

Analysis of Cyclin D3-cdk4 Complexes in Fibroblasts Expressing and Lacking p27^{kip1} and p21^{cip1}

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Our studies examined the effects of p27^{kip1} and p21^{cip1} on the assembly and activity of cyclin D3-cdk4 complexes and determined the composition of the cyclin D3 pool in cells containing and lacking these cyclin-dependent kinase inhibitors. We found that catalytically active cyclin D3-cdk4 complexes were present in fibroblasts derived from p27^{kip1}-p21^{cip1}-null mice and that immunodepletion of extracts of wild-type cells with antibody to p27^{kip1} and/or p21^{cip1} removed cyclin D3 protein but not cyclin D3-associated activity. Similar results were observed in experiments assaying cyclin D1-cdk4 activity. Data obtained using mixed cell extracts demonstrated that p27^{kip1} interacted with cyclin D3-cdk4 complexes in vitro and that this interaction was paralleled by a loss of cyclin D3-cdk4 activity. In p27^{kip1}-p21^{cip1}-deficient cells, the cyclin D3 pool consisted primarily of cyclin D3 monomers, whereas in wild-type cells, the majority of cyclin D3 molecules were complexed to cdk4 and either p27^{kip1} or p21^{cip1} or were monomeric. We conclude that neither p27^{kip1} nor p21^{cip1} is required for the formation of cyclin D3-cdk4 complexes and that cyclin D3-cdk4 complexes containing p27^{kip1} or p21^{cip1} are inactive. We suggest that only a minor portion of the total cyclin D3 pool accounts for all of the cyclin D3-cdk4 activity in the cell regardless of whether the cell contains p27^{kip1} and p21^{cip1}.

Cell cycle progression is regulated by an ordered sequence of events that includes the activation of the cyclin-dependent kinases (cdk's) (37, 43). Activation of the cdk's requires both their association with cyclins, whose levels fluctuate during the cell cycle, and their phosphorylation at specific threonine residues by cdk-activating kinase, a constitutively expressed enzyme (46). cdk's also interact with a group of proteins collectively termed cdk inhibitors (CKIs); CKI levels, like cyclin levels, vary during the cell cycle and thus contribute to the timing of cdk activation (44, 45). Traverse of G₀/G₁ and entry into S phase is controlled by the sequential activation of complexes containing the D cyclins and cdk4 or cdk6, cyclin E and cdk2, and cyclin A and cdk2. This series of events is initiated by mitogen-induced increases in the expression of the D cyclins (D1, D2, and D3) and the formation of active cyclin D-cdk4 (or cdk6) complexes in mid-G₁ (30, 50). D cyclin-containing complexes phosphorylate the antioncogene Rb, as do cyclin E-cdk2 complexes, which become active in late G₁ due, at least in part, to decreases in CKI levels (16, 20, 22, 29, 49). When phosphorylated by these kinases, Rb no longer represses the activity of the E2F transcription factors, and a variety of E2F target genes, including those encoding cyclin E, cyclin A, and several DNA replication enzymes, are expressed (6, 13, 48). At this point, cells pass through the restriction point in late G₁ and, in a manner dependent on cyclin E-cdk2 and cyclin A-cdk2 activity, enter and traverse S phase (36).

Two classes of CKIs have been defined: the INK proteins, which block activation of D cyclin-containing complexes (41), and the Cip/Kip proteins, which target D-, E-, and A-containing complexes (21). The INK family consists of p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}, and the Cip/Kip family is composed of p21^{cip1}, p27^{kip1}, and p57^{kip2}. The INK proteins

interact with monomeric cdk4 or cdk6, whereas the Cip/Kip proteins associate with cyclin-cdk complexes. Of the Cip/Kip proteins, p27^{kip1} is thought to be the primary modulator of proliferative status in most cell types, where it functions to induce and maintain the quiescent state in response to suboptimal mitogenic stimuli and growth-inhibitory agents (10). In support of this role, numerous studies have shown that p27^{kip1} accumulates in serum-starved and density-arrested cells and that mitogen-triggered decreases in its levels are required for the resumption of G₀/G₁ traverse (1, 8, 14, 32, 34, 39, 40, 49). Moreover, as described by Coats et al. (9) and Rivard et al. (40), ablation of p27^{kip1} expression by antisense mRNA retards the entry of serum-starved cells into G₀, and due to higher percentages of cycling cells and the consequent enlargement of all internal organs, mice lacking p27^{kip1} are larger than their control littermates (18, 24, 33).

In addition to phosphorylating Rb, the D cyclins and their cdk partners also promote proliferation by a noncatalytic process that involves sequestration of p27^{kip1} (44). This model proposes that cdk2 activation is dependent on both a mitogen-induced reduction in overall p27^{kip1} levels and the titration of residual p27^{kip1} molecules by cyclin D-cdk complexes. In line with the latter function, previous studies suggest that antiproliferative agents such as transforming growth factor β and lovastatin induce the formation of inactive p27^{kip1}-bound cdk2 complexes by decreasing the size of the cyclin D-cdk reservoir (17, 39). In addition to sequestering p27^{kip1}, cyclin D-cdk complexes also facilitate cdk2 activation by titrating the levels of p21^{cip1} and p57^{kip2} (25, 44, 45). The role of these CKIs in mitogen-regulated cell proliferation is, however, unclear. p57^{kip2}, which is expressed in a tissue-specific manner, is thought to participate in differentiation and development (27), whereas p21^{cip1} has been linked primarily with radiation-induced growth arrest (15). Interestingly, levels of p21^{cip1} often increase after mitogenic stimulation, and regulation of cdk2 activity by p21^{cip1} in cycling cells (rather than restimulated quiescent cells) has been proposed elsewhere (19, 28, 34).

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While the capacity of p27^{kip1} to inhibit cdk2 activity is well established (44), its effects on the activity of the D cyclin-associated cdk's are controversial. Cheng et al. (7, 8), for example, found that antibody to p27^{kip1} removed cdk4 activity from cell extracts and that ablation of p27^{kip1} expression reduced the association of cyclins D1 and D2 with cdk4. These data suggest that p27^{kip1} acts as an enabler rather than an inhibitor of cdk4 activity and provide a mechanism by which D cyclin complexes can simultaneously fulfill their sequestration and enzymatic requirements. Consistent with the data of Cheng et al. (7), Blain et al. (3) reported that in vitro-assembled complexes containing cyclin D2, cdk4, and low levels of p27^{kip1} were catalytically active. However, in this study, cyclin D2 efficiently interacted with cdk4 in the absence of p27^{kip1}. Thus, according to these findings, p27^{kip1} neither prevents nor promotes cdk4 activity. On the other hand, LaBaer et al. (25) found that p27^{kip1} stabilized the interaction of cdk4 with cyclins D1, D2, and D3 and that ectopically expressed p27^{kip1} inhibited cyclin D1-cdk4 activity regardless of expression level. Repression of cyclin D1-cdk4 activity by p27^{kip1} has also been observed in recombinant systems (47), in fibroblasts inducibly expressing p27^{kip1} (52), and in macrophages treated with agents (e.g., cyclic AMP analogs) that increase endogenous p27^{kip1} levels (23). Whether the conflicting results obtained in past studies reflect differences in cell type, assay conditions, or other factors is not known. The effects of p21^{cip1} on the assembly and activity of D cyclin-containing complexes also remain to be resolved (3, 25, 51).

We have suggested previously that p27^{kip1} inhibits the activity of cyclin D3-cdk4 complexes in mouse fibroblasts (14, 52). The studies presented here further explore the effects of p27^{kip1}, as well as p21^{cip1}, on this process. Given the controversy regarding the role of the Cip/Kip proteins in the regulation of D cyclin-cdk activity, we used a variety of approaches, both in vivo and in vitro, to address this issue and to establish a mechanism by which the D cyclins contribute to both Rb phosphorylation and cdk2 activation. Our data show that cyclin D3 associates with cdk4 in the absence of both p27^{kip1} and p21^{cip1} and that cyclin D3-cdk4 complexes containing these CKIs are catalytically inactive. We suggest that different segments of the cyclin D3 pool are responsible for Rb phosphorylation and p27^{kip1}-p21^{cip1} sequestration and that enzymatically active cyclin D3-cdk4 complexes comprise only a small portion of this pool. As a result, cells contain a large reservoir of cyclin D3 molecules that facilitate cdk2 activation by forming stable ternary complexes with cdk4 and either p27^{kip1} or p21^{cip1}. Additional data indicate that cyclin D1-cdk4 activity is regulated by p27^{kip1} and p21^{cip1} in a manner similar to that of cyclin D3-cdk4 activity.

