Differential Interaction of the Cyclin-dependent Kinase (Cdk) Inhibitor p27Kip1 with Cyclin A-Cdk2 and Cyclin D2-Cdk4*

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Although p27Kip1 has been considered a general inhibitor of G1 and S phase cyclin-dependent kinases, we report that the interaction of p27 with two such kinases, cyclin A-Cdk2 and cyclin D-Cdk4, is different. In Mv1Lu cells containing a p27 inducible system, a 6-fold increase over the basal p27 level completely inhibited Cdk4 and cell cycle progression. In contrast, the same or a larger increase in p27 levels did not inhibit Cdk4 or its homologue Cdk6, despite extensive binding to these kinases. A p27-cyclin A-Cdk2 complex formed in vitro was essentially inactive, whereas a p27-cyclin D2-Cdk4 complex was active as a retinoblastoma kinase and served as a substrate for the Cdk-activating kinase Cak. High concentrations of p27 inhibited cyclin D2-Cdk4, apparently by conversion of active complexes into inactive ones by the binding of additional p27 molecules. In contrast to their differential interaction, cyclin A-Cdk2 and cyclin D2-Cdk4 were similarly inhibited by bound p21Cip1/Waf1. Roles of cyclin A-Cdk2 and cyclin D2-Cdk4 as p27 targets and cyclin D2-Cdk4 as a p27 reservoir may result from the differential ability of bound p27 to inhibit the kinase subunit in these complexes.

Cell cycle transitions are controlled by the action of the cyclin-dependent kinases (Cdk) and their activating subunits, the cyclins (1, 2). In mammalian cells, cyclin D-Cdk4 or -Cdk6, cyclin E-Cdk2, and cyclin A-Cdk2 act sequentially during the G1/S transition and are required for cell cycle progression through this period. Cdk activity is tightly regulated by a combination of mechanisms, including changes in the cyclin or Cdk levels, phosphorylation of positive and negative regulatory sites, and interaction with stchiomolecular inhibitors (3). The latter in particular act as mediators of a wide range of antimitogenic signals. However, their specific functions are still poorly understood.

Two families of stchiomolecular Cdk inhibitors have been described (4). The Ink4 family, which includes p16Ink4a (5), p15Ink4b (6), p18Ink4c (7), and p19Ink4d (8, 9), specifically inhibits cyclin E-Cdk2, and cyclin A-Cdk2 in vitro (14, 15, 33). In vivo, p27 mediates inhibition of cyclin E-Cdk2 in cells that are exposed to transforming growth factor-β, lovastatin, rapamycin, vitamin D3, cell-to-cell contact, or lack of anchorage (26–28, 34–40). p27 may also inhibit cyclin D2-Cdk4, as shown with macrophages containing elevated levels of cAMP (25).

However, the notion that Cdks are equivalent targets of p27 and the concept that inhibitor-Cdk interactions are simply governed by their relative abundance in the cell are challenged by various observations. In vitro, p27 is a more effective inhibitor of cyclin E-Cdk2 than of cyclin D-Cdk4 (14, 15, 33). During periods of proliferation as well as during exit from the cell cycle, it has been observed that p27 shuttles between Cdk4/6 and Cdk2 complexes even though the levels of p27, Cdk2, and Cdk4 may remain constant (28, 37, 41). Furthermore, although biochemical and structural evidence (23) argues that the p27-cyclin A-Cdk2 complex is inactive, p27 immunoprecipitated from proliferating human B cell lymphoma was shown to be associated with retinoblastoma protein (Rb) kinase activity that could be significantly depleted with antibodies against Cdk6 (41). These observations have raised the possibility that in some conditions at least, p27 may interact differently with Cdk2 and Cdk4/6 complexes, with p27 binding not necessarily causing Cdk4/6 inhibition. In the pres-
ent work, we have investigated the idea whether p27-associated Cdk4 or Cdk6 complexes might exist as active kinases in vitro as well as in vivo. Here we report on the existence of p27-cyclin D-Cdk4 complexes that are largely active, whereas similar complexes of p27 with cyclin A-Cdk2 are essentially inactive.

EXPERIMENTAL PROCEDURES

Analysis of Tet-p27 Cells—Mv1Lu cells expressing a tetracycline-regulated p27 expression system (37) were grown to 90% confluency in the presence of 1 μg/ml tetracycline. The culture medium was then switched to media containing different tetracycline concentrations. After 18 h, the cells were lysed in hypotonic buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin) as described previously (42). Cell lysates were immunoprecipitated with the appropriate antibody for 3–16 h at 4 °C. Complexes bound to protein A-Sepharose were washed 4 times with hypotonic buffer (42) and separated by SDS-PAGE. The 1.5 mg of total cell extract protein was used for immunoprecipitation followed by Western analysis. Western blotting with Cdk4 and Cdk6 antibodies, and assayed for Rb kinase activity and Rb kinase activity (Fig. 1, f). Tet-p27 is a derivative of the mink lung epithelial cell line (Mv1Lu) that expresses human p27 under the control of a tetracycline transactivator (37, 48). In media containing a high concentration of tetracycline (1 μg/ml) Tet-p27 cells do not express exogenous p27, and their basal level of endogenous p27 is similar to the level in parental Mv1Lu cells (37). In proliferating Mv1Lu and Tet-p27 cells, p27 is bound to cyclin D-Cdk4 (Fig. 1c) and, to a lesser extent, cyclin D-Cdk6 (Fig. 1d) and cyclin A-Cdk2 complexes (Fig. 1e). By lowering the tetracycline concentration, the p27 level in Tet-p27 cells can be gradually increased up to 25-fold over basal (Fig. 1b), with a concomitant increase in the level of p27-bound Cdk2 or Cdk2-bound p27 (Fig. 1e; Ref. 37), a complete loss of Cdk2-associated histone H1 kinase activity and Rb kinase activity (Fig. 1, f and g), and inhibition of DNA synthesis (Fig. 1e; Ref. 37). A 6-fold increase in p27 levels is sufficient to completely inhibit Cdk2-associated kinase activity on both substrates (Fig. 1, f and g). As cyclin E-Cdk2 levels remain constant during this treatment, the loss of Cdk2 kinase activity is due to its increased association with p27. p27 immunoprecipitates did not contain histone H1 kinase activity even when derived from cells not overexpressing p27 (Fig. 1i), in which the level of Cdk2-associated kinase activity was high. Among G1, Cdkks, Cdk2, Cdk4, and its close isoform Cdk6, all have Rb kinase activity in vitro, whereas only Cdk2 has histone H1 kinase activity (49). This suggested that p27-associated Cdk2 is largely inactive, an observation consistent with previous results (23, 41).

