Inhibition of Cdk4 Activity Enhances Translation of p27kip1 in Quiescent Rb-negative Cells

Received for publication, July 26, 2002, and in revised form, January 31, 2003
Published, JBC Papers in Press, February 3, 2003, DOI 10.1074/jbc.M207530200

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We show in this work that the inhibition of Cdk4 (6) in Rb−/− 3T3 cells enhances the accumulation of the p27kip1 cyclin-dependent kinase inhibitor when these cells are induced into quiescence. Two different forms of inhibition of Cdk4 (6), namely overexpression of the Cdk4 (6) inhibitor p16 and overexpression of a dominant negative mutant of Cdk4 (Cdk4N158), result in this effect. This suggests that the relevant activity of Cdk4 (6) that has to be inactivated in this setting is its kinase activity. The accumulation of p27kip1 is due to enhanced translation of the protein, mediated by the 3′-untranslated region of the p27 mRNA. Moreover, the cells that overexpress p16ink4a or Cdk4N158 show a delay in G1 when made quiescent and restimulated to proliferate. This delay is overcome by transfection of a plasmid expressing antisense p27kip1, which shows that the accumulation of p27kip1 in these cells is related to their G1 delay. In summary, we report a new functional link between two important cell cycle regulators, Cdk4 and p27kip1, and provide a mechanistic explanation to the previously reported epistatic relations between these two proteins.

Progression through the mammalian cell cycle is controlled by the sequential activation of a series of cell cycle-dependent kinases (Cdks) (1). The Cdks active in G1 include the cyclin E-Cdk2 complex and cyclin D complexes with Cdk4 and Cdk6 (2). The enzymatic activity of a Cdk can be regulated at three levels (3): cyclin binding and activation, subunit phosphorylation, and inhibition by one of a group of small regulatory proteins, called Cdk inhibitors (4).

The cyclin D1-Cdk4 complex plays an important role in oncogenesis. Cyclin D1 and Cdk4 genes are often amplified or overexpressed in many types of cancer (5). Besides, experimental overexpression of cyclin D1 can induce oncogenic transformation both in cultured cells and in transgenic mice (6, 7). The importance of cyclin D1-Cdk4 (and of cyclin D-Cdk4 (6) complexes in general) in tumorigenesis is further demonstrated by the frequent deregulation of the downstream effectors and upstream regulators of the complex (8). One of the targets of cyclin D-Cdk4 (6) is Rb, the product of a known tumor suppressor gene, p16ink4a (referred to hereafter as p16), a regulator of cyclin D-Cdk4 (6), is also an important tumor suppressor gene product. It is noteworthy that very few human tumors have mutations in more than one of these elements. For example, mutations in p16 and Rb seem to be mutually exclusive, with very few tumors carrying mutations in both genes (9). This has been taken as evidence that p16-cyclin D-Cdk4-pRB form part of a biological pathway. Alteration of this path contributes to the oncogenic phenotype of the cancer cell, but once the path is altered, the mutation of another element confers no further selective advantage to the cell bearing it.

Despite the relevance of the cyclin D-Cdk4 (6) complex in tumorigenesis, its role in cell cycle progression is still not clear. This complex can in principle play two different roles in this respect. On one side, it has a kinase activity per se that is known to phosphorylate some substrates, at least pRB and its two related proteins, p107 and p130 (10–13). On the other hand, it can bind to the kinase inhibitors p27 and p21 and sequester them from Cdk2-containing complexes (13, 14). The relative importance of these two activities in cell cycle progression is still not completely settled.