MATERIALS AND METHODS

Cell culture and preparation of MEFs. BALB/c 3T3 mouse fibroblasts (clone A31) were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 50 U of penicillin per ml, 50 µg of streptomycin per ml, and 10% calf serum. Experiments were done on either exponentially growing cells or cells arrested at confluency for 4 to 5 days. Density-arrested cells were stimulated to reenter the cell cycle by refeeding with fresh medium containing 10% calf serum and 10 ng of platelet-derived growth factor per ml. Mice lacking the entire coding regions for p21^{cip1} (p21^{-/-}) and for both p27^{kip1} and p21^{cip1} (p27/p21^{-/-}) were obtained from Tyler Jacks (4) and James Roberts (18), respectively. Mice lacking a region within the N-terminal cyclin-cdk binding domain of p27^{kip1} (p27N^{-/-}, provided by Andrew Koff [24]) were crossed with p21^{-/-} mice to generate p21/p27N^{-/-} double-knockout mice. Cyclin D1-null mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mouse embryo fibroblasts (MEFs) were prepared from 15- or 16-day-old embryos. Following removal of the head and internal organs, embryos were minced and plated individually in 100-mm-diameter tissue culture dishes containing medium supplemented with 10% fetal calf serum.

Preparation of cell extracts, immunoprecipitation, and immunoblotting. Cultures were rinsed twice in ice-cold phosphate-buffered saline, harvested by scraping, and collected by centrifugation. The pellets were resuspended in lysis buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, 0.1 mM sodium orthovanadate, 0.5 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 µg of leupeptin per ml, and 1 mM dithiothreitol), vortexed, and incubated on ice for 30 min. Insoluble material was removed by centrifugation. For immunoprecipitations, cell extracts (80 to 350 µg) were incubated with the indicated antibody for 1 to 2 h at 4°C with gentle agitation. Immune complexes were recovered with protein A-agarose beads (1 to 2 h, 4°C) and washed twice with lysis buffer. For Western analysis, cell extracts (40 to 80 µg) or immune complexes were boiled in Laemmli buffer (20% glycerol, 3% sodium dodecyl sulfate [SDS], 4% β-mercaptoethanol, 0.5% bromophenol blue) and separated on 10 or 11% SDS-polyacrylamide gels. Resolved proteins were electrophoretically transferred to nitrocellulose. Membranes were blocked in PBST (phosphate-buffered saline plus 0.1% Tween 20) containing 5% instant milk and incubated with antibody in PBST for 2 h at room temperature. Proteins recognized by the antibody were detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody as specified by the manufacturer (Pierce, Rockford, Ill.). In experiments involving immunodepletion, protein removal was confirmed by Western blotting.

In vitro kinase assays. Immune complexes were washed twice with lysis buffer and once with 2× kinase reaction buffer (100 mM HEPES [pH 7.5], 20 mM MgCl₂, 10 mM MnCl₂, 20 mM dithiothreitol). Washed complexes were resuspended in 1× kinase reaction buffer (50 mM HEPES [pH 7.5], 10 mM MgCl₂, 5 mM MnCl₂, 10 mM dithiothreitol) containing 10 µCi of [³²P]ATP, 10 µM ATP, and 1 µg of glutathione S-transferase (GST)-Rb and incubated for 30 min at 30°C. Reactions were stopped by boiling for 4 min in Laemmli buffer, and proteins were resolved on 11% SDS gels. Phosphoproteins were visualized by autoradiography.

Reagents and antibodies. Platelet-derived growth factor was purchased from Peppo (Rocky Hill, N.J.). Cyclin D3, cdk6, and Stat3 polyclonal antibodies were obtained from Santa Cruz (Santa Cruz, Calif.). p21^{cip1} polyclonal antibody was purchased from PharMingen (San Diego, Calif.), and cdk4 and cyclin D3 monoclonal antibodies were obtained from Transduction Laboratories (Lexington, Ky.). Cyclin A monoclonal antibody was from Neomarker (Union City, Calif.). Polyclonal antibodies to cdk4, cyclin A, cyclin D3, and p27^{kip1} were prepared by us as described previously (1, 14). Polyclonal antibody to cyclin D1 was generated against a C-terminal peptide (EVEEEAGLACTPTDVRDVI). Flavopiridol was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute.

RESULTS

Cyclin D3-cdk4-p27^{kip1} complexes are inactive in vivo. Unlike cyclin D1, which is up-regulated in response to mitogenic stimulation, cyclin D3 is expressed constitutively throughout the BALB/c 3T3 cell cycle, as is cdk4, the predominant cyclin D3 catalytic partner in these cells (14). Moreover, cyclin D3 is complexed to cdk4 in both proliferating and G₀-arrested cells. Cyclin D3-cdk4 activity, however, is restricted to growing cells, and its repression in quiescent cells may reflect the presence of inhibitory proteins. Levels of p27^{kip1} are low in cycling cells and high in quiescent cells (1, 14, 49), and this inverse relationship between the amount of p27^{kip1} and the activity of cyclin D3-associated cdk4 prompted us to examine the role of p27^{kip1} in the regulation of this activity. Initial experiments assessed the interaction of p27^{kip1} with cyclin D3 in quiescent versus stimulated cells. Density-arrested BALB/c 3T3 cells received fresh medium containing 10% serum and 10 ng of platelet-derived growth factor per ml, and cell lysates were prepared at various times thereafter and immunoprecipitated with antibody to p27^{kip1}. Immune complexes were Western blotted with antibody to cyclin D3 or p27^{kip1}. To determine the amount of cyclin D3 not associated with p27^{kip1}, p27^{kip1}-depleted supernatants were immunoprecipitated and immunoblotted with antibody to cyclin D3. As shown in Fig. 1, essentially all of the cyclin D3 in unstimulated cells was complexed to p27^{kip1} (compare Fig. 1B and C). The amount of cyclin D3 associated with p27^{kip1} decreased progressively with time (Fig. 1C), as did total levels of p27^{kip1} (Fig. 1D), and was accompanied by an increase in the amount of p27^{kip1}-free cyclin D3 (Fig. 1B). In addition, Western blotting of unfractionated cell lysates reaffirmed that

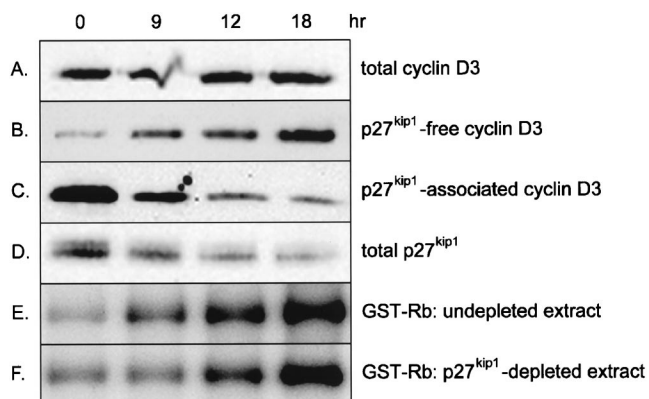


FIG. 1. Lack of effect of $p27^{kip1}$ immunodepletion on cyclin D3-associated kinase activity in BALB/c 3T3 cells. Density-arrested BALB/c 3T3 cells were mitogenically stimulated with 10 ng of platelet-derived growth factor per ml and 10% serum and harvested at the indicated times. (A) Cell lysates (40 μ g) were immunoblotted with antibody to cyclin D3. (B and C) Cell lysates (40 μ g) were incubated with antibody to $p27^{kip1}$. Immune complexes were pelleted with protein A-agarose beads and immunoblotted with antibody to cyclin D3 (C). The $p27^{kip1}$ -depleted supernatant was immunoprecipitated and immunoblotted with cyclin D3 antibody (B). (D) Cell lysates (40 μ g) were immunoblotted with antibody to $p27^{kip1}$. (E and F) Cell lysates were immunoprecipitated with pre-immune serum (E) or antibody to $p27^{kip1}$ (F). Immune complexes were removed by centrifugation with protein A-agarose beads, and supernatants were immunoprecipitated with cyclin D3 antibody. Immunoprecipitated material was assayed for cyclin D3-associated kinase activity using GST-Rb as substrate.

total levels of cyclin D3 were unaltered during the time course (Fig. 1A). These findings show that BALB/c 3T3 cells contain two distinct pools of cyclin D3, one with and one without $p27^{kip1}$, and that the relative proportions of these pools are governed by the amount of $p27^{kip1}$ in the cell in a manner that promotes expansion of the $p27^{kip1}$ -free pool as cells traverse G_0/G_1 .

