Molecular Biology of the Cell
Vol. 6, 1197–1213, September 1995

Redistribution of the CDK Inhibitor p27 between Different Cyclin·CDK Complexes in the Mouse Fibroblast Cell Cycle and in Cells Arrested with Lovastatin or Ultraviolet Irradiation

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Submitted January 31, 1995; Accepted June 30, 1995
Monitoring Editor: Tim Hunt

The cyclin-dependent kinase (CDK) inhibitor p27 binds and inhibits the kinase activity of several CDKs. Here we report an analysis of the behavior and partners of p27 in Swiss 3T3 mouse fibroblasts during normal mitotic cell cycle progression, as well as in cells arrested at different stages in the cycle by growth factor deprivation, lovastatin treatment, or ultraviolet (UV) irradiation. We found that the level of p27 is elevated in cells arrested in G0 by growth factor deprivation or contact inhibition. In G0, p27 was predominantly monomeric, although some portion was associated with residual cyclin A·Cdk2. During G1, all of p27 was associated with cyclin D1·Cdk4 and was then redistributed to cyclin A·Cdk2 as cells entered S phase. The loss of the monomeric p27 pool as cyclins accumulate in G1 is consistent with the in vivo and in vitro data showing that p27 binds better to cyclin·CDK complexes than to monomeric CDKs. In growing cells, the majority of p27 was associated with cyclin D1 and the level of p27 was significantly lower than the level of cyclin D1. In cells arrested in G1 with lovastatin, cyclin D1 was degraded and p27 was redistributed to cyclin A·Cdk2. In contrast to p21 (which is a p27-related CDK inhibitor and is induced by UV irradiation), the level of p27 was reduced after UV irradiation, but because cyclin D1 was degraded more rapidly than p27, there was a transient increase in binding of p27 to cyclin A·Cdk2. These data suggest that cyclin D1·Cdk4 acts as a reservoir for p27, and p27 is redistributed from cyclin D1·Cdk4 to cyclin A·Cdk2 complexes during S phase, or when cells are arrested by growth factor deprivation, lovastatin treatment, or UV irradiation. It is likely that a similar principle of redistribution of p27 is used by the cell in other instances of cell cycle arrest.

INTRODUCTION

The eukaryotic cell cycle is driven by cyclin-dependent kinases (CDK)[1] in association with specific activating subunits known as cyclins. The archetypal CDK family member Cdc2 is mainly associated with B-type cyclins and regulates M phase (King *et al.*, 1994). Cdk2 is associated with cyclin E and cyclin A, and cyclin E·Cdk2 and cyclin A·Cdk2 complexes are believed to control the G1-S transition and S phase, respectively (Heichman and Roberts, 1994; Sherr, 1994). Cdk4 and Cdk6 are associated with the D-type cyclins and are important for G1 progression (Sherr, 1994).

CDKs are regulated by an intricate network of positive and negative regulators (Morgan, 1995). CDK kinase activity depends absolutely on binding to a cyclin subunit. On binding to cyclin, the cyclin·CDK complex has only very low kinase activity (Connell-

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[1] Abbreviations used: BSA, bovine serum albumin; CAK, CDK-activating kinase; CDK, cyclin-dependent kinase; FACS, fluorescence-activated cell sorter; GSH, glutathione; GST, glutathione-*S*-transferase; PA, *Staphylococcus aureus* protein A; PCNA, proliferating cell nuclear antigen; Rb, retinoblastoma gene product; UV, ultraviolet.

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Crowley *et al.*, 1993); full CDK activation requires phosphorylation on a threonine residue in the CDK catalytic domain. Phosphorylation of this threonine residue is mediated by a CDK-activating kinase (CAK) (Poon *et al.*, 1993; Solomon *et al.*, 1993; Fisher and Morgan, 1994; Mäkelä *et al.*, 1994; Matsuoka *et al.*, 1994). CDKs are negatively regulated by phosphorylation on T14 and Y15. The protein kinase that phosphorylates T14 is unknown, but there is evidence that it is a membrane-associated kinase (Kornbluth *et al.*, 1994). Y15 is phosphorylated by Wee1 and dephosphorylated by Cdc25 (Coleman and Dunphy, 1994).

Recently, a new class of protein inhibitors has been discovered that can bind to CDKs and inhibit their kinase activity; these inhibitors may constitute a further mechanism that regulates CDK activity and cell cycle progression (Elledge and Harper, 1994; Hunter and Pines, 1994). The mammalian CDK inhibitors identified so far fall into two classes based on sequence homology: those that are related to p16 and those that are related to p21. The CDK inhibitor p16 was identified as a Cdk4-associated protein in some transformed cells (Serrano *et al.*, 1993) and found to be a specific inhibitor for Cdk4 and Cdk6 (Hannon and Beach, 1994). p16 appears to inhibit Cdk4 and Cdk6 by displacing cyclin D (Parry *et al.*, 1995). The p16 (*MTS1*) locus is altered at high frequency in tumor cell lines and at a lower but significant frequency in primary tumors (Hunter and Pines, 1994). Recently, another p16-related CDK inhibitor p15 has been identified and shown to be specific for Cdk4 and Cdk6 (Hannon and Beach, 1994). Unlike p16, the abundance of p15 is increased more than 30-fold after TGFβ treatment of HaCaT keratinocytes (Hannon and Beach, 1994). Two further members of the p16 family, p18 and p19, were recently identified and are able to bind and inhibit Cdk4 and Cdk6 (Guan *et al.*, 1994; Chan *et al.*, 1995; Hirai *et al.*, 1995). The level of p19 is low in G1 and peaks in S phase (Hirai *et al.*, 1995).

The CDK inhibitor p21 (also called Cap1, Cip1, Pic1, Sdi1, and WAF1) was identified in several different ways. It was found as a CDK-associated protein with inhibitory activity toward several CDKs (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a), as a gene induced by the tumor suppressor p53 (El-Deiry *et al.*, 1993), and as a mRNA that increased in abundance in senescent cells (Noda *et al.*, 1994). p21 inhibits a wide variety of cyclin·CDK complexes including cyclin D·Cdk4/6 and cyclin A/E·Cdk2 complexes. Unlike the p16 family of inhibitors, p21 does not appear to inhibit CDK by displacing the cyclin subunit, and more than one molecule p21 may be needed for the inhibition of a single CDK molecule (Zhang *et al.*, 1994; Harper *et al.*, 1995). p21 has also been shown to bind to proliferating cell nuclear antigen (PCNA) and to inhibit DNA replication (Waga *et al.*, 1994), but DNA repair is not affected by p21 (Li *et al.*, 1994a). DNA damage caused by agents like irradiation increases the amount of p53, which would then increase the amount of p21 and in turn inhibit the kinase activity of cyclin D·Cdk4/6 and cyclin A/E·Cdk2, stopping entry into S phase (Dulic *et al.*, 1994; El-Deiry *et al.*, 1994; Li *et al.*, 1994b).

The CDK inhibitor p27 (also called Kip1) is 42% identical to p21 in its N-terminal region (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). Like p21, p27 inhibits a wide variety of cyclin·CDK complexes, and overexpression of p27 blocks cell cycle progression in G1 phase (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). p27 appears to be involved in cell cycle arrest caused by TGFβ treatment in some cell lines (Polyak *et al.*, 1994a). TGFβ has been shown to repress cyclin D or Cdk4 synthesis (Ewen *et al.*, 1993; Ko *et al.*, 1995) and to induce $p15^{INK4B}$ synthesis (Hannon and Beach, 1994); this may release p27 that was bound to cyclin D·Cdk4 and allow it to inhibit cyclin E·Cdk2. Similarly, a p27-like inhibitor has been implicated in cell cycle arrest caused by cell-cell contact and lovastatin (Hengst *et al.*, 1994; Slingerland *et al.*, 1994). In murine macrophages blocked in G1 by cAMP, the level of p27 is increased and apparently blocks the activating phosphorylation of Cdk2 by CAK (Kato *et al.*, 1994). Recently, it has been reported that during interleukin-2–induced T lymphocyte mitogenesis the level of p27 is high in quiescent cells but diminishes to undetectable levels as cells enter the cell cycle (Nourse *et al.*, 1994), suggesting that p27 may control the kinase activity of CDKs during the transition from quiescence to S phase in T lymphocytes.

Another p21/p27-related CDK-inhibitor $p57^{Kip2}$ has recently been cloned and was found mainly in terminally differentiated cells (Lee *et al.*, 1995; Matsuoka *et al.*, 1995).

Here we report the level and behavior of p27 in Swiss 3T3 mouse fibroblasts during the normal mitotic cell cycle and in cells arrested in G0 by growth factor deprivation or contact inhibition, in G1 by lovastatin, and at multiple points of the cell cycle by UV irradiation. In growing cells, p27 was predominantly associated with cyclin D1·Cdk4. During normal cell cycle progression, p27 was associated with cyclin D1·Cdk4 in G1, and on entry into S, cyclin D1 dissociated from Cdk4 and p27 was released to form a complex with cyclin A·Cdk2. Upon progression through M phase, p27 was dissociated from cyclin A·Cdk2 and then reassociated with cyclin D1·Cdk4 in the next G1. In all three cell cycle–arrested states we studied, the proportion of p27 that was bound to cyclin A·Cdk2 was elevated, due to a decrease in cyclin D1 and redistribution of p27 to cyclin A·Cdk2. Our data show that p27 is redistributed between Cdk4- and Cdk2-cyclin complexes in normal cell cycle progression and upon cell cycle arrest, and thus p27 may contribute to the

control of different CDK activities at different points in the cell cycle.

MATERIALS AND METHODS

Constructs

GST-cdk2 K33R in pGEX-2T and H10-PA-cyclin A in pET16b were constructed as described (Poon *et al.*, 1993). PA-cyclin D3-H6 in pET21b was constructed by amplification of the cyclin D3 coding region from a human cyclin D3 clone (provided by K. Yamashita) with polymerase chain reaction using the following primers: 5′-GGGGATCCCATGGAGCTGCTGTGTTGCGA-3′ and 5′-CCAGGGCTCGAGGTGTATGGCTGTGACATC-3′. The polymerase chain reaction fragment was cut with *Bam*HI and *Xho*I, and ligated into *Bam*HI- and *Xho*I-cut PA-H6 in pET21b (Poon and Hunt, 1994). Human p27-H6 in pET21a was kindly provided by J. Massagué. GST-Rb was provided by M. Howell. For in vitro translation in a mRNA-dependent rabbit reticulocyte lysate, *Xenopus* cdk4 in pET16b was provided by M. Cockerill and *Xenopus* cdk2 in pET8c was as described (Kobayashi *et al.*, 1991). GST-p27 was constructed as described (Toyoshima and Hunter, 1994).

