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The Proto-oncogene c-*myc* Acts through the Cyclin-dependent Kinase (Cdk) Inhibitor $p27^{Kip1}$ to Facilitate the Activation of Cdk4/6 and Early G_1 Phase Progression*

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Progression through the early G_1 phase of the cell cycle requires mitogenic stimulation, which ultimately leads to the activation of cyclin-dependent kinases 4 and 6 (Cdk4/6). Cdk4/6 activity is promoted by D-type cyclins and opposed by Cdk inhibitor proteins. Loss of c-*myc* proto-oncogene function results in a defect in the activation of Cdk4/6. c-*myc*$^{-/-}$ cells express elevated levels of the Cdk inhibitor $p27^{Kip1}$ and reduced levels of Cdk7, the catalytic subunit of Cdk-activating kinase. We show here that in normal (c-*myc*$^{+/+}$) cells, the majority of cyclin D-Cdk4/6 complexes are assembled with p27 and remain inactive during cell cycle progression; their function is presumably to sequester p27 from Cdk2 complexes. A small fraction of Cdk4/6 protein was found in lower molecular mass catalytically active complexes. Conditional overexpression of p27 in c-*myc*$^{+/+}$ cells caused inhibition of Cdk4/6 activity and elicited defects in G_0-to-S phase progression very similar to those seen in c-*myc*$^{-/-}$ cells. Overexpression of cyclin D1 in c-*myc*$^{-/-}$ cells rescued the defect in Cdk4/6 activity, indicating that the limiting factor is the number of cyclin D-Cdk4/6 complexes. Cdk-activating kinase did not rescue Cdk4/6 activity. We propose that the defect in Cdk4/6 activity in c-*myc*$^{-/-}$ cells is caused by the elevated levels of p27, which convert the low abundance activable cyclin D-Cdk4/6 complexes into unactivable complexes containing higher stoichiometries of p27. These observations establish p27 as a physiologically relevant regulator of cyclin D-Cdk4/6 activity as well as mechanistically a target of c-Myc action and provide a model by which c-Myc influences the early-to-mid G_1 phase transition.

D-type cyclins are the first cyclins to be expressed during the early-to-mid G_1 phase of the cell cycle (1). Cyclin D-Cdk4/6 [1] complexes are believed to mediate a key signaling connection between the extracellular environment and the intrinsic cell cycle clock. Inappropriate activation of cyclin D-Cdk4/6 complexes and the consequent hyperphosphorylation of the retinoblastoma protein (Rb) are common events in a variety of human tumors (2, 3). The activation of cyclin E-Cdk2 complexes has been implicated as the major function of cyclin D-Cdk4/6 complexes (4). The activation process of cyclin E-Cdk2 complexes by cyclin D-Cdk4/6 has been shown to involve both catalytic and stoichiometric mechanisms, *viz.* the phosphorylation of Rb and the sequestration of the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, respectively (1). In normal cells, however, both cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes appear to be required in sequential fashion to elicit the hyperphosphorylation and inactivation of Rb (5).

The expression of the c-*myc* proto-oncogene is closely correlated with proliferation, and removal of growth factors at any point in the cell cycle results in its prompt down-regulation (6, 7). c-*myc* is not expressed in quiescent cells, but is rapidly induced by growth factors (8, 9); and ectopic expression in quiescent cells can elicit entry into S phase (10, 11). Overexpression of c-Myc in growing cells leads to reduced growth factor requirements and a shortened G_1 phase (12), whereas reduced expression causes lengthening of the cell cycle (13). c-Myc has been likened to a cell-autonomous rheostat, with engineered changes in its expression resulting in incremental changes in proliferation largely independent of the outside environment (14).

A striking parallel between the cyclin D and c-Myc pathways is that both act as growth factor sensors by channeling environmental cues to drive the cell cycle engine. However, the manner in which they may be mechanistically connected is not understood. We used gene targeting to eliminate c-*myc* expression in an immortalized rat fibroblast cell line (15). The c-*myc*$^{-/-}$ cells are viable, but display a significant lengthening of both G_1 and G_2, resulting in a 3-fold reduced proliferation rate. Analysis of key cell cycle regulatory components showed that the absence of c-Myc coordinately reduced the activity of all cyclin-Cdk complexes (16). The expression of the p27 protein was elevated 2–3-fold, and the expression of Cdk7 was reduced by a similar factor. During entry of quiescent cells into the cell cycle, the earliest and largest defect was a >10-fold reduction of cyclin D-Cdk4/6 activity, which resulted in a significant delay in the phosphorylation of Rb. Although the expression of Cdk4 is reduced in c-*myc*$^{-/-}$ cells (17), the defect in Cdk4/6 activity during the G_0-to-S phase transition is significantly greater (16). In this study, we report an analysis of the role of p27 and Cdk7 in the regulation of Cdk4/6 activity in the presence and absence of c-Myc.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—TGR-1 is a subclone of the Rat-1 cell line, and HO15.19 is a c-*myc*-null derivative constructed by sequen-

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[1] The abbreviations used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester; tTa, tetracycline-controlled transactivator; CAK, Cdk-activating kinase.