A marked increase in p27 levels led only to a small (less than 2-fold) increase in the amount of p27-bound Cdk4 in the Tet-p27 cells (Fig. 1c), suggesting that most of the cyclin D-Cdk4 present in proliferating Mv1Lu cells is already bound to p27. A larger increase was observed in the level of p27-bound Cdk6 (Fig. 1d), indicating an interesting difference in the ability of Cdk4 and Cdk6 to interact with p27. p27 immunoprecipitated from Tet-p27 cells was associated with Rb kinase activity (Fig. 2).
Lanes 1–4 Cdk4 immunoblotting. Immunoprecipitated with p27 antiserum (experiment, Mv1Lu cell extracts were subjected to four cycles of immunodepletion, followed by kinase assay. 

Rb kinase activity associated with the p27 immunocomplexes was then determined. Due to a lack of suitable antibodies against the mink Rb, the Rb kinase activity in p27 immunocomplexes is due to bound Cdk4 and/or Cdk6.

p27 was associated with Rb kinase activity even when precipitated from cells that contained enough p27 to cause a complete inhibition of Cdk2 (Fig. 1, f and i, lanes 2–7). The presence of Rb kinase activity in p27 complexes from these cells correlated with the presence of hyperphosphorylated Rb protein in the cells, as indicated by the levels of the slow migrating Rb band in Western immunoblotting of cell lysates with Rb antibodies (Fig. 1h). Rb hyperphosphorylation did decrease at the highest p27 concentration, and this correlated with a decrease in p27-associated Rb kinase activity. These results therefore suggest that a large portion of Cdk4 in the exponentially growing cells is bound to p27, that p27-bound Cdk4 or Cdk6 can be active as kinases, and that p27 may not effectively inhibit these kinases in vivo.

Different Susceptibility of Cdk2 and Cdk4 to Inhibition by p27 In Vitro—In an attempt to explain the above described phenomena, we analyzed the ability of p27 to inhibit Cdk2 and Cdk4. We measured the ability of bacterially expressed p27 to inhibit the Rb kinase activity of baculovirally expressed cyclin A-Cdk2 or cyclin D2-Cdk4 in insect cell extracts (46). Both cyclin A-Cdk2 and cyclin D2-Cdk4 extracts contained a similar amount of catalytically inactive complexes, as determined by their ability to be further activated by the addition of exogenous Cak (see below). Rb kinase assays were conducted under conditions of Rb substrate excess, in the linear range of the kinase reaction, and using two different concentrations of the kinases. The use of the higher concentration allowed the visualization of p27- and Cdk-associated complexes by immunoblotting analysis. Although a 100-fold higher p27 concentration range was needed to achieve the same level of inhibition when the 100-fold higher cyclin-Cdk concentration was assayed, the inhibition profile was the same in both cases (Fig. 2a). The p27 inhibition profile was also the same when Cdk immunoprecipitated complexes were assayed (Fig. 3b).

An important qualitative difference between Cdk2 and Cdk4 complexes was observed when the extent of Cdk inhibition by p27 was compared with the extent of Cdk association with p27 (Fig. 2, b and d). With cyclin A-Cdk2, the inhibition of kinase activity was proportional to p27 binding; Cdk2 inhibition and binding to p27 were both half-maximal at similar p27 concentrations. In contrast to this proportionality, p27 did not significantly inhibit Cdk4 until it bound a near-maximal amount of Cdk4 (Fig. 2, a, b, and d). The concentration of p27 required for maximal Cdk4 inhibition was approximately 4-fold higher than the concentration of p27 required for a maximal level of Cdk4 binding. When separately expressed, cyclin D2 and Cdk4 bound to p27 very weakly compared with the cyclin D2-Cdk4 complex (data not shown). Under our experimental conditions, p27 did not promote the assembly of cyclin D2-Cdk4 complexes from free components as reported by others (50), as a constant amount of cyclin D2 was bound to Cdk4 in the presence of increasing amounts of p27 (Fig. 2c). Thus, whereas the cyclin A-Cdk2 complex is inhibited if p27 is bound, p27 can bind to cyclin D2-Cdk4 and not cause significant inhibition.

