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The p21^{Cip1} and p27^{Kip1} CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts

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The widely prevailing view that the cyclin-dependent kinase inhibitors (CKIs) are solely negative regulators of cyclin-dependent kinases (CDKs) is challenged here by observations that normal up-regulation of cyclin D–CDK4 in mitogen-stimulated fibroblasts depends redundantly upon p21^{Cip1} and p27^{Kip1}. Primary mouse embryonic fibroblasts that lack genes encoding both p21 and p27 fail to assemble detectable amounts of cyclin D–CDK complexes, express cyclin D proteins at much reduced levels, and are unable to efficiently direct cyclin D proteins to the cell nucleus. Restoration of CKI function reverses all three defects and thereby restores cyclin D activity to normal physiological levels. In the absence of both CKIs, the severe reduction in cyclin D-dependent kinase activity was well tolerated and had no overt effects on the cell cycle.

Keywords: CDK4/cell cycle/D-type cyclins/p21^{Cip1}/p27^{Kip1}

Introduction

Regulation of mammalian cell proliferation by extracellular mitogens is governed through receptor-mediated signaling circuits which ultimately converge on the cell cycle machinery driven by cyclin-dependent kinases (CDKs) and opposed by CDK inhibitors (CKIs) (Sherr and Roberts, 1995). One important example is receptor-activated Ras signaling, which governs the accumulation of cyclin D1–CDK4 complexes by at least three independent but complementary pathways: gene transcription, protein association and protein stabilization. First, Ras signaling promotes transcription of the cyclin D1 gene via a kinase cascade that depends upon the sequential activities of Ras, Raf-1, mitogen-activated protein kinase kinase (MEK1) and mitogen-activated protein kinases (MAPKs), also referred to as extracellular signal-regulated protein kinases (ERKs) (Albanese *et al.*, 1995; Lavoie *et al.*, 1996; Winston *et al.*, 1996; Aktas *et al.*, 1997; Kerkhoff and Rapp, 1997; Weber *et al.*, 1997). Signaling through this same pathway is also sufficient to promote assembly of cyclin D1–CDK4 complexes (Cheng *et al.*, 1998), although the physiological target of ERK phosphorylation that mediates this process has not been defined. Finally, proteasomal degradation of cyclin D1 is triggered by its phosphorylation on a single threonine residue (Thr286) by glycogen synthase kinase-3β (Diehl *et al.*, 1997, 1998), a process antagonized by signaling through a separate Ras-dependent pathway involving phosphatidylinositol 3-kinase (PI3K) and protein kinase B (also called Akt) (Boudewijn *et al.*, 1995; Cross *et al.*, 1995; Franke *et al.*, 1995, 1997; Klinghoffer *et al.*, 1996; Dudek *et al.*, 1997; Vanhaesebroeck *et al.*, 1997). In the continued presence of mitogenic signals, cyclin D1–CDK4 complexes assemble and accumulate throughout G_1 phase, enter the nucleus and undergo phosphorylation by CDK-activating kinase (CAK) to yield active holoenzymes. One key function of the cyclin D-dependent kinases is to initiate phosphorylation of the retinoblastoma protein (Rb), thereby helping to cancel its activity as a transcriptional repressor of a bank of genes, including cyclins E and A, whose activities are required for S phase entry (Weinberg, 1995; Sherr, 1996).

A separate, non-catalytic action of cyclin D-dependent kinases is the sequestration of CKIs, including p27^{Kip1} and p21^{Cip1} (Sherr and Roberts, 1995). The Cip/Kip proteins interact with a variety of cyclin–CDK complexes through a conserved N-terminal domain that contains both cyclin and CDK binding sites (Toyoshima and Hunter, 1994; Chen *et al.*, 1995, 1996; Luo *et al.*, 1995; Nakanishi *et al.*, 1995; Lin *et al.*, 1996; Russo *et al.*, 1996). Cyclin D-dependent CDKs isolated from mammalian cells appear to be less susceptible to Cip/Kip-mediated inhibition than are other classes of cyclin–CDKs (Soos *et al.*, 1996; Blain *et al.*, 1997; LaBaer *et al.*, 1997), and sequestration of p21^{Cip1} and p27^{Kip1} into higher order complexes with cyclin D-dependent kinases during G_1 phase helps to relieve cyclin E–CDK2 from their constraint, thereby facilitating its activation later in G_1 phase. This ability to 'titrate' CKIs therefore sets a dependency of cyclin E-dependent kinase on the mitogen-stimulated assembly of cyclin D-dependent kinases. Cyclin E–CDK2 collaborates with cyclin D-dependent kinases to phosphorylate Rb (Hatakeyama *et al.*, 1994; Mittnacht *et al.*, 1994; Lee *et al.*, 1996; Kelly *et al.*, 1998; Lundberg and Weinberg, 1998), phosphorylates p27^{Kip1} to trigger its degradation (Sheaff *et al.*, 1997; Vlach *et al.*, 1997), and may target other proteins whose modifications trigger origin firing and DNA replication per se (Stillman, 1996; Krude *et al.*, 1997).

Although it is generally assumed that CKIs act solely to retard G_1 progression, the fact that they can be found in complexes with active cyclin–CDKs (Zhang *et al.*, 1994; Soos *et al.*, 1996; Blain *et al.*, 1997; LaBaer *et al.*, 1997) raises the possibility that they may also act as positive regulators. Intriguingly, LaBaer *et al.* (1997) demonstrated that p21^{Cip1} could promote the assembly of active cyclin D1–CDK4 complexes and, in addition, could

provide a localization signal for their nuclear import. However, the fact that p21 nullizygous mice undergo normal development and do not seem to have a significant deficiency in cyclin D-dependent kinase function (Brugarolas *et al.*, 1995; Deng *et al.*, 1995) leaves open the question of whether the CKIs are normal physiological regulators of cyclin D–CDK assembly. In this study, we have used primary mouse embryo fibroblast (MEF) strains deficient in p21, p27 or both to study their roles in governing the activities of cyclin D–CDK holoenzymes.

Results

Impaired assembly of cyclin D–CDK4 complexes in MEFs lacking p21 and p27

Cell lysates from asynchronously proliferating MEFs derived from wild-type mice, p21- and p27-null mice and from animals lacking both genes were precipitated with antibodies to cyclin D1 or CDK4. Precipitated proteins were resolved on denaturing polyacrylamide gels, transferred to nitrocellulose membranes and blotted with the cognate antibodies to quantitate cyclin D1 and CDK4 levels, respectively, or with the reciprocal antibodies to score for the presence of cyclin D1–CDK4 complexes (Figure 1A). In early passage (p5) wild-type MEFs, ~40% of the total CDK4 (lane 1, K4 blot) co-precipitated with antibodies to D1 (lane 2, K4 blot). A smaller percentage of the total D1 pool (lane 2, D1 blot) co-precipitated with CDK4 (lane 1, D1 blot). However, antibodies to full-length recombinant CDK4 used in this experiment preferentially detect free versus cyclin D1-bound catalytic subunits, so the amount of D1 detected in CDK4 immunoprecipitates underestimates the extent of complex formation. Generally equivalent levels of CDK4 were expressed in MEF strains lacking one or both CKIs (Figure 1A, lanes 3, 5 and 7). In contrast, the overall levels of cyclin D1 were significantly lowered in cells lacking either p21 or p27 (D1 blot, lanes 6 and 8 versus lane 2) and were decreased at least 10-fold in lysates of cells lacking both CKIs (lane 4). Cyclin D1–CDK4 complexes were recovered at similarly reduced levels from lysates of p21-null and p27-null MEFs (lanes 5–8), but at this level of resolution, no cyclin D1–CDK4 complexes were detected in immune precipitates from cells lacking both CKIs (lanes 3 and 4).

Reduced cyclin D1–CDK4 complex formation in double-null MEFs may have simply reflected the lower levels of cyclin D1 expressed in these cells. However, several lines of evidence indicate that this is not the explanation. First, when cell lysates were normalized so that each contained comparable amounts of cyclin D1 protein, the levels of CDK4 that co-precipitated with cyclin D1 were again found to be decreased in p21-null or p27-null cells (Figure 1B, lanes 2 and 3) and were very much reduced in p21/p27 double-null MEFs, whether the cells were in early (p7) or late (p15) passage (Figure 1B, lanes 4 and 6). We estimated that p7 double-null cells contained $<10\%$ of the D1–CDK4 complexes detected in age-matched wild-type MEFs. Secondly and more importantly, infection of p21/p27 double-null MEFs for 48 h with a retrovirus encoding Flag epitope-tagged cyclin D1 restored high levels of D1 expression but not D1–CDK4 complex formation (Figure 1C). This demonstrated directly that the lower levels of cyclin D1 expressed in the p21/p27 double-null cells do not account for the defect observed in cyclin D1–CDK4 assembly.

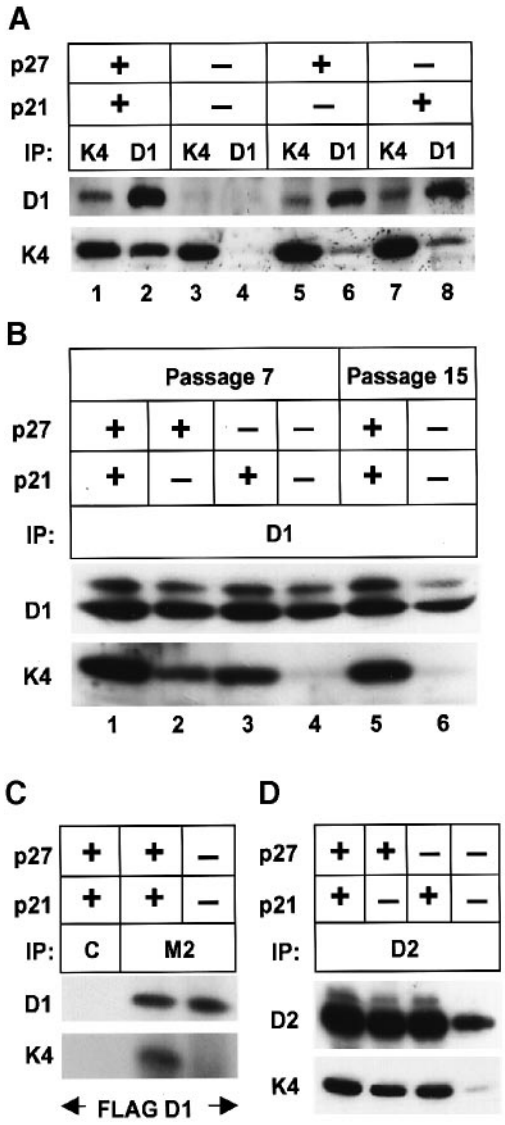

Fig. 1. Impaired assembly of cyclin D–CDK4 complexes in MEFs lacking p21 and p27. (**A**) Cell lysates (500 μg total protein per lane) from MEFs of the indicated genotypes were immunoprecipitated (IP) with antibodies to CDK4 (K4) or cyclin D1 and the separated proteins were blotted with the cognate or reciprocal antibodies. (**B**) Cell lysates were normalized for cyclin D1 abundance, and D1 immune precipitates were blotted with antibodies to cyclin D1 or CDK4. Two electrophoretic forms of cyclin D1 detected in this experiment can be routinely observed when separation conditions are sufficiently stringent (Matsushime *et al.*, 1991); both are phosphoproteins and the nature of the differences between them remains unclear (Diehl *et al.*, 1998). (**C**) Wild-type or p21/p27 double-null MEFs were infected with retrovirus encoding Flag-tagged cyclin D1. Cell lysates prepared 48 h post-infection were precipitated with a control monoclonal antibody (C) or with antibodies to the Flag epitope (M2), and the separated proteins were blotted with antibodies to cyclin D1 or CDK4. (**D**) Cell lysates from MEFs with the indicated genotypes were precipitated with antibodies to cyclin D2 and the separated proteins were blotted with antibodies to cyclin D2 or CDK4. All immunoblots were visualized using enhanced chemiluminescence.