Several experiments support the idea that an important role of cyclin D-Cdk4 (6) in cell cycle progression is to be a titrator of Cdk2 inhibitors. The Cdk4 (6) inhibitor p16 not only can inhibit the Cdk4 and Cdk6 kinase activity; it also disrupts cyclin D-Cdk4 (6) complexes and displaces the p27 bound by these complexes, leaving it free to inhibit Cdk2 (14). This indirect inhibition of Cdk2 is essential for p16-mediated growth arrest (13). A dominant negative mutant of Cdk4, Cdk4N158 (dnCdk4), can inhibit the kinase activity of endogenous Cdk4, but it does not induce displacement of p27 to Cdk2 complexes, and it does not induce growth arrest when overexpressed (13, 15). Moreover, overexpression of cyclin E can override the growth arrest imposed by p16 (16). These results suggest that growth arrest mediated by p16 is due to its inhibition of cyclin E-Cdk2 complexes. Fibroblasts derived from Cdk4 knockout mice show a delay in G1 progression when stimulated to proliferate from quiescence that has also been suggested to be related to the displacement of p27 to Cdk2-containing complexes (17). Furthermore, substitution of the cyclin D1 gene with cyclin E restores the defects caused by cyclin D1 deficiency in mice, showing that if there is cyclin E activity, cyclin D1 is dispensable (18).

On the other hand, some experiments suggest that the kinase activity of cyclin D-Cdk4 (6) plays a role in cell cycle progression, even if it is not essential for the completion of the cell cycle. Specifically, overexpression of dnCdk4 induces a delay in G1 progression in quiescent cells stimulated to proliferate (13), a result that we have reproduced in our system.
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However, the mechanism that mediates this effect has not been studied in detail.

p27kip1 (p27) is a Cdk inhibitor that plays a role in the establishment of quiescence. The levels of p27 are elevated in quiescence (19), although the exact mechanisms that mediate this induction are still not completely understood. The levels of this protein can be regulated in several different ways (19–24). The best studied of them is the modulation of its half-life. Nevertheless, regulation of p27 translation has also been suggested as an important means of p27 induction during quiescence (19).

In this work, we report that inhibition of Cdk4 (6) kinase activity induces accumulation of p27 in quiescent cells and a delay in cell cycle progression through G1 in serum-stimulated cells. p27 accumulation is produced by enhanced translational activity of its mRNA, an effect mediated by the 3′-untranslated region of the p27 mRNA. These results show that the kinase activity of Cdk4 (6) is important for cell cycle progression at least in part because it modulates the translation efficiency of an important cell cycle regulator, p27.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—


cells were stained with propidium iodide, and DNA fluorescence was measured with a Becton Dickinson device. To determine incorporation of 5-bromo-2′-deoxyuridine (BrdUrd), cells plated on coverslips were incubated with BrdUrd for 1 h, fixed, and processed for immunofluorescence according to the manufacturer’s instructions (in situ cell proliferation kit; Roche Molecular Biochemicals).

Cell Cycle Analysis—For flow cytometry analysis of DNA content, cells were stained with propidium iodide, and DNA fluorescence was measured with a Becton Dickinson device. To determine incorporation of 5-bromo-2′-deoxyuridine (BrdU), cells plated on coverslips were incubated with BrdU for 1 h, fixed, and processed for immunofluorescence according to the manufacturer’s instructions (in situ cell proliferation kit; Roche Molecular Biochemicals).

Transfections and Retroviral Infections—For retroviral infections, human p16 or Cdk446156 were cloned into the retroviral vector pBABE-puro by standard techniques. The resulting construct was transfected into Phoenix cells. Retroviral supernatants were obtained and used to infect Rh+/− 3T3 cells. Rh+/− 3T3 cells were selected in 4.5 μg/ml puromycin, and clones were isolated from the resulting populations by limiting dilution. Transient transfections were performed with FuGene 6 Transfection Reagent (Roche Molecular Biosciences) according to the manufacturer’s instructions, using the plasmids pRcCMV-p16, pCMV dncdk4 (15), and pCMVβ (Clontech), together with the SVL series of vectors (27). Cells were harvested in 200 μl of reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were assayed with standard methods. The p27 depletion experiments were performed using the pCEC-ASp27 plasmid (34).

Antibodies—The following antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were used: anti-Cdk4 (sc-280); cyclin D1 (sc-450); cyclin E (sc-481); p107 (sc-318); human p16 (sc-468); Cdk2 (sc-163); p27kip1 (sc-528); Sp1 (sc-59).

