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Repression of p27^{kip1} Synthesis by Platelet-Derived Growth Factor in BALB/c 3T3 Cells

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We have investigated the regulation of p27^{kip1}, a cyclin-dependent kinase inhibitor, in BALB/c 3T3 cells during growth factor-stimulated transition from quiescence (G_0) to a proliferative (G_1) state. The level of p27^{kip1} protein falls dramatically after mitogenic stimulation and is accompanied by a decrease in cyclin E associated p27^{kip1}, as well as a transient increase in cyclin D1-associated p27^{kip1} that later declines concomitantly with the loss of total p27^{kip1}. Analysis of metabolically labelled cells revealed that cyclin D2, cyclin D3, and cdk4 were also partnered with p27^{kip1} in quiescent BALB/c 3T3 cells and that this association decreased after platelet-derived growth factor (PDGF) treatment. Furthermore, the decline in p27^{kip1} and reduced association with cyclin D3, initiated by the addition of PDGF but not plasma-derived factors, suggested that these changes are involved in competence, the first step in the exit from G_0. Synthesis of p27^{kip1} as determined by incorporation of [^{35}S]methionine was repressed upon mitogenic stimulation, and PDGF was sufficient to elicit this repression within 2 to 3 h. Pulse-chase experiments demonstrated the reduced rate of synthesis was not the result of an increased rate of degradation. Full repression of p27^{kip1} synthesis required the continued presence of PDGF and failed to occur in the presence of the RNA polymerase inhibitor 5,6-dichlorobenzimidazole riboside. These characteristics demonstrate that repression was a late effect of PDGF and was consistent with our finding that conditional expression of activated H-*ras* did not affect synthesis of p27^{kip1}. Northern (RNA) analysis of p27^{kip1} mRNA revealed that the repression was not accompanied by a corresponding decrease in p27^{kip1} mRNA, suggesting that the PDGF-regulated decrease in p27^{kip1} expression occurred through a translational mechanism.

Under normal circumstances, proliferation of mammalian cells is a highly controlled process. This control is achieved, in part, by growth factors and cytokines that exert either mitogenic and/or antiproliferative effects in a cell-specific manner (1). Binding of growth-regulatory ligands results in the activation of signaling pathways that trigger direct alteration of specific metabolic pathways as well as immediate and delayed changes in gene expression (65). Mitogenic stimulation initiates a program of sequential synthesis of proteins, termed cyclins, that complex with and activate cyclin-dependent kinases (cdks) (9, 29–31, 39, 52, 54, 64, 67), enzymes that modulate key regulatory events leading to progression through and transitions between different stages of the cell cycle (7, 13, 20, 21, 47, 55, 64). The order of cyclin synthesis during cell cycle traverse has been examined in a variety of cells, including T cells, macrophages, epithelial cells, and fibroblasts (16, 30, 31, 44, 45, 50, 52). The D-type cyclins, which complex with cdk4 and cdk6, are the first cyclins synthesized during the cell cycle. They are detected in mid-G_1 and are believed to function as Rb kinases (42, 44, 45, 67), thus overcoming the growth-suppressive functions of Rb to allow progression through the G_1/S transition (15, 25, 43, 61). The activation of D-type cyclins is followed by activation of cyclin E and cyclin A, both partnered with cdk2, and active at the G_1/S boundary and during S phase, respectively (10, 18, 31, 34, 35, 52). It has been reported cyclin E is also capable of activating cdk3 (41), a PSTAIRE kinase closely related to cdk2 and cdc2 (4). Rb has been identified as a physiologically relevant substrate for cyclin D1 activity during G_1. Similar physiological substrates and functions for cyclin E and cyclin A activities are relatively less characterized, although it has been recently demonstrated that cyclin E is required for initiation of DNA replication in *Xenopus* interphase extracts and suggested that cyclin A may control the switch from initiation to elongation (24).

Control of cyclin activity is known to occur through at least two mechanisms. The first involves the effects of growth-promoting signals on cyclin synthesis and perhaps on the assembly of cyclin-cdk complexes. For example, mitogenic stimulation leads to synthesis and accumulation of cyclin D1, allowing the activation of cdk4 or cdk6 (46, 67, 74). Accumulating evidence indicates the resulting cyclin D1 activity is responsible for the activation of cyclin E and cyclin A, perhaps through the activation of E2F, which is bound and rendered inactive by hypophosphorylated Rb (8, 51, 76). Once activated, cyclins are responsive to a second control mechanism, exemplified by antiproliferative signals such as transforming growth factor β (32, 58, 59, 62). The decrease in cdk activity through the action of transforming growth factor β is enforced primarily through the synthesis and activation of cdk inhibitor (CKI) proteins (6, 40, 59). A variety of CKI proteins that are present in a context appropriate for growth arrest have been identified; this finding has led to the notion that negative control of cyclin-cdk complexes represents an important means to regulate the cell cycle machinery (12, 16, 40, 59, 63). Importantly, inhibition afforded by CKIs is important not only to arrest growing cells but also to prevent growth of arrested cells.

CKIs fall into two broad categories: those specific for cdk4 or cdk6 (INK4s) and those able to inhibit all of the known cdks. In addition to being specific for cdk4 and cdk6, the INK4

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proteins thus far characterized, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, all have a highly conserved motif consisting of repeated ankyrin motifs (23, 66, 75). The significance of multiple proteins to inhibit cdk4 and cdk6 activities remains unclear, although it has been suggested that specific INK4 proteins may result in subtly different effects on activity or may respond to different environmental stimuli (68). The second class of inhibitors, including $p21^{Cip1}$ (also called WAF1, SDI, and CAP20) (11, 22), $p27^{kip1}$ (59, 69), and $p57^{kip2}$ (33, 41) all have a conserved region near the amino terminus which has been demonstrated to be necessary and sufficient for binding and inhibiting cdk2 (5, 19, 48). The carboxy-terminal region of $p21^{Cip1}$ allows it to associate with proliferating cell nuclear antigen a processivity subunit of the DNA polymerase δ holoenzyme (5, 19, 37, 49, 71). Binding of $p21^{Cip1}$ to proliferating cell nuclear antigen inhibits the processivity of polymerization but does not affect excision repair (17). Thus, it has been suggested that $p21^{Cip1}$ may serve to coordinate DNA replication with cell cycle progression.

