Differential Modulation of G1 Cyclins and the Cdk Inhibitor p27kip1 by Platelet-derived Growth Factor and Plasma Factors in Density-arrested Fibroblasts*

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The growth of nontransformed eukaryotic cells is controlled by environmental cues which govern the transition from G1 into S phase. In mammalian cells, growth regulatory signals from serum-derived growth factors are integrated during a late G1 event called the restriction point (1). Successful execution of this event commits cells to another round of DNA replication, at which time cell cycle progression becomes independent of extracellular mitogens.

Growth factor-stimulated proliferation is achieved, at least in part, by a modulation of the cell cycle machinery consisting of the cyclin-dependent kinases (Cdns) and their regulatory subunits (2, 3). While a single kinase, p34cdc2, is sufficient to drive progression through the major cell cycle regulatory points at the G1/S and G2/M transition phases in yeast (4), multiple distinct Cdc2-related kinases have been identified in higher eukaryotes (5–7). The involvement of many of these kinases in cell cycle regulation has yet to be established. However, Cdk4 becomes active as a retinoblastoma protein kinase during mid G1 (8), and overexpression of Cdk4 in epithelial cells reduces the requirement for serum-derived growth factors and confers resistance to TGF-β-mediated growth inhibition (9). Furthermore, Cdk2 rescues the growth-arrested phenotype of Cdc2-deficient Saccharomyces cerevisiae yeast mutants (5), and ablation of Cdk2 activity prevents the onset of DNA replication in mammalian cells (10, 11). Thus, growth stimulatory pathways initiated by extracellular signals must ultimately engage and activate one or more of the G1-specific Cdk proteins.

Cdk activation is positively regulated by periodic association with cyclin subunits (2, 3). Cdk4 complexes with the D-type cyclins, while Cdk2 primarily associates with cyclin E and cyclin A. Ectopic overexpression of either cyclin D1 or cyclin E accelerates progression through G1 and reduces the proliferative requirement for serum-derived growth factors (12–14). Conversely, abolition of cyclin D1 or cyclin E activity through the use of neutralizing antibodies or antisense oligonucleotides effectively blocks entry into S phase (15, 16). Ablation of cyclin A function also prevents DNA replication (17, 18) and disrupts the checkpoint control pathway that couples mitotic initiation to the completion of DNA synthesis (19). However, it appears that the cyclin A-Cdk2 complex may function at a point distal to restriction point traversal. Although the mitogen-dependent expression of cyclin D genes is well defined in mammalian cells (20–22), growth factor regulation of cyclin E and cyclin A is incompletely understood.

Cyclin interaction with its catalytic partner is necessary but not sufficient for kinase activation. Nonfunctional cyclin-Cdk complexes have been shown to accumulate in serum-stimulated senescent fibroblasts (23) as well as cells that have been growth inhibited by exposure to TGF-β (24), γ radiation (25), or agents which elevate intracellular levels of cAMP (26). Activation of assembled cyclin/Cdk holoenzymes is negatively regulated by direct interaction with Cdk inhibitory proteins, termed CKIs (27). Several of these inhibitors function as intracellular effectors of antiproliferative environmental signals. In S. cerevisiae, cell cycle arrest in response to mating pheromone α is mediated by FAR1, a protein which inactivates the yeast Cdc2 kinase (28). In mammalian cells, DNA damaging agents such as transforming growth factor; PDGF, platelet-derived growth factor; PPP, platelet-poor plasma.
as µ radiation induce the expression of p53, which transactivates the promoter of the Cdk inhibitor, p21cip1 (29). Furthermore, TGF-β-dependent inhibition of Cdk2 activation and S phase entry is mediated by the inhibitor, p27kip1 (30). Recently it has been shown that p27 expression is down-regulated after interleukin 2 stimulation of T lymphocytes (31), suggesting that Kip1 may serve as a common target of both positive and negative growth regulatory pathways.

Kip1 activity may also be regulated through association with cellular proteins such as Cdk4. p27kip1 associates with the cyclin D-Cdk4 complex in a cell cycle-dependent manner (32), and treatment of human keratinocytes with the antiproliferative agent TGF-β leads to a redistribution of p27kip from Cdk4 to Cdk2, correlating with an inhibition of Cdk2 activity and cell cycle arrest (33). Baculovirus-produced cyclin D2-Cdk4 complexes can facilitate activation of Cdk2 in vitro (34), suggesting that a sequestering of Kip1 by Cdk4 may also be an important component of the mitogenic response to environmental proliferative signals. However, this hypothesis has not been rigorously tested in vivo.