tial gene targeting (15). HO15.19 derivatives expressing ectopic cyclin D1 were constructed by retrovirus vector transduction of full-length murine or human cyclin D1 cDNA (16). Rat1p27 cells express p27 under the control of the tTA (18) and were obtained from Bruno Amati (19). Cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37 °C in an atmosphere of 5% CO_2. Rat1p27 cells were grown in the presence of 2 μg/ml tetracycline to block the expression of p27. Great care was taken that cultures were cycling asynchronously and were in a rapid and exponential phase of growth. Briefly, cells were always split at subconfluent densities (<50%) and at relatively low dilution (1:10 for c-*myc*$^{+/+}$ and 1:4 for c-*myc*$^{-/-}$ cells). Cultures can thus be maintained continuously at densities of 10–50% confluence (to avoid any contact inhibition), and the relatively frequent passaging (every 3–4 days) and media changes maintain a rapid growth rate. This regimen was followed for a minimum of two passages before cells were harvested for biochemical experiments. In G_0 synchronization experiments, Rat1p27 cells were trypsinized and replated in the presence of 2 μg/ml tetracycline (p27-OFF condition) or 0 μg/ml tetracycline (p27-ON condition) 12–16 h prior to serum starvation (0.25% calf serum), which was initiated in 95% confluent cultures and maintained for 48 h. To induce cell cycle reentry, cells were trypsinized and replated at 50% confluence in the presence of 10% calf serum. Tetracycline concentrations were kept constant throughout the starvation and restimulation periods. Flow cytometry was performed as indicated (15). Labeling of cells with CFSE was performed as described (20, 21). Cells were labeled in suspension for brief periods of time (5 min) and then replated under standard exponential phase culture conditions. Cells were cultured for a minimum of one cell cycle (24 h for c-*myc*$^{+/+}$ and 48 h for c-*myc*$^{-/-}$ cells) to allow the clearing of unincorporated dye before the dye dilution experiments were commenced.

Immunoblotting and Antibodies—Samples for immunoblotting were prepared by direct rapid lysis in Laemmli sample buffer and analyzed as described (14, 16, 22). The sources of antibodies were as follows: Pharmingen, Rb (14001A); and Santa Cruz Biotechnology, cyclin D1 (sc-450), cyclin A (sc-596), cyclin B1 (sc-245), Cdk4 (sc-260), Cdk2 (sc-163), Cdk6 (sc-177), and p27 (sc-528 and sc-1641).

Immunoprecipitation Kinase Assays—Kinase assays were performed as described (16, 23). Briefly, cultures at the indicated time points were harvested with trypsin; washed with ice-cold Dulbecco's phosphate-buffered saline; and lysed for 2 h at 4 °C in buffer containing 50 mM HEPES (pH 8), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Tween 20, and protease and phosphatase inhibitors. Protein concentrations were determined with the Bio-Rad protein assay. Cyclin D1 was immunoprecipitated from 500 μg of extract with 1 μg of anti-cyclin D1 antibody and 20 μl of Gammabind G-Sepharose beads (Amersham Biosciences). Cdk2 was immunoprecipitated from 200 μg of extract with 1 μg of anti-Cdk2 antibody and 20 μl of protein A-agarose beads (Sigma). 1 μg of glutathione *S*-transferase-Rb (24) and 2 μg of histone H1 (Roche Molecular Biochemicals) were used as substrates to assay Cdk4 and Cdk2 activities, respectively. Kinase reactions were displayed by SDS-PAGE and analyzed with a PhosphorImager. CAK activation assays of Cdk4 and Cdk2 complexes were performed as described (16).

Size Exclusion Chromatography—TGR-1 or HO15.19 cultures in exponential growth were lysed as described for kinase assays. 1 mg of total protein (300 μl of extract) was chromatographed on a Superdex 200 column (24-ml bed volume) using a fast protein liquid chromatography system (Amersham Biosciences) at a flow rate of 0.5 ml/min. The column was run in lysis buffer and calibrated with gel filtration standards (Bio-Rad). 500-μl fractions were collected; 80 μl of each fraction was analyzed by immunoblotting, and the remainder was immunoprecipitated and assayed for Cdk kinase activity as described above.

RESULTS

The Majority of Cdk4 Is Found in Inactive Complexes in Both c-myc$^{+/+}$ *and c-myc*$^{-/-}$ *Cells*—The composition and activity of cyclin D1-Cdk4 complexes were examined in growing c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells by size exclusion chromatography. Both cultures were cycling asynchronously in a rapid and exponential phase of growth (see "Materials and Methods"). Column fractions were immunoprecipitated with anti-cyclin D1 antibody; assayed for Cdk4 kinase activity; and then immunoblotted with antibody to cyclin D1, Cdk4, or p27 (Fig. 1). In both c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells, the Cdk4 and cyclin D1 proteins coeluted as a broad peak between 50 and 200 kDa. The p27 protein eluted as a sharper peak between 100 and 200 kDa. Cdk4 activity peaked in two fractions between 70 and 100 kDa and was reduced ~3-fold in c-*myc*$^{-/-}$ cells. The elution profile of Cdk6 was the same as that of Cdk4 (data not shown). The Rat-1 cells under study here express very low levels of cyclins D2 and D3 (16), which precluded their analysis.

Densitometric analysis of the immunoblots revealed that in c-*myc*$^{+/+}$ cells, ~80% of the Cdk4 protein was found in 125–200-kDa complexes that were catalytically inactive and comigrated with the peak of the p27 protein. p27 thus appears to be capable of both binding and inhibiting the activity of cyclin D1-Cdk4 complexes. Only a relatively small fraction of the Cdk4 protein (20%) was found in lower molecular mass (70–100 kDa) catalytically active complexes that migrated adjacent to the major peak of the Cdk4, cyclin D1, and p27 proteins. Although the resolution of the columns did not allow us to determine whether the lower molecular mass catalytically active complexes were free of p27 or contained low stoichiometries of p27, several reports in the literature indicate that both p27 and p21 promote the assembly of cyclin D-Cdk4/6 complexes at low stoichiometries without inhibiting the Rb kinase activity, but inhibit the activity at higher stoichiometries (25–27). The c-*myc*$^{-/-}$ elution profile contained clearly elevated levels of p27, and the peak was broader. The levels of both the cyclin D1 and Cdk4 proteins were also slightly elevated in c-*myc*$^{-/-}$ cells despite the fact that the mRNAs are down-regulated (16, 17), suggesting that p27 stabilizes cyclin D1-Cdk4 complexes. This observation is consistent with previous reports that the half-life of the cyclin D1 protein is increased in both p27 and p21 complexes (27–29).

