF + 10% PPP. Cyclin D3 activity, however, was observed only in cultures treated with PDGF in the presence of PPP (Figure 4D), supporting

the hypothesis that the removal of p27^{kip1} from the cyclin D3 complex is required, but not sufficient, for activation of the cyclin D3/cdk4 Rb kinase. Thus, the induction of cyclin D3-associated Rb kinase activity during the mitogenic stimulation of BALB/c 3T3 cells appears to result from a two-step activation. The first step is the removal of p27^{kip1} from the ternary p27^{kip1}/cyclin D3/cdk4 complex, which is under the control of PDGF. The second step is the activation of the cyclin D/cdk4 complex, which is dependent on the presence of factors in PPP or regulated by traverse of the cell cycle.

p27^{kip1}-associated Rb Kinase Remains Elevated Throughout the Cell Cycle

Recently, p27^{kip1}-immune complexes were shown to have Rb-associated kinase activity in hematopoietic (Soos *et al.*, 1996) and epithelial (Hauser *et al.*, 1997) cells. We detected p27^{kip1}-specific kinase activity in BALB/c 3T3 fibroblasts extracts by assaying p27^{kip1}-immune complexes for phosphotransferase activity with GST-Rb as substrate. As shown in Figure 5A, a p27^{kip1} Rb kinase was present in quiescent cells and continued to be detectable in mitogenically stimulated cells, although this activity appeared to decrease somewhat after 9–12 h of stimulation. To determine the cyclin on which p27^{kip1} kinase activity was dependent, we immunodepleted either cyclin D1, D2, D3, cdk4, or cyclin E and tested the immunodepleted extracts for the presence of p27^{kip1} Rb kinase activity (Figure 5B). These experiments revealed that the p27^{kip1} Rb kinase was most dependent on cyclin D3 and cdk4 (Figure 5B).

To examine whether the dependency of p27^{kip1} kinase activity on cyclin D3 was periodic, p27^{kip1} activity was determined with or without prior cyclin D3 depletion in extracts prepared from quiescent cells and cells mitogenically stimulated for various times up to 18 h. Each extract was divided into two portions: the first utilized to measure p27^{kip1} Rb kinase activity (Figure 5C, lanes 1–6) and the second to measure cyclin D3 kinase activity (Figure 5C, lanes 7–12). In addition, the second group of extracts was assayed for p27^{kip1} activity after removal of cyclin D3 (Figure 5C, lanes 13–18). In accord with the results shown above, the Rb kinase activity associated with p27^{kip1} remained relatively constant, while the cyclin D3 Rb kinase activity was not observed until 12 h of mitogenic stimulation. In addition, the p27^{kip1} activity was uniformly removed by cyclin D3 immunodepletion throughout the cell cycle. Note that the dependence of p27^{kip1} activity on cyclin D3/cdk4 was observed (lanes 13–15) even though no cyclin D3 activity could be detected in cells until 12 h after stimulation (lane 13/14).

