The subcellular locations of p15^{Ink4b} and p27^{Kip1} coordinate their inhibitory interactions with cdk4 and cdk2

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In dividing cells, p27^{Kip1} is predominantly bound to cyclin D-cdk4 without inhibiting this kinase. Upon being induced by TGF- β or with a conditional expression system in lung epithelial cells, p15^{Ink4b} binds to and inhibits the cyclin D-dependent kinases, prevents p27 binding to these cdk complexes, and promotes p27 binding and inhibition of cyclin-cdk2. In vitro, however, p15 prevents p27 binding only if it has access to cyclin D-cdk4 first. We present evidence that the different subcellular location of p15 and p27 ensures the prior access of p15 to cdk4. In the cell, p15 is localized mostly in the cytoplasm, whereas p27 is nuclear. p15 prevails over p27 or a p27 construct consisting of the cdk inhibitory domain tagged with a nuclear localization signal. However, when p15 and p27 are forced to reside in the same subcellular location, either the cytoplasm or the nucleus, p15 no longer prevents p27 from binding to cdk4. These properties allow p15 and p27 to coordinately inhibit cdk4 and cdk2.

[**Key Words:** p15^{Ink4b}; p27^{Kip1}; cdk inhibitors; cyclin D-cdk4/6; subcellular location]

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Cyclin-dependent kinases (cdks) that control G₁ and S-phase progression in mammalian cells are regulated by two families of cdk inhibitors, the Ink4 family and the Cip/Kip family (for review, see Sherr and Roberts 1995). Ink4 proteins and Cip/Kip1 proteins are structurally very distinct and interact with cyclins and cdks in completely different ways. The Ink4 proteins (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}) consist of tandem repeats of an ankyrin-like sequence, whereas Cip/Kip proteins (p21^{Cip1}/Waf1, p27^{Kip1}, and p57^{Kip2}) have a homologous amino-terminal domain that contains contiguous cyclin- and cdk-binding regions. The Ink4 proteins bind specifically to cdk4 and its close homolog cdk6, which are kinases responsible for the first wave of cdk activity during the cell cycle, starting in mid-G₁ phase. Cip/Kip proteins, on the other hand, bind to a wide range of cyclin-cdk complexes including cyclin D-cdk4/6, cyclin E-cdk2, which is active in late G₁ phase, and cyclin A-cdk2, which is active at the G₁/S transition and throughout S phase. Each of the Ink4 and Cip/Kip genes has a distinct response pattern to antimitogenic cytokines (Hannon and Beach 1994; Hengst et al. 1994; Kato et al. 1994; Nourse et al. 1994; Polyak et al. 1994a; Slingerland et al. 1994; Poon et al. 1995; Reynisdóttir et al. 1995; Hengst and Reed 1996), differentiation agents (Steinman et al. 1994; Halevy et al. 1995; Parker et al.

1995; Liu et al. 1996), the p53 tumor suppressor protein (El-Deiry et al. 1993, 1994) cell-cell contact (Polyak et al. 1994a; Slingerland et al. 1994; Toyoshima and Hunter 1994; Poon et al. 1995; Hengst and Reed 1996), cell anchorage to extracellular matrix (Fang et al. 1996; Zhu, et al. 1996), or events leading to cell senescence (Noda et al. 1994; Hara et al. 1996). p16, and possibly p15, are tumor suppressor genes whereas the other inhibitors appear not to be (for review, Hall and Peters 1996). In spite of these differences in structural, biochemical, and regulatory properties, little is known that would explain the requirement of two families of cdk inhibitors for proper control of cell cycle progression and arrest.

We have focused on this problem through studies on the antiproliferative response to transforming growth factor- β (TGF- β). This response in lung epithelial cells and keratinocytes involves an interplay between p15^{Ink4b} and p27^{Kip1} (as well as p21^{Cip1} in keratinocytes) (Hannon and Beach 1994; Reynisdóttir et al. 1995). When growth-arrested by contact inhibition or mitogen withdrawal, these cells accumulate in G₀ with a high level of p27. Upon restimulation with mitogen, p27 levels decline but remain sufficiently high such that they could block cdk2 activity if they were exclusively available to cdk2 complexes (Reynisdóttir et al. 1995). However, p27 in proliferating cells is found predominantly in complexes with cyclin D-cdk4/6 (Toyoshima and Hunter 1994; Poon et al. 1995; Soos et al. 1996). These complexes are active, perhaps as a result of p27 being bound

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to the cyclin subunit without establishing a blocking interaction with the cdk subunit. TGF- β addition to these cells does not increase the total level of p27, but induces a redistribution of p27 from cyclin D-cdk4/6 complexes to cyclin E-cdk2, inhibiting cdk2 (Reynisdóttir et al. 1995). It has been suggested that this redistribution is the result of a rapid and strong induction of p15 by TGF- β , and a model in which an Ink4 protein (p15) and a Cip/Kip protein (p27) cooperate to suppress multiple G₁ cdks has been proposed based on these findings (Reynisdóttir et al. 1995).

In the course of the studies to test this model, we observed that p15 can compete with p27 for binding to cyclin D-cdk4/6 *in vivo* without dissociating this complex. Examination of this process *in vitro* revealed that unless p15 is added first, p27 prevails over p15 in binding to cyclin D-cdk4. These observations raised the question of how a surge in p15 levels may prevent pre-existing p27 from binding to cyclin D-cdk4/6 complexes in the cell. An answer is proposed based on the fact that p15 is distributed throughout the cell whereas p27 is predominantly nuclear.

Results

Growth arrest in a p15-inducible system

In exponentially growing Mv1Lu mink lung epithelial cells p27 is bound predominantly to cyclin D-cdk4/6 without inhibiting the kinase activity of these complexes (S.W. Blain, E. Montalvo, and J. Massagué, in prep.). Addition of TGF- β to these cells causes a loss of cdk4/6-bound p27, an increase in p27 binding to cdk2, cdk2 inhibition, and G₁ arrest (Reynisdóttir et al. 1995). To test the hypothesis that all these events may result from the rapid increase in p15 expression induced by TGF- β , we generated a Mv1Lu cell derivative, Tet-p15, that expresses the human *p15* gene under negative control of the tetracycline *trans*-activator (Gossen and Bujard 1992). Tet-p15 cells do not express p15 in the presence of 1 μ g/ml of tetracycline but express a high level of exogenous p15 when placed in tetracycline-free medium (Fig. 1A). This increase was half-maximal after 9 hr and maximal after 14 hr in tetracycline-free medium (data not shown). Tetracycline removal slightly increased cyclin D levels but did not alter the levels of p27 or G₁ cdks in Tet-p15 cells and did not induce p15 in control transfectants (Figs. 1A and 2A).