A recent report indicates that c-Myc may affect the frequency of productive cell cycles (30). This raises the possibility that the cultures under examination here may be mixtures of cycling and non-cycling cells and that the observed changes in Cdk4 activity may be caused by variable fractions of cycling cells. To address this issue, we performed a careful analysis of proliferation rates (Fig. 1*B*); the resultant growth curves show that both cultures were kinetically in exponential phase. Only if a constant fraction of cells were leaving the cycle at each division would the bulk culture still give the appearance of exponential kinetics. To further examine whether the cultures were composed of discrete cohorts of cycling and non-cycling cells, we monitored the dilution of the vital dye CFSE for several days under our standard exponential phase growth conditions. CFSE is a fluorescent dye that penetrates cell membranes and is metabolized and trapped within cells. The dye is evenly distributed to daughter cells, so fluorescence intensity decreases by half with each cell division. This method has been widely used in immunology (31) and neurobiology (32) to track cells both *in vitro* and *in vivo* for up to 10 generations. Dye dilution was found to be completely uniform in both c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cell lines (Fig. 1*C*). In this experiment, cohorts of non-cycling (or slowly cycling) cells would be visualized as discrete peaks (or shoulders) at higher fluorescence intensity values. However, the peaks were found to be symmetrical and of the same width in both cultures at all time points. Furthermore, the rate of dye dilution (decrease in fluorescence intensity as a function of time) was completely consistent with the doubling times measured by standard growth curves. We therefore conclude that under our conditions, both c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cultures are uniformly composed of continuously cycling cells.

Because the majority of assembled cyclin D1-Cdk4 complexes in normal (c-*myc*$^{+/+}$) cells appeared to be catalytically inactive, we examined the assembly and activation process during entry into S phase. Quiescent cells were induced to