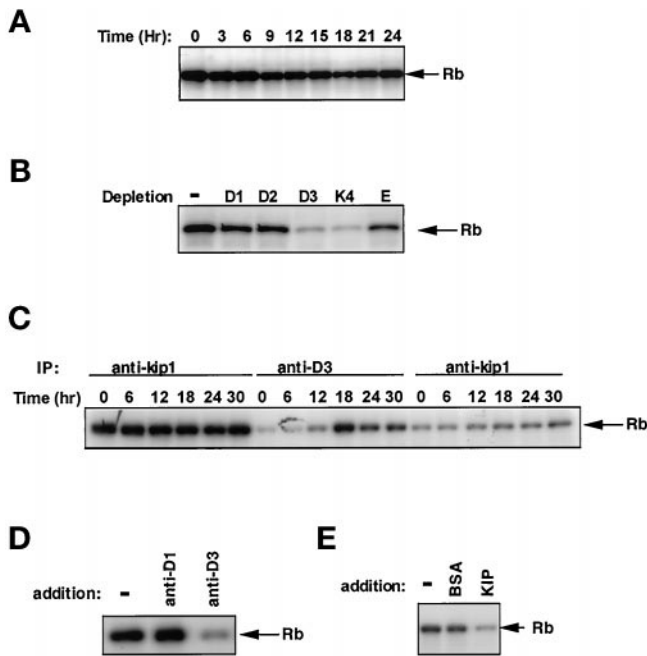


Figure 5. p27^{kip1}-associated kinase activity in BALB/c 3T3 fibroblasts. Quiescent, density-arrested cells were mitogenically stimulated by the addition of fresh DMEM supplemented with 10% CS and 10 ng/ml PDGF-BB, and then harvested at the times indicated. (A) Cell extracts containing 40 μ g of total proteins were subjected to immunoprecipitation with p27^{kip1} antibody-linked beads. The resulting immune complexes were tested for the presence of kinase activity in vitro with a GST-Rb substrate. Phosphorylated GST-Rb was separated on 11% SDS-PAGE and visualized by autoradiography. (B) Equal portions, containing 40 μ g protein, of an extract prepared from quiescent cells were subjected to immunodepletion with cyclin D1, D2, and D3, Cdk4, or cyclin E antibody-linked beads (indicated above individual lanes). The immunodepleted cell extracts were subjected to immunoprecipitation with p27^{kip1} antibody-linked beads, and immunoprecipitates were used to measure in vitro kinase activity using GST-Rb as a substrate. Phosphorylated GST-Rb was separated on 11% SDS-PAGE and visualized by autoradiography. (C) Cell extracts were subjected to immunoprecipitation with p27^{kip1} antibody (anti-kip1, left) and cyclin D3 antibody (anti-D3). After centrifugation, the supernatants without cyclin D3 were subject to second immunoprecipitation with p27^{kip1} antibody (anti-kip1, right). Immunoprecipitates were used to measure in vitro kinase activity using GST-Rb as a substrate. Phosphorylated GST-Rb was separated on 11% SDS-PAGE and visualized by autoradiography. (D) Immune complexes prepared with p27^{kip1} antibody and a quiescent cell extract were tested for their response to the addition of antibodies specific for cyclin D1 and cyclin D3. Immunoprecipitates were incubated in the presence or absence of 1 μ l of monoclonal cyclin D1 or D3 antibodies (as indicated above individual lanes) for 30 min in 250 μ l of the lysis buffer, washed twice with the lysis buffer, and used to measure in vitro kinase activity using GST-Rb as a substrate. Phosphorylated GST-Rb was separated on 11% SDS-PAGE and visualized by autoradiography. (E) The cyclin D3-associated Rb kinase activity is sensitive to inhibition by p27^{kip1}. Cyclin D3-immune complexes, prepared from stimulated cell extracts, were incubated at 37°C without any addition, with the addition of 100 ng BSA, or the addition of 100 ng recombinant His₆-p27^{kip1}. After incubation, each sample was washed once in lysis buffer and then washed once in kinase buffer before GST-Rb kinase activity was measured as described above.

From the results presented above, it is clear that untreated cells and cells treated with PPP contained a cyclin D3 population that is entirely bound to p27^{kip1} since immunodepletion with p27^{kip1}-specific antibody completely removed cyclin D3 (Figure 4C). However, cells treated with PDGF in the presence or absence of PPP had, in addition, a distinct pool of cyclin D3 that was not associated with p27^{kip1}. Since cyclin D3 immunodepletion resulted in the removal of p27^{kip1}-associated kinase activity, the p27^{kip1} Rb kinase must represent the former pool. The second pool of cyclin D3, not associated with p27^{kip1}, is responsible for the cyclin D3 activity, since cyclin D3 activity can be recovered from p27^{kip1}-depleted extracts prepared from stimulated cells. Because the p27^{kip1} Rb kinase is present and dependent on cyclin D3 in quiescent cells, when cyclin D3 kinase cannot be detected (Figure 5A), we determined whether the addition of cyclin D3 antibodies to p27^{kip1}-immune complexes could mask the Rb kinase activity (Figure 5D). The results demonstrate that inhibition of Rb kinase activity resulted from the addition of an antibody to cyclin D3 but not from the addition of an antibody to cyclin D1 and suggest that the function of cyclin D3 in a ternary p27^{kip1}/cyclin D3/cdk4 complex is extremely sensitive to the steric constraints imposed by antibody binding. The effect of anticyclin D3 antibody-directed inhibition was not specific for the antiserum that is shown, and similar results were also obtained with a monoclonal antibody to cyclin D3 (our unpublished observations). In addition, it is interesting to note that when recombinant p27^{kip1} was added to active cyclin D3/cdk4 complexes prepared from stimulated cells, the activity was greatly diminished (Figure 5E), demonstrating the active cyclin D3/cdk4 complex can be inhibited by p27^{kip1} in vitro.