Expression and Purification of Recombinant Proteins

Expression and purification of histidine-tagged proteins from bacteria with nickel-NTA-agarose chromatography was as described (Poon and Hunt, 1994). Expression and purification of GST-fusion proteins from bacteria using GSH-Sepharose chromatography was as described (Poon *et al.*, 1993)

Growth and Synchronization of Swiss 3T3 Cells

Swiss 3T3 fibroblasts were grown in 15-cm plates in DMEM supplemented with 10% v/v fetal calf serum in a humidified incubator at 37°C, in 10% CO_2 until the cultures reached about 50% confluence. The medium was then changed to DMEM with 0.2% v/v fetal calf serum for 72 h. Cells were released from the G0 arrest by supplying the cells with DMEM containing 10% v/v fetal calf serum. Samples were taken at 3-h intervals for 27 h after release. At each time point, the cells were washed with phosphate-buffered saline (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) and scraped off the plates. The cells were harvested by centrifugation and mixed with twice the pellet volume of a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2% Nonidet P-40, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine, 10 μg/ml pepstatin, and 10 μg/ml soybean trypsin inhibitor. The suspension was incubated at 4°C for 45 min and cell debris was removed by centrifugation in a microfuge at 4°C for 30 min. The protein concentration of the cell lysate was measured with bicinchoninic acid protein assay system (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard.

Swiss 3T3 cells were arrested at M phase by treating 50% confluent cells with fresh DMEM medium containing 0.1 μg/ml nocodazole for 12 h. To harvest the M phase-arrested cells, the medium was removed and 10 ml of phosphate-buffered saline was added to the plate followed by vortexing for 2 min. The cells in the buffer were collected by centrifugation and replated in fresh medium. Lovastatin treatment of Swiss 3T3 cells was modified from the method described by Keyomarsi *et al.* (1991). Semi-confluent cells were incubated with fresh DMEM medium containing 20 μM lovastatin for 36 h, and the cells were then harvested and extracts were prepared as described above.

UV-Irradiation of Cells

Swiss 3T3 or $p53^{-/-}$ mouse embryonic fibroblasts were grown to semi-confluence in DMEM supplemented with 10% v/v fetal calf serum in a humidified incubator at 37°C in 10% CO_2. The medium was removed and the cells were irradiated with 50 J/m^2 of UV in a Stratalinker UV crosslinker apparatus (Stratagene, La Jolla, CA). Fresh medium was then added to the cells and cell extracts were prepared at different time points as described above.

Gel Filtration Chromatography

Samples (200 μl) were applied onto a Superose 12 HR10/30 column (Pharmacia, Piscataway, NJ) and subjected to fast-performance liquid chromatography with the buffer 50 mM NH_4HCO_3, pH 7.5, 5 mM NaCl, 0.1 mM EDTA with a flow rate of 0.4 ml/min at 4°C. Fractions were collected at 1 fraction/min. The fractions were lyophilized in a Speedvac and dissolved in 30 μl of sodium dodecyl sulfate (SDS)-sample buffer. Molecular mass standards for the gel filtration column were as follows: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), and soybean trypsin inhibitor (31 kDa).

Histone H1 Kinase, Rb Kinase, and CAK Assays

Immunoprecipitates equilibrated with kinase buffer (80 mM Na-β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $Mg(OAc)_2$, and 1 mM dithiothreitol) were mixed with 10 μl kinase buffer containing 50 μM ATP, 1.25 μCi [γ-^{32}P]ATP, 1 μg histone H1, or 2 μg of GST-Rb. The samples were incubated at 23°C for 30 min and the reactions were terminated by addition of 30 μl of SDS-sample buffer. CAK assays were performed by immunoprecipitation with anti-Cdk7(MO15) antibody followed by phosphorylation of a kinase-inactive mutant of GST-cdk2 (K33R) as described before (Poon *et al.*, 1994). The samples were subjected to SDS-poylacrylamide gel electrophoresis (PAGE) and phosphorylation was quantitated with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Antibodies and Immunological Methods

Anti-Cdc2 monoclonal antibody was raised against the C-terminal half of *Xenopus* Cdc2 as described by Kobayashi *et al.* (1991); this antibody also recognizes mouse Cdc2. Rabbit anti-Cdk2 antiserum, rabbit anti-cyclin B1 antiserum, and rabbit anti-Cdk7(MO15) antiserum were as described (Poon *et al.*, 1994). Rabbit anti-mouse Cdk4 antisera were gifts from C. Sherr, H. Kiyokawa, and P. Marks. Anti-cyclin D1 and anti-cyclin D2 monoclonal antibodies were kindly provided by C. Sherr. Rabbit anti-p21 antisera were obtained from M. Howell and from PharMingen (San Diego, CA). Rabbit anti-cyclin E antiserum was a gift from J. Roberts. Anti-cyclin A monoclonal antibody was obtained from J. Gannon. Anti-p53 monoclonal antibody (Ab122) was provided by Amy Tsou. Rabbit anti-mouse p27 antisera were either raised against the C-terminal peptide of mouse p27 or against full length GST-p27 (Toyoshima and Hunter, 1994). Anti-full length GST-p27 antiserum was used in this paper unless stated otherwise.

Immunoblotting was performed as previously described using enhanced chemiluminescence detection (Amersham, Arlington Heights, IL) (Poon *et al.*, 1993), except for immunoblotting of immunoprecipitated samples, in which both immunoprecipitation and immunoblotting were performed with antibodies raised in rabbits, horseradish peroxidase-conjugated protein A (Amersham) was used instead of secondary antibodies, and 10 mM *N*-ethyl maleimide was used instead of 2-mercaptoethanol in the SDS-sample buffer. Secondary antibodies were as follows: horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibodies were obtained from Amersham. Immunoprecipitation was performed as previously described (Poon *et al.*, 1993).

For quantitation of p27, cyclins, and CDKs, the corresponding proteins were overexpressed in *Escherichia coli* and quantitated after SDS-gel electrophoresis by staining with Coomassie and scanning the stained gel with a densitometer using serially diluted BSA

samples as standards. The amounts of the endogenous proteins in Swiss 3T3 cell extracts were then quantitated by immunoblotting by comparison with serial dilutions of the bacterially expressed protein standards run on the same gel. All the antibodies, including the anti-p27 protein antiserum, gave a linear dose response signal over the range of Swiss 3T3 cell protein concentrations used.

Immunodepletion was performed on 400 μg of cell extract. The cell extract was diluted to 100 μl in bead buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 2 μg/ml aprotinin, 15 μg/ml benzamidine, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor) and added to 15 μl of protein A-Sepharose or protein G-Sepharose prebound with 3 μl of antiserum or 1 μg of monoclonal antibody, respectively. After incubation at 4°C for 60 min with end-to-end rotation, the supernatant was removed and immunodepletion was repeated a further two times. After the third immundepletion, the supernatant was diluted to 1 mg/ml in SDS-sample buffer and 10 μl was loaded onto SDS-PAGE. The beads from each immunodepletion step were washed four times (250 μl each time) with bead buffer, dissolved in 30 μl of SDS-sample buffer, and 10 μl of this was applied to SDS-PAGE. In the course of doing these experiments, we found that when immunoblotting with some monoclonal antibodies, there can be a significant masking of the signal in total cell lysate in comparison to purified protein, such as that isolated in an immunoprecipitate (our unpublished observation). Thus, it may not be valid to make a direct quantitative comparison between a protein in total cell extract and after isolation by immunoprecipitation when using immunoblotting for detection.

RESULTS

p27 Binds to Different Cyclin-CDK Complexes in the Normal Cell Cycle of Swiss 3T3 Cells

To investigate possible roles for p27 in normal cell cycle control, the protein level and cyclin/CDK partners of p27 were examined in the Swiss 3T3 mouse fibroblast cell line in a quiescent state following serum starvation, and during cell cycle progression after serum stimulation. Swiss 3T3 cells in G0 were obtained by incubation in medium containing 0.2% fetal calf serum for 72 h. The cells were released into the cell cycle by supplying the cells with medium containing 10% fetal calf serum. Fluorescence-activated cell sorter (FACS) analysis of these cells at different times after serum stimulation showed that >85% of cells entered the cycle and that entry into S phase occurred reasonably synchronously (Figure 1A). Cell extracts were prepared from cells taken at different time points and the level of p27, various cyclins, and CDKs during the cell cycle were detected by immunoblotting with specific antibodies (Figure 1B). Cyclin B1 was present at a high level at 21–24 h after serum stimulation, and it was destroyed between 24 and 27 h. Together with the result from FACS analysis, this indicated that the cells went through one complete cell cycle synchronously. Cyclin A was synthesized slightly earlier than cyclin B1. Cyclin D1 was detected as early as 6 h, peaked at 12 h, and remained high during the rest of the cell cycle. Cyclin E peaked at 12–15 h after serum stimulation. No great fluctuation in the levels of Cdk2 and Cdk4 was detected in the cell cycle. The level of p27 was relatively high in G0 and decreased slowly after serum stimulation as cells progressed through G1, and was maintained without significant variation for the rest of the cell cycle (Figure 1B, top panel). We found that the rate of turnover of p27 is similar between G0 and G1, but the rate of synthesis of p27 was higher in G0 than in G1 (our unpublished observations). Similarly, no significant variation of the level of p27 was observed when growing Swiss 3T3 cells were blocked at G2/M with nocodazole and then released into the cell cycle by shake off and replating (Figure 1D). Essentially the same increase in p27 level was found in Swiss 3T3 cells arrested by contact inhibition (our unpublished observations).