To determine if $p27^{kip1}$ -associated cyclin D3 complexes were catalytically active, $p27^{kip1}$ -depleted and undepleted cell extracts were immunoprecipitated with antibody to cyclin D3, and in vitro kinase assays were performed on immune complexes using GST-Rb as substrate. As shown in Fig. 1E, cyclin D3-cdk4 activity was low in undepleted extracts of quiescent cells and increased significantly upon mitogenic stimulation in parallel with the decrease in $p27^{kip1}$ levels. Immunodepletion of $p27^{kip1}$ prior to assay had no effect on either the extent or the timing of cyclin D3-cdk4 activation (compare Fig. 1E and F), thus indicating that cyclin D3 complexes that contain $p27^{kip1}$ do not contribute substantially to this process. These data suggest that cyclin D3-cdk4 activity is restricted to $p27^{kip1}$ -free complexes, which are present in stimulated but not quiescent BALB/c 3T3 cells.

Experiments similar to those described above were also performed on MEFs prepared from $p21^{cip1}$ -null mice. Although $p27^{kip1}$ antibody coprecipitated cyclin D3 protein (~50% of total) from extracts of mitogenically stimulated $p21^{-/-}$ MEFs, it did not remove GST-Rb-phosphorylating activity as determined in cyclin D3 immunoprecipitates (Fig. 2). As shown in Fig. 2A, levels of cyclin D3-cdk4 activity were similar in both mock-depleted and $p27^{kip1}$ -depleted extracts of $p21^{-/-}$ MEFs. This result indicates that cyclin D3-cdk4 complexes lacking both $p27^{kip1}$ and $p21^{cip1}$ are enzymatically active and account for most if not all of the cyclin D3-cdk4 activity in the cell.

Cyclin D3-cdk4 activity in cells lacking both $p27^{kip1}$ and $p21^{cip1}$. Previous studies (7) did not detect Rb kinase activity in either cyclin D1 or cdk4 immune complexes derived from MEFs lacking both $p27^{kip1}$ and $p21^{cip1}$. On the other hand, we

observed cyclin D3-cdk4 activity in BALB/c 3T3 extracts immunodepleted of both $p27^{kip1}$ and $p21^{cip1}$ and thus predict that this activity should be readily apparent in $p27^{kip1}$ - $p21^{cip1}$ double-null cells. Two mouse models were used to test this premise: one in which the entire coding regions of $p27^{kip1}$ and $p21^{cip1}$ were eliminated (termed $p27/p21^{-/-}$) and the other in which the entire coding region of $p21^{cip1}$ and the N-terminal cyclin-cdk binding domain of $p27^{kip1}$ (termed $p27N/p21^{-/-}$) were deleted (4, 18, 24). Cell lysates were prepared from asynchronously growing wild-type, $p27/p21^{-/-}$, and $p27N/p21^{-/-}$ MEFs, and cyclin D3-associated kinase activity was determined. Although less than that of wild-type MEFs, considerable cyclin D3-cdk4 activity was seen in cells derived from both types of knockout mice (Fig. 3A and E). In accord with this observation, antibody to cdk4 coprecipitated cyclin D3 from extracts of both $p27/p21^{-/-}$ and $p27N/p21^{-/-}$ cells, albeit to a reduced extent compared to that for wild-type cells (Fig. 3D and H). These findings demonstrate that cyclin D3-cdk4 complexes are formed and become active in the absence of both $p27^{kip1}$ and $p21^{cip1}$. The lower levels of cyclin D3-cdk4 association and activity in the double-null cells may reflect the lower levels of cyclin D3 (Fig. 3B and F) and cdk4 (Fig. 3C and G) in these cells.

Additional experiments were performed to conclusively demonstrate that the GST-Rb-phosphorylating activity seen in our experiments on $p27^{kip1}$ - $p21^{cip1}$ -deficient MEFs was indeed representative of cyclin D3-cdk4 activity. In the first set of experiments, extracts of asynchronously cycling $p27/p21^{-/-}$ cells were immunoprecipitated with a panel of different antibodies, and the capacity of the precipitated material to phosphorylate GST-Rb was assessed. A very low level of GST-Rb phosphorylation was observed in mock precipitates ("no antibody") and in precipitates of lysates preincubated with pre-immune serum or with antibody to Stat3, a transcription factor that does not possess kinase activity or associate with cyclin-cdk complexes (Fig. 4A, top panel) (11). Immune complexes prepared using antibodies to $p27^{kip1}$, $p21^{cip1}$, or cdk6 also exhibited basal levels of GST-Rb phosphorylation. On the other hand, kinase activity resulting in greater than basal amounts of GST-Rb phosphorylation was evident in immunoprecipitates obtained with two different antibodies to cyclin D3, as well as with an antibody to cdk4. Increased activity correlated with the appearance of cyclin D3 in the immune complex (Fig. 4A, bottom panel), and neither increased activity nor cyclin D3 protein was seen in extracts precipitated with cyclin D3 anti-

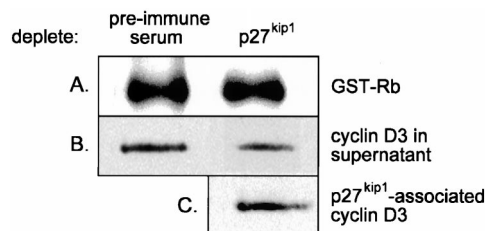


FIG. 2. Lack of effect of $p27^{kip1}$ immunodepletion on cyclin D3-associated kinase activity in $p21^{-/-}$ MEFs. Confluent $p21^{-/-}$ MEFs were incubated in medium containing 0.1% serum for 30 h and subsequently stimulated with 10 ng of PDGF per ml and 10% serum for 18 h. Cell lysates were immunoprecipitated with preimmune serum (mock depletion, left panel) or antibody to $p27^{kip1}$ (right panel). Immune complexes were removed by centrifugation with protein A-agarose beads and immunoblotted with monoclonal antibody to cyclin D3 (C). Supernatants were immunoprecipitated with polyclonal antibody to cyclin D3, and immunoprecipitated material was assayed for cyclin D3-associated kinase activity using GST-Rb as substrate (A) or immunoblotted with monoclonal antibody to cyclin D3 (B).