P27^{KIP1} Alters the Cyclin D3/cdk4 Rb Substrate Specificity

Our data reveal that cyclin D3/cdk4-dependent kinase activity in mitogenically stimulated cells resided in two separate pools: one pool was not associated with p27^{kip1}, and one pool clearly was associated with p27^{kip1}. The cyclin D3/cdk4 activity that was not associated with p27^{kip1} could be readily immunoprecipitated and measured with an antibody to cyclin D3. The second pool of cyclin D3/cdk4 was associated with p27^{kip1} and did not display Rb kinase activity when immunoprecipitated or treated with antibodies to cyclin D3; however, this complex did display Rb kinase activity when prepared with an antibody to p27^{kip1}. The data in Figure 5D indicated that the association of p27^{kip1} with the cyclin D3/cdk4 complex resulted in a structural alteration such that the presence of cyclin D3 antibodies inhibited kinase activity. To investigate whether p27^{kip1} association may have

functional consequences, we sought to determine whether the association of p27^{kip1} with the cyclin D3/Cdk4 complex altered the *in vitro* substrate specificity with an Rb substrate.

Extracts prepared from density-arrested cells and from cells that had been mitogenically stimulated for 18 h were used to isolate p27^{kip1}- and cyclin D3-immune complexes, respectively, and these complexes were used to phosphorylate Rb *in vitro*. The phosphorylated Rb product from each reaction was analyzed by tryptic phosphopeptide mapping to compare the sites of phosphorylation. Figure 6 shows a comparison of the phosphopeptide maps obtained with p27^{kip1}-phosphorylated Rb (Figure 6A) and the cyclin D3-phosphorylated Rb (Figure 6B). The arrows in each panel indicate peptides that are differentially phosphorylated by the two complexes. Arrow 1 indicates a phosphopeptide that is greatly diminished in the phosphopeptide map from cyclin D3/cdk4-phosphorylated Rb, while arrow 2 indicates a phosphopeptide present in cyclin D3/cdk4-phosphorylated Rb (arrow 2) that is diminished in the Rb phosphorylated by the p27^{kip1}/cyclin D3/cdk4 activity (Figure 6A). These data clearly demonstrate that the association of p27^{kip1} with the cyclin D3/cdk4 altered the phosphorylation specificity, as determined by a change in the relative level of phosphorylation at two sites. The observation that one peptide was preferentially phosphorylated by each kinase complex indicated that the alteration was indeed related to specificity and not simply a consequence of reduced activity. It is unlikely that the difference in the specificity could be explained by the presence of a minor contaminating kinase species since immunodepletion of cdk4 results in the removal of both p27^{kip1}-associated (Figure 5B) and cyclin D3-associated (Figure 2C) activity. The differences in the phosphorylation specificity between p27^{kip1}/cyclin D3/cdk4 and cyclin D3/cdk4 were studied further in a second type of assay utilizing peptides with sequences derived from Rb protein. Figure 7 shows the phosphorylation of these individual peptides by the two different complexes. In the active complex containing p27^{kip1}, peptide G is utilized as a substrate more than twice as efficiently as peptide J, while in the active cyclin D3 complex (without p27^{kip1}), peptides G and J are equally active as substrates.