Given that the level of p27 is essentially constant in the cell cycle (apart from an increase in G0), we next investigated whether the proteins that interact with p27 vary during the cell cycle. p27 was immunoprecipitated from equal amounts of cell extract from different time points after release from G0; cyclins and CDKs that associated with the p27 immunoprecipitates were detected by immunoblotting with specific antibodies. Immunoblotting with anti-Cdk4 antibody (Figure 1C, top panel) revealed that the amount of Cdk4 that bound to p27 was very low in G0. On entry into G1, the amount of Cdk4 that associated with p27 increased and peaked around 9–12 h; it then decreased to an undetectable level for the rest of the cell cycle. The p27-associated Cdk4 increased again when the cells exited from M phase and entered the next cycle (24–27 h). Immunoblotting with anti-cyclin D1 antibody revealed that the amount of cyclin D1 that associated with p27 followed a similar pattern to Cdk4 (Figure 1C) (multiple bands of cyclin D1 were usually observed after immunoprecipitation, probably due to protein degradation that occurred during immunoprecipitation). This periodic association between p27 and cyclin D1·Cdk4 was rather surprising, because the total level of cyclin D1 and Cdk4 did not fluctuate in a similar manner (Figure 1A, see below). Intriguingly, we found that in G0 cells, p27 was associated with Cdk2 and cyclin A (Figure 1C), despite the fact that the amounts of Cdk2 and cyclin A in the total G0 cell lysate were very low (Figure 1B) (but detectable when the immunoblots were overexposed). The amount of cyclin A and Cdk2 that bound to p27 diminished as cyclin D1·Cdk4 complexes appeared in G1, and peaked again at 21 h, roughly concomitant with the peak of cyclin A in the total cell lysate.

These data indicated that p27 could bind to the low amounts of cyclin A·Cdk2 that persisted in serum-starved cells. Presumably, the increased level of p27 and the lack of cyclin D1·Cdk4 in G0 cells contributed to the availability of p27 to bind to the residual cyclin A·Cdk2. Hence, p27 may be important for the inhibition of the cyclin·CDK complexes that linger after cells enter quiescence.

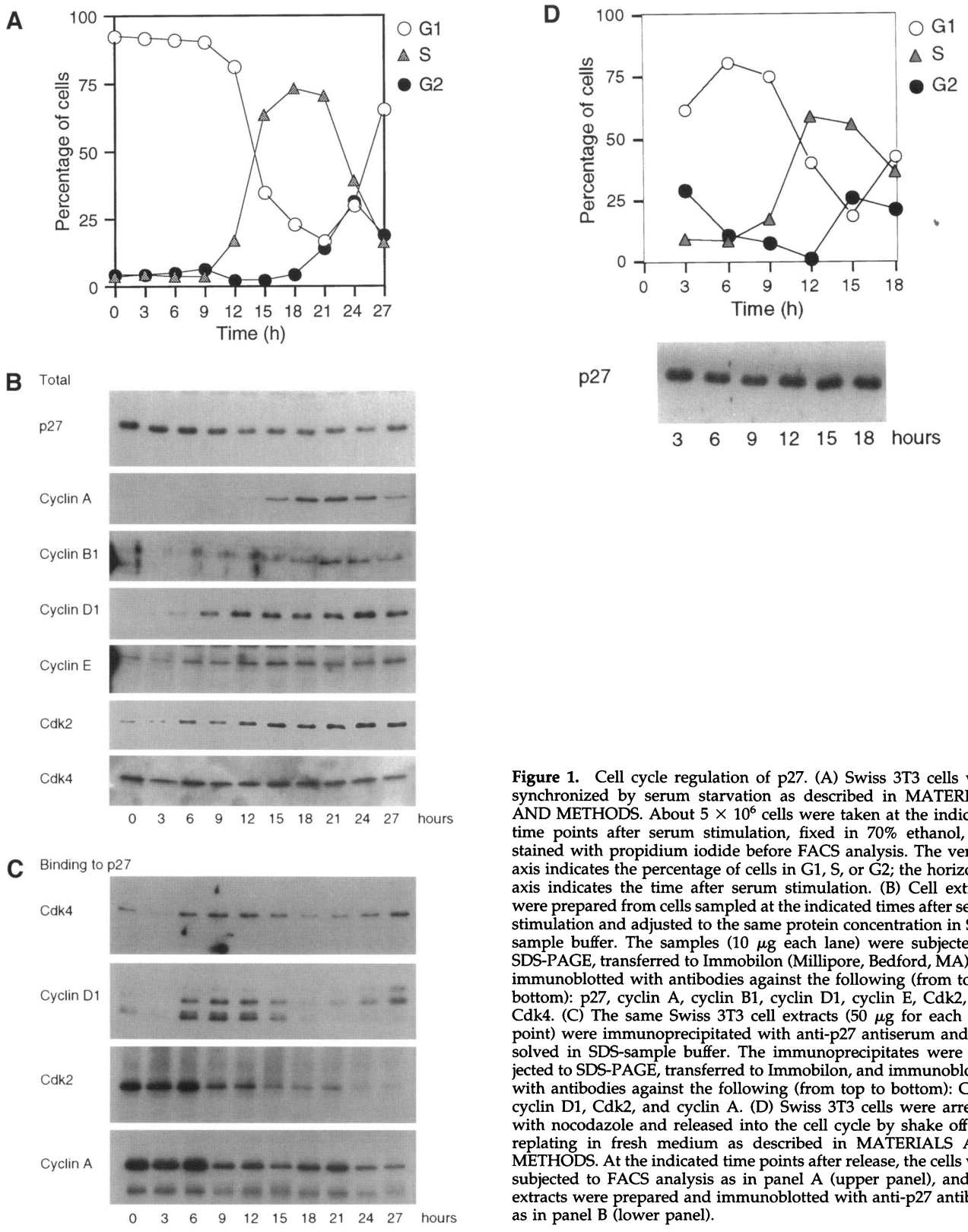

Figure 1. Cell cycle regulation of p27. (A) Swiss 3T3 cells were synchronized by serum starvation as described in MATERIALS AND METHODS. About 5×10^6 cells were taken at the indicated time points after serum stimulation, fixed in 70% ethanol, and stained with propidium iodide before FACS analysis. The vertical axis indicates the percentage of cells in G1, S, or G2; the horizontal axis indicates the time after serum stimulation. (B) Cell extracts were prepared from cells sampled at the indicated times after serum stimulation and adjusted to the same protein concentration in SDS-sample buffer. The samples (10 μg each lane) were subjected to SDS-PAGE, transferred to Immobilon (Millipore, Bedford, MA) and immunoblotted with antibodies against the following (from top to bottom): p27, cyclin A, cyclin B1, cyclin D1, cyclin E, Cdk2, and Cdk4. (C) The same Swiss 3T3 cell extracts (50 μg for each time point) were immunoprecipitated with anti-p27 antiserum and dissolved in SDS-sample buffer. The immunoprecipitates were subjected to SDS-PAGE, transferred to Immobilon, and immunoblotted with antibodies against the following (from top to bottom): Cdk4, cyclin D1, Cdk2, and cyclin A. (D) Swiss 3T3 cells were arrested with nocodazole and released into the cell cycle by shake off and replating in fresh medium as described in MATERIALS AND METHODS. At the indicated time points after release, the cells were subjected to FACS analysis as in panel A (upper panel), and cell extracts were prepared and immunoblotted with anti-p27 antibody as in panel B (lower panel).

p27 Has Higher Affinity for Cyclin-CDK Complex than for Monomeric CDK

Because there seemed to be a correlation between the amount of cyclin present and the binding of the corresponding cyclin·CDK complex to p27, we investigated whether the binding of CDKs to p27 is affected by cyclins in vitro. Cdk2 and Cdk4 were translated in an mRNA-dependent rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The translated Cdk2 and Cdk4 alone, or in complex with purified bacterially expressed PA-cyclin A or PA-cyclin D3, respectively, were tested for their ability to bind recombinant GST-p27. Figure 2 shows that Cdk2 and Cdk4 could bind to GST-p27 on their own, but the binding was much more efficient in the presence of the cyclin partners.

Although binding of Cdk4 to p27 was stimulated by cyclin D in vitro, and the total levels of cyclin D1 and Cdk4 remained high during S and G2, very little cyclin D1·Cdk4 was associated with p27 during these periods (Figure 1). But when we investigated the association between cyclin D1 and Cdk4 in the same cell cycle samples, we discovered that cyclin D1 was indeed dissociated from Cdk4 during S and G2 (Figure 3). This datum supports the notion that p27 is released from Cdk4 when cyclin D1 is dissociated from the complex, and the released p27 was then available to bind cyclin A·Cdk2 during S phase.

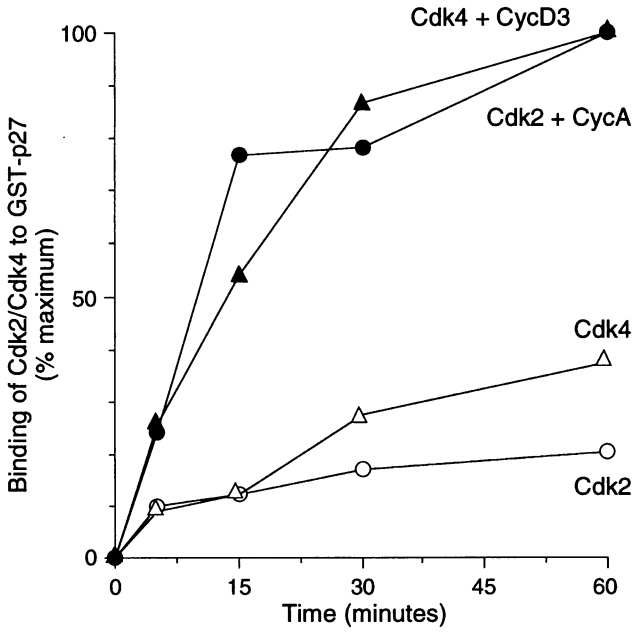

Figure 2. Cyclins and CDKs bind p27 cooperatively. Cdk2 and Cdk4 were translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The translated Cdk2 (2 μl of reaction mix) was mixed with either buffer or 100 ng of purified PA-cyclin A expressed in bacteria. Likewise the translated Cdk4 was mixed with either buffer or 100 ng purified PA-cyclin D3 expressed in bacteria. The mixture was incubated with 1 μg of GST-p27 prebound to GSH-agarose beads (10 μl) in 50 μl of buffer. At different time points, the beads were washed and dissolved in SDS-PAGE. The samples were applied to SDS-PAGE and the Cdk2 or Cdk4 that associated with GST-p27 was quantitated with a Phosphorimager. Symbols: Cdk2 (○); Cdk4 (△); Cdk2 with PA-cyclin A (●); and Cdk4 with PA-cyclin D3 (▲).

The Majority of p27 Is Free in G0 but Partnered in the Cell Cycle

To see what fraction of p27 is associated with other proteins in the cell cycle, we investigated the distribution of p27 after fractionation by gel filtration chromatography. In cell extracts made from asynchronously growing Swiss 3T3 cells, all the p27 migrated as a single population at ~200 kDa (Figure 4A). Immunoblotting of the same fractions with antibodies against Cdk2 revealed that the p27 comigrated with the cyclin-complexed form of Cdk2 but not the monomeric form (Figure 4B). p27 also comigrated with cyclin A and cyclin D1 on the gel filtration column (Figure 4, C and D), although the cyclins migrated as a much broader peak, probably due to their presence in many different complexes.