Balb/c 3T3 fibroblasts are a nontransformed mouse cell line that has been extensively characterized with regard to the proliferative requirements for specific serum-derived growth factors (34). Platelet-derived growth factor (PDGF) acts early in the cell cycle to stimulate the G0 to G1 transition and render cells competent to respond to progression factors contained in platelet-poor plasma (PPP). Sequential exposure of quiescent fibroblasts to PDGF and PPP is sufficient to stimulate traverse of the restriction point and initiate commitment to DNA synthesis. Using the Balb/c 3T3 fibroblast system, we have examined the molecular mechanisms by which growth regulatory signals such as PDGF and plasma factors cooperatively activate the Cdk2 kinase during late G1. Our results suggest that both a PDGF-dependent association of Kip1 with Cdk4 and a plasma-dependent reduction in Kip1 levels are essential for the activation of cyclin E- and cyclin A-dependent kinases.

MATERIALS AND METHODS

Cell Culture—Balb/c 3T3 mouse embryo fibroblasts (clone A31) were cultured in a water-jacketed incubator with a humidified atmosphere (5% CO2, 95% air) maintained at 37°C. Experimental cultures were grown in 100-mm Petri dishes using Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated bovine calf serum (Colorado Serum Company), 4 mM l-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin. Cells were used 3–4 days after density arrest. PDGF (BB) was purchased from Biosource International, Camarillo, CA, and plasma was obtained from the Southwest Blood Bank, Tampa, FL.

Immunoblots—Cultures were rinsed twice in ice-cold phosphate-buffered saline and scraped in 600 µl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glyc erol, 1 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 10 µg/ml glycophosphatase, 1 mM NaF, 0.1 mM sodium vanadate). Whole cell extracts were sonicated twice, 10 s each, and insoluble material was removed by centrifugation for 5 min at 12,000 × g. Lysates were stored 1–14 days at −70°C. For Western analysis, extracts were thawed on ice, and protein concentrations were determined by Bradford assay. 100 µg of protein/sample were boiled in 3 ml of Laemmli buffer (20% glycerol, 3% sodium dodecyl sulfate, 4% β-mer captoethanol, 10 mM EDTA; 0.05% bromphenol blue) and separated on a 7–12% SDS-polyacrylamide gel. Gels were electrophoretically transferred to nitrocellulose (Bio-Rad), and blots were probed with the following polyclonal antibodies: cyclin D1 (G. Peters, Imperial Cancer Research Fund), cyclin A (E. Leff, Mayo Clinic), cyclin E (Santa Cruz Biotechnologies), Cdk4 (S. Hansks, Vanderbilt University), or Kip1 (Pharmingen). Proteins were detected using the electrochemiluminescence system per instructions of the manufacturer (Amersham Corp.).

Immunoprecipitations and Kinase Assays—Balb/c 3T3 cells lysates were prepared exactly as described for the preparation of immunoblots. Kinase activities were immunoprecipitated from 100 µg of extract using rabbit polyclonal antisera (approximately 4 ha at 4°C). Immunoprecipitates were brought down with protein A-agarose (30 min at 4°C), and pellets were washed twice in immunoblot lysis buffer and twice in kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol), Pellets were incubated in 50 µl of kinase buffer containing 5 µM ATP, 100 µg/ml histone H1, and 0.1 µCi/ml [γ-32P]ATP for 5 min at 30°C. Reactions were stopped by boiling 3 min in 50 µl of 2 × Laemmli buffer, and separated on a 10% polyacrylamide gel. Histone phosphorylation was visualized by autoradiography (0.5–3 h at −70°C with intensifying screens) and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Inhibition experiments were performed by mixing extracts of proliferating cells containing active cyclin A-kinase complexes with inhibitor-containing extracts for 1 h at 37°C prior to immunoprecipitation of cyclin A. Density-arrested Balb/c 3T3 cells were stimulated with 20 ng/ml PDGF with 10% calf serum in Dulbecco’s modified Eagle’s medium for 18 h then harvested for proliferating cell extracts. Unless noted otherwise, extracts were mixed in a 1:1 ratio of protein (100 µg:100 µg).