In contrast to $p21^{Cip1}$, which is induced as cells enter the cell cycle (38), $p27^{kip1}$ is present at high levels in quiescent 3T3 fibroblasts and decreases after mitogenic stimulation (2, 60). An earlier study of BALB/c fibroblasts demonstrated that under conditions in which density-arrested cells are stimulated to undergo a single round of cell cycle traverse, a low level of $p27^{kip1}$ persists during interphase and increases only after cells complete mitosis and exit the cell cycle (2). $p27^{kip1}$ is found associated with cyclin E in a variety of cell types during quiescence (2, 16, 50, 58), and it has been proposed that removal of $p27^{kip1}$ from the cyclin-cdk complexes is an essential step for S-phase entry. T-cell antigen receptor signaling results in the continued presence of $p27^{kip1}$ in cyclin E complexes (16); its removal requires costimulation with interleukin-2, thus explaining the need for two signals to effect proliferation. Similarly, it has recently been demonstrated that both platelet-derived growth factor (PDGF) and platelet-poor plasma (PPP) are required in BALB/c 3T3 cells to attain minimal levels of $p27^{kip1}$ (72). Stimulation of BALB/c 3T3 cells with PDGF alone resulted in a decrease of $p27^{kip1}$, although it was not as dramatic as that observed in combination with PPP. Cyclin D1 sequesters $p27^{kip1}$, providing an alternative or additional mechanism for the activation of cyclin E-cdk2 in some cell types (58, 59, 63, 69). The decrease of $p27^{kip1}$ in T cells and fibroblasts and its sequestration by cyclin D1 jointly serve to reduce its inhibitory activity on cyclin-cdk activities, thus effecting growth regulation.

The relationship of $p27^{kip1}$ activity to its cellular concentration includes the regulation of the quantity of $p27^{kip1}$ and its association with other proteins. The regulation of $p27^{kip1}$ levels is rendered somewhat more enigmatic by the finding that the level of mRNA encoding this protein remains relatively constant throughout the cell cycle. Similarly, it has been reported that the rate of synthesis is constant; however, a recent report has demonstrated that the rate of $p27^{kip1}$ degradation is subject to periodic fluctuations (53). Since there is evidence that at least one role of $p27^{kip1}$ is to limit cyclin activities under non-growth-promoting extracellular conditions (16, 50, 58, 59), we have used quiescent BALB/c 3T3 cells to study the regulation of $p27^{kip1}$ synthesis and its association with G_1 cyclins.

We report our findings that $p27^{kip1}$ protein synthesis was halted after exposure to PDGF and that the accumulated protein was subject to mitogen-dependent degradation, although this effect is more subtle than what has been previously demonstrated. Concomitant with the change in $p27^{kip1}$ levels, there is a corresponding decrease in the $p27^{kip1}$ protein associated with cyclin E and an increase in its association with cyclin D1. We demonstrate that $p27^{kip1}$ is associated with cyclin D3 in quiescent BALB/c 3T3 cells and that this association is lost after mitogenic stimulation. These data support the hypothesis that $p27^{kip1}$ synthesis, at least in BALB/c 3T3 cells, occurs predominantly during quiescence, and repression of $p27^{kip1}$ synthesis as well as a change in its association profile with D-type cyclins is regulated by PDGF. Furthermore, since PDGF is sufficient to produce these changes, we suggest they are important in releasing quiescent cells for entry into the cell cycle.

MATERIALS AND METHODS

Cell culture. BALB/c 3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum in a humidified atmosphere (5% CO_2) at 37°C. Density-arrested quiescent cultures were prepared as previously described (57). Cells were harvested by scraping after adherent cells were washed twice with phosphate-buffered saline (PBS) and then were concentrated by centrifugation, and pellets were stored frozen.

Chemicals and reagents. Biological buffers, detergents, and inorganic molecules were purchased from Sigma (St. Louis, Mo.) or Fisher (Pittsburgh, Pa.). Cell culture media, antibiotics, and protein A-agarose beads were from Life Technologies (Gaithersburg, Md.), and PDGF B homodimer (PDGF-BB) was purchased from BioSource (Camarillo, Calif.). Nitrocellulose paper and reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad (Hercules, Calif.). Anti-rabbit horseradish peroxidase, enhanced chemiluminescence reagents, and radioisotopes were purchased from Amersham (Arlington Heights, Ill.). Autoradiographic film was purchased from Kodak (Rochester, N.Y.).

Preparation of cell lysates and Western blotting (immunoblotting). Frozen pellets were thawed on ice, resuspended in immunoprecipitation buffer [IP buffer; 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2), 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM orthovanadate, 0.5 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 μg of leupeptin per ml, and 1 mM dithiothreitol], vortexed vigorously, and stored on ice for 20 min. Following centrifugation to remove cellular debris, the crude cell extract (10 μg) was separated on SDS–10 or 12% discontinuous polyacrylamide gels, and the separated proteins were transferred to a nitrocellulose membrane by electrophoretic blotting. To identify $p27^{kip1}$ in specific cyclin complexes, immunoprecipitation was performed on 50 μg of protein as described previously (2). Prestained molecular weight markers (Sigma) were used to verify the efficiency of the transfer. Nonspecific binding was prevented by blocking the membrane in BLOTTO (5% dry milk in 1× PBS-Tween 20 [PBS-T; 1× PBS, 0.1% Tween 20]) and incubated with the primary antibody (1:1,000 to 1:5,000 dilution in PBS-T) for 1 h at room temperature. Primary antibodies used in this study were anti-cyclin D1 (sc-450), anti-cyclin D2 (sc-182), and anti-cyclin D3 (sc-183) from Santa Cruz Biotechnology; anti-cyclin E (directed against an amino-terminal peptide and directed against a recombinant human fusion protein); anti-cdk4 (directed against a carboxy-terminal peptide antigen [the kind gift of Ed Leof] and directed against the fusion protein [the kind gift of Steve Hanks]); anti-cdk2 (against a carboxy-terminal peptide and against a recombinant human cdk2 fusion protein); and anti-$p27^{kip1}$, described below. After being washed in PBS-T, the membranes were incubated with anti-rabbit–horseradish peroxidase (1:10,000) for 1 h, washed, and visualized by enhanced chemiluminescence as recommended by the supplier.

The $p27^{kip1}$ coding sequences were amplified by PCR from first-strand cDNA generated from RNA derived from quiescent BALB/c 3T3 cells. The oligonucleotide primers used for amplification spanned the sequence from nucleotides 218 to 235 (5′ primer) and 796 to 811 (3′ primer) (numbering is according to Toyoshima and Hunter [70]). The amplified fragment was cloned into the *Escherichia coli* expression vector pET30b (Novagen) and verified to encode $p27^{kip1}$ by dideoxy sequencing. Production of the recombinant His_6 tag fusion protein was induced by treatment of plasmid containing bacteria with 0.1 mM isopropyl-thiogalactopyranoside (IPTG), and the fusion protein was purified on an Ni chelating resin as recommended by the supplier. The fusion protein migrated with an apparent molecular mass of approximately 30 kDa and was found to be recognized by several commercial polyclonal antibodies against $p27^{kip1}$. Rabbit sera containing anti-$p27^{kip1}$ antibodies were generated by Rockland, Inc. (Gilbertsville, Pa.).