It has been shown that p27 is sequestered by heat-labile factors and that p27 itself is heat resistant (Hengst *et al.*, 1994; Polyak *et al.*, 1994a). Hence, we boiled the cell extracts before loading onto a gel filtration column to see whether the mobility of p27 was affected. Boiling the extracts converted all of the p27 to a population migrating at ~45 kDa (Figure 4E). Purified, bacterially expressed p27 (tagged with six histidines) also migrated at ~45 kDa (Figure 4F). (The fact that denatured p27 and bacterially expressed p27 both migrated at ~45 kDa suggests that monomeric p27 has an elongated shape and migrates anomalously upon gel filtration). These data suggested that in growing cells, the majority of p27 is associated with other proteins, presumably including cyclin·CDK complexes. The comigration of p27 with cyclins and the complexed form of Cdk2 is consistent with the idea that most of the p27 is associated with these proteins (see below), and that p27 has higher affinity for cyclin·Cdk2 than for monomeric Cdk2 (see Figure 2).

We next investigated p27 by gel filtration chromatography of synchronized cell extracts. Serum-starved

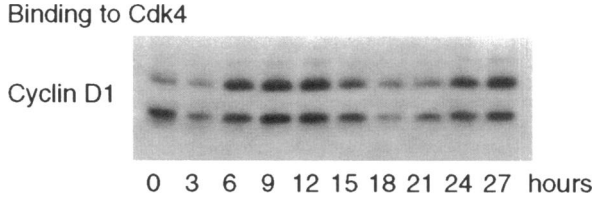

Figure 3. Cyclin D1 dissociates from Cdk4 around S phase. Exactly the same cell extracts as in Figure 1 were immunoprecipitated with anti-Cdk4 antiserum. The immunoprecipitates were then immunoblotted with anti-cyclin D1 antiserum.

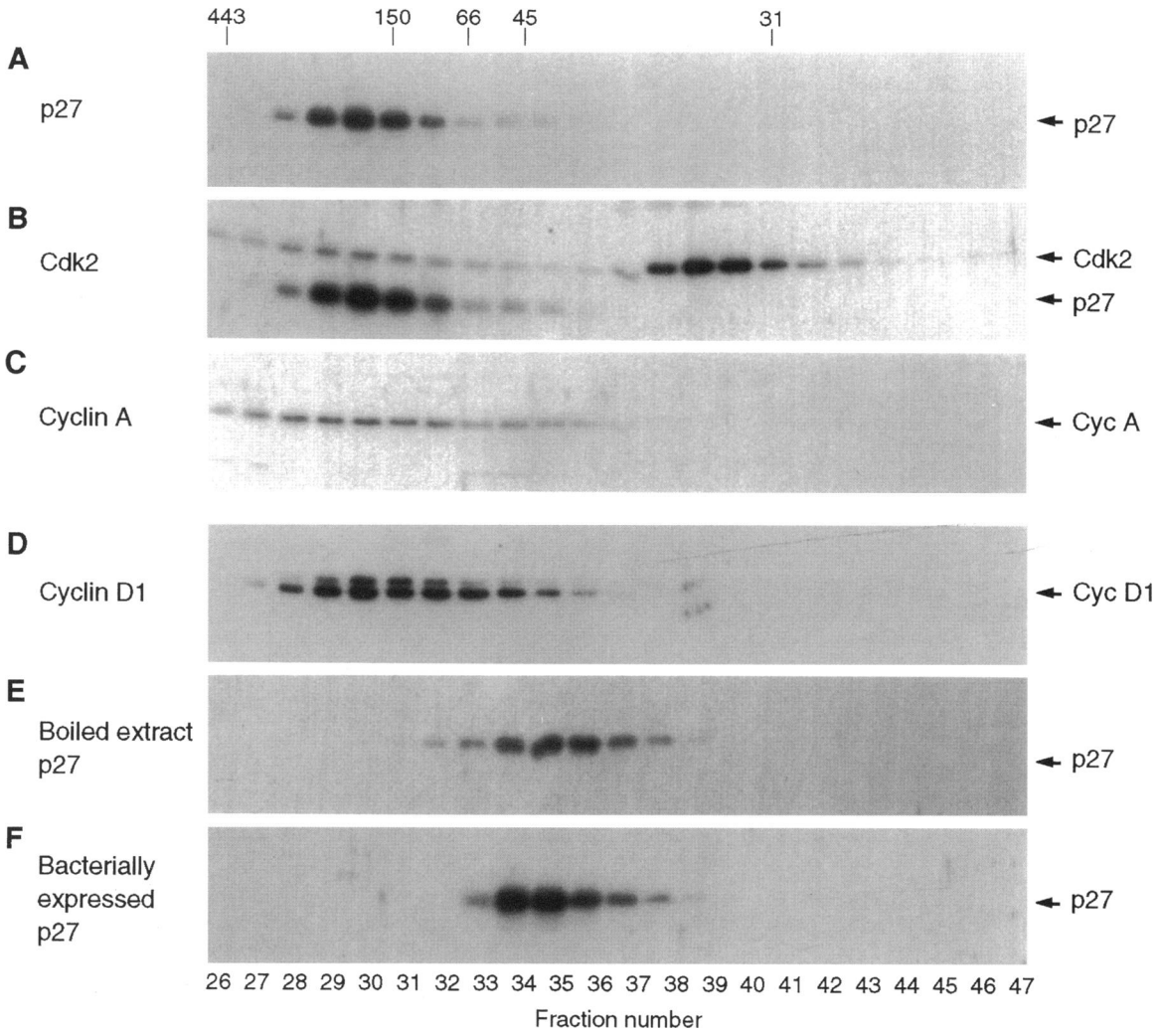

Figure 4. Fractionation of p27 with gel filtration chromatography. Asynchronous Swiss 3T3 cell extract was fractionated on a Superose 12 gel filtration column as described in MATERIALS AND METHODS. The fractions were subjected to SDS-PAGE, transferred to Immobilon, and immunoblotted with antibodies against the following: (A) p27; (B) Cdk2, after immunoblotting with anti-p27 antibody without stripping the blot; (C) cyclin A; and (D) cyclin D1. (E) An asynchronous 3T3 cell extract was heated to 100°C for 5 min and insoluble material was removed by centrifugation in a microfuge for 15 min at 4°C. The supernatant was then loaded onto a gel filtration column and the fractions were immunoblotted with anti-p27 antibody. (F) p27 tagged with six histidine residues was expressed in bacteria and purified as described in MATERIALS AND METHODS. The purified p27-H6 was loaded onto a gel filtration column and the fractions were immunoblotted with anti-p27 antibody. Fraction numbers are shown at the bottom and the positions of molecular mass markers in kDa are shown at the top.

Swiss 3T3 cells were released into the cell cycle synchronously (see Figure 1). Cell extracts made from cells in G0 and at various times after serum stimulation were fractionated by gel filtration chromatography. p27 from cells arrested in G0 was fractionated into two peaks, with sizes of ~200 and ~45 kDa (Figure 5A). These two populations of p27 comigrated with the p27 seen in asynchronous cell extracts (200 kDa, Figure 4A) and the monomeric p27 (45 kDa, Figure 4E). More than half of the p27 was found in the monomeric form in these G0 cells. Fractionation of G1 cell extracts (9 h after serum stimulation) revealed that the monomeric form of p27 (45 kDa) had disappeared and nearly all of the p27 was in the 200-kDa form (Figure 5B). In S phase cell extracts (18 h after serum stimulation), the bulk of the p27 was in the 200-kDa form, but a small amount of p27 was detected in the monomeric form (Figure 5C). It was difficult to pinpoint the exact time of M phase in these serum-stimulated cells, but in extracts made from nocodazole-arrested M phase cells, the majority of p27 was in the 200-kDa peak with a small amount of monomeric p27 being apparent (Figure 5D). These data suggest that most of the p27 is in monomeric form in G0 cells, but

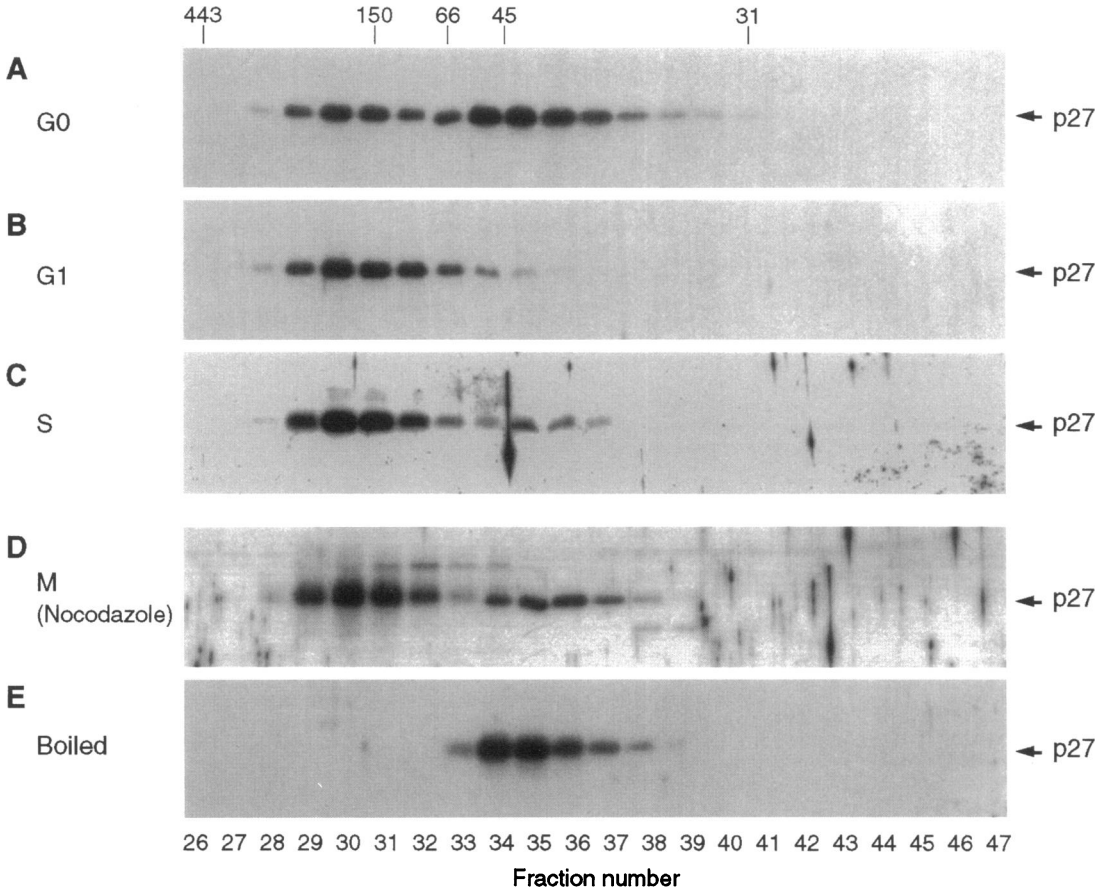

Figure 5. Gel filtration chromatography of extracts at different phases of the cell cycle. Swiss 3T3 cell extracts derived from the following: (A) serum-starved, G0-arrested cells; (B) cells at 9 h after serum stimulation; (C) cells at 18 h after serum stimulation; and (D) nocodazole-arrested cells were subjected to gel filtration chromatography as described in MATERIALS AND METHODS. The fractions were immunoblotted with anti-p27 antibody. (E) Fractionation of boiled cell extracts (the same panel as in Figure 4E) was included for comparison. Fraction numbers are shown at the bottom and the positions of molecular mass markers in kDa are shown at the top.