Immunoprecipitations and Western Blot Analysis—Cells were lysed in EBC buffer (50 mM Tris, pH 8, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM orthovanadate, aprotinin, 50 mM NaF) and cleared by centrifugation. For immunoprecipitation, equal protein quantities were incubated with appropriate antibodies. Immune complexes were collected with protein G-Sepharose beads (AP Biotech) and washed three times with EBC buffer. Resuspended samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Detection of the immune complexes was performed with a chemiluminescence assay (Tropix) according to the manufacturer’s instructions. For detection of proteins in cell lysates, a 100-μg sample of the total cell lysate was separated on SDS-polyacrylamide gels and processed for Western blotting.

Protein Labeling—Cells were growth-arrested and then preincubated for 1 h in methionine- and cysteine-deficient Dulbecco’s modified Eagle’s medium, pulse-labeled for 90 min with fresh medium containing 1.5 μCi/ml Redivue Promix L-35S (AP Biotech), and then incubated in Dulbecco’s modified Eagle’s medium. At the time points shown, samples of cells were collected and lysed in radioimmune precipitation buffer (in phosphate-buffered saline: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Protein concentrations were determined by Bradford assay (Bio-Rad), and equal amounts of protein extracts were used for protein immunoprecipitation. Samples were resolved by SDS-PAGE (12%), and the gels were transferred to nitrocellulose membranes and exposed.

Kinase Assays—Cyclin E kinase assays were performed as described (28). Cdk4 kinase assays and preparation of substrate glutathione S-transferase-RB were carried out essentially as described by Matsushima et al. (29). In both assays, the phosphorylated substrate was detected initially by autoradiography and quantitated by cutting bands off the gel and measuring their radioactivity.

RNA Analysis—Northern blot was performed by standard techniques using the appropriate cDNA probes. 18 S RNA analysis was performed with an oligonucleotide probe as described (30).

Luciferase, β-galactosidase, and hypoxanthine phosphoribosyltransferase mRNAs were quantitated by reverse transcriptase-PCR analysis, using oligonucleotides derived from the coding sequence of the three genes for the PCR.

RESULTS

We began this work trying to determine whether p16 overexpression had an effect in cell cycle progression in Rh-negative cells, even if this effect was not a complete arrest of the cycle. To this end, we have used Rh+/− 3T3 cells as a model system (25). This is a cell line derived from mice in which the Rh gene has been deleted by genetic targeting (31). Contrary to Rh-negative tumor cells, Rh+/− 3T3 cells show cell cycle regulation similar to normal Rh-positive cells. Specifically, they can be arrested in G0 when serum-deprived and can also be contact-inhibited (25). Therefore, they seem to be a good model to test cell cycle regulation of Rh-negative cells.

Rh+/− 3T3 cells have endogenous p16 (data not shown). Nevertheless, they also have cyclin D1-Cdk4 complexes formed in a cell cycle-dependent fashion and active as a kinase (see Fig. 1A, B and C, first two lanes of each). However, both the amount of cyclin D1-Cdk4 complexes and the activity of Cdk4 as a kinase are clearly lower in Rh+/− 3T3 cells than in NIH3T3 cells, which are known to be functionally p16-negative (data not shown). We infected Rh+/− 3T3 cells with a retrovirus encoding human p16 and isolated individual colonies from the transduced populations. These clones expressed the exogenous p16 at high levels (Fig. 1A). The two clones shown in Fig. 1A were chosen for in-depth study of their cell cycle regulation. These cells had disrupted cyclin D1-Cdk4 complexes compared with parent cells (Fig. 1B), and their Cdk4 activity was not detectable above background levels, defined as the kinase activity of immunoprecipitates in which the anti-Cdk4 antibody had been preincubated in the presence of a Cdk4-blocking peptide (Fig. 1C). We repeated this experiment three times. The average kinase activity of Cdk4 in p16.1 cells was 5.5% that of control Rh−/− 3T3 cells, with an upper 95% confidence interval of 22.2%, whereas the kinase activity of p16.2 cells was 0.67% that of control Rh−/− 3T3 cells, with an upper 95% confidence interval of 4.2%.