RESULTS

Induction of Cyclin E- and Cyclin A-Associated Kinase Activities in Balb/c 3T3 Fibroblasts—To examine the expression of cyclin E and cyclin A after mitogenic stimulation, density-arrested Balb/c 3T3 fibroblasts were treated with PDGF, PPP, or both. Whole cell extracts were harvested after 16 h (for cyclin E) or 21 h (for cyclin A) and analyzed by immunoblotting (Fig. 1). Quiescent Balb/c 3T3 cells contained undetectable amounts of cyclin A protein, while low levels of cyclin E were typically observed in unstimulated fibroblasts. A weak induction of both cyclin A and cyclin E expression was observed after exposure to PDGF, a factor which stimulates cell cycle entry from quiescence but does not support traverse of the late G1 restriction point or subsequent entry into S phase. Although plasma by itself did not affect cyclin expression, the abundance of both cyclin A and cyclin E was increased to maximal levels in fibroblasts stimulated with a combination of PDGF and PPP. In contrast to the cyclins, Cdk2, the catalytic subunit of cyclins A and E, was relatively abundant in quiescent cells and underwent only a modest increase after growth factor stimulation (data not shown).

Although exposure to PDGF stimulated limited expression of cyclin A protein in nonproliferating cells, it was not sufficient to induce cyclin A-associated kinase activity (Fig. 1). Similarly, cyclin E-Cdk complexes immunoprecipitated from PDGF- or plasma-treated cells failed to phosphorylate histone H1 above basal levels. Identical results were also observed when Cdk2 was immunoprecipitated from fibroblasts treated with PDGF or plasma alone (data not shown). However, cyclin A- and
A-CDK complexes to measure inhibition in the following experiments.

Although exposure of quiescent fibroblasts to plasma alone had no effect on inhibitor levels, stimulation of density-arrested Balb/c 3T3 cells with either PDGF or a combination of PDGF and PPP resulted in a nearly identical reduction of free cyclin A/CDK inhibitory activity that was biphasic in nature (Fig. 2B). Activity of the CDK inhibitor(s) was rapidly and dramatically reduced by approximately 75% between 2 and 6h after treatment with PDGF or PDGF/PPP (Fig. 2C). After this time, free inhibitor activity continued to decline at a more gradual rate until it was completely abolished by 12–15 h poststimulation, a point coincident with S phase entry and normal cyclin A activation in those cells exposed to a full complement of growth factors. Down-regulation of free inhibitory activity persisted for at least 24 h after initial mitogen stimulation.

The majority of CDK inhibitory activity could be restored to growth factor-treated cell lysates when samples were heat-treated prior to incubation with proliferating cell extracts (Fig. 2B). These results suggest that a CDK inhibitor present in both quiescent and stimulated Balb/c 3T3 fibroblasts was reversibly masked by interaction with a heat-labile factor after exposure to PDGF: p27kip1 had previously been shown by others to be heat-stable in other cells (30). However, boiled extracts of stimulated cells were less effective in inhibiting the cyclin A-CDK complex than identically treated extracts of quiescent cells. Therefore, a decrease in the abundance of inhibitory factors may also contribute to the apparent down-regulation of inhibitory activity after growth factor treatment. Inhibition from boiled extracts decreased slowly starting 6–9 h after stimulation (Fig. 2C). However, in boiled extracts from cells treated with PDGF alone, decline in activity was transient, and the ability to inhibit cyclin A-CDK complexes returned to basal levels at later time points. In contrast, total cellular inhibitory activity continued to decline over the time course of the experiment when cells were exposed to both PDGF and PPP.

In order to identify the factor responsible for cyclin A/CDK inactivation, boiled lysates from quiescent and stimulated Balb/c 3T3 cells were preclarified with antibodies to p27kip1 prior to use in mixing experiments. Immunodepletion of Kip1 from extracts of both nonstimulated cells and cells treated with PDGF and PPP for 24 h eliminated essentially all inhibitory activity toward the cyclin A-CDK complex (Fig. 3A). Immunoprecipitation of [35S]methionine-labeled cells and Western blot analysis using the anti-p27 antibody indicated that this antibody did not recognize p21cip1 or p57kip2 (data not shown). These data suggest that Kip1 is the primary negative regulator of cyclin A-dependent kinase activity in Balb/c 3T3 fibroblasts.