In tetracycline medium, the proliferation rate of control and Tet-p15 cells was the same. Removal of tetracycline inhibited the proliferation of Tet-p15 cells but not that of control cells (Fig. 1B). This effect was similar to that caused by TGF- β in Mv1Lu cells (Laiho et al. 1990) or by TGF- β in Tet-p15 cells in tetracycline (data not shown). In tetracycline-free medium, Tet-p15 cells became arrested with the retinoblastoma susceptibility gene product (Rb) in the hypophosphorylated state (Fig. 1D, bottom panel) and with a G₁ DNA content (data not shown). This confirmed that p15 induction is sufficient to inhibit Rb kinases and cause a G₁ arrest.

We compared the level of exogenous p15 induced by tetracycline removal with the level of endogenous p15 induced by TGF- β addition. Because antibodies that recognize the endogenous (mink) p15 were not available, the levels of p15 were compared by immunoprecipitating cdk6 from [³⁵S]methionine-labeled cells and quantitating the amount of associated p15 (Fig. 1C). Tet-p15 cells incubated in the absence of tetracycline to induce the expression of exogenous p15, or incubated with tetracycline plus TGF- β , to induce the expression of endogenous p15, yielded cdk6-associated proteins of ~15 kD that were absent in negative controls (Fig. 1C). Assuming a similar methionine content in human and mink p15, the level of cdk6-bound p15 induced by tetracycline removal was only 1.5- to 2-fold higher than that induced by TGF- β addition. The fact that similar levels of p15 were induced under both conditions confirmed the suitability of Tet-p15 cells for studies on the function of p15.

p15 interaction with cyclin D-cdk4/6 complexes

Levels of cdk- and cyclin-bound p15 in Tet-p15 cells were determined by immunoprecipitating individual components and immunoblotting the precipitates with p15 antibodies. The levels of p15 bound to cdk4 and cdk6 were proportional to the total level of p15 in the cell (Fig. 1A,D). p15 was also found in association with cyclin D1 and D2 (Fig. 1D) (cyclin D3 is not expressed in these cells). Because Ink4 proteins can interact with free cdk4 or cdk6 but not with free D cyclins, the coprecipitation of p15 with D cyclins presumably reflects p15 binding to cyclin D-cdk4/6 complexes.

This result was surprising because p16^{Ink4a} binding to cdk4 *in vivo* has been reported to inhibit the association of cdk4 with cyclin D (Parry et al. 1995). Cyclins D1 and D2 have a half-life of <1 hr, even when bound to cdks (Matsushime et al. 1992; Bates et al. 1994). The maintenance of steady-state cyclin D-cdk4/6 levels requires a rapid rate of assembly of new cyclin D-cdk4/6 complexes (Matsushime et al. 1992; Bates et al. 1994). If p15 interfered with the stability or assembly of cyclin D-cdk4/6, it would cause a rapid depletion of these complexes in the cell. To investigate this point, cdk4 and cdk6 were immunoprecipitated from Tet-p15 cells that were exponentially growing or growth-arrested by either tetracycline removal or TGF- β addition (18 hr). Immunoblotting of these precipitates with cyclin D antibodies showed that the levels of cdk4- and cdk6-bound cyclins D1 and D2 were similar under all three conditions (Fig. 2A; data not shown). Upon prolonged exposure to TGF- β (> 20 hr), a reduction in cyclin D-cdk4 complexes was observed occasionally (data not shown). Thus, levels of p15 that blocked cell proliferation were bound to cyclin D-cdk4/6 without causing any detectable dissociation of these complexes.

The ability of p15 to bind and inhibit cyclin D-cdk4/6 without causing their dissociation was confirmed *in vitro*. Insect cell lysates containing recombinant cyclin D2 and cdk4 were incubated with various concentrations of bacterially expressed human p15. Cdk4 was then

Figure 1. Overexpression of p15 leads to inhibition of Rb kinase activity and growth arrest. Tet-p15 cells and control cells were incubated for 16 hr in medium containing the indicated concentrations of tetracycline. (A) Cleared whole-cell extract (0.2 mg of protein) was subjected to Western immunoblotting with antibodies against p15 or p27. (B) The rate of DNA synthesis was measured by adding ^{125}I -labeled deoxyuridine (dU) to the medium for the last 2 hr of incubation. Deoxyuridine incorporation is expressed as percent relative to controls incubated in the presence of 1 $\mu\text{g}/\text{ml}$ of tetracycline. (C) Lysates from Tet-p15 cells grown in medium containing [^{35}S]methionine, 100 pM of TGF- β , and/or the indicated concentrations of tetracycline were immunoprecipitated with a cdk6 antibody or normal rabbit serum (NRS). The associated proteins were analyzed by SDS-PAGE and fluorography. (D) Lysates (5 mg of protein) were immunoprecipitated with antibodies against cdk4, cdk6, cyclin D1, or cyclin D2. The immunoprecipitates were subjected to Western immunoblotting with a p15 antibody. The Rb kinase activity was measured by Western immunoblotting of whole-cell lysates (0.2 mg of protein) using an Rb antibody. (Rb^{phos}) The hyperphosphorylated Rb form.

immunoprecipitated and analyzed for the presence of associated p15, cyclin D2, and Rb kinase activity (Fig. 3A). These results show that p15 bound to and inhibited cdk4 without causing dissociation of cyclin D2.

Direct inhibition of p27 binding to cyclin D-cdk4/6 by p15

Next, we investigated whether the observed loss of cdk4- and cdk6-bound p27 in response to TGF- β (Reynisdóttir et al. 1995) could be reproduced by inducing p15 expression. Tet-p15 cells growing in high tetracycline medium or growth arrested by either tetracycline removal or TGF- β addition were lysed and the proteins were precipitated with cdk4 or cdk6 antibodies. Immunoblotting of these precipitates with p27 antibodies showed that p15 induction caused a loss of cdk4- and cdk6-bound p27 as extensive as that caused by TGF- β (Fig. 2A). Immunoprecipitation of p27 from tetracycline-free Tet-p15 cells and immunoblotting of these complexes with p15 antibodies provided little evidence of p27 association with p15 (data not shown). Therefore, p27-cyclin D-cdk4/6-p15 complexes were unstable or absent.