Metabolic labeling of cells with [^{35}S]methionine and immunoprecipitation. For labeling newly synthesized proteins, we used Dulbecco modified Eagle medium lacking cysteine and methionine (Life Technologies) and Tran^{35}S-label (Amersham). After incubation of cells as indicated in the figure legends, cells were rinsed with cysteine-methionine-free medium containing identical supplements and then incubated in 1.5 ml (for each 60-mm-diameter plate) of the same medium containing 100 μCi of Trans^{35}S-label. Labeling was allowed to proceed for 1 to 2 h as indicated in the figure legends. Cell extracts were prepared as described above, and incorporation was measured by scintillation counting of trichloroacetic acid-precipitated material (8% trichloroacetic acid followed by an

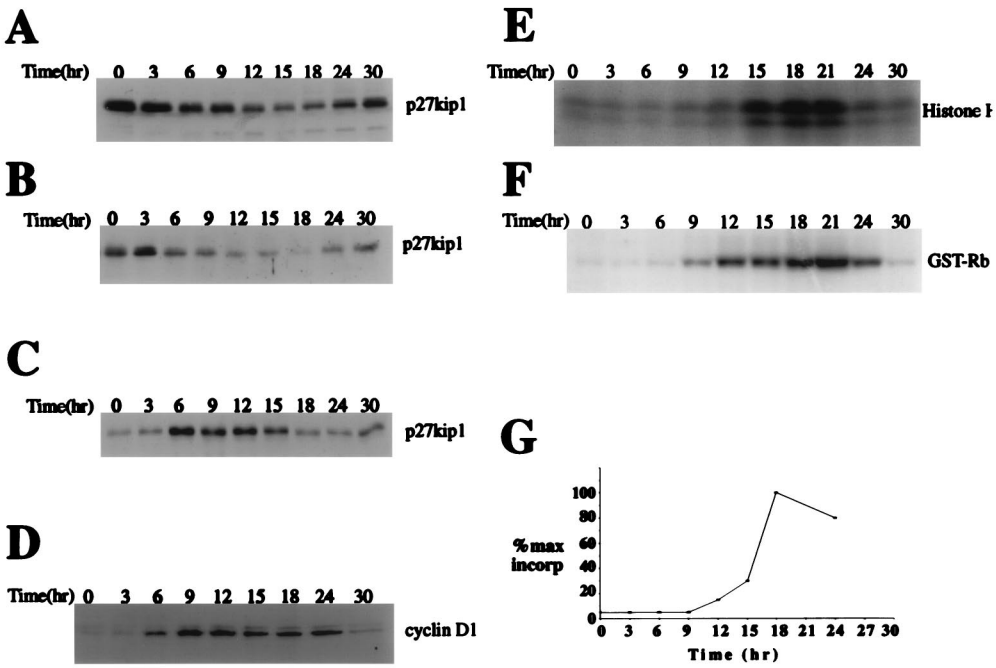

FIG. 1. Periodic association of p27^{kip1} with cyclin E and cyclin D1 during cell cycle traverse. Quiescent BALB/c 3T3 cells cultured in complete medium were stimulated to undergo cell cycle traverse by replacement of the spent medium with fresh complete medium (Dulbecco modified Eagle medium plus 10% fetal calf serum) containing 20 ng of PDGF-BB per ml. Extracts derived from cells harvested at times indicated above the lanes were assayed for total p27^{kip1} by Western blotting (A), p27^{kip1} content in cyclin E immunoprecipitates (B), and p27^{kip1} content in cyclin D1 immunoprecipitates (C). The level of cyclin D1 was determined and is shown in panel D. The kinase activities of parallel samples were determined with histone H1 (cyclin E [E]) and a recombinant glutathione *S*-transferase (GST)–Rb fusion protein (cyclin D1 [F]). DNA synthesis, measured by determining [^{3}H]thymidine incorporation after a 1-h pulse at the indicated times, is shown in panel G.

acetone wash) from a portion (5 to 10 μl) of the sample. Immunoprecipitations were conducted with material equivalent to 10^6 cpm per sample in 500 μl of IP buffer (final volume). Antibodies (or serum) were added at a dilution of 1:500 to 1:1,000, and the samples were incubated at 4°C with rocking from 1 to 6 h. After addition of protein A-agarose beads (Life Technologies), the samples were rocked for an additional 1 to 2 h. Immune complexes were harvested by pelleting, and for samples requiring second immunoprecipitations, the supernatants were removed to a fresh tube. Pelleted immune complexes were washed four to five times by resuspension in 1 ml of IP buffer followed by vigorous vortexing. The radioimmunoprecipitation (RIPA) buffer used for more stringent washing contained 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, and 50 mM Tris-Cl (pH 7.5). The final pellets were resuspended in 1× SDS-PAGE loading buffer, heated, and then separated on SDS–11% polyacrylamide gels. After electrophoresis was completed, gels were fixed in methanol-acetic acid (30%/10%) for 1 h and either dried immediately (for autoradiography) or equilibrated with dimethyl sulfoxide followed by dimethyl sulfoxide–2,5-diphenyloxazole (PPO) before drying (for fluorography). Dried gels were exposed on Kodak X-AR film and also on a Molecular Dynamics PhosphorImager for quantitation.

Northern (RNA) hybridization. Total RNA was isolated with RNAzol essentially as described by the manufacturer. Twenty micrograms of RNA was denatured by heating (95°C, 2 min) in 1× morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS [pH 5.5], 5 mM sodium acetate, 1 mM EDTA) containing 50% formamide, 5% formaldehyde, and 5% glycerol. Separation was accomplished with a 1.5% agarose gel made with 1× MOPS buffer and 0.3% formaldehyde. After separation, RNA was transferred to a nylon membrane (MSI) in a vacuum blotter (Bio-Rad). Transferred RNA was fixed to the membrane in a Stratalinker (Stratagene). Probes were generated by in vitro transcription of a pT3/T7α18 plasmid (Life Technologies) containing the p27^{kip1} cDNA with T3 RNA polymerase in the presence of [α-^{32}P]CTP, generating an antisense RNA probe covering the coding sequence. Hybridizations with 10^7 cpm of labeled probe were performed for 16 h in 5% SDS and 0.125 M sodium phosphate at 69°C. After hybridization, membranes were washed consecutively in 1% SDS with 25 mM sodium phosphate and 2.5 mM sodium phosphate at 69°C. Washed membranes were subjected to autoradiography and also developed on a PhosphorImager for quantitation.

RESULTS

Effect of mitogenic stimulation on p27^{kip1} and its association with cyclin D1 and cyclin E. To correlate the magnitude of change in the amount of p27^{kip1} during cell cycle traverse, we assayed both the level of total p27^{kip1} protein present in cells (Fig. 1A) and the extent of entry into S phase at various times after mitogenic stimulation (Fig. 1G). Incorporation of [^{3}H]thymidine was detected at 12 h and reached a maximum at 18 h after stimulation. The results clearly demonstrated that the level of p27^{kip1} protein was maximal in quiescent cells, began to decline between 3 and 6 h after stimulation, and continued to decrease, reaching a minimum 15 to 18 h after stimulation, corresponding to a point at which DNA synthesis had reached a maximum. The decline in p27^{kip1} protein was consistent with the difference that we had previously described between quiescent and logarithmically growing BALB/c 3T3 cells (2). p27^{kip1} began to reaccumulate between 24 and 30 h, consistent with completion of the cell cycle and reentry into quiescence (2).

