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D-Type Cyclin-Dependent Kinase Activity in Mammalian Cells

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D-type cyclin-dependent kinase activities have not so far been detected in mammalian cells. Lysis of rodent fibroblasts, mouse macrophages, or myeloid cells with Tween 20 followed by precipitation with antibodies to cyclins D1, D2, and D3 or to their major catalytic partner, cyclin-dependent kinase 4 (cdk4), yielded kinase activities in immune complexes which readily phosphorylated the retinoblastoma protein (pRb) but not histone H1 or casein. Virtually all cyclin D1-dependent kinase activity in proliferating macrophages and fibroblasts could be attributed to cdk4. When quiescent cells were stimulated by growth factors to enter the cell cycle, cyclin D1-dependent kinase activity was first detected in mid G_1, reached a maximum near the G_1/S transition, and remained elevated in proliferating cells. The rate of appearance of kinase activity during G_1 phase lagged significantly behind cyclin induction and correlated with the more delayed accumulation of cdk4 and formation of cyclin D1-cdk4 complexes. Thus, cyclin D1-associated kinase activity was not detected during the G_0-to-G_1 transition, which occurs within the first few hours following growth factor stimulation. Rodent fibroblasts engineered to constitutively overexpress either cyclin D1 alone or cyclin D3 together with cdk4 exhibited greatly elevated cyclin D-dependent kinase activity, which remained absent in quiescent cells but rose to supraphysiologic levels as cells progressed through G_1. Therefore, despite continued enforced overproduction of cyclins and cdk4, the assembly of cyclin D-cdk4 complexes and the appearance of their kinase activities remained dependent upon serum stimulation, indicating that upstream regulators must govern formation of the active enzymes.

D- and E-type cyclins govern the rate of progression of mammalian cells through the first gap phase (G_1) of the cell cycle and enforce the commitment of cells to replicate their chromosomal DNA (reviewed in reference 44). When quiescent cells enter the cell cycle, both classes of cyclins are synthesized during G_1 phase and associate with cyclin-dependent kinases (cdks) to form holoenzymes whose activities are presumed to facilitate entry into S phase.

Cyclin E, first identified by complementation of G_1 cyclin deficiency in yeasts (22, 25), is synthesized in late G_1 phase and associates with cdk2 to generate a histone H1 kinase that is maximally active during the G_1-to-S phase transition in mammalian cells (10, 23). The enforced overexpression of cyclin E in fibroblasts shortens their G_1 interval, decreases (but does not eliminate) their dependency on serum growth factors, and leads to a reduction in their size, indicating that cyclin E can be rate limiting for G_1 progression (33). Its catalytic partner, cdk2, is also necessary for S-phase entry (34, 48), but it remains unclear whether this is due to its association with cyclin E and/or with cyclin A, which is expressed somewhat later during the cell cycle and steadily accumulates during the S and G_2 phases (30, 38, 39). The activity of the cyclin E-cdk2 complex is most likely subject to both positive and negative regulation. For example, phosphorylation of cyclin A- or B-bound cdc2 and cdk2 by a cdk-activating kinase can markedly increase their kinase activity (6, 14, 41, 45, 46). Conversely, a titratable stoichiometric inhibitor of the cyclin E-cdk2 complex has recently been identified in cells growth arrested by either contact inhibition or treatment with transforming growth factor β (TGF-β) (40). Therefore, cyclin E-cdk2 complex formation per se appears insufficient for maximal holoenzyme activity, and upstream signals that influence the synthesis of the catalytic and regulatory subunits, their assembly, and post-translational modifications might all contribute to the cell's rate of entry into S phase.

Three D-type cyclins (D1, D2, and D3) identified in the context of the delayed early response to growth factor stimulation (27) are differentially and combinatorially expressed in mammalian cells (1, 21, 27, 28, 32, 53). Cyclin D1 can complement G_1 cyclin-deficient yeasts (25, 54), and its locus is a known target of chromosomal translocations in human malignancies (31, 52). In general, the D-type cyclins are induced earlier in G_1 than cyclin E and can form complexes with cdk2, -4, -5, and -6 (4, 26, 29, 55). Although cdk4 appears to be their most prominent partner in macrophages and fibroblasts (26, 55), complexes with cdk6 predominate in peripheral blood T cells (29). Like cyclin E, overexpression of cyclin D1 in mammalian fibroblasts shortens G_1, reduces cell size, and decreases the cells' serum dependency (42). Conversely, cells microinjected during the G_1 interval with cyclin D1 antisense vectors or with specific antibodies to the D1 protein are inhibited from entering S phase, but injections performed at or after the G_1/S transition are without effect (3, 42). At least in fibroblasts, then, cyclin D1 is both necessary and rate limiting for G_1 progression. TGF-β arrests cells in late G_1 by inhibiting the expression of cdk4 (13) and the activity of the cyclin E-cdk2 complex (24). Overexpression of cdk4, but not cdk2, in epithelial cells can abrogate the ability of TGF-β to arrest cell cycle progression and leads to reactivation of cyclin E-cdk2 histone H1 kinase activity (13). Therefore, the similar phenotypes induced by cyclin D and E overexpression in fibroblasts may reflect a dependency of cyclin E-cdk2 activity on the earlier formation of cyclin D-cdk4 complexes, thereby temporally ordering the activity of the holoenzymes as cells progress through G_1 (13, 40).

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When cyclin-cdk complexes are reconstituted in insect cells, each of the D-type cyclins can activate cdk4 (19) and cdk6 (29), whereas D2 and D3, but not D1, can also functionally interact with cdk2 (12). Complexes between cdk2 and cyclins A or E readily phosphorylate histone H1, but histone H1 is a relatively poor substrate for reconstituted cyclin D-cdk4 and cyclin D-cdk6 complexes, which preferentially phosphorylate the retinoblastoma protein, pRb (19, 26, 29). This appears to be due, at least in part, to the ability of the D-type cyclins to bind to pRb directly (7, 12, 19). Paradoxically, cyclin D-cdk4 complexes recovered by immunoprecipitation from mammalian cell lysates have not yielded either histone H1 or pRb kinase activity when assayed in vitro, and this has greatly hampered efforts to determine their timing of action and their interactions with putative physiologic substrates, pRb among them. We now show that these limitations are technical in nature and that cyclin D-cdk4 kinases recovered from mammalian cells are activated during G_1 phase after quiescent cells have entered the cell cycle.

MATERIALS AND METHODS

Cells and culture conditions. Murine BAC1.2F5A macrophages were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25% L-cell-conditioned medium as a source of colony-stimulating factor 1 (CSF-1) (27, 47). Mouse myeloid 32DCl3 cells (15) and derivatives overexpressing D-type cyclins (20) were grown in Iscove's modified medium with 20% FBS, glutamine, antibiotics, and 25% WEHI-3B-conditioned medium as a source of interleukin-3. The derivation and properties of mouse NIH 3T3 and rat-2 fibroblasts overexpressing D-type cyclins and cdk4 have been described (42). G418-resistant rat-2 cells transfected with a control vector encoding neomycin (*neo*) resistance were used as controls in analyses of endogenous cyclin D1-associated kinase activity. NIH 3T3 cells stably overproducing cyclin D3 and cdk4 were developed by using identical transfection and selection procedures, employing pRc/RSV-neo vectors to express the cDNAs (Invitrogen, San Diego, Calif.) and drug selection in 0.8 mg of G418 (Geneticin; GIBCO/BRL, Gaithersburg, Md.) per ml to obtain polyclonal populations.

Antibodies. Antisera and monoclonal antibodies (MAbs) used in these studies are summarized in Table 1. Polyvalent rabbit antisera to mouse cyclins D1 (R_E and R_R), D2 (R_S, and R_T), and D3 (R_M) and to cdk4 (R_Y) were generated to bacterially produced polypeptides (26, 27). All sera were raised to full-length proteins except for R_R, which was prepared to a C-terminally truncated cyclin D1 mutant, lacking amino acid residues 215 to 295. The antisera to cyclins D1 and D2 cross-react with one another but preferentially precipitate the cognate proteins, whereas those to cyclin D3 do not detect D1 or D2 (26, 27). The antiserum to cdk4 is highly specific and both precipitates and blots authentic $p34^{cdk4}$ from rodent, human, and mink cells (13, 26). An antiserum (R_Z) to a synthetic C-terminal peptide of mouse cdk4 (NH_2-ISAFRALQHSYLHKEESDAE) conjugated to keyhole limpet hemocyanin was prepared as previously described (9); this antiserum does not cross-react with cdc2, cdk2, or cdk5 transcribed in vitro or expressed in insect Sf9 cells and detects only $p34^{cdk4}$ in metabolically labeled lysates of rodent macrophages and fibroblasts. A specific antiserum to a C-terminal peptide of cdk2 was prepared by similar methods (34, 35). Mouse and rat MAbs to intact proteins made in bacteria were raised and have been characterized in detail elsewhere (50).