a portion of the p27 is complexed with other proteins (presumably including cyclin A·Cdk2 as shown in Figure 1). As soon as the cells enter G1, nearly all of the p27 becomes complexed.

The Majority of p27 Is Associated with Cyclin D1 in Growing Cells

To get an idea of what fractions of the different cyclins were bound to p27 in asynchronously growing cells, we attempted to deplete p27 from cell extracts to see how much cyclin was also depleted. Cell extracts were sequentially immunodepleted three times with either normal rabbit serum or antiserum against p27. The amount of p27 that was immunoprecipitated or remained in the cell extracts was visualized by immunoblotting with anti-p27 antibody. Figure 6A shows that p27 was immunoprecipitated by anti-p27 antiserum (lanes 4–6) but not by normal rabbit serum (lanes 1–3). There was essentially no p27 left in the extract depleted with anti-p27 antiserum by comparison with the extract depleted with normal serum (Figure 6, compare lanes 7 and 8; note that 40 times more extract was used for the immunoprecipitated samples). When the same samples were immunoblotted with anti-cyclin A or anti-cyclin D1 antiserum, we found that both cyclin A and cyclin D1 were co-immunoprecipitated with p27, but there was little change in the levels of cyclin A and D1 between extracts depleted with normal serum and with anti-p27 antiserum (Figure 6, lanes 7 and 8). Identical results were obtained with anti-p27 antiserum raised against the full length mouse p27 or against the C-terminal peptide of mouse p27. Although Figure 6A clearly shows that cyclins were indeed co-immunoprecipitated with p27, we cannot exclude the possibility that some cyclins were dislodged from p27 by the anti-p27 antibodies.

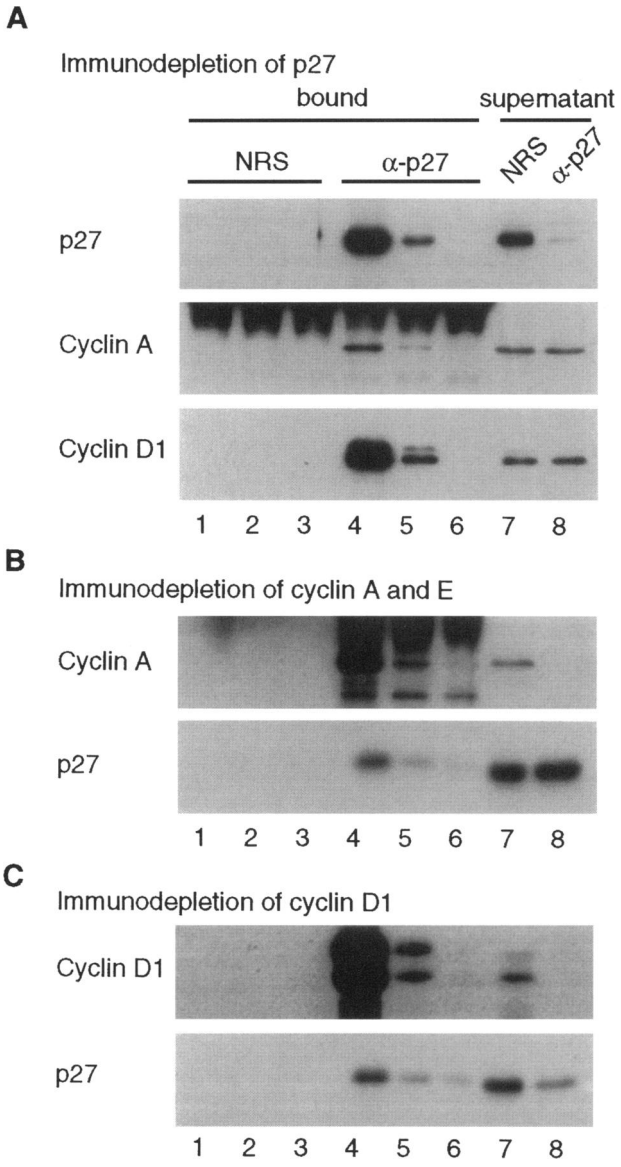

Figure 6. Immunodepletion of p27 from growing Swiss 3T3 cells. (A) Cell extracts (400 μg) prepared from asynchronously growing Swiss 3T3 cells were sequentially immunodepleted three times with either normal rabbit serum (NRS) (lanes 1–3) or with antiserum against p27 (lanes 4–6) as described in MATERIALS AND METHODS and the immunoprecipitates (lanes 1–6), and analyzed by SDS-PAGE. The supernatants (10 μg) immunodepleted with NRS (lane 7) and with anti-p27 antiserum (lane 8) were run in parallel. The samples were then immunoblotted with anti-p27 (top panel), anti-cyclin A (middle panel), and anti-cyclin D1 antibodies (bottom panel). (B) As in panel A, except that immunodepletion was performed with anti-cyclin A and anti-cyclin E antibodies. The samples were then immunoblotted with anti-cyclin A (upper panel) or anti-p27 antibodies (lower panel). (C) As in panel A, except that immunodepletion was performed with anti-cyclin D1 antibodies. The samples were then immunoblotted with anti-cyclin D1 (upper panel) or anti-p27 antibodies (lower panel).

We next performed the converse experiment by immunodepleting either cyclin A and E together or cyclin D1 from cell extracts to see how much p27 was also removed. We found that very little p27 was depleted with anti-cyclin A plus E antibodies (Figure 6B), but, on the other hand, most of the p27 was depleted with anti-cyclin D1 antibody (Figure 6C). Similar results were obtained when the extracts were immunodepleted with antisera against Cdk2 and Cdk4, respectively (our unpublished observations). These data indicate that although p27 was associated with both cyclin A and D1, most of the p27 was associated with cyclin D1, as described previously (Toyoshima and Hunter, 1994). Furthermore, only a very small proportion of cyclin A and cyclin D1 was bound to p27.

Using bacterially expressed proteins as standards, the relative amount of p27, cyclin A, cyclin D1, Cdk2, and Cdk4 in cell extracts derived from growing Swiss 3T3 cells was quantitated by immunoblotting. We found that these cell extracts typically contained the following amount of each protein: p27 (14 nM); cyclin A (8 nM); cyclin D1 (97 nM); Cdk2 (75 nM); and Cdk4 (510 nM). The relative amount of p27, cyclin D1, Cdk2, and Cdk4 was probably representative for the whole cell cycle because their levels did not vary significantly (Figure 1). However, the level of cyclin A fluctuated in the cell cycle (Figure 1), and we have measured the peak level of cyclin A as about 3–4 times the level in asynchronized cell extracts. Hence, we expected that immunoclearing of p27 would deplete at most only about 15% of the cyclin D1, but all of the cyclin A if p27 bound to either of the cyclins predominantly. The fact that clearing with anti-p27 antibodies depleted little cyclin A is consistent with p27 being mainly associated with cyclin D1 in growing cells. The failure of anti-p27 antibodies to deplete a significant amount of cyclin D1 is consistent with the level of p27 being significantly lower than that of cyclin D1 in growing cells. Finally, it should be noted that, based on their relative levels, p27 is theoretically capable of inhibiting a substantial portion of cyclin A-associated kinase, but very little of the cyclin D1-associated kinase.

Redistribution of p27 in Lovastatin-arrested Swiss 3T3 Cells

Studying situations where cell cycle progression is artificially blocked may shed some light on the mechanism and role of the redistribution of p27 among different CDKs in the cell cycle, and the possible role of p27 in cell cycle arrest. Lovastatin is a competitive inhibitor of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) and has been shown to reversibly arrest a wide variety of cells in G1 (Keyomarsi *et al.*, 1991). Moreover, a p27-like factor has been implied to be involved in G1 arrest induced by lovastatin in HeLa

cells (Hengst *et al.*, 1994). Therefore we compared the level and kinase activity of different cell cycle components between asynchronously growing Swiss 3T3 cells and cells arrested with lovastatin. We also used another chemical cell cycle blocker, nocodazole, which arrests cells in M phase, to act as a comparison to the lovastatin arrest.

Lovastatin caused a G1 arrest in Swiss 3T3 cells (FACS analysis revealed that the percentage of cells in G1 went from 57% in growing cells to 92% in lovastatin-treated cells). Figure 7A shows that the kinase activities associated with Cdc2, Cdk2, and Cdk4 were all down-regulated in lovastatin-arrested cells. The kinase activities associated with their cyclin partners (cyclin A, D1, and E) were likewise diminished (our unpublished observations). In nocodazole-arrested cells, by contrast, the kinase activities of Cdc2 and Cdk4 were highly elevated, whereas that of Cdk2 was reduced. The CAK activity, measured with a kinase-inactive GST-Cdk2 as a substrate, was not decreased by lovastatin. Immunoblotting with specific antibodies revealed that the protein level of Cdc2 was reduced, but the levels of Cdk2 and Cdk4 remained high after lovastatin treatment (Figure 7B). The level of cyclin D1 was greatly reduced in cells treated with lovastatin. The level of cyclin A and E did not vary significantly after lovastatin treatment, although the activity of cyclin A·Cdk2 was diminished.