The relative abundance of p27^{kip1} protein in quiescent cells has been suggested to provide regulatory restraint for progression through the cell cycle. This is thought to be the result of its cdk-inhibitory activity, which has been demonstrated in vitro with a variety of cyclin-cdk complexes. As cyclin E was previously identified as one such target which is complexed with p27^{kip1} in quiescent but not proliferating cells (2, 16, 50, 58, 72), we sought to determine whether the change in total p27^{kip1} levels during cell cycle traverse reflected the relative level associated with cyclin E. Immunoprecipitated cyclin E complexes were examined at various intervals during cell cycle traverse for associated p27^{kip1} protein by immunoblotting. The results in Fig. 1B established that the level of p27^{kip1} associated with cyclin E rapidly decreased between 3 and 6 h, thereafter continuing a slower decline, reaching a minimum level at 12 h, ahead of the time at which cyclin E activity could be detected (Fig. 1E). The level of cyclin E protein in these cells remained

relatively constant over this period (2), indicating the ratio of p27^{kip1} to cyclin E continually decreased. Thus, the decrease in cyclin E-associated p27^{kip1} largely mirrored the decrease in total p27^{kip1} but could not fully account for cyclin E activation, as the level of associated p27^{kip1} protein continually declined between 6 and 12 h, while the activity was apparent only after 12 h.

It has been reported that in several cell types, cyclin D_1-cdk4 complexes induced after mitogenic stimulation sequester p27^{kip1}, and it was believed that this association was necessary to allow activation of cyclin E-cdk2 complexes (26, 58, 60, 68, 72). The ability of p27^{kip1} to inhibit both the cyclin-activating kinase (CAK)-mediated cdk4 phosphorylation and the catalytic activity of cyclin D_1-cdk4 complexes (26) led to the suggestion that the cyclin D1-cdk4 activity appeared only after the inhibitory threshold had been exceeded by the availability of cyclin-cdk complexes (68, 72). To examine cyclin D1-cdk4 association with p27^{kip1} in BALB/c 3T3 cells, anti-cyclin D1 immunoprecipitates from cells harvested at various times after stimulation were examined for the presence of p27^{kip1} (Fig. 1C). We observed that p27^{kip1} was associated with cyclin D1 in a periodic fashion, peaking between 6 and 12 h after stimulation, declining between 12 and 15 h, and reaching a minimal level by 18 h, even though cyclin D1 protein persisted until 24 h (Fig. 1D). Cyclin D1 associated Rb kinase activity appeared between 9 and 12 h (Fig. 1F), a point at which substantial p27^{kip1} was still associated with cyclin D1, departing somewhat from the notion p27-cyclin-cdk complexes are catalytically inactive. Alternatively, it is possible a portion of the cyclin D1 remained uncomplexed and was the fraction able to exhibit kinase activity. It is interesting that the association between cyclin D1 and p27^{kip1} began at a point where the cyclin E-p27^{kip1} association was drastically reduced (compare the 6-h samples in Fig. 1B and C) and the decline in the cyclin D1-p27^{kip1} association mirrored the decrease in total p27^{kip1} observed during the interval of 9 to 15 h.

p27^{kip1} is synthesized in quiescent cells and is complexed with D-type cyclins. The experiments described above made extensive use of an antibody prepared against a full-length fusion protein derived from murine p27^{kip1} cDNA; therefore, to ascertain the efficacy and specificity of this antibody, we performed immunoprecipitations with extracts of quiescent cells metabolically labeled with [^{35}S]methionine (Fig. 2A). In addition to a 27-kDa species, four additional bands migrating with apparent molecular masses of between 30 and 36 kDa were present. To demonstrate that these species were associated with p27^{kip1}, we compared the immunoprecipitation patterns obtained with preimmune serum and with immune serum blocked with an excess of the recombinant p27^{kip1} antigen used for immunization. Neither of these control precipitations contained p27^{kip1} or the three additional bands, demonstrating that precipitation of these additional species was dependent on immunoprecipitation of p27^{kip1}. To probe the relative strengths of these associations, anti-p27^{kip1} immune complexes were washed in a more denaturing buffer (RIPA buffer). Use of the more denaturing wash regimen resulted in the loss of the 30- to 36-kDa protein-associated species, while the majority of p27^{kip1} was retained. Thus, these data clearly identify the additional species as p27^{kip1}-complexed proteins and not proteins bearing epitopes that were cross-reacting with our antibody preparation. Similar results have been obtained with an antibody directed against a C-terminal peptide sequence of p27^{kip1} (unpublished observations).

Since a similar examination of p27^{kip1} immune serum identifying comparable p27^{kip1}-associated proteins as cdks, has recently been published (63), we examined their identities in more detail (Fig. 2B). We found that p27^{kip1} in extracts prepared from quiescent cells was associated not only with cyclin E and cdk2 but with D-type cyclins and cdk4 as well (reference 72 and unpublished data). Anti-p27^{kip1} immune complexes derived from metabolically labeled extracts precleared with preimmune serum resulted in a profile identical to that of anti-p27^{kip1} immune complexes derived from naive extracts (Fig. 2B). Furthermore, our conditions resulted in quantitative recovery of p27^{kip1} (data not shown), and a second sequential immunoprecipitation with anti-p27^{kip1} gave a profile indistinguishable from that obtained with preimmune serum (Fig. 2B). Immunoprecipitation with antibodies specific for the D-type cyclins, cyclin E, cdk2, and cdk4 revealed that the coimmunoprecipitating species represent cyclin D1, D2, D3, and cdk4. Immunoprecipitation with anti-cyclin D1 yielded a species comigrating with the slowest of the complexed proteins, and a sequential immunoprecipitation with anti-p27^{kip1} yielded a profile similar to that obtained with a naive extract with the exception of the topmost band. Anti-cyclin D2, which also cross-reacts with cyclin D1, immunoprecipitated the second band, while anti-cyclin D3 and anti-cdk4 immunoprecipitated the upper and lower components of the fastest-migrating species, a closely spaced doublet. Additionally, neither cdk2- nor cyclin E-directed antibodies removed any of these species, and a sequential precipitation with anti-p27^{kip1} gave a profile like that observed in the second and third lanes. These data show while almost all of the labeled D-type cyclins were complexed with p27^{kip1}, there was a substantial amount of labeled cdk4