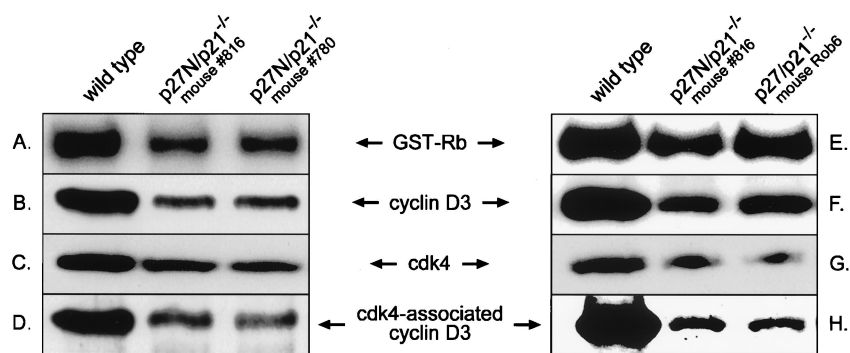


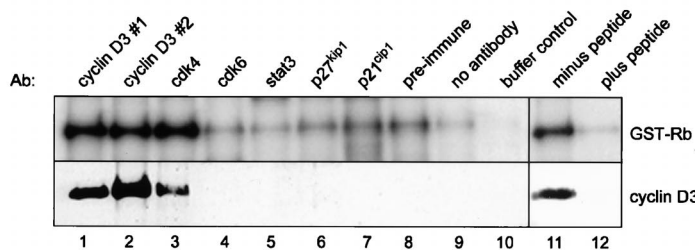
FIG. 3. Cyclin D3-cdk4 association and activity in MEFs lacking both p27^{kip1} and p21^{cip1}. Asynchronously cycling wild-type MEFs, p27N/p21^{-/-} MEFs (derived from mice 816 and 780), and p27/p21^{-/-} MEFs (derived from mouse Rob6) were harvested and assayed as follows. (A and E) Cell lysates were immunoprecipitated with antibody to cyclin D3, and immune complexes were assayed for kinase activity using GST-Rb as substrate. (B and F) Cell lysates (80 μg) were immunoblotted with antibody to cyclin D3. (C and G) Cell lysates (80 μg) were immunoblotted with antibody to cdk4 and immunoblotted with antibody to cyclin D3. As different amounts of lysate were used for the assays in panels B and F versus those in panels D and H, the results obtained cannot be compared on a quantitative basis.

body prebound to the immunizing peptide (Fig. 4A, compares lanes 11 and 12). These results demonstrate that a background level of Rb-phosphorylating activity is present in immune complexes regardless of the antibody used for precipitation. Despite this background, an increase in activity that specifically

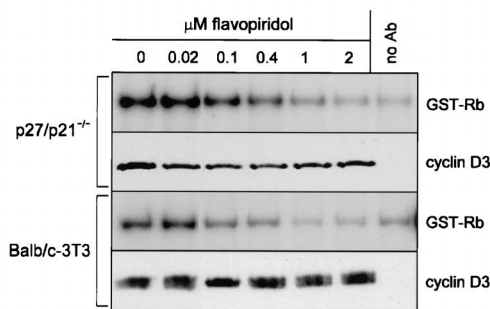
reflects the presence of cyclin D3 in the immune complex is observed.

In a second set of experiments, extracts of exponentially proliferating p27/p21^{-/-} cells (or, as a positive control, BALB/c 3T3 cells) were immunoprecipitated with antibody to

A.



B.



C.

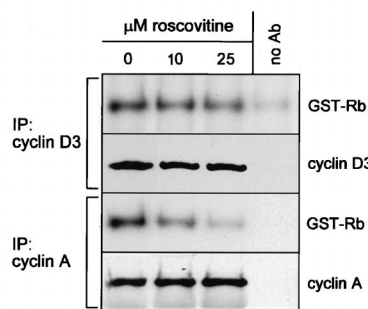


FIG. 4. Verification of cyclin D3-associated cdk4 activity as the GST-Rb-phosphorylating activity in p27^{kip1}-p21^{cip1}-deficient MEFs. (A) Lysates of exponentially proliferating p27/p21^{-/-} MEFs were immunoprecipitated with the indicated antibodies (lanes 1 to 7) or preimmune serum (lane 8) or were mock precipitated (lane 9). Immune complexes were assayed for kinase activity using GST-Rb as substrate (top panel) or were immunoblotted with antibody to cyclin D3 (bottom panel). The buffer control (lane 10) contained kinase buffer in place of resuspended immune complex. Cyclin D3 antibodies 1 and 2 were purchased from Santa Cruz and prepared by us, respectively. Cell extracts were also immunoprecipitated with antibody to cyclin D3 (Santa Cruz) that had been preincubated with either buffer alone (lane 11) or the peptide against which the antibody was generated (lane 12). (B) Cell extracts prepared from logarithmically growing p27/p21^{-/-} MEFs (top panels) and BALB/c 3T3 cells (bottom panels) were immunoprecipitated with antibody to cyclin D3. Immunoprecipitated material was resuspended in kinase buffer containing the indicated concentrations of flavopiridol and incubated for 30 min at 30°C. Immune complexes were assayed for cyclin D3-associated activity using GST-Rb as substrate or were immunoblotted with antibody to cyclin D3. (C) Cell extracts prepared from asynchronously cycling p27/p21^{-/-} cells were immunoprecipitated with antibody to cyclin D3 (top panels) or cyclin A (bottom panels). Immune complexes were resuspended in kinase buffer containing the indicated concentrations of roscovitine and incubated for 30 min at 30°C. Immunoprecipitated material was assayed for kinase activity using GST-Rb as substrate or was immunoblotted with antibody to cyclin D3 (cyclin D3 immunoprecipitates) or cyclin A (cyclin A immunoprecipitates). Ab, antibody; IP, immunoprecipitation.

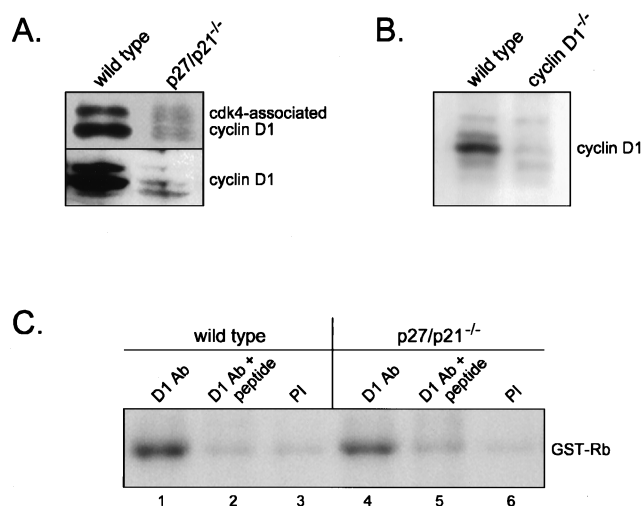


FIG. 5. Cyclin D1-cdk4 activity in $p27/p21^{-/-}$ MEFs. (A) Cell lysates of growing wild-type and $p27/p21^{-/-}$ MEFs were immunoprecipitated with antibody to cdk4 and immunoblotted with antibody to cyclin D1 (top panel) or were only immunoblotted with antibody to cyclin D1 (bottom panel). (B) Extracts of wild-type and cyclin D1-deficient MEFs were immunoblotted with antibody to cyclin D1. (C) Extracts of wild-type and $p27/p21^{-/-}$ MEFs were immunoprecipitated with antibody to cyclin D1 (lanes 1 and 4), preimmune serum (PI) (lanes 3 and 6), or cyclin D1 antibody that had been preincubated with the peptide against which the antibody was generated (lanes 2 and 5). Immune complexes were assayed for kinase activity using GST-Rb as substrate. Ab, antibody.

cyclin D3, and immune complexes were incubated with the cdk4 inhibitor flavopiridol (5) prior to determination of kinase activity. For both cell lines, addition of flavopiridol to immune complexes resulted in a dose-dependent decrease in activity, with essentially complete inhibition occurring at 2 μ M (Fig. 4B). Flavopiridol also inhibits the activity of cdk2 (12); however, as assessed in immune complexes derived from $p27/p21^{-/-}$ MEFs, the cdk2 inhibitor roscovitine (2, 31) had no effect on cyclin D3-associated activity (Fig. 4C). In contrast, cyclin A-associated activity (presumably cdk2) was markedly repressed by roscovitine. As confirmed by Western blotting, equal levels of cyclin D3 and cyclin A were present in each sample. Although additional unsuspected actions of flavopiridol cannot be excluded, the susceptibility of cyclin D3-associated activity to flavopiridol (but not roscovitine) strongly suggests that cdk4 is the catalytic entity responsible for this activity in $p27/p21^{-/-}$ cells. This supposition is supported by the presence of both cyclin D3 protein and GST-Rb-phosphorylating activity in cdk4 immune complexes prepared from double-null cells (Fig. 4A, lane 3). Although cdk6 is a potential target of flavopiridol, we did not detect cdk6 activity in $p27/p21^{-/-}$ MEFs (Fig. 4A, lane 4). A similar finding was reported by Cheng et al. (7).