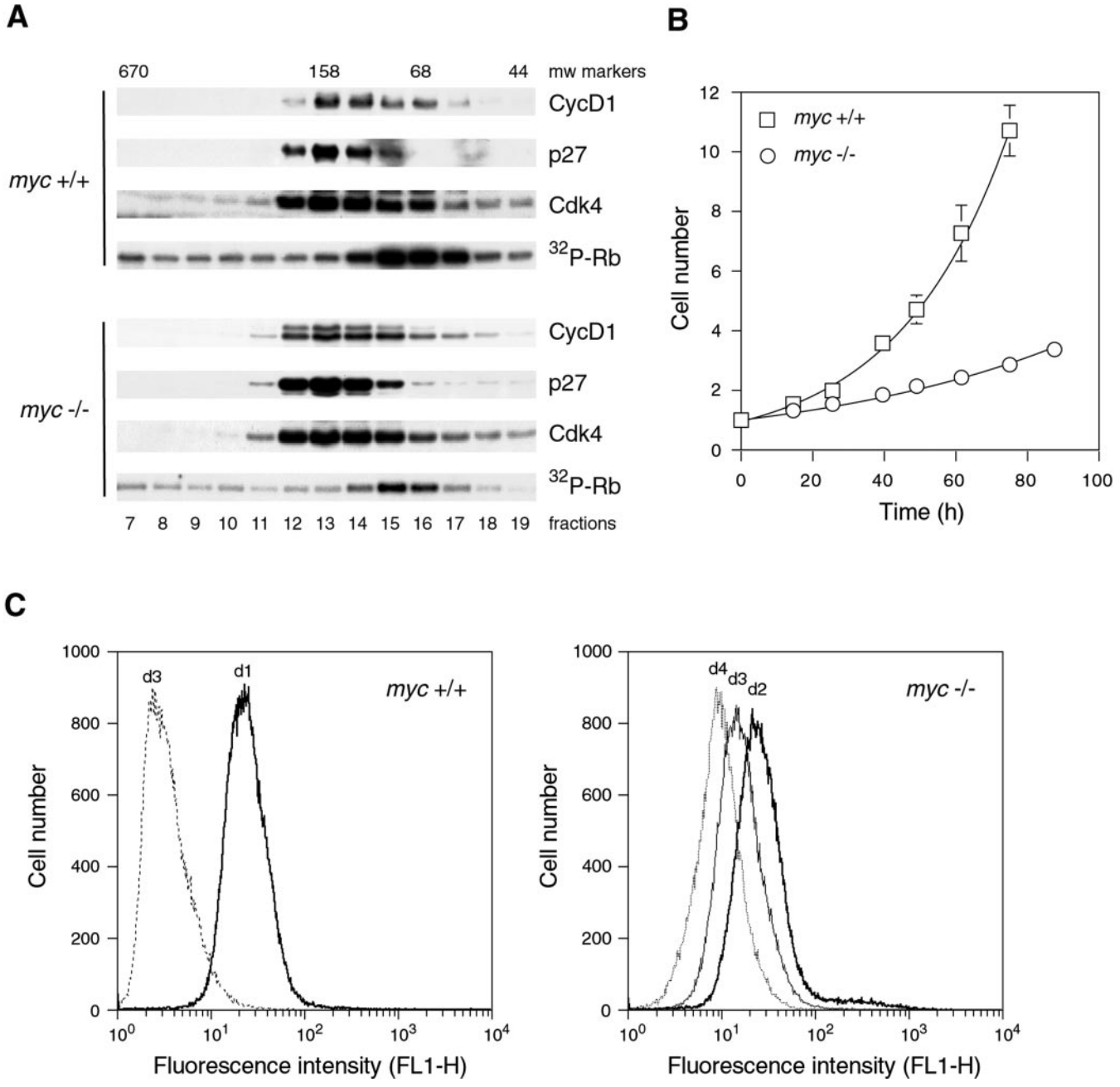

FIG. 1. **Composition and activity of cyclin D-Cdk4 complexes in cycling cells.** *A*, gel filtration profiles. Extracts of exponentially growing c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells were chromatographed on a Superdex 200 column. Cultures were kept continuously in exponential phase growth for a minimum of six doublings prior to harvest. Individual fractions were immunoprecipitated with anti-cyclin D1 antibody and either immunoblotted as indicated or assayed for Rb kinase activity (^{32}*P-Rb*). The experiment was repeated three times with consistent results. *CycD1*, cyclin D1. *B*, proliferation profiles of exponentially cycling cultures. Growth curves were generated on cultures maintained under the conditions described for *A*. Curve fits of the experimental data points yielded exponential functions with R^2 values of 0.997 and 0.975 for c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells, respectively. Doubling times calculated from these functions were 20.5 and 52 h for c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells, respectively. *C*, CFSE dye dilution profiles of exponentially cycling cultures. Cultures maintained in exponential phase under the conditions described for *A* were labeled with CFSE and replated at 20–30% confluent densities. Dye dilution was monitored on successive days (*d*) using flow cytometry. The mean intensity values of the peaks (in arbitrary units) were as follows: for c-*myc*$^{+/+}$ cells, 22 (day 1) and 3 (day 3); and for c-*myc*$^{-/-}$ cells, 21 (day 2), 14 (day 3), and 9 (day 4). The calculated dye dilution half-times for these values are 16 h (c-*myc*$^{+/+}$ cells) and 40 h (c-*myc*$^{-/-}$ cells).

enter the cell cycle; and samples were collected at 2-h intervals, immunoprecipitated with anti-cyclin D1 antibody, assayed for Rb kinase activity, and then immunoblotted with antibodies to the known components of the complexes (Fig. 2). The cyclin D1, Cdk4, and p27 proteins appeared abruptly and concomitantly in the immunoprecipitates at the 6-h time point. This time coincides with the induction of cyclin D1 mRNA expression (16). The proliferating cell nuclear antigen and Cdk6 proteins appeared in the cyclin D1 immunoprecipitates with kinetics that followed closely those of Cdk4 and p27 (data not shown). In contrast, the appearance of Rb kinase activity was significantly delayed and was not fully induced until the 12-h time point in c-*myc*$^{+/+}$ cells (Fig. 2*A*, *left panel*). Examination of c-*myc*$^{-/-}$ cells revealed a very similar profile, except that the abundance of all the components was somewhat increased and that the Rb kinase activity was greatly reduced (Fig. 2*B*, *left panel*). A densitometric quantification of the immunoblots of cyclin D1, Cdk4, and p27 revealed that all proteins were present in the immunoprecipitates at low basal levels in quiescent cells and remained relatively constant at the 2- and 4-h time points (Fig.

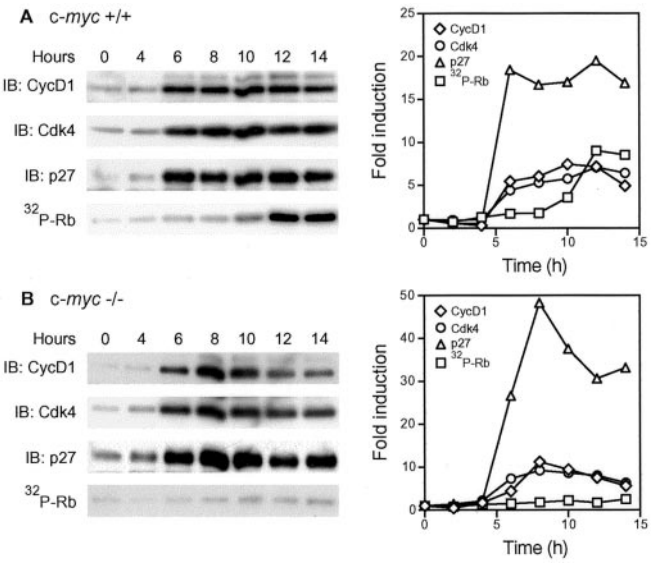

FIG. 2. **Assembly and activation of cyclin D1-Cdk4 complexes during G_0-to-S phase progression.** *A*, c-*myc*$^{+/+}$ cells; *B*, c-*myc*$^{-/-}$ cells. Cell cycle entry of quiescent cells was initiated at 0 h, and samples were collected at the indicated time points. *Left panels*, extracts were immunoprecipitated with anti-cyclin D1 antibody and either immunoblotted (*IB*) as indicated or assayed for Rb kinase activity (*^{32}P-Rb*). *Right panels*, the data in the *left panels* were subjected to densitometric analysis. The experiment was repeated twice with consistent results. *CycD1*, cyclin D1.

2, *A* and *B*, *right panels*). The maximum induction ratios were as follows: cyclin D1, 7-fold in c-*myc*$^{+/+}$ cells and 11-fold in c-*myc*$^{-/-}$ cells; Cdk4, 6-fold in c-*myc*$^{+/+}$ cells and 9-fold in c-*myc*$^{-/-}$ cells; and p27, 19-fold in c-*myc*$^{+/+}$ cells and 48-fold in c-*myc*$^{-/-}$ cells. Gel filtration chromatography of extracts collected at the time of peak activity (12–14 h) revealed profiles very similar to those shown in Fig. 1 (data not shown). c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells express equivalent amounts of the Cdk inhibitors p15^{INK4b} and p16^{INK4a} (19), and neither express detectable levels of p18^{INK4c} and p19^{INK4d} (data not shown). These results are consistent with the hypothesis that the majority of the newly synthesized cyclin D1 protein is rapidly assembled with Cdk4/6 and multiple molecules of p27. Of particular interest is the observation that complexes are apparently fully assembled at early times, but not activated until much later. The fact that the complexes are eventually activated without significant changes in overall stoichiometry further corroborates the interpretation that only a small fraction of total complexes become catalytically active.