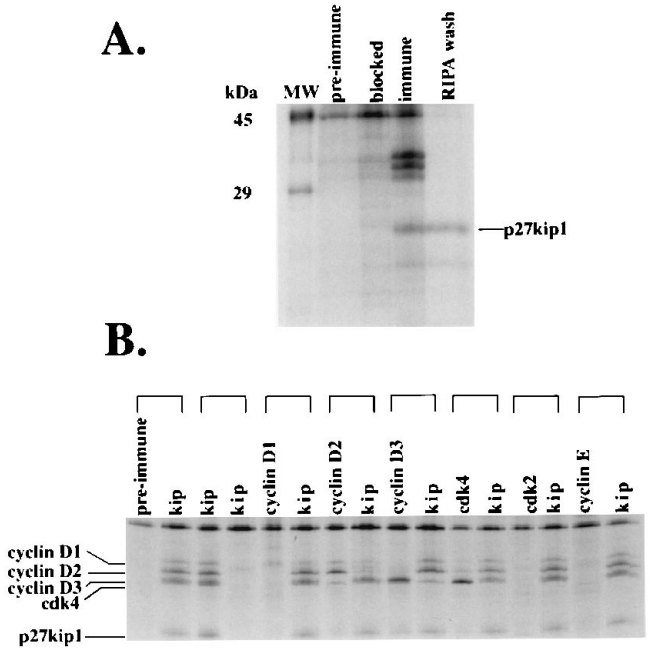

FIG. 2. Cyclins D1, D2, and D3 and cdk4 are coimmunoprecipitated with p27^{kip1}. Labeled cell extracts derived from quiescent cells cultured in [^{35}S]methionine for 1 h were subjected to immunoprecipitation with the indicated antibodies. (A) Labeled extracts immunoprecipitated with preimmune serum, anti-p27^{kip1} serum in the presence of 5 μg of recombinant p27^{kip1} protein, and anti-p27^{kip1} washed in IP buffer or in RIPA buffer. Lane MW, molecular weight markers. (B) Labeled extracts were subjected to sequential immunoprecipitations (brackets indicate the first and second immune complexes) with antibodies directed against p27^{kip1}, cyclins D1, D2, D3, and E, cdk2, and cdk4 (indicated above the lanes). The supernatants were saved, anti-p27^{kip1} serum was added, and the resulting complexes were harvested (lanes labeled kip). The immune complexes were separated on SDS–11% polyacrylamide gels, and the labeled proteins were detected by fluorography.

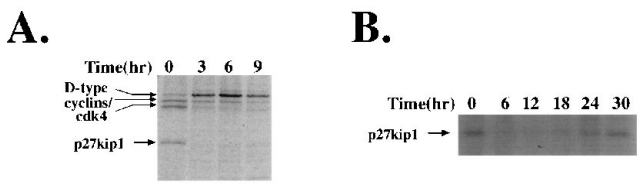

FIG. 3. Synthesis of p27^{kip1} is repressed upon mitogenic stimulation. Quiescent cells stimulated as described in the legend to Fig. 1 were metabolically labeled with [^{35}S]methionine for 1 h beginning at the times indicated above the lanes. Independent cultures labeled at 6-h intervals were used for the data shown in panel B. Extracts derived from these cultures were subjected to immunoprecipitation with anti-p27^{kip1} antibody, the resulting samples were separated on SDS–11% polyacrylamide gels, and labeled proteins were detected by autoradiography. Quantitation was accomplished by also exposing the dried gels on a Molecular Dynamics PhosphorImager.

not associated with p27^{kip1}. Similarly, the amount of labeled cdk4 associated with the D-type cyclins appeared to be less than the amount detected with the cdk4-specific antibody. The level of labeled D-type cyclins found in anti-cdk4 complexes was consistent with a relatively modest association of newly synthesized subunits. It cannot be excluded that the results that we obtained were due to the specific antibodies used, although independently derived anti-cyclin D1 and cdk4 antibodies gave similar results (data not shown).

Synthesis of p27^{kip1} is repressed in PDGF-stimulated cells. While the immunoblotting data presented in Fig. 1 clearly demonstrated a marked reduction in p27^{kip1}, it does not address the means by which the reduction in p27^{kip1} levels was achieved. Regulation of p27^{kip1} expression has not been definitively described, and studies from a variety of cell types have led to the belief several mechanisms may exist (68). To ascertain whether the amount of p27^{kip1} was being controlled through an alteration in the rate of synthesis, rate of degradation, or a combination thereof, we conducted metabolic labeling experiments on quiescent and proliferating cells (Fig. 3A). Clearly, the characteristic synthesis of p27^{kip1} found in quiescent cells declines within 3 h after mitogenic stimulation. There was no appreciable [^{35}S]methionine incorporation into p27^{kip1} until 24 h after exposure to mitogens (Fig. 3B). These data suggest that p27^{kip1} synthesis in BALB/c 3T3 fibroblasts, in contrast to what has been reported for other cell types, was strongly repressed upon entry into the cell cycle. The reappearance of p27^{kip1} synthesis at the later time points probably represents an induction of p27^{kip1} synthesis corresponding to reentry into quiescence. Indeed, the labeling observed at 30 h revealed that p27^{kip1} synthesis returned to a level comparable to that observed during quiescence. The synthesis of p27^{kip1} corresponded to the same period during which the majority of p27^{kip1} accumulation occurred in cells after undergoing a single round of cell cycle traverse (2). Not that there was a rapid loss of newly synthesized cyclin D3 associated with p27^{kip1} accompanied by an increase in the association of p27^{kip1} with cyclin D1 over the interval of 0 to 9 h (Fig. 3A). Certainly, these data are consistent with the notion that p27^{kip1} is found in a variety of cyclin complexes during cell cycle traverse and support a model in which mitogenic stimulation initiates the loss of cyclin D3-p27^{kip1} complexes concomitantly with an increase in cyclin D1-p27^{kip1} complexes.

The interval in which p27^{kip1} synthesis was repressed occurred early in cell cycle entry, promoting us to examine if the apparently decreased level of p27^{kip1} synthesis was shown in Fig. 4 required PDGF, PPP, or both. The entry into and traverse of the cell cycle by density-arrested BALB/c fibroblasts has been characterized to occur in a two-step, multi-growth factor-dependent manner referred to as competence-progression (56, 57). Transient exposure of density-arrested cells to PDGF generates a population able to proliferate in response to the presence of PPP. Density-arrested cells stimulated with 10% PPP fail to become competent and remain quiescent, while those treated with PDGF alone become competent but fail to undergo G_1 traverse (56, 57). As shown in Fig. 4, p27^{kip1} synthesis in 10% PPP remains relatively constant. Addition of PDGF, in the presence or absence of PPP, however, produces a rapid decline in accumulation of labeled p27^{kip1} apparent within 2 h of stimulation. Thus, PDGF was sufficient to both repress synthesis of p27^{kip1} and initiate the loss of newly synthesized cyclin D3 from p27^{kip1} immune complexes (Fig. 3A).

To determine whether the decrease in the rate of [^{35}S]methionine incorporation into p27^{kip1} was due to an increased rate of turnover, pulse-chase experiments were performed by metabolically labeling quiescent cells and then incubating them in a large excess of unlabeled methionine in the presence of either PDGF, PPP, or both. It is apparent from Fig. 5A the amounts of labeled p27^{kip1} remaining were similar in all three conditions for a 3-h chase. Thus, the decreased amount of labeled p27^{kip1} observed in the experiments depicted in Fig. 3 and 4 could not be accounted for by an increased rate of degradation. Quantitation of these data revealed there was an increase in the rate of degradation after 6 h when both PDGF and PPP were present (Fig. 5B). Even though there was a difference in p27^{kip1} stability between quiescent and actively proliferating cells, as in the presence of both PDGF and PPP, our data suggest that the change in stability becomes manifest subsequent to the repression of p27^{kip1} synthesis, providing a two-step mechanism for setting p27^{kip1} levels. These data also demonstrated that the association of p27^{kip1} and cyclin D3 synthesized in quiescent BALB/c 3T3 cells was subject to a larger decrease when PDGF was present than in PPP alone. It may be noted that cdk4 remains present in anti-p27^{kip1}' immune complexes, even in proliferating cells.