Different Specific Activity of p27-bound Cdk2 and Cdk4—These results were consistent with the observation made with Tet-p27 cells that once bound, p27 may inhibit cyclin D-Cdk4 less effectively than it inhibits cyclin A-Cdk2. To confirm this phenomenon, we immunoprecipitated p27-cyclin-Cdk complexes.
plexes using anti-p27 antibodies and assayed their Rb kinase activity (Fig. 3). The p27-cyclin D2-Cdk4 complexes had Rb kinase activity (Fig. 3c) when obtained from mixtures that were not fully inhibited by the added p27 (Fig. 3a and b, middle lanes). As the amount of added p27 was increased, so did the amount of p27-bound Cdk2 or Cdk4 at the high concentration range determined by immunoblot analysis of p27 immunoprecipitates with Cdk2 (left) and Cdk4 (right) antisera. c, the amount of Cdk2-bound cyclin A (left) or Cdk4-bound cyclin D2 (right) at the high concentration range in the presence of increasing concentrations of p27 was determined by immunoblot analysis of Cdk2 or Cdk4 immunoprecipitates with cyclin A (left) and cyclin D2 (right) antisera. d, inhibition of cyclin A-Cdk2 and cyclin D2-Cdk4 kinase activity by p27 (Rb phosphorylation) plotted against the amount of p27-bound Cdk2 or Cdk4 detected at different concentrations of p27. Rb phosphorylation data taken from a (closed squares and circles) and densitometric quantitation of the Cdk2 or Cdk4 band in b (bars) are shown in the same panel for comparison. The p27-bound Cdk2 and Cdk4 are expressed as a percent of the maximal p27 association with each respective complex.

**Fig. 2. In vitro inhibition of cyclin A-Cdk2 and cyclin D2-Cdk4 complexes.** a, inhibition of cyclin A-Cdk2 (left) and cyclin D2-Cdk4 (right) Rb kinase activity by p27. The indicated low range (top scale) and high range (bottom scale) of p27 concentrations were added to low or high concentrations of cyclin A-Cdk2 and cyclin D2-Cdk4, respectively, and assayed for phosphorylation of GST-Rb. Radioactivity incorporated into GST-Rb was quantified and plotted as a percentage relative to controls without p27 and is the average of four separate experiments. The lower range of kinase was inhibited at p27 concentrations similar to those previously published (12, 14, 15). The profile of inhibition between the different concentrations of kinase was the same, enabling the higher concentration range to be used in immunoblot analysis of the complexes in b, b, the amount of p27-bound Cdk2 or Cdk4 at the high concentration range was determined by immunoblot analysis of p27 immunoprecipitates with Cdk2 (left) and Cdk4 (right) antisera. c, the amount of Cdk2-bound cyclin A (left) or Cdk4-bound cyclin D2 (right) at the high concentration range in the presence of increasing concentrations of p27 was determined by immunoblot analysis of Cdk2 or Cdk4 immunoprecipitates with cyclin A (left) and cyclin D2 (right) antisera. d, inhibition of cyclin A-Cdk2 and cyclin D2-Cdk4 kinase activity by p27 (Rb phosphorylation) plotted against the amount of p27-bound Cdk2 or Cdk4 detected at different concentrations of p27. Rb phosphorylation data taken from a (closed squares and circles) and densitometric quantitation of the Cdk2 or Cdk4 band in b (bars) are shown in the same panel for comparison. The p27-bound Cdk2 and Cdk4 are expressed as a percent of the maximal p27 association with each respective complex.

Weak kinase activity was also present in the p27-cyclin A-Cdk2 complex as manifested by its ability to phosphorylate not only Rb but also the associated p27 (Fig. 3c). p27 phosphorylation was observed in kinase assays using complexes precipitated with p27 antibodies (Fig. 3c) or with Cdk2 antibodies (data not shown) and required the presence of both cyclin A and Cdk2. Interestingly, cyclin D2-Cdk4 did not phosphorylate p27. p27 phosphorylation by Cdk2 was partially inhibited by a T to A mutation in the Cdk consensus site TPKK present near the C terminus of p27 (14) (data not shown). We did not determine the stoichiometry of the p27 phosphorylation, and thus it may constitute a small percentage of the total bound p27.

The presence of kinase activity in a p27-cyclin A-Cdk2 complex was in discordance with our observation that Cdk2 inhibition is proportional to p27 binding. This discrepancy was resolved by comparing the kinase activity of free and p27-bound cyclin A-Cdk2. When normalized by Western immunoblotting with anti-Cdk2 antibodies, p27-bound cyclin A-Cdk2 was found to be 50-fold less active than free cyclin A-Cdk2, as visualized by different film exposures of the same gel (Fig. 4a, left panel) and data not shown). p27-cyclin A-Cdk2 complexes generated in the presence of 10, 20, or 40 nM p27 all had the same low specific activity (data not shown). The three-dimensional structure of the p27-cyclin A-Cdk2 complex reveals that
with cyclin D2-Cdk4 in vitro. Activity was determined by analyzing Rb kinase activity of the incubation mixtures in solution (a), immunocomplexes after precipitation with Cdk antibodies (b), or after precipitation with p27 antibodies (c). The position of phosphorylated p27 is noted in c. The higher concentrations of kinase and inhibitor were used in these assays.

**FIG. 3.** Rb kinase activity of cyclin A-Cdk2 and cyclin D2-Cdk4 in the presence of p27. Activity was determined by analyzing Rb kinase activity of the incubation mixtures in solution (a), immunocomplexes after precipitation with Cdk antibodies (b), or after precipitation with p27 antibodies (c). The position of phosphorylated p27 is noted in c. The higher concentrations of kinase and inhibitor were used in these assays.

separate regions of p27 interact with the cyclin and the Cdk (23). On the Cdk, p27 interacts with the active site in a manner that is incompatible with catalytic activity. Although p27 could bind to cyclin A without contacting the Cdk2 subunit, the structure argues that Cdk2 inhibition is strongly favored upon p27 binding to the cyclin subunit (23). This is consistent with our result that the p27-cyclin A-Cdk2 complex is largely inactive.