To determine whether p15 binding to cyclin D-cdk4 directly prevents p27 binding, we incubated a fixed amount of recombinant cyclin D2-cdk4 with increasing amounts of p15 prior to addition of a fixed amount (790 ng) of p27. As determined in preliminary experiments, this amount of p27 was able to bind a maximal amount of cyclin D2-cdk4 in this mixture. Preincubation of cyclin D2-cdk4 with p15 inhibited the subsequent binding of p27 (Fig. 3B), suggesting that the inhibition of p27 binding observed in vivo may be a direct result of p15 binding to cyclin D-cdk4/6. To confirm this conclusion,

we performed transfection experiments using a highly transfectable Mv1Lu subclone (R-1B/L17; Cárcamo et al. 1994), which can be used to assess the effect of transiently transfected genes (Polyak et al. 1994a; Lee et al. 1995). The cells were transfected with p15 and/or p27 and either cdk4 or cdk4-R24C. The latter is a mutant form of cdk4, which is found in hereditary melanoma (Wölfel et al. 1995). It is defective in p15 binding, but it can still bind p27 (Wölfel et al. 1995). Consistent with its ability to prevail over p27, p15 inhibited the binding of cotransfected p27 to cdk4 (Figs. 3C and 8A). However, p15 did not inhibit the binding of p27 to cdk4-R24C (Fig. 3C, cf. lanes 4 and 8), indicating that inhibition of p27 binding is a direct result of the p15 interaction with cdk4.

p15-induced redistribution of p27

Immunoprecipitation of cdk2 from Tet-p15 cells and immunoblotting of these complexes with p27 antibodies showed a 3.5-fold increase in cdk2-bound p27 upon shifting the cultures to tetracycline-free medium (Fig. 2B). Although this was a modest increase, previous work using a tetracycline-regulated p27 inducible system in Mv1Lu cells showed that a 3- to 4-fold increase in p27 levels is sufficient to inhibit cdk2-associated kinase activity and cell proliferation (Reynisdóttir et al. 1995). p15 induction in Tet-p15 cells also resulted in an inhibition of cdk2-associated kinase activity, as measured by using either histone H1 or GST-Rb fusion protein as a substrate (Fig. 2B). Cyclin E and cdk2 levels remained unaffected under these conditions, whereas cyclin A levels became undetectable in cells that were growth-arrested by tetracycline removal or TGF- β addition (data not

Figure 2. p15 induction causes dissociation of p27, but not cyclin D, from cdk4 and cdk6. (A) Tet-p15 cells were incubated in medium containing 1 μ g/ml of tetracycline, in tetracycline-free medium, or in medium including both 1 μ g/ml of tetracycline and 100 pM TGF- β . Lysates (5 mg of protein) were immunoprecipitated with cdk4, cdk6, or cyclin D2 antibodies. The total levels of these proteins, and the coprecipitating (bound) proteins were determined by immunoblotting with the corresponding antibodies. The total levels of p27 were examined by Western immunoblotting of lysate (0.2 mg of protein) using a p27 antibody. (B) Tet-p15 cells were incubated with the indicated concentrations of tetracycline. Lysates were immunoprecipitated with a cdk2 antibody and the immunoprecipitates analyzed by immunoblotting with a p27 antibody. The cdk2-bound p27 was quantitated using a densitometer. The cdk2-associated kinase activity was measured by immunoprecipitating lysates with a cdk2-specific antibody or NRS and incubating the immunoprecipitates with [γ - 32 P]ATP and either histone H1 or GST-Rb as substrates.

shown). Loss of cyclin A expression may be secondary to cdk4/6 inhibition and G₁ arrest (Henglein et al. 1994). Thus, p15 induction is sufficient to induce redistribution of p27 from cdk4/6 to cyclin E-cdk2, leading to cdk2 inhibition. We conclude that p15 is a mediator of these effects in TGF- β -treated cells.

Inhibition of p27 binding requires prior occupancy of cdk4 by p15

To further analyze the competition between p15 and p27

for binding to cyclin D-cdk4, we determined whether addition of p15 could dissociate p27 from preformed p27-cyclin D2-cdk4 complexes. Addition of excess p15 (at a molar ratio of p15:p27 up to 16.7:1) to preformed p27-cyclin D2-cdk4 complexes did not cause p27 disso-

Figure 3. p15 prevents p27 binding to cyclin D-cdk4/6. (A) Increasing concentrations of recombinant p15 were incubated for 20 min on ice with insect cell lysates (1 μ l) containing cdk4 and cyclin D2. The resulting complexes were immunoprecipitated with a cdk4 antibody. The kinase activity associated with these precipitates was measured using [γ - 32 P]ATP and GST-Rb fusion protein as substrate, and was quantitated using a PhosphorImager. The cdk4 kinase activity obtained in the absence of p15 is set as 100%. (B) Insect cell lysates (1 μ l) containing recombinant cyclin D2 and cdk4 (lanes 1–8) or cdk4 alone (lane 9) were incubated for 20 min on ice with the indicated concentrations of recombinant p15. Recombinant p27 (790 ng) was then added and the incubation continued for 20 min. (C) Mv1Lu cells (R-1B/L17 subclone) were transiently transfected with pCMV-based vectors encoding cdk4, cdk4-R24C, p27, and p15. Whole-cell extracts were subjected to Western immunoblotting with cdk4-, p27-, and p15-specific antibodies. A cdk4-specific antibody was used to capture the complexes, which were subjected to Western immunoblotting with cyclin D2 (A), p27 (B,C), or p15 (A–C) antibodies. For unknown reasons, the level of expression of cdk4-R24C was lower than that of wild-type cdk4. However, note that the ratio of cdk4 to p27, in lanes 3 and 4, was the same with both cdk4 constructs.

ciation (Fig. 4A). Under our assay conditions, maximal binding of p27 or p15 to cyclin D2-cdk4 was achieved in <1 min of incubation in each case (data not shown). To confirm that prior binding of p15 to cyclin D2-cdk4 is