Restoration of *in Vitro* p27^{kip1}Rb Kinase Activity in Fibroblasts Derived from p27^{kip1}^{-/-} Mice Using Recombinant p27^{kip1}

To test whether p27^{kip1} complexes containing cyclin D3/cdk4 could be formed by the addition of recombinant p27^{kip1} protein, we utilized dermal fibroblasts isolated from newborn mice with a *kip*^{-/-} phenotype (Kiyokawa *et al.*, 1996). Density-arrested cultures were mitogenically stimulated with fresh medium contain-

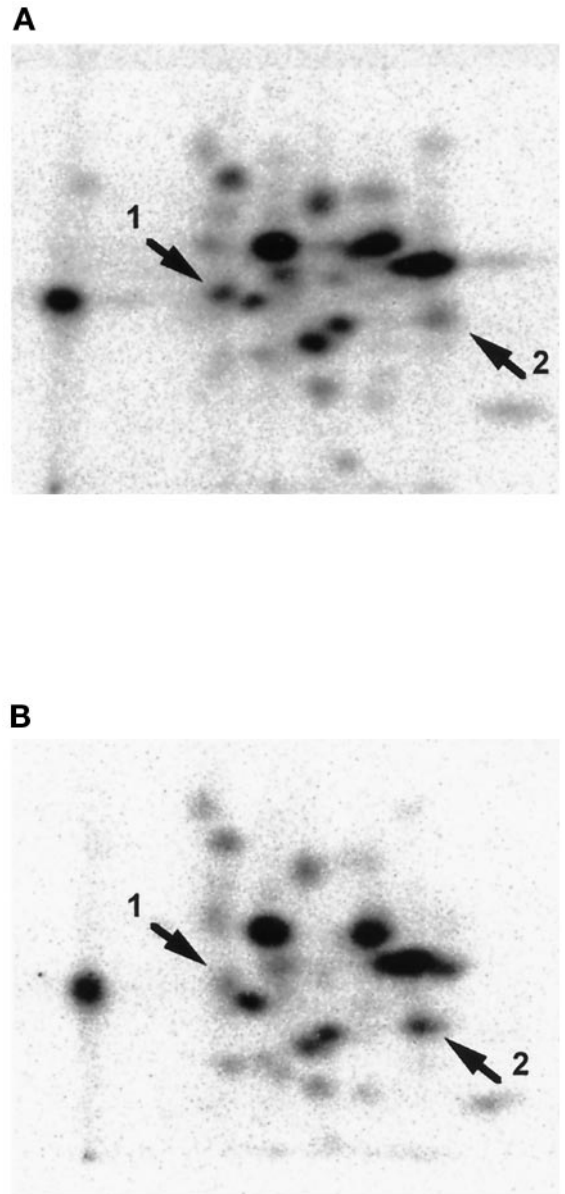


Figure 6. Tryptic phosphopeptide analysis of substrate specificities of p27^{kip1}-associated kinase and cyclin D3-associated kinase. Immune complexes prepared with p27^{kip1} antibody and a quiescent cell extract (A) and immune complexes prepared with cyclin D3 antibody and a stimulated cell extract (B) were utilized for *in vitro* phosphorylation of GST-Rb. Phosphorylated GST-Rb was purified with SDS-PAGE, digested with trypsin, separated on cellulose TLC plates, and visualized by autoradiography, as described in MATERIALS AND METHODS. To allow quantitative comparison of phosphopeptide maps, exposures were also performed with a Molecular Dynamics PhosphorImager. Arrowheads indicate peptides that were differentially phosphorylated by the two immune complexes.

ing 10% CS and PDGF (10 ng/ml). Extracts prepared from stimulated p27^{kip1}^{-/-} cells were cleared of possible p27^{kip1} cross-reacting materials and were then

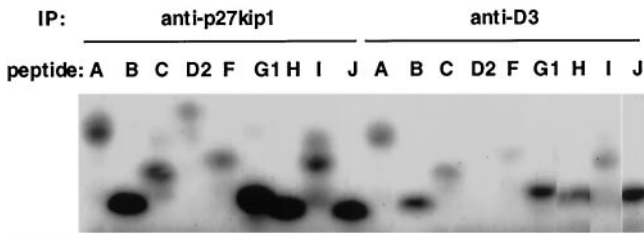


Figure 7. Phosphorylation of synthetic peptides, derived from Rb sequences, by cyclin D3/cdk4 and cyclin D3/cdk4/p27^{kip1}. Immune complexes prepared as outlined in the legend for Figure 6 were prepared and utilized to phosphorylate synthetic peptides in vitro. The peptide nomenclature corresponds to that reported previously (Kitagawa *et al.*, 1996). Phosphorylated peptides were separated on a 16.5% T/6% C Tricine-SDS-polyacrylamide gel and visualized by autoradiography as described in MATERIALS AND METHODS. To allow quantitative comparisons, dried gels were also exposed on a Molecular Dynamics PhosphorImager, and the resulting images were analyzed with ImageQuant software.