TABLE 1. Antibodies to D-type cyclins and cdks[a]

Antibody	Antigen	Specificity
Rabbit		
E (R_E)	Bacterial D1	D1 > D2; no D3
R (R_R)	Bacterial D1 (amino acids 1–214)	D1 > D2; no D3
S (R_S)	Bacterial D2	D2 > D1; no D3
T (R_T)	Bacterial D2	D2 > D1; no D3
M (R_M)	Bacterial D3	D3 only
Y (R_Y)	Bacterial CDK4	CDK4 >> CDK2 or CDC2
Z (R_Z)	CDK4 (C-terminal peptide)	CDK4 only
Mouse MAb D1-72-13G	Bacterial D1	Rodent D1 only
Rat		
MAbs D1-17A6, D1-10C3, and D1 18B6	Bacterial D1	D1 > D2; no D3
MAb D2-34B1-3	Bacterial D2	D2 >> D1; no D3
MAb D2-34B4-7	Bacterial D2	D2 only
MAbs D3-18B6-10B, D3-32A1-7, D3-21A2-2, D3-23D6-6, D3-22B5-7, and D3-24B4-2	Bacterial D3	D3 only

[a] The derivation and specificity of all MAbs have been described elsewhere (50). The symbols > and >> indicate their relative degree of cross-reactivity to heterologous D cyclins and cdks.

Rat MAbs to mouse cyclin D1 and cyclin D2 weakly cross-react with epitopes in mouse cyclins D2 and D1, respectively, whereas all rat MAbs to cyclin D3 recognize only the cognate D3 protein. The rat MAbs react strongly with their rodent and human counterparts. A highly specific mouse MAb to cyclin D1 (D1-72-13G) reacts only with the cognate rodent protein but does not cross-react with cyclins D2 and D3 and is biologically active in arresting G_1 progression in fibroblasts after microinjection (42). Because of its specificity for D1 alone, this MAb was used for immunodepletion analyses (see Fig. 3) and for studies of cyclin D1-dependent kinase activity throughout the cell cycle (see Fig. 4 to 6). However, in fibroblasts and macrophages, similar data can be obtained by using other D1 antisera which support kinase activity.

Immune complex kinase assay. Cells were suspended at 1×10^6 to 5×10^6/ml in IP buffer [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.1% Tween-20] containing 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 20 U of aprotinin per ml, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate (all protease inhibitors are from Sigma Chemicals, St. Louis, Mo.) and sonicated at 4°C (Virsonic 475, full microtip power two times for 10 s each time). Lysates were clarified by centrifugation at 10,000 × *g* for 5 min, and the supernatants were precipitated for 2 to 6 h at 4°C with protein A-Sepharose beads precoated with saturating

amounts of the indicated antibodies. When MAbs were used, protein A-Sepharose was pretreated with rabbit anti-mouse or anti-rat immunoglobulin G (Cappel Organon, Durham, N.C.) to provide a suitable affinity matrix. Immunoprecipitated proteins on beads were washed four times with 1 ml of IP buffer and twice with 50 mM HEPES (pH 7.5) containing 1 mM DTT. The beads were suspended in 30 μl of kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT) containing substrate (either 0.2 μg of soluble glutathione *S*-transferase–pRb fusion protein [see below], 1 μg of histone H1 [Boehringer Mannheim, Indianapolis, Ind.], or 1 μg of casein [Sigma]) and 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP (NEN Dupont, Boston, Mass.; 6,000 Ci/mmol). After incubation for 30 min at 30°C with occasional mixing, the samples were boiled in polyacrylamide gel sample buffer containing sodium dodecyl sulfate (SDS) and separated by electrophoresis (2). Phosphorylated proteins were visualized by autoradiography of the dried slab gels.

For preparation of pRb substrate, *Escherichia coli* transformed with pGEX-Rb (379-928) (12) was grown to saturation overnight, diluted 1:10 in L broth, and incubated at 37°C for 2 h. Glutathione *S*-transferase–pRb fusion protein was induced by addition of 0.2 mM isopropylthioglycoside to the culture for 3 h, after which cells were recovered by centrifugation (5,000 × *g* for 5 min) at 4°C and lysed on ice by sonication in a 1/10 volume of NETN buffer (50 mM Tris HCl [pH 7.5], 120 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40 [NP-40]). Cleared lysates were mixed with glutathione-Sepharose 4B (Pharmacia, Piscataway, N.J.) and incubated for 1 to 18 h (as convenient) at 4°C. Beads were washed three times with NETN buffer and twice with kinase buffer, and the pRb fusion proteins were released at 4°C by incubation in kinase buffer containing 2 mM reduced glutathione (Sigma). The concentration and purity of soluble pRb were estimated by Coomassie blue staining of electrophoretically separated proteins on denaturing polyacrylamide gels in comparison to protein standards of known concentration.

Cell cycle analysis. For flow cytometric analysis (FACS) of DNA content, approximately 10^6 cells were suspended in 1 ml of a solution of 0.1% sodium citrate and 0.1% Triton X-100 containing 50 μg of propidium iodide per ml and treated for 30 min at room temperature with 1 μg of RNase (Calbiochem, San Diego, Calif.) per ml. DNA fluorescence was measured with a Coulter EPICS 753 flow cytometer, and the percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined (27). Estimates of S-phase fractions by this method correlate very well with those made on the basis of incorporation of 5-bromodeoxyuridine into replicating nuclear DNA (42). To analyze cyclin and cdk expression and kinase activity in synchronized cells, BAC1.2F5A macrophages arrested in early G_1 by CSF-1 starvation for 16 h were restimulated with the growth factor to reenter the cycle (27, 49). Fibroblasts made quiescent by contact inhibition and serum starvation in medium containing 0.1% FBS for 36 h were trypsinized and replated at subconfluent densities in complete medium containing 10% FBS. Cells progressing through the cycle were harvested at the intervals indicated in the figures.

Protein analysis. For detection of cyclins and cdks throughout the cell cycle, unlabeled cell lysates were immunoprecipitated with the indicated antisera, and denatured immune complexes were separated on gels, transferred to nitrocellulose, and immunoblotted (8) with antisera to the same proteins, by using ^{125}I-protein A (Amersham, Arlington Heights, Ill.) to visualize sites of antibody binding. Detection of cyclin-cdk complexes was achieved by immunoprecipitation with antiserum to one subunit (e.g., anti-cyclin) and blotting with antiserum to the other (e.g., anti-cdk) or vice versa. When indicated, the levels of D-type cyclins were also quantitated by direct immunoblotting analysis with MAbs detected by ECL enhanced chemiluminescence (Amersham), according to the manufacturer's instructions.

RESULTS

D-type cyclin-associated kinase activity in immune complexes. Antibodies to D-type cyclins and to their associated cdks can immunoprecipitate the cognate proteins and complexes between them from detergent lysates of mammalian cell lines, but many such reagents have not so far proven useful in recovering protein kinase activity in immune complexes. These have included polyvalent rabbit antisera raised against bacterially produced cyclins D1, D2, D3, and cdk4; antisera to a synthetic peptide representing the carboxyl-terminal amino acids of cdk4; and a panel of MAbs to the mouse D-type cyclins (Table 1). Negative results were previously obtained by using several substrates, including histone H1, the bacterially produced retinoblastoma protein (pRb), casein, myelin basic protein, synthetic copolymers, or a synthetic peptide containing canonical p34^{cdc2} recognition sites (26, 27).

In contrast, extracts of *Spodoptera frugiperda* (Sf9) insect cells coinfected with baculovirus vectors encoding cdk4 and D-type cyclins contain readily detected cyclin D-dependent kinase activity able to phosphorylate pRb (19, 26). We subsequently found that addition of detergents to these Sf9 lysates followed by precipitation of D-type cyclin-cdk4 complexes with polyvalent antisera or MAbs directed to the cyclins yielded significantly less pRb kinase activity than expected in the resulting immune complexes. SDS, sodium deoxycholate, Triton X-100, and NP-40 at concentrations as low as 0.1% all reduced the recovery of cyclin D-dependent kinase activity, whereas 0.1% Tween 20 appeared to be noninhibitory. Although SDS and sodium deoxycholate interfered with recovery of cyclin D-cdk4 complexes, NP-40 did not, and the subsequent removal of detergents by washing of immune complexes did not regenerate full enzyme activity (data not shown).