PDGF-mediated repression of p27^{kip1} synthesis requires prolonged exposure to PDGF. Activation of many components important in obtaining a proliferative response, such as *ras*, can be detected within minutes after addition of PDGF. In contrast, repression of p27^{kip1} was mediated 2 h after mitogenic stimulation. Quiescent BALB/c 3T3 cells require a 4-h treatment with PDGF to become competent, and it remains unknown what downstream effects require the longer (4-h) treatment with this growth factor. To ascertain whether the repression of p27^{kip1} synthesis required the continued presence of PDGF or could be induced with a brief treatment, p27^{kip1} synthesis was examined in cells stimulated with PDGF for various times (Fig. 6A). Removal of PDGF after 1 h gave incomplete repression of p27^{kip1} synthesis compared with the

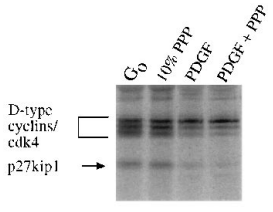

FIG. 4. PDGF repression of p27^{kip1} synthesis. Quiescent cells (G_0) stimulated for 2 h with either 10% PPP, PDGF-BB alone, or PDGF-BB plus 10% PPP were labeled with [^{35}S]methionine for 1 h. Extracts derived from harvested cells were subjected to immunoprecipitation with anti-p27^{kip1} antibody, and the immune complexes were separated on SDS-polyacrylamide gels. Labeled proteins were visualized by detection on a PhosphorImager.

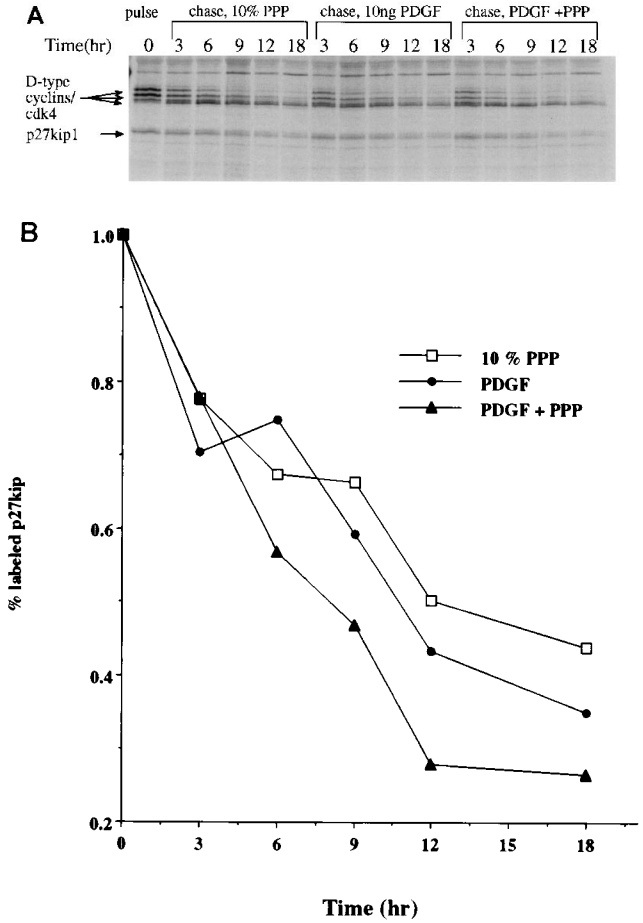

FIG. 5. Stability of $p27^{kip1}$ in cells under different mitogenic conditions. (A) Quiescent cells were labeled with [^{35}S]methionine for 1 h, after which the labeling medium was replaced with fresh medium (containing methionine and cysteine) containing 10% PPP, PDGF-BB, or PDGF-BB plus 10% PPP. Extracts derived from cells harvested at the times indicated above the lanes were subjected to immunoprecipitation with anti-$p27^{kip1}$ antibody. Labeled proteins present in the immune complex were separated on SDS-polyacrylamide gels and visualized by autoradiography. Dried gels were also exposed on a PhosphorImager to provide quantitative data. (B) Graphical representation of the quantitative data for the labeled $p27^{kip1}$ remaining in each sample.

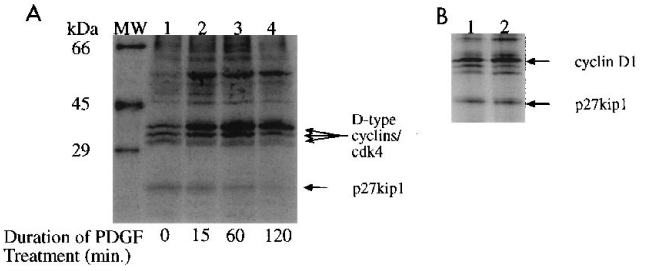

FIG. 6. Continued presence of PDGF is required for repression of $p27^{kip1}$. Quiescent cells were left untreated in 10% PPP (lane 1) or stimulated by the addition of PDGF-BB (lanes 2 to 4) for 15 min (lane 2), 1 h (lane 3), or the duration of the experiment (label 4). Cells transiently exposed to PDGF were washed twice and placed to medium containing 10% PPP. Two hours from the time of initial exposure to PDGF, the spent medium was exchanged for cysteine- and methionine-free medium containing 10% PPP in the presence (lane 4) or absence (lanes 1 to 3) of PDGF-BB and labeled as described in Materials and Methods. Cell extracts were analyzed for the presence of $p27^{kip1}$ by immunoprecipitation followed by SDS-PAGE and fluorography. Lane MW, molecular weight markers. (B) Expression of oncogenic *ras* fails to repress $p27^{kip1}$ synthesis. AC-3 cells, a BALB/c 3T3-derived cell line that expresses H-*ras* under the control of a dexamethasone-inducible promoter (71), were serum starved in the absence (lane 1) or presence (lane 2) of dexamethasone. After 16 h, both cultures were labeled as outlined in Materials and Methods. Labeled cells were harvested, and extracts were analyzed for $p27^{kip1}$ by immunoprecipitation followed by SDS-PAGE and fluorography. Note that the AC-3 cells exhibit a basal level of cyclin D1 higher than that observed in the parental BALB/c 3T3 cells, caused by a higher basal level of *ras* activity.

control in which PDGF was not removed. It was somewhat surprising that a short treatment did not result in repression, indicating that this effect of PDGF required completion of early response pathways. This pattern was in contrast to that observed for cyclin D1, for which increased synthesis is required only transient exposure to PDGF and was observed after a 15-min treatment.

The contrasting patterns for the temporal dependence on PDGF for repression of $p27^{kip1}$ and induction of cyclin D1 motivated us to explore the dependence of these two processes on the function of *ras*, which is activated as an immediate-early response to PDGF treatment. It has been demonstrated by this laboratory and others that cyclin D1 synthesis is induced in cells ectopically expressing oncogenic *ras* in the absence of mitogenic stimulation (3, 36, 73). Thus, we examined if activation of *ras* could cause repression of $p27^{kip1}$. Quiescent cells harboring an oncogenic *ras* under the control of a dexamethasone-inducible promoter were analyzed to determine $p27^{kip1}$ synthesis in the presence or absence of inducer, and we found that levels of $p27^{kip1}$ synthesis were similar under the two conditions (Fig. 6B).

