Molecular Biology of the Cell
Vol. 8, 1815–1827, September 1997

p27Kip1 Induces an Accumulation of the Repressor Complexes of E2F and Inhibits Expression of the E2F-regulated Genes

Pavel Shiyanov,* Steven Hayes,* Ningyu Chen,† Dimitri G. Pestov,† Lester F. Lau,† and Pradip Raychaudhuri*‡

*Department of Biochemistry, University of Illinois at Chicago, Chicago, Illinois 60612; and
†Department of Genetics, University of Illinois at Chicago, Chicago, Illinois 60607

Submitted November 15, 1996; Accepted June 12, 1997
Monitoring Editor: Keith R. Yamamoto

p27Kip1 is an inhibitor of the cyclin-dependent kinases and it plays an inhibitory role in the progression of cell cycle through G_1 phase. To investigate the mechanism of cell cycle inhibition by p27Kip1, we constructed a cell line that inducibly expresses p27Kip1 upon addition of isopropyl-1-thio-β-D-galactopyranoside in the culture medium. Isopropyl-1-thio-β-D-galactopyranoside-induced expression of p27Kip1 in these cells causes a specific reduction in the expression of the E2F-regulated genes such as cyclin E, cyclin A, and dihydrofolate reductase. The reduction in the expression of these genes correlates with the p27Kip1-induced accumulation of the repressor complexes of the E2F family of factors (E2Fs). Our previous studies indicated that p21WAF1 could disrupt the interaction between cyclin/cyclin-dependent kinase 2 (cdk2) and the E2F repressor complexes E2F–p130 and E2F–p107. We show that p27Kip1, like p21WAF1, disrupts cyclin/cdk2-containing complexes of E2F–p130 leading to the accumulation of the E2F–p130 complexes, which is found in growth-arrested cells. In transient transfection assays, expression of p27Kip1 specifically inhibits transcription of a promoter containing E2F-binding sites. Mutants of p27Kip1 harboring changes in the cyclin- and cdk2-binding motifs are deficient in inhibiting transcription from the E2F sites containing reporter gene. Moreover, these mutants of p27Kip1 are also impaired in disrupting the interaction between cyclin/cdk2 and the repressor complexes of E2Fs. Taken together, these observations suggest that p27Kip1 reduces expression of the E2F-regulated genes by generating repressor complexes of E2Fs. Furthermore, the results also demonstrate that p27Kip1 inhibits expression of cyclin A and cyclin E, which are critical for progression through the G_1-S phases.

INTRODUCTION

The cyclin-dependent kinase inhibitor p27Kip1 is believed to play an important role in controlling the entry and exit from the cell cycle (Roberts *et al.*, 1994; Sherr and Roberts, 1995). p27Kip1 was discovered as an inhibitory activity induced by antimitogenic signals (Koff *et al.*, 1993; Firpo *et al.*, 1994; Polyak *et al.*, 1994a,b; Slingerland *et al.*, 1994; Toyoshima and Hunter, 1994). p27 expression increases in cells grown to high density or in those deprived of serum mitogens (Coats *et al.*, 1996). Antimitogenic agents such as cyclic AMP and rapamycin also stimulate expression of p27 (Kato *et al.*, 1994; Nourse *et al.*, 1994). Increased expression of p27 correlates with cell cycle arrest at G_1 (Coats *et al.*, 1996). Moreover, p27 has been shown to cooperate with an INK4 family cyclin-dependent kinase (cdk) inhibitor, p15, to induce a G_1 arrest in response to transforming growth factor β (Reynisdottir *et al.*, 1995). Experiments with an antisense oligonucleotide that specifically reduces p27 indicated a role of p27 in the restriction point control of the fibroblast cell cycle (Coats *et al.*, 1996). Targeted gene disruption experiments also indicated an antiprolifera-

‡ Corresponding author.

tive role of p27. Mice lacking the *p27* gene exhibit, besides other defects, enhanced growth, multiorgan hyperplasia, and tumorigenesis (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996). Also deletion of the *p27* gene, like deletion of the retinoblastoma (*Rb*) gene, caused development of pituitary tumors, suggesting the possibility of an overlap between the growth suppression pathways of *p27* and *Rb* (Fero *et al.*, 1996).

p27Kip1 inhibits cell growth by binding to and inhibiting the function of the cdks (Roberts *et al.*, 1994; Toyoshima and Hunter, 1994; Sherr and Roberts, 1995). For example, cell cycle arrest in immortalized mouse fibroblasts correlates with a down-regulation of the cyclin E-cdk2 and cyclin A-cdk2 activities, and that coincides with an increased binding of p27 to these kinases (Coats *et al.*, 1996). However, the molecular events downstream of cdk inhibition are not fully understood. The cdk kinases have been shown to phosphorylate the Rb family proteins and the members of the E2F family factors (Nevins, 1992; Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Weinberg, 1995 and references therein). Because Rb and E2F families of proteins regulate transcription through the E2F-binding site, it is possible that an increased level of p27 would modify activities of these proteins and alter expression of the E2F-regulated genes. The E2F family of proteins (E2Fs) control expression of several genes that are important for progression through G_1 (Nevins, 1992; DeGregori *et al.*, 1995). Therefore, determination of a link between p27 and the E2F family of proteins will provide insight into the events downstream of cdk inhibition.

E2Fs bind to the Rb family proteins, Rb, p130, and p107, to form complexes that are believed to be repressors of E2F-dependent transcription (Nevins, 1992; Zhu *et al.*, 1993; Vairo *et al.*, 1995; Weintraub *et al.*, 1995; Shiyanov *et al.*, 1996). Rb has been shown to bind E2F1, E2F2, E2F3, and E2F4 (Ikeda *et al.*, 1996; Moberg *et al.*, 1996 and references therein) whereas p107 and p130 bind E2F4 and E2F5 (Sardet *et al.*, 1995; Vairo *et al.*, 1995). It is speculated that these repressor complexes of E2Fs inhibit expression of genes involved in cell cycle progression (Nevins, 1992; Zhu *et al.*, 1993; Sellers *et al.*, 1995; Shiyanov *et al.*, 1996; Vairo *et al.*, 1995; Weintraub *et al.*, 1995). Moreover, several lines of evidence have indirectly implicated these repressors as downstream targets of the cdk inhibitors. It has been suggested that the interaction between Rb and E2Fs is inhibited by the cdk kinase-mediated phosphorylation of Rb (Nevins, 1992; Weinberg, 1995; Fero *et al.* 1996). Therefore, an inhibition of the cdk kinases would increase the interaction between Rb and E2Fs, leading to a repression of the E2F-regulated genes. However, in vivo evidence for an increased accumulation of the E2F–Rb complex in response to cdk inhibitors is not available.

A variety of growth-arrested cells contain E2Fs bound to the Rb family protein p130. A recent study indicated that the E2F–p130 complex is a marker for cells in G_0 (Smith *et al.*, 1996). The E2F–p130 complex, like the E2F–Rb complex, is believed to be a negative regulator of E2F-dependent transcription (Cobrinik *et al.*, 1993; Vairo *et al.*, 1995; Shiyanov *et al.*, 1996). Progression through G_1 correlates with a cyclin-dependent binding of cdk2 to the E2F–p130 complex (Cobrinik *et al.*, 1993; Vairo *et al.*, 1995; Shiyanov *et al.*, 1996; Smith *et al.*, 1996). In S-phase, the p130-containing complex is replaced by p107-containing complex (Cobrinik *et al.*, 1993; Moberg *et al.*, 1996; Shiyanov *et al.*, 1996; Smith *et al.*, 1996). In vitro studies with reconstituted E2F–p107–cyclin A–cdk2 complex indicated an incubation with ATP causes release of free E2F (Zhu *et al.*, 1995). Thus, it is possible that cdk2 binding eventually leads to a loss of the repressor complexes involving p107 or p130. Recent studies also indicated that the cdk2-containing complexes of E2F are targets of the cdk inhibitors. For example, activation of p53 causes a disruption of the interaction between cdk2 and the E2F–p130 complex, which correlates with an expression of the cdk inhibitor p21 (Shiyanov *et al.*, 1996). Moreover, an in vitro incubation of p21 with a partially purified preparation of the E2F–p130–cyclin–cdk2 complex resulted in a dissociation of the cdk2-containing complex, leading to the accumulation of the E2F–p130 complex (Shiyanov *et al.*, 1996). Similar results were also obtained with the E2F–p107–cyclin–cdk2 complex (Afshari *et al.*, 1996).

p27 exhibits structural and functional similarities with p21 (Toyoshima and Hunter, 1994). Moreover, an exit from the cell cycle in fibroblasts is accompanied by the accumulation of both p27 and the E2F–p130 complex. Therefore, we investigated whether p27 plays a role in the accumulation of the E2F–p130 complex. Here, we provide evidence that p27 disrupts the interaction between E2F–p130 and cdk2, leading to an accumulation of the E2F–p130 complex. Accumulation of the E2F–p130 complex correlates with a loss of expression of several E2F-regulated genes. In transient transfection assays, expression of p27 specifically inhibits transcription from promoters containing E2F-binding sites. Moreover, mutants of p27 harboring changes in the cyclin- and cdk2-binding motifs were impaired in their ability to inhibit E2F-regulated transcription, which also correlated with their inability to generate the repressor complexes of E2F.