Immunoblotting of whole cell extracts demonstrated that Kip1 expression was influenced by both PDGF and plasma factors (Fig. 3B). The p27 protein was relatively abundant in quiescent Balb/c 3T3 cells but was moderately reduced in response to treatment with PDGF. The PDGF-mediated reduction in Kip levels reached a nadir 12–18 h after stimulation; however, Kip1 expression increased by 24 h, correlating temporally with a return of total cellular inhibitory activity to the maximal basal level observed in nonstimulated cells (Fig. 2B). In contrast, fibroblasts stimulated with PDGF in the presence of plasma displayed a more dramatic and prolonged reduction in Kip1 expression. By 24 h after stimulation, cells treated with PDGF and PPP contained less than 50% of the p27 expressed in cells receiving PDGF alone (Fig. 3C). Kinetics of the plasma-dependent decline in Kip1 levels at later time points closely paralleled the reduction of inhibitory activity detected in boiled cell lysates. These data show that p27 expression is differen-
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**Fig. 3. Growth factor regulation of Kip1 expression.** A, Kip1 protein was immunodepleted from boiled extracts of quiescent and stimulated Balb/c 3T3 fibroblasts (cells were stimulated 21 h in 25 ng/ml PDGF-BB and 10% PPP). Depleted (+) and nondepleted (−) extracts were then mixed with lysates of proliferating cells, and cyclin A-associated histone H1 kinase activity was determined after immunoprecipitation. Control extracts of proliferating cells (lane 1) were mixed with lysis buffer. B, density-arrested cells were stimulated with 25 ng/ml PDGF-BB in the presence and absence of 10% PPP. At the time indicated, extracts were isolated, immunoblotted, and probed with a polyclonal antibody to Kip1. C, Kip1 protein was detected as in B, and levels in cells stimulated 24 h with PDGF or PDGF + PPP were quantitated by laser densitometry. Data points represent the average of three independent experiments.

Although Kip1 levels were dramatically reduced after stimulation with PDGF and PPP, under no condition was Kip1 expression completely abolished. The data presented in Fig. 2 indicate that the low level of Kip1 protein present in cells stimulated with PDGF and PPP was sufficient to inhibit the majority of cyclin A-dependent kinase activity when released from a masking factor by heat treatment. These results imply that the PDGF-mediated association of p27 with a heat labile silencing factor is likely to be essential for the removal of Kip1 inhibitory activity.

Requirement for Protein Synthesis during Down-regulation of Free Inhibitory Activity—To further examine the mechanism by which growth factors decreased the level of free inhibitory activity in Balb/c 3T3 cells, density-arrested fibroblasts were stimulated with PDGF and PPP in the presence of cycloheximide (Fig. 4A). Whole cell extracts of cells exposed to PDGF and PPP were immunoprecipitated from mixed extracts and histone H1 kinase activity was determined after immunoprecipitation. Control extracts of proliferating cells were mixed with lysis buffer (+, lane 1) or non-treated quiescent cell extract (Q, lane 2). Cyclin A was immunoprecipitated from mixed extracts and histone H1 kinase activity was determined. C, cycloheximide was added to quiescent Balb/c 3T3 cells stimulated with PDGF in the presence or absence of PPP as described in A. After 12 h, extracts were isolated, immunoblotted, and probed with polyclonal antibodies Kip1 and cyclin D1.

**Fig. 4. Effect of cycloheximide on regulation of Kip1.** A, 10 µg/ml cycloheximide (CHX) or 100 µM 5,6-dichlorobenzimidazole (DRB) were added to density-arrested Balb/C 3T3 cells stimulated with 25 ng/ml PDGF-BB in the presence and absence of 10% PPP. After 12 h, whole cell extracts were isolated and mixed with proliferating cell lysates. Cyclin A was immunoprecipitated from mixed extracts, and histone H1 kinase activity was determined. Control extracts of proliferating cells were mixed with lysis buffer (+, lane 1) or non-treated quiescent cell extract (Q, lane 2). B, density-arrested cells were treated with PDGF and PPP as above. At the indicated times after stimulation, cycloheximide was added to culture medium. Cells represented in lane 8 received no cycloheximide. 12 h after initial exposure to mitogens, extracts were prepared and mixed with proliferating cell lysates. Control extracts of proliferating cells were mixed with lysis buffer (lanes 1) or nontreated quiescent cell extract (Q, lane 2). Cyclin A was immunoprecipitated from mixed extracts and histone H1 kinase activity was determined. C, cycloheximide was added to quiescent Balb/C 3T3 cells stimulated with PDGF in the presence or absence of PPP as described in A. After 12 h, extracts were isolated, immunoblotted, and probed with polyclonal antibodies Kip1 and cyclin D1.

To determine whether protein synthesis inhibition affected the growth factor-mediated decrease in p27 expression, lysates from quiescent Balb/c 3T3 cells stimulated in the presence and absence of cycloheximide were immunoblotted and probed with...
FIG. 5. Inhibitor activity is sequestered by Cdk4 in quiescent Balb/c 3T3 cells. A, various amounts of boiled or nonboiled (no pre-treat) quiescent cell extracts were incubated with 100 μg of proliferating cell extract (stimulated 21 h with 25 ng/ml PDGF-BB and 10% PPP). Cyclin A was immunoprecipitated from mixed extracts, and histone H1 kinase activity was determined. Control extracts of proliferating cells (−, lane 1) were incubated with lysis buffer. B, cyclin A-dependent kinase activity measured in A was quantitated using a PhosphorImager and graphed as a percentage of maximum activity. Data points represent the average of three separate experiments. C, normal rabbit serum (NRS) or antibodies to cyclin E, cyclin A, or Cdk4 immobilized on protein A-agarose beads were used in immunoprecipitation of quiescent cell extracts. The beads and the supernatant were separated, boiled, and assayed for inhibition of cyclin A-associated kinase activity.

