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Density-dependent Growth Inhibition of Fibroblasts Ectopically Expressing $p27^{kip1}$

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The cyclin/cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$ is thought to be responsible for the onset and maintenance of the quiescent state. It is possible, however, that cells respond differently to $p27^{kip1}$ in different conditions, and using a BALB/c-3T3 cell line (termed p27-47) that inducibly expresses high levels of this protein, we show that the effect of $p27^{kip1}$ on cell cycle traverse is determined by cell density. We found that ectopic expression of $p27^{kip1}$ blocked the proliferation of p27-47 cells at high density but had little effect on the growth of cells at low density whether exponentially cycling or stimulated from quiescence. Regardless of cell density, the activities of cdk4 and cdk2 were markedly repressed by $p27^{kip1}$ expression, as was the cdk4-dependent dissociation of E2F4/p130 complexes. Infection of cells with SV40, a DNA tumor virus known to abrogate formation of p130- and Rb-containing complexes, allowed dense cultures to proliferate in the presence of supraphysiological amounts of $p27^{kip1}$ but did not stimulate cell cycle traverse when cultures were cotreated with the potent cdk2 inhibitor roscovitine. Our data suggest that residual levels of cyclin/cdk activity persist in $p27^{kip1}$-expressing p27-47 cells and are sufficient for the growth of low-density cells and of high-density cells infected with SV40, and that effective disruption of p130 and/or Rb complexes is obligatory for the proliferation of high-density cultures.

INTRODUCTION

Cell cycle progression is governed by the ordered activation of cyclin-dependent kinases (cdks) and the consequent serine/threonine phosphorylation of target proteins (Sherr, 1996). Cdks require association with cyclins for activity, and different cyclin/cdk combinations are active during, and thus mediate traverse of, distinct portions of the cell cycle. In mammalian cells, the D cyclins (D1–D3) pair with cdk4 or cdk6 and implement traverse of G_1. Cyclin E/cdk2 complexes act at the G_1–S boundary, and cyclin A in association with cdk2 and cyclin B with cdc2 modulate passage through S phase and entry into mitosis, respectively. In general, cdk levels are invariant during the cell cycle, whereas those of the cyclins fluctuate, because of timed synthesis and degradation, and thus account in part for the periodicity of cyclin/cdk assembly and activation. In addition to cyclins, cdks also interact with a group of proteins collectively termed cdk inhibitors (CKIs); CKI levels, like cyclin levels, vary during the cell cycle and thus contribute to the timing of cyclin/cdk activation (Sherr and Roberts, 1999). In addition to protein interaction, cdk activity is also controlled by phosphorylation; cdk-activating kinase, for example, phosphorylates and thus activates cdks (Solomon *et al.*, 1993). In contrast to cyclins and CKIs, however, cdk-activating kinase is present at similar amounts in all phases of the cell cycle (Tassan *et al.*, 1994).

Studies assessing the mitogen-dependent reentry of G_0-arrested cells into the proliferative cycle have further defined the events leading to and resulting from cyclin/cdk activation during G_0–G_1. In response to mitogenic stimulation, the expression of the D cyclins increases (Matsushime *et al.*, 1991; Winston and Pledger, 1993), and newly synthesized D cyclins combine with preexisting cdk4 or cdk6. The resultant complexes become active in mid-G_1 (Matsushime *et al.*, 1994) and phosphorylate the antioncogene Rb and the Rb-related protein p130 (Matsushime *et al.*, 1992; Ewen *et al.*, 1993; Kato *et al.*, 1993; Dong *et al.*, 1998a). Phosphorylation of these proteins is required for cells to pass through the "restriction point" in late G_1 and commit to cell division (Planas-Silva and Weinberg, 1997) and results in their release from the E2F transcription factors (Chellappan *et al.*, 1991) and the consequent expression of a variety of E2F target genes, including those encoding cyclin E, cyclin A,

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and several enzymes required for DNA replication (DeGregori *et al.*, 1995). Once synthesized, cyclins E and A associate with cdk2, and activated cdk2 maintains Rb in a hyperphosphorylated state (Hinds *et al.*, 1992; Hatakeyama *et al.*, 1994; Lundberg and Weinberg, 1998) and initiates yet-to-be-identified Rb-independent events required for continued G_1–S progression (Resnitzky and Reed, 1995; Hofmann and Livingston, 1996).