MATERIALS AND METHODS

Construction of the IPTG-inducible LAP3-p27 Cell Line

LAP3-p27 is a single-cell clone derived from LAP3 cells (Pestov and Lau, 1994) cotransfected with pHyg (Sudgen *et al.*, 1985) and pX12-p27, which contains the mouse p27 cDNA under the control of the

isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible promoter in pX12. The mouse p27 cDNA clone was a kind gift from T. Hunter (Salk Institute, La Jolla, CA). pX12 is slightly modified from the pX11 vector (Pestov and Lau, 1994), resulting in a lower level of background expression. Stably transfected cells were selected in medium containing hygromycin (150 μg/ml, Life Technologies, Gaithersburg, MD) for 2 weeks; single-cell clones were obtained by limit dilution and tested for IPTG-inducible expression of p27. LAP3-p27 showed a low background level of p27 expression, high inducibility, and growth inhibition when p27 was expressed using the SETGAP assay (Pestov and Lau, 1994).

Cell Extracts and Gel Retardation Assays

Whole-cell extracts were prepared following a previously described procedure (Shiyanov *et al.*, 1996). E2F complexes were assayed by using a previously published procedure (Shiyanov *et al.*, 1996). Briefly, the extracts were incubated in reaction mixtures (30 μl total volume) containing 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 30 μg/ml salmon sperm DNA, 0.01% Nonidet P-40, and 0.2 ng of ^{32}P-labeled E2F-specific DNA probe that contained a single E2F-binding site (Shiyanov *et al.*, 1996). After an incubation at room temperature for 20 min, 4 μl of 20% Ficoll were added. Aliquots (7.5 μl) of the reaction mixtures were analyzed by gel retardation assays as described before (Shiyanov *et al.*, 1996).

Assays of cdk2 and cdk4 Kinase Activities

The cdk2 and cdk4 kinase activities in the immunoprecipitates obtained with specific antibodies were performed following a previously described procedure (Morozov *et al.*, 1997).

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting were performed following previously described procedures (Shiyanov *et al.*, 1996). p130 and cdk2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DP1 antibody was a kind gift from Chin-Lee Wu (Ed Harlow's laboratory at the Massachusetts General Hospital Cancer Center, Harvard University, Boston, MA).

RNase Protection Assay

RNase T2 protection assays using antisense RNA probes and total cellular RNA were performed following a previously described procedure (Morozov *et al.*, 1994; Lee *et al.*, 1996). The antisense probe for the dihydrofolate reductase (DHFR)-mRNA was obtained by subcloning a *Eco*RI-*Hin*dIII DHFR-fragment from pST410 (a kind gift from P.J. Farhnam, University of Wisconsin, Madison, WI) in pGEM 3Z(+) vector and by transcribing with SP6 RNA polymerase. The cyclin D1 probe was obtained by digesting the plasmid pcBZ054 (a gift from C. Sherr, St. Jude Children's Research Hospital, Memphis, TN) with *Nco*I and by transcribing with T3 RNA polymerase. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was described before (Morozov *et al.*, 1994; Lee *et al.* 1996).

Northern Blots

Northern blot assays for cyclin E and cyclin A mRNAs were performed following a previously described procedure (Morozov *et al.*, 1995). Briefly, 10 μg of total cellular RNA were separated by 1.2% agarose-formaldehyde (0.41 M) gels followed by blotting to Nylon-1 (Life Technologies). The blots were probed with labeled DNA obtained by nick translation of human cyclin E, cyclin A cDNAs, or mouse c-myc cDNA.

Construction of p27 Mutants

p27 mutants harboring alanine substitutions within the cyclin A-binding site between residues 30 and 33 (p27 mC) or within the cdk2-binding site between residues 60 and 64 (p27 mK) were constructed by site-directed mutagenesis using a two-step polymerase chain reaction (PCR) procedure. Two overlapping internal oligoprimers containing the altered sequences in opposite orientation along with outside primers were used in two separate PCRs. p27 cDNA was used as the template. The PCR products were gel purified, combined, and used as template for the second PCR. The outside primers were used to obtain the full-length products. The outside primers contained *Eco*RI and *Bam*HI sites. The PCR products were digested with *Eco*RI-*Bam*HI and cloned in-frame with glutathione *S*-transferase (GST) in pGEX-KG or into the mammalian expression vector pcDNA3 (Invitrogen). For p27 mC, the internal primers were TGACGGGGCCGGCGGCAGCTGCGCAGGCGGAA and TTCCGCCTGCGCAGCTGCCGCCGGCCCGGTCA. For p27 mK, the internal primers were GCTTATGATTCTGAGCGGCG-GCAGCTGCCTTGCGCTGA, TCAGCGCAAGGCAGCTGCCGCC-GCTCAGAATCATAAGC. The outside primers used in the PCR were CGCGGATCCATGTCAAACGTGAGAGTGTC (upstream) and CGGAATTCATTACGTCTGGCGTCGAAGG (downstream). The mutations were confirmed by DNA sequencing.

To obtain GST-p27, a full-length cDNA fragment corresponding to mouse p27 was subcloned into pGEX-2T in-frame with GST. The fusion protein was purified from bacterial culture induced with IPTG, using a procedure described for GST-E7 purification (Arroyo *et al.*, 1993).

Flow Cytometry

Nuclei from approximately 5 million cells were isolated using a detergent-trypsin method and stained with propidium iodide (Vindelov *et al.*, 1983). The flow cytometry analysis was performed in the Research Resources Center University of Illinois at Chicago with the kind help of Dr. K. Hagen. The calculation of percentage of cells in different phases of the cell cycle was done using a Multicycle AV program (Phoenix Flow Systems, San Diego, CA).

RESULTS

Construction of a Cell Line That Inducibly Expresses p27Kip1

To investigate the mechanism of cell cycle inhibition by p27, we sought to construct a cell line that expresses p27 in an on/off manner. Construction of the LAP3-p27 cell line is described in the MATERIALS AND METHODS. Briefly, an IPTG-inducible mammalian expression system that utilizes the chimeric protein LAP267 was used (Labow *et al.*, 1990). LAP267 in the presence of IPTG activates transcription from a promoter linked to the lac operator sequence (Labow *et al.* 1990). A recently described lac operator containing plasmid pX12 (Pestov and Lau, 1994) was used to express p27. p27 expression in this cell line can be induced by adding IPTG (1 mM) to the culture medium. A Western blot assay for p27 expression after IPTG induction is shown in Figure 1 (upper panel). Addition of IPTG or the expression of p27 did not significantly alter the level of the p21 protein in these cells. The slight increase shown in Figure 1 was not reproducible. However, there was a very reproducible reduction in the cdk2 and cdk4 kinase activities by the expression of p27 (Figure 1, lower panels). The cdk2 activity was more sensitive than cdk4 activity to inhibition by the p27 expression. These results indicated

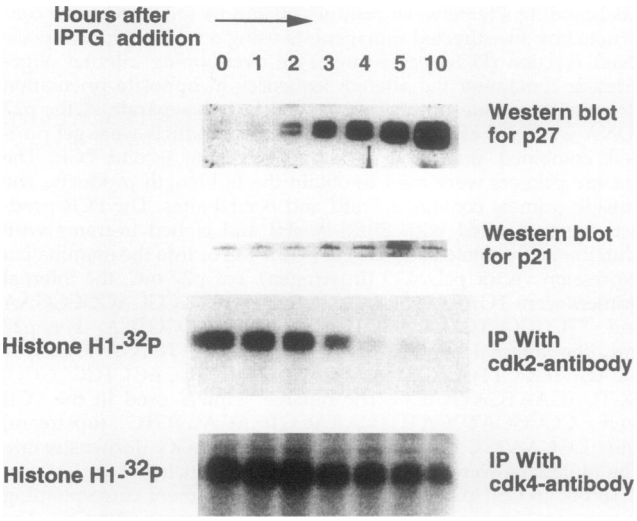

Figure 1. Expression of p27 in LAP3-p27 cells reduces the activities of cdk2 and cdk4. LAP3-p27 cells were induced with IPTG. At the indicated time points, cells were harvested and extracts were prepared for Western blots and immunoprecipitation experiments as described in MATERIALS AND METHODS. Western blots (two upper panels) were performed with 50 μg of the extracts. The blots were probed with p27 antibody or p21 antibody. cdk2 and cdk4 antibodies were used to immunoprecipitate the respective kinases from 100 μg of the extracts. The immunoprecipitates were used in histone H1 kinase assays in the presence of a protein kinase A inhibitor as described before (Morozov *et al.*, 1997).