The opposite result was obtained with the p27-cyclin D2-Cdk4 complex. When normalized by Western immunoblotting with anti-cyclin D2 antibodies, this complex retained a level of kinase activity that was comparable to that of free cyclin D2-Cdk4 (Fig. 4a, right panel). This was confirmed by performing a time course of Rb phosphorylation with equal amounts of free or p27-associated cyclin D2-Cdk4 complex (Fig. 4b). To rule out the possibility that the Cdk4 antibody itself was inhibitory, we compared the kinase activity of free and antibody-bound kinases (Fig. 4c). When normalized by Western immunoblotting with anti-cyclin D2 antibodies, antibody-bound cyclin D2-Cdk4 was as active as free cyclin D2-Cdk4. p27 was not dissociating from the cyclin D2-Cdk4 complex during the kinase assay, as analysis of the supernatant following the assay did not reveal the presence of free cyclin D2 or Cdk4 (data not shown). Collectively, these results suggest that p27 binding to cyclin A-Cdk2 results in a 50-fold inhibition of this complex, whereas under similar conditions, p27 can bind to cyclin D2-Cdk4 without causing a discernible decrease in the Rb kinase activity of this complex.

To determine if the ability of p27 to bind to cyclin D2-Cdk4 complexes without causing inhibition could be extended to other Cip/Kip inhibitors, we analyzed p21 in a similar assay (Fig. 4d), using non-inhibitory concentrations of p21 for cyclin A-Cdk2 or cyclin D2-Cdk4, respectively (data not shown). When normalized by Western immunoblotting with anti-Cdk2 antibodies, the p21-cyclin A-Cdk2 complex only retained 25% of the activity observed with the free complex (Fig. 4d, left panel), consistent with previous results (33, 51). Thus, while this complex has kinase activity, it is significantly impaired as compared with the free complex. However, when normalized by Western immunoblotting with anti-cyclin D2 antibodies, p21-bound cyclin D2-Cdk4 was found to be about 30-fold less active than free cyclin D2-Cdk4 (Fig. 4d, right panel). p21 association with cyclin D2-Cdk4 in vitro appears to cause inhibition of this complex at all concentrations and thus is more similar to the p27-cyclin A-Cdk2 interaction. Therefore, the ability of p27 to bind to cyclin D2-Cdk4 in a non-inhibitory fashion does not appear to be a hallmark of all Cip/Kip inhibitors but rather is specific for the p27-cyclin D2-Cdk4 interaction.

**Analysis of Cyclin D2-Cdk4 Complexes Purified by Gel Filtration**—As the above described experiments were performed with insect cell lysates, we wanted to eliminate possible interference of cyclin D2-Cdk4 aggregates or free components in the reactions. To this end, we subjected the lysates to gel filtration chromatography on Superose 12 and analyzed fractions by anti-cyclin D2 immunoblotting, anti-Cdk4 immunoblotting, and Rb kinase assays (Fig. 5, a and b). To determine the elution profile of free cyclin D2 and Cdk4, we separately subjected cyclin D2 and Cdk4 proteins produced by single baculovirus infections to gel filtration. When cyclin D2 was expressed alone, the majority of the protein migrated as an aggregate (>440 kDa) in the void volume (Fig. 5, a and b; void volume of 8.3 ml ended at fraction 16). Less than 10% of the total cyclin D2 eluted at the size expected of free cyclin. On the contrary, Cdk4 eluted at the expected size in fraction 27. Cyclin D2 or Cdk4 expressed alone had no significant Rb kinase activity (data not shown). When derived from coinfected lysate, both cyclin D2 and Cdk4 eluted with the size expected of the binary complex (66 kDa), in fraction 25, and coeluted with peak Rb kinase activity (Fig. 5, a and b). Other proteins were present in this fraction, as judged by SDS-PAGE and Coomassie Blue staining (data not shown). Some monomeric Cdk4 was detected in the coinfected lysates, but this limited amount was separable from the binary complex. Note that all of the cyclin D2 appeared in the fractions containing the binary complex, suggesting that cyclin D2 expression was limiting for cyclin D2-Cdk4 complex formation in our insect cell infection conditions, and suggesting also that cyclin D2 did not form aggregates when bound to Cdk4. Importantly, addition of p27 to the cyclin D2-Cdk4 sample followed by analysis of the resulting p27-cyclin D2-Cdk4 complexes demonstrated that these complexes had Rb kinase activity (Fig. 5c). When normalized by immunoblotting with anti-cyclin D2 antibodies, the p27-cyclin D2-Cdk4 complex formed at low p27 concentrations had a level of kinase activity comparable to that of the cyclin D2-Cdk4 complex (Fig. 5c). This confirmed that the presence of Rb kinase activity in p27-cyclin D2-Cdk4 was intrinsic to this ternary complex and not the result of p27 binding to cyclin D2-Cdk4 aggregates.