Figure 4. Prior occupancy of cyclin D-cdk4 by p15 prevents p27 binding. (A) Cyclin D2-cdk4 (lanes 1–6) or cdk4 (lanes 7,8) were incubated for 20 min with no p27 (lane 1) or with a fixed amount of recombinant p27 (90 ng; lanes 2–8). The indicated concentrations of p15 were then added, and incubations continued for 20 min. (B) Cyclin D2-cdk4 (lanes 1–8) was incubated without (lane 1) or with recombinant p15 (200 ng; lanes 2–8) for the indicated time. Then, recombinant p27 (790 ng; lanes 1–8) was added and the incubation continued for an additional 10 min. (C) Cyclin D2-cdk4 was incubated without p15 (lanes 4,9) or with p15 added to the lysate 1 min prior (lanes 1,5), at the same time (lanes 2,6), 1 min after (lanes 3,7), or 5 min after (lane 8) p27 addition. Once p27 or p27(N) was added, the incubation continued for 10 min. (D) Cyclin D2-cdk4 (lanes 1–8) or cdk4 (lane 9) was incubated in the absence (lanes 1–4) or presence (lanes 5–9) of 4 mM ATP and 7 mM MgCl₂ for 10 min. p15 was not added (lanes 4,8,9) or added to the complexes 1 min prior (lanes 1,5), at the same time (lanes 2,6) or 1 min after (lanes 3,7) p27 addition. Once p27 was added the incubation continued for 10 min. (E) Cyclin D2-cdk4 (lanes 1–7) and cdk4 (lane 8) were incubated without (lanes 1,5–8) or with (lanes 2–4) recombinant p15 for 10 min prior to incubation with the indicated concentrations of p27 (lanes 2–8) for 10 min. In all the experiments shown, 1 μ l of insect cell lysate was used. Cdk4 was immunoprecipitated, and the amount of associated p27 or cyclin D2 was determined by Western immunoblotting of these precipitates with p27 or cyclin D2 antibodies.

required for inhibition of p27 binding, p15 was added to cyclin D2-cdk4 0 to 60 sec before p27 addition (Fig. 4B). Remarkably, p15 did not inhibit p27 binding when the two were added simultaneously to cyclin D2-cdk4. Inhibition of p27 binding was significant when p15 was added 5 sec before p27 and nearly complete when added 60 sec before p27 (Fig. 4B). A similar binding pattern was observed with the amino-terminal Cdk inhibitory domain of p27 (Fig. 4C).

The crystal structure of the p27-cyclin A-cdk2 complex reveals that p27 competes for the ATP binding site in cdk2 (Russo et al. 1996). Therefore, we tested whether the interaction between p27 and cyclin D2-cdk4 might be weaker and more susceptible to inhibition by p15 when analyzed in the presence of competing ATP. However, addition of physiological concentrations of ATP and Mg²⁺ did not decrease the ability of p27 to bind cyclin D2-cdk4 in the presence of simultaneously added p15 (Fig. 4D).

The insect cell lysates used in these experiments contain free as well as associated cyclin D2 and cdk4. Addition of p27 increased somewhat (approximately two-fold) the amount of cyclin D2-cdk4 complex recovered from the mixtures at the end of the incubation (Fig. 4E, cf. lanes 5–7 with lane 1). Interestingly, this increase was inhibited by prior addition of p15 (Fig. 4E, cf. lanes 2–4 with lanes 5–7). However, this effect does not alter the interpretation of the above results.

Distinct subcellular localization of p15 and p27

Because p15 can prevent p27 binding to cyclin D-cdk4/6 complexes in vivo and in vitro without dissociating these complexes, and because simultaneous exposure of cyclin D2-cdk4 to p15 and p27 results predominantly in binding of p27, we reasoned that in order to inhibit p27 binding to cyclin D-cdk4/6 complexes in vivo, p15 must have access to these complexes before p27 does. The high turnover of p27-cyclin D-cdk4/6 complexes would then ensure their rapid replacement with cyclin D-cdk4/6-p15. We therefore examined the possibility that p15 might reside in a cellular compartment that contains cdk4 but not p27. The subcellular distribution of p15, p27, and cdk4 was determined by immunostaining of Mv1Lu cell lines expressing these proteins under tetracycline control. After tetracycline removal, specific immunostaining appeared in the cytoplasm and nucleus of Tet-p15 and Tet-Cdk4 cells, whereas Tet-p27 cells stained predominantly in the nucleus (Fig. 5). Because the p15 levels expressed in Tet-p15 cells in the absence of tetracycline are similar to those induced by TGF- β (see Fig. 1C), the presence of p15 in the cytoplasm can be considered physiologically relevant. Previously, cdk4 has been observed throughout the cell associating with p50^{Cdc37} and Hsp90 in the cytoplasm (Stepanova et al. 1996) and forming active complexes with cyclin D, most likely in the nucleus (Baldin et al. 1993).

Similar results were obtained by indirect immunofluorescence of transiently transfected Mv1Lu cells that

Figure 5. Subcellular location of p15, p27, and cdk4 in Tet-induced cells. Tet-p15, Tet-p27, and Tet-cdk4 cells were incubated in tetracycline-free medium (–) or in medium containing 1 μ g/ml tetracycline (+). The cells were stained with antibodies specific for p15, p27, and cdk4, followed by staining with a biotinylated secondary antibody, then by avidin and biotin, and finally by diaminobenzidine.

were used to study various combinations of transfected proteins (Fig. 6). In agreement with previous results (Baldin et al. 1993), cyclin D1 accumulated in the nucleus when transfected alone. When cyclin D1 and cdk4 were cotransfected, both proteins stained predominantly in the nucleus but also in the cytoplasm (Fig. 6). When cotransfected, cdk4 and p15 did not alter each other's stain-

ing pattern (Fig. 6; data not shown). However, when cdk4 was cotransfected with p27, the cdk4 immunostaining became predominantly nuclear. Cotransfection of p15 prevented the p27-induced nuclear accumulation of cdk4. We generated a p27 construct with a carboxy-terminal truncation [p27(1–151)] that eliminates the putative nuclear localization signal starting at amino acid 152 but retains the cyclin and cdk interaction domains (Fig. 7; Polyak et al. 1994b). It stained throughout the cytoplasm and the nucleus, and, furthermore, it did not induce nuclear accumulation of cdk4 (Fig. 6). These results are consistent with the idea that p15 may gain access to cdk4 in the cytoplasm without competition by p27.

Dependence of cdk4 binding on subcellular location

Based on the subcellular distribution patterns, cytoplasmic p15 might be able to prevail over p27 by binding to free cdk4 or to cyclin D–cdk4 in the cytoplasm. To test the role of subcellular localization as a determinant of inhibitor–cdk interactions, we generated various p27 and p15 constructs (Fig. 7), which were transfected into Mv1Lu cells. Overexpression of wild-type p27 or the amino-terminal half of p27, in these cells causes growth inhibition whereas overexpression of the carboxy-terminal half of p27 does not (Luo et al. 1995). Overexpression of p27(1–151) was also growth inhibitory in these cells (data not shown).