Because coinfected Sf9 cells express as much as 1,000-fold more cyclin and cdk4 than proliferating mammalian cells, we reasoned that we might be more likely to recover cyclin D-dependent kinases from mammalian cells engineered to overexpress the proteins. If such cells were mechanically disrupted in hypotonic buffers lacking detergent and then salt was added to extract nuclear proteins, immunoprecipitates prepared with antibodies to D-type cyclins or to cdk4 contained unacceptably high levels of contaminating nonspecific kinase activity. However, when such cells were lysed with 0.1% Tween 20 (but not SDS, sodium deoxycholate, or NP-40) and precipitated with antibodies to the cyclins, we retrieved readily detectable pRb kinase activity in the immune complexes (Fig. 1). As shown in Fig. 1A, polyvalent antisera (lanes 2 and 3) and a single mouse MAb directed to cyclin D1 (lane 7) supported pRb kinase activity in immune complexes, whereas three rat MAbs to D1 (lanes 8 to 10) or a cross-reactive MAb to D2 (lane 11) did not. An antiserum to the C terminus of cdk4 also supported enzymatic activity (lane 5) compared with one raised to the entire bacterially produced cdk4 protein (lane 4). Similar data were obtained with cells overproducing both cdk4 and cyclins D2 (Fig. 1B) or D3 (Fig. 1C), although in these cases, several of the rat MAbs as well as polyvalent rabbit antisera were effective in recovering active enzyme complexes (see Fig. 1B, lane 9 for D2, and Fig. 1C, lanes 7 to 11, 13, and 14 for D3). It seems likely that the nature and concentration of

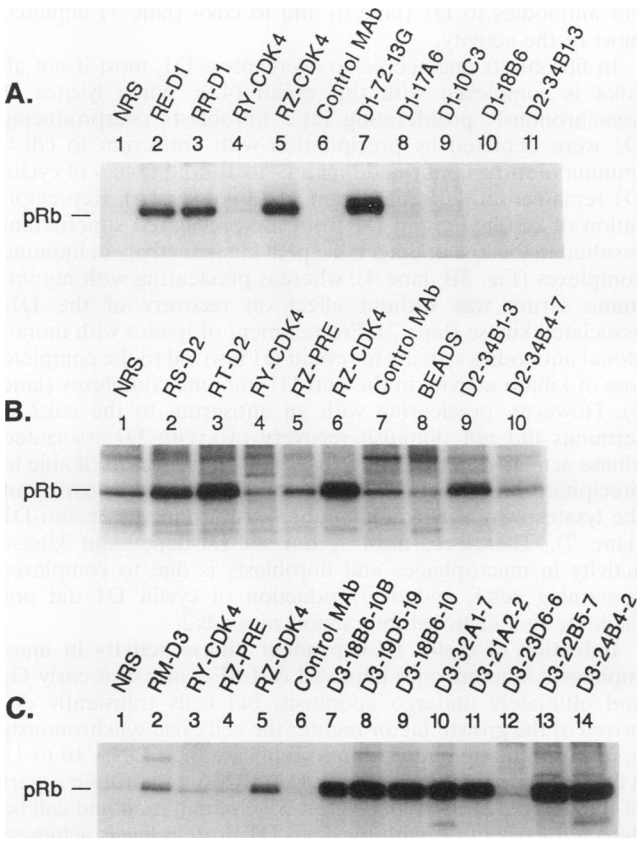

FIG. 1. Cyclin D-associated kinase activity in immune complexes. Extracts of 32Dcl3 myeloid cells engineered to overexpress cyclin D1 (A) (20) or NIH 3T3 cells overexpressing cdk4 and cyclins D2 (B) (42) or D3 (C) were immunoprecipitated with the indicated antibodies (see Table 1), with NRS or preimmune rabbit serum (RZ-PRE), with an irrelevant MAb to the human CSF-1 receptor (Control MAb), or with beads alone (BEADS). Immune complexes were assayed for pRb kinase activity. All autoradiographic exposure times were 1 h.

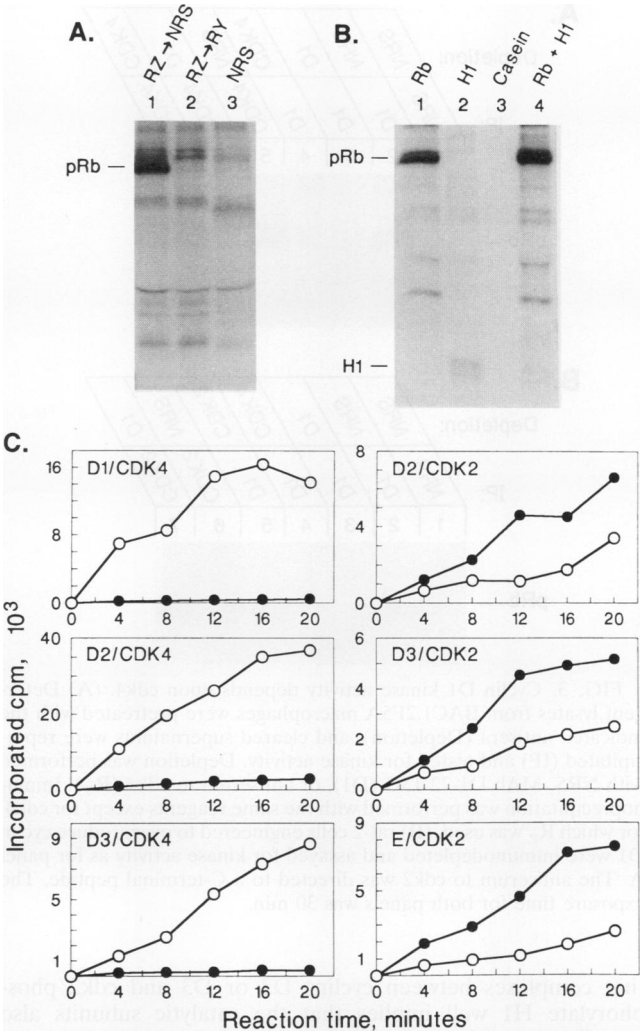

FIG. 2. Properties of the cyclin D-cdk4 kinase. (A) Lysates of 32Dcl3 myeloid cells overexpressing cyclin D1 were precipitated with an antiserum to the C terminus of cdk4 (R_Z, lanes 1 and 2) or with NRS (lane 3). The precipitates were washed and resuspended (arrows) in NRS or in antiserum directed to the entire cdk4 polypeptide (R_Y). Following incubation at 4°C for 1 h, the precipitates were rewashed and assayed for pRb kinase activity. (B) Immune complexes from the same cells were prepared by using MAb D1-72-13G to cyclin D1 and assayed for kinase activity against pRb (lane 1), histone H1 (lane 2), casein (lane 3), or a mixture of pRb and H1 in a 1:5 ratio (lane 4). Autoradiographic exposures for panels A and B were 4 h. (C) The indicated cyclin-cdk complexes reconstituted in insect cells were tested for their ability to phosphorylate pRb (open circles) or histone H1 (closed circles). Kinase reactions were initiated by using empirically determined, limiting enzyme concentrations and an excess of substrate (either 2.5 μg of histone H1 or 1.25 μg of pRb). Samples recovered at the indicated times were separated on denaturing gels, and radioactivity incorporated into labeled substrates was measured by liquid scintillation of the excised bands.

detergents are critically important in extracting stably assembled cyclin D-cdk complexes from cell nuclei, where they are normally compartmentalized (3).

At least in some cases, antibodies used to precipitate the cyclin D-cdk4 complexes can inhibit kinase activity. Enzymatically active complexes were recovered from cells overexpressing cyclin D1 by use of an antibody directed to the cdk4 C terminus (R_Z) (Fig. 1A, lane 5). The washed complexes were resuspended either in antiserum to the intact cdk4 protein (R_Y) or in nonimmune rabbit serum (NRS), and the beads were then rewashed and tested for pRb kinase activity (Fig. 2A). No kinase activity was precipitated from the cells by using nonimmune serum (lane 3), nor did it inhibit the pRb kinase activity recovered with antiserum to the cdk4 C terminus (lane 1). Under identical conditions, the antiserum to intact cdk4 inhibited the pRb kinase reaction (lane 2). In agreement, precipitates prepared with antiserum R_Y did not support kinase activity in cells overproducing cyclin D1, D2, or D3 (Fig. 1).