Conditional Overexpression of p27 Results in a Phenotype Resembling Loss of c-Myc—The observation that the majority of cyclin D1-Cdk4/6 complexes appear to be bound by multiple molecules of p27 and are not activated even in c-*myc*$^{+/+}$ cells predicts that increasing the p27 pool will inhibit the remaining active complexes. Transient overexpression of p27 has been shown to cause G_1 arrest (33). A stable Rat-1-derived cell line, Rat1p27 (19), in which p27 expression is controlled by the tTA tetracycline-controlled transactivator (18), proliferates normally in the presence of 2 μg/ml tetracycline. Withdrawal of tetracycline, which induces p27 expression, reduced proliferation ~2-fold (data not shown). To analyze the consequences of elevated p27 expression during progression from G_0 to S phase, it was necessary to prepare quiescent cells with increased levels of p27. This was accomplished by removing tetracycline from the medium 12–16 h prior to as well as during a standard 48-h serum deprivation period. Cells thus treated were quiescent and displayed maximum p27 induction. Cell cycle re-entry was elicited by serum stimulation in the continued absence of tetracycline. Control cultures were treated identically, but were supplemented with 2 μg/ml tetracycline throughout the regimen to keep the p27 transgene inactive.

Analysis of cell cycle progression by flow cytometry (Fig. 3, *A* and *B*) showed normal S phase entry in the presence of tetracycline (p27-OFF) at 12 h after serum stimulation, the same as in parental Rat-1 cells. In the absence of tetracycline (p27-ON), the S phase peaks were significantly diminished as well as delayed to 16–18 h. This profile was similar to that seen in c-*myc*$^{-/-}$ cells, which began to enter S phase at 20–22 h. As previously reported (15), bromodeoxyuridine labeling analyses showed that the entire cell population was delayed in S phase entry and that entry was spread over a longer time period, rather than a portion of the culture failing to enter the cell cycle. Immunoblot analysis of Rat1p27 cells showed clearly elevated levels of the p27 protein in quiescent cells in the absence of tetracycline (p27-ON), but not in its presence (p27-OFF) (Fig. 3*C*). The elevated levels of p27 under the p27-ON condition persisted for >24 h after serum stimulation, but were reduced at later times, which may account for the leakiness of the block and continued proliferation in the absence of tetracycline, albeit at reduced rates. In agreement with the previous results, the levels of cyclin D1 were also noticeably increased in the presence of elevated p27.

Given that increased p27 expression impeded S phase entry, we investigated the extent to which the molecular landmarks of this transition resembled those seen in c-*myc*$^{-/-}$ cells. The absence of c-Myc during the G_0-to-S phase transition results in significantly reduced (>10-fold) Cdk4/6 activity, delayed phosphorylation of Rb, delayed and dampened activation of Cdk2, and delayed induction of cyclin A (16). Under the p27-OFF condition, the onset of Rb phosphorylation was first evident at 4 h, whereas under the p27-ON condition, significant Rb phosphorylation was minimum even at the 12-h time point (Fig. 3*C*). 24 h after serum stimulation, Rb phosphorylation was still appreciably defective. In comparison, Rb phosphorylation is first detectable at 8 h in c-*myc*$^{-/-}$ cells and at 6 h in parental c-*myc*$^{+/+}$ cells (16). Rb phosphorylation thus appears to be even more defective in the presence of excess p27 than in the absence of c-Myc.

The composition and activity of Cdk4 complexes were examined during the G_0-to-S phase transition under the p27-OFF and p27-ON conditions and compared with the total expression of the constituent proteins. Immunoblot analysis (Fig. 4*A*) showed that total cyclin D1 levels were highest early in the transition (6 h) and declined at later times (18 and 30 h) under the p27-OFF condition; in the p27-ON cells, cyclin D1 levels were elevated throughout and peaked at much later times (18 h). Cdk4 and Cdk2 levels were constant throughout the time course under both conditions. Cdk2 showed a shift to higher mobility indicative of CAK phosphorylation at 18 h in p27-OFF cells, but not until 30 h in p27-ON cells. This result is consistent with observations that p27 can antagonize the phosphorylation of Cdk complexes by CAK (34, 35). Immunoprecipitation with anti-cyclin D1 antibody (Fig. 4*B*) from extracts of p27-OFF cells showed the expected induction of cyclin D1 early in the transition (6 h), and both the Cdk4 and p27 proteins were efficiently co-immunoprecipitated at this time. Cdk4 activity was strongly induced at later times. Under the p27-ON condition, cyclin D1-Cdk4 complexes were more abundant, were present at high levels at much later times, and at all times contained high levels of the p27 protein. The activity of the complexes was low throughout, although a weak induction was evident at 18 h. The defect in Cdk4 activation in the p27-ON cells was ~15-fold in this experiment.