MEFs also express cyclin D2 (Figure 1D, lane 1) but little detectable cyclin D3 (data not shown). Compared with wild-type MEFs, cyclin D2 levels were reduced by ~30% in cells lacking either p21 (lane 2) or p27 (lane 3)

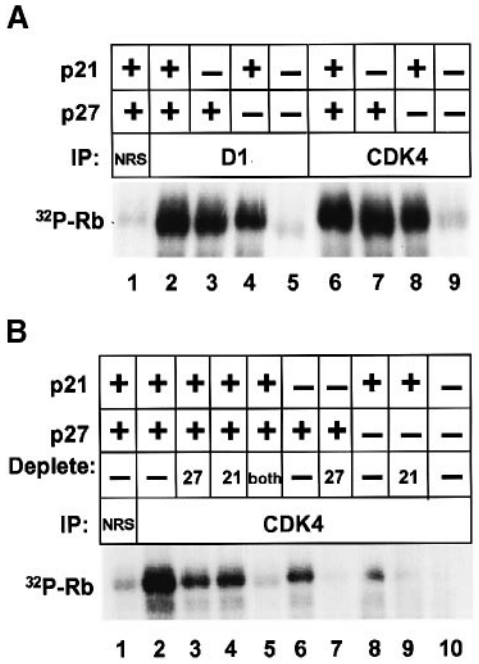

Fig. 2. Cyclin D1 and CDK4-dependent Rb kinase activity in MEFs lacking p21 and p27. (**A**) Lysates from MEFs of the indicated genotypes were precipitated with non-immune rabbit serum (NRS) or with antibodies to cyclin D1 or CDK4. Resulting complexes were assayed for kinase activity using GST-Rb as the substrate. (**B**) Lysates from cells of the indicated genotype were depleted of p21, p27 or both, and then precipitated with antibodies to CDK4 or control NRS. Washed immune complexes were assayed for Rb kinase activity.

and, like D1, were significantly decreased in cells lacking both CKIs (lane 4). Despite the fact that the D2 signal in Figure 1D exceeds the D1 signal in Figure 1A and B, quantitation of the two cyclins by comparison with recombinant protein standards indicated that the level of D1 exceeds that of D2 by 2- to 3-fold (data not shown). Cyclin D2–CDK4 complexes were readily detected in wild-type MEFs and in those lacking either CKI but were significantly reduced in cells lacking both inhibitors (Figure 1D, K4 blot). In agreement with previous data showing that CDK4 is the predominant partner of D-type cyclins in rodent fibroblasts (Matsushime *et al.*, 1994), virtually no cyclin D1–CDK6 or D2–CDK6 complexes were detected in MEFs.

D-type cyclin binding is essential for activating CDK4 kinase activity (Matsushime *et al.*, 1992). Since association of D-type cyclins with CDK4 was significantly compromised in the p21/p27 double null MEFs, both cyclin D1-dependent and total CDK4 kinase activity were measured in these cells (Figure 2A). Cell lysates from proliferating MEFs were precipitated with either non-immune rabbit serum (NRS), antibody to cyclin D1 or antibody to CDK4, and the resulting immune complexes were assayed for kinase activity using recombinant GST-Rb as the substrate. Note that CDK4-dependent kinase activity should include contributions from both D1- and D2-containing holoenzymes (Figure 1). Active cyclin D1- and CDK4-dependent Rb kinase activities were detected in precipitates from wild-type MEFs (Figure 2A, lanes 2 and 6), p21-null MEFs (lanes 3 and 7) and p27-null MEFs (lanes 4 and 8). In contrast, only background levels of kinase activity were detected in immune complexes recovered from p21/p27 double-null MEFs (lanes 5 and 9), consistent with observations that few cyclin D–CDK4 complexes were formed (Figure 1).

Cell lysates from wild-type MEFs were subjected to two rounds of immunodepletion using antisera to p21, p27 or to both, and CDK4 kinase activity was measured using glutathione *S*-transferase (GST)-Rb as the substrate. Removal of p27 (Figure 2B, lane 3) or p21 (lane 4) from lysates of wild-type MEFs partially reduced CDK4 kinase activity, whereas elimination of both p21 and p27 (lane 5) completely depleted the activity. Similarly, removal of p27 from lysates of p21-null MEFs (lane 7) or vice versa (lane 9) depleted all CDK4 kinase activity from these lysates. Therefore, both p21- and p27-associated cyclin D–CDK complexes retain activity (Zhang *et al.*, 1994; Soos *et al.*, 1996; Blain *et al.*, 1997; LaBaer *et al.*, 1997), consistent with results that either p21 or p27 is required for efficient assembly of active cyclin D–CDK4 complexes.

p21 or p27 promotes assembly of stable cyclin D1–CDK4 complexes in double-null MEFs

One prediction is that reintroduction of p21 or p27 into double-null MEFs should increase the assembly of cyclin D1–CDK4 complexes. We infected these cells either with a control retrovirus encoding the T-cell co-receptor CD8 or with retroviruses encoding either p21 or p27 (Figure 3A). Lysates prepared from MEFs infected for 48 h were precipitated with antibodies to cyclin D1 or CDK4, and assayed for complex formation. Ectopic expression of either p21 (Figure 3A, lane 3) or p27 (lane 4) but not CD8 (lane 1) increased cyclin D1–CDK4 complex formation in p21/p27 double-null MEFs. The N-terminal portion of p27, which contains the cyclin and CDK binding sites, is sufficient to promote the stable association of cyclin D1 and CDK4, whereas the C-terminal half of p27 is inactive in this assembly assay (data not shown). Ectopic expression of another CDK inhibitor, $p16^{INK4a}$, which binds to CDK4 or CDK6 but not to D cyclins (Serrano *et al.*, 1993), did not promote assembly of cyclin D1–CDK4 in these cells (data not shown). Ectopic expression of p21 or p27 not only increased the assembly of cyclin D–CDK4 complexes but also increased the overall levels of cyclin D1 in p21/p27 double-null MEFs to levels that approached those in wild-type MEFs (Figure 3A, lanes 3 and 4 versus lane 2).

Cyclin D1 is a labile protein (Matsushime *et al.*, 1992), and its rapid proteolytic degradation is triggered by phosphorylation on Thr286 (Diehl *et al.*, 1997). By interacting with both cyclin D1 and CDK4, p21 and p27 might slow cyclin D1 turnover, possibly by promoting nuclear localization of the complexes (see below) and/or by interfering with cyclin D1 phosphorylation. Double-null MEFs were infected for 48 h with a retrovirus encoding p27, and cells were metabolically labeled with [^{35}S]methionine for 30 min. Medium containing labeled methionine was removed, and cells were incubated in complete medium containing a 100-fold excess of unlabeled methionine. Cell lysates prepared after different periods of 'chase' were precipitated with the monoclonal antibody to cyclin D1, and the labeled proteins were resolved on a denaturing gel (Figure 3B). The half-life of cyclin D1 in p21/p27 double-null MEFs in several such experiments was calculated to be 15 min (Figure 3B, lanes

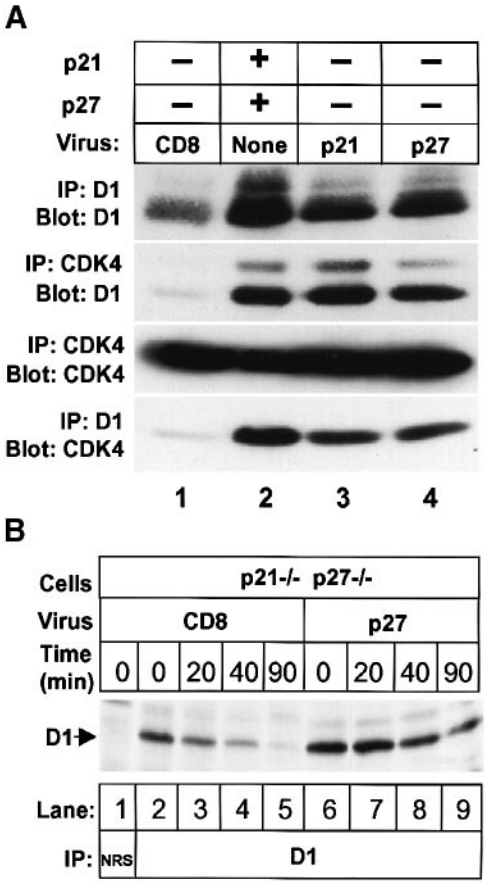

Fig. 3. Reconstitution of cyclin D1–CDK4 complexes *in vivo* and restabilization of cyclin. (**A**) Wild-type MEFs or those lacking both p21 and p27 were infected for 48 h with control virus (CD8) or with viruses encoding p21 or p27. Cells were lysed and immunoprecipitated with antibodies to cyclin D1 or CDK4. Separated immune complexes were then blotted with the cognate or reciprocal antibodies, and sites of antibody binding were detected by enhanced chemiluminescence. (**B**) MEFs lacking both p21 and p27 were infected with a control retrovirus encoding CD8 or with a virus encoding p27. Two days post-infection, cells were pulse-labeled for 30 min with [^{35}S]methionine and then 'chased' in the presence of 100-fold excess of unlabeled methionine for the indicated times. Lysates normalized for protein concentration were precipitated with a monoclonal antibody to cyclin D1, and the labeled proteins were resolved on a denaturing gel, which was dried and subjected to autoradiography.