Cyclin D-cdk4 complexes engineered in Sf9 insect cells phosphorylate pRb much better than histone H1 (12, 19, 26). Figure 2C shows the kinetics of kinase reactions performed with different holoenzymes assembled in Sf9 cells. Although complexes composed of D-type cyclins and cdk4 showed an almost absolute preference for pRb over histone H1 (Fig. 2C, left panels), those containing cdk2, as well as the cyclin E-cdk2 complex, preferred histone H1 (right panels). Cyclin D1 differs from cyclins D2 and D3 in not forming functional complexes with cdk2 in this system (12). Although the substrate preference of cyclin D-cdk4 complexes may in part reflect the ability of D-type cyclins to interact directly with pRb (12, 19), the fact

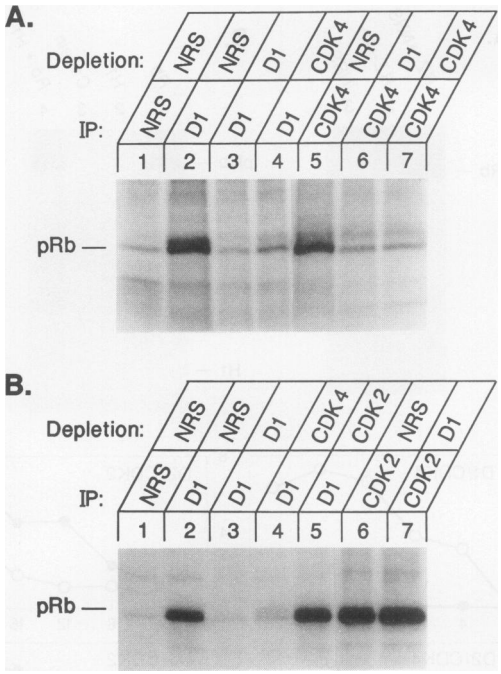

FIG. 3. Cyclin D1 kinase activity depends upon cdk4. (A) Detergent lysates from BAC1.2F5A macrophages were pretreated with the indicated antisera (Depletion), and cleared supernatants were reprecipitated (IP) and tested for kinase activity. Depletion was performed with NRS, MAb D1-72-13G (D1), or antiserum to cdk4 (R_Y). Immunoprecipitation was performed with the same reagents except for cdk4, for which R_Z was used. (B) rat-2 cells engineered to overproduce cyclin D1 were immunodepleted and assayed for kinase activity as for panel A. The antiserum to cdk2 was directed to a C-terminal peptide. The exposure time for both panels was 30 min.

that complexes between cyclins D2 or D3 and cdk2 phosphorylate H1 well implies that the catalytic subunits also contribute to substrate specificity. Similarly, cyclin D-associated kinase activity recovered in immune complexes from mammalian cell lysates (Fig. 2B) was more reactive toward pRb (lane 1) than to histone H1 (lane 2) and did not phosphorylate casein at all (lane 3). When the bacterially produced pRb fusion protein was mixed with a fivefold molar excess of histone H1 and tested in the assay, pRb was again the preferred substrate (lane 4). Similar data were obtained with cyclins D2 and D3, and the kinetics of pRb versus histone H1 phosphorylation obtained with the immunoprecipitated enzymes from mammalian cells recapitulated those observed with the enzymes reconstituted in insect cells (data not shown). We therefore continued to use pRb in all subsequent assays.

Cyclin D-dependent kinase activity in macrophages and fibroblasts is primarily due to cdk4. cdk4 is the major partner of D-type cyclin complexes in macrophages and fibroblasts (26, 55), but it cannot be activated in insect cells by cyclin A, B, or E (19). We therefore tested whether antisera to cdk4 could deplete D1 cyclin-dependent kinase activity from mouse macrophages and vice versa (Fig. 3A). Preclearing of mouse macrophage lysates with NRS had no effect on the recovery of kinase activity precipitated with a MAb to D1 (Fig. 3A, lane 2), but pretreatment with the MAb itself removed the activity (lane 3). The vast majority of kinase activity was also removed by rabbit antiserum to cdk4 (lane 4). Reciprocally, pretreatment with NRS did not affect the recovery of kinase activity precipitated with an antiserum to the cdk4 C terminus (lane 5), but antibodies to D1 (lane 6) and to cdk4 (lane 7) depleted most of the activity.

In fibroblasts engineered to overexpress D1, most if not all cdk4 is complexed with this cyclin (42). When lysates of asynchronously proliferating rat-2 fibroblasts overproducing D1 were depleted by precipitation with antiserum to cdk4, immunoblotting confirmed that a 6- to 10-fold excess of cyclin D1 remained in the supernatant (data not shown). Reprecipitation of residual cyclin D1 from the precleared supernatant resulted in the loss of detectable pRb kinase activity in immune complexes (Fig. 3B, lane 4), whereas preclearing with nonimmune serum was without effect on recovery of the D1-associated kinase (lane 2). Pretreatment of lysates with monoclonal antibodies specific for cyclin D1 also led to the complete loss of kinase activity in the anti-D1 immune complexes (lane 3). However, preclearing with an antiserum to the cdk2 C terminus did not diminish recovery of cyclin D1-associated kinase activity (lane 5). The antiserum to cdk2 was itself able to precipitate high levels of kinase activity, after preclearing of the lysates with either nonimmune serum (lane 6) or anti-D1 (lane 7). Therefore, most if not all D1-dependent kinase activity in macrophages and fibroblasts is due to complexes containing cdk4, and overproduction of cyclin D1 did not facilitate its functional interaction with cdk2.

Induction of cyclin D1-dependent kinase activity in macrophages. Macrophages deprived of CSF-1 arrest in early G_1 and ultimately undergo apoptosis, but cells transiently deprived of the growth factor reenter the cell cycle synchronously upon restimulation and begin to replicate their DNA 10 to 12 h later (49). Expression of cyclin D1 mRNA and protein is part of the delayed early response to CSF-1 stimulation and can be detected early in G_1 with maximal D1 protein levels achieved by mid-G_1 phase (Fig. 4A) (27). In these cells, the total D1 protein level oscillates minimally (less than twofold) throughout subsequent cell cycles, with its highest expression observed near the G_1/S transition (Fig. 4, compare top and bottom, 9 and 12 h). Two forms of cyclin D1 can be detected in immunoblots, but the species of slower mobility is more pronounced at the G_1/S boundary (Fig. 4A) (27). Both forms are phosphoproteins, and the biochemical differences that distinguish them have not been defined. In contrast, cdk4 is expressed at very low levels in CSF-1-starved cells and increases following CSF-1 stimulation, reaching maximal levels by early S phase (Fig. 4B) (26). Note that D1 is induced several hours prior to cdk4 (Fig. 4A and B) and that both proteins continue to be synthesized throughout the cell cycle as long as CSF-1 is present (26, 27). Withdrawal of CSF-1 at any time during the cycle leads to the rapid loss of cyclin D1 (half-life, ca. 20 min) and to a slower decline in cdk4 levels (half-life, ca. 4 h) (26, 27).

As might be expected from these results, the rate of complex formation between cyclin D1 and cdk4 is limited by the slower appearance of the catalytic subunit during G_1. When cyclin D1 immunoprecipitates were separated on denaturing gels, transferred to nitrocellulose, and blotted with antiserum to cdk4, the rate of complex formation closely corresponded to the accumulation of cdk4 during G_1 (Fig. 4C). Reciprocal experiments in which cdk4 immunoprecipitates were separated and blotted with antibodies to cyclin D1 rendered virtually identical results (Fig. 4D). Both forms of cyclin D1 normally detected in macrophages formed complexes with cdk4.

Assays of cyclin D1-associated kinase activity throughout the first cycle after growth arrest indicated that cells deprived of CSF-1 lacked enzyme activity (time 0), but the kinase was induced during mid-G_1 phase and, once induced, remained elevated (Fig. 4E). Kinase activity appeared not to significantly