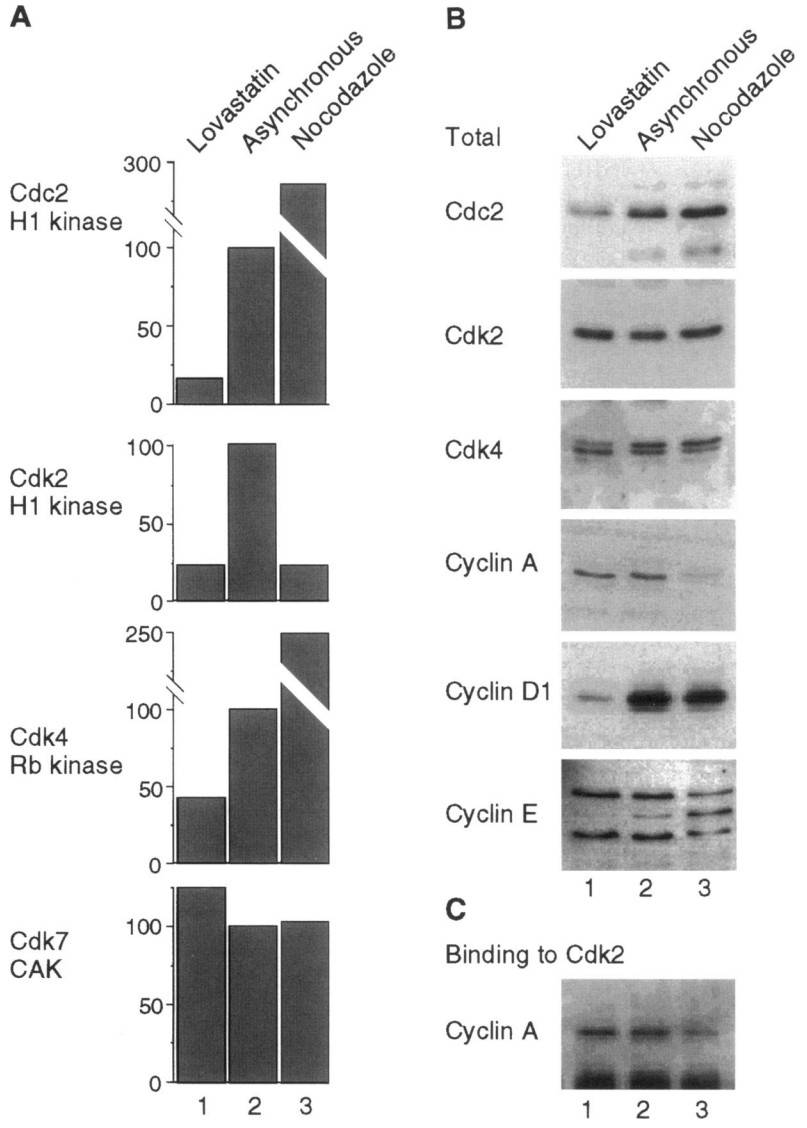

Figure 7. Lovastatin arrest of Swiss 3T3 cells. Cell extracts from asynchronously growing Swiss 3T3 cells (lane 2), lovastatin-arrested cells (lane 1), or nocodazole-arrested cells (lane 3) were prepared as described in MATERIALS AND METHODS. (A) Equal amounts of cell extract (50 μg) were immunoprecipitated with antibodies against the following CDKs (from top to bottom): Cdc2, Cdk2, Cdk4, and Cdk7. The kinase activity of the immunoprecipitates were assayed using histone H1 (for Cdc2 and Cdk2), GST-Rb (for Cdk4), or GST-cdk2 K33R (for Cdk7) as substrates. The degree of phosphorylation was expressed as a percentage of the phosphorylation found in asynchronous cells. (B) Equal amounts of cell extract (10 μg) were immunoblotted with antibodies against the following (from top to bottom): Cdc2, Cdk2, Cdk4, cyclin A, cyclin D1, or cyclin E. (C) Equal amounts of cell extract (50 μg) were immunoprecipitated with anti-Cdk2 antibody. The samples were immunoblotted with anti-cyclin A antibodies.

Cyclin A was co-immunoprecipitated with Cdk2 in lovastatin-treated cells (Figure 7C). Thus, the lack of cyclin A·Cdk2 activity was not due to their inability to form a complex. Furthermore, the CAK activity remained high in lovastatin-arrested cells. Hence, it is conceivable that the lack of cyclin A·Cdk2 activity could be due to an inhibitory factor(s). In cells arrested with lovastatin, the total level of p27 did not alter significantly (Figure 8A), but a significantly higher amount of p27·cyclin A complex was formed after lovastatin treatment with a concomitant decrease in the amount of p27·cyclin D1 complex (Figure 8B). It appears that lovastatin caused the degradation of cyclin D1 in Swiss 3T3 cells, which releases extra p27 that can associate with cyclin A·Cdk2 and may contribute to the decrease in its kinase activity and cell cycle arrest.

Redistribution of p27 in UV-Irradiated Swiss 3T3 Cells

The p27-related inhibitor p21 is transcriptionally regulated by p53 (El-Deiry *et al.*, 1993). Radiation damage of cells induces p53, which in turn leads to an increase in p21 level and inhibition of cell cycle progression at various points of the cell cycle (Dulić *et al.*, 1994; El-Deiry *et al.*, 1994; Li *et al.*, 1994b). We investigated whether the level of p27 was also affected in cells arrested by UV irradiation. Swiss 3T3 cells were irradiated with UV and cell extracts were prepared at different times after irradiation. Equal amounts of cell extract were immunoblotted with antibodies against p27 and various cell cycle regulatory proteins (Figure 9A). We found that p53 was induced strongly by UV irradiation in these cells. Using monoclonal antibodies that distinguish between wild-type and mutant p53, we found that Swiss 3T3 cells contained wild-type p53 (our unpublished observations). There was also a concomitant increase in the level of p21 as expected. In contrast, the level of p27 decreased after UV irradiation (at 24 h there was a small increase in p27, but this was probably because many cells went into apoptosis at that time and only relatively healthy cells remained on the dish). The decrease in Cdc2, Cdk2, and Cdk4 was relatively slight or slow after irradiation, whereas the catalytic component of CAK, Cdk7, was diminished. Cyclin A level decreased but did not disappear completely; cyclin E remained relatively constant. It is interesting that there was a transient mobility shift of cyclin A at 2–6 h after irradiation (see DISCUSSION). The D-type cyclins were destroyed much more rapidly (cyclin D1 was destroyed within 2 h; the cyclin D2 level was fairly low in these cells; and cyclin D3 was not detectable) than cyclin A and E, and p27. We found that both the Cdk2-associated histone H1 kinase activity, and the Cdk4-associated Rb kinase activity were rapidly diminished (Figure 9B). We further showed that when mouse fibroblasts that have both copies of the p53 gene deleted ($p53^{-/-}$ homozygote) were irradiated with UV, the level of p27 was also reduced with similar kinetics (Figure 9C). Therefore, unlike p21, p27 is not induced by UV and probably is not regulated by p53.

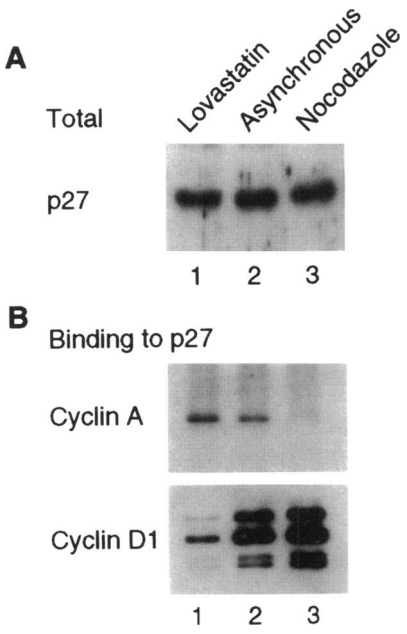

Figure 8. Lovastatin-induced p27-cyclin A association. Cell extracts from asynchronously growing Swiss 3T3 cells (lane 2), lovastatin-arrested cells (lane 1), or nocodazole-arrested cells (lane 3) were prepared as described in MATERIALS AND METHODS. (A) Ten micrograms of cell extract were immunoblotted with anti-p27 antiserum. (B) Equal amounts of cell extract (50 μg) were immunoprecipitated with anti-p27 antiserum and the immunoprecipitates were immunoblotted with anti-cyclin A antibodies (upper panel), or anti-cyclin D1 antibodies (lower panel).

To detect the cyclins and CDKs that associated with p27 after UV irradiation, cell extracts made at different times after irradiation were immunoprecipitated with anti-p27 antiserum followed by immunoblotting with anti-cyclin and anti-CDK antibodies (Figure 10A). Both Cdk4 and cyclin D1 were found in the p27 immunoprecipitates before irradiation, but their presence in the p27 immunoprecipitates was rapidly reduced after irradiation (Figure 10A); this correlates faithfully with the total level of cyclin D1 in the cell extracts. In contrast, the Cdk2 and cyclin A that associated with p27 increased after UV irradiation, peaked at 2 h, and then decreased to undetectable level after 12 h (Figure 10A). The converse experiment was performed by immunoprecipitation with anti-Cdk2 or anti-Cdk4 antibodies followed by immunoblotting with anti-p27 antiserum (Figure 10B). This confirmed that there was a rapid surge in binding of p27 to Cdk2 after irradiation, followed by a return to a low level.