2–5), which is shorter than that in wild-type cells ($t_{1/2}$ = 25 min) (Matsushime *et al.*, 1992; Diehl *et al.*, 1997, 1998). In contrast, in cells infected with p27-virus, the half-life of D1 exceeded 40 min (Figure 3B, lanes 6–9). Metabolic labeling experiments indicated that the relatively low level of cyclin D1 in p21/p27 double-null MEFs also reflects a 3-fold reduced rate of D1 synthesis versus that in wild-type MEFs (data not shown). Cyclin D1 synthesis was only modestly increased following acute infection of the cells with the p27 retrovirus (Figure 3B, compare lane 6 with lane 2), so the restoration of D1 levels following reintroduction of p21 or p27 (Figure 3) primarily reflects increased D1 stability.

Association of CDK4 with Cdc37 and INK4 proteins in p21/p27 double-null cells

Although CDK4 in mouse fibroblasts has a half-life of ~4 h (Matsushime *et al.*, 1992), unassembled CDK4 subunits are unstable, and the levels of monomeric CDK4 are very low (Dai *et al.*, 1996; Stepanova *et al.*, 1996). Similarly, the vast majority of CDK6 subunits are bound to other molecules (Mahony *et al.*, 1998). CDK4 requires association with Hsp90/Cdc37 for stabilization, suggesting that the latter acts as a chaperone for the proper folding of kinase subunits (Dai *et al.*, 1996; Stepanova *et al.*, 1996). High molecular weight complexes containing Hsp90, Cdc37 and CDK4 or CDK6 are cytoplasmic and do not contain D-type cyclins, so assembly of cyclin D with CDK4, the nuclear translocation of these complexes, and their activation by CAK presumably occur as later steps. We considered the possibility that in p21/p27 double-null cells, the lack of complexes between CDK4 and cyclins D1 and D2 might result in a greater association of CDK4 subunits with Cdc37. Instead, the level of Cdc37-bound CDK4 was lower in double-null cells than in wild-type cells (Figure 4D), suggesting that most CDK4 was complexed with other molecules or remained monomeric.

Apart from interacting with D-type cyclins, CDK4 and CDK6 can independently associate with INK4 proteins (Serrano *et al.*, 1993; Guan *et al.*, 1994; Hannon and Beach, 1994; Chan *et al.*, 1995; Hirai *et al.*, 1995). CDK–INK4 complexes are stable, lack Cdc37, cannot assemble with cyclins, and therefore appear inaccessible for enzymatic activation (Parry *et al.*, 1995; Stepanova *et al.*, 1996). It is therefore presumed that CDK4 can achieve alternative fates after release from the chaperone complex, either assembling with D-type cyclins or being inactivated through INK4 binding. Possibly, p21 and p27 might facilitate assembly of CDK4 and D-type cyclins by blocking the ability of INK4 proteins to sequester CDK4 in an inactive pool. We therefore studied the expression of the four different INK4 family members ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) and compared their associations with CDK4 in wild-type and p21/p27 double-null MEFs.

Individual INK4 proteins were depleted from cell lysates by two sequential immunoprecipitations, and the levels of CDK4 associated with each INK4 family member were determined by blotting the precipitated proteins with antibodies to CDK4 (Figure 4A, lanes 1 and 2). Lysates depleted of individual INK4 proteins were then precipitated and blotted with antibodies to CDK4, in order to estimate the levels of residual CDK4 that remained unassociated with INK4 proteins (Figure 4A, lanes 3). Wild-type MEFs expressed significant amounts of CDK4 in complexes with $p16^{INK4a}$, $p15^{INK4b}$ and $p18^{INK4c}$ (Figure 4A). In cells lacking both p21 and p27, CDK4 associated with the same three INK4 family members, although in comparison with wild-type MEFs, less CDK4 was bound to $p18^{INK4c}$ and more was bound to $p15^{INK4b}$ (Figure 4B). In both MEF strains, more CDK4 was complexed with $p16^{INK4a}$ than with other INK4 family members, and no association with $p19^{INK4d}$ was detected.

To determine the relative pools of total INK4-bound and -unbound CDK4, MEF lysates were depleted with mixtures of antibodies directed to all four INK4 family members, and the above analysis was repeated (Figure 4C). From several such experiments, we estimated that in wild-type cells, ~40% of the total CDK4 pool stably associated with INK4 proteins. As expected, cyclin D1 co-precipitated only with those CDK4 molecules that were not bound to INK4 proteins (+/+ cells, lanes 3).

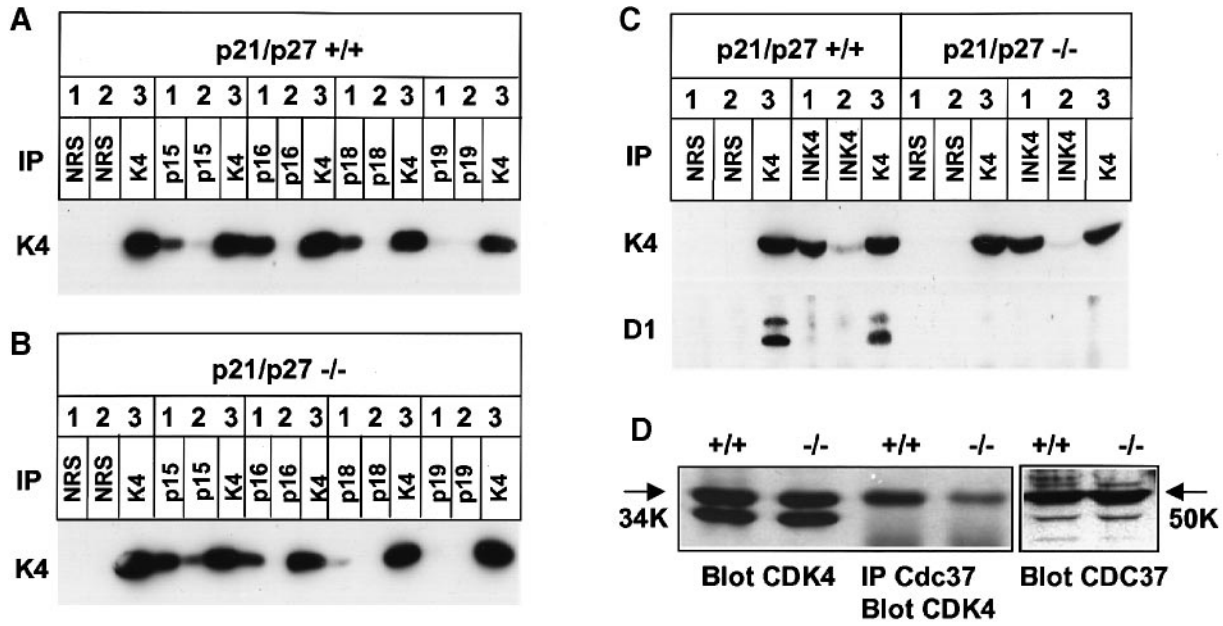

Fig. 4. Association of CDK4 with INK4 proteins and Cdc37. Cell lysates from wild-type (**A**) or p21/p27 double-null cells (**B**) were sequentially depleted by two rounds of precipitation with non-immune rabbit serum (NRS) or with antibodies to the designated INK4 proteins (lanes 1 and 2) and then precipitated with anti-CDK4 (lanes 3). All recovered proteins were immunoblotted with anti-CDK4. (**C**) Experiments were performed as above, except that immunodepletion was carried out with a mixture of antibodies to all four INK4 family members prior to blotting of precipitated proteins with anti-CDK4 and anti-D1. (**D**) Cells of the indicated genotype were lysed and equal quantities of protein (100 μg) were resolved on denaturing gels and immunoblotted directly with anti-CDK4 (lanes 1 and 2) or rabbit anti-Cdc37 (lanes 5 and 6; produced by J.A.D. and C.J.S. to recombinant mouse protein synthesized in bacteria, unpublished). The arrows indicate the position of authentic CDK4 (34 kDa, left) and Cdc37 (50 kDa, right). The faster-migrating band detected with commercial polyclonal antibodies to mouse CDK4 used in this experiment (Santa Cruz Biotechnology) is not observed using antisera raised in our laboratory (R_Y or R_Z; Matsushime *et al.*, 1994). To quantitate complex formation, 5-fold more lysate protein (500 μg/lane) precipitated with anti-Cdc37 was separated on denaturing gels and blotted with anti-CDK4 (lanes 3 and 4).

Importantly, all enzymatically active CDK4 is bound to D-type cyclins, and this fraction also contained associated p21 and p27 molecules (Figure 2). In p21/p27 double-null cells, the INK4-bound CDK4 fraction was increased to ~50–60%; D1–CDK4 complexes were again not detected even though a substantial pool of non-INK4-bound CDK4 remained (–/– cells, lanes 3). Therefore, in wild-type MEFs, ~40% of CDK4 is associated with cyclin D1 and 10–15% is associated with D2 (Figure 1), ~40% is associated with INK4 proteins (Figure 4C), and much of the remainder is bound to Cdc37 (Figure 4D; see figure legend for amounts of protein loading per lane). In p21/p27 double-null cells, little CDK4 binding to D cyclins was detected (Figure 1), ~60% was bound to INK4 proteins (Figure 4C) and <10% was complexed to Cdc37. Therefore, a substantial fraction of CDK4 must either remain monomeric or is associated with as yet unidentified molecules. This indicates that p21 and p27 do not simply compete with INK4 proteins in directing cyclin D–CDK assembly.

p21 or p27 can facilitate nuclear accumulation of cyclin D1

Cyclin D1 normally accumulates in the nuclei of cells during G_1 phase but relocalizes to the cytoplasm during S phase (Baldin *et al.*, 1993). Although cyclin D1 has no obvious nuclear import signal, p21 family members can direct the nuclear localization of cyclin D1–CDK4 complexes (Diehl and Sherr, 1997; LaBaer *et al.*, 1997), raising the possibility that cyclin D1 might not be able to enter the nucleus in MEFs lacking both p21 and p27. When asynchronously proliferating wild-type MEFs were stained with antibody to cyclin D1, 61% of the cells exhibited strong nuclear fluorescence (Table I). Fluorescence-activated cell sorter (FACS) analysis of DNA content indicated that 52% of the total asynchronously proliferating population were in G_1 phase, and in agree-

Table I. Subcellular localization of cyclin D1 in cells lacking CKIs

Genotype		% Nuclear	% Nuclear and cytoplasmic	% Cytoplasmic
p21	p27			
+	+	61 ± 4	21 ± 3	18 ± 3
–	+	49 ± 4	23 ± 3	28 ± 4
+	–	46 ± 4	26 ± 4	28 ± 4
–	–	38 ± 3	28 ± 3	34 ± 4
Flag-tagged D1 retrovirus				
+	+	59 ± 5	23 ± 2	18 ± 2
–	–	7 ± 3	64 ± 5	29 ± 4
Flag-tagged D1 (T286A) retrovirus				
+	+	82 ± 5	14 ± 2	4 ± 1
–	–	77 ± 7	17 ± 5	6 ± 2

Proliferating wild-type MEFs and those lacking p21, p27 or both were stained with monoclonal antibody to cyclin D1 and scored by immunofluorescence for the presence of nuclear and/or cytoplasmic D1. Cells infected with retroviruses encoding Flag-tagged D1 or the D1 (T286A) mutant that is stable and remains in the nucleus throughout interphase were studied similarly. Because ectopic expression of D1 greatly exceeded that of the endogenous protein, no background signals were detected at the exposures used. At least 500 cells were counted per experiment and the results show mean ± SD from three such experiments.