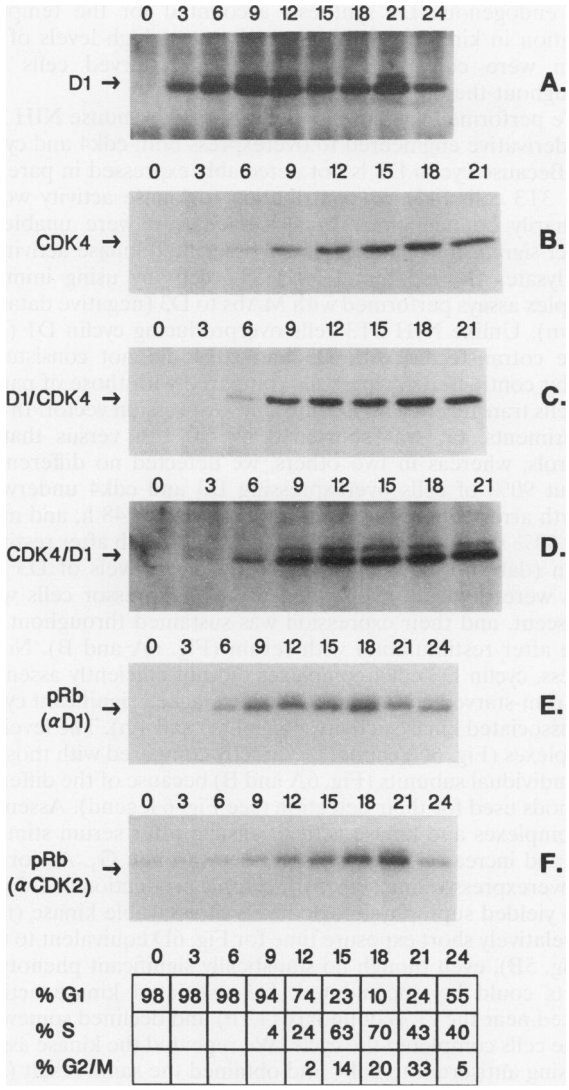

FIG. 4. Kinetics of appearance of cyclin D1 (A), cdk4 (B), D1-cdk4 complexes (C and D), D1-associated pRb kinase (E), and cdk2-associated pRb kinase (F) following CSF-1 stimulation of growth factor-deprived macrophages. The intervals (in hours) following stimulation are indicated at the tops of the panels, and the distribution of cells in different phases of the cell cycle is indicated at the bottom of the figure. (A and B) Lysates prepared at indicated times were immunoprecipitated and blotted with R_E (anti-D1) and R_Y (anti-cdk4), respectively; (C and D) lysates precipitated with one of the antisera were blotted with the other in the order shown (left to right) in the margin; (E) pRb kinase activity performed with immune complexes (αD1) prepared with MAb D1-72-13G; (F) assays performed with antiserum (αCDK2) to the cdk2 C terminus. Exposure times (^{125}I-protein A detection) for panels A to D are 18 h, and those for panels E and F (^{32}P) are 2 h and 30 min, respectively. The gels shown in panels A, E, and F were performed by using narrower combs.

oscillate throughout subsequent cycles, but it rapidly declined in parallel with cyclin D1 when CSF-1 was withdrawn, regardless of position in the cell cycle (data not shown). Note that for transiently starved and restimulated cells in which good synchrony can be achieved, maximal kinase activity was initially observed by 12 h after stimulation (Fig. 4E), corresponding to the rate of formation of cyclin D1-cdk4 complexes (Figs. 4C and D). However, D1-associated kinase activity appeared to be subtly biphasic with a very minimal decline observed in S phase (15 h) followed by a small but reproducible rise in late S and G_2 phases (18 h) and falling again during peak mitosis (21 h). Although unimpressive in magnitude, the latter oscillations in kinase activity later in the cycle were observed reproducibly in four independent experiments using MAb D1-72-13G, antiserum R_E, or an antiserum directed to a cyclin D1 C-terminal peptide. Immunodepletion assays (similar to those whose results are shown in Fig. 3) performed with cells synchronized in late S/G_2 again suggested that most D1-dependent pRb kinase activity was due to cdk4, but given the limits of the assays, we cannot exclude minor contributions from other catalytic partners. Unlike the kinase activity associated with cyclin D1, pRb kinase assays (or histone H1 kinase assays [data not shown]) performed with immune complexes prepared with an antiserum to cdk2 yielded very different kinetics throughout the cell cycle (Fig. 4F). cdk2-associated kinase activity was detected late in G_1 but increased steadily as cells moved toward M phase. It is likely that this activity is initially due to holoenzyme complexes involving cyclin E, which form late in G_1 (10, 23), and cyclin A, which is first synthesized near the G_1/S transition and accumulates until cells enter mitosis (11, 35, 38, 39, 43).

Cyclin D1-associated kinase activity in serum-stimulated fibroblasts. Similar data for quiescent rat-2 fibroblasts stimulated to reenter the cell cycle with serum were obtained (Fig. 5A, top). Here, not all cells reentered the cycle, their generation time was shorter, and the degree of synchrony obtained was therefore lower (Fig. 5A, bottom). The major D-type cyclin expressed in rat-2 cells (42), as in other fibroblast lines (3, 53), is also D1, again in complexes with cdk4 (55). The expression of cyclin D1 tends to be more periodic in fibroblasts than in macrophages, with maximal levels achieved late in G_1, followed by a more appreciable decrease as cells enter S phase (3, 53). Immunofluorescence studies revealed that D1 accumulates in the nucleus during G_1 phase, but nuclear staining is lost as cells replicate their DNA (3). We observed that D1-associated pRb kinase activity was virtually absent in quiescent cells (Fig. 5A, top, 0 h) but was induced by serum, rising abruptly before the G_1/S transition (6 to 9 h) and increasing somewhat more as cells progressed through the cycle. The high levels of kinase observed by the end of the experiment are typical of those in asynchronously proliferating cells (data not shown). Immune complex kinase assays performed with control beads rendered only background levels of pRb kinase activity throughout the cell cycle (Fig. 5A, middle). The subtle oscillations in D1-associated kinase observed in macrophages (Fig. 4E) were not seen in rat-2 cells.

Kinase activity in cells overexpressing D-type cyclins and cdk4. Fibroblasts overexpressing cyclin D1 exhibit contracted G_1 intervals (42). If the rate of increase of cyclin D1-dependent kinase during reentry into the cell cycle is normally determined by the rates of synthesis of both cyclin D1 and cdk4, we would assume that cells engineered to constitutively overexpress one or both subunits would not only exhibit significantly higher levels of kinase activity but might also manifest this activity earlier in G_1.

Figure 5B shows that rat-2 cells overproducing five to eightfold-more D1 than counterparts transfected with a control *neo* vector (42) also expressed much higher levels of cyclin D1-associated kinase activity (note the 1-h exposure for Fig. 5B, top, versus 8 h in Fig. 5A, top), whereas very little activity was detected with control beads (Fig. 5B, middle). As previously reported, it was much more difficult to arrest the growth of these cells by serum starvation (Fig. 5B, bottom). Even so, the kinase was induced during G_1 (Fig. 5B, top). It is unlikely

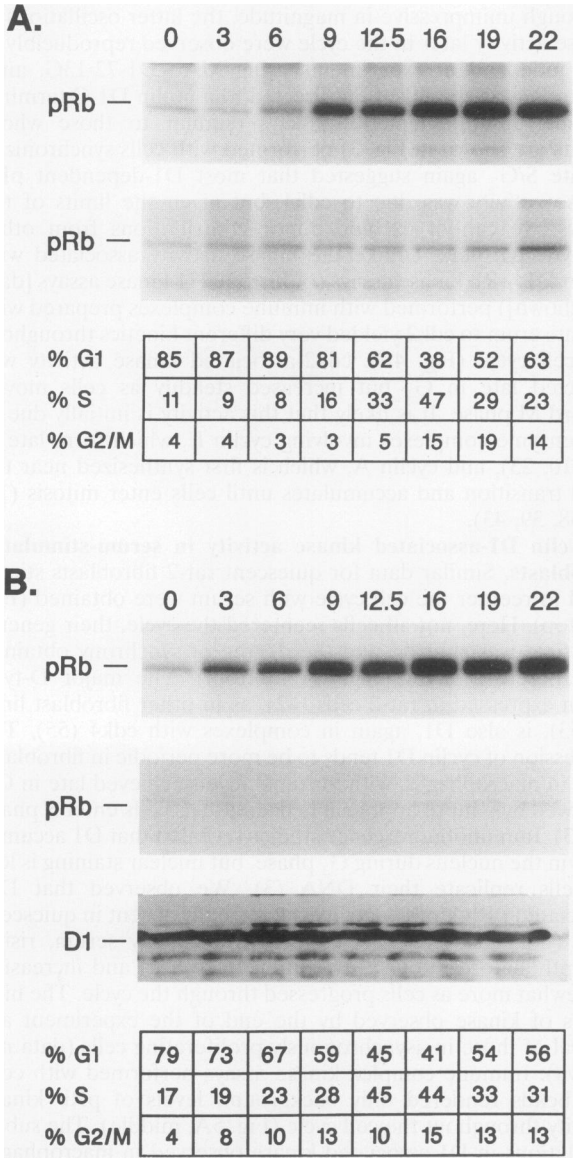

FIG. 5. Kinetics of appearance of cyclin D1-associated kinase in rat-2 fibroblasts. Quiescent cells (time zero) were stimulated to reenter the cell cycle, and lysates prepared at the indicated times (in hours) were immunoprecipitated with MAb D1-72-13G and assayed for pRb kinase activity (top strips in both panels). The middle strips show results with control beads prepared with an irrelevant antibody. Results with control rat-2 cells (A) are contrasted with those of derivatives engineered to overexpress cyclin D1 (ca. five- to eightfold increase at the protein level) (B). The latter cells were not completely quiescent at the start of the experiment because of their reduced serum dependence (42). Note the different autoradiographic exposure times in panels A (8 h) and B (1 h). (B) The lower strip shows direct immunoblotting analysis of constitutively expressed cyclin D1 performed with MAb D1-72-13G, using the same cell lysates shown above; D1 was detected by enhanced chemiluminescence. Cell cycle distributions determined by FACS analysis are indicated below both panels. In all experiments, lysates from 2×10^6 cell equivalents were loaded per lane.

that endogenous D1 synthesis accounted for the temporal variation in kinase activity, given that very high levels of the cyclin were constitutively expressed in starved cells and throughout the cycle (Fig. 5C).