Table 1. Ectopic expression of p27 at the G_1-S-phase boundary inhibits progression into S-phase[a]

Condition	G_0-G_1	S	G_2-M
	92.2	3.2	4.6
No serum induction	91.9	4.1	4.1
16-h serum induction	23.5	70.1	6.4
Without IPTG	27.9	67.6	4.5
16-h serum induction	62.9	22.4	14.7
With IPTG added in 7 h	58.9	24.3	16.8
55-h serum induction	90.0	7.3	2.7
With IPTG added at 7 h	88.6	8.8	2.6

[a] The LAP3-p27 cells were grown to 80% confluence and then incubated in medium containing no serum. Following serum starvation for 48 h, cells were stimulated with fetal bovine serum (10%). After 7 h of serum stimulation, some of the plates received IPTG (1mM final). The cells were harvested 16 h after serum stimulation and subjected to flow cytometric analysis as described in MATERIALS AND METHODS. The data are presented as the percentage of cells in each phase of the cell cycle. The two sets of numbers corresponded to two independent experiments.

that the exogenous p27 expressed in the LAP3-p27 cells was functionally active.

p27 has been shown to inhibit progression into the S-phase (Reynisdottir *et al.*, 1995; Coats *et al.*, 1996). We analyzed the effect of p27 expression on the serum-stimulated progression into the S-phase. The LAP3-p27 cells are derived from NIH 3T3 cells and these cells can be synchronized to G_0 by serum starvation. Stimulation of the G_0-arrested cells by serum supplementation induces entry into the S-phase (Table 1). The IPTG-induced expression of p27 in the serum-stimulated cells caused an inhibition of the progression into the S-phase. A significant population of cells remained arrested in G_1 (Table 1). A continued incubation with IPTG increased the population of cells in G_0-G_1 (compare the 16-h and 55-h time points). These cells remained arrested for at least 55 h and very little or no apoptosis was detected during this time (our unpublished observations).

p27Kip1 Causes an Accumulation of the Repressor Complexes of E2F

Recent studies indicated that p21 could disrupt the interaction between cdk2 and the E2F–p130 or E2F–p107 complex, leading to an accumulation of the repressor complexes of E2F (Zhu *et al.*, 1995; Shiyanov *et al.*, 1996). The N-terminal half of p21 exhibits extensive structural similarities with p27 (Toyoshima and Hunter, 1994). Therefore, we investigated whether p27 could disrupt the interaction between cdk2 and the E2F–p130 complex. Because we cannot distinguish between E2F4 and E2F5, which bind to p130, the generic term E2F has been used to refer to these two members of E2F family. The LAP3-p27 cells were incubated in medium containing IPTG for various lengths of time. Extracts from these cells were analyzed for the interaction between cdk2 and p130, as well as cdk2 and DP1 (the DNA-binding partner of E2Fs) by coimmunoprecipitation assays. Extracts prepared from cells that were incubated with IPTG for 0 to 2 h exhibited interaction between cdk2 and DP1 or p130 (Figure 2). However, incubation with IPTG longer than 2 h caused a significant reduction in the coprecipitation of cdk2 with p130 and DP1, which will be consistent with a loss of interaction between cdk2 and the E2F–p130 complex (Figure 2). Moreover, the loss of interaction between cdk2 and p130 or DP1 coincided with the expression of p27 (Figure 2). In addition, consistent with the observation of Zerfass-Thome *et al.* (1997), we observed a loss of the interaction between cdk2 and p107 by the expression of p27 (our unpublished results). Effects of p27 on the E2F complexes were also analyzed by gel retardation assays. Consistent with the coimmunoprecipitation data, extracts with higher levels of p27 exhibited a loss of the cdk2-containing E2F–p130 complex and an accumulation of the E2F–p130 complex (our unpublished results, and see Figure 6).

The recombinant p27 protein was used to determine whether p27 can directly dissociate the interaction

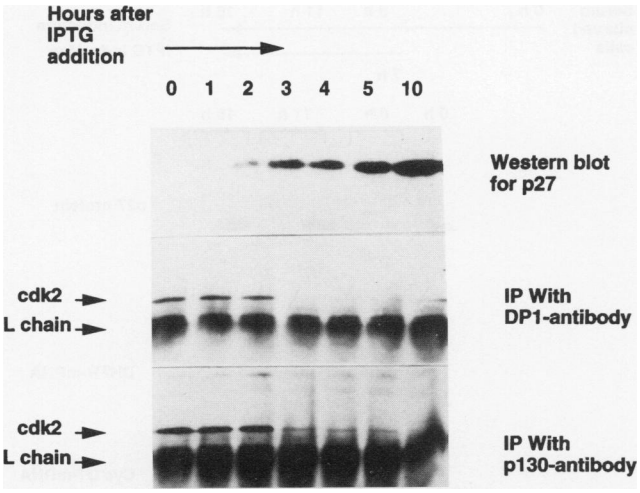

Figure 2. Expression of p27 coincides with a reduction of the interaction between cdk2 and E2F or p130. The LAP3-p27 cells were induced for p27 expression by adding IPTG (1 mM) to the culture medium. Cells were harvested at the indicated time points after the IPTG induction. Upper panel, cell extracts (25 μg) were analyzed for p27 expression by Western blot assays. The blot was probed with a p27 antibody from Santa Cruz (C-19; 1:200 final dilution), and it was developed by ECL reagents. The extracts (150 μg) were also analyzed for an association between DP1 and cdk2 or p130 and cdk2. The immunoprecipitates obtained with DP1 antibody (middle panel) or p130 antibody (lower panel) were analyzed by Western blot assays for the presence of cdk2 following previously described procedures (Shiyanov *et al.*, 1996).

between cdk2 and the E2F–p130 complex. Whole-cell extracts from serum-starved and serum-stimulated cells were analyzed for E2F complexes in the presence or absence of recombinant p27 by a gel retardation assay (Figure 3A). Bands 2 and 3 were characterized previously and they corresponded to E2F–p130 and E2F–p130–cyclin–cdk2 complexes, respectively (Shiyanov *et al.*, 1996). The composition of the E2F complex corresponding to band 1 is yet to be determined; however, it is mainly found in the cytosol (Shiyanov and Raychaudhuri, unpublished observation). Incubation with p27 resulted in a loss of the E2F–p130–cyclin–cdk2 complex with a concomitant appearance of the E2F–p130 complex (Figure 3A, lane 2). The effects of recombinant p27 on the interaction between cdk2 and the E2F–p130 complex was also analyzed by coimmunoprecipitation assays. Antibodies against p130 or DP1 (a subunit of E2F) coprecipitated cdk2 from the extracts of serum-stimulated cells because the E2F–p130 complex in these extracts remains bound to cdk2 (Figure 3B). An incubation of the serum-stimulated cell extracts with GST-p27, but not GST, caused a loss of coprecipitation of cdk2 with p130 or DP1 (Figure 3B). These results would be consistent with the notion that, like p21, p27 disrupts the interaction between cdk2 and the E2F–p130 complex, leading to an accumulation of the E2F–p130 complex.

p27 Specifically Reduces Expression of the E2F-regulated Genes

The E2F–p130 complex is found mainly in growth-arrested cells, and it was suggested that this complex might be involved in actively repressing the E2F-regulated genes in growth-arrested cells (Vairo *et al.*, 1995). One prediction of this hypothesis is that, if we generate this complex at a time when E2F-regulated genes are actively transcribing, it would cause a repression. The E2F-regulated genes are expressed between mid-G_1 to early S-phase. Therefore, we used a synchronized population of the LAP3-p27 cells to induce p27 expression and generate the E2F–p130 complex at the G_1-S-phase boundary. The LAP3-p27 cells were synchronized to G_0 by serum starvation. The cells were stimulated by adding serum, and after 7 h of serum induction, IPTG was added to induce expression of p27. Addition of IPTG after 7 h of serum induction would cause an accumulation of p27 at the G_1-S-phase boundary because p27 expression takes 3 h after IPTG addition (Figure 2), and these cells enter S-phase about 11–12 h after serum stimulation (our unpublished results). Cells, incubated with or without IPTG, were harvested at 8, 11, and 16 h after serum stimulation. Total cellular RNA was analyzed for the expression of DHFR, cyclin D1, cyclin E, cyclin A, c-myc, and GAPDH. DHFR is a much studied E2F-regulated gene (Blake *et al.*, 1989; Means *et al.*, 1992; Slansky *et al.*, 1993). Cyclins E and A are also shown to be induced by E2F1 (DeGregori *et al.*, 1995; Ohtani *et al.*., 1995). The cyclin D1 promoter contains E2F-binding sites and in transfection assays is stimulated by coexpression of E2F1 (Ohtani *et al.*, 1995). However, the endogenous cyclin D1 gene was not induced by E2F1 (DeGregori *et al.*, 1995; Ohtani *et al.*, 1995). Similarly, the c-myc promoter contains E2F-binding site; however, it is not clear whether it is an E2F-inducible gene (DeGregori *et al.*, 1995). GAPDH is not known to be an E2F-regulated gene.