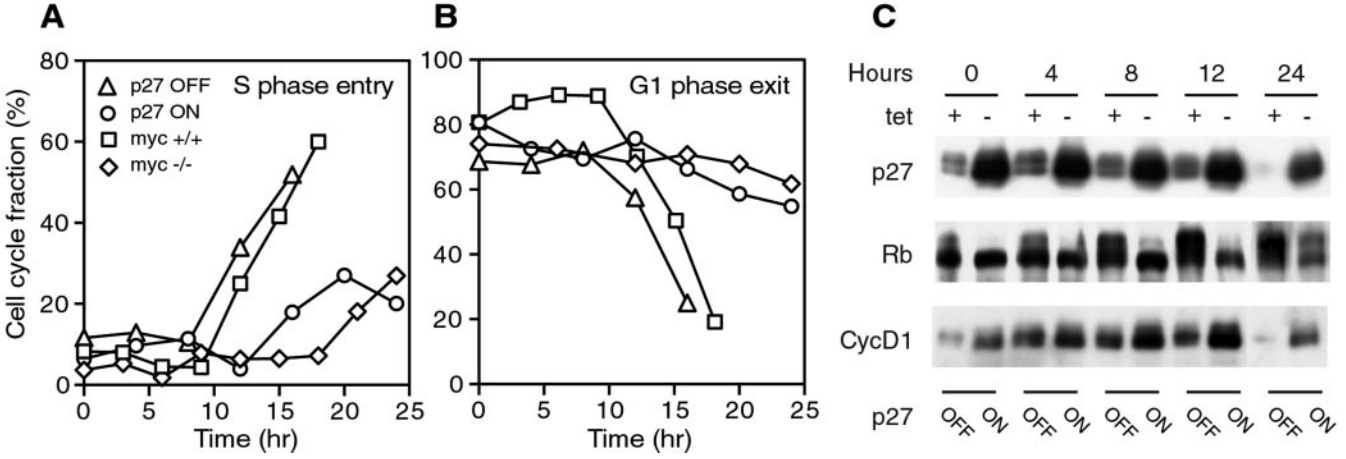

FIG. 3. **Conditional expression of p27 delays S phase entry and Rb phosphorylation.** *A* and *B*, cell cycle entry of quiescent cells was initiated at 0 h, and samples were collected at the indicated time points and analyzed by flow cytometry. *C*, Rat1p27 cells were treated as indicated in *A* and *B*, and samples were analyzed by immunoblotting. *tet*, tetracycline; *CycD1*, cyclin D1.

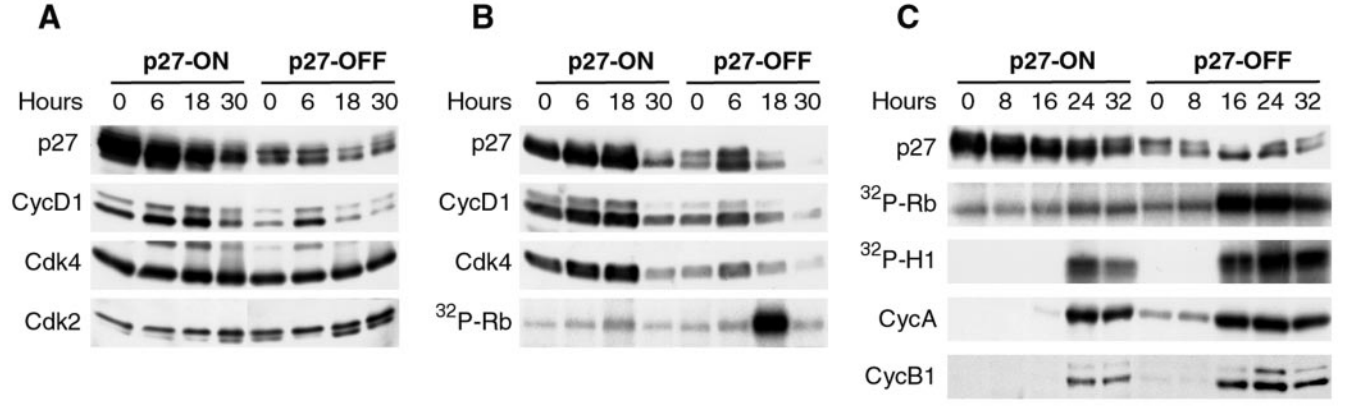

FIG. 4. **Composition and activity of Cdk4 and Cdk2 complexes under p27-ON and p27-OFF conditions.** Cell cycle entry of quiescent cells was initiated at 0 h, and samples were collected at the indicated time points. *A*, shown are the results from immunoblot analysis. *CycD1*, cyclin D1. *B*, extracts were immunoprecipitated with anti-cyclin D1 antibody and either immunoblotted as indicated or assayed for Rb kinase activity (*^{32}P-Rb*). *C*, Cdk4 and Cdk2 activities were assayed in cyclin D1 immunoprecipitates (*^{32}P-Rb*) and Cdk2 immunoprecipitates (*^{32}-H1*), respectively. The expression of p27, cyclin A (*CycA*), and cyclin B1 (*CycB1*) was determined by immunoblotting.

A comparison of Cdk4 and Cdk2 activities and the induction of cyclins A and B1 were monitored in a separate experiment (Fig. 4*C*). In p27-OFF cells, strong induction of both Cdk4 and Cdk6 activities was detected at 16 h, whereas neither activity was apparent in p27-ON cells until 24 h. The defect in activation in p27-ON cells was 5–7-fold for both Cdk4 and Cdk2; however, Cdk4 activity was barely elevated above the background level in p27-ON cells, whereas Cdk2 activity was substantially induced. Thus, the defect in activation is more pronounced for Cdk4 than for Cdk2. Both cyclins A and B1 were detected at 16 h in p27-OFF cells, but not until 24 h in p27-ON cells. In summary, elevated levels of p27 cause a significant delay in the G_0-to-S phase transition that is characterized by a strong defect in Cdk4 activation and Rb phosphorylation and a delay in the activation of Cdk2 and the expression of cyclins A and B1. These molecular phenotypes are very similar to those caused by the absence of c-Myc (16).

Cyclin D1 (but Not Cdk7) Rescues Cdk4 Activity in c-myc$^{-/-}$ Cells—The hypothesis that increased p27 expression contributes to the Cdk4 defect seen in c-*myc*$^{-/-}$ cells by binding to the small fraction of potentially activable cyclin D1-Cdk4/6 complexes and converting them to unactivable complexes containing higher stoichiometries of p27 predicts that increasing the pool of cyclin D will rescue Cdk4 activity by allowing the assembly of additional complexes. To test this prediction, we constructed numerous clonal cell lines ectopically expressing a murine or human cyclin D1 transgene (16). All cell lines were screened by immunoblotting for expression of the exogenous cyclin D1 protein, and clones with a moderate level of overexpression were chosen. Kinase assays of two representative clones showed that the increase in cyclin D1 expression completely rescued Cdk4 activity, but did not rescue Cdk2 activity (Fig. 5*A*).