We performed similar experiments using a mouse NIH 3T3 cell derivative engineered to overexpress both cdk4 and cyclin D3. Because cyclin D3 is not detectably expressed in parental NIH 3T3 cells (42), its contribution to kinase activity would ordinarily be negligible. In agreement, we were unable to detect significant endogenous D3-associated kinase activity in cell lysates from parental NIH 3T3 cells by using immune complex assays performed with MAbs to D3 (negative data not shown). Unlike NIH 3T3 cells overproducing cyclin D1 (42), those cotransfected with D3 and cdk4 did not consistently exhibit contracted G_1 intervals compared with those of parental cells transfected with a control *neo* expression vector. In two experiments, G_1 was shortened by ca. 1 h versus that in controls, whereas in two others, we detected no differences. About 90% of cells overexpressing D3 and cdk4 underwent growth arrest when starved in 0.1% serum for 48 h, and more than 60% synchronously entered S phase by 12 h after restimulation (data not shown). Importantly, high levels of D3 and cdk4 were detected even when the overexpressor cells were quiescent, and their expression was sustained throughout the cycle after restimulation with serum (Fig. 6A and B). Nonetheless, cyclin D3-cdk4 complexes did not efficiently assemble in serum-starved cells (Fig. 6C), which lacked significant cyclin D3-associated kinase activity (Fig. 6D, 0 and 4 h). The levels of complexes (Fig. 6C) cannot be directly compared with those of the individual subunits (Fig. 6A and B) because of the different methods used for their detection (see Fig. 6 legend). Assembly of complexes and kinase activity ensued after serum stimulation and increased as cells progressed through G_1. As for the D1 overexpressor line (Fig. 5B), ectopic production of D3 plus cdk4 yielded supraphysiologic levels of assayable kinase (note the relatively short exposure time for Fig. 6D equivalent to that in Fig. 5B), even though no statistically significant phenotypic effects could be documented. D3-associated kinase activity peaked near the G_1/S boundary (12 h) and declined somewhat as the cells completed the cycle. We repeated the kinase assays by using antiserum to cdk4 and obtained the same result (Fig. 6E). Together, these findings indicate that the formation of the catalytically active complexes must be subject to additional rate-limiting controls.

DISCUSSION

An assay for cyclin D-dependent kinases in mammalian cells. The major impediment in characterizing the manner by which D-type cyclins function during the cell cycle has been the inability to measure their associated kinase activities in mammalian cells. Although the D-type cyclins form complexes with known cdks in vivo (4, 20, 26, 29, 55) and can directly activate certain of these catalytic subunits in artificially reconstituted systems (12, 19, 26, 29), direct evidence supporting such a role in mammalian cells has been conspicuously lacking. We found that three variables are of prime importance in recovering active holoenzymes containing cdk4. First, by reconstituting recombinant cyclin D-cdk4 complexes in Sf9 insect cells and attempting to recover their enzyme activity in immune complexes, we recognized that certain detergents would inhibit the recovery of cyclin D-associated kinase activity from mammalian cell lysates. Of the detergents tested, Tween 20 afforded the best results. Second, unlike other functional cyclin-cdk complexes which readily phosphorylate histone H1, reconstituted cyclin D-cdk4 complexes have a strong substrate prefer-

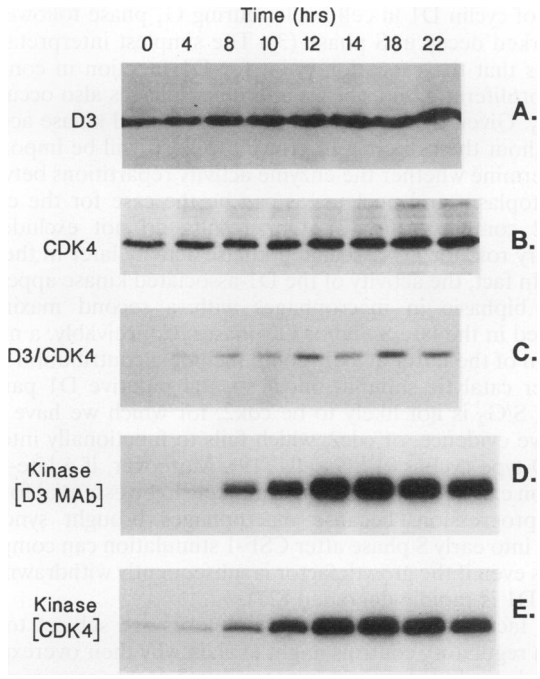

FIG. 6. Kinetics of appearance of cyclin D3-cdk4 activity in NIH 3T3 cells overproducing both subunits. The experiment was performed as described in the legend to Fig. 5, by using 5×10^5 cell equivalents per lane in each assay. (A and B) Direct immunoblotting of cyclin D3 and cdk4, respectively, as detected by enhanced chemiluminescence. (C) To study complex formation between the two subunits, lysates prepared at the indicated intervals were precipitated with antibodies to cyclin D3 (MAb D3-18B6-10B) and blotted with antiserum to cdk4 (R_Y). Detection of sites of antibody binding was performed with ^{125}I-protein A (exposure time, 14 h). Because of the different methods used, intensity of the signals in panel C cannot be normalized to those in panels A and B. Kinase activity was determined in immune complexes prepared with the MAb to D3 (D) or with antiserum (R_Z) to the cdk4 C terminus (E). The autoradiographic exposure times for panels D and E were 1 h. Background kinase activity determined with control beads was equivalent to those observed at the 0-h time points throughout the experiment.

ence for pRb over histone H1 (26), in part reflecting a propensity of the D-type cyclins to bind to pRb directly (7, 12, 19), and this was also observed for holoenzymes recovered from mammalian cells. Finally, not all antibodies directed to D-type cyclins or to cdk4 are capable of supporting kinase activity in immune complexes, and at least some can directly inhibit the enzymes. Under optimized conditions, this assay is sensitive, convenient, highly specific, and can be used to detect kinase activity associated with each of the D-type cyclins.

Using an antiserum directed to a C-terminal peptide of cyclin D1 (TPTDVRDVDI, residues 286 to 295), Hall et al. (16) detected low levels of associated histone H1 kinase activity in immunoprecipitates recovered from detergent lysates of rat epithelial and human osteosarcoma cells. Their lysis buffers contained 0.3% NP-40 and 0.2% Triton X-100 (5), which in our hands inhibited cyclin D1-associated kinase activity recovered with several other polyvalent and anti-peptide antibodies and MAbs. Moreover, D1-associated kinase activity was attributed to complexes containing cdk2 and cdc2, whereas we have been unable to reconstitute D1-associated pRb or histone H1 kinase activity with either of these catalytic subunits (12, 19). The anti-peptide serum to the cyclin D1 C terminus, but not an antiserum directed to the entire cyclin D1 protein, coprecipitated another 46-kDa protein, which Hall and coworkers designated cyclin X (51). The latter species differs from bona fide cyclin D1 in its timing of expression, in its immunoreactivity and primary structure, and in its preferential association with cdk2. When partially purified, this protein exhibited significantly greater histone H1 kinase activity than authentic cyclin D1 isolated from the same cells, suggesting that the kinase activity attributed to cyclin D1 in these studies may reside with cyclin X. None of our antibodies precipitated or immunoblotted this 46-kDa protein.

Cyclin D1 preferentially activates cdk4 in macrophages and fibroblasts. In CSF-1-dependent macrophages in which cyclin D3 is not expressed and D2 is synthesized at very low levels and in rat-2 cells in which ectopically expressed cyclin D1 was synthesized in significant excess over endogenous cdk4, immunodepletion of detergent lysates with antiserum to cdk4 removed virtually all cyclin D1-associated pRb kinase activity (and vice versa); depletion of cdk2 was without detectable effect. In such cells, then, most if not all cyclin D1-associated cdk activity could be attributed to cdk4, and excess D1 was unable to detectably activate other cdks. In Sf9 cells engineered to produce mammalian cyclin-cdk complexes, cdk4 can be activated by each of the D-type cyclins but not by cyclins A, B1, or E, whereas cdc2 (and cdk5 [18]) do not functionally interact with D-type cyclins in this system (19). However, cyclins D2 and D3, but not D1, can functionally interact with cdk2 in Sf9 cells (12), leaving open the possibility that in those mammalian cells which predominately express cyclins D2 and D3 (e.g., T lymphocytes or early myeloid precursors [1, 20, 21, 27, 29]), at least some cyclin D-associated kinase activity might be due to cdk2. Moreover, Meyerson and Harlow (29) provide accompanying evidence that the major D-type cyclin catalytic partner in T cells is cdk6. Observations that cyclin D1 can form complexes with cdk2 in fibroblasts (55) suggest that it might direct cdk2 to other substrates or alternatively act as a cdk2 inhibitor. D-type cyclins might also form functional complexes with yet other novel cdks whose inability to phosphorylate either histone H1 or pRb could hamper their detection.