**Activation of p27-cyclin D2-Cdk4 by Cak**—In addition to the effect of p27 on activated cyclin-D2-Cdk complexes, it has been reported that p27 binding may prevent activation of these complexes by the kinase Cak (11, 25). To investigate whether p27 binding has this effect on cyclin D2-Cdk4 in vitro, we examined the ability of Cak (cyclin H-Cdk7) (52–54) to activate cyclin D2-Cdk4 in the presence or absence of p27. Baculovirally expressed cyclin D2-Cdk4 and cyclin A-Cdk2 are not fully activated, presumably because of insufficient Cak activity in the insect cells. Cyclin D2-Cdk4 preparations were incubated with no p27, a low, non-inhibitory concentration of p27, or a high, inhibitory concentration of p27 (Fig. 6; refer to Fig. 2). Aliquots of these mixtures were precipitated with anti-Cdk4 or anti-p27.
**p27Kip1 Inhibition of Cdk2 and Cdk4**

|          | Cyclin A-ckd2 | Cyclin D2-ckd4 |
|----------|---------------|---------------|
| **a**<br>Immunoprecipitate | p27 addition | - | - | - |
|          | α-p27 | α-cyc A | α-p27 | α-cdk4 |
| Kinase activity | Rb | - | Rb | - |
| Immunoblot | cdk2 | cyc D2 | | |
|          | Time, min. | 5 | 10 | 20 | 30 |

**b**<br>α-cdk4<br>α-p27<br>Time, min. | 5 | 10 | 20 | 30 |

**c**<br>**Cyclin A-ckd2**<br>cyclin D2-Cdk4<br>Immunoprecipitate | p21 addition | - | - | - |
|          | α-p21 | α-cyc A | α-p21 | α-cdk4 |
| Kinase activity | Rb | - | Rb | - |
| Immunoblot | cdk2 | cyc D2 | | |

**d**<br>**Immunoprecipitate**<br>α-p21<br>α-cyc A<br>α-p21<br>α-cdk4<br>Kinase activity | Rb | - | Rb | - |

**Fig. 4. Kinase activity of free and p27-bound Cdk2 and Cdk4 complexes.** *a*, left panel, an amount of p27-bound cyclin A-Cdk2 (formed with 20 nM p27) was compared with the same amount of the corresponding p27-free cyclin-Cdk complex. The free cyclin A-Cdk2 complexes was isolated by immunoprecipitation of cyclin A. Western immunoblot analysis of the component that was not used for immunoprecipitation (Cdk2) (bottom) served as a measure of the amount of complex present in the kinase reactions (top). *a*, right panel, an amount of p27-bound cyclin D2-Cdk4 (formed with 160 nM p27) was compared with the same amount of the corresponding p27-free cyclin-Cdk complex. The free cyclin D2-Cdk4 complex was isolated by immunoprecipitation. The free cyclin D2-Cdk4 complexes was isolated by immunoprecipitation of cyclin A. Western immunoblot analysis of the component that was not used for immunoprecipitation (cyclin D2) (bottom) served as a measure of the amount of complex present in the kinase reactions (top). *b*, equivalent amounts of free or p27-bound cyclin D2-Cdk4 complexes (normalized by cyclin D2 immunoblotting as in *a*) were subjected to Rb kinase assays for the indicated times. *c*, an amount of Cdk4 antibody-bound cyclin D2-Cdk4 was compared with the same amount of antibody-free cyclin D2-Cdk4 complex. Western immunoblot analysis of cyclin D2 (bottom) served as a measure of the amount of complex present in the kinase reactions (top). *d*, a, left panel, an amount of p21-bound cyclin A-Cdk2 (formed with 20 nM p27) was compared with the same amount of the corresponding p21-free cyclin-Cdk complex. The free cyclin A-Cdk2 complexes were isolated by immunoprecipitation of cyclin A. *d*, right panel, an amount of p21-bound cyclin D2-Cdk4 (formed with 250 nM p21) was compared with the same amount of the corresponding p21-free cyclin-Cdk complex. The free cyclin A-Cdk2 complexes were treated with Cak (data not shown), p27 complexes formed with a high concentration of p27 lacked Rb kinase activity and did not gain activity upon incubation with Cak (Fig. 6), either because this high concentration of p27 prevented activation by Cak or inhibited Cak-activated complexes. However, incubation with a low concentration of p27 did not decrease Cdk4 activation by Cak (Fig. 6, compare top lanes 2 and 5). Furthermore, the Rb kinase activity of the p27 complexes formed under these conditions was also increased 10-fold when these complexes were incubated with Cak (Fig. 6; compare bottom lanes 2 and 5). Thus, p27 can bind to cyclin D2-Cdk4 in a manner that interferes neither with the activation of this complex by Cak nor with the Rb kinase activity of the activated complex.

**Cdk4 Inhibition by Supra-stoichiometric Binding of p27—** Although it appeared that p27-cyclin D2-Cdk4 complexes could be active both *in vivo* and *in vitro*, the above results also suggested that two different types of p27-cyclin D2-Cdk4 complexes can be formed *in vitro*, one having Rb kinase activity and the other, which is obtained at higher p27 concentrations, lacking Rb kinase activity. Therefore, we analyzed the composition of the inactive p27-cyclin D2-Cdk4 complexes formed at high concentrations of p27 *in vitro*. We determined the levels of p27-bound cyclin D2 and Cdk4-bound cyclin D2 and p27 achieved over a range of p27 concentrations added to a fixed amount of cyclin D2 and Cdk4 (Fig. 7a). p27 did not promote assembly of cyclin D2-Cdk4 complexes, as Cdk4 bound cyclin D2 was constant in the presence of increasing p27 concentrations (Fig. 7a, top panel). The level of Cdk4-associated p27 (Fig. 7a, middle panel) continued to increase well after the level of p27-associated cyclin D2 (Fig. 7a, bottom panel) had reached a plateau. This suggested that the same amount of cyclin D2-Cdk4 was binding more p27. The progressive increase in p27 binding to Cdk4 (Fig. 7a, middle) eventually reached a plateau when complete inhibition of Cdk4 was achieved (refer to Fig. 2a).