Cdk7 is expressed at reduced levels in c-*myc*$^{-/-}$ cells (16). Because cyclin D1-Cdk4/6 complexes in c-*myc*$^{-/-}$ cells are assembled at essentially normal levels in early G_1, but subsequently fail to be activated, we investigated whether phosphorylation by CAK may be limiting. Cyclin D1-Cdk4/6 complexes were immunoprecipitated from c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells at successive times during the G_0-to-S phase transition, incubated with active recombinant CAK, and subsequently assayed for Rb kinase activity (Fig. 5*B*). CAK did not increase the activity of cyclin D1-Cdk4/6 complexes at any time point in either c-*myc*$^{+/+}$ or c-*myc*$^{-/-}$ cells. The CAK preparation was catalytically active because it strongly activated purified recombinant cyclin A-Cdk2 complexes under the same assay conditions. CAK also activates native Cdk2 complexes immunoprecipitated from Rat-1 cells (16). We also ectopically expressed Cdk7 in c-*myc*$^{-/-}$ cells using the same strategy as that used for cyclin D1. Stable clonal cell lines were isolated, and restoration of Cdk7 expression levels to those seen in c-*myc*$^{+/+}$ cells was demonstrated by immunoblotting. However, in this case, nei-

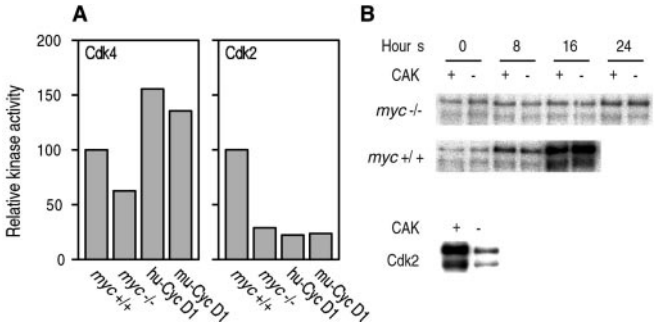

FIG. 5. **Effect of cyclin D1 and CAK on Cdk4 activity.** *A*, the indicated cell lines were grown exponentially, and extracts were assayed for Cdk4 and Cdk2 activities as described in the legend to Fig. 4*C*. The cell line *hu-CycD1* (where *hu* is human, and *CycD1* is cyclin D1) was previously referred to as HO15D2 (16). The cell line *mu-CycD1* (where *mu* is murine) was constructed in an analogous fashion. The experiment was repeated twice with consistent results. *B*, cell cycle entry of quiescent cells was initiated at 0 h; samples were collected at the indicated time points; and extracts were immunoprecipitated with anti-cyclin D1 antibody, incubated in the presence or absence of catalytically active CAK, and subsequently assayed for Rb kinase activity. *Lower panel*, activation of recombinant cyclin A-Cdk2 assayed by histone H1 kinase activity.

ther the growth rate nor Rb phosphorylation was rescued (data not shown).

DISCUSSION

During the entry of quiescent cells into the cell cycle, a transition that is acutely dependent on strong and sustained mitogenic signaling, the earliest and largest defect in c-*myc*$^{-/-}$ cells is a >10-fold reduction of cyclin D-Cdk4/6 activity. The magnitude of the activity defect cannot be explained by the relatively modest effects on cyclin D1 and Cdk4 expression (16, 17). In fact, we show here that although c-*myc*-null cells assembled slightly more cyclin D1-Cdk4/6 complexes than normal cells, the complexes remained largely inactive.

Several studies have indicated that members of the Cip/Kip family of Cdk inhibitors are required for the assembly of cyclin D-Cdk4/6 complexes (25–29, 36–38). The picture emerging from *in vitro* studies is that both p27 and p21 promote the assembly of the complexes at low (1:1) stoichiometries without inhibiting the Rb kinase activity, but inhibit the activity at higher stoichiometries (25–27). p27 can also interfere with the activation of cyclin D-Cdk4/6 complexes by CAK. The experiments reported here involving conditional expression of p27 show that elevated p27 expression can potently inhibit the *in vivo* activity of cyclin D1-Cdk4/6 complexes. In fact, during G_0-to-S phase progression, elevated expression of p27 elicited a remarkable molecular phenocopy of the c-*myc* loss-of-function phenotype: an early large defect in Cdk4/6 activity, a delay in Rb phosphorylation, a subsequent delay in the induction of cyclin E and Cdk2 activities, and finally a delay in cyclin A expression and S phase entry. Although both Cdk4/6 and Cdk2 activities were affected, the Cdk4/6 defect was earlier and larger in magnitude, exactly as seen in c-*myc*-null cells.

It is likely that c-Myc can affect the expression level of the p27 protein by several mechanisms. One mechanism is the promotion of p27 degradation by ubiquitin-mediated proteolysis (39). Because the degradation of p27 is triggered by cyclin E-Cdk2 phosphorylation, it is unlikely that these mechanisms are operative in early G_1 at the time when cyclin D-Cdk4/6 complexes are being assembled and activated. c-Myc can also influence the expression of the p27 mRNA, which is not downregulated normally in c-*myc*$^{-/-}$ cells after mitogenic stimulation of quiescent cells (16). This study did not, however, address whether these effects are transcriptional or post-transcriptional. Repression of some (but not all) genes by c-Myc has been shown to involve the Inr promoter element (40); the p27 promoter contains an Inr element, and one study suggested that the repression of p27 by c-Myc may be mediated in part through this site (41). However, the mechanisms by which c-Myc affects the expression of the p27 gene need to be further investigated.