Cyclin D1-cdk4 activity in $p27/p21^{-/-}$ MEFs. Although cdk4 interacted with cyclin D3 in cells lacking $p27^{kip1}$ and $p21^{cip1}$, it is possible that it does not associate with other D cyclins in the absence of these CKIs. To test this, we examined the association of cdk4 with cyclin D1 in wild-type and $p27/p21^{-/-}$ MEFs by immunoprecipitation-immunoblot analysis. As shown in Fig. 5A, cyclin D1-cdk4 complexes were present in $p27/p21^{-/-}$ cells, albeit at lower levels than in wild-type cells. Total amounts of cyclin D1 were substantially reduced in the double-null cells and thus may limit the extent of cyclin D1-cdk4 complex formation in these cells. Using various amounts of cell extract, we determined that cyclin D1 levels were \sim 15-fold

lower in $p27/p21^{-/-}$ MEFs (data not shown). The cyclin D1 antibody used in these experiments was prepared in our laboratory, and Western analysis of cell extracts derived from cyclin D1^{+/+} and cyclin D1^{-/-} MEFs confirmed that this antibody specifically recognizes cyclin D1 (Fig. 5B). To determine if cyclin D1-cdk4 complexes in $p27/p21^{-/-}$ MEFs were enzymatically active, kinase assays were performed on cell extracts immunoprecipitated with cyclin D1 antibody or, as negative controls, preimmune serum or cyclin D1 antibody plus blocking peptide. A low level of kinase activity was observed in the negative controls for both wild-type and $p27/p21^{-/-}$ MEFs (Fig. 5C). Kinase activity greater than background levels was, however, clearly evident in cyclin D1 immune complexes prepared from wild-type cells and, to a somewhat lesser extent, $p27/p21^{-/-}$ cells. Additional experiments showed that depletion of $p27^{kip1}$ and $p21^{cip1}$ from extracts of wild-type cells did not remove cyclin D1-cdk4 activity, as assessed in both cyclin D1 (data not shown) and cdk4 (see Fig. 8C) immunoprecipitates. Collectively, these results suggest that the activity of cyclin D1 complexes is regulated similarly to that of cyclin D3 complexes. Both complexes are present and active in cells lacking $p27^{kip1}$ and $p21^{cip1}$ and are not active when bound to $p27^{kip1}$ or $p21^{cip1}$.

Inhibition of cyclin D3-cdk4 activity by $p27^{kip1}$ in vitro. The data presented in Fig. 1 and 2 demonstrate that cyclin D3-cdk4- $p27^{kip1}$ complexes are inactive in vivo. As a corollary to these studies, we also examined the effect of exogenously supplied $p27^{kip1}$ on cyclin D3-cdk4 activity in vitro. As our source of $p27^{kip1}$, we used extracts of G₀-arrested BALB/c 3T3 cells. As we reported previously (49), these extracts contain a large pool of $p27^{kip1}$ molecules that are not bound to cyclin-cdk complexes. In addition, we boiled G₀ extracts to release cyclin-cdk-sequestered $p27^{kip1}$, and it is noted that boiling also results in a cyclin D3-containing precipitate, which is removed by centrifugation. Different amounts of boiled and clarified G₀ extracts were mixed with extracts of exponentially growing $p27/p21^{-/-}$ cells; as shown above (Fig. 3), these cells contain active cyclin D3-cdk4 complexes. After a 30-min incubation at 30°C, cyclin D3 (or for comparative purposes, cyclin A) was immunoprecipitated from mixed cell extracts, and the amounts of coprecipitated $p27^{kip1}$ and kinase activity were determined.

As shown in Fig. 6, addition of boiled and clarified G₀ extracts to extracts of growing $p27/p21^{-/-}$ cells resulted in the association of $p27^{kip1}$ with cyclin D3-containing complexes (Fig. 6B) and the repression of cyclin D3-cdk4 activity (Fig. 6A). The extent of inhibition of cyclin D3-cdk4 activity was directly proportional to the amount of $p27^{kip1}$ bound to cyclin D3-cdk4 complexes, with maximal interaction and inactivation occurring at a 1:1 ratio of G₀ extract to growing cell extract (lane 5). In terms of dose dependency, and although less striking, inhibition of cyclin D3-cdk4 activity by G₀ extract was comparable to that of cyclin A-cdk2 activity (Fig. 6C and D). We have shown previously that the inhibitory activity in G₀ extracts is removed by antibody to $p27^{kip1}$ (49). Thus, similar to results obtained in vivo, $p27^{kip1}$ inhibits the activity of cyclin D3-cdk4 complexes in vitro. We also found that recombinant $p27^{kip1}$ repressed cyclin D3-cdk4 activity when added to extracts of proliferating $p27/p21^{-/-}$ cells (T. K. Bagui and W. J. Pledger, unpublished data).

Limited amounts of cyclin D3-cdk4 complexes in $p27^{kip1}$ - $p21^{cip1}$ -deficient MEFs. Additional experiments assessed the stoichiometry of cyclin D3 binding to $p27^{kip1}$ in mixed cell extracts. In these experiments, as in those above, extracts of exponentially proliferating $p27/p21^{-/-}$ MEFs were combined with boiled and clarified extracts of G₀-arrested BALB/c 3T3 cells. As shown in Fig. 7A (top panel), similar amounts of

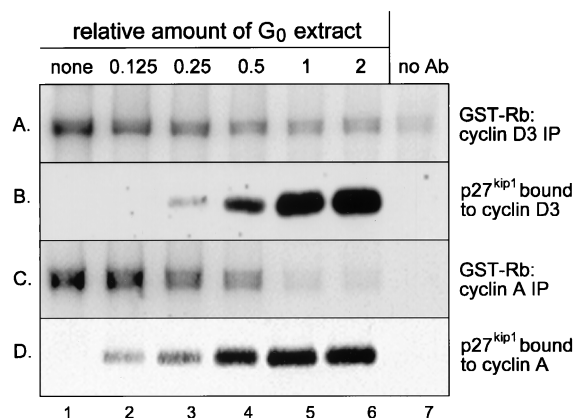


FIG. 6. Inhibition of cyclin D3-CDK4 activity by p27^{kip1} in mixed cell extracts. Extracts of density-arrested BALB/c 3T3 cells were boiled for 5 min and clarified by centrifugation (designated "G₀ extracts"). G₀ extracts were mixed at the indicated ratios with extracts (200 μg) of proliferating p27/p21^{-/-} cells and incubated at 30°C for 30 min. Mixed cell extracts were immunoprecipitated with antibody to cyclin D3 (A and B) or cyclin A (C and D). Immune complexes were assayed for cyclin D3-associated activity (A) or cyclin A-associated activity (C) or were immunoblotted with antibody to p27^{kip1} (B and D). Based on comparisons with known amounts of recombinant p27^{kip1}, 100 μg of G₀ extract contains approximately 7 ng of p27^{kip1}. Ab, antibody. IP, immunoprecipitation.

cyclin D3 were present in mixed cell extracts regardless of the amount of G₀ extract added. This observation verifies the removal of cyclin D3 from boiled G₀ extracts and thus indicates that extracts of growing p27/p21^{-/-} MEFs are the sole source of cyclin D3 in mixed cell extracts. As shown in Fig. 7A (middle panel) and similar to data presented in Fig. 6, cyclin D3 and p27^{kip1} interacted *in vitro*. However, even in conditions in which cyclin D3-CDK4 activity was maximally inhibited (Fig. 6, lanes 5 and 6), only a low percentage of the total cyclin D3 pool was associated with p27^{kip1} (Fig. 7A, compare top and middle panels). Consistent with this observation, immunodepletion of p27^{kip1} from mixed cell extracts had no discernible effect on cyclin D3 levels (bottom panel), again indicating that the amount of cyclin D3 bound to p27^{kip1} was limited. These data demonstrate that p27/p21^{-/-} cells contain a large pool of cyclin D3 molecules that do not bind p27^{kip1}.