The kinase activity of cyclin E-containing complexes in serum-stimulated cells overexpressing p16 was clearly lower than in parent cells, but it was not completely inhibited (Fig. 1D). In p16 overexpressers, p27 was displaced from cyclin D-containing complexes in p16-overexpressing cells (Fig. 1E). We concluded that p16 was acting as described (13, 14), displacing p27 from cyclin D to cyclin E, and this was contributing to the inhibition of cyclin E-dependent kinase activity. Overexpression of p16 also affected the phosphorylation of a cyclin D-Cdk4 target, the Rh-related protein p130 (data not shown), which shows that the inhibition of this complex was functionally significant in vivo as well as in vitro.
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We then studied the effect of p16 overexpression on the cell cycle of proliferating Rb-negative cells. Specifically, we determined the length of G<sub>1</sub> in control and p16 cells. For this, we synchronized cells by nocodazole treatment followed by mitotic shake off and measured DNA synthesis at different times after mitotic release by incorporation of BrdUrd. Fig. 2A shows that the behavior of control and p16 cells was identical. However, the situation was different in cells that had been made quiescent and then stimulated to proliferate. As shown in Fig. 2B, when cells were synchronized using this protocol and analyzed by flow cytometry, those that overexpressed p16 progressed through the cell cycle clearly slower than control cells. In fact, at 23 h after stimulation, between 25 and 30% of control cells had already attained the 4n DNA content characteristic of G<sub>2</sub>M cells, but there was no enrichment of cells with 4n DNA content in cells overexpressing p16, even 26 h after serum stimulation. The same difference was seen when we analyzed another p16-overexpressing colony, compared with a different control (data not shown). We also determined the moment of the beginning of DNA synthesis by measuring BrdUrd incorporation in control and p16 cells, at different times after serum stimulation. We used the two colonies that had been analyzed previously, together with a third colony, and another control (a puromycin-resistant colony from an infection of Rb<sup>−/−</sup> 3T3 cells with empty pBABEpuro). This experiment showed that the delay occurred before S phase entry in p16 cells (Fig. 2C). Furthermore, the effect was evident in the three colonies analyzed, which showed a delay of several h with respect to the two control cell lines (Rb<sup>−/−</sup> 3T3 and puro), despite the slight variability in S phase entry between these two. We concluded that p16 induced a marked delay in cell cycle progression in serum-stimulated, Rb-negative cells.

The delay in cell cycle progression in serum-stimulated cells we observed was very similar to that reported in cells overexpressing a dominant negative Cdk4 mutant or derived from Cdk4<sup>−/−</sup> mouse embryos (13, 17). We wanted to know whether the effect could be reproduced by affecting only the kinase activity of Cdk4. We infected Rb<sup>−/−</sup> cells with Cdk4<sup>N158S</sup>, a dominant negative Cdk4 (dnCdk4) that has been shown to inhibit the kinase activity of Cdk4 but not to affect the distribution of p27 between Cdk4 and Cdk2 complexes (13) and studied the cell cycle of the resulting colonies. Inhibition of Cdk4 kinase activity had the same effect on the cell cycle as overexpression of p16. Cells overexpressing a dominant negative version of the kinase showed a delay in cell cycle progression when serum-stimulated and entered S phase several h later than control cells (Fig. 3).