The ability of p15, p27, and their derivatives to interact with cotransfected cdk4 in Mv1Lu cells was con-

Figure 6. Subcellular location of p15, p27, p27(1–151), cyclin D, and cdk4. Mv1Lu cells (subclone R-1B/L17) were transfected transiently with vectors encoding p15, p27, p27(1–151), cyclin D1, or cdk4, or cotransfected with these vectors, as indicated. Transfections with the empty vector pCMV5 were used as a negative control. The cells were stained with antibodies against p15, p27, cyclin D1, or cdk4, as indicated, followed by staining with a fluorescein-conjugated anti-rabbit antibody (p15 and cdk4) or fluorescein-conjugated anti-mouse antibody (p27 and cyclin D1).

Localization

C	N
+/-	+++
++	+
+/-	+++
++	++
+	++

Figure 7. Schematic representation of the p27 and p15 constructs. The cyclin-cdk binding domain and the putative bipartite NLS of p27 are indicated. The hatched box represents the SV40 T antigen nuclear localization signal (PKKKRKV) that was added to the carboxyl terminus of p27(1-151) and wild-type p15.

firmed in each case by immunoprecipitation of cdk4 followed by immunoblotting of the precipitates with p15 or p27 antibodies. Although p15 was able to inhibit the binding of cotransfected p27 to cdk4 (see Figs. 3C and 8A), it failed to inhibit the binding of cotransfected p27(1-151) to cdk4 (Fig. 8A). Addition of an SV40 large T antigen nuclear localization signal (NLS) to the carboxyl terminus of p27(1-151) restored the nuclear localization of this protein (Fig. 7) and the ability of p15 to prevail in binding to cdk4 (Fig. 8A). Addition of an SV40 large T antigen nuclear localization signal (NLS) to the carboxyl terminus of p15 forced this protein to reside preferentially in the nucleus (Fig. 7). p15-NLS was able to bind cotransfected cdk4 (Fig. 8B). However, its ability to prevail over p27 was eliminated: A cotransfected p27 strongly diminished the binding of cdk4 to p15-NLS. Furthermore, a cotransfected p27(1-151) completely eliminated cdk4 binding to p15-NLS (Fig. 8B). In contrast to wild-type p15, p15-NLS did not cause G_1 arrest when transfected into R1B/L17 cells (data not shown). Thus, the normal or enforced cytoplasmic localization of either inhibitor provides it with a clear advantage in binding cdk4.

Discussion

The discovery of two families of cdk inhibitors, one specific for cdk4 and cdk6 and the other with a wider spectrum of target cdks, raises questions about the specific role of each family in cell cycle control. To address these questions, we have focused on the extensive changes that occur in the cdk interaction with p15 and p27 during a TGF- β -induced cell cycle arrest in Mv1Lu cells. Here, we describe mechanisms underlying the central features of this TGF- β response, namely, the increased binding of p15 to cdk4 and cdk6, the concomitant loss of cdk4/6-bound p27, and the increase in p27 binding to cdk2 complexes. Furthermore, the present work sheds light on how p15 and p27 affect the interaction of each other with target cdks and the critical role that the subcellular location of the inhibitors plays in this process.

p15 induction recapitulates TGF- β action

In dividing Mv1Lu cells and other cell types, p27 binds predominantly to cyclin D-cdk4 without inhibiting the activity of this complex (Poon et al. 1995; Soos et al.

1996; S. Blain, E. Montalvo, and J. Massagué, in prep.). The increase in p15 levels induced by TGF- β addition to these cells leads to a replacement of active p27-cyclin

Figure 8. The subcellular location of p15 determines whether it prevails over p27 in binding to cdk4. (A,B) R1B/L17 cells were cotransfected transiently with vectors encoding the indicated products. Cleared whole-cell lysates were immunoprecipitated with a cdk4 antibody and these precipitates (bottom panels) or whole cell lysates (top panels) were probed by sequential Western immunoblotting with antibodies against p15, p27, or cdk4. (A) Lanes 7-9 are from an experiment separate from the one shown in lanes 1-6. It included the same controls, which are omitted here because of space constraints.

D-cdk4 complexes with inactive cyclin D-cdk4-p15 complexes (Reynisdóttir et al. 1995). Concomitantly, cyclin E-cdk2 complexes bind p27 and become inhibited, and G₁ progression comes to a halt (Polyak et al. 1994a). It has been proposed that all these changes are mediated by the surge in p15 expression (Reynisdóttir et al. 1995). We have now verified this with the use of a Mv1Lu cell derivative, the Tet-p15 cell line, that can be induced to express levels of p15 similar to those normally reached in response to TGF- β . p15 induction and binding to cdk4/6 in Tet-p15 cells causes a loss of cdk4/6-bound p27, an increase in p27 binding to cdk2, an inhibition of cyclin E-cdk2, a loss of cyclin A expression, and G₁ arrest. Induction of p15, therefore, recapitulates all these antimitogenic effects of TGF- β .

The increase in cdk2-bound p27 observed upon induction of p15 results not from an increase in the total level of p27 but from an elimination of p27 binding to cdk4 and cdk6. In vitro, preincubation of cyclin D-cdk4 with p15 prevents the subsequent binding of p27. Likewise, in Tet-p15 cells or in transiently transfected cells, p15 binding to cyclin D-cdk4 prevents the binding of p27. Cdk4-R24C, which fails to bind p15, does not lose bound p27 under similar conditions. As with TGF- β addition, the artificial induction of p15 leads to a redistribution of p27 from cdk4 to cdk2 complexes. Unable to bind cyclin D-cdk4 when p15 is present, the displaced p27 binds to cyclin E-cdk2, inhibiting this complex. In effect, p15 mobilizes p27 from a cdk4/6 reservoir toward an inhibitory interaction with cdk2.

p15 interaction with cyclin D-cdk4/6: inhibition without dissociation

In vitro, p16^{Ink4a} or p19^{Ink4d} can interact with free cdk4 or cdk6 (for review, see Sherr and Roberts 1995) and, under certain conditions, at least, they can interact with cyclin D-cdk4/6 complexes as well (Hirai et al. 1995). In cell extracts, however, p16 has been found in association with cdk4 but not cyclin D (Serrano et al. 1993; Xiong et al. 1993; Guan et al. 1994; Hannon and Beach 1994; Hirai et al. 1995; Parry et al. 1995). Based on these observations, it has been suggested that Ink4-bound cdk4 may be unable to subsequently associate with cyclin D (Hirai et al. 1995; Parry et al. 1995). Because D cyclins turn over much more rapidly than cdk4 (Matsushime et al. 1992; Bates et al. 1994), this would lead to a rapid depletion of cyclin D-cdk4 complexes when Ink4 inhibitors are expressed. However, in at least one case, ternary complexes between p19-cyclin D-cdk4 were formed that lacked Rb kinase activity (Hirai et al. 1995). Our results argue that p15 also forms inactive p15-cyclin D-cdk4/6 complexes and this form is more prevalent than p15-cdk4/6 complexes in the cell.