antibodies to Kip1 (Fig. 4C). After a 12 h stimulation, no detectable difference in Kip1 levels was observed in cells exposed to cycloheximide and PDGF or PDGF/PPP compared with cells treated with growth factors alone. In contrast, the addition of cycloheximide completely abolished PDGF-induced expression of cyclin D1 in the same cells. Thus, the effect of cycloheximide on mitogen-dependent reduction in Cdk inhibitory activity is not mediated at the level of Kip1 expression. These results demonstrate that the decline in p27 levels achieved after PDGF stimulation is not sufficient to effect a decrease in Cdk inhibition.

Interaction of Kip1 with Cdk4—The moderate reduction in p27 expression stimulated by PDGF during early time points was not accompanied by a corresponding decrease in total cellular inhibitory activity observed in boiled lysates. Failure to detect modulation of inhibitory activity could be due to the presence of saturating amounts of Kip1 in our mixing experiments. Although titration of nontreated extracts indicated that experiments using these lysates were performed within a linear range of inhibition, boiling of extracts could, in principle, release additional stores of inhibitor if a pool of Kip1 was sequestered in density-arrested cells. Dose response studies indicated that 100 μg of extract from nontreated quiescent Balb/c 3T3 cells was sufficient to maximally inhibit cyclin A-dependent kinase activity immunoprecipitated from 100 μg of proliferating cell extract (Fig. 5, A and B). However, an identical inhibition of the cyclin A-Cdk complex was obtained using only 50 μg of heat-treated cell extracts. These results suggest an excess of Kip1 exists in quiescent Balb/c 3T3 fibroblasts; however, approximately half of the functional p27 protein was sequestered in an inactive state.

Since many Cdk inhibitors were originally isolated by virtue of their ability to bind cyclin-Cdk complexes, potential candidate molecules which might sequester p27 in quiescent fibroblasts include the cyclin and Cdk proteins themselves. To test this possibility, extracts of nonstimulated Balb/c 3T3 cells were incubated with either normal rabbit serum or antibodies to cyclin E, cyclin A, or Cdk4 (Fig. 5C). The antibodies were then immobilized on protein A-agarose beads and removed from the lysate by centrifugation. Both the boiled supernatant and the boiled eluate of the immunoprecipitated pellet were then assayed for inhibition toward a cyclin A-Cdk complex. Of the antibodies used, the Cdk4 immunoprecipitate was found to contain the highest level of Cdk inhibitory activity, while a smaller portion of inhibitor was associated with cyclin E. The amount of inhibitory activity released from the Cdk4 complex after heat treatment was comparable to the activity remaining in the supernatant after immunodepletion of the Cdk4 kinase. These results suggest that the majority of sequestered inhibitor in density-arrested Balb/c 3T3 fibroblasts is associated with Cdk4.

In quiescent Balb/c 3T3 fibroblasts, approximately 50% of the total cellular Kip1 protein is sequestered, while the remainder exists in a free active state. However, within 12 h after stimulation with PDGF, all remaining p27 is sequestered. Previously, we have demonstrated that expression of the growth regulatory Cdk4 subunit, cyclin D1, is induced by PDGF during this time period (21). As cyclin D1 has been reported to associate with the Kip1 inhibitor both in vitro (8) and in vivo (34), we examined the role of D1 during PDGF-dependent down-regulation of free inhibitory activity. While Cdk4 levels did not fluctuate during cell cycle progression, cyclin D1 was undetectable in quiescent cells and increased dramatically upon growth factor stimulation (Fig. 6A). D1 expression was elevated within 3 h after exposure to PDGF/PPP, coincident with the onset of a decline in free Cdk inhibitory activity. Further increases in cyclin D1 levels were inversely proportional to the reduction of Cdk inhibition until after 12 h when D1 levels decreased. Immunoprecipitation of D1 after a 12-h exposure to PDGF coprecipitated both the Cdk4 kinase and p27 (Fig. 6B). These results suggest that the cyclin D1-Cdk4 complex may regulate the availability of functional Kip1 protein in growth-stimulated fibroblasts.