We then studied the regulation of cell cycle-regulated genes and proteins in cells overexpressing p16 or dnCdk4. The most striking difference we found between these cells and controls related to p27. The levels of this protein were higher in quiescent than in proliferating cells, as has been described (see Fig. 4C). Surprisingly, its levels in quiescence were higher in p16 and dnCdk4 cells than in parental cells (Fig. 4A) or cells that had been transduced with an empty vector (Fig. 4C). Given that the levels of this protein are induced upon entry into quiescence, we determined whether the inhibition of Cdk4 activity influenced the withdrawal of cells from the cell cycle when serum was removed. We measured BrdUrd incorporation by immunofluorescence in cells at different times after serum withdrawal. A slightly higher percentage of parental cells (Rb<sup>−/−</sup> cells) incorporated BrdUrd in the first 12 h of serum deprivation, compared with cells without Cdk4 activity. Nevertheless, there was no significant difference between cells transduced with p16 or dnCdk4 and cells transduced with an empty vector (puro cells). At later times, the differences were no longer evident, and from 48 h onward, there were no cells positive for BrdUrd in any of the populations tested (Fig. 4B). Despite the fact that the differences in parental and transduced cells in serum deprivation were very small, we were concerned about the effects that difference might have on the
regulation of p27. For this reason, we used a puromycin-resistant cell line (puro) as a control in the following experiments. We performed a time course of the induction of the p27 in puro cells and two cell lines overexpressing either dnCdk4 or p16 (Fig. 4C). The induction of p27 with respect to proliferating cells was evident after only 3 h of serum withdrawal in the three cell lines, but there was no appreciable difference between the control and transduced cells until 48 h of serum deprivation had passed. We concluded that the higher levels of p27 in cells overexpressing p16 or dnCdk4 were unlikely to be a consequence of different rates of cell cycle withdrawal upon serum deprivation.

We then began to study the mechanisms that mediate the induction of p27 in p16 and dnCdk4 cell lines. We first determined the levels of the p27 mRNA in quiescent parental and transduced cells. To our surprise, the levels of p27 mRNA were clearly lower in quiescent p16 and dnCdk4 cells than in parental cells (Fig. 5A). We are at present studying the reason for this difference. Nevertheless, it is clear that the high levels of p27 in transduced cells are not a consequence of higher levels of p27 mRNA.

It is known that an important mechanism of regulation of the levels of p27 depends on the modulation of its half-life. We suspected that the effect we were seeing was due to differences in protein stability and determined the half-life of p27 in our cells with a pulse-chase experiment using medium labeled with \([35S]\)methionine and \([35S]\)cysteine (Fig. 5B). p27 was very stable in the three cell lines analyzed, consistent with previous reports on its half-life in serum-deprived cells (23, 32). The experiment clearly showed that the differences in p27 protein levels were not the consequence of its differential stability.

InFig. 2. Overexpression of p16 does not affect cell cycle progression of continuously proliferating Rb-negative cells but delays G1 progression from quiescence. A, lack of effect of p16 in proliferating cells. Rb\(^{−/−}\) 3T3 and colonies p16.1, p16.2, and p16.3, were synchronized by treatment with nocodazole, followed by mitotic shake off. Synchronized cells were pulse-labeled with BrdUrd (BrdU) at the indicated times after replating, and the percentage of BrdUrd incorporating cells was estimated by immunofluorescence. B, cell cycle delay in quiescent cells stimulated to proliferate. Confluent Rb\(^{−/−}\) 3T3 cells and colonies p16.1, p16.2, and p16.3, were serum-deprived and restimulated with serum. The DNA content of individual cells was determined by flow cytometry after staining with propidium iodide at the times poststimulation indicated. C, lengthening of G1 in stimulated quiescent cells. Confluent Rb\(^{−/−}\) 3T3 cells, a colony transfected with an empty vector (puro), and three p16-overexpressing clones (p16.1, p16.2, and p16.3), were serum-deprived and restimulated with serum. Cells were pulse-labeled with BrdUrd at the times poststimulation indicated, and the percentage of BrdUrd-incorporating cells was estimated by immunofluorescence.

In Fig. 3. Overexpression of a dominant negative mutant of Cdk4 delays G1 progression in serum-stimulated Rb-negative cells. Confluent Rb\(^{−/−}\) 3T3 cells and two clones overexpressing Cdk4\(^{N158}\) (dn.1 and dn.2) were serum-deprived and restimulated with serum. Cells were pulse-labeled with BrdUrd (BrdU) at the times poststimulation indicated, and the percentage of BrdUrd-incorporating cells was estimated by immunofluorescence.
fact, the half-life of p27 was longer in puro cells (>12 h), than in either p16.1 cells (half-life of 8 h) or Cdk4dn1 cells (half-life of 7.5 h). Surprisingly, however, the intensity of the band at time 0 was clearly higher in transduced cells than in control cells, which suggested an effect in protein translation. The intensity of p27 labeling after a 90-min incubation in [35S]methionine and [35S]cysteine is also shown in Fig. 5C. The lack of enhancement in the labeling of an unrelated protein, the transcription factor Sp1, shows that the effect is specific to p27.