Expression of DHFR, cyclin D1, and GAPDH mRNAs were analyzed by RNase T2 protection assays. As expected, serum stimulation resulted in an increase in the expression of the DHFR and cyclin D1 mRNAs (Figure 4, − lanes). A slight increase in the expression of GAPDH mRNA was also detected (Figure 4, − lanes). Expression of p27 caused a specific reduction of the DHFR mRNA (Figure 4, + lanes). No significant reduction of the mRNA corresponding to cyclin D1 or GAPDH was observed. Expression of cyclin E and cyclin A mRNAs was analyzed by Northern blots. Probes derived from human cyclin E and cyclin A cDNAs were used. These probes were previously shown to detect mouse cyclin E and cyclin A mRNAs in Northern blot assays (DeGregori *et al.*, 1995). As can be seen in Figure 5 (− lanes), serum stimulation induced the expression of cyclin E and cyclin A mRNAs

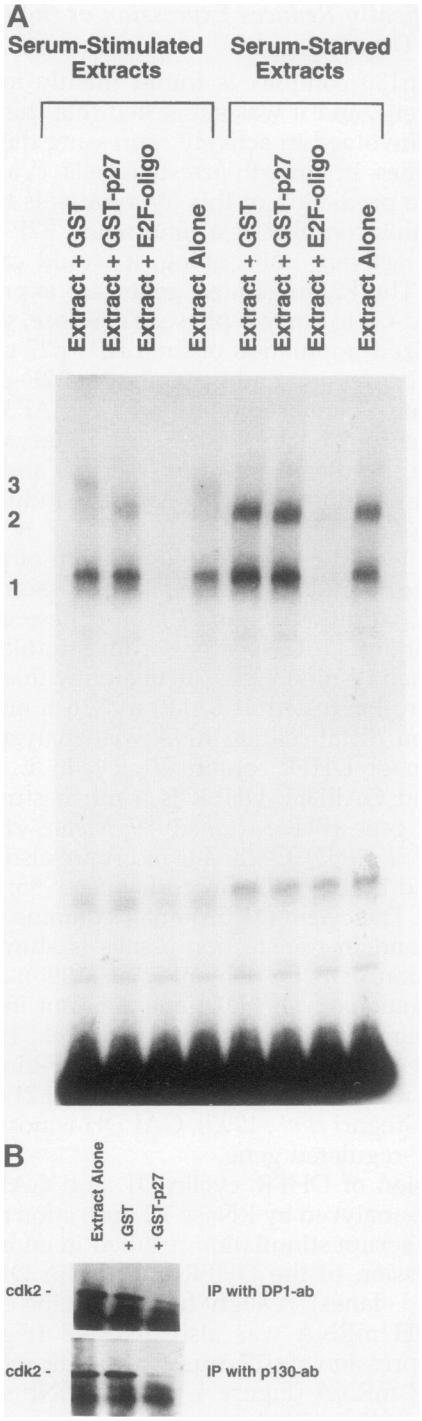

Figure 3. p27 disrupts the interaction between cdk2 and the E2F–p130 complex in vitro. (A) Whole-cell extracts (10 μg) from serum-stimulated (8 h of stimulation with 10% fetal bovine serum; lanes 1–4) and serum-starved (lanes 5–8) NIH 3T3 cells were subjected to gel retardation assays in the presence of components necessary for E2F-specific DNA binding as described in MATERIALS AND METHODS. Where indicated the reaction mixtures also contained an E2F-specific oligo competitor (50 ng), GST-p27 fusion protein (50 ng), or GST (100 ng). Aliquots of the reaction mixtures were analyzed by gel retardation assays as described in MATERIALS AND METHODS. Bands marked as 2 and 3 were characterized before and they corresponded to the E2F–p130 and E2F–p130–cyclin–cdk2 complexes, respectively (Shiyanov *et al.*, 1996). The composition of the band marked as 1 is unknown. (B) Extracts (200 μg) from serum-stimulated NIH 3T3 cells were incubated alone (Extract Alone) or with 1.5 μg of GST (+GST) or with 1 μg of GST-p27 fusion protein (+GST-p27) at 4°C for 30 min. The incubation mixtures were then subjected to immunoprecipitation with DP1 or p130 antibody. The immunoprecipitates were analyzed for the presence of cdk2 by Western blots. The blots were probed with a cdk2 antibody and developed with ECL as described before (Shiyanov *et al.*, 1996).

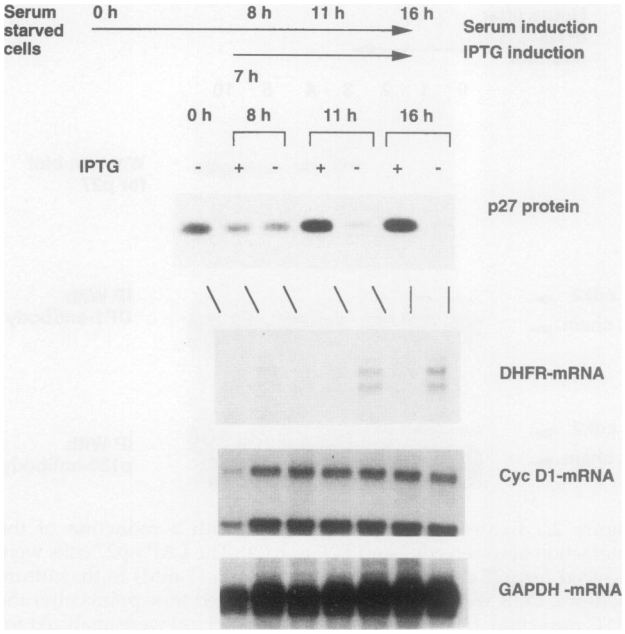

Figure 4. p27 expression coincides with an inhibition of the *DHFR* gene expression. LAP3-p27 cells were grown to 80% confluence and then maintained in medium without serum for 48 h. The synchronized cells were stimulated by adding fetal bovine serum (10%). Seven hours after serum stimulation, IPTG (1 mM final concentration) was added to a set of plates and the incubation was continued. At the indicated time points, cells were harvested, and cell extracts were analyzed for p27 expression by a Western blot assay (upper panel) as described in Figure 2. Lower panels, total cellular RNA from the harvested cells was subjected to RNase T2 protection assays using antisense RNA probes corresponding to DHFR, cyclin D1, and GAPDH as described in MATERIALS AND METHODS. The DHFR probe, which corresponded to −365 to +61 of the *DHFR* gene, generated a doublet of around 61 nts; although in primer extension assays a single band corresponding to the correct initiation site was seen (our unpublished results).

in NIH 3T3 cells. The expression of cyclin E reached maximum level at the 11-h time point, which is consistent with the fact that cyclin E is a G1 cyclin. The expression of cyclin A, which is an S-phase cyclin, reached peak level at the 16-h time point. Interestingly, expression of p27 in these serum-stimulated

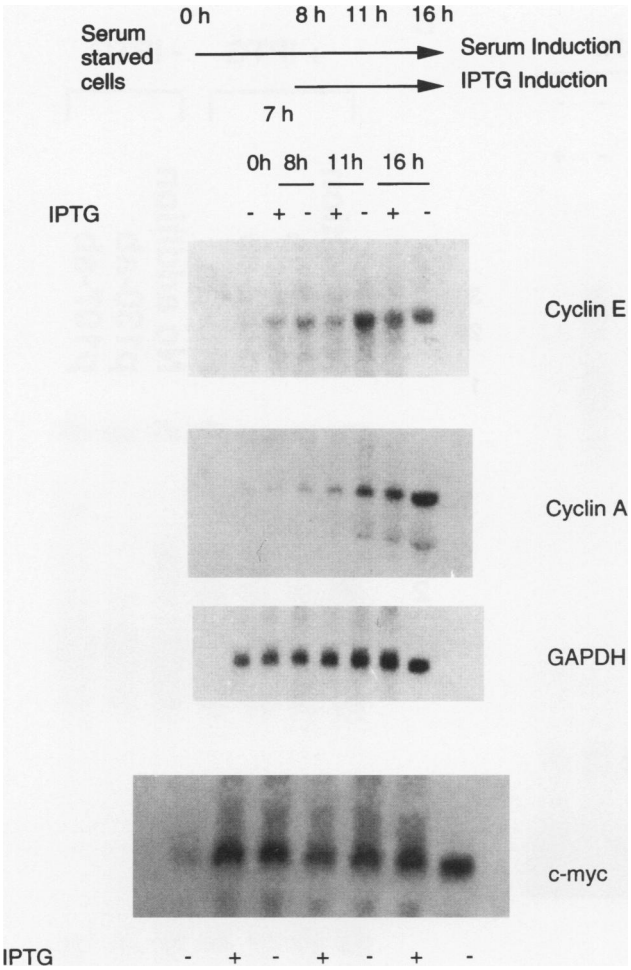

Figure 5. p27 expression causes a reduction in the levels of cyclin E and cyclin A mRNAs. The LAP3-p27 cells were synchronized by serum starvation. Cells were stimulated by adding 10% fetal bovine serum in the medium. After 7 h of serum stimulation, IPTG (1 mM) was added to the medium (+ lanes only). Cells were harvested at the indicated time points, and total cellular RNA (10 μg) was subjected to Northern blot analysis. The blots were probed with ^{32}P-labeled DNA probe derived from cyclin E, cyclin A, GAPDH, or c-myc cDNA as described in MATERIALS AND METHODS.

cells reduced the expression of cyclin E and cyclin A genes (compare the − and + lanes in Figure 5). This observation suggests that p27 not only reduces the activity of the cyclin kinases, it also reduces the expression of cyclins E and A that are essential for the progression into S-phase. We did not detect any significant reduction of the c-*myc* gene expression (Figure 5).