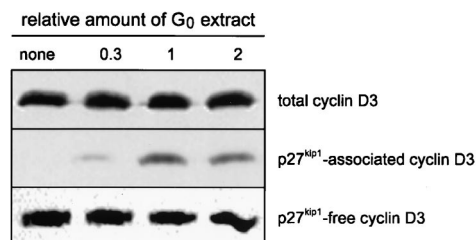
Although p27^{kip1} binds with high affinity to cyclin-CDK complexes, it does not interact efficiently with individual cyclin or CDK subunits (38, 39, 42). Thus, it is possible that the cyclin D3 pool in p27/p21^{-/-} MEFs that does not bind p27^{kip1} consists of cyclin D3 monomers (defined as cyclin D3 not bound to CDK4). To test this, extracts of proliferating p27/p21^{-/-} cells and boiled, clarified extracts of quiescent BALB/c 3T3 cells were combined, and the amounts of CDK4-associated cyclin D3 in mock-depleted and p27^{kip1}-depleted mixed cell extracts were determined. Although comprising only a small fraction of the total cyclin D3 pool, cyclin D3-CDK4 complexes were evident in mock-depleted extracts (Fig. 7B, compare lanes 1 and 2). In contrast, cyclin D3-CDK4 complexes were not detectable in mixed cell extracts depleted of p27^{kip1} (lane 4). Removal of p27^{kip1}, however, had little effect on total levels of cyclin D3 (compare lanes 1 and 3) or CDK4 (data not shown). These results indicate that most of the cyclin D3 molecules in p27/p21^{-/-} cells are not bound to CDK4 and thus are unavailable for interaction with p27^{kip1}.

Active cyclin D3-CDK4 complexes comprise only a minor portion of the cyclin D3 pool in wild-type MEFs. The observation that only a minor portion of total cyclin D3 molecules are complexed to CDK4 in p27/p21^{-/-} cells implies that only a

minor portion of the total cyclin D3 pool is (or can be) active. Thus, one would predict that cyclin D3-CDK4 activity in p27/p21^{-/-} cells would be, at best, barely detectable. However, as shown in Fig. 3, considerable cyclin D3-CDK4 activity was observed in two different types of p27^{kip1}-p21^{cip1}-deficient MEFs. As an explanation of this paradox, we considered the possibility that the cyclin D3 pool in wild-type cells consists primarily of cyclin D3 monomers and inactive complexes (e.g., cyclin D3-CDK4-p27^{kip1}). Thus, in both p27/p21^{-/-} and p27/p21^{+/+} cells, only a seemingly inconsequential fraction of total cyclin D3 molecules would account for all of the cyclin D3-associated activity in the cells. To test this possibility, we determined the relative amounts of cyclin D3 monomers and of binary (cyclin D3-CDK4) and ternary (cyclin D3-CDK4-CKI) cyclin D3 complexes in asynchronously growing wild-type MEFs. In these experiments, cell extracts were immunodepleted with antibody to p27^{kip1}, p21^{cip1}, or both, and depleted extracts were immunoprecipitated with antibody to cyclin D3 (to assess cyclin D3) or CDK4 (to assess CDK4-associated cyclin D3); immunoprecipitated material was immunoblotted with antibody to cyclin D3.

As shown in Fig. 8A, the amount of cyclin D3 precipitated by cyclin D3 antibody (lane 5) was only slightly higher than that coprecipitated by CDK4 antibody (lane 1). This finding indicates that, unlike p27/p21^{-/-} cells, most (although not all) of the cyclin D3 molecules in p27/p21^{+/+} cells are complexed to CDK4. The CDK4-associated cyclin D3 pool was reduced by ~50% following depletion of either p27^{kip1} or p21^{cip1} (lanes 2 and 3) and was essentially undetectable following removal of both

A.



B.

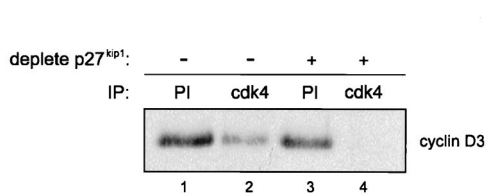


FIG. 7. High levels of uncomplexed cyclin D3 in p27/p21^{-/-} MEFs. (A) Boiled and clarified extracts of G₀-arrested BALB/c 3T3 cells were mixed with extracts of exponentially growing p27/p21^{-/-} cells at the indicated ratios and incubated for 30 min at 30°C. (Top panel) Mixed cell extracts were immunoprecipitated and immunoblotted with antibody to cyclin D3. (Middle panel) Mixed cell extracts were immunoprecipitated with antibody to p27^{kip1} and immunoblotted with antibody to cyclin D3. (Bottom panel) Mixed cell extracts were immunodepleted of p27^{kip1}, and depleted extracts were immunoprecipitated and immunoblotted with antibody to cyclin D3. (B) Mixed cell extracts were prepared and incubated as for panel A and were immunodepleted of p27^{kip1} (+) or mock depleted (-). Depleted extracts were immunoprecipitated (IP) with preimmune serum (PI) or antibody to CDK4 and immunoblotted with antibody to cyclin D3.

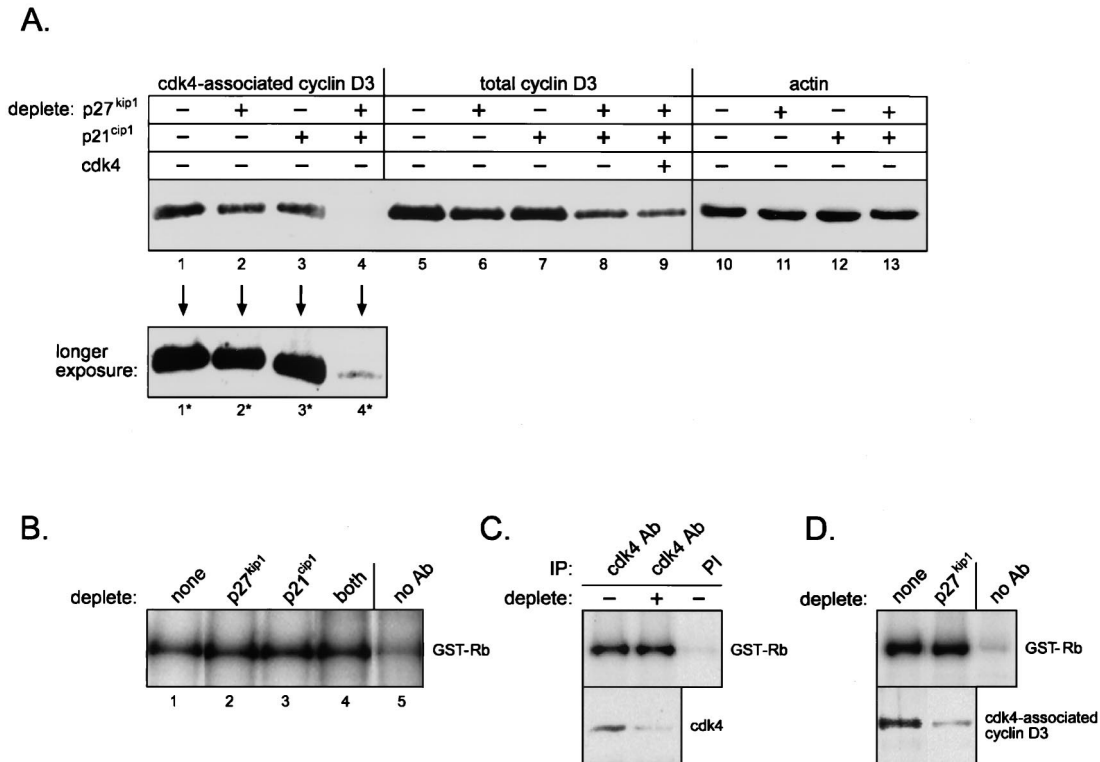


FIG. 8. Low levels of binary cyclin D3-cdk4 complexes in wild-type MEFs. (A) Extracts of growing wild-type MEFs were immunodepleted of p27^{kip1}, p21^{cip1}, or cdk4 or were mock depleted. Depleted extracts were immunoprecipitated with antibody to cdk4 (lanes 1 to 4) or cyclin D3 (lanes 5 to 9) and immunoblotted with antibody to cyclin D3. Depleted extracts were also immunoblotted with antibody to actin (lanes 10 to 13). A longer exposure of lanes 1 to 4 is also shown (lanes denoted by asterisks). (B) Extracts were immunodepleted of p27^{kip1}, p21^{cip1}, or both or were mock depleted. Depleted extracts were immunoprecipitated with antibody to cyclin D3 and assayed for kinase activity using GST-Rb as substrate. (C) Extracts of wild-type MEFs were immunodepleted using preimmune serum (-) or antibodies to p27^{kip1} and p21^{cip1} (+). Depleted extracts were immunoprecipitated with antibody to cdk4 or preimmune serum (PI), and immune complexes were assayed for kinase activity (top panel) or immunoblotted with cdk4 antibody (bottom panel). (D) Extracts of growing p21^{-/-} MEFs were depleted of p27^{kip1} or mock depleted. Depleted extracts were immunoprecipitated with antibody to cyclin D3 for determination of kinase activity (top panel) or were immunoprecipitated with antibody to cdk4 and immunoblotted with antibody to cyclin D3 (bottom panel). Ab, antibody. IP, immunoprecipitation.