Translation of p27 has been proposed as an important mechanism of regulation of this protein (22). Using translation reporter plasmids with the untranslated regions of p27 flanking a luciferase reporter gene, Vidal et al. (33) have identified a region in the 3'-untranslated region (UTR) of p27 that responds to extracellular signals and may be at least partially responsible for the accumulation of p27 upon serum deprivation. We have used the assay system they have developed to confirm the effect of the inhibition of Cdk4 on translation of p27. The reporter plasmids we have used express the luciferase gene under the control of the SV40 early promoter, SV40 polyadenylation signal, and the 5'-UTRs (nucleotides 303–466) of the p27 mRNA (5'-plasmid); the 3'-UTR (nucleotides 1063–2404) of p27 mRNA (3'-plasmid); or both UTRs (5',3'-plasmid). We have transfected these plasmids in control cells and cells overexpressing dnCdk4 or p16, together with a β-galactosidase expression vector, and compared the corrected luciferase activity of this plasmid in the different cells to that of a plasmid expressing luciferase under the same promoter but without the
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**DISCUSSION**

In this work, we describe a new link between two important cell cycle regulators. Inhibition of Cdk4 enhances the accumulation of \( p27 \) in quiescence, and this is important in the lengthening of \( G_1 \) after quiescent cells are stimulated to proliferate.

Both p16 and a dominant negative mutant of Cdk4 have the same effect, suggesting that the important activity of Cdk4 (or Cdk6) that has to be inhibited to produce the effect on \( p27 \) is its kinase activity and not the ability of cyclin D-Cdk4 (6) complexes to sequester p27. The kinase activity of Cdk4 had been shown to be important for the phosphorylation of several cell cycle-related substrates, the most relevant of which are pRB and the Rb-related proteins. Despite this, the only effect on cell cycle regulation of the inhibition of Cdk4 kinase activity that had been described is the lengthening of \( G_1 \) in the reentry in the cell cycle after quiescence (13). We propose in this work a mechanism for this effect, namely the enhancement of \( p27 \) accumulation in quiescent cells.

Lowering of the levels of \( p27 \) by antisense technology in cells without Cdk4 activity shortens the length of \( G_1 \) after quiescent cells are stimulated to proliferate, which shows that the enhancement of the accumulation of \( p27 \) in quiescence is related
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![Diagram](https://i.imgur.com/3827.png)

**Fig. 7.** Transfection of antisense p27 reverts the cell cycle delay in p16 and dnCdk4 cells. A, p16.2 cells were cotransfected with CMV-β-galactosidase and pECE or pECE ASp27 (ASp27). 24 h after transfection cells were serum deprived and restimulated to proliferate in the presence of BrdUrd (BrdU). At the times indicated, cells were fixed and stained for β-galactosidase, and BrdUrd. The percentage of β-galactosidase-positive cells that had incorporated BrdUrd at each time point is indicated. The percentage of BrdUrd-positive untransfected cells seen in the same experiment is also shown (p16.2). B, the same experiment as in A was performed in a dnCdk4-expressing cell line (dn.2).

The higher levels of p27 in cells without Cdk4 activity are due to a translational effect mediated by the 3′-UTR of p27 mRNA. In contrast, the levels of p27 mRNA are lower in cells without Cdk4 activity when induced into quiescence, and the half-life of the protein is not prolonged by the inhibition of Cdk4. Translation of p27 mRNA has been proposed to be an important mechanism for the accumulation of the protein in quiescent cells, and the 3′-UTR of the messenger of p27 has also been shown to be regulated by extracellular signals. Specifically, Vidal et al. have identified a region of 300 bp in the 3′-UTR that is responsible of the accumulation of p27 after inhibition of Rho activity (33). It will be interesting to further delineate the region of p27 mRNA modulated by Cdk4 and study the relation between the Rho and the Cdk4 pathways.