To determine whether the p27-mediated reduction of the DHFR, cyclin E, and cyclin A mRNAs was related to the accumulation of the E2F–p130 complex, whole-cell extracts were prepared after stimulation of the cells with serum and IPTG as in Figures 4 and 5. The extracts were analyzed by gel retardation assays (Figure 6A). Expression of p27 resulted in a loss of the cdk2-containing E2F complex and an accumulation of a complex that migrated like the E2F–p130 complex. Moreover, a p130 antibody specifically recognized the complex accumulated at the 11-h time point as a function of p27 expression (Figure 6B). This accumulation of the E2F–p130 complex correlated with the reduction of the DHFR, cyclin E, and cyclin A mRNA (compare lanes with and without IPTG in Figures 4, 5, and 6). DHFR, cyclin E, and cyclin A were previously shown to be E2F-regulated genes (DeGregori *et al.*, 1995; Ohtani *et al.*, 1995). Therefore, this observation would support the notion that p27, by inducing accumulation of the E2F–p130 complex, reduces expression of these E2F-regulated genes. Band 3 generated by the S-phase extract also contained E2F–p107–cyclin–cdk2 (Shiyanov *et al.*, 1996). Therefore, the effects on the expression in S-phase might be mediated by both E2F–p130 and E2F–p107 complexes.

Expression of p27 Inhibits Transcription from a Reporter Gene Containing E2F-binding Sites

Results presented above show a strong correlation between the p27-mediated accumulation of the repressor complexes of E2F and a reduction in the expression of the E2F-regulated genes, suggesting that p27 inhibits transcription of the E2F-regulated genes. To obtain further evidence along that line, we analyzed the effect of p27 expression on reporter genes containing E2F-binding sites. Two *CAT* gene reporter constructs (Figure 7A, and see Shiyanov *et al.*, 1996), which are identical except that they contain two copies of the E2F- or CRE-binding sites, were used in this analysis. The reporter genes were transfected into NIH 3T3 cells along with a plasmid expressing p27. To correct for the variability in transfection efficiencies, a plasmid expressing β-galactosidase was cotransfected. The results of three independent transfection experiments are shown in Figure 7B. Expression of p27 caused only a marginal reduction of the *CAT* gene expression from the CRE sites containing reporter genes, whereas the E2F sites containing reporter genes was severely repressed by the expression of p27 (compare the p27 lanes in Figure 7B).

p27 binds to cyclin/cdk2 through sequences in the N-terminal half of the p27 (Toyoshima and Hunter, 1994). This region of p27 exhibits about 42% sequence identity with p21 (Toyoshima and Hunter, 1994). Recent studies from several laboratories indicated that p21 possesses distinct cyclin- and cdk2-binding motifs. We observed that the disruption of the E2F–p130–cyclin–cdk2 complex, which was purified from cell extracts, by p21 depends upon the N-terminal cyclin- and cdk2-binding motifs. Moreover, in the context of this endogenous complex, both motifs are required for the inhibition of cdk2-mediated phosphorylation of

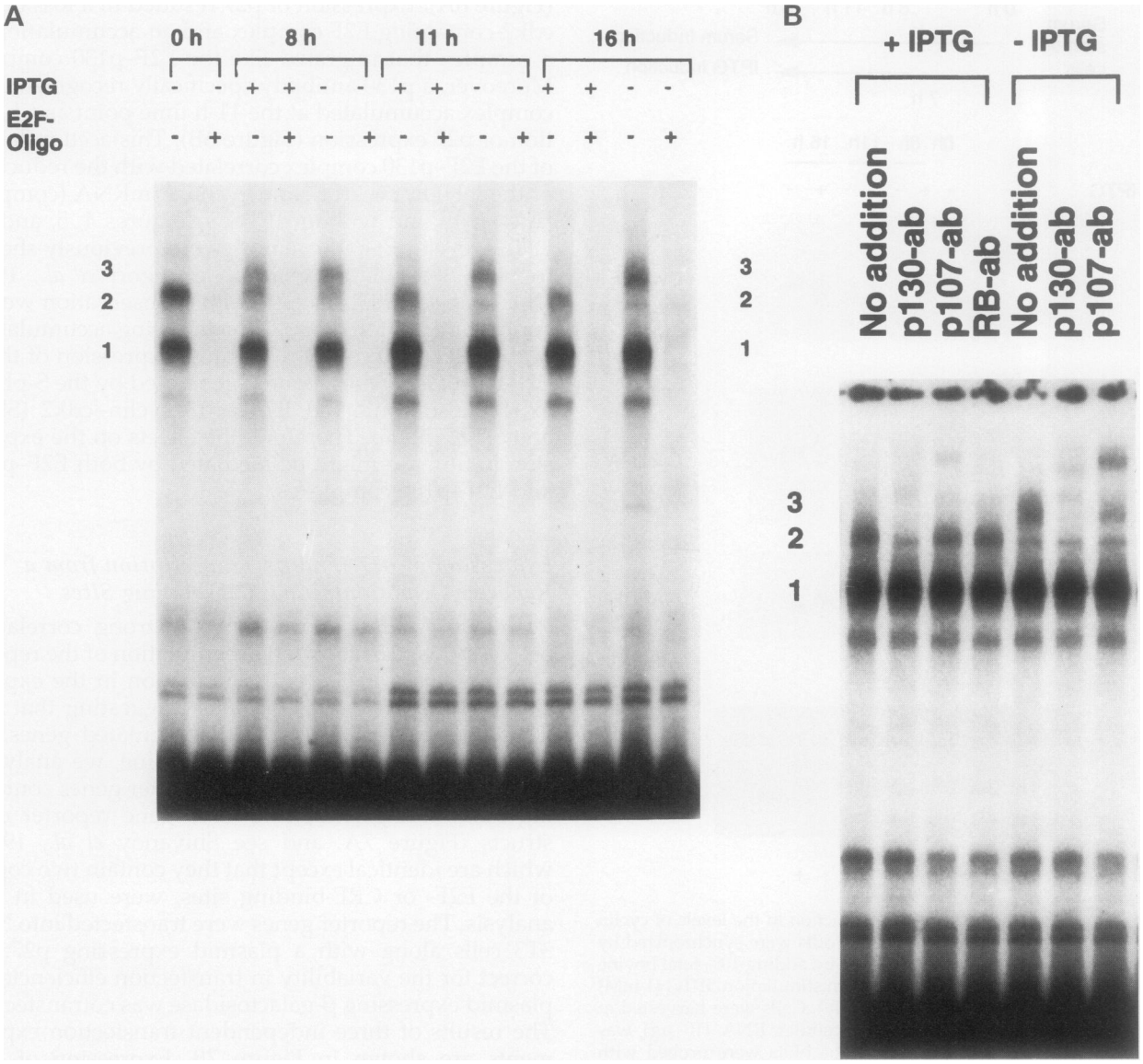

Figure 6. p27-mediated reduction of the E2F-regulated genes correlates with an accumulation of the E2F–p130 complex. (A) LAP3-p27 cells were synchronized, serum stimulated, and induced with IPTG as in the experiments described in Figures 3 and 4. Cells were harvested at the indicated time points after serum induction with or without IPTG. Whole-cell extracts (10 μg) were analyzed by gel retardation assays in the presence (+) or absence (−) of specific DNA competitor as described in MATERIALS AND METHODS. (B) The extracts from the 11-h time point were analyzed by gel retardation assay in the presence of the indicated antibodies. p130 antibody was obtained from Santa Cruz. Rb antibody was obtained from PharMingen (San Diego, CA), and the p107 antibody was a kind gift from E. Harlow's laboratory at MGH. The E2F-specific DNA-binding assays were performed as in Figure 6A except that following an incubation for DNA-binding antibodies (0.1 μg) were added to the reaction mixtures and the incubation was continued for another 30 min. Aliquots of the incubation mixtures were analyzed by gel retardation assay as described before (Shiyanov *et al.*, 1996).