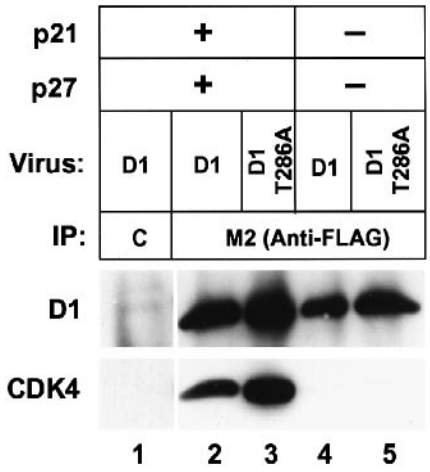

Fig. 5. Assembly of ectopically expressed cyclin D1 and D1 (T286A) with CDK4. Wild-type and double-null MEFs were infected with retrovirus encoding Flag-tagged cyclin D1 or D1 (T286A) for 48 h. Lysates were then precipitated with a control monoclonal antibody (C) or with the monoclonal antibody to the flag epitope (M2), and the resulting precipitates were resolved on a denaturing gel and transferred to nitrocellulose. Proteins were visualized by enhanced chemiluminescence using a monoclonal antibody to D1 or antibodies to CDK4.

ment with others' results (Baldin *et al.*, 1993), double-labeling with BrdU for 2 h prior to staining indicated that ~90% of cells exhibiting bright nuclear cyclin D1 fluorescence were not in S phase (data not shown). Although a smaller fraction of cells lacking p21, p27 or both exhibited exclusively nuclear staining, the lower levels of cyclin D1 expressed were still able to enter the nucleus (Table I). In agreement with immunoblotting results (Figure 1), the intensity of cyclin D1 staining in p21/p27 double-null MEFs was much lower than that of wild-type MEFs (as judged by the need for a 6-fold increase in exposure time to obtain an almost comparable signal). Hence, the CDK inhibitors are not strictly required for cyclin D1 nuclear import. Moreover, the fact that a significant fraction of cyclin D1 was detected in the nucleus of double-null cells (Table I) whereas >95% failed to assemble with CDK4 (Figure 1) suggests that stable association with catalytic subunits is also not essential for D1 nuclear import.

We next used infection with a retrovirus encoding Flag-tagged D1 to increase cyclin D levels in double-null cells. After infection for 36 h, similar amounts of Flag-D1 were expressed in both wild-type and the double-null MEFs, as demonstrated by immunoprecipitation with M2 antibodies to the tag followed by immunoblotting with antibodies to D1 (Figure 5, lanes 2 versus 4, D1 blot). These levels of ectopically expressed cyclin D1 exceeded the endogenous level of cyclin D1 in double-null MEFs by >10-fold (data not shown). Most infected wild-type MEFs (59%) displayed an exclusively nuclear cyclin D1 staining pattern (Table I). In marked contrast, <7% of p21/p27 double-null MEFs exhibited exclusively nuclear cyclin D1 staining, and instead, the cells displayed both nuclear and cytoplasmic staining or exclusively cytoplasmic staining (Table I). Therefore, under conditions in which cyclin D1 was restored, an absence of p21 and p27 limited D1 nuclear accumulation. Importantly, ectopically expressed Flag-tagged cyclin D1 was still unable to assemble with CDK4 in cells lacking both p21 and p27 (Figure 5, lane 4, CDK4 blot; also see Figure 1C).

These experiments left open the possibility that the ability of p21 and p27 to promote assembly of cyclin D1–CDK4 complexes was an indirect effect of their directing cyclin D1 to the nucleus. Accordingly, we took advantage of a mutant form of cyclin D1 (T286A) that contains an alanine for threonine-286 substitution, is remarkably stable ($t_{1/2}$ >3 h) (Diehl *et al.*, 1997), and remains in the nucleus throughout the cell cycle (Diehl *et al.*, 1998). When infected with a vector encoding D1 (T286A), both wild-type MEFs (82%) and p21/p27 double-null MEFs (77%) displayed an exclusively nuclear staining pattern of the D1 mutant (Table I), reinforcing the concept that D1 can enter the nucleus in the absence of both p21 and p27. Moreover, the abundance of cyclin D1 (T286A) was similar in both wild-type and double-null MEFs (Figure 5, lanes 3 and 5). Even under these conditions, D1 (T286A) could not assemble with CDK4 (Figure 5, lane 5, CDK4 blot). This argues that CKIs do not ensure assembly by simply contributing a nuclear import signal, in agreement with results obtained with mutants of p21 (LaBaer *et al.*, 1997) and p27 (not shown) that lack the signal sequences. These results also confirm that decreased assembly of cyclin D1–CDK4 complexes is not simply secondary to the decreased abundance of cyclin D1 in p21/p27 double-null MEFs.

Rb phosphorylation in p21/p27 double-null cells

In normal MEFs, phosphorylation of Rb is triggered by cyclin D-dependent kinases and is probably completed by cyclin E–CDK2 (and/or cyclin A–CDK2) as cells enter S phase. Since active cyclin D-dependent kinase activity was not detected in the p21/p27 double-null MEFs (Figure 2), we studied the kinetics of Rb phosphorylation in these cells. MEFs arrested by contact inhibition and serum starvation were trypsinized and reseeded at lower density in complete medium containing 10% fetal bovine serum (FBS). Cell lysates prepared at different times thereafter were precipitated with antibody to Rb, and the resulting immunoprecipitates were resolved on a denaturing gel, transferred to nitrocellulose and blotted with the cognate antibodies. The percentage of cells in S phase was estimated by flow cytometric analysis of DNA content. As cells approached S phase, Rb appeared to undergo phosphorylation, as manifested by its characteristic retardation in electrophoretic mobility on denaturing gels (Figure 6A). The kinetics of Rb phosphorylation and the rate of cell cycle progression were quite similar in p21/p27 double-null and wild-type MEFs. Hence, in the absence of the CKIs, resident cyclin-dependent kinases are sufficient to phosphorylate Rb, canceling its growth suppressive function as cells exit G_1 phase.

Although the levels of cyclin D-dependent kinase activity expressed in p21/p27 double-null cells were reduced below the limits of detection (Figure 2), several lines of evidence suggest that there may be some residual kinase activity. First, using antibodies that detect Rb phosphorylated on Ser780, a site reported to be specifically phosphorylated by cyclin D-dependent kinases (Kitagawa *et al.*, 1996), forms of Rb phosphorylated on this residue could be detected in cycling (lanes 4 and 6) but not in quiescent (lane 5) p21/p27 double-null cells (Figure 6B).

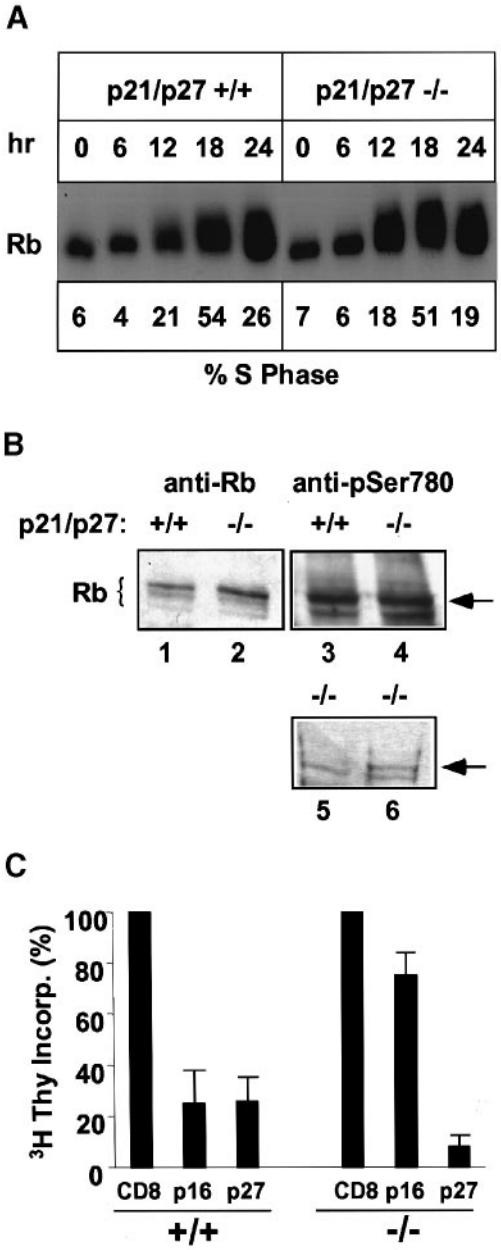

Fig. 6. Rb status and p16-induced arrest. (**A**) Wild-type and double-null MEFs made quiescent by contact inhibition and serum starvation were trypsinized, reseeded and stimulated to enter the cell cycle in complete medium containing 10% FBS. Cell lysates prepared at different times thereafter were immunoprecipitated with polyclonal antibodies to mouse Rb, separated on denaturing gels and blotted with monoclonal antibody to Rb. The position of the hypophosphorylated form of Rb is indicated by the signal in starved cells, whereas additional phosphorylation is connoted by the retardation in Rb's mobility as cells progress through the division cycle. The fraction of cells in S phase was determined by flow cytometric analysis of DNA content. Cells entered S phase by ~12 h and were predominately in G_2/M by the completion of the experiment. (**B**) Rb precipitated from quiescent (lane 5) or proliferating (all other lanes) MEFs of the indicated genotypes was immunoblotted with anti-Rb (lanes 1 and 2) or with an antibody that specifically detects an epitope containing pSer780 (lanes 3–6) (Kitagawa *et al.*, 1996). The position of the pSer780 form of Rb is indicated by arrows in the right margin. (**C**) MEFs infected for 24 h with a control CD8 retrovirus or with vectors encoding CD8 plus either $p16^{INK4a}$ or p27 were scored for [^{3}H]thymidine incorporation (2 h pulse). Results with the control CD8 vector were normalized to 100%. Standard errors (bars) were calculated from several independent experiments.