mixed with recombinant p27^{kip1} protein. After a 30-min incubation at 37°C, the exogenous p27^{kip1} protein was recovered by immunoprecipitation and assayed for p27^{kip1} Rb kinase activity. The data shown in Figure 8 demonstrate that incubation of p27^{kip1} in a cell extract containing active cyclin D3 complexes can result in the formation of a p27^{kip1} Rb kinase activity in a cyclin D3-dependent manner.

DISCUSSION

The data presented in this report demonstrate the cell cycle regulation of cdk4 activity by cyclin D3. The control of cdk4 activity through cyclin D1 has been extensively studied (Xiong *et al.*, 1991; Matsushime *et*

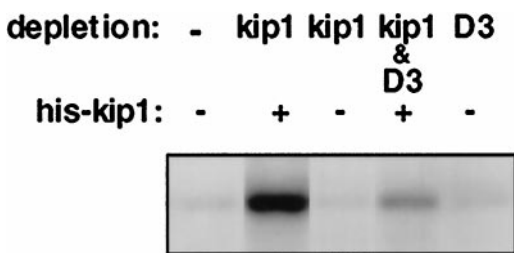


Figure 8. Restoration of p27^{kip1} kinase activity with recombinant p27^{kip1}. Dermal fibroblasts were isolated from newborn mice with a kip^{-/-} phenotype (Koff *et al.*, 1996). Density-arrested cultures were stimulated with fresh medium containing 10% CS serum and 10 ng/ml PDGF for 20 h. Cell extracts containing 40 µg of protein were subjected to immunodepletion with p27^{kip1}, cyclin D3, p27^{kip1} plus cyclin D3 antibodies, or used without any further treatment. Immunodepleted cell extracts were incubated in the presence or absence of recombinant kip protein for 30 min at 30°C and immunoprecipitated with p27^{kip1} antibody-linked beads. Immunoprecipitates were analyzed for in vitro kinase activity using GST-Rb as a substrate. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography.

et al., 1992, 1994; Won *et al.*, 1992; Baldin *et al.*, 1993; Quelle *et al.*, 1993; Winston and Pledger, 1993); however, there is considerably less work on its control through the additional D-type cyclins D2 and D3, which are also present in a variety of cell types (Kato and Sherr, 1993; Meyerson and Harlow, 1994; Polyak *et al.*, 1994a; Hauser *et al.*, 1997). We have shown that cyclin D3 protein was present in both growing and nongrowing BALB/c 3T3 cells, although D3-associated Rb kinase activity was only detectable in growing cells. This observation provides a marked contrast with cyclin D1, which is absent in quiescent cells and accumulates upon entering the growth cycle, correlating with increased activity (Agrawal *et al.*, 1996; Winston *et al.*, 1996). By examining cyclin D3 complexes for the presence of catalytic and inhibitory subunits, we have demonstrated that the periodic activity can be explained, in part, by the accumulation of p27^{kip1} in quiescent cells that binds quantitatively to cyclin D3/cdk4 complexes. Once inactivated in this manner, removal of p27^{kip1} is not sufficient to regain activity, which is accomplished only under culture conditions that promote S-phase entry. Curiously, while the cyclin D3/cdk4 complexes isolated from quiescent cells do not exhibit in vitro kinase activity, the corresponding p27^{kip1}-immune complexes do exhibit in vitro activity. Furthermore, this activity is dependent on cyclin D3/cdk4 and can thus be depleted with antibodies to either protein. These data suggested that p27^{kip1} may not only inhibit the cyclin D3/cdk4 complexes per se, but also modify the activity. This hypothesis was confirmed using both a recombinant Rb substrate as well as peptide substrates derived from Rb sequences known to be phosphorylated by cdk4. In both types of assays, p27^{kip1} complexes derived from quiescent cells and dependent on cyclin D3/cdk4 for activity were found to exhibit different phosphorylation specificities than the active cyclin D3/cdk4 complexes isolated from growing cells. Thus, these data are consistent with the notion that cyclin D3/cdk4 activity may serve dual functions during cell cycle traverse, the first in a binary form that is present during growth and a second function in a ternary complex with p27^{kip1} that modifies its substrate specificity.