The inhibition of Cdk4 kinase activity cannot stimulate the translation of p27 alone. Cells need to be made quiescent for the effect to be evident. At first sight, this might be due to the instability of p27 protein in proliferating cells, which could preclude the accumulation of the protein even if translation was enhanced. Nevertheless, transfection experiments with the translational reporter plasmids show that in proliferating cells, the inhibition of Cdk4 cannot enhance the translation of p27. This suggests that the effect of Cdk4 on the translation of p27 needs a second signal, probably the lack of growth factor signaling characteristic of quiescence. Furthermore, the effect on p27 is only evident after 48 h of serum deprivation, a time when cells do not have any detectable cyclin D-Cdk4 complexes or Cdk4 activity (Fig. 1). In our view, this suggests that the effect of Cdk4 on translation of p27 is indirect and mediated by an as yet unidentified factor or factors with a long half-life. We expect that further analysis of the relation between Cdk4 and p27 will shed some light on this issue.

The activity of cyclin D-Cdk4 complexes had been previously reported to be more important in cells growing at a suboptimal rate than in actively growing cells. For example, embryonic stem cells do not have a detectable Cdk4 activity, and are refractory to p16 expression, when they are actively proliferating. When they are induced to differentiate, the kinase activity of Cdk4 is activated, and they become susceptible to p16 overexpression (38). Furthermore, when all Rb family members are inactivated in embryonic stem cells, there is no consequence while they retain a high proliferative state, but their differentiation is inhibited (39). Likewise, and as stated above, fibroblasts derived from mice in which the Cdk4 gene is inactivated by gene targeting grow normally when continuously proliferating but show a delay in S phase entry when serum-deprived and restimulated to proliferate (17). Our results are consistent with this view of Cdk4 activity being important in nonproliferating cells and provide a possible explanation for the effect. Translation of p27 is modulated by Cdk4 activity in conjunction with growth factor-generated signals, and this influences the regulation of the cell cycle.

Overexpression of p16 has been previously shown to result in a stimulation of translation of another important cell cycle regulator, the Cdk inhibitor p21 (40). There are several differences between that effect and the one reported here. Only p16 has been shown to enhance translation of p21, which suggests that the relevant function of Cdk4 that has to be inactivated in this case is its ability to sequester p27. Besides, the induction of p21 occurred even in proliferating cells and contributed to the p16-mediated growth arrest. Despite these differences, it will be interesting to delineate the regions of p21 mRNA responsible for its translational induction and compare them with those of p27 with an equivalent function.

Previous reports have underscored the relation between cyclin D-Cdk4 complexes and p27. Relevantly, Tsutsui et al. have shown that proliferative defects in *in vitro* cultured *Cd4*−/− mouse embryo fibroblasts can be corrected by deletion of *p27* (17), and both Geng et al. (41) and Tong et al. (42) have reported that deletion of the p27 gene can overcome the developmental abnormalities characteristic of *cyclin D1*−/− mice. These results have been explained in terms of the importance of redistribution of p27 from Cdk4- to Cdk2-containing complexes. Our results provide an alternative explanation. The rescue of the cell cycle effects of a dominant negative Cdk4, a mutant that does not induce redistribution of p27 to Cdk2 complexes, by
antisense p27, suggests that the relevance of this inhibitor as a downstream target of Cdk4 may be related to its accumulation in cells without Cdk4 activity when quiescent, rather than to the redistribution of the existing p27 to Cdk2 complexes.

In summary, we have described a new function of the kinase activity of Cdk4, important for the effects that its inhibition has on the cell cycle regulation of cells and related to the accumulation of the Cdk inhibitor p27 during quiescence. In depth study of the pathway leading from Cdk4 to the translation of p27 will yield important insights into how cells withdraw from the cell cycle when deprived of growth factors.

Acknowledgments—We are grateful to G. Peters, R. Watson, T. Jacks, and A. Koff for materials and reagents, to A. Vidal for helpful discussions, and to C. Pombo and V. Arce for critically reading the paper.

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*J. Biol. Chem.* 2003, 278:12688-12695.
doi: 10.1074/jbc.M207530200 originally published online February 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M207530200

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