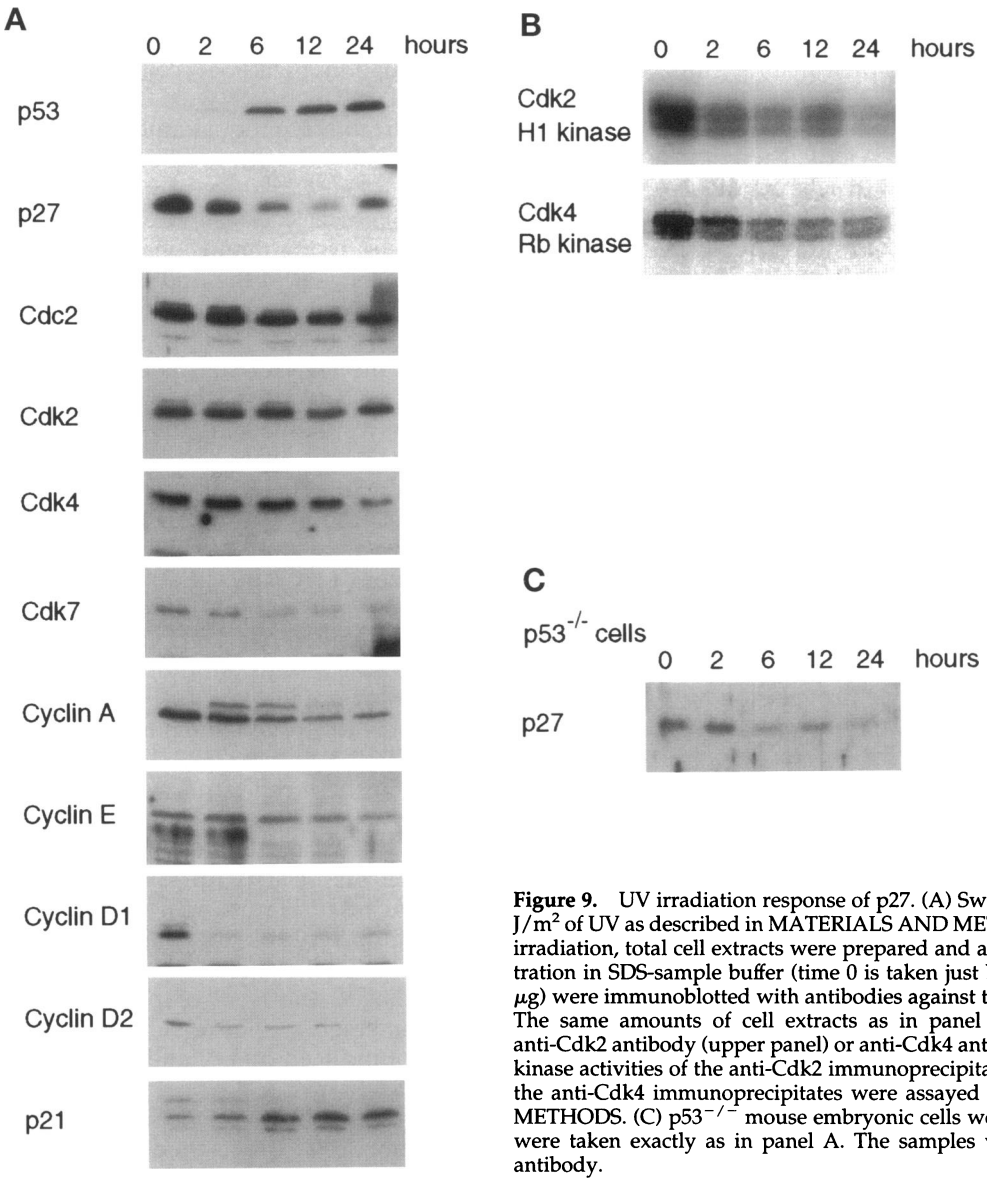

Figure 9. UV irradiation response of p27. (A) Swiss 3T3 cells were irradiated with 50 J/m^2 of UV as described in MATERIALS AND METHODS. At the indicated times after irradiation, total cell extracts were prepared and adjusted to the same protein concentration in SDS-sample buffer (time 0 is taken just before irradiation). The samples (10 μg) were immunoblotted with antibodies against the proteins indicated on the left. (B) The same amounts of cell extracts as in panel A were immunoprecipitated with anti-Cdk2 antibody (upper panel) or anti-Cdk4 antibody (lower panel). The histone H1 kinase activities of the anti-Cdk2 immunoprecipitates or the GST-Rb kinase activity of the anti-Cdk4 immunoprecipitates were assayed as described in MATERIALS AND METHODS. (C) p53$^{-/-}$ mouse embryonic cells were irradiated with UV and samples were taken exactly as in panel A. The samples were immunoblotted with anti-p27 antibody.

The increase in association between p27 and Cdk2 after UV irradiation was somewhat unexpected given that the total level of p27 was reduced. It is likely that the very rapid loss of cyclin D1 after irradiation released p27 from Cdk4, and that this "free" p27 was able to bind to cyclin A·Cdk2. This rapid p27-Cdk2 binding response may play a role in the rapid loss of Cdk2-associated histone H1 kinase activity after irradiation (Figure 9B), which was comparable to the rate of loss of Cdk4-associated Rb kinase activity. This rapid loss of Cdk2-associated kinase activity was probably not explicable by the loss of cyclin partners (the amount of both cyclin A and E stayed relatively high) nor the increase in p21 alone, because substantial levels of p53 and p21 did not accumulate until 6 h after irradiation.

DISCUSSION

One underlying conclusion from these studies is that p27 is able to shift between Cdk4 and Cdk2 in the normal cell cycle and in arrested cells. For example, p27 is redistributed from cyclin D1·Cdk4 to cyclin A·Cdk2 during the G1-S transition in the normal cell cycle, in lovastatin-arrested cells, and immediately after UV irradiation. Thus, when cyclin D1·CDK levels are high, p27 is found associated with these complexes, and when cyclin D1·CDK complex levels fall, p27 is able to associate with cyclin·Cdk2 complexes.

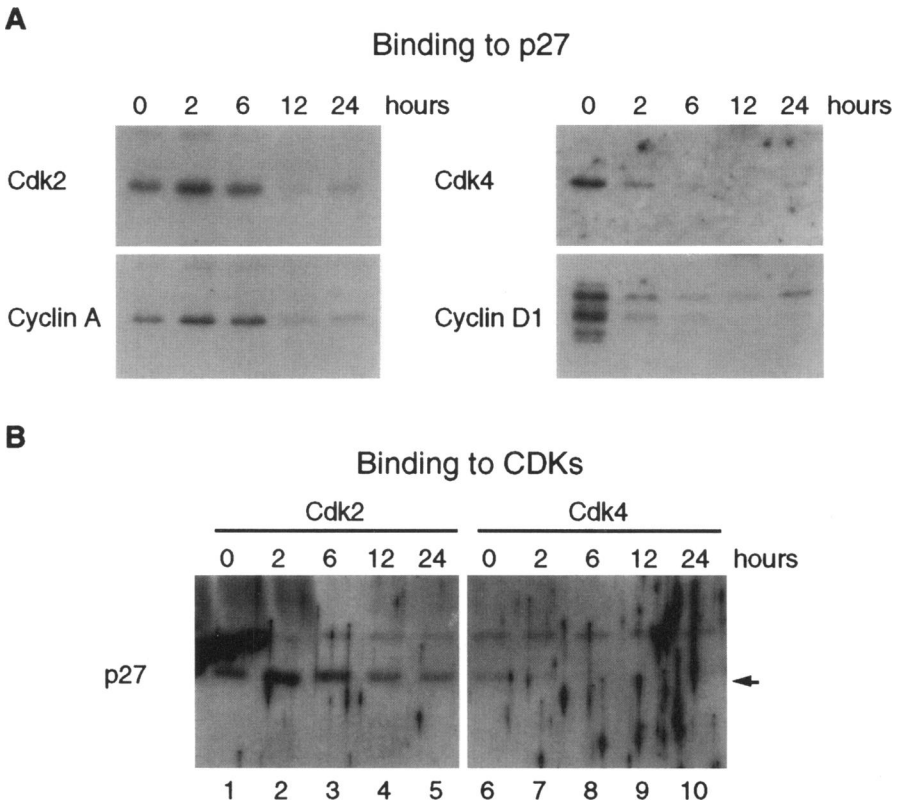

Figure 10. Association of p27 with cyclin·CDK after UV damage. (A) Swiss 3T3 cells were irradiated with UV as described in MATERIALS AND METHODS. At the indicated times, total cell extracts were prepared and the protein concentrations were measured (time 0 is taken just before irradiation). Equal amounts of cell extract (50 μg) were immunoprecipitated with anti-p27 antiserum and immunoblotted with antibodies raised against Cdk2, cyclin A, Cdk4, and cyclin D1 as indicated. (B) Equal amounts of cell extract (50 μg) taken at the indicated times after UV treatment as in panel A were immunoprecipitated with anti-Cdk2 antibodies (lanes 1–5) or anti-Cdk4 antibodies (lanes 6–10). The samples were then immunoblotted with anti-p27 antiserum. The arrow indicates the position of p27.

Experiments described here suggest that p27 is mainly associated with cyclin D1·Cdk4, and that the abundance of p27 is low in comparison with cyclin D1·Cdk4 in growing Swiss 3T3 cells. The low abundance of p27 in the cell may also explain why p27 was not observed in immunoprecipitates of cyclins and CDKs before (Xiong *et al.*, 1992, 1993b). It is possible that cyclin D1·Cdk4 complexes act as a reservoir for p27. On destruction of cyclin D1 (as in serum-starved cells, lovastatin treatment, or UV irradiation), or dissociation of cyclin D1 from Cdk4 (as in G1-S), p27 is released and redistributes to other cyclin·CDK complexes like cyclin A·Cdk2. An unanswered question is whether the increased association between p27 and Cdk2 plays a physiologically significant role in the regulation of Cdk2 kinase activity and G1/S phase progression and in cell cycle arrest. In addition, it is important to know whether the binding of p27 to cyclin D1·Cdk4, which occurs during G1, plays a part in controlling G1 progression.

p27 as an Inhibitor for the Residual CDKs in G0

Previously, we did not observe a large increase in p27 level in serum-starved Swiss 3T3 cells (Toyoshima and Hunter, 1994), whereas in the present study we found that the level of p27 was elevated as a result of growth factor deprivation or contact inhibition (Figure 1). We suspect that our earlier result was due to the fact that the cells had not achieved a true resting state (0.5% serum was used to induce quiescence instead of the 0.2% serum used here). More than half of the p27 population in G0 cells was monomeric (Figure 5), but surprisingly, we found that some of the p27 in these cells was associated with cyclin A·Cdk2. The efficient binding of the residual cyclin A·Cdk2 to p27 may be due to the increase in p27 level and the absence of cyclin D1·Cdk4 complexes in G0 cells. Hence it is possible that p27 may be important in ensuring that there is no residual cyclin·CDK kinase activity in growth-arrested cells. In some respects our results are similar to those observed during interleukin-2-induced exit of T lymphocytes from quiescence into S phase (Nourse *et al.*, 1994). The level of p27 in G0 T lymphocytes is high and quickly diminishes as cells enter S phase, but the difference in abundance of p27 between G0 and S in T lymphocytes is much more striking than in Swiss 3T3 cells. A similar role for the inhibition of CDK activity in resting cells has been suggested for p27-related CDK inhibitors p21 (Halevy *et al.*, 1995; Parker *et al.*, 1995; Skapek *et al.*, 1995) and $p57^{Kip2}$ (Lee *et al.*, 1995; Matsuoka *et al.*, 1995).

p27 as a Buffer for CDKs in the Cell Cycle

When quiescent cells are stimulated to enter the cell cycle, cyclin D1 is expressed and forms a complex with Cdk4 (and presumably with Cdk6 too, but this was not investigated here). Cyclin D1·Cdk4 is associated with p27 during G1, probably saturating the p27, because no free p27 was observed by gel filtration (Figure 5) and other cyclins are not expressed this early in the cell cycle. It has been proposed that p27 acts as a physiological buffer for cyclin·CDK complexes in G1, such that cyclin·CDKs are not active until their levels exceed that of p27 (Polyak *et al.*, 1994a). It is possible that p27 may serve as a buffer for cyclin D1·Cdk4 in early G1, but as indicated above, we estimate that the level of p27 is far less than the maximum level of cyclin D1·CDK complexes in G1, and thus the buffering effect of p27 would be modest in Swiss 3T3 cells. Furthermore, if more than one molecule of p27 is required for the inhibition of cyclin·CDK activity, as in the case for p21, it is unlikely any cyclin D1·Cdk4 is inhibited by p27 in G1.