The simplest interpretation of this phenomenon is that a p27-cyclin D2-Cdk4 complex can become inhibited *in vitro* by binding additional p27 molecules. A similar conclusion was previously reached in studies on the related Cdk inhibitor p21Cip1/Waf1 which can form p21-cyclin A-Cdk2 complexes that retain kinase activity (33, 51). To obtain direct evidence for the binding of multiple p27 molecules to a cyclin D2-Cdk4 complex, we used two recombinant forms of p27, one tagged with the Flag epitope (p27Flag) and the other tagged with hexahistidine (p27His). p27His-cyclin D2-Cdk4 complexes were formed at increasing concentrations of p27His and isolated by binding to metal-agarose beads. The ability of these immobilized complexes to bind additional p27 was then assayed by incubation with a fixed concentration of p27Flag followed by detection by anti-Flag Western immunoblotting (Fig. 7b). p27Flag-Cdk2-Cdk4 complexes formed in the presence of low concentrations of p27Flag were able to bind to p27His, whereas complexes formed at higher concentrations of p27His were not (Fig. 7b). The ability of an additional molecule of p27 to bind to the immunoprecipitated complexes and the immunocomplexes incubated with or without purified Cak. Incubation with Cak increased 10-fold the Rb kinase activity of Cdk4 complexes (Fig. 6; compare top lanes 1 and 4), suggesting that a significant fraction of the original complexes had not undergone activation by Cak. A similar 10-fold increase in Rb kinase activity was seen when cyclin A-Cdk2 complexes were treated with Cak (data not shown). p27 complexes formed with a high concentration of p27 lacked Rb kinase activity and did not gain activity upon incubation with Cak (Fig. 6), either because this high concentration of p27 prevented activation by Cak or inhibited Cak-activated complexes. However, incubation with a low concentration of p27 did not decrease Cdk4 activation by Cak (Fig. 6, compare top lanes 2 and 5). Furthermore, the Rb kinase activity of the p27 complexes formed under these conditions was also increased 10-fold when these complexes were incubated with Cak (Fig. 6; compare bottom lanes 2 and 5). Thus, p27 can bind to cyclin D2-Cdk4 in a manner that interferes neither with the activation of this complex by Cak nor with the Rb kinase activity of the activated complex.
Precipitated complexes only occurred within those complexes that contained submaximal Cdk4 binding (Fig. 7b, lanes 2–4). Immobilized p27His alone did not bind p27Flag (data not shown), indicating that under these conditions, p27Flag binding was mediated by p27His-associated cyclin D2-Cdk4. Thus, although the cyclin A-Cdk2 complex in Tet-p27 cells, in vitro, and in crystallinelographic studies (23) appears to be inhibited by binding of a single p27 molecule, an active p27-cyclin D2-Cdk4 can bind and be inhibited by an additional p27 molecule under our in vitro assay conditions.

FIG. 5. Activity of cyclin D2-Cdk4 complexes purified by gel filtration. a, fractions from gel filtration of Cdk4 alone, cyclin D2 alone, or cyclin D2-Cdk4 from coinfected baculoviral extracts were analyzed by Western immunoblot analysis with Cdk4 or cyclin D2 antibodies. Fractions were assayed for phosphorylation of GST-Rb. b, densitometric quantitation of the Cdk4 or cyclin D2 band in a was plotted as a percent of maximal immunoreactivity. Open circles correspond to immunoreactivity from Cdk4 or cyclin D2 from single baculoviral infections, and closed circles correspond to immunoreactivity detected in fractions from coinfected cyclin D2-Cdk4. Radioactivity incorporated into GST-Rb by cyclin D2-Cdk4 coinfected complexes was quantified and plotted as a percentage of maximal phosphorylation (closed triangles). Molecular mass standards are indicated at the top of the panel. c, left, Rb kinase activity of cyclin D2-Cdk4 complexes partially purified by gel filtration in the presence of p27, as determined by analyzing Rb kinase activity in solution (top) or after precipitation with p27 antibodies (middle). The amount of Cdk4-bound cyclin D2 in the presence of p27 was determined by immunoblot analysis of Cdk4 immunoprecipitates with cyclin D2 antiserum (bottom). Molecular mass standards are indicated at the top of the panel.

FIG. 6. Cdk activation of cyclin D2-Cdk4 complexes. Cyclin D2-Cdk4 insect cell lysates were incubated with the indicated concentrations of p27 and immunoprecipitated with Cdk4 antiserum (top) or p27 antiserum (bottom). The precipitates were incubated with or without Cak (cyclin H-Cdk7) before they were assayed for Rb kinase activity.

The ability of p27 and related proteins to act as Cdk inhibitors and as repressors of cell proliferation is well established. Overexpression of p27 leads to cell cycle arrest (14, 15), and antisense inhibition of p27 expression can prevent quiescence upon growth factor withdrawal (55, 56). p27 levels increase during cell cycle arrest in response to cell-cell contact (34), growth inhibitory agents (25–27), inducers of terminal differentiation (38, 57), loss of anchorage (39, 40), and during neuronal differentiation in vivo (29). Further evidence for a physiological role of p27 as a negative regulator of growth is provided by the phenotype of generalized organomegaly and increased body size observed in p27 null mice (58–60).
precludes catalytic activity (23). In the cell, even a modest increase in p27 levels can completely inhibit all measurable Cdk2 kinase activity (Refs. 37, 41, 61, and present work). Furthermore, a p27-cyclin A-Cdk2 complex produced in vitro is 50-fold less active as an Rb kinase than a cyclin A-Cdk2 complex. These present and previous results therefore support the conclusion that Cdk2 complexes, and the cyclin A-Cdk2 complex in particular, are prime targets for inhibition by p27.