Although periodic cyclin D1 activity has been demonstrated to primarily result from periodic cyclin D1 synthesis, it is clear from our data that cyclin D3 is present throughout the growth cycle in BALB/c 3T3 cells. Furthermore, we have found that the association of cyclin D3 with cdk4 also remains constant and thus can explain neither the loss of activity in quiescent cells nor the appearance of activity upon subsequent mitogenic stimulation. In contrast, the periodic association of p27^{kip1} with cyclin D3 renders this cdk inhibitor a likely candidate for mediating regulation of cyclin D3 activity. The Kip/Cip family of proteins was

initially described as proteins capable of inhibiting both cdk2 and cdk4 (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). Recent studies, however, directed at a more detailed understanding of the role of p27^{kip1} in regulation of D-cyclin/cdk4 activity, have provided data that, if not conflicting, remain to be reconciled. In accord with our results, Kato *et al.* (1997) found that cyclin D1/cdk4 complexes found in serum-starved RAT-1 cells were inactive due to associated p27^{kip1}, primarily resulting in complexes that did not undergo CAK-mediated cdk4 phosphorylation, a property that was previously described for p27^{kip1} *in vitro* (Matsuoka *et al.*, 1994). Furthermore, while both p27^{kip1} and p21^{Cip1} are active in promoting the assembly of cyclin D/cdk4 complexes, those complexes formed by p21^{Cip1} resulted in complexes with kinase activity, whereas those formed by p27^{kip1} did not (LaBaer *et al.*, 1997). The latter observation is again consistent with the notion that p27^{kip1} is a physiological inhibitor of cdk4. Other reports, however, have questioned the ability of p27^{kip1} to effect inhibition under physiological conditions, demonstrating active cyclin D/cdk4 activity in the presence of micromolar concentrations of p27^{kip1} (Blain *et al.*, 1997). Together, these findings leave the role of p27^{kip1} in the regulation of cyclin D/cdk4 complexes unclear. Our experiments indicate that the amount of cyclin D3/cdk4 activity in BALB/c-3T3 cells is inversely correlated with amount of p27^{kip1} bound to cyclin D3/cdk4 (Figures 1 and 2), and we demonstrated that the inhibitory effect of p27^{kip1} could be observed *in vitro* by addition of recombinant p27^{kip1} protein to cyclin D3 complexes (Figure 5E). Our data, therefore, support the hypothesis that p27^{kip1} is a physiological cdk4 inhibitor, at least in the case of cyclin D3. We note that much of the work reporting the effect of p27^{kip1} on cdk4 activity has primarily utilized cyclin D1 and cyclin D2 (Polyak *et al.*, 1994a; Blain *et al.*, 1997; Kato *et al.*, 1997; LaBaer *et al.*, 1997; Reynisdottir and Massague, 1997). Thus, our results may also indicate a difference in the specificity of p27^{kip1} with respect to a particular cyclin subunit.

Previous studies showed that both PDGF and PPP are required for stimulation of BALB/c 3T3 cells to enter the cell cycle, and our results indicate that activation of cyclin D3/cdk4 requires both mitogenic components. While it has been established that PDGF is sufficient to result in a reduction of the total level of p27^{kip1} (Agrawal *et al.*, 1996; Winston *et al.*, 1996), we demonstrate here that PDGF stimulation also results in the removal of p27^{kip1} from cyclin D3 complexes. However, p27^{kip1}-free cyclin D3/Cdk4 complexes generated by PDGF treatment alone were not active and, therefore, we concluded that a PPP-dependent factor(s) or cell cycle progression was required for activating the cyclin D3/cdk4 complex. This is consistent with the finding that CAK is required for activat-

ing cyclin D complexes after removal of p27^{kip1} (Kato *et al.*, 1997). However, since CAK has been shown to have constitutive activity (Matsuoka *et al.*, 1994), the lack of activity in the cyclin D3/cdk4 complexes that are free of p27^{kip1} suggests that another cell cycle-regulated event must be required to activate the cyclin D3/cdk4 complex at the appropriate time within the traverse of G1.