Second, when we infected double-null cells with a retrovirus encoding $p16^{INK4a}$, we observed inhibition of S phase entry, albeit not nearly to the same extent as that observed with the wild-type MEFs (Figure 6C). As expected, both MEF strains remained highly sensitive to growth arrest by a retrovirus encoding p27. Since, unlike p27, INK4 proteins appear to specifically target CDK4 and CDK6, these data imply that p21/p27 double-null cells are not entirely devoid of cyclin D-dependent kinase activity.

We also measured total CDK2 or cyclin E-dependent kinase activity in lysates of the various MEF strains, using either histone H1 or GST-Rb as the substrate (Figure 7). Although the levels of CDK2 were comparable in the different cell strains (Figure 7D), both CDK2 and cyclin E-dependent kinase activity were enhanced in p21/p27 double-null MEFs, as compared with those in wild-type MEFs (Figure 7A–C). Therefore, in the absence of p21 and p27, unopposed CDK2 activity may compensate for the severe reduction in CDK4 function. In agreement, p21- and p27-null MEFs are not significantly perturbed in cell cycle progression and exhibit generation times and S phase fractions similar to those of wild-type cells (Figure 6A and data not shown).

Assembly of cyclin D–CDK complexes in tissues from double-null mice

Although the above studies were performed with primary MEFs, a clear prediction is that assembly of different cyclin D-dependent kinases would be perturbed in many other cell types. Extracts of whole liver from wild-type and double-null mice expressed all three D-type cyclins together with CDK6 (Figure 8, lanes 1 and 2). When these were immunoprecipitated with a mixture of antibodies to cyclins D1, D2 and D3 and then immunoblotted with antibodies to CDK6, significantly fewer cyclin D–CDK6 complexes were observed in liver from double-null animals. Similar results were obtained with CDK4 in complexes with D1 (data not shown). T lymphocytes primarily express cyclins D2 and D3 in conjunction with CDK6 (Meyerson and Harlow, 1994), and thymic extracts from double-null mice also contained much lower levels of cyclin D–CDK6 complexes than those from wild-type animals (Figure 8, lanes 3 and 4). Assembly of cyclin D–CDK4 complexes was only modestly reduced in extracts of kidney and heart from double-null mice (data not shown). Therefore, despite the combinatorial nature of expressed cyclin D–CDK complexes in different tissues and the potential participation of other CKIs such as $p57^{Kip2}$ in the assembly process, assembly of cyclin D-dependent kinases was impaired *in vivo*.

Discussion

CDK inhibitors of the Cip/Kip family, including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, negatively regulate cell cycle progression and enforce cell cycle arrest when expressed at high levels (Elledge and Harper, 1994; Sherr and Roberts, 1995). Our results provide a different perspective in showing that p21 and p27 are necessary for certain processes that positively regulate cell cycle progression: cyclin D assembly with CDK4, its stability and its nuclear localization. Hence, the generally prevailing view of Cip/Kip proteins as universal inhibitors of CDKs appears to

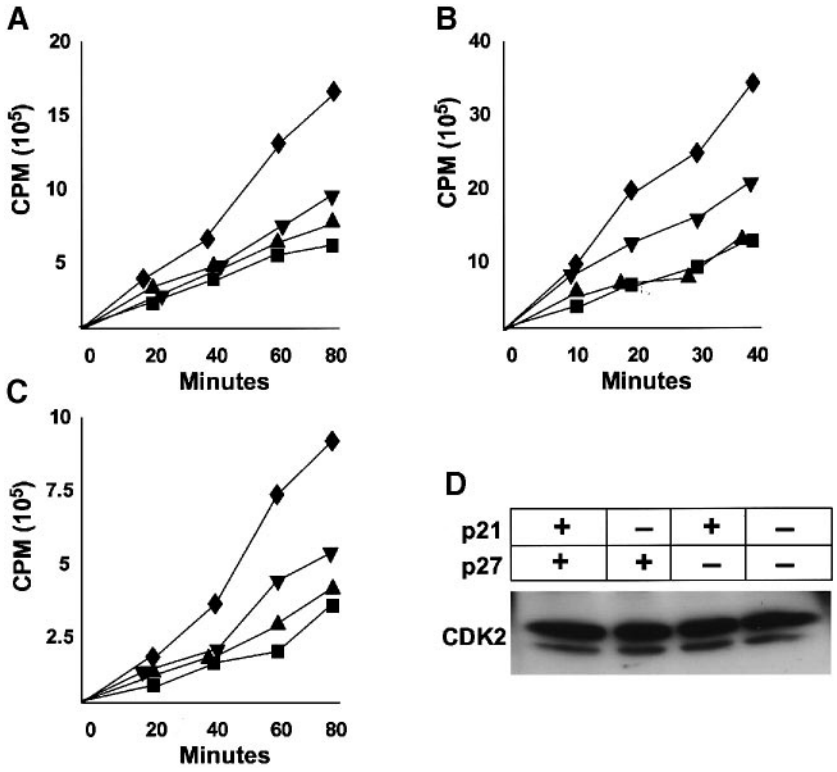

Fig. 7. CDK2 activities in MEFs lacking p21 and/or p27. Lysates from proliferating MEFs were precipitated with antibodies to CDK2 (**A** and **B**) or to cyclin E (**C**), and immune complex kinase assays were performed using histone H1 (A and C) or GST-Rb (B) as substrates. All reactions were stopped at the times indicated by heating samples to 85°C for 5 min in gel sample buffer containing SDS. Labeled proteins were resolved on denaturing gels, which were dried and subjected to autoradiography. Excised slices containing substrates were rehydrated and radioactivity was determined by liquid scintillation. Results are plotted in (A–C) for wild-type MEFs (squares), p21-null MEFs (triangles), p27-null MEFs (inverted triangles) and double-null MEFs (diamonds). (**D**) CDK2 protein in MEFs of the indicated genotype was detected by immunoblotting with the cognate antibody. Equal aliquots of lysate were separated per lane, and sites of antibody binding were detected by enhanced chemiluminescence.

portray too simple a picture of their regulatory effects on the cell cycle. A more accurate representation is that these CKIs are activators of CDK4 and inhibitors of CDK2. This reformulation of the role of Cip/Kip proteins places their inhibitory effect on cell proliferation more specifically at the pivotal transition in the cell cycle between the cyclin D-mediated responses to extrinsic mitogenic cues and the CDK2-mediated progression from G_1 to S phase.

Stable association of p21 and p27 with active cyclin D–CDK4 complexes in vivo

The solved structure of p27 in a complex with cyclin A–CDK2 illustrates that a single p27 molecule can bind to both the cyclin and CDK subunit and can disrupt the CDK to dismantle its ATP binding site (Russo *et al.*, 1996). Although no analogous structure of a cyclin D–CDK–CKI complex is yet available, p21 and p27 were found to be potent inhibitors of binary cyclin D–CDK4 complexes assembled from recombinant protein subunits (Harper *et al.*, 1993; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). However, the idea that p21 and p27 differentially regulate various cyclin–CDK complexes is consistent with data that cyclin E–CDK2 and cyclin A–CDK2 are much more susceptible to inhibition by these CKIs than are cyclin D-dependent kinases *in vivo* (Soos *et al.*, 1996; Blain *et al.*, 1997; LaBaer *et al.*, 1997). As also observed in our studies, enzymatically active cyclin D–CDK4 complexes can be depleted from mammalian cell lysates with antibodies to these CKIs. In turn, immune complexes prepared in this manner retain Rb but not histone H1 kinase activity (Soos *et al.*, 1996), a feature of substrate specificity that distinguishes cyclin D-dependent holoenzymes from cyclin A-, B- and E-dependent kinases (Matsushime *et al.*, 1992). Therefore, p21 and p27 remain

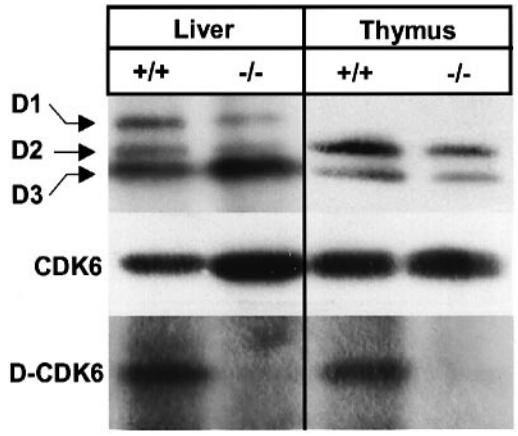

Fig. 8. Cyclin D–CDK assembly in mouse tissues. Tissue extracts from liver and thymus of wild-type or p21/p27 double-null mice were precipitated with a mixture of antibodies to cyclins D1, D2 and D3 (top panel) or anti-CDK6 (middle panel), and precipitated proteins separated on denaturing gels were blotted with the same antibodies. In parallel, cyclin D precipitates were blotted with anti-CDK6 (bottom) to score for complex formation. Proteins were detected by enhanced chemiluminescence using exposure times of 10 s (top, middle) and 60 s (bottom).

stably bound to active holoenzyme complexes during the cell cycle.

How can cyclin D–CDK complexes containing p21 and p27 retain activity? One possibility is that higher order cyclin D–CDK–CKI complexes recovered from mammalian cells contain additional components that protect the core binary enzyme from CKI-mediated inhibition (see, for example, Zhang *et al.*, 1994). Consistent with this idea, the mass of active cyclin D–CDK6 complexes recovered from T cells has been estimated at 150–170 kDa (Mahony *et al.*, 1998). Post-translational modifications of the included subunits might also alter their activities. This is not to say that Cip/Kip proteins cannot, under certain circumstances, inhibit CDK4 and CDK6. For example, in response to negative regulators of G_1 progression such as cAMP or TGF-β, the accumulation of Cip/Kip proteins can occlude cyclin D–CDK activation by CAK (Kato *et al.*, 1994; Polyak *et al.*, 1994; Aprelikova *et al.*, 1995). Clearly, the nature of the active holoenzymes expressed in mammalian cells needs to be better clarified.

The fact that Cip/Kip proteins enter into stable, enzymatically active complexes with cyclin D and CDK4 subunits highlights a second, non-catalytic function of cyclin D–CDKs: to 'titrate' p21 and p27, thereby freeing other CDKs from their constraint. In the face of an inhibitory threshold set by the CKIs, the latter process sets a dependency of CDK2 activity on the mitogen-stimulated assembly of cyclin D–CDK complexes, thereby coordinating the sequential activities of these enzymes as quiescent cells enter the cycle and progress toward S phase.

Cip/Kip regulation of cyclin D–CDK assembly

The loss of both p21 and p27 in MEF strains decreased the steady-state levels of assembled cyclin D–CDK4 complexes >10-fold and lowered cyclin D- and CDK4-associated Rb kinase activities to undetectable levels. The turnover of D-type cyclins was accelerated in cells lacking both CKIs and their overall levels were diminished. Nonetheless, the reduction in cyclin D levels per se did not account for their failure to assemble with catalytic subunits because ectopically overexpressed cyclin D1, whether located primarily in the nucleus or cytoplasm, was also unable to associate with CDK4 in this setting. Conversely, reintroduction of p21 or p27 into double-null cells reconstituted the formation of cyclin D–CDK complexes and increased the stability of cyclin D1.