CKIs (lane 4). However, longer exposures of the Western blot demonstrated the presence of cyclin D3 in this lane (lane 4*). Thus, nearly all of the cyclin D3-cdk4 complexes in wild-type MEFs contain either p27^{kip1} or p21^{cip1}. Analysis of the total cyclin D3 pool confirmed that the majority of cyclin D3 molecules were associated with p27^{kip1} or p21^{cip1} (lanes 6 and 7) and directly demonstrated the presence of a small amount of cyclin D3 monomers (lane 9). Together, these data demonstrate that the cyclin D3 pool in exponentially growing wild-type MEFs consists primarily of cyclin D3-cdk4-CKI complexes, contains a lesser subfraction of cyclin D3 monomers, and also includes a minute amount of cyclin D3-cdk4 complexes that are not associated with either p27^{kip1} or p21^{cip1}. Consistent with the low percentage of binary complexes, the amount of cyclin D3 observed after depletion of p27^{kip1} and p21^{cip1} (representative of cyclin D3 plus cyclin D3-cdk4 [lane 8]) was only marginally higher than the amount of cyclin D3 observed after depletion of p27^{kip1}, p21^{cip1}, and cdk4 (representative of cyclin D3 alone [lane 9]). To ensure that our immunodepletion protocol did not nonspecifically remove proteins that do not associate with p27^{kip1} or p21^{cip1}, depleted extracts were immunoblotted with antibody to actin. As shown in Fig. 8A, actin levels were comparable in control extracts (lane 10) and in extracts depleted of p27^{kip1}, p21^{cip1}, or both (lanes 11 to 14).

Although nearly undetectable, binary cyclin D3-cdk4 complexes accounted for essentially all of the cyclin D3-associated

activity in extracts of wild-type MEFs. As shown in Fig. 8B, cyclin D3 immunoprecipitates containing only binary complexes (i.e., not containing p27^{kip1} or p21^{cip1} [lane 4]) exhibited levels of activity similar to those of immunoprecipitates derived from mock-depleted extracts (lane 1). In addition, the inability of p21^{cip1} antibody to remove activity (lane 3) indicates that cyclin D3-cdk4 complexes containing p21^{cip1}, like those containing p27^{kip1}, are inactive. The lack of effect of p27^{kip1}-p21^{cip1} depletion on D cyclin-associated activity was also apparent in experiments in which kinase activity was determined in cdk4 (rather than cyclin D3) immune complexes (Fig. 8C). Despite the removal of cdk4 protein by p27^{kip1}-p21^{cip1} depletion, levels of cdk4 activity were similar in control and depleted extracts. Because cdk4 interacts with cyclins D1, D2, and D3, all of which are present in MEFs (this study and reference 7), the data in Fig. 8C indicate that cdk4 activity is inhibited by p27^{kip1} and p21^{cip1} regardless of which D cyclin is present in the complex.

Similar experiments were done on logarithmically growing p21^{-/-} MEFs. As shown in Fig. 8D (bottom panel), levels of cdk4-associated cyclin D3 were reduced substantially by p27^{kip1} depletion, thus indicating that most of the cyclin D3-cdk4 complexes in these cells are bound to p27^{kip1}. As seen with wild-type MEFs, removal of p27^{kip1}-associated cyclin D3-cdk4 complexes from extracts of p21^{-/-} MEFs was not accompanied by a corresponding loss of cyclin D3-cdk4 activity (top panel). These observations, coupled with those presented above, dem-

onstrate that irrespective of whether MEFs contain p27^{kip1} and/or p21^{cip1}, the majority of cyclin D3 molecules in the cell are not present in enzymatically active complexes.

DISCUSSION

Our studies establish a model of cyclin D3-*cdk4* activation in which p27^{kip1} and p21^{cip1} play inhibitory roles. Our data show that cyclin D3-*cdk4* complexes containing these CKIs are catalytically inactive both in vivo and in vitro. Moreover, these CKIs are not required for the formation of cyclin D3-*cdk4* complexes, as has been proposed previously (7). Both p27^{kip1} and p21^{cip1} may, however, enhance the stability of these complexes (see below). We also found that enzymatically active cyclin D3-*cdk4* complexes comprised only a small fraction of the total cyclin D3 pool regardless of whether cells contained p27^{kip1} and p21^{cip1}. We conclude that *cdk2* activation in wild-type cells is implemented by the presence of a large pool of cyclin D3 molecules that (with *cdk4*) sequester p27^{kip1} and p21^{cip1}. On the other hand, in p27^{kip1}-p21^{cip1}-deficient cells, the inherent instability of cyclin D3-*cdk4* complexes limits the extent of cyclin D3-*cdk4* activity and thus prevents unregulated cell proliferation.

Using two different mouse models, we found that MEFs lacking both p27^{kip1} and p21^{cip1} contained cyclin D3-*cdk4* complexes and exhibited cyclin D3-*cdk4* activity. Levels of both parameters were lower in double-null cells than in wild-type cells, as were levels of cyclin D3 and *cdk4*. We also observed cyclin D3-*cdk4* activity in p27^{kip1}-immunodepleted extracts of p21^{-/-} MEFs and in p27^{kip1}-p21^{cip1}-depleted extracts of wild-type MEFs. These data clearly demonstrate that neither p27^{kip1} nor p21^{cip1} is required for the assembly or activation of cyclin D3-*cdk4* complexes. These CKIs were also dispensable for cyclin D1-*cdk4* activation; as shown above, substantial amounts of cyclin D1-associated activity were seen in p27/p21^{-/-} cells. Based on these results, and although not examined in this study, it is likely that cyclin D2-*cdk4* complexes are also present and active in p27/p21^{-/-} MEFs.

In a previous investigation, Cheng et al. (7) found that *cdk4* did not appreciably associate with cyclin D1 or cyclin D2 (cyclin D3 was not examined) in p27/p21^{-/-} MEFs. Moreover, these investigators did not detect *cdk4* activity in p27/p21^{-/-} cells by in vitro kinase assay (i.e., by the method used in our study). However, residual levels of *cdk4* activity were apparent in other assays (e.g., Western blotting of cell lysates with an antibody that recognizes D cyclin-specific Rb phosphorylation). Thus, both our study and that of Cheng et al. (7) demonstrate that D cyclin complexes are active, at least to some extent, in MEFs lacking p27^{kip1} and p21^{cip1}. The differences between these studies appear to be more quantitative than qualitative, with Cheng et al. (7) showing a more severe reduction in *cdk4* activity in the double-null cells than was seen by us. Because p27^{kip1}-p21^{cip1}-deficient MEFs proliferate and are susceptible to p16^{INK4a}-mediated growth inhibition (7), it is evident that these cells retain a level of D cyclin-associated activity that, while less than that of wild-type MEFs, is sufficient for cell cycle traverse.

Based on the apparent absence of cyclin D-*cdk4* complexes from p27/p21^{-/-} MEFs, Cheng et al. (7) concluded that these CKIs were required for the efficient assembly of these complexes and, consequently, functioned as activators of cyclin D-dependent kinases. Consistent with this role, Cheng et al. (7) found that p27^{kip1} and p21^{cip1} did not inhibit *cdk4* activity when bound to *cdk4* complexes, a finding that has been both corroborated (3, 25, 35, 50) and challenged (25). Our data show that cyclin D-*cdk4* complexes containing p27^{kip1} or

p21^{cip1} are not active, and thus it is unlikely that the formation of these complexes would be dependent on their association with proteins that would ultimately prevent their activation.