To examine the effect of a cyclin D/Cdk4/Kip1 interaction on free inhibitory activity, cyclin D1 and Cdk4 were immunoprecipitated from extracts of cells stimulated with PDGF or PDGF/PPP for various times. Both the supernatant and the pellet were then boiled and assayed for inhibition of cyclin A-depend-
plasma (Fig. 7) blasts that were stimulated with PDGF in the presence of the cyclin D1-Cdk4 complex has a greater effect on Kip1 availability in PPP-treated cells due to the lower abundance of the supernatant after immunodepletion of Cdk4 or cyclin D1. Alternatively, treated with PDGF and PPP.

100°C. Eluate of the boiled pellet was assayed for inhibition of cyclin kinase activity. As expected, heat treatment of either the cyclin D1 and Cdk4 were immunoprecipitated. Antibodies were immobilized on protein A-agarose beads and removed from the lysate by centrifugation. Pelleted beads were washed extensively and heated to 100 °C. Eluate of the boiled pellet was assayed for inhibition of cyclin A-dependent kinase activity. B, supernatants of cyclin D1 and Cdk4 immunoprecipitations described in A were boiled and assayed for inhibition of cyclin A/kinase activity. Immunoprecipitations of cyclin D1 and Cdk4 were performed on extracts of quiescent cells and cells that were stimulated for 21 h. C, kinase activity measured in A and B at 0 and 21-h time points was quantitated using a PhosphorImager. Data are expressed as a percentage of the decrease from control activity immunoprecipitated from proliferating cell extracts mixed with lysis buffer.

ent kinase activity. As expected, heat treatment of either the Cdk4 or cyclin D1 immunoprecipitates released an activity that efficiently inactivated the cyclin A-Cdk complex (Fig. 7A). Cdk inhibitory activity was complexed with the Cdk4 kinase for at least 21 h after exposure to PDGF. However, a marked reduction in the amount of inhibitor bound to Cdk4 was observed in cells stimulated in the presence of plasma, particularly at the later time points of 18 and 21 h. The identical phenomenon was observed when cyclin D1 was immunoprecipitated from cells treated with PDGF and PPP.

We next measured the inhibitory activity remaining in the supernatant after immunodepletion of Cdk4 or cyclin D1. Although a considerable amount of inhibitory activity associated with Cdk4 in quiescent fibroblasts, sufficient inhibitory activity remained in the lysate after the removal of either Cdk4 or D1 to maximally inhibit the cyclin A/Cdk enzyme (Figs. 5C and 7B). In contrast, Cdk inhibition was decreased when cyclin D1 or Cdk4 was depleted from extracts of PDGF-treated cells. However, the most striking reduction of inhibitory activity was observed after D1 or Cdk4 was cleared from lysates of fibroblasts that were stimulated with PDGF in the presence of plasma (Fig. 7C). These data demonstrate that, while Cdk4 interacts with p27 in both quiescent and stimulated fibroblasts, the cyclin D1-Cdk4 complex has a greater effect on Kip1 availability in PPP-treated cells due to the lower abundance of the inhibitor under this condition.