Assembly of D-type cyclins with CDK4 requires several steps. Proper folding of CDK4 relies on the chaperone function of a cytoplasmic complex that includes Hsp90/Cdc37 (Dai *et al.*, 1996; Stepanova *et al.*, 1996). Once released from this complex, CDK4 can either enter into complexes with a D-type cyclin or can accumulate in enzymatically inactive binary complexes with an INK4 protein. The fate of CDK4 is largely determined by the availability of cyclin D subunits, which accumulate in response to mitogenic signaling, but how p21 and p27 contribute mechanistically to the assembly process remains unclear. Both of the latter CKIs contain distinct binding sites for CDKs and cyclins, which enable them to contact both subunits simultaneously (Toyoshima and Hunter, 1994; Chen *et al.*, 1995, 1996; Luo *et al.*, 1995; Nakanishi *et al.*, 1995; Lin *et al.*, 1996; Russo *et al.*, 1996). Hence, their binding to cyclin D and CDK4 might stabilize complex formation. Interactions between p21/p27 and CDK4/CDK6 might also prevent INK4 binding to the catalytic subunits (Reynisdottir and Massagué, 1997), diverting CDK4 into cyclin D-containing complexes and facilitating assembly through a less direct competitive mechanism. Cells lacking p27 and p21 would be expected to accumulate more CDK4–INK4 complexes, and under these conditions, cyclin D1 should be destabilized (Bates *et al.*, 1994; Parry *et al.*, 1995). Increased binding of CDK4 to INK4 proteins was indeed observed, but its association with Cdc37 was diminished relative to that in wild-type cells. A significant fraction (~30%) of CDK4 remained unbound to INK4 proteins or to Cdc37 in double-null MEFs, arguing that mechanisms other than competition between p21/p27 and INK4 proteins for CDK4 binding play a role in the assembly process.

Both the levels and assembly promoting activities of p21 and p27 are governed by mitogenic signals. For instance, p21 is frequently induced in cells entering the cycle from a quiescent state, whereas p27 levels are generally high in quiescent cells but fall prior to their entry into S phase (Firpo *et al.*, 1994; Kato *et al.*, 1994; Nourse *et al.*, 1994). Hence, p27 should not be limiting in promoting the association of cyclin D1 with CDK4 as cells enter the cycle, but its presence in a quiescent cell is still incapable of assisting assembly of cyclin–CDK complexes (Matsushime *et al.*, 1994). While it might be argued that the level of cyclin D1 in quiescent cells is simply too low to allow its entry into complexes with CDK4, ectopically expressed D1 subunits are also unable to assemble with CDK4 in serum-starved fibroblasts (Matsushime *et al.*, 1994) and require signals via the Ras-Raf1-MEK-ERK kinase cascade to ensure complex formation (Cheng *et al.*, 1998). One scenario is that p27 or p21 is subject to phosphorylation by ERKs, and that only the appropriately modified forms of the CKIs are able to promote cyclin D–CDK complex formation. Both of these CKIs are phosphoproteins, and p27 can be phosphorylated on serine by ERK1 (Zhang *et al.*, 1994; Alessandrini *et al.*, 1997). However, induction of enzymatically active MEK1 in NIH 3T3 fibroblasts was unaccompanied by detectable phosphorylation of p21 or p27. Also, a mutant version of p27 in which the ERK1 phosphorylation site was changed to alanine was still able to promote cyclin D1–CDK4 assembly (data not shown). Other possibilities are that cyclin D and CDK4 must be phosphorylated prior to assembly, or that the chaperone activity of the Cdc37/hsp90 complex can be regulated by ERKs.

Several factors are likely to contribute to added complexity in living animals. First, different cell types exhibit varying levels of Cip/Kip proteins *in vivo*, so the extent of assembly promoting activity attributed to each of these CKIs likely varies between tissues. Double-null mice continue to express cyclin D–CDK4 complexes, albeit at reduced levels, in many tissues. Although $p57^{Kip2}$ seems not to be required for assembly of cyclin D-dependent kinases in cultured MEFs, it may well promote assembly in other tissues. The possibility that new more distantly related Cip/Kip family members may be identified and be shown to play a role in cyclin–CDK assembly cannot be formally excluded. Secondly, while MEFs preferentially synthesize cyclin D1 and D2 in complexes with CDK4,

other cell types such as T lymphocytes, for example, express D3 instead of D1 and much more CDK6 than CDK4 (Ajchenbaum *et al.*, 1993; Meyerson and Harlow, 1994). Therefore, the six cyclin D-dependent kinases (containing D1, D2 or D3 with CDK4 or CDK6) are likely to be differentially regulated by Cip/Kip family members. Thirdly, the levels of cyclin D–CDK4 complexes were only modestly reduced in MEFs lacking p21 or p27, as compared with those in cells lacking both CKIs. Possibly, the loss of one of the CKIs facilitates compensation by the other. Finally, CKIs themselves are regulated by signals that affect cell proliferation, organismal development and cell differentiation (Elledge and Harper, 1994; Sherr and Roberts, 1995). As a singular example, the *Cip1* gene is directly regulated by p53 (El-Deiry *et al.*, 1993; Dulic *et al.*, 1994), and in cancer cells containing mutant p53, the levels of p21 are generally reduced making other CKIs more likely to promote cyclin D–CDK assembly under such circumstances.

CKIs affect cyclin D stability and nuclear localization

Cyclin D1 accumulates in the nucleus during G_1 phase but redistributes into the cytoplasm during S phase (Baldin *et al.*, 1993). D-type cyclins and CDK4 lack obvious nuclear localization signals (NLSs), and p21 or p27 can promote the nuclear import of cyclin D1–CDK4 through putative NLSs at their C-termini (LaBaer *et al.*, 1997). When cyclin D1 was ectopically overexpressed in wild-type MEFs, most of it localized to the nucleus, but in p21/p27 double-null cells, it was largely confined to the cytoplasm. Therefore, under conditions in which cyclin D1 levels are relatively high, the absence of p21 and p27 can limit its nuclear accumulation. However, assembly of cyclin D–CDK complexes per se does not drive their nuclear uptake, since endogenous cyclin D1 expressed at much reduced levels in p21/p27 double-null cells was able to accumulate in the nucleus, albeit less efficiently. Moreover, a cyclin D1 (T156A) mutant that assembles with CDK4 remains largely cytoplasmic, although its nuclear entry can be enforced by p21 overexpression (Diehl and Sherr, 1997). Conversely, the D1 (T286A) mutant is preferentially retained in the nucleus even in p21/p27 double-null cells where it does not undergo assembly. Indeed, this underscores the fact that assembly is inhibited in the absence of CKIs irrespective of whether D1 is predominately cytoplasmic or nuclear. We conclude that cyclin D–CDK assembly and nuclear uptake are separable functions to which the CKIs contribute independently.

The overall levels of cyclins D1 and D2 were significantly reduced in MEFs lacking both p21 and p27, but cyclin D1 was stabilized and its levels were restored when either CKI was reintroduced into the double-null cells. How might the presence of CKIs affect cyclin D stability? Phosphorylation of cyclin D1 on Thr286 targets it for degradation, and elimination of this threonine markedly increases the half-life of cyclin D1 in proliferating cells ($t_{1/2} > 3$ h) (Diehl *et al.*, 1997). Cyclins D2 and D3 appear subject to similar controls (our unpublished data). One possibility, then, is that binding of p21 or p27 to cyclin D1–CDK4 complexes can partially suppress D1 phosphorylation on Thr286, thereby helping to stabilize the cyclin. Phosphorylation of cyclin D1 on Thr286 is mediated by GSK-3β, and although cyclin D1 turnover in proliferating cells is relatively rapid ($t_{1/2}$ = 25 min), decreased signaling through the Ras-PI3K-Akt pathway activates GSK-3β and further shortens the half-life of cyclin D1 to ~12 min (Diehl *et al.*, 1998). Moreover, overexpression of an active, but not kinase-defective form of GSK-3β in mouse fibroblasts causes a redistribution of cyclin D1 from the cell nucleus to the cytoplasm (Diehl *et al.*, 1998). Therefore, by suppressing Thr286 phosphorylation, CKIs might affect the stability of cyclin D1 via two mechanisms: by promoting its nuclear retention and by preventing its targeting to proteasomes.

Consequences of Cip/Kip loss during the cell division cycle

Remarkably, MEFs lacking p21 and p27 did not exhibit overtly aberrant cell cycles. Indeed, our failure to detect cyclin D-dependent kinase activity in cells lacking both p21 and p27 raised the possibility that these kinases are not required for cell cycle progression. In an attempt to test directly whether p21/p27 double-null cells lack all cyclin D-dependent kinase activity, we infected these cells with retroviruses encoding the CDK4- and CDK6-specific inhibitor, $p16^{INK4a}$. Proteins of the INK4 family are presumed to act specifically as inhibitors of cyclin D-dependent kinases and are only able to arrest cells that retain Rb function (Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995). Despite the absence of detectable cyclin D-dependent kinase activity, p21/p27 double-null MEFs retained some sensitivity to the cell cycle inhibitory effects of overexpressed $p16^{INK4a}$. Even more inhibition was obtained using adenovirus vectors that programmed higher levels of ectopic protein expression (data not shown). In addition, Rb was phosphorylated on at least one site that is preferentially recognized by cyclin D-dependent kinases. Therefore, cells lacking p21 and p27 might well express cryptic cyclin D-dependent kinase activity. In short, while we were unable to resolve whether cyclin D-dependent kinases are dispensable for the division cycle in Rb-positive cells lacking Cip/Kip proteins, it is evident that such cells tolerate a significant reduction in enzyme activity.

Although mice lacking cyclins D1 or D2 (or both) exhibit focal developmental anomalies (Fantl *et al.*, 1995; Sicinski *et al.*, 1995, 1996), two of the three D-type cyclins are dispensable for most cell divisions in the life of these animals. Indeed, if the key functions of cyclin D-dependent kinases are to phosphorylate Rb and to titrate CDK inhibitors, then their loss in p21/p27 double-null cells should be well tolerated. In the absence of these CKIs, the titration function of cyclin D-dependent kinases would be superfluous, and unopposed cyclin E–CDK2 and cyclin A–CDK2 activities might be sufficient to phosphorylate Rb. Based on changes in its electrophoretic mobility, Rb phosphorylation increased as p21/p27-null cells approached the G_1/S boundary, and the kinetics of cell cycle progression were remarkably similar to those of wild-type MEFs. Together, our results argue that p21 and p27 positively regulate the assembly, stability and nuclear localization of D-type cyclins. In turn, the activities of cyclin E–CDK2 and A–CDK2 are normally opposed by these CKIs, but in their absence, the latter enzymes

are likely to compensate for loss of cyclin D-dependent kinase activity.