Temporal expression and regulation of cyclin D-associated kinases. Early-G_1-arrested, CSF-1-deprived macrophages and quiescent, serum-starved fibroblasts do not express detectable cyclin D1-associated kinase activity, but the enzyme is induced once cells reenter the cell cycle and initially reaches a maximum near the G_1/S transition. Very similar kinetics of activation were observed in T lymphocytes for cyclin D-dependent complexes containing cdk6 (29). This argues strongly against a role for cyclin D-associated kinases during the G_0-to-G_1 transition, which occurs in the first few hours after growth factor stimulation (36). Importantly, the synthesis of cyclin D1 during G_1 phase preceded the appearance of cyclin D1-associated kinase activity, which temporally corresponded to the induction of cdk4 and its movement into complexes with D1. Thus, a functional execution point for cyclin D1 is likely to occur late in G_1, consistent with conclusions culled from microinjection experiments (3, 42). Enforced overexpression of cyclin D1, which, as shown here, led to significant increases in kinase activity, shortens the G_0-to-S phase transition of rat-2 or mouse NIH 3T3 cells by several hours (42), but the results above imply that cdk4 synthesis is also rate limiting during G_1, at least for cells reentering the cycle from a quiescent state.

The maintenance of cyclin D1-cdk4 complexes in continuously proliferating cells depends upon persistent growth factor-induced signals (3, 26–28, 53), but because cdk4 turns over much more slowly than D1 (26), it is less likely to be as rapid or sensitive a sensor. However, TGF-β arrests mink epithelial

cells in G_1 by interfering with cdk4 synthesis but not that of cyclin D1, D2, E, or cdk2 (13, 24). The enforced expression of cdk4 in such cells can override the TGF-β block, enabling subsequent activation of the cyclin E-cdk2 complex, pRb phosphorylation, and S-phase entry (13). Therefore, the synthesis of both cdk4 and cyclin D1 can be independently governed by upstream regulatory controls which determine their rates of synthesis and, ultimately, their assembly into functional holoenzyme complexes.

Although constitutive overexpression of either cyclin D1 alone or D3 together with cdk4 in proliferating fibroblasts led to very significant overall increases in D-type cyclin-dependent kinase activity relative to the endogenous levels, the kinases remained inactive in quiescent cells. Entry into the cell cycle was accompanied by an increase in both cyclin D-cdk4 complexes and kinase activity as cells approached the G_1/S boundary. For NIH 3T3 cells overexpressing cyclin D3 and cdk4, the increase in kinase activity during G_1 clearly cannot be attributed to the expression of endogenous D-type cyclins, because the parental cells synthesize negligible quantities of cyclin D3, and the MAb used to immunoprecipitate D3-cdk4 complexes from the overexpressors does not cross-react with D2 or D1 (50). Assembly of active cyclin D-cdk4 complexes therefore appears to be subject to posttranslational controls, and the abundance or activity of their regulator(s) must also be rate limiting for the G_0-to-S phase transition.

A surprising result was that ectopic expression of the cyclin D3-cdk4 complex, as opposed to D1-cdk4 (42), did not consistently affect the rate of G_1 progression. Perhaps, the D1- and D3-containing holoenzymes exhibit distinct preferences for physiologic substrates, and these are simply not reflected in immune complex kinase assays performed with pRb. A conceptually analogous result was previously obtained with early myeloid cells which normally express D2 and D3 but not D1; overexpression of D2 and D3 perturbed their ability to differentiate to neutrophils in response to granulocyte CSF, whereas D1 was without effect (20). Together, these results buttress the view that D-type cyclins are unlikely to be redundant (44).

A candidate positive regulator of the cyclin D-cdk4 complex is the cdk-activating kinase, which might trigger its activity by phosphorylating cyclin D-bound cdk4 on threonine 172 (analogous in context and position to Thr-160 and Thr-161 in cdk2 and cdc2, respectively). cdk-activating kinase itself contains a catalytic subunit (alias MO15) which is distantly related to known cdks, and like the latter, it appears to require another regulatory subunit(s) for its activity (14, 41, 45). We have evidence that cdk4 bound to D-type cyclins is phosphorylated on threonine 172 and activated through this mechanism when the two subunits are coexpressed and assembled in intact Sf9 cells (18). However, additional factors, present either in Sf9 or in mammalian cell lysates, are required for efficient cyclin D-cdk4 assembly. Negative regulators of the cyclin D-cdk4 complex, analogous to those that govern cyclin E-cdk2 activity (24, 40), may also exist. In keeping with this possibility, cdk4 forms stable complexes in vivo with a series of low-molecular-weight proteins whose functions have not yet been determined (55, 56). On the basis of results here with cells overproducing cdk4 and D-type cyclins, one or more of these putative regulators of the holoenzyme complexes are also likely to be responsive to growth factor-induced signals.

In proliferating macrophages and fibroblasts, oscillations of the cyclin D1 and cdk4 proteins are not readily apparent, although both components are somewhat more abundant during late G_1 (3, 26, 27). However, immunofluorescence staining of asynchronously replicating, 5-bromodeoxyuridine-labeled human fibroblast strains revealed an intense accumulation of cyclin D1 in cell nuclei during G_1 phase followed by its marked decay in S phase (3). The simplest interpretation, then, is that the execution point for D1 function in continuously proliferating fibroblasts and macrophages also occurs in late G_1. Given the persistence of D1-associated kinase activity throughout the cell cycle in growing cells, it will be important to determine whether the enzyme activity repartitions between the cytoplasm and nucleus, as can be the case for the cyclin B-cdc2 complex (17, 37). Our results do not exclude an ancillary role for D1-associated kinase activity later in the cell cycle. In fact, the activity of the D1-associated kinase appeared subtly biphasic in macrophages with a second maximum achieved in the late S and/or G_2 phases. Conceivably, a minor fraction of the latter activity could include a contribution from another catalytic subunit, but if so, the putative D1 partner during S/G_2 is not likely to be cdk2, for which we have only negative evidence, or cdc2, which fails to functionally interact with D-type cyclins in Sf9 cells (19). Moreover, if a late-S/G_2 function exists for cyclin D1, it is unlikely to be essential for cell cycle progression, because macrophages brought synchronously into early S phase after CSF-1 stimulation can complete mitosis even if the growth factor is subsequently withdrawn and cyclin D1 is rapidly degraded (27).

The fact that cyclin D-cdk4 complexes are subject to upstream regulatory controls might explain why their overexpression reduces but does not bypass growth factor requirements for progression through G_1. In turn, several lines of evidence now suggest that the activity of cyclin E-cdk2 is regulated, at least in part, by formation of the D-cdk4 complex (13, 40). The relative timing and interdependence of the cyclin D-cdk4 and cyclin E-cdk2 kinases are likely to be important in ensuring an orderly phosphorylation of physiologic substrates as cells commit to DNA synthesis and concomitantly lose their requirement for mitogenic growth factors until they complete division and reenter G_1 (36).

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REFERENCES

1. **Ajchenbaum, F., K. Ando, J. A. DeCaprio, and J. D. Griffin.** 1993. Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes. J. Biol. Chem. **268**:4113–4119.
2. **Anderson, S. J., M. A. Gonda, C. W. Rettenmier, and C. J. Sherr.** 1984. Subcellular localization of glycoproteins encoded by the viral oncogene v-*fms*. J. Virol. **51**:730–741.
3. **Baldin, V., J. Likas, M. J. Marcote, M. Pagano, J. Bartek, and G. Draetta.** 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. **7**:812–821.
4. **Bates, S., L. Bonetta, D. MacAllan, D. Parry, A. Holder, C. Dickson, and G. Peters.** 1994. CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. Oncogene **9**:71–79.
5. **Carbonaro-Hall, D., R. Williams, L. Wu, D. Warburton, M.**