Interaction of Kip1 with Cyclin E in Growth-stimulated Cells—Our results suggest that, in the absence of plasma, p27 is sufficiently abundant to bind and inactivate cyclin-Cdk complexes assembled in response to PDGF stimulation. To determine whether Kip1 associated with cyclin E-dependent kinases in growth-stimulated fibroblasts, we immunoprecipitated cyclin E from extracts of cells treated with PDGF or PDGF/PPP. Immunoprecipitates were then immunoblotted and probed for the presence of p27 (Fig. 8). While Kip1 coprecipitated with inactive cyclin E-Cdk complexes in both quiescent and PDGF-treated cells, nearly undetectable levels of Kip1 complexed with the active cyclin E/Cdk holoenzyme in cells exposed to both PDGF and plasma. Consistent with this observation, a high level of Cdk inhibitory activity could be dissociated from cyclin E protein immunoprecipitated from lysates of PDGF-treated cells, while considerably less inhibitory activity interacted with cyclin E after exposure to plasma. These results support the hypothesis that most of the Kip1 protein remaining in PDGF/PPP-stimulated cells is titrated away from Cdk2 by the cyclin D1-Cdk4 complex. Effect of cAMP on Growth Factor-dependent Regulation of Kip1—Previously, it has been shown that growth-stimulated macrophages treated with cAMP analogs arrest during mid G1 due, at least in part, to a failure to down-regulate Kip1 expression (26). Balb/c 3T3 fibroblasts are also growth-inhibited by chronic elevation of intracellular cAMP levels (35). To examine the effect of cAMP on cyclin A/Cdk2 inhibitor activity in 3T3 cells, density-arrested fibroblasts were treated with PDGF or PDGF/PPP in the presence and absence of the cAMP-inducing agent cholera toxin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Whole cell extracts of stimulated fibroblasts were immunoblotted and probed with antibody to cyclin D1 (Fig. 9). PDGF-mediated expression of cyclin D1 was severely reduced by the addition of cAMP inducing agents to the stimulation medium. In contrast, elevation of cAMP levels did not prevent the normal decline in Kip1 protein levels after a 12-h exposure to PDGF (data not shown). In addition, cAMP antagonized the growth factor-dependent elimination of free Cdk inhibitory activity. These results suggest that cAMP does not interfere with the early component of Kip1 down-regulation in Balb/c 3T3 fibroblasts. However, because these cells eventu-
which govern the G0/G1 transition directly engage the cell cycle dependent kinases and their cyclin subunits. Previously we activated by these factors must ultimately impinge upon the cyclin-of the semitogens and their cognate receptors are incompletely derived progression factors (34). While the downstream targets the sequential and synergistic action of PDGF and plasma-dependent reduction of Kip1 at later time points could be overcome the threshold of inhibition by p27kip1 and allow kinase activation. However, addition of plasma to PDGF-treated cells stimulated maximal cyclin expression and an overall reduction of Kip1 levels, thereby promoting the activation of cyclin associated Cdkks. Thus, distinct proliferative signals from both PDGF and PPP converge upon common targets which regulate cell cycle progression from G1 into S phase in 3T3 cells. Therefore, sequestering of free Kip1 protein after growth factor stimulation is likely to be essential for the activation of cyclin E- and cyclin A-associated kinases. Previously it has been shown that cyclin D-Cdk4 complexes compete with cyclin E-Cdk2 and cyclin A-Cdk2 for binding of the Kip1 inhibitor in vitro (30). These data suggest that growth factor-mediated assembly of cyclin D/Cdk4 holoenzymes and consequent association with p27 may facilitate activation of Cdk2 later in the cell cycle. Consistent with this hypothesis, treatment of epithelial cells with TGF-β elevates the synthesis of p15 which displaces Kip1 from cyclin D4. p27kip1 is then available to bind and inhibit Cdk2 (33). In Balb/c 3T3 fibroblasts stimulated with PDGF either in the presence or absence of plasma, a substantial down-regulation of Kip1 was associated with cyclin D4. As a consequence of this Kip1/Cdk4 interaction, the cellular pool of inhibitory activity was depleted by greater than 50%. Our results suggest that cyclin D4 is an integral component of a mitogen-stimulated feed-forward mechanism which promotes activation of Cdk2 in Balb/c 3T3 cells.

DISCUSSION

Progression through the Balb/c 3T3 cell cycle is regulated by the sequential and synergistic action of PDGF and plasma-derived progression factors (34). While the downstream targets of these mitogens and their cognate receptors are incompletely defined, it is clear that the growth regulatory pathways activated by these factors must ultimately impinge upon the cyclin-dependent kinases and their cyclin subunits. Previously we have demonstrated that PDGF and other competence agents which govern the G1/G0 transition directly engage the cell cycle machinery via modulation of cyclin D1 expression (21). Here it is shown that PDGF and plasma factors cooperatively induce the cyclin E- and cyclin A-dependent kinase activities required for traverse of late G1 and the initiation of DNA replication. Activation of the PDGF receptor resulted in a limited induction of cyclin E and cyclin A expression that was not sufficient to overcome the threshold of inhibition by p27kip1 and allow kinase activation. However, addition of plasma to PDGF-treated cells stimulated maximal cyclin expression and an overall reduction of Kip1 levels, thereby promoting the activation of cyclin associated Cdkks. Thus, distinct proliferative signals from both PDGF and PPP converge upon common targets which regulate cell cycle progression from G1 into S phase in 3T3 fibroblasts. These results provide a molecular basis of how competence and progression factors might synergistically stimulate cell growth through unique modulation of the activities of specific Cdk kinases during the traverse of G1.