The Cdk inhibitory activity of p27 extends to cyclin D-dependent kinases. Cell cycle entry in macrophages is prevented by cyclic AMP which maintains the high level of p27 present in the quiescent state and inhibits cyclin D-Cdk4 activation by Cak (25). Transient transfection of p27 in U2OS human tumor cells causes inhibition of cotransfected cyclin D1-Cdk4 (50). p27 Cak (25). Transient transfection of p27 in U2OS human tumor cells causes inhibition of cotransfected cyclin D1-Cdk4 (50). p27 can inhibit cyclin D-Cdk prepared from recombinant sources (14, 15, 33, and present work). This notwithstanding, recent reports and the present results provide evidence that in other cell types or under other in vitro conditions, p27 is not an effective inhibitor of cyclin D-Cdk4 even though it binds to this complex. In proliferating mink lung epithelial cells, human keratinocytes (37), mouse fibroblasts (15, 28), rat fibroblasts (61), and Manca B cell lymphoma (41), cyclin D-Cdk4 is associated with a large proportion of the p27 present in the cell. Further evidence comes from the present analysis of the effect of p27 on cyclin D-dependent kinases in the Tet-p27 inducible cell line. In these cells, p27 levels that cause full inhibition of Cdk2 fail to inhibit Cdk4/6. Tet-p27 cells that have been growth-arrested by p27 with full inhibition of Cdk2 still yield high levels of p27-associated Rb kinase activity and contain phosphorylated Rb protein. The p27-associated Rb kinase activity under these conditions is attributable to bound Cdk4 and Cdk6, as determined by Cdk4/6 immunodepletion experiments. The concentration of p27 needed to inhibit cyclin D-Cdk4/6 in Tet-p27 cells is significantly higher than that needed to inhibit Cdk2. Although under certain experimental conditions it may be possible to raise the level of exogenous p27 high enough to inhibit cyclin D-Cdk4, this concentration may not normally be available in the parental cells. We therefore conclude that in these cells p27 normally acts as a cyclin A-Cdk2 inhibitor but not as a cyclin D-Cdk4/6 inhibitor. The latter role may fall on the Ink4 family of selective Cdk4 inhibitors (4, 5, 37, 43).

The results of our experiments using recombinant proteins provide further evidence that p27 interacts with and inhibits cyclin D-Cdk4 and cyclin A-Cdk2 differently. p27-cyclin A-Cdk2 complexes are essentially inactive, whereas p27-cyclin D2-Cdk4 complexes formed at low concentrations of p27 retain Rb kinase activity. Furthermore, the specific activity of these p27-cyclin D2-Cdk4 complexes is similar to that of cyclin D2-Cdk4. As obtained from baculovirally infected insect lysates, cyclin D2-Cdk4 is not fully activated by Cak since a 10-fold further activation is achieved by incubation with Cak in vitro. A similar increase in activity is obtained by incubation of p27-cyclin D2-Cdk4 complexes with Cak, implying that p27-cyclin D2-Cdk4 not only remains active but it also remains susceptible to Cdk4 activation by Cak.

The p27-cyclin A-Cdk2 complex contains residual kinase activity and can phosphorylate the associated p27 at a Cdk consensus site. This activity may result from an incomplete block of the Cdk2 binding site by bound p27. However, while measurable, the level of kinase activity in p27-cyclin A-Cdk2 complexes appears negligible when compared with the activity of free cyclin A-Cdk2. Yet, the underlying phenomenon, incomplete inhibition of a cyclin-Cdk by bound p27, is a prevalent feature in the interaction of p27 with cyclin D2-Cdk4. Higher concentrations of p27 can inhibit cyclin D2-Cdk4 complexes (12, 14, 15, and present work). Under our conditions, this appears to involve the conversion of an active p27-cyclin D2-Cdk4 complex into an inactive one by the binding of additional p27 molecules. A similar conclusion was previously reached in studies on the related Cdk inhibitor p21Cip1/Waf1 which can...
may represent two extremes of the interaction between a Cip/Kip inhibitor and a cyclin-Cdk complex. Each individual interaction of p21, p27, and p57 with various cyclin-Cdks may be characterized by a different balance between the two states represented in Fig. 8. Indeed, p21-cyclin A-Cdk2 complexes are not completely inactive but retain approximately 25% of the activity seen with the free cyclin A-Cdk2 complex, consistent with previous results (33, 51). However, p21 association with cyclin D2-Cdk4 in vitro appears to cause extensive inhibition of this complex at all concentrations, consistent with the in vivo observations reported by others (64, 65) concerning the ability of p21 to inhibit cyclin D-Cdk4 complexes. Thus, the p21-cyclin D2-Cdk4 complex is more similar to the p27-cyclin A-Cdk2 interaction.

Our present results stand in contrast with the previously observed ability of p27 to inhibit cyclin D-Cdk4 in macrophages stimulated with agents that raised cyclic AMP levels (25), in which p27 was able to block Cak activation of cyclin D-Cdk4. One potential explanation for this discrepancy is that in cyclic AMP-stimulated macrophages p27 levels might be sufficiently high to achieve binding of multiple molecules to cyclin D-Cdk4, as observed in our in vitro conditions. Alternatively, an as yet unknown factor might modify the ability of p27 to bind cyclin D-Cdk4 in an inhibitory mode. Such a factor might enhance the ability of bound p27 to block the Cdk4 subunit in a p27-cyclin D-Cdk4 complex. There is precedent for the idea that diverse proteins associate with cyclin D-Cdk4 (42, 66–68).