The conclusion that p15 inhibits cyclin D-cdk4/6 without causing the dissociation of this complex is supported by the results of adding p15 to preformed cyclin D2-cdk4 complexes in vitro as well as activating p15 expression in Tet-p15 cells. The extent of association of cyclins D1 and D2 with cdk4 or cdk6 in Tet-p15 cells is

the same regardless of the p15 levels in the cell. Levels of p15 that are sufficient to cause a complete inhibition of Rb phosphorylation and G₁ arrest do not decrease the level of cyclin D-cdk4/6 complexes recovered from the cell. Under these conditions, p15 is associated with cyclins D1 and D2 as well as cdk4 and cdk6, as determined by its coimmunoprecipitation with these various proteins. Because p15 does not bind to isolated D cyclins, these results suggest that cdk4 and cdk6 act as a bridge between p15 and the cyclins. Furthermore, because the half-life of cyclin D is short, we infer that p15 binding may allow normal turnover with reassociation of new cyclin D with p15-cdk4. Alternatively, p15 binding might prevent dissociation of cyclin D from cdk4/cdk6.

Mutual exclusion and the advantage of prior occupancy

In vitro, p15 binding to cyclin D2-cdk4 prevents the subsequent binding of p27. By incubating p27-cyclin D2-cdk4 complexes with p15, we have been able to recover quaternary complexes containing both inhibitors (Reynisdóttir and Massagué, unpubl.). However, the yield of such complexes is very low, suggesting that they are unstable. Because p15 binding does not cause dissociation of the cyclin D-cdk4/6 complex in vivo or in vitro, it appears that p15 binding and p27 binding to cdk4 are mutually exclusive.

A basis for this phenomenon is suggested by the crystal structure of a p27-cyclin A-cdk2 complex (Russo et al. 1996). In this structure, p27 contacts cyclin A and cdk2 separately. The cyclin contact is a docking interaction via the LFG sequence motif of p27, which is a set of amino acids that are necessary for the interaction with cyclin A (Russo et al. 1996). The residues forming the LFG-binding pocket in cyclin A are conserved in D cyclins (Jeffrey et al. 1995), so it is likely that p27 contacts cyclin D in a similar way. On cdk2, p27 makes extensive contacts with the small lobe and the catalytic cleft, blocking the kinase. Because a p27-cyclin D-cdk4 complex is active catalytically, the contacts of p27 with cdk4 must be different, or weaker, than those with cdk2, but may also involve the small lobe of the kinase. Missense mutations in this region of cdk4 impair p15 binding without affecting kinase activity (Wölfel et al. 1995). Therefore, p15 and p27 may bind to overlapping regions of cdk4 and block the binding of each other.

The results of the vitro experiments demonstrate that it is not simply the quantity of p27 or p15 that determines which inhibitor will bind to a cyclin D-cdk4 complex, but the order of exposure of cdk4 to the inhibitors. Although we are unable to measure the physiological levels of p15, and are thus unable to compare the levels of p15 and p27 in TGF- β -treated cells, this problem was addressed using in vitro experiments. They revealed that to prevent p27 binding, p15 must have prior access to a cyclin D-cdk4 complex. p27 binding is not prevented by the simultaneous addition of p15, even when p15 is added in a large molar excess over p27, suggesting that

the association rate of p27 with the complex is faster than that of p15. In spite of this, p27 is prevented from binding to cyclin D–cdk4/6 by prior addition of p15. Therefore, once the cyclin D–cdk4–p15 complex has formed in vitro, it appears to be very stable.

Subcellular location determines cdk interaction with inhibitors

The above observations argue that in order to inhibit p27 binding to cyclin D–cdk4 in vivo, p15 must be able to bind this complex before p27 does. If p27 is present in the Tet–p15 cells before induction of p15 occurs, how could p15 prevent the binding of p27 to cyclin D–cdk4? Because p15 can recognize free cdk4 whereas p27 can recognize only cyclin D–cdk4, p15 might prevail over p27 by binding cdk4 before cdk4 binds cyclin D. In this model, the continued turnover of cyclin D would ensure the progressive replacement of p27 by p15. However, this model alone would not explain the present observations. Our evidence suggests that the different subcellular localizations of p15 and p27 play a key role in ensuring the prevalence of p15 over p27. As shown previously by p27 immunostaining in tissue (Lee et al. 1996), p27 in Mv1Lu cells is predominantly nuclear. In contrast, the p15 is distributed throughout the cytoplasm and nucleus. Cdk4 is cytoplasmic and nuclear when transfected alone or with p15, but becomes predominantly nuclear when cotransfected with p27. Cotransfection of p15 prevents both the interaction of p27 with cdk4 and the accumulation of cdk4 staining in the nucleus. Thus, the dominance of p15 over p27 is reflected in the immunostaining pattern of cotransfected cdk4.

The role of subcellular location as a determinant of cdk4 interaction with these inhibitors is illustrated best by the results of experiments in which we forced p15 into the nucleus and p27 into the cytoplasm. A truncated p27 lacking a carboxy-terminal nuclear retention region is located in the cytoplasm and is no longer inhibited by p15 from binding cdk4. Likewise, p15 fused to a heterologous nuclear localization signal no longer inhibits cdk4 binding to p27. That is, whenever p15 and p27 are in the same compartment, p15 fails to inhibit p27 binding to cdk4.