The amount of Kip1 available to bind and inactivate cyclin E- and cyclin A-associated kinases was regulated in a mitogen-dependent fashion by at least two distinct mechanisms: 1) active Kip1 protein was sequestered by Cdk4 which repressed inhibition toward cyclin A-Cdk complexes in lysate mixing experiments, and 2) total protein levels of Kip1 were decreased. Reduction of Kip1 expression occurred in two phases that were differentially regulated by PDGF and PPP. Exposure of quiescent fibroblasts to PDGF stimulated a moderate decline in p27 levels that began within 6 h of mitogen stimulation. Although in several cell types Kip1 expression is elevated in response to antiproliferative signals such as TGF-β (33), this early component of Kip1 elimination was not affected by inhibitors of Balb/c 3T3 cell growth such as cAMP and cycloheximide. However, the PDGF-mediated removal of free Cdk inhibitory activity was prevented under conditions of protein synthesis inhibition. Therefore, the down-regulation of Kip1 levels achieved after treatment with PDGF alone was not sufficient to ablate inhibition of the cyclin A/Cdk enzyme as determined in the in vitro assays. In contrast, stimulation of PDGF-treated cells with PPP resulted in a more pronounced decline in p27 expression, particularly at later time points when cyclin A-associated kinase activity was maximal. Thus, the greatest decrease in p27 was observed under conditions that stimulated DNA synthesis. This plasma-dependent reduction of Kip1 levels, together with the PDGF-mediated inactivation of Kip1 by Cdk4, critically limited the interaction of Cdk inhibitors with cyclin E and cyclin A kinase partners.

Dissociation of Kip1 from labile proteins after heat treatment revealed that enough inhibitor was present to reduce cyclin A-dependent kinase activity by 70–80%, even after maximal down-regulation of Kip1 expression in plasma-treated cells. Therefore, sequestering of free Kip1 protein after growth factor stimulation is likely to be essential for the activation of cyclin E- and cyclin A-associated kinases. Previously it has been shown that cyclin D-Cdk4 complexes compete with cyclin E-Cdk2 and cyclin A-Cdk2 for binding of the Kip1 inhibitor in vitro (30). These data suggest that growth factor-mediated assembly of cyclin D/Cdk4 holoenzymes and consequent association with p27 may facilitate activation of Cdk2 later in the cell cycle. Consistent with this hypothesis, treatment of epithelial cells with TGF-β elevates the synthesis of p15 which displaces Kip1 from cyclin D4. p27kip1 is then available to bind and inhibit Cdk2 (33). In Balb/c 3T3 fibroblasts stimulated with PDGF either in the presence or absence of plasma, a substantial proportion of Kip1 was associated with cyclin D4. As a consequence of this Kip1/Cdk4 interaction, the cellular pool of inhibitory activity was depleted by greater than 50%. Our results suggest that cyclin D4 is an integral component of a mitogen-stimulated feed-forward mechanism which promotes activation of Cdk2 in Balb/c 3T3 cells.

The affinity of Cdk4 for Kip1 in vitro is increased by association with a cyclin subunit (36). Cdk4 assembles combinatorially with three-dimensional-type cyclins which are differentially induced in various cell types. Transcripts for all three of the D cyclins are expressed during the G1 phase of the Balb/c 3T3 cell cycle; however, only cyclin D1 is up-regulated in response to PDGF (21). The kinetics of cyclin D1 increase temporally correlated with a reduction in free inhibitor levels after PDGF stimulation. Furthermore, D1 expression was first detected during a window of time when down-regulation of inhibitory activity is absolutely dependent on new protein synthesis. As cyclin D1 levels increased, removal of Cdk inhibitory activity became less sensitive to protein synthesis inhibition. Immunoprecipitation of cyclin D1 during peak expression coprecipitated Kip1 protein, and boiling of the immunoprecipitate released a considerable amount of Cdk inhibitory activity. Comparison of D1 and Cdk4 immunoprecipitates revealed a nearly identical pattern of association with the inhibitor, suggesting that cyclin D4 modulates Kip1 availability in growth-stimulated cells was primarily effected by complexes containing cyclin D1.

However, down-regulation of Cdk inhibition may not be strictly dependent on cyclin D1. Treatment of Balb/c 3T3 cells with cAMP-inducing agents inhibited D1 expression, but only weakly antagonized the removal of free Cdk inhibitory activity...
after PDGF stimulation. Presently, it is not known whether Cdk4 expressed under these conditions sequesters Kip1 in association with another cyclin partner, or whether the small amount of D2 induced in the presence of cAMP is sufficient to modulate Kip1 availability. However, Cdk4 was found to bind a large amount of inhibitory activity in quiescent cells despite the absence of cyclin D1 protein. Transcripts for both cyclin D2 and cyclin D3 are relatively abundant in density-arrested Balb/c 3T3 fibroblasts (21), and cyclin D2–Cdk4 complexes compete more effectively for Kip1 binding in vitro than do cyclin D1–Cdk4 complexes (36). Thus, the Cdk4/p27 interaction may be directed by various cyclin partners during different stages of the cell cycle. One consequence of Kip1 association with Cdk4 in quiescent cells may be to maintain Cdk4 in an inactive state until normal cell cycle progression is initiated in response to growth factor stimulation.

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