p130 (Robles, Shiyanov, Raychaudhuri, and Adami, unpublished observation). Therefore, we constructed two mutants of p27: one (p27 mC) containing a substitution of four amino acids in the cyclin-binding motif and another (p27 mK) containing a substitution of five amino acids in the cdk2-binding motif (see Figure 7A). These mutants were used in transient transfection assays. As can be seen in Figure 7B, these two mutants were significantly impaired compared with the wild-type protein in inhibiting the E2F-regulated transcription (compare lanes marked p27, p27 mC, and p27 mK). There was no defect in the expression of the mutant proteins (Figure 7C). Moreover, these two mutants were unable to disrupt the interac-

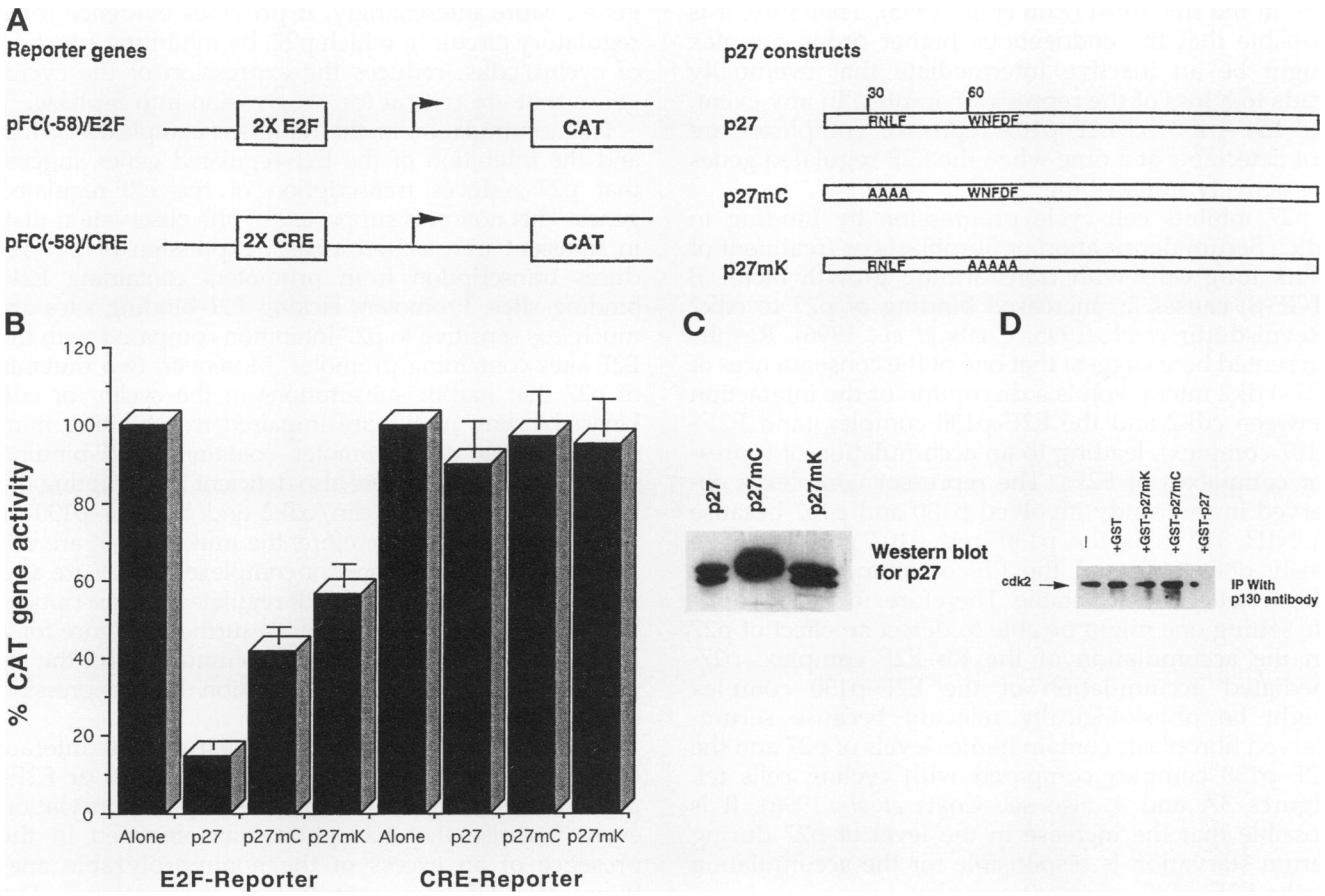

Figure 7. Mutations in the cyclin-cdk2-binding motifs impair p27's ability to inhibit E2F-regulated transcription. (A) Schematic diagram of the reporter *CAT* genes and the mutant p27 proteins. The reporter *CAT* genes have been described before (Shiyanov *et al.*, 1996). The substitutions in p27 mC and p27 mK are shown. Construction of the mutants are described in MATERIALS AND METHODS. (B) Mammalian expression vectors containing the p27 cDNAs (2 μg) were transfected into NIH 3T3 cells along with 5 μg of the reporter *CAT* gene encoding plasmids as described before (Shiyanov *et al.*, 1996). A plasmid expressing β-galactosidase was also included in the transfection mixtures to normalize for the transfection efficiencies. The *CAT* gene activities obtained with the two reporter genes were determined. Conversion levels for each promoter construct without the presence of p27 were normalized to 100%. The result represents an average of three experiments. (C) A Western blot assay was performed to detect expression of the wild-type and mutant p27 proteins in the transfected cells. Extracts (100 μg) from the cells transfected with the three p27 expression vectors (see Figure 7A) were subjected to Western blot assay. The blot was probed with p27 antibody and developed with ECL. (D) GST fusion proteins containing the wild-type or mutant p27 were assayed for their ability to disrupt the interaction between cdk2 and p130 by a coimmunoprecipitation assay. Fifty micrograms of extracts from NIH 3T3 cells were incubated alone (−) or with the indicated GST proteins (50 ng). After an incubation at room temperature for 20 min, the incubation mixtures were subjected to immunoprecipitation with p130 antibody. The immunoprecipitates were assayed for the presence of cdk2 by a Western blot assay. The band corresponding to cdk2 is indicated by an arrow.

tion between cdk2 and p130 as determined by a coimmunoprecipitation assay (Figure 7D). Taken together, these results suggest that p27 specifically inhibits transcription of the E2F-regulated genes by inducing accumulation of the repressor complexes of E2Fs.

DISCUSSION

The E2F family factors E2F-4 and E2F-5 bind to the Rb-related proteins p107 and p130 (Beijersbergen *et al.*, 1994; Sardet *et al.*, 1995; Vairo *et al.*, 1995). Because the p130- and p107-containing complexes of E2F-4 and E2F-5 are difficult to distinguish (Smith *et al.*, 1996), we have used the generic name E2F to refer to E2F-4 and E2F-5. It has been suggested that the p107- and the p130-containing complexes, like the E2F–Rb complex, are repressors of E2F-regulated genes (Beijersbergen *et al.*, 1994; Sardet *et al.*, 1995; Vairo *et al.*, 1995; Starostik *et al.*, 1996). Extracts of mitogen-stimulated cells contain the E2F–p130 and E2F–p107 complexes in higher order forms that contain cyclin-cdk2 (Cobrinik *et al.* 1993; Beijersbergen *et al.*, 1994; Shiyanov *et al.*, 1996; Smith *et al.*, 1996; Vairo *et al.*, 1995). An in vitro incubation of the higher order complex, reconstituted with active cyclin A-cdk2 kinase, resulted in a release of

E2F in the free form (Zhu *et al.*, 1995). Therefore, it is possible that the endogenous higher order complex might be an inactive intermediate that eventually leads to a loss of the repressor complex. In any event, the E2F–p130 or E2F–p107 repressor complexes are not detectable at a time when the E2F-regulated genes are actively transcribing.

p27 inhibits cell cycle progression by binding to cdk2. Serum deprivation of fibroblasts or treatment of mink lung cells with transforming growth factor β (TGF-β) causes an increased binding of p27 to cdk2 (Reynisdottir *et al.*, 1995; Coats *et al.*, 1996). Results presented here suggest that one of the consequences of p27–cdk2 interaction is a disruption of the interaction between cdk2 and the E2F–p130 complex (and E2F–p107 complex), leading to an accumulation of repressor complexes of E2Fs. The repressor complexes observed in this study involved p130 and p107 because in NIH 3T3 cells the p130 and p107 pathways are easily detectable and the Rb-containing complex of E2F is not easily detectable. Therefore, in an appropriate setting one might be able to detect an effect of p27 on the accumulation of the Rb–E2F complex. p27-mediated accumulation of the E2F–p130 complex might be physiologically relevant because serum-starved fibroblasts contain higher levels of p27 and the E2F–p130 complex compared with cycling cells (cf. Figures 3A and 4, also see Coats *et al.*, 1996). It is possible that the increase in the level of p27 during serum starvation is responsible for the accumulation of the E2F–p130 repressor complex.