A model suggested by these observations is shown in Figure 9. In this model, proliferating cells expressing p27 contain this protein bound predominantly to cyclin D–cdk4/6 complexes. These complexes are dissociated as a result of the high turnover rate of cyclin D. Cdk4/6 released from these complexes may diffuse to the cytoplasm and reassemble with new cyclin D and, subsequently, with p27 in the nucleus (Fig. 9A). This equilibrium is altered by an increase in p15 levels: The appearance of p15 in the cytoplasm leads to its association with free cdk4 or newly formed cyclin D–cdk4 complexes before these complexes become exposed to p27 in the nucleus. If cyclin D turnover continues, these cyclin D–cdk4/6–p15 complexes may yield cdk4/6–p15 that may remain as such or associate with new cyclin D, providing an additional advantage to p15 over p27. The

Figure 9. Schematic representation of the subcellular location of p15 and p27, and their interaction with the cdk4. (A) In proliferating cells, p27 is found in association with cyclin D–cdk4 complexes, which are active. The p27–cyclin D–cdk4 complexes turn over rapidly as a result of the short half-life of cyclin D. Cdk4 may diffuse to the cytoplasm, where it reassembles with newly synthesized cyclin D and, subsequently, in the nucleus, with p27. p27 is not found in complex with cyclin E–cdk2, which is an active complex. (B) Upon induction of p15 by TGF- β or in an inducible system, there are high levels of p15 in the cytoplasm. As a result of the turnover of p27–cyclin D–cdk4 complexes, p15 interacts with free cdk4 or newly formed cyclin D–cdk4 complexes in the cytoplasm before the complexes encounter p27 in the nucleus. Because the interaction of p15 and p27 with cyclin D–cdk4 is mutually exclusive, the active p27–cyclin D–cdk4 complexes are replaced by inactive cyclin D–cdk4–p15 complexes. The displaced p27 interacts with cyclin E–cdk2 complexes rendering them inactive. As cyclin D levels decline in the arrested cells, the cyclin D–cdk4–p15 complexes give way to binary cdk4–p15 complexes. The combined action of the two inhibitors inactivates the G₁ cdk4 leading to a G₁ growth arrest. The inactive cdk complexes are depicted in black. For simplicity, cyclin D–cdk6 is not depicted in the schematic.

replacement of p27–cyclin D–cdk4/6 complexes with inactive cyclin D–cdk4/6–p15 complexes leads, secondarily, to cyclin E–cdk2 inhibition by the displaced p27 (Fig. 9B). Binary p15–cdk4/6 complexes might form eventually as cyclin D expression declines in the arrested cells. It has been shown previously in mammalian cells that subcellular location may be important in the regulation of cdc2 by the Wee1 kinase and the Cdc25 phosphatase (Heald et al. 1993). In addition, it may play a role for the regulation of cyclin E–cdk2 assembly. Recently it was demonstrated that in fibroblasts growth-arrested by serum starvation cdk2 was distributed in both the cytoplasm and the nucleus, but upon serum stimulation

cdk2 was found exclusively in the nucleus (Bresnahan et al. 1996). The present work suggests that the coordinated regulation of G₁ cdk is dependent on the different subcellular location of the two families of cdk inhibitors.

Materials and methods

Inducible p15 expression system

The human *p15^{Ink4b}* cDNA was amplified by PCR using pET21a(+)-hp15 and two flanking primers. The fragment was cloned into the *Xba*I site of pUHD10-3 hygromycin (Reynisdóttir et al. 1995) and verified by sequencing. The mink lung epithelial cell line Mv1Lu tTA (Reynisdóttir et al. 1995) was transfected with pUHD10-3 hygromycin-hp15 using the lipofectin procedure according to the manufacturer's protocol (GIBCO-BRL). p15 inducible clones were selected as described previously (Reynisdóttir et al. 1995). The p15 inducible clone 14.159 was further subcloned by end dilution, obtaining a cell line that was designated Tet-p15. These cells are routinely maintained in MEM, 10% fetal calf serum (FCS), 1 mg/ml of G418, 0.3 mg/ml of hygromycin, and 1 µg/ml of tetracycline.

Growth inhibition assays were performed as described previously (Laiho et al. 1990), except the cells were incubated in medium containing various concentrations of tetracycline.

Antibodies

Anti-p15 antibody (C-20) was from Santa Cruz Biotechnology. Anti-cyclin D1 antibody was a generous gift from M. Pagano (Mitotix) or from Neomarkers (DCS-6). Anti-cyclin D2 antibodies were from Santa Cruz Biotechnology or Neomarkers. Anti-cdk6 antibody was a generous gift from M. Meyerson and E. Harlow (Meyerson and Harlow 1994) or from Santa Cruz Biotechnology. Anti-Rb antibody (14001) was from Pharmingen. The anti-p27 antibody was a generous gift from A. Koff, Neomarkers, or described previously (Reynisdóttir et al. 1995). The anti-cdk4 antisera were from Santa Cruz Biotechnology, Pharmingen, Upstate Biotechnology, or raised in rabbits using a synthetic murine cdk4 carboxy-terminal peptide (CKRISAFRALQHSYLHKEESDAE; purchased from Chiron). The anti-cdk2 antiserum was raised in rabbits by immunizing rabbits using a synthetic human cdk2 carboxy-terminal peptide (CDVTKPVPHRLRL; purchased from Chiron) according to standard procedures (Harlow and Lane 1988). The whole goat serum was purchased from CAPPEL and the biotinylated goat anti-rabbit secondary antibody and the avidin and biotin were from Vector Laboratories, Inc.

Immunoprecipitation and immunoblotting assays

The Tet-p15 cells were grown to near confluency in the presence of 1 µg/ml of tetracycline and then split 1:3 into medium containing the desired concentration of tetracycline. The cells were incubated for 16–20 hr and then harvested by trypsinization. The cell pellet was lysed according to published procedures (Matsushime et al. 1994). Precleared lysates were immunoprecipitated with the appropriate antibody for 3–16 hr at 4°C. Complexes bound to protein A- or G-Sepharose were washed four times with IP buffer (Matsushime et al. 1994) and separated by SDS-PAGE.

For immunoblot analyses, immunoprecipitates or 0.2 mg of precleared lysate were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon-P; Millipore). The blots were incubated with the primary antibody for 1 hr followed by 1 hr incubation with a secondary antibody. Protein A-conjugated horseradish peroxidase (Sigma) was used as a secondary reagent for immunoprecipitated proteins to avoid binding to immuno-

globulin light chains. The bound antibodies were visualized by enhanced chemiluminescence (Amersham).

Kinase activity assays

Kinase assays were performed using lysates from Tet-p15 cells grown under the conditions outlined above. To measure cdk2 kinase activity, 0.2 mg (for H1 kinase activity) or 0.5 mg (for Rb kinase activity) of lysate protein was immunoprecipitated and analyzed as described (Matsushime et al. 1994) using as substrate 1 mg/ml of histone H1 or the carboxy-terminal part of pRb (GST-Rb fusion: pRb amino acids 773–928). To examine the cdk4/6 Rb kinase activity, whole cell lysates (0.2 mg) were resolved by 6% SDS-PAGE. Western blotting was performed using a pRb-specific antibody that recognizes both hypo- and hyperphosphorylated Rb.