How could a 3-fold increase in the steady-state level of the p27 protein result in a >10-fold defect in Cdk4/6 activity? A key observation that shed light on this mechanism was that ~80% of the Cdk4/6 protein was found in inactive complexes that contained high stoichiometries of p27. Gel filtration experiments showed that Cdk4/6 migrated as a broad peak of ~160 kDa that comigrated with the peak of cyclin D1. However, Rb kinase activity migrated as a distinct peak of 70–100 kDa, and the distribution of p27 was skewed toward the higher molecular mass inactive complexes. Although the resolution of the columns did not allow us to determine whether the active complexes were free of p27, it is clear that the higher molecular mass complexes contained more abundant p27 (compare fractions 16 and 13 in Fig. 1) and were inactive as Rb kinases. Thus, even in c-*myc*$^{+/+}$ cells, only a small fraction of the total cyclin D-Cdk4/6 complexes become activated. The column profile in c-*myc*$^{-/-}$ cells was qualitatively very similar; but levels of p27 were elevated, the peak was broader, and the kinase activity of the 70–100-kDa fractions was reduced.

During the G_0-to-S phase transition, cyclin D1 and Cdk4/6 were rapidly assembled in early G_1 into complexes that contained abundant p27, whereas the appearance of Rb kinase activity was delayed by several hours. Again, c-*myc*$^{-/-}$ cells displayed the same profile; the abundance of Cdk4/6 complexes was somewhat increased; and Rb kinase activity was greatly reduced. The absence of a significant change in the stoichiometry of the complexes in either c-*myc*$^{+/+}$ or c-*myc*$^{-/-}$ cells during the activation process suggests that activation does not involve rearrangement of the complexes, but is likely dependent on additional events such as phosphorylation by CAK of pre-existing complexes that contain a few (or no) molecules of p27.

Taken together, the gel filtration data and the G_0-to-S phase induction profiles suggest the following model. Mitogenic signaling induces the expression of cyclin D, which, with the aid of p27, is rapidly assembled with Cdk4/6 and transported into the nucleus. The majority of the nuclear cyclin D-Cdk4/6 complexes are bound to multiple molecules of p27 and remain inactive throughout G_1. As previously suggested (1), the physiological function of these complexes would be to eliminate the free pool of p21 and p27 or even to actively sequester these Cdk inhibitors from the low levels of cyclin E-Cdk2 complexes present in early G_1 to facilitate their subsequent activation. A small fraction of cyclin D-Cdk4/6 complexes would be bound to only one (or no) p27 molecule and could thus become activated later in G_1. The key prediction of this model is that even a modest increase in overall p27 levels could convert a significant fraction of the low abundance activable complexes into unactivable complexes containing higher stoichiometries of p27.

This model is supported by the observation that even a modest overproduction of cyclin D1 in c-*myc*$^{-/-}$ cells can completely rescue complex activity. This is because cyclin D levels are limiting for the assembly of cyclin D-Cdk4/6 complexes, and overexpression of cyclin D1 results in the assembly of additional complexes. The observations that CAK activity is not limiting for the activation of cyclin D1-Cdk4/6 complexes in either c-*myc*$^{+/+}$ or c-*myc*$^{-/-}$ cells, that the expression of Cdc25A is not affected in c-*myc*$^{-/-}$ cells (42), and that the activation of cyclin D1-Cdk4/6 complexes is initiated normally in both

c-*myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells at the same time after serum stimulation (~12 h) (19) are all consistent with the interpretation that the major reason for the observed Cdk4/6 activity defect is the assembly of fewer potentially active complexes. Furthermore, in light of the recent report that the p21 gene is silenced in Rat-1 cells by promoter methylation (43), this model also provides an attractive explanation of why c-*myc*$^{-/-}$ Rat-1 cells are capable of proliferation, albeit at greatly reduced rates.

However, it also needs to be stressed that p27 is unlikely to be the only c-Myc target relevant to regulation of cell cycle progression. For example, although the expression of p27 in the conditional Rat1p27 cell line was higher than that seen in c-*myc*$^{-/-}$ cells, the proliferation defect during either exponential growth or the G_0-to-S phase transition was not as severe in Rat1p27 cells as in c-*myc*$^{-/-}$ cells. Similarly, if the only function of c-Myc were to promote the activity of Cdk4/6 complexes, overexpression of cyclin D would be expected to rescue cell cycle progression as well as Cdk2 activity, which is not the case. Although CAK is not limiting for Cdk4/6 activity, it appears to be limiting for Cdk2 activity (16) and may thus in part explain the effects of c-Myc on cell cycle progression in late G_1. The facts that cyclin D1 overexpression rescues Cdk4/6 activity, but cyclin E overexpression does not rescue Cdk2 activity, are in agreement with this interpretation (data not shown). Furthermore, the lack of rescue by cyclin E is not consistent with the possibility that the major bottleneck in c-*myc*$^{-/-}$ cells is the failure of cyclin D-Cdk4/6 complexes to sufficiently sequester the elevated levels of p27. By extension, this would then argue in favor of a functional role for Cdk4/6 catalytic activity during G_1 phase progression.

The function of the cyclin D pathway is subverted to a greater or lesser extent in most (if not all) cancer cells and derived cell lines. Given the importance of this signaling connection between the extracellular environment and the intrinsic cell cycle clock, it is of interest that c-Myc regulates the expression (17) as well as the activity of cyclin D-Cdk4/6 complexes by multiple mechanisms. The work reported here establishes p27 as a physiologically relevant regulator of cyclin D-Cdk4/6 activity as well as a target of c-Myc and provides a mechanistic model by which c-Myc influences the early-to-mid G_1 phase transition. Additional targets of c-Myc relevant to cell cycle regulation will undoubtedly be discovered; however, regulation of Cdk4/6 activity may provide a direct link between the ability of c-Myc to promote tumorigenesis and cell cycle progression.

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The Proto-oncogene c-*myc* Acts through the Cyclin-dependent Kinase (Cdk) Inhibitor p27 ^{Kip1} to Facilitate the Activation of Cdk4/6 and Early G_1 Phase Progression

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