An effect on the E2F complexes predicts that the expression of the E2F-regulated genes will be altered. DHFR is one of the most well-characterized E2F-regulated genes (Blake *et al.*, 1989; Means *et al.*, 1992; Slansky *et al.*, 1993). In NIH 3T3 cells, the *DHFR* gene is expressed at the G_1-S-phase boundary, which correlates with a decrease in p27 expression (see Slansky *et al.*, 1993 and see Figure 4, lanes without IPTG). Ectopic expression of p27 at the G_1-S-phase boundary caused a reduction of the DHFR mRNA. These observations suggest that the expression of the *DHFR* gene can be controlled by p27. Moreover, repression of the *DHFR* gene correlated with an accumulation of the E2F–p130 repressor complex. This result implied that the E2F-regulated genes might be specific targets of the p27 growth suppression pathway.

Recent studies indicated that the promoter regions of the *cyclins E* and *A* genes contain E2F-binding sites (Ohtani *et al.*, 1995). Moreover, these genes can be activated by a recombinant adenovirus that expresses E2F-1 (DeGregori *et al.*, 1995; Ohtani *et al.*, 1995). Therefore, we analyzed the effects of p27 on the expression of these genes. Expression of p27 caused a reduction in the levels of cyclins E and A mRNAs (Figure 5). This observation is consistent with the notion that p27 reduces expression of the E2F-regulated genes. More interestingly, it provides evidence for a regulatory circuit in which p27, by inhibiting function of cyclin/cdks, reduces the expression of the cyclin genes that are critical for progression into S-phase.

The accumulation of the repressor complexes of E2F and the inhibition of the E2F-regulated genes suggest that p27 reduces transcription of the E2F-regulated genes. This notion is supported by the observation that, in transient transfection assays, expression of p27 reduces transcription from promoters containing E2F-binding sites. Promoters lacking E2F-binding sites are much less sensitive to p27 inhibition compared with the E2F sites containing promoter. Moreover, two mutants of p27 that harbor substitutions in the cyclin- or cdk kinase-binding motifs are impaired in inhibiting transcription from the promoter containing E2F-binding sites. These mutants were also deficient in disrupting the interaction between cyclin/cdk2 and the E2F–p130 or E2F–p107 complex. Therefore, the mutants that are unable to generate the repressor complexes of E2F are also impaired in inhibiting E2F-regulated transcription. These experiments, thus, provide further evidence for a link between the p27-mediated accumulation of the repressor complex of E2F and inhibition of the expression of the E2F-regulated genes.

The mechanism by which p27 disrupts the interaction between cyclin-cdk2 and the E2F–p130 or E2F–p107 complex does not involve a phosphorylation event because the disruption was observed in the presence of an excess of the nonhydrolyzable analogue of ATP (our unpublished observations). The observation that both cyclin- and cdk2-binding motifs in p27 are required for the disruption process is consistent with our studies on p21. p21, unlike p27, contains a second cyclin-binding motif near the C terminus (Adams *et al.*, 1996; Chen *et al.*, 1996; Ball *et al.*, 1997). However, we observed that the N-terminal cyclin-binding site and the cdk2 motif in p21 are sufficient to disrupt the interaction between the cyclin-cdk2 and the repressor forms of E2F (Robles, Shiyanov, Raychaudhuri, and Adami, unpublished results, also see Foteder *et al.*, 1996). The E2F complexes studied here contain E2F4 and E2F5. These two members of the E2F family, unlike E2F1, E2F2, and E2F3, lack any cyclin-binding motif (Krek *et al.*, 1994). Cyclin-cdk2 associate with E2F4 and E2F5 through their interactions with p130 or p107. p130 and p107 contain a cyclin-binding motif which is homologous to that found in p21 or p27 (Zhu *et al.*, 1995; Adams *et al.*, 1996; Shiyanov *et al.*, 1996). Therefore, the cyclin-binding motif in p27 or p21 should be able to compete with p107 or p130 for an interaction with the cyclins, which might be involved in the mechanism of disruption of the E2F complexes described in this study. This model is also consistent with the observation of Adams *et al.* (1996), who showed that a synthetic peptide corresponding to the cyclin A-binding motif in E2F1 could

inhibit the interaction between the cyclins and substrates such as E2F1 and the Rb family of proteins. However, our studies with mutant p27 indicate a role of the cdk2-binding motif, in addition to the cyclin-binding motif, in the disruption of the interaction between E2F–p130 and the cyclin–cdk2 complex. The difference may be a reflection of the fact that we analyzed the effect of p27 on a preformed cellular enzyme–substrate complex. It appears that in the context of the E2F–p130–cyclin–cdk2 or E2F–p107–cyclin–cdk2 complex, an effective competition, which leads to a disruption of the complex, depends upon retention of both cyclin and cdk2 motifs in p27. We propose that p27 transiently interacts with the E2F–p130–cyclin–cdk2 complexes by binding to cdk2, which localizes the cyclin-binding motif in p27 in the vicinity of the cyclin-p130 interface within the complex. The transient interaction is followed by a competition between p130 and p27 for cyclin binding, leading to a dissociation of the E2F–p130–cyclin–cdk2 complex. This scenario does not readily account for the observation that more than a stoichiometric amount of p27 is needed for its inhibition function (Zhang *et al.*, 1994; LaBaer *et al.*, 1997). It is possible that p27 forms multimers, which would be a more effective competitor. However, evidence for multimeric p27 is not available. It is also possible that an excess of p27 is needed to maintain the equilibrium in favor of the dissociation of the enzyme–substrate complex. Clearly, further biochemical studies will be necessary to determine the mechanism by which p27 dissociates the interaction between cyclin-cdk2 and the E2F–p130 complex.

The results presented here also provide further insights into the mechanism by which p27 controls the entry and progression through the cell cycle. It was shown that the ability of growth factors to stimulate progression of mouse BALB/c-3T3 fibroblasts through the restriction point correlated with their ability to reduce expression of p27 (Coats *et al.*, 1996). It is to be noted, however, that additional mechanisms exist that control the activity of p27 in G_1. Poon *et al.* (1995), by using Swiss 3T3 cells, demonstrated that the growth factor-induced expression of cyclin D is sufficient to titrate out all available p27 in the form of the cyclin D–cdk–p27 complex. Nevertheless, it is generally believed that, in the absence of growth stimulus, p27 plays an important role in maintaining a G_0 state by inhibiting the residual cdk kinases (Poon *et al.*, 1995). The observation that p27 inhibits cyclin gene expression provide evidence for an additional mechanism by which p27 controls entry and progression through the cell cycle. A p27-mediated inhibition of *cyclin E* and *cyclin A* gene expression would lower the levels of available cyclins needed to be inhibited by p27, thus allowing a more effective control of the cell cycle progression. Moreover, the observation that p27 induces accumulation of the repressor complex of E2F with a consequent loss of *cyclin E* and *cyclin A* gene expression suggests a role for the repressor complexes of E2F in controlling entry into the cell cycle. We suggest that the repressor complexes of E2F are downstream mediators in the mechanism by which p27 controls entry into the cell cycle or controls the G_1 restriction point in immortalized mouse fibroblast. This will also be consistent with the observation that ectopic expression of E2F-1 in quiescent fibroblasts induced S-phase entry without requiring any growth factor (Johnson *et al.*, 1993). Moreover, a viral oncoprotein that dissolves the repressor complexes of E2F could overcome G_1 arrest induced by serum starvation (Morozov *et al.*, 1997).

ACKNOWLEDGMENTS

We thank Dr. T. Hunter (The Salk Institute) for the cDNA clone of mouse p27. The authors are also grateful to Drs. S. Bagchi, R. Costa, and G. Adami at the University of Illinois for critically reviewing the manuscript. This work was supported by grants from the American Cancer Society to P.R. (ACS-VM-138 and ACS-BE219) and a grant from the National Institutes of Health (CA-52220) to L.F.L.

REFERENCES

Adams, P.D., Sellers, W.R., Sharma, S.K., Wu, A.D., Nalin, C.M., and Kaelin, W.G., Jr. (1996). Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol. Cell. Biol. *16*, 6623–6633.

Afshari, C.A., Nichols, M.A., Xiong, Y., and Mudryj, M. (1996). A role for a p21–E2F interaction during senescence arrest of normal human fibroblasts. Cell Growth & Differ. *7*, 979–988.

Arroyo, M., Bagchi, S., and Raychaudhuri, P. (1993). Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. Mol. Cell. Biol. *13*, 6537–6546.

Ball, K.L., lain, S., Fahraeus, R., Smythe, C., and Lane, D.P. (1997). Cell-cycle arrest and inhibition of cdk4 activity by small peptides based on the carboxy-terminal domain of p21WAF1. Curr Biol. *1*, 71–80.