In S phase, cyclin D1 was dissociated from Cdk4 (Figure 3), and this could account for the observed dissociation of p27 from cyclin D1 and Cdk4. The dissociation of cyclin D1 and Cdk4 may be due to the expression of p16 family of CDK inhibitors during S phase that can bind to Cdk4 and dislodge cyclin D (Parry *et al.*, 1995). In support of this, the level of p19 was found to increase in S phase (Hirai *et al.*, 1995). The released p27 is then free to bind to cyclin A/E·Cdk2 complexes, which more or less follow the level of cyclin A, and peak before M phase (Figure 1). Hence after dissociation from cyclin D1·Cdk4, p27 may serve as a buffer for Cdk2 activity during S phase. The dissociation of cyclin D1 and Cdk4 might also provide an explanation for the observation that cyclin D1 is excluded from the nucleus during S phase (Pagano *et al.*, 1994) if cyclin D1 requires association with Cdk4 or p27 to remain in the nucleus. In contrast to the level of cyclin D1, we estimated that the level of cyclin A is comparable to that of p27, so p27 may have a more significant buffering effect for cyclin A·Cdk2 than for cyclin D1·Cdk4. As cells pass through M phase and enter the next G1, cyclin A is degraded and cyclin D1·Cdk4 complexes are formed, allowing p27 to associate again with cyclin D1·Cdk4. It is interesting that during S phase a small fraction of p27 can be found in the monomeric form; it is possible that immediately after p27 is released from cyclin D·Cdk4, the amount of cyclin A has not been accumulated to a sufficient level to overcome all the p27.

p27 as an Inhibitor for CDKs in G1 Arrest

Unlike p21, the level of p27 does not seem to be regulated by p53 or UV irradiation. The p27 level was diminished after UV irradiation (Figure 9), but cyclin D1 was destroyed even more rapidly after irradiation (Figure 9), as also reported by Pagano *et al.* (1994). This means that the rate of release of p27 from Cdk4 was quicker than the rate of loss of total p27, and so there was a net increase in the p27 available to bind to cyclin A·Cdk2. As discussed earlier, this transient but rapid binding of p27 to cyclin A·Cdk2 may contribute to the rapid loss of Cdk2-associated kinase activity. This may be important because there is a considerable lag before p21 accumulates as a result of transcriptional induction by p53. Also unlike p21, we did not detect any association between p27 and PCNA (our unpublished observations).

It is interesting that the timing of the mobility shift of cyclin A seen after UV irradiation corresponds to the binding of p27 to cyclin A·Cdk2 (Figure 9). We found that p27 only binds to the lower form of cyclin A and that addition of recombinant p27 to cell extracts does not induce the shift of cyclin A (our unpublished observations).

During lovastatin- and UV irradiation-induced arrest, p27 was redistributed from cyclin D1·Cdk4 to cyclin A·Cdk2. Conceivably, either on its own or in conjunction with other CDK inhibitors, p27 is able to inhibit Cdk2 kinase activity and block cell cycle progression, because overexpression of p27 does arrest cells in G1 (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). But it is unlikely that shutting off Cdk2 kinase activity is the only factor responsible for cell cycle arrest. In both cases, cyclin D1 is obliterated very rapidly, and it is possible that this alone is capable of stopping the cell cycle. We imagine that inhibition of Cdk2 by the redistributed p27 may be important for arresting cells that have already passed the cyclin D1·Cdk4 control point.

In growing cells, p27 is predominantly associated with cyclin D1·Cdk4 and the level of p27 is significantly lower than cyclin D1·Cdk4. After lovastatin treatment or UV irradiation, cyclin D1 is degraded and p27 is redistributed to cyclin A·Cdk2 and this may contribute to the loss in kinase activity of cyclin A·Cdk2. In Swiss 3T3 cells, the level of p27 is comparable to that of cyclin A, so when all of the p27 is redistributed from cyclin D1 to cyclin A, a significant proportion of cyclin A·Cdk2 can theoretically be inhibited by p27. However, we think that the amount of redistributed p27 alone may not be sufficient (or is not available) to overcome and inhibit the peak of cyclin A·Cdk2. In support of this, we found that in lovastatin-arrested cells, most of the p27 was depleted by immunodepletion of cyclin A (compare to asynchronously growing cells in Figure 6), but cyclin A was not depleted significantly by immunodepletion of p27 (our unpublished observations). Hence, it is likely that p27 is only part of the mechanism that contributes to the inhibition of Cdk2 (although we should caution that this interpretation relies on cyclins not being dis-

placed from p27 by the anti-p27 antibodies used in these experiments). Similarly, it is unclear whether in TGFβ-arrested cells, p27 is important in inhibition of Cdk2, or whether other inhibitors like p15 or p21 are more significant (Hannon and Beach, 1994; Datto *et al.*, 1995). We have not investigated the behavior of cyclin E binding to p27, mainly because cyclin E signals were masked by the IgG heavy chains after immunoprecipitation. Moreover, the level of cyclin E is rather low in comparison to cyclin A in Swiss 3T3 cells, and most of the Cdk2 kinase activity detected was probably due to cyclin A·Cdk2. Hence we do not exclude the possibility that p27 may play a significant role in inhibition of cyclin E·Cdk2 activity during cell cycle arrest. It is also interesting to note that in human WI38 fibroblasts, the majority of the active Cdk2 is associated with p21 (Harper *et al.*, 1995); hence maybe a small increase in the level of p27 is sufficient to achieve more than one p21/p27 molecule per Cdk2 molecule and inhibits its kinase activity. In conclusion, it is possible that one role of cyclin D1 in the cell is to provide a reservoir of p27 that can be redistributed to inhibit Cdk2 when cyclin D1 is degraded.

As a result of this study we now have a good picture of the changes in abundance of p27 and its partners during the cell cycle in Swiss 3T3 mouse fibroblasts. To what extent the regulation of p27 is the same in mouse fibroblasts and in other cell types is unclear. It is already apparent, however, that the fluctuation in p27 levels is much greater in primary human T lymphocytes than in mouse fibroblasts during exit from G0 (Nourse *et al.*, 1994). The fluctuation in p27 levels also appears to be greater in HL60 human promyelocytic leukemia and HeLa human cervical carcinoma cells, with a pronounced trough in early G1, a peak in late G1 and a decline as cells enter S phase (L. Hengst and S. Reed, personal communication). Based on the changes in p27 abundance during G1 and S phase, Hengst and Reed propose that p27 may regulate the activation of cyclin E·Cdk2, which is needed for entry into S phase. Interestingly, when we used the anti-p27 antibodies developed by Hengst and Reed to analyze our Swiss 3T3 cell cycle samples, there was also a slight decrease in the apparent level of p27 after G1 phase that was not observed with either our anti-p27 protein or peptide antibodies (our unpublished observations); the reason for the variable ability of different antibodies to recognize p27 as cells enter S phase is under investigation. Results similar to ours showing little variation in the level of p27 in the cell cycle have been observed in MANCA human Burkitt's lymphoma cell line (A. Koff, personal communication). However, it is clear that the absolute levels of p27 vary widely between different cell types, and that there are also cell type differences in the accumulation of p27 in response to cell cycle arrest. Thus, p27 levels increase in lovastatin-arrested HeLa cells (Hengst *et al.*, 1994), whereas this is not the case in Swiss 3T3 cells. Despite these differences in the detailed regulation of p27 during the cell cycle, the one common feature in all the systems examined so far is that p27 levels rise significantly in G0, suggesting that it may play an important role in maintaining the quiescent state.

p27 can associate with many different cyclin·CDK complexes at different times in the cell cycle, and many different CDK-inhibitors can associate with a particular cyclin·CDK. It is not clear what governs the ability of p27 to bind to specific complexes, and this could be due to post-translational modification of p27 and its partners as well as differences in subcellular localization. It is also possible that p27 acts in conjunction with other CDK inhibitors to regulate cell cycle progression, with there being a complex interplay of multiple cyclin·CDKs competing for p27, and multiple CDK-inhibitors competing with p27 for binding to cyclin·CDKs. Ultimately a study of cells lacking the p27 gene will provide useful information on the function of p27.

ACKNOWLEDGMENTS

We thank Andy Koff, Ludger Hengst, and Steve Reed for communicating their unpublished results. We also thank Amy Tsou for the anti-p53 monoclonal antibody, $p53^{-/-}$ mouse embryonic fibroblast cells, and help with the UV experiments. We are extremely grateful to C. Sherr for anti-Cdk4, anti-cyclin D1, and anti-cyclin D2 antibodies; M. Ohtsubo and J. Roberts for anti-cyclin E antiserum; J. Massagué for the human p27 clone; H. Kiyokawa and P. Marks for anti-Cdk4 antiserum; M. Howell, M. Cockerill, J. Gannon, and T. Hunt for the GST-Rb and Cdk4 clones, and for the anti-p21, anti-Cdc2 and anti-cyclin A antibodies. Lovastatin was kindly provided by A.W. Alberts (Merck Research Laboratories). This work was supported by the United States Public Health Service grants CA-14195 and CA-39780 to T.H. R.Y.C.P. is a European Molecular Biology Organisation long term fellow. H.T. is a fellow of the National Cancer Institute-Japanese Foundation for Cancer Research Training Program. T.H. is an American Cancer Society Research Professor.

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