Materials and methods

Cells and culture conditions

Mouse embryonic fibroblasts (MEFs) from animals deficient in $p21^{Cip1}$, $p27^{Kip1}$ or both CKIs were established as described previously (Zindy *et al*., 1997) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids, 55 μM 2-mercaptoethanol and 100 U/ml each of penicillin and streptomycin. To make them quiescent, confluent MEFs were washed twice with phosphate-buffered saline (PBS) and cultured in serum-depleted medium [DMEM with 0.1% FBS, 0.04% bovine serum albumin (BSA), glutamine, penicillin and streptomycin] for 18 h. Quiescent cells were trypsinized, re-plated at low density and stimulated with complete medium containing 10% FBS to enter the division cycle, and entry into S phase was monitored by estimating the DNA content of propidium iodide-stained nuclei using fluorescence-activated flow cytometry (Matsushime *et al.*, 1991). The 293T retrovirus packaging cell line and helper virus plasmid (Pear *et al.*, 1993) were obtained from C.Sawyers (University of California, Los Angeles) with permission from David Baltimore (California Institute of Technology).

Immunoblotting, immunoprecipitation and immunofluorescence

Immunoblotting of cyclin D1, cyclin D2, CDK4, CDK6, p21, p27 and the detection of cyclin D–CDK complexes was performed as described previously (Cheng *et al.*, 1998). INK4 proteins were precipitated from MEFs or mouse tissues as described (Zindy *et al.*, 1997). For detection of Rb protein, cells were disrupted in lysis buffer containing 50 mM HEPES pH 7.0, 0.5% Nonidet P-40, 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 4 μg/ml aprotinin, 4 μg/ml pepstatin (protease inhibitors from Sigma Chemicals, St Louis, MO), 0.5 mM sodium orthovanadate, 5 mM sodium fluoride, and 50 mM β-glycerophosphate. Lysates were clarified by centrifugation, and protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL). Protein (2 mg) was precipitated with rabbit antibody to Rb (SC-050, Santa Cruz, CA) and collected with 30 μl of protein A–Sepharose (Pharmacia Biotechnology, Uppsala, Sweden). After three washes in lysis buffer, immune complexes resuspended in 30 μl gel sample buffer [50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 1 mM dithiothreitol (DTT), 0.1% Bromophenol Blue], were electrophoretically resolved on denaturing polyacrylamide gels, transferred to nitrocellulose and probed with the mouse monoclonal antibody to Rb (14001A, Pharmingen, CA). Rb protein phosphorylated on Ser780 was detected by direct immunoblotting using purified antibodies specifically reactive with a phosphorylated epitope (Kitagawa *et al.*, 1996). Metabolic labeling and measurements of cyclin D1 turnover were performed as previously described (Diehl *et al.*, 1997). Indirect immunofluorescence was also carried out according to previous methods (Diehl and Sherr, 1997) except that the cells were fixed with 3.7% paraformaldehyde at room temperature for 15–20 min and permeabilized with acetone for 5 min at –20°C.

Protein kinase assays

Immune complex kinase assays using 5 μg recombinant GST-Rb as substrate were performed (Matsushime *et al.*, 1994) using 500 μg total lysate protein per reaction and immunoprecipitation with either rabbit anti-peptide serum (R_Z) directed to the CDK4 C-terminus or monoclonal anti-cyclin D1 (D1-72-13G) derived previously (Matsushime *et al.*, 1994; Vallance *et al.*, 1994). For immunodepletion, cell lysates were first subjected to two rounds of immunoprecipitation with rabbit anti-p21, rabbit anti-p27 or both, and proteins remaining in the supernatant were precipitated with anti-CDK4 (serum R_Z); the Rb kinase activity of resulting CDK4 immune complexes was then determined as above. To measure CDK2 activity, cells were lysed in 50 mM Tris–HCl pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 10 mM β-glycerophosphate, 0.5 mM sodium vanadate, 1 mM sodium fluoride, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, and 4 μg/ml pepstatin. Clarified lysates (200 μg protein per sample) were precipitated for 3 h at 4°C with antisera to cyclin E (M-20) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or CDK2 (sera R_{CC} plus R_{DD}) (Matsuoka *et al.*, 1994) plus 20 μl protein A–Sepharose. Immune complexes were washed twice with the same lysis buffer and twice with kinase buffer (50 mM HEPES pH 8.0, 10 mM $MgCl_2$, 1 mM DTT). Reaction mixtures (50 μl kinase buffer) contained 30 μM ATP with 10 μCi [γ-^{32}P]ATP plus 10 μg histone H1 or 5 μg GST-Rb. All reactions were stopped by adding 1/3 volume 3× gel sample buffer and heating at 85°C for 5 min. Labeled proteins were resolved on denaturing polyacrylamide gels, which were dried and subjected to autoradiography.

Virus production and infection

Human kidney 293T cells were transfected (Chen and Okayama, 1987) with 15 μg of ecotropic helper retrovirus plasmid plus 15 μg of SRα vector DNA encoding p21, p27, $p16^{INK4a}$ or Flag-tagged cyclin D1 (WT) and cyclin D1 (T286A). Cell supernatants containing infectious retroviral pseudotypes were harvested 24–60 h post-transfection, pooled on ice and filtered (0.45 μm membrane). Virus infections of exponentially growing MEFs were performed in a 9% CO_2 atmosphere with 5 ml of virus-containing culture supernatants plus 10 μg/ml polybrene (Sigma, St Louis, MO) for each 100 mm diameter culture dish. After 5 h, 10 ml fresh medium was added, and medium was changed 24 h later. Cells were harvested 48 h after infection, and the percentage of cells in S phase was determined by flow cytometric analysis of DNA content (Matsushime *et al.*, 1991) or by incorporation of [^{3}H]thymidine into replicating cell DNA (Kamijo *et al.*, 1997).

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References

Ajchenbaum,F., Ando,K., DeCaprio,J.A. and Griffin,J.D. (1993) Independent regulation of human D-type cyclin gene expression during G_1 phase in primary human T lymphocytes. *J. Biol. Chem.*, **268**, 4113–4119.

Aktas,H., Cai,H. and Cooper,G.M. (1997) Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the cdk inhibitor $p27^{Kip1}$. *Mol. Cell. Biol.*, **17**, 3850–3857.

Albanese,C., Johnson,J., Watanabe,G., Eklund,N., Vu,D., Arnold,A. and Pestell,R.G. (1995) Transforming $p21^{ras}$ mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J. Biol. Chem.*, **270**, 23589–23597.

Alessandrini,A., Chiaur,D.S. and Pagano,M. (1997) Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation. *Leukemia*, **11**, 342–345.

Aprelikova,O., Xiong,Y. and Liu,E.T. (1995) Both p16 and p21 families of the cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. *J. Biol. Chem.*, **270**, 18195–18197.

Baldin,V., Lukas,J., Marcote,M.J., Pagano,M. and Draetta,G. (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev.*, **7**, 812–821.

Bates,S., Parry,D., Bonetta,L., Vousden,K., Dickson,C. and Peters,G. (1994) Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. *Oncogene*, **9**, 1633–1640.

Blain,S.W., Montalvo,E. and Massague,J. (1997) Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$ with cyclin A–Cdk2 and cyclin D2–Cdk4. *J. Biol. Chem.*, **272**, 25863–25872.

Boudewijn,M., Burgering,Th. and Coffer,P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*, **376**, 599–602.

Brugarolas,J., Chandrasekaran,C., Gordon,J.I., Beach,D., Jacks,T. and Hannon,G.J. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature*, **377**, 552–557.

Chan,F.K.M., Zhang,J., Cheng,L., Shapiro,D.N. and Winoto,A. (1995) Identification of human/mouse p19, a novel CDK4/CDK6 inhibitor with homology to $p16^{INK4}$. *Mol. Cell. Biol.*, **15**, 2682–2688.

Chen,C. and Okayama,H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752.

Chen,J., Jackson,P.K., Kirschner,M.W. and Dutta,A. (1995) Separate domains of p21 involved in the inhibition of cdk kinase and PCNA. *Nature*, **374**, 386–388.

Chen,J., Saha,P., Kornbluth,S., Dynlacht,B.D. and Dutta,A. (1996) Cyclin-binding motifs are essential for the function of $p21^{CIP1}$. *Mol. Cell. Biol.*, **16**, 4673–4682.

Cheng,M., Sexl,V., Sherr,C.J. and Roussel,M.F. (1998) Assembly of cyclin D-dependent kinase and titration of $p27^{Kip1}$ regulated by mitogen-activated protein kinase kinase (MEK1). *Proc. Natl Acad. Sci. USA*, **95**, 1091–1096.

Cross,D.A.E., Alessi,D.R., Cohen,P., Andjelkovich,M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, **378**, 785–789.

Dai,K., Kobayashi,R. and Beach,D. (1996) Physical interaction of mammalian CDC37 with CDK4. *J. Biol. Chem.*, **271**, 22030–22034.

Deng,C., Zhang,P., Harper,J.W., Elledge,S.J. and Leder,P. (1995) Mice lacking $p21^{Cip1/Waf1}$ undergo normal development, but are defective in G_1 checkpoint control. *Cell*, **82**, 675–684.

Diehl,J.A. and Sherr,C.J. (1997) A dominant-negative cyclin D1 mutant prevents nuclear import of cyclin-dependent kinase-4 (CDK4) and its phosphorylation by CDK-activating kinase. *Mol. Cell. Biol.*, **17**, 7362–7374.

Diehl,J.A., Zindy,F. and Sherr,C.J. (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.*, **11**, 957–972.

Diehl,J.A., Cheng,M., Roussel,M.F. and Sherr,C.J. (1998) Glycogen Synthase Kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.*, **12**, 3499–3511.

Dudek,H., Datta,S.R., Franke,T.F., Birnbaum,M.J., Yao,R., Cooper,G.M., Segal,R.A., Kaplan,D.A. and Greenberg,M.E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science*, **275**, 661–665.

Dulic,V., Kaufmann,W.K., Wilson,S.J., Tlsty,T.D., Lees,E., Harper,J.W., Elledge,S.J. and Reed,S.I. (1994) p53-Dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G_1 arrest. *Cell*, **76**, 1013–1023.

El-Deiry,W.S. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Elledge,S.J. and Harper,J.W. (1994) CDK inhibitors: on the threshold of check points and development. *Curr. Opin. Cell Biol.*, **6**, 847–852.