Beijersbergen, R.L., Kerkhoven, R.M., Zhu, L., Carlee, L., Voorhoeve, P.M., and Bernards, R. (1994). E2F-4, a new member of the *E2F* gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. *8*, 2680–2690.

Blake, M., and Azizkhan, J.C. (1989). Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. *9*, 4994–5002.

Chen, J., Saha, P., Kornbluth, S., Dynlacht, B.D., and Dutta, A. (1996). Cyclin-binding motifs are essential for the function of p21Cip1. Mol. Cell. Biol. *16*, 4673–4682.

Coats, S., Flanagan, W.M., Nourse, J., and Roberts, J.M. (1996). Requirement of $p27^{kip1}$ for restriction point control of the fibroblast cell cycle. Science *272*, 877–880.

Cobrinik, D., Whyte, P., Peeper, D., Jacks, T., and Weinberg, R. (1993). Cell cycle-specific association of E2F with p130 E1A-binding protein. Genes Dev. *7*, 2392–2404.

DeGregori, J., Kowalik, T., and Nevins, J.R. (1995). Cellular target for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol. Cell. Biol. *15*, 4215–4224.

Dynlacht, B.D., Flores, O., Lees, J.A., and Harlow, E. (1994). Differential regulation of E2F trans-activation by cyclin/cdk2 complexes. Genes Dev. *8*, 1772–1786.

Fero, M.L., *et al.* (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{kip1}$-deficient mice. Cell *85*, 733–744.

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

Foteder, R., Fitzgerald, P., Rouselle, T., Cannella, D., Doree, M., Messier, H., and Foteder, A. (1996). p21 contains independent binding sites for cyclin and cdk2: both sites are required to inhibit cdk2 kinase activity. Oncogene *12*, 2155–2164.

Johnson, D.G., Schwarz, J.K., Cress, W.D., and Nevins, J.R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349–352.

Ikeda, M., Jakoi.L., and Nevins, J.R. (1996). A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA *93*, 3215–3220.

Kato, J., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. (1994). Cyclin AMP-induced G1 phase arrest mediated by an inhibitor ($p27^{kip1}$) of cyclin-dependent kinase 4 activation. Cell *79*, 487–496.

Kiyokawa, H., *et al.* (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of $p27^{kip1}$. Cell *85*, 721–732.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J., and Massague, J. (1993). Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-β. Science *260*, 536–539.

Krek, W., Ewen, M.E., Shirodkar, S., Arany, Z., Kaelin, W.G., and Livingston, D.M. (1994). Negative regulation of the growth promoting transcription factor E2F1 by a stably bound cyclin A-dependent protein kinase. Cell *78*, 161–172.

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

Labow, M.A., Baim, S.B., Shenk, T., and Levine, A.J. (1990). Conversion of the lac repressor into an allosterically regulated transcriptional activator for mammalian cells. Mol. Cell. Biol. *10*, 3343–3356.

Lee, M.-H., Mori, S., and Raychaudhuri, P. (1996). Trans-activation by the hnRNP K protein involves an increase in RNA synthesis from the reporter genes. J. Biol. Chem. *271*, 3420–3427.

Means, A.L., Slansky, J.E., McMahon, S.L., Knuth, M.W., and Farhnam, P.J. (1992). The HIP1 binding site is required for growth regulation of the dihydrofolate reductase gene promoter. Mol. Cell. Biol. *12*, 1054–1063.

Moberg, K., Starz, M.A., and Lees, J.A. (1996). E2F4 switches from p130 to p107 and pRb in response to cell cycle entry. Mol. Cell. Biol. *16*, 1436–1449.

Morozov, A., Phelps, W.C., and Raychaudhuri, P. (1994). Activation of the c-*fos* gene by the HPV16 oncoproteins depends upon the cAMP-response element at −60. J. Biol. Chem. *269*, 18434–18440.

Morozov, A., Subjeck, J., and Raychaudhuri, P. (1995). HPV16 E7 oncoprotein induces expression of a 110 kD heat shock protein. FEBS Lett. *371*, 214–218.

Morozov, A., Shiyanov, P., Barr, E., Leiden, J.M., and Raychaudhuri, P. (1997). Accumulation of the HPV16 E7 protein bypasses G_1 arrest induced by serum-starvation and by the cell cycle inhibitor p21. J. Virol. *71*, 3451–3457.

Nevins, J.R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science *258*, 424–429.

Nourse, J., Firpo, E., Flanagan, M., Meyerson, M., Polyak, K., Lee, M.-H., Massague, J., Crabtree, G., and Roberts, J. (1994). IL-2 mediated elimination of the $p27^{kip1}$ cyclin-Cdk kinase inhibitor prevented by rapamycin. Nature *372*, 570–573.

Ohtani, K., DeGregori, J., and Nevins, J.R. (1995). Regulation of the *cyclin E* gene by transcription factor E2F1. Proc. Natl. Acad. Sci. USA *92*, 12146–12150.

Pestov, D.G., and Lau, L.F. (1994). Genetic selection of growth-inhibitory sequences in mammalian cells. Proc. Natl. Acad. Sci. USA *91*, 12549–12553.

Polyak, K., Kato, J.-Y., Solomon, M.J., Sherr, C., Massague, J., Roberts, J.M., and Koff, A. (1994a). $P27^{kip1}$, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. *8*, 9–22.

Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J., Tempst, P., and Massague, J. (1994b). Cloning of $p27^{kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*, 59–66.

Poon, R.Y.C., Toyoshima, H., and Hunter T. (1995). Redistribution of the CDK inhibitor between cyclin-CDK complexes in the mouse fibroblast cell cycle and in cells arrested with Lovastatin or ultraviolet irradiation. Mol. Biol. Cell *6*, 1197–1213.

Reynisdottir, I., Polyak, K., Lavarone, A., and Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Dev. *9*, 1831–1845.#

Roberts, J.M., Koff, A., Polyak, K., Firpo, E., Colins, S., Ohtsubo, M., and Massague, J. (1994). Cyclins, Cdks, and cyclin kinase inhibitors. Cold Spring Harbor Symp. Quant. Biol. *59*, 31–38.

Sardet, C., Vidal, M., Cobrinik, C., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R. (1995). E2F-4, and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA *92*, 2403–2407.

Sellers, W.R., Rodgers, J.W., and Kaelin, W.G., Jr. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA *92*, 11544–11548.

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

Shiyanov, P., Bagchi, S., Adami, G.R., Kokontis, J., Hay, N., Arroyo, M., Morozov, A., and Raychaudhuri, P. (1996). P21 disrupts the interaction between cdk2 and the E2F–p130 complex. Mol. Cell. Biol. *16*, 737–744.

Slansky, J.E., Li, Y., Kaelin, W.G., and Farnahm, P.J. (1993). A protein synthesis dependent increase in E2F1 mRNA correlates with growth regulation of the DHFR promoter. Mol. Cell. Biol. *13*, 1610–1618.

Slingerland, J., Hengst, L., Pan, C., Alexander, D., Stampfer, M., and Reed, S. (1994). A novel inhibitor of cyclin-cdk activity detected in transforming growth factor b-arrested epithelial cells. Mol. Cell. Biol. *14*, 3683–3694.

Smith, E.J., Leone, G., DeGregori, J., Jakoi, L., and Nevins, J.R. (1996). The accumulation of an E2F-p130 transcriptional repressor distinguishes a G_0 cell state from a G_1 cell state. Mol. Cell. Biol. *16*, 6965–6976.

Starostik, P., Chow, K.N.B., and Dean, D. (1996). Transcriptional repression and growth suppression by the p107 pocket protein. Mol. Cell. Biol. *16*, 3606–3614.

Sudgen, B., Marsh, K., and Yates, J. (1985). A vector that replicates as a plasmid and can be efficiently selected in B-Lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. *5*, 410–413.

Toyoshima, H., and Hunter, T. (1994). P27, a novel inhibitor of G_1 cyclin-Cdk protein kinase activity, is related to p21. Cell *78*, 67–74.

Vairo, G., Livingston, D.M., and Ginsberg, D. (1995). Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma family members. Genes Dev. *9*, 869–881.

Vindelov, L.L., Christensen, I.J., and Nissen, N.I. (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry *3*, 323–327.

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

Weintraub, S.J., Chow, K.N.B., Luo, R.X., Zhang, S.H., He, S., and Dean, D. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. Nature *375*, 812–815.

Zerfass-Thome, K., Schulze, A., Zwerschke, W., Vogt, B., Helin, K., Bartek, J., Henglein, B., and Jansen-Durr, P. (1997). p27Kip1 blocks cyclin E-dependent transactivation of cyclin A gene expression. Mol. Cell. Biol. *17*, 407–415.

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

Zhu, L., Harlow, E., and Dynlacht, B.D. (1995). P107 uses a p21 CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. Genes Dev. *9*, 1740–1752.

Zhu, L., van den Huevel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D.M., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. *7*, 1111–1125.