The repression of $p27^{kip1}$ synthesis occurred as a relatively late event after PDGF treatment, was not mimicked by induction of H-*ras*, and required the continued presence of PDGF. These characteristics suggested that a factor(s) other than or in addition to *ras* was functioning 2 h after PDGF treatment but did not discern if early effects brought about by PDGF treatment were required. We tested for this possibility by blocking RNA synthesis with the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole riboside (DRB) and measured the decline in $p27^{kip1}$ synthesis after exposure to PDGF (Fig. 7). There was clearly a lack of repression if mRNA synthesis was blocked, indicating that an mRNA-encoded component(s) involved in $p27^{kip1}$ repression must be synthesized after PDGF stimulation. There was no induction of cyclin D1 synthesis in response to PDGF if mRNA synthesis was inhibited, consistent with previous reports demonstrating that increased levels of this protein after mitogenic stimulation results from an increased level of its mRNA.

The level of $p27^{kip1}$ mRNA is constant in BALB/c 3T3 cells. Interestingly, there was no effect of DRB on $p27^{kip1}$ synthesis in the absence of mitogenic induction. This observation sup-

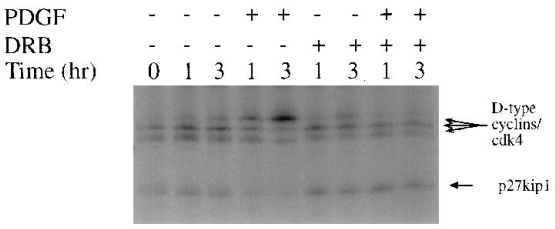

FIG. 7. Inhibition of mRNA synthesis prevents repression of $p27^{kip1}$. Quiescent cells were not treated or treated with 100 μM DRB. Cells were then either not stimulated or stimulated with PDGF-BB. Labeling was performed for 2 h after a 1- or 3-h incubation (indicated above the lanes), and cell extracts were examined for $p27^{kip1}$ by immunoprecipitation followed by SDS-PAGE and fluorography.

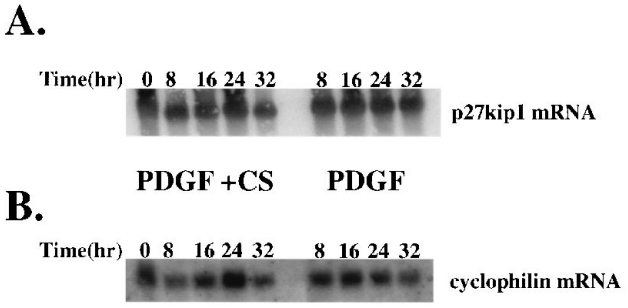

FIG. 8. The level of p27^{kip1} mRNA remains constant throughout the cell cycle. RNA prepared from cells stimulated from quiescence with PDGF-BB–10% fetal calf serum (CS) (left) or PDGF-BB alone (right) and harvested at 8-h intervals (indicated above the lanes) was separated on a 1.5% formaldehyde agarose gel and transferred to a nylon membrane. The membrane was subjected to hybridization with a ^{32}P-labeled probe specific for either p27^{kip1} (A) or cyclophilin (B). After washing, the membrane was subjected to autoradiography to detect the presence of specific mRNA.

ports the notion that p27^{kip1} mRNA is not limiting or subject to rapid turnover in quiescent cells. To probe directly whether the loss of p27^{kip1} synthesis was accompanied by transcriptional repression, Northern hybridizations were performed on mRNA isolated from quiescent and stimulated cells (Fig. 8). The results indicated that p27^{kip1} mRNA in BALB/c 3T3 cells appeared relatively constant, as was demonstrated in a variety of cell types (59, 69). We considered the possibility that transient repression of p27^{kip1} mRNA synthesis is sufficient to cause a decrease in synthesis of the protein, and therefore analysis of a progressing population may not reveal a decrease in mRNA. Cells stimulated with PDGF alone have lower p27^{kip1} protein levels than quiescent cells, and this reduced level of protein persists for periods extending past 20 h (72). Thus, such a treatment may result in a more homogeneous population blocked at a point that prevents reaccumulation of p27^{kip1}, perhaps through a lasting repression of synthesis. Such cells, however, fail to display a correspondingly reduced level of mRNA, and a constant level was observed for at least 32 h after PDGF treatment. Thus, while the examination of p27^{kip1} protein levels, with respect to both accumulation and net synthesis over different intervals, demonstrated repression resulting from PDGF treatment, this effect was not mediated through a curtailment of mRNA synthesis.

DISCUSSION

The role of p27^{kip1} in maintaining the quiescent state and regulating G_1 transit is currently described through its ability to inhibit the cyclin-cdk complexes that provide essential functions prior to and during S phase. The manner in which this protein is regulated through mitogenic signaling is not known. We have shown that the synthesis of p27^{kip1} was sharply reduced after mitogenic stimulation with PDGF. This reduced synthesis was not accompanied by a corresponding reduction in mRNA levels, indicating that the decreased p27^{kip1} synthesis is controlled at a posttranscriptional level, probably through a translational mechanism. It is likely that this repression represents a key feature in the exit from quiescence or formation of competence, permitting a passive rate of decline in the level of p27^{kip1} and supporting the idea that the continued synthesis of this inhibitor is required to maintain a quiescent state. Furthermore, PDGF-induced reduction in synthesis was accompanied by a change in the association pattern of with cyclin E and cyclin D1, generally consistent with previous studies of proliferating cells (16, 50, 59, 60, 63, 70). In addition, we describe a decrease in the level of newly synthesized cyclin D3 associated with p27^{kip1} following mitogenic stimulation. While our studies do not directly address the mechanism by which the rearrangement of existing p27^{kip1} is effected, our data support the hypothesis that this distribution is regulated, perhaps through cyclin synthesis, and not simply the consequence of a change in the concentration of p27^{kip1} protein.

A strong interaction of cyclin D1 and p27^{kip1} has been well studied in both murine macrophages and mink lung epithelial cells (26, 63) and more recently in Swiss 3T3 fibroblasts (60). As observed in the latter report, we demonstrated that the association of p27^{kip1} with cyclin D1 in BALB/c 3T3 fibroblasts is periodic, peaking between 6 and 12 h after stimulation and declining between 15 and 18 h. The decline in association occurred during a period in which the level of p27^{kip1} protein was rapidly declining, perhaps providing an explanation for the association pattern that we observed. The activity of cyclin D1 becomes apparent during the period in which the cyclin D1-p27^{kip1} association is maximal. We cannot exclude the possibility that a portion of the cyclin D1 remains in a form devoid of p27^{kip1}; however, preclearing with antibodies directed against cyclin D1 removed only a portion of the total p27^{kip1} present (unpublished observations). These observations certainly raise the possibility that cyclin D1 is not completely inhibited by p27^{kip1} in vivo, although previous studies have demonstrated that it potently inhibits D-type cyclin-cdk4 complexes in vitro (59, 69). The possibility that CKIs have additional roles has been suggested previously; the demonstration that p21^{Cip1} regulates cyclin A-cdk2 as a function of its molar concentration in the cyclin complex (77) and a recent report describing its ability to recruit p107, a member of the Rb family of growth inhibitors, into cyclin complexes (78) represent significant contributions to our understanding in this regard. Thus, it is possible p27^{kip1} is likewise recruited by cyclin D1-containing complexes not only to provide a reduction in free p27^{kip1}, but also to fulfill a functional role, perhaps modulating or participating in an activity of cyclin D1-cdk4.