Fantl,V., Stamp,G., Andrews,A., Rosewell,I. and Dickson,C. (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev.*, **9**, 2364–2372.

Firpo,E.J., Koff,A., Solomon,M.J. and Roberts,J.M. (1994) Inactivation of a cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol. Cell. Biol.*, **14**, 4889–4901.

Franke,T.F., Yang,T.O., Chan,K., Datta,A., Kazlauskas,D.K., Morrison,D.K., Kaplan,D.R. and Tsichlis,P.N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, **81**, 727–736.

Franke,T.F., Kaplan,D.R., Cantley,L.C. and Toker,A. (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science*, **275**, 665–668.

Guan,K.-L., Jenkins,C.W., Li,Y., Nichols,M.A., Wu,X., O'Keefe,C.L., Matera,A.G. and Xiong,Y. (1994) Growth suppression by p18, a $p16^{INK4A/MTS1}$- and $p15^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev.*, **8**, 2939–2952.

Hannon,G.J. and Beach,D. (1994) $p15^{INK4B}$ is a potential effector of TGFβ-induced cell cycle arrest. *Nature*, **371**, 257–261.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Hatakeyama,M., Brill,J.A., Fink,G.R. and Weinberg,R.A. (1994) Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev.*, **8**, 1759–1771.

Hirai,H., Roussel,M.F., Kato,J.-Y., Ashmun,R.A. and Sherr,C.J. (1995) Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases. *Mol. Cell. Biol.*, **15**, 2672–2681.

Kamijo,T., Zindy,F., Roussel,M.F., Quelle,D.E., Downing,J.R., Ashmun,R.A., Grosveld,G. and Sherr,C.J. (1997) Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell*, **91**, 649–659.

Kato,J., Matsuoka,M., Polyak,K., Massagué,J. and Sherr,C.J. (1994) Cyclic AMP-induced G_1 phase arrest mediated by an inhibitor ($p27^{Kip1}$) of cyclin-dependent kinase-4 activation. *Cell*, **79**, 487–496.

Kelly,B.L., Wolfe,K.G. and Roberts,J.M. (1998) Identification of a substrate-targeting domain in cyclin E necessary for phosphorylation of the retinoblastoma protein. *Proc. Natl Acad. Sci. USA*, **95**, 2535–2540.

Kerkhoff,E. and Rapp,U.R. (1997) Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. *Mol. Cell. Biol.*, **17**, 2576–2586.

Kitagawa,M. *et al.* (1996) The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *EMBO J.*, **15**, 7060–7069.

Klinghoffer,R.A., Duckworth,B., Valius,M., Cantley,L. and Kazlauskas,A. (1996) Platelet-derived growth factor-dependent activation of phosphatidylinositol 3-kinase is regulated by receptor binding of SH2-domain-containing proteins which influence ras activity. *Mol. Cell. Biol.*, **16**, 5905–5914.

Koh,J., Enders,G.H., Dynlacht,B.D. and Harlow,E. (1995) Tumour-derived p16 alleles encoding proteins defective in cell cycle inhibition. *Nature*, **375**, 506–510.

Krude,T., Jackman,M., Pines,J. and Laskey,R.A. (1997) Cyclin/cdk-dependent initiation of DNA replication in a human cell-free system. *Cell*, **88**, 109–119.

LaBaer,J., Garrett,M.D., Stevenson,L.F., Slingerland,J.M., Sandhu,C., Chou,H.S., Fattaey,A. and Harlow,E. (1997) New functional activities for the p21 family of CDK inhibitors. *Genes Dev.*, **11**, 847–862.

Lavoie,J.N., L'Allemain,G., Brunet,A., Müller,R. and Pouysségur,J. (1996) Cyclin D1 expression is regulated positively by the p42/$p44^{MAPK}$ and negatively by the $p38/HOG^{MAPK}$ pathway. *J. Biol. Chem.*, **271**, 20608–20616.

Lee,M.-H., Nikolic,M., Bapista,C.A., Lai,E., Tsai,L.-H. and Massagué,J. (1996) The brain-specific activator p35 allows Cdk5 to escape inhibition by $p27^{Kip1}$ in neurons. *Proc. Natl Acad. Sci. USA*, **93**, 3259–3263.

Lin,J., Reichner,C., Wu,X. and Levine,A.J. (1996) Analysis of wild-type and mutant $p21^{WAF-1}$ gene activities. *Mol. Biol. Cell*, **16**, 1786–1793.

Lukas,J., Parry,D., Aagaard,L., Mann,D.J., Bartkova,J., Strauss,M., Peters,G. and Bartek,J. (1995) Retinoblastoma protein-dependent cell cycle inhibition by the tumor suppressor p16. *Nature*, **375**, 503–506.

Lundberg,A.S. and Weinberg,R.A. (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin–CDK complexes. *Mol. Cell. Biol.*, **18**, 753–761.

Luo,Y., Hurwitz,J. and Massagué,J. (1995) Cell cycle inhibition mediated by functionally independent CDK and PCNA inhibitory domains in $p21^{CIP1}$. *Nature*, **375**, 159–161.

Mahony,D., Parry,D.A. and Lees,E. (1998) Active cdk6 complexes are predominantly nuclear and represent only a minority of the cdk6 in T cells. *Oncogene*, **13**, 603–611.

Matsuoka,M., Kato,J., Fisher,R.P., Morgan,D.O. and Sherr,C.J. (1994) Activation of cyclin-dependent kinase-4 (CDK4) by mouse MO15-associated kinase. *Mol. Cell. Biol.*, **14**, 7265–7275.

Matsushime,H., Roussel,M.F., Ashmun,R.A. and Sherr,C.J. (1991) Colony-stimulating factor 1 regulates novel cyclins during the G_1 phase of the cell cycle. *Cell*, **65**, 701–713.

Matsushime,H., Ewen,M.E., Strom,D.K., Kato,J.-Y., Hanks,S.K., Roussel,M.F. and Sherr,C.J. (1992) Identification and properties of an atypical catalytic subunit ($p34^{PSKJ3}$/CDK4) for mammalian D-type G_1 cyclins. *Cell*, **71**, 323–334.

Matsushime,H., Quelle,D.E., Shurtleff,S.A., Shibuya,M., Sherr,C.J. and Kato,J.-Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066–2076.

Medema,R.H., Herrera,R.E., Lam,F. and Weinberg,R.A. (1995) Growth suppression by $p16^{ink4}$ requires functional retinoblastoma protein. *Proc. Natl Acad. Sci. USA*, **92**, 6289–6293.

Meyerson,M. and Harlow,E. (1994) Identification of a G1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.*, **14**, 2077–2086.

Mittnacht,S., Lees,J.A., Desai,D., Harlow,E., Morgan,D.O. and Weinberg,R.A. (1994) Distinct sub-populations of the retinoblastoma protein show a distinct pattern of phosphorylation. *EMBO J.*, **13**, 118–127.

Nakanishi,M., Robetorge,R.S., Adami,G.R., Pereira-Smith,O.M. and Smith,J.R. (1995) Identification of the active region of the DNA synthesis inhibitory gene $p21^{Sdi1/CIP1/WAF1}$. *EMBO J.*, **14**, 555–563.

Nourse,J., Firpo,E., Flanagan,W.M., Coats,S., Polyak,K., Lee,M.H., Massagué,J., Crabtree,G.R. and Roberts,J.M. (1994) Interleukin-2-mediated elimination of $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*, **372**, 570–573.

Parry,D., Bates,S., Mann,D.J. and Peters,G. (1995) Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumour suppressor gene product. *EMBO J.*, **14**, 503–511.

Pear,W.S., NoPan,M.L., Scott,M.L. and Baltimore,D. (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl Acad. Sci. USA*, **90**, 8392–8396.

Polyak,K., Lee,M.-H., Erdjument-Bromage,H., Koff,A., Roberts,J.M., Tempst,P. and Massagué,J. (1994) Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, **78**, 59–66.

Reynisdóttir,I. and Massagué,J. (1997) The subcellular locations of $p15^{INK4b}$ and $p27^{Kip1}$ coordinate their inhibitory interactions with cdk4 and cdk2. *Genes Dev.*, **11**, 492–503.

Russo,A.A., Jeffrey,P.D., Patten,A.K., Massagué,J. and Pavletich,N.P. (1996) Crystal structure of the p27Kip1 cyclin-dependent kinase inhibitor bound to the cyclin A–cdk2 complex. *Nature*, **382**, 325–331.

Serrano,M., Hannon,G.J. and Beach,D. (1993) A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704–707.

Sheaff,R., Groudine,M., Gordon,M., Roberts,J. and Clurman,B. (1997) Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev.*, **11**, 1464–1478.

Sherr,C.J. (1996) Cancer cell cycles. *Science*, **274**, 1672–1677.

Sherr,C.J. and Roberts,J.M. (1995) Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.*, **9**, 1149–1163.

Sicinski,P. *et al.* (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell*, **82**, 621–630.

Sicinski,P. *et al.* (1996) Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature*, **384**, 470–471.

Soos,T.J. *et al.* (1996) Formation of p27–CDK complexes during the human mitotic cell cycle. *Cell Growth Differ.*, **7**, 135–146.

Stepanova,L., Leng,X., Parker,S.B. and Harper,J.W. (1996) Mammalian $p50^{Cdc37}$ is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes Dev.*, **10**, 1491–1502.

Stillman,B. (1996) Cell cycle control of DNA replication. *Science*, **274**, 1659–1664.

Toyoshima,H. and Hunter,T. (1994) p27, a novel inhibitor of G_1 cyclin/cdk protein kinase activity, is related to p21. *Cell*, **78**, 67–74.

Vallance,S.J., Lee,H.-M., Roussel,M.F., Shurtleff,S.A., Kato,J.-Y., Strom,D.K. and Sherr,C.J. (1994) Monoclonal antibodies to mammalian D-type G_1 cyclins. *Hybridoma*, **13**, 37–44.

Vanhaesebroeck,B., Leevers,S.J., Panayotou,G. and Waterfield,M. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci*., **22**, 267–272.

Vlach,J., Hennecke,S. and Amati,B. (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. *EMBO J.*, **16**, 5334–5344.

Weber,J.D., Raben,D.M., Phillips,P.J. and Baldassare,J.J. (1997) Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G_1 phase. *Biochem. J*., **326**, 61–68.

Weinberg,R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323–330.

Winston,J.T., Coats,S.R., Wang,Y.-Z. and Pledger,W.J. (1996) Regulation of the cell cycle machinery by oncogenic *ras*. *Oncogene*, **12**, 127–134.

Zhang,H., Hannon,G.J. and Beach,D. (1994) p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev.*, **8**, 1750–1758.

Zindy,F., Quelle,D.E., Roussel,M.F. and Sherr,C.J. (1997) Expression of the $p16^{INK4a}$ tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene*, **15**, 203–211.

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