In our studies, two approaches were used to determine the effect of p27^{kip1} on the activity of cyclin D3-*cdk4* complexes. In the in vitro approach, we examined the effect of exogenously added p27^{kip1} on cyclin D3-*cdk4* activity in mixed cell extracts. Our results show that p27^{kip1} interacts with cyclin D3-*cdk4* complexes in vitro and that this interaction is accompanied by a decrease in cyclin D3-*cdk4* activity. p27^{kip1} also repressed the activity of cyclin A-*cdk2* complexes present in mixed cell extracts, and the loss of both activities occurred at similar concentrations of p27^{kip1}. In the in vivo approach, we found that mitogenic stimulation of quiescent BALB/c 3T3 cells resulted in a decrease in cyclin D3-p27^{kip1} association and a corresponding increase in cyclin D3-*cdk4* activity. More importantly, immunodepletion of extracts of stimulated or cycling cells with p27^{kip1} antibody did not remove cyclin D3-*cdk4* activity, thus indicating that active cyclin D3-*cdk4* complexes do not contain p27^{kip1}. Depletion of p21^{cip1}, either alone or with p27^{kip1}, also had no effect on cyclin D3-*cdk4* activity. Moreover, additional experiments examining kinase activity in cyclin D1 and *cdk4* immune complexes indicate that p27^{kip1} and p21^{cip1} inhibit the activity of all D cyclin-*cdk4* complexes.

Our in vivo data are in agreement with a previous study (25) showing undiminished levels of cyclin D1-*cdk4* activity in p27^{kip1}-depleted extracts of U2OS cells. On the other hand, Cheng et al. (7) reported that p27^{kip1} depletion of wild-type MEF extracts significantly reduced *cdk4* activity. The reason for these differences is not known at present. In our studies, we tested a variety of different cyclin D1 antibodies and found that the capacity of these antibodies to recognize low amounts of cyclin D1 protein and activity varied widely. Thus, it is possible that the disparate results obtained here and in past studies reflect, at least in part, the antibodies used. As shown above and in a previous publication (14), p27^{kip1} is associated with cyclin D3 (and thus with *cdk4*) in quiescent BALB/c 3T3 cells. If cyclin D3-*cdk4*-p27^{kip1} complexes are active as suggested by Cheng et al. (7), one would expect to see cyclin D3-*cdk4* activity in growth-arrested BALB/c 3T3 cells, which is not the case. On the other hand, the restriction of cyclin D3-*cdk4* activity to p27^{kip1}-free complexes, as proposed here, provides a means by which the activity of constitutively expressed D cyclin complexes can be repressed in noncycling cells. The lack of cyclin D1-*cdk4* activity in serum-starved NIH 3T3 cells ectopically expressing cyclin D1 was also thought to result from p27^{kip1}-mediated inhibition (26).

Additional studies compared the composition of the cyclin D3 pools in logarithmically growing wild-type and p27/p21^{-/-} MEFs. We found that the majority of cyclin D3 molecules in wild-type cells were complexed to *cdk4* and either p27^{kip1} or p21^{cip1}. Wild-type MEFs also contained a small subpopulation of cyclin D3 monomers (i.e., cyclin D3 molecules not bound to p27^{kip1}, p21^{cip1}, or *cdk4*) and a nearly imperceptible amount of binary cyclin D3-*cdk4* complexes. Although comprising only a minor fraction of the total cyclin D3-*cdk4* pool, binary cyclin D3-*cdk4* complexes appeared to be the sole source of cyclin D3-*cdk4* activity in wild-type MEFs. As shown above, cyclin D3 complexes containing p27^{kip1} or p21^{cip1} were not active (i.e., removal of these complexes did not deplete activity), and cyclin D3 monomers have no intrinsic kinase activity. We suggest that cyclin D3 fulfills its sequestration requirement in wild-type cells by devoting the major portion of its *cdk*-associated pool to p27^{kip1}-p21^{cip1} interaction. We also note that proliferating wild-type MEFs contained approximately equal amounts of cyclin D3-*cdk4*-p27^{kip1} and cyclin D3-*cdk4*-p21^{cip1}. Whereas

G₀ entrance and exit is controlled primarily by p27^{kip1} (10), this finding, in agreement with previous data (34), suggests that G₁ traverse in cycling cells is regulated by both p27^{kip1} and p21^{cip1}.

In contrast to p27/p21^{+/+} MEFs, the majority of cyclin D3 molecules in p27/p21^{-/-} MEFs were not coupled to cdk4. This finding implies that in the absence of p27^{kip1} and p21^{cip1}, cyclin D3-cdk4 complexes are not readily formed or are not stable. The preferential association of p27^{kip1} and p21^{cip1} with cyclin-cdk complexes as opposed to individual subunits (37, 38, 42) argues against a role of these CKIs in complex assembly. Moreover, using purified proteins, LaBaer et al. (25) found that p27^{kip1} and p21^{cip1} significantly increased the stability of cyclin D1-cdk4 complexes but had little effect on complex formation. Regardless of the mechanism involved, the limited association of cyclin D3 with cdk4 in p27/p21^{-/-} cells indicates that in these cells, as in wild-type cells, only a small fraction of the total cyclin D3 pool supplies the enzymatic activity required for Rb phosphorylation and G₁ traverse. These findings suggest that loss of p27^{kip1} and/or p21^{cip1} would not necessarily affect the absolute amount of cyclin D-cdk4 activity in the cell; i.e., loss of these CKIs would simply convert catalytically inactive ternary complexes to catalytically inactive monomers. In our studies, cyclin D3-cdk4 activity was lower in p27/p21^{-/-} cells than in wild-type cells; this may simply reflect the reduced levels of cyclin D3 and cdk4 in the double-knockout cells. Decreased amounts of cyclin D1 and cyclin D2 were also observed in p27/p21^{-/-} cells, the result perhaps of accelerated degradation due to glycogen synthase kinase-3 β -mediated phosphorylation (7). Removal of p27^{kip1} and p21^{cip1} from cells obviates the need for high levels of cyclin D-cdk4 complexes, and in response to elimination of these CKIs, cells may readjust their levels of D cyclins and their cdk partners.

In our experiments on mixed cell extracts, enhancement of cyclin D3-cdk4 complex formation by exogenously added p27^{kip1} was not observed. In contrast, in studies by others (25), both p27^{kip1} and p21^{cip1} promoted cyclin D-cdk4 assembly in vitro systems. In the cell, an equilibrium most likely exists between the monomeric and binary forms of the D cyclins and their cdk partners. As noted above, binary complexes have higher dissociation rates than do ternary complexes containing p27^{kip1} or p21^{cip1} (25). Generation of stable ternary complexes, however, presumably requires the initial association of cyclin and cdk and the subsequent interaction of p27^{kip1} or p21^{cip1} with the preassembled cyclin D-cdk complex. It is possible, therefore, that the capacity of the Cip/Kip proteins to promote assembly in vitro reflects the capacity of the D cyclins and cdk4 or cdk6 to transiently dimerize, a phenomenon that may be governed by assay conditions; such differences would account for the differences between our results and those of others (25).

Lastly, it is emphasized that, although cyclin D-cdk4 complexes may be more stable when bound to p27^{kip1} or p21^{cip1}, cyclin D-associated kinases are active only in the absence of these CKIs. We suggest that the D cyclins fulfill their sequestration requirement in wild-type cells by devoting the major portion of their cdk-associated pool to p27^{kip1}-p21^{cip1} interaction. The remainder of this pool is sufficient to supply all the necessary activity required for Rb phosphorylation. In addition, in wild-type cells, cyclin D-cdk4 activation indicates that levels of free p27^{kip1} and p21^{cip1} are reduced sufficiently to allow activation of cdk2-containing complexes; thus, cyclin D-cdk4 activation serves as a permissive signal for cdk2 activation. In cells lacking p27^{kip1} and p21^{cip1}, levels of cyclin D-cdk4 activity are kept in check by the inherent instability of binary cyclin D-cdk4 complexes. Thus, irrespective of whether p27^{kip1}

and p21^{cip1} are present in cells, mechanisms capable of limiting the extent and duration of cyclin D-cdk4 activity are operative.

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