In light of the foregoing discussion, the decreased level of cyclin D3 associated with p27^{kip1} after stimulation is noteworthy. Specific roles for cyclin D3 have not been described; however, it is reported to be induced during late G1 and S phase in a variety of cell types. This protein is induced during differentiation of myoblasts, erythroleukemic cells, and neuroblasts (27, 28). Our results indicate that its activity may be subject to p27^{kip1} regulation in quiescent cells. Since the newly synthesized cyclin D3 associated with p27^{kip1} decreases after stimulation, it might be surmised that a growth-promoting activity of cyclin D3 is being unmasked. Interestingly, it has recently been reported that p27^{kip1} immune complexes contain substantial Rb kinase activity and that cyclin D3 can be detected in those complexes (69). We have found similar activity in p27^{kip1}, immune complexes derived from quiescent BALB/c 3T3 cells, although we have not determined its functional significance.

Another significant conclusion from our data was provided by the comparison of labeled proteins associated with p27^{kip1} after PDGF stimulation in either the presence or the absence of PPP (Fig. 4). The distribution remains qualitatively similar in either condition, which strongly supports the notion that initiation of these events is a relatively early event in cell cycle entry and thus correlates with the formation of competence. Indeed, we are able to detect the shift of p27^{kip1} from cyclin D3 to cyclin D1 in as little as 4 h after treatment with only PDGF (unpublished data). It will be of interest to determine if this shift is mediated directly through PDGF or occurs as a consequence of a shift in cyclin synthesis.

The progressive loss of $p27^{kip1}$ from cyclin E complexes during G_1 transit reported here was similar to that previously described for T cells (50). This feature was speculated to reflect an important event in the activation of cyclin E in these cells. While additional work with human fibroblasts revealed that this progressive loss from cyclin E complexes was not a universal theme (52), the presence of $p27^{kip1}$ in cyclin E complexes derived from quiescent cells would provide a straightforward mechanism for regulating cyclin E activity. Our data reveal that the majority of $p27^{kip1}$ bound to cyclin E is lost within 6 h of stimulation, without any appreciable increase in associated kinase activity. A similar partial loss of cyclin E-associated $p27^{kip1}$ can be achieved by treatment with PDGF alone, without concomitant G_1 traverse or the development of cyclin E activity (72). However, in the absence of PPP, the decline in cyclin E-associated $p27^{kip1}$ ceases at an intermediate level severalfold higher than that observed in the presence of PPP. These observations raise the question of whether the portion of $p27^{kip1}$ lost during the first 6 h represents a molar excess of inhibitor in the cyclin E complexes, or whether the inhibition afforded by the cyclin E bound $p27^{kip1}$ was simply a fortification of another mechanism(s) holding cyclin E activity in check.

The reduced synthesis of $p27^{kip1}$ resulting from mitogenic stimulation was a somewhat unanticipated result. The demonstration PDGF was sufficient to elicit repression, combined with the $p27^{kip1}$ association with cyclins discussed above, provides a strong link between competence and $p27^{kip1}$. It appears that the inhibitor, which has been considered a major regulatory component of the cyclin-based cell cycle machinery, may be a major target of PDGF-stimulated pathways. In combination with increased degradation observed in actively proliferating cells (Fig. 6 and reference 53), these data support a two-step mechanism for maximal decline in $p27^{kip1}$ levels, an initial lowered rate of synthesis resulting from treatment with PDGF, and a subsequent increase in the degradation in the presence of PPP. Such a model is also consistent with a recent study demonstrating that PDGF causes a decline in $p27^{kip1}$ levels which is not as pronounced as that observed when PPP is present (72). The rate at which synthesis decreases in these cells after PDGF treatment (2 to 3 h) does not support a direct link between PDGF signaling and repression. Rather, it is more likely that a protein synthesized or activated as an early or immediate-early event after PDGF treatment is more directly responsible. This hypothesis was supported by several lines of evidence: the requirement for the continued presence of PDGF for repression to occur, the inability of oncogenic *ras* to provide maximal repression of synthesis, and the ability to preclude repression by inhibiting mRNA synthesis with DRB. This model predicts the existence of two important regulatory elements: a *cis* element contained on the $p27^{kip1}$ mRNA and a factor(s) able to recognize this element and effect a decrease in $p27^{kip1}$ synthesis. While this model is speculative, we note that a similar mechanism exists for mediating negative growth control induced by transforming growth factor β through the repression of a positively acting cyclin component, cdk4 (14). We are currently examining $p27^{kip1}$ sequences for the ability to impart a PDGF-induced repression. The identification of such a signal should allow the development of a screen for proteins able to bind this signal.

Finally, we caution that while the data in Fig. 3 and 5 strongly support our model that PDGF treatment leads to decreased $p27^{kip1}$ synthesis, there remains an untested possibility, albeit slight, that $p27^{kip1}$ is synthesized after stimulation but enters a pool distinct from the $p27^{kip1}$ synthesized during quiescence. This newly synthesized pool of $p27^{kip1}$ would have to be subject to extremely rapid degradation, which necessitates the existence of an additional factor able to distinguish between protein in these two pools and implies that $p27^{kip1}$ synthesized during quiescence is lost at a relatively constant rate.

A reduced level of $p27^{kip1}$ in a proliferative state has now been demonstrated for a wide variety of cell types. While examination of these studies may reveal primarily quantitative differences at first glance, there are important qualitative differences as well. For example, the observation that $p27^{kip1}$ is able to exert an inhibitory effect on a wide variety of cyclin-cdk complexes would require a masking component to be present throughout the growth cycle. The relatively low level of protein found at times of peak cyclin E activity in the fibroblasts that we examined in this study would preclude the necessity for such a component to mask its activity past the point at which $p27^{kip1}$ levels have reached their minimum. As cyclin D1-cdk4 complexes have been described as the major $p27^{kip1}$ "sink" during G_1 traverse, this latter alternative is concordant with the reports that cyclin D1 is present during G_1 and degraded thereafter, a finding also true for BALB/c 3T3 cells (17a). We cannot rule out the possibility of the existence of some additional factor(s) whose masking ability may be manifest after this period. The decline of $p27^{kip1}$ protein, in the absence of additional making components, therefore, would be a hallmark event for G_1 progression. Our study demonstrates that this decline is fueled, in part, by a strong and rapid repression of $p27^{kip1}$ synthesis by PDGF.

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