p27^{kip1}-associated kinase activity that could phosphorylate pRb but not histone H1 was first described in MANCA cells, a hematopoietic B-cell line (Soos *et al.*, 1996). The MANCA p27^{kip1} kinase activity appeared to be dependent on cdk6, since a cdk6-specific antibody was able to deplete 50% of the activity. Therefore, it was proposed that p27^{kip1}-associated kinase activity was derived from cdk6 and that p27^{kip1} was incapable of inhibiting the cyclin D/cdk6. Since cdk6 has not been found in BALB/c 3T3 mouse fibroblasts and since there is a constant high p27^{kip1}-associated Rb kinase activity in the cell throughout the cell cycle, we explored the derivation of the activity. In our experiments, more than 70% of the p27^{kip1} Rb kinase activity could be depleted by cyclin D3-specific and cdk4-specific antibodies, but was not affected by depletion with D1, D2, or E-specific antibodies (Figure 5B). Therefore, in BALB/c-3T3 fibroblasts, p27^{kip1}-associated kinase activity is derived from cyclin D3/cdk4 and not cdk6. Interestingly, in quiescent cells we could measure kinase activity in p27^{kip1}-immune complexes but not in cyclin D3-immune complexes. Further experiments indicated that the binding of a cyclin D3-specific antibody itself partially inhibited the kinase activity of the p27^{kip1} immunocomplex, suggesting that a conformational change may occur upon the binding of p27^{kip1} to cyclin D3/cdk4 (Figure 5D). It is possible that the sensitivity of the ternary complex to inhibition by cyclin D3 antibodies could also explain the loss of activity we observed after addition of recombinant p27^{kip1} protein to cyclin D3-immune complexes, although we note that several studies have demonstrated the inhibition of D-cyclin-dependent cdk4 activity in assays with purified proteins.

Because the p27^{kip1} Rb kinase activity did not change while the amount of ternary complexes decreased substantially (see Figures 3 and 4), we propose that only a portion of the complex (p27^{kip1}/cyclin D3/cdk4) is an active kinase in quiescent cells. This decline in the amount of ternary complexes is most likely a reflection of the reduced level of p27^{kip1} that occurs after mitogenic stimulation since the level of cdk4 and cyclin D3 remained relatively constant under these conditions. We note that these data could also be explained by a decreased specific activity of ternary complexes present in quiescent cells as compared with ternary complexes present in mitogenically stimulated cells. This latter possibility would nonetheless require an additional modification to con-

vert the lower-specific activity form to the higher-specific activity form. The reaction required to activate this ternary complex is not known and may represent a novel activation mechanism, since it is known that CAK is unable to phosphorylate cdk4 complexes containing p27^{k_{ip}1} (Matsuoka *et al.*, 1994).

In light of the role of p27^{k_{ip}1} as a growth suppressor, it might be expected that a p27^{k_{ip}1}-associated kinase would have different characteristics than a binary cyclin D3/cdk4 complex. To address whether such a difference might be detected as an alteration in substrate specificity, we performed tryptic phosphopeptide mapping of *in vitro* phosphorylated Rb and also compared catalytic efficiency with a panel of peptide substrates. We demonstrated that p27^{k_{ip}1}-associated kinase has a different substrate specificity than that of cyclin D3-dependent kinase in experiments using either an Rb substrate or peptides as substrates (Figures 6 and 7). The results from both types of experiments demonstrated an alteration in phosphorylation patterns between p27^{k_{ip}1}/cyclin D3/cdk4 and cyclin D3/cdk4 complexes, indicating that the binding of p27^{k_{ip}1} can result not only in inhibition of cdk activity but also modify the target specificity.

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