Zeichner-David, M. MacDougall, V. Tolo, and F. Hall. 1993. G1 expression and multistage dynamics of cyclin A in human osteosarcoma cells. Oncogene **8:**1649–1659.
6. **Desai, D., Y. Gu, and D. O. Morgan.** 1992. Activation of human cyclin-dependent kinases in vitro. Mol. Biol. Cell **3:**571–582.
7. **Dowdy, S. F., P. W. Hinds, K. Louis, S. I. Reed, A. Arnold, and R. A. Weinberg.** 1993. Physical interactions of the retinoblastoma protein with human cyclins. Cell **73:**499–511.
8. **Downing, J. R., C. W. Rettenmier, and C. J. Sherr.** 1988. Ligand-induced tyrosine kinase activity of the colony stimulating factor-1 receptor in a murine macrophage cell line. Mol. Cell. Biol. **8:**1795–1799.
9. **Downing, J. R., S. A. Shurtleff, and C. J. Sherr.** 1991. Peptide antisera to human colony-stimulating factor 1 receptor detect ligand-induced conformational changes and a binding site for phosphatidylinositol 3-kinase. Mol. Cell. Biol. **11:**2489–2495.
10. **Dulic, V., E. Lees, and S. I. Reed.** 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. Science **257:**1958–1961.
11. **Elledge, S. J., R. Richman, F. L. Hall, R. T. Williams, N. Lodgson, and J. W. Harper.** 1992. *CDK2* encodes a 33-kDa cyclin A-associated protein kinase and is expressed before *CDC2* in the cell cycle. Proc. Natl. Acad. Sci. USA **89:**2907–2911.
12. **Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J.-Y. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73:**487–497.
13. **Ewen, M. E., H. K. Sluss, L. L. Whitehouse, and D. M. Livingston.** 1993. TGF-β inhibition of cdk4 synthesis is linked to cell cycle arrest. Cell **74:**1009–1020.
14. **Fesquet, D., J.-C. Labbé, J. Derancourt, J.-P. Capony, S. Galas, F. Girard, T. Lorca, J. Shuttleworth, M. Dorée, and J.-C. Cavadore.** 1993. The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologous. EMBO J. **12:**3111–3121.
15. **Greenberger, J. S., M. A. Sakakeeny, R. J. Humphries, C. J. Eaves, and R. J. Eckner.** 1983. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA **80:**2931–2943.
16. **Hall, F. L., R. T. Williams, L. Wu, F. Wu, D. A. Carbonaro-Hall, J. W. Harper, and D. Warburton.** 1993. Two potentially oncogenic cyclins, cyclin A and cyclin D1, share common properties of subunit configuration, tyrosine phosphorylation and physical association with the Rb protein. Oncogene **8:**1377–1384.
17. **Heald, R., M. McLoughlin, and F. McKeon.** 1993. Human Wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. Cell **74:**463–474.
18. **Kato, J.-Y., M. Matsuoka, D. Strom, D. E. Quelle, and C. J. Sherr.** Unpublished data.
19. **Kato, J.-Y., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr.** 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase, CDK4. Genes Dev. **7:**331–342.
20. **Kato, J.-Y., and C. J. Sherr.** 1993. Inhibition of granulocyte differentiation by G1 cyclins, D2 and D3, but not D1. Proc. Natl. Acad. Sci. USA **90:**11513–11517.
21. **Kiyokawa, H., X. Busquets, C. T. Powell, L. Ngo, R. A. Rifkind, and P. A. Marks.** 1992. Cloning of a D-type cyclin from murine erythroleukemia cells. Proc. Natl. Acad. Sci. USA **89:**2444–2447.
22. **Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J. M. Roberts.** 1991. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell **66:**1217–1228.
23. **Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza, and J. M. Roberts.** 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science **257:**1689–1694.
24. **Koff, A., M. Ohtsuki, K. Polyak, J. M. Roberts, and J. Massague.** 1993. Negative regulation of G1 progression in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-β. Science **260:**536–539.
25. **Lew, D. J., V. Dulic, and S. I. Reed.** 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell **66:**1197–1206.
26. **Matsushime, H., M. E. Ewen, D. K. Strom, J.-Y. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr.** 1992. Identification and properties of an atypical catalytic subunit ($p34^{PSKJ3}$/CDK4) for mammalian D-type G1 cyclins. Cell **71:**323–334.
27. **Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr.** 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell **65:**701–713.
28. **Matsushime, H., M. F. Roussel, and C. J. Sherr.** 1991. Novel mammalian cyclin (CYL) genes expressed during G_1. Cold Spring Harbor Symp. Quant. Biol. **56:**69–74.
29. **Meyerson, M., and E. Harlow.** 1994. Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14:**2077–2086.
30. **Minshull, J., R. Golsteyn, C. Hill, and T. Hunt.** 1990. The A- and B-type cyclin associated cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. EMBO J. **9:**2865–2875.
31. **Motokura, T., T. Bloom, H. G. Kim, H. Juppner, J. V. Ruderman, H. M. Kronenberg, and A. Arnold.** 1991. A novel cyclin encoded by a *bcl*1-linked candidate oncogene. Nature (London) **350:**512–515.
32. **Motokura, T., K. Keyomarsi, H. M. Kronenberg, and A. Arnold.** 1992. Cloning and characterization of human cyclin D3, a cDNA closely related in sequence to the PRAD1/cyclin D1 proto-oncogene. J. Biol. Chem. **267:**20412–20415.
33. **Ohtsubo, M., and J. M. Roberts.** 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science **259:**1908–1912.
34. **Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek, and G. Draetta.** 1993. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. J. Cell Biol. **121:**101–111.
35. **Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta.** 1992. Cyclin A is required at two points in the human cell cycle. EMBO J. **11:**961–971.
36. **Pardee, A. B.** 1989. G1 events and regulation of cell proliferation. Science **246:**603–608.
37. **Pines, J., and T. Hunter.** 1989. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell **58:**833–846.
38. **Pines, J., and T. Hunter.** 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature (London) **346:**760–763.
39. **Pines, J., and T. Hunter.** 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol. **115:**1–17.
40. **Polyak, K., J.-Y. Kato, M. J. Solomon, C. J. Sherr, J. Massague, J. M. Roberts, and A. Koff.** $p27^{Kip1}$, a cyclin-cdk inhibitor, links TGF-β and contact inhibition to cell cycle arrest. Genes Dev., in press.
41. **Poon, R. Y. C., K. Yamashita, J. P. Adamczewski, T. Hunt, and J. Shuttleworth.** 1993. The cdc2-related protein $p40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $p33^{cdk2}$ and $p34^{cdc2}$. EMBO J. **12:**3123–3132.
42. **Quelle, D. E., R. A. Ashmun, S. A. Shurtleff, J.-Y. Kato, D. Bar-Sagi, M. F. Roussel, and C. J. Sherr.** 1993. Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. Genes Dev. **7:**1559–1571.
43. **Rosenblatt, J., Y. Gu, and D. O. Morgan.** 1992. Human cyclin-dependent kinase 2 (CDK2) is activated during the S and G2 phases of the cell cycle and associates with cyclin A. Proc. Natl. Acad. Sci. USA **89:**2824–2828.
44. **Sherr, C. J.** 1993. Mammalian G1 cyclins. Cell **73:**1059–1065.
45. **Solomon, M. J., J. W. Harper, and J. Shuttleworth.** 1993. CAK, the $p34^{cdc2}$ activating kinase, contains a protein identical or closely related to $p40^{MO15}$. EMBO J. **12:**3133–3142.
46. **Solomon, M. J., T. Lee, and M. W. Kirschner.** 1992. Role of phosphorylation in $p34^{cdc2}$ activation: identification of an activating kinase. Mol. Biol. Cell **3:**13–27.
47. **Stanley, E. R.** 1986. The macrophage colony stimulating factor, CSF-1. Methods Enzymol. **116:**564–587.
48. **Tsai, L.-H., E. Lees, B. Faha, E. Harlow, and K. Riabowol.** 1993. The cdk2 kinase is required for the G1-to-S transition in mamma-

lian cells. Oncogene **8:**1593–1602.
49. **Tushinski, R. J., and E. R. Stanley.** 1985. The regulation of mononuclear phagocyte entry into S phase by the colony stimulating factor CSF-1. J. Cell. Physiol. **122:**221–228.
50. **Vallance, S. J., H.-M. Lee, M. F. Roussel, S. A. Shurtleff, J.-Y. Kato, D. K. Strom, and C. J. Sherr.** Monoclonal antibodies to mammalian D-type G1 cyclins. Hybridoma, in press.
51. **Williams, R. T., L. Wu, D. A. Carbonaro-Hall, V. T. Tolo, and F. L. Hall.** 1993. Identification of a novel cyclin-like protein in human tumor cells. J. Biol. Chem. **268:**8871–8880.
52. **Withers, D. A., R. C. Harvey, J. B. Faust, O. Melnyk, K. Carey, and T. C. Meeker.** 1991. Characterization of a candidate *bcl-1* gene. Mol. Cell. Biol. **11:**4846–4853.
53. **Won, K.-A., Y. Xiong, D. Beach, and M. Gilman.** 1992. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA **89:**9910–9914.
54. **Xiong, Y., T. Connolly, B. Futcher, and D. Beach.** 1991. Human D-type cyclin. Cell **65:**691–699.
55. **Xiong, Y., H. Zhang, and D. Beach.** 1992. D-type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell **71:**505–514.
56. **Xiong, Y., H. Zhang, and D. Beach.** 1993. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev. **7:**1572–1583.