An implication of the present findings is that, in the cell, the outcome of an interaction between p27 and G1/S cyclin-Cdk complexes will be determined by the distribution of p27 between inhibitable targets such as cyclin A-Cdk2 and a reservoir such as cyclin D-Cdk4, which sequesters p27 while remaining active. The differential interaction of p27 with cyclin D-Cdk4 and cyclin A-Cdk2 may fulfill several purposes. It may help maintain the two types of Cdk complexes in an active state during periods of growth. It may also allow a concerted inhibition of cyclin A-Cdk2 by mobilization of p27 from cyclin D-Cdk4 complexes. This could occur when the cellular levels of cyclin D and/or Cdk4 decline upon mitogen deprivation (69–72), contact inhibition (28, 37), or loss of anchorage in non-transformed cells (39, 73). Likewise, a displacement of p27 from cyclin D-Cdk4 complexes by elevated InK4 inhibitors during a response to transforming growth factor-β (37, 43) or by other mechanisms during cell cycle progression (28) could lead to a coordinate inhibition of cyclin A-Cdk2.

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REFERENCES

1. Hunter, T., and Pines, J. (1994) Cell 79, 573–582
2. Sherr, C. J. (1994) Cell 79, 551–555
3. Morgan, D. O. (1995) Nature 374, 131–134
4. Sherr, C. J., and Roberts, J. M. (1995) Genes Dev. 9, 1149–1163
5. Serrano, M., Hannon, G. J., and Beach, D. (1993) Nature 366, 704–707
6. Hannon, G. J., and Beach, D. (1994) Nature 371, 257–261
7. Guan, K.-L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O’Keefe, C. L., Matera, A. G., and Xiong, Y. (1994) Genes Dev. 8, 2939–2952
8. Chan, F. K. M., Zhang, J., Cheng, L., Shapiro, D. N., and Winoto, A. (1995) Mol. Cell. Biol. 15, 2682–2688
9. Hirai, H., Roussell, M. F., Kato, J.-Y., Ashmun, R. A., and Sherr, C. J. (1995) Mol. Cell. Biol. 15, 2672–2681
10. El-Dahr, S. W., Tóth, V., Velusescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
11. Gu, Y., Turek, C. W., and Morgan, D. O. (1993) Nature 366, 707–710
12. Harper, J. W., Adam, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) Cell 75, 805–816
13. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) Exp. Cell Res. 211, 90–98
14. Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. (1994) Cell 78, 59–66
15. Toyoshima, H., and Hunter, T. (1994) Cell 78, 67–74

p27Kip1 Inhibition of Cdk2 and Cdk4

Fig. 8. Schematic representation of the model for CKI association and inhibition of cyclin A-Cdk2 and cyclin D2-Cdk4 complexes. In the three-dimensional structure of the p27-cyclin A-Cdk2 complex, p27 anchors on the cyclin via the conserved LFG motif and inserts into the ATP binding site of Cdk2 via the FY region (23). For simplicity, only the cyclin-Cdk interaction domain of p27 is depicted. These contacts are schematically represented in State B. Binding of p27 to the cyclin subunit in State C is not sufficient for Cdk inhibition. This state is not favored in the p27-cyclin A-Cdk2 complex but may be prevalent in the p27-cyclin D2-Cdk4 complex. As a result, p27-cyclin A-Cdk2 complexes are essentially inactive, whereas p27-cyclin D2-Cdk4 complexes are largely active. In contrast, p21-cyclin D2-Cdk4 complexes are essentially inactive and would be represented by State B. Inactivation of a p27-cyclin D2-Cdk4 complex at high p27 concentrations involves the binding of additional p27 molecules to cyclin D-Cdk4. Thus, the relative activity of a CKI-cyclin-Cdk complex would be determined by the type of interaction (State B or C) with the respective CKI.

form p21-cyclin A-Cdk2 complexes that retain kinase activity (33, 51). Evidence for the conversion of p27-cyclin D2-Cdk4 complexes from an active to an inactive form is provided by saturation analysis of these complexes and the use of differently tagged p27 molecules. A similar phenomenon has been observed in the interaction of p27 with cyclin D2-Cdk6 in vitro.3

A possible basis for these different interactions is provided by the three-dimensional structure of p27 in complex with cyclin A-Cdk2. This structure suggests that binding of a single molecule of p27 is sufficient for inhibition of Cdk2 kinase activity (23), which is consistent with our results. However, this structure also reveals that p27 has separate binding sites for the cyclin and the Cdk subunits. Binding to the cyclin subunit is primarily a docking interaction that brings the inhibitor to the cyclin-Cdk complex. This interaction is mediated by a p27 sequence (CRNLFG) known as the “LFG motif” and does not affect the Cdk subunit (23). A similar sequence motif is present in certain Cdk substrates and favors the association and phosphorylation of these substrates by Cdk2 (62, 63). In contrast, the contact between p27, via the “FY” motif, and the Cdk2 subunit is a blocking interaction that disrupts the configuration of the active site and occludes the ATP binding site (23). As suggested in the model (Fig. 8), p27 binding to the cyclin subunit may not always lead to an inhibitory contact with the Cdk subunit. Cyclin A-bound p27 may effectively block the Cdk active site (Fig. 8, State B), whereas cyclin B-bound p27 may not (Fig. 8, State C). A single molecule of p27 bound to cyclin D may be sterically hindered from reaching the Cdk4 site, or its access may be blocked by other associating factors. Only at high p27 concentrations might the Cdk4 site be filled by an additional molecule of p27 causing inhibition.

The p27-cyclin A-Cdk2 and p27-cyclin D2-Cdk4 complexes

3 S. W. Blain and J. Massagué, unpublished work.