Metabolic labeling

After growing the Tet-p15 cells in medium containing the indicated concentration of tetracycline for 17.5 hr, the cells were incubated for 30 min in methionine-free medium and then for 2 hr in medium containing the indicated tetracycline concentration and 200 µCi/ml of [³⁵S]methionine (Trans-label, ICN). For treatment with TGF-β, the Tet-p15 cells were maintained in medium containing 1 µg/ml of tetracycline and 100 pM TGF-β for 8 hr. They received [³⁵S]methionine for the last 2 hr of incubation. Cells were collected by trypsinization and lysed in hypotonic buffer, as described above, and by forcing the cell suspension through a 27-gauge needle several times. The supernatant was collected after centrifugation for 10 min at 14,000 rpm. In metabolic labeling assays, half of the lysate from a 150-mm dish was used for the cdk6 immunoprecipitations.

In vitro cdk association and kinase assays

Hi5 insect cells (Pharmingen) were infected with a baculovirus encoding cdk4 or coinfecting with baculoviruses encoding cdk4 and cyclin D2. Insect cell extracts were prepared as described (Desai et al. 1992). Recombinant murine p27, human p27(N), and human p15 were expressed and purified as described previously (p15, Reynisdóttir et al. 1995; murine p27, Polyak et al. 1994b; human p27(N), Luo et al. 1995). Cyclin D2-cdk4 insect cell lysate (1 µl) was mixed with either recombinant p15 or recombinant p27 in a 50-µl reaction volume adjusted with modified hypotonic buffer (50 mM HEPES, at pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% Tween 20), and incubated on ice for 20 min, or as indicated. Then, p27 or p15 was added and the incubation continued for additional 10 or 20 min on ice. The volume was brought to 500 µl with modified hypotonic buffer and the complexes were immunoprecipitated with an antibody specific for the carboxyl terminus of cdk4. After binding to protein A-Sepharose and several washes in modified hypotonic buffer, the complexes were analyzed by 15% SDS-PAGE and immunoblotted with antibodies specific for cdk4, cyclin D2, p27, or p15. The kinase activity of these cdk4 immunoprecipitates was determined as described above for cdk2.

Cell transfection and immunofluorescence analysis

The Mv1Lu mink lung epithelial cell subclone R-1B/L17 (Cárcamo et al. 1994) was maintained in MEM, 10% FCS and 0.5 mM histidinol. The cells were seeded at a density of 5×10^5 cells per 60-mm dish. A day later the cells were transfected transiently with the DEAE-dextran transfection method as described previously (Attisano et al. 1993). The cells were transfected with pCMV5 alone or with pCMV5-derived constructs encoding murine p27 (Polyak et al. 1994b), murine p27(1–151), murine p27(1–151)-NLS, human p15 (Reynisdóttir et al. 1995), human p15-NLS,

human cyclin D1, murine cdk4 (Matsushime et al. 1992), and human cdk4-R24C (Wölfel et al. 1995). The p27 DNA construct encoding amino acids 1–151 was generated by PCR and by cloning into the *HindIII* and *BamHI* restriction sites of pCMV5. The NLS of SV40 T antigen was attached to the carboxyl terminus of p27(1–151) and wild-type p15 by PCR and subsequent cloning into pCMV5. The cyclin D1 encoding the *NotI* fragment from pAA13–21 (Xiong et al. 1991) was cloned into the *BamHI* site of pCMV5. The cells were transfected with either a single DNA plasmid or with a combination of p15, p15–NLS, p27, p27(1–151), p27(1–151)–NLS, cdk4, and cdk4–R24C as indicated. Twenty-four hours post-transfection, the cells were trypsinized and seeded into four well chamber slides (Nunc) at a density of 1×10^5 cells per well. The cells were subjected to immunostaining 24 hr later. This procedure was performed at room temperature. After removal of the medium and a wash with $1 \times$ PBS, a large volume of 50:50 methanol/acetone solution was added to the slide chambers for 2 min. Following four washes in $1 \times$ PBS, the cells were incubated in $1 \times$ PBS containing 10% FCS for 20 min. After the blocking step, the cells were washed in PBS and the primary antibody was added. The primary antibodies anti-p15 (1:100), anti-p27 (1:200; Neomarkers), anti-cdk4 (1:150; this work), and anti-cyclin D1 (1:200; DCS6, Neomarkers) were diluted in 200 μ l of 3% bovine serum albumin (BSA)–PBS. After a 60-min incubation and four washes in $1 \times$ PBS, the cells were incubated for 30 min with the secondary antibodies. The goat anti-mouse and goat anti-rabbit fluorescein-conjugated antibodies (Jackson ImmunoResearch Laboratories) were diluted 1:100 in 3% BSA–PBS. Coverslips were added with permount after four washes in $1 \times$ PBS. The cells were analyzed on a Zeiss microscope and the images were recorded on a Kodak Ektachrome 400 film. The slide films were scanned in a Nikon LS-1000 slide scanner and the composite figure (Fig. 6) was generated with the computer program Adobe Photoshop 3.0. Immunoprecipitations from transiently transfected cells were performed as described for Tet–p15 cells.

Immunostaining of Tet cells

The murine CDK4 *HindIII*–*XbaI* encoding cDNA fragment from pRc/RSV (Matsushime et al. 1992) was cloned into the *XbaI* site of pUHD10–3–hygromycin (Reynisdóttir et al. 1995) by blunt-end ligation. Tet–cdk4 cells were generated by transfecting tTA–neo Mv1Lu cells as described for p15 above. Tet–p15, Tet–p27, and Tet–cdk4 cells were split into chamber slides. They were maintained in tetracycline-free medium or tetracycline-containing medium (1 μ g/ml) for 18 hr. The cells were fixed in 10% formalin for 10 min and washed with tap water and PBS before incubation in 0.01% H_2O_2 –PBS for 15 min. After washing with PBS and blocking with 10% goat serum for 30 min, the cells were incubated with the primary antibodies for 1–2 hr [anti-p15 (1:500), anti-p27 (1:10,000, from A. Koff), and anti-cdk4 (1:1000, from Santa Cruz Biotechnology)]. The cells were washed in PBS and incubated for 30 min in biotinylated goat anti-rabbit secondary antibody diluted 1:1000 in PBS. After more washes in PBS, the cells were incubated for 30 min in a 1:25 dilution of avidin and biotin in PBS. The cells were washed in PBS, stained with diaminobenzidine, washed in water, and counterstained with hematoxylin. Finally, coverslips were mounted with permount. The slides were analyzed and pictures taken as described above.

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The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2.

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