Two classes of CKIs have been defined: the INK proteins, which block activation of cdk4 and cdk6, and the Cip/Kip proteins, which target several cyclin/cdk complexes (Sherr and Roberts, 1995; Sherr and Roberts, 1999). The Cip/Kip family consists of three members, $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$, all of which contain conserved sequences in their amino-terminal domains that mediate interaction with both cyclins and cdks. Via their capacity to inhibit cyclin/cdk activity, all three Cip/Kip proteins are growth inhibitory when present in cells at high levels; each of these proteins, however, signals growth arrest in distinct circumstances. $p27^{kip1}$, for example, is thought to act as a "growth factor sensor" that induces and maintains G_0 arrest in response to limiting mitogenic stimuli or antiproliferative agents. Initially identified as an inhibitor of cyclin E/cdk2 activity in contact-inhibited and transforming growth factor β (TGFβ)-treated mink lung epithelial cells (Polyak *et al.*, 1994a; Slingerland *et al.*, 1994), $p27^{kip1}$ binds complexes containing the D, E, and A cyclins and their cdk partners but does not interact (or interacts poorly) with monomeric cyclin or cdk subunits (Polyak *et al.*, 1994a; Poon *et al.*, 1995; Russo *et al.*, 1996). $p27^{kip1}$ is present at higher levels in quiescent compared with cycling cells, and its levels decrease upon mitogenic stimulation because of both its translational repression and accelerated degradation (Kato *et al.*, 1994; Nourse *et al.*, 1994; Agrawal *et al.*, 1995, 1996; Pagano *et al.*, 1995; Poon *et al.*, 1995; Hengst and Reed, 1996; Rivard *et al.*, 1996; Winston *et al.*, 1996b; Millard *et al.*, 1997). Treatment of G_0-arrested cultures with agents that prevent the loss of $p27^{kip1}$ (e.g., rapamycin and cAMP analogues) precludes mitogen-induced G_0–G_1 traverse (Kato *et al.*, 1994; Nourse *et al.*, 1994), as does ectopic expression of $p27^{kip1}$ (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994; Rivard *et al.*, 1996). Conversely, $p27^{kip1}$ levels increase when exponentially growing cells cease proliferation in response to serum withdrawal (Polyak *et al.*, 1994a; Coats *et al.*, 1996; Dong *et al.*, 1998b), and the inability of mitogen-depleted cells to enter G_0 in the absence of $p27^{kip1}$ has been described (Coats *et al.*, 1996; Rivard *et al.*, 1996). Rather than altering the abundance of $p27^{kip1}$, antiproliferative agents such as TGFβ and lovastatin are thought to inhibit cell cycle traverse by increasing the interaction of $p27^{kip1}$ with cdk2-containing complexes (Polyak *et al.*, 1994a; Poon *et al.*, 1995).

Because of higher percentages of cycling cells and consequent enlargement of all internal organs, mice lacking $p27^{kip1}$ are larger than their control littermates (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). Surprisingly, however, lymphocytes and fibroblasts derived from $p27^{kip1}$-deficient mice retain the capacity to growth arrest in response to TGFβ, rapamycin, and serum deprivation (Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). Taken together, these findings suggest that although $p27^{kip1}$ is not obligatory for cell cycle arrest, perhaps because of the actions of other CKIs (Coats *et al.*, 1999), its lack during development delays the exit of cells from the cell cycle, thus allowing continued proliferation in the absence of optimal mitogenic stimuli. Along these lines, previous studies have shown that hamster fibroblasts transfected with antisense $p27^{kip1}$ cDNA remain in the cell cycle for several generations when placed in a medium that does not support the growth of $p27^{kip1}$-containing cells (Rivard *et al.*, 1996). Similarly, mouse fibroblasts transfected with Jab1, a protein that interacts with and accelerates the degradation of $p27^{kip1}$, exhibit a reduced dependence on serum for growth (Tomoda *et al.*, 1999). These findings suggest that $p27^{kip1}$ sets the "signal threshold" required for cell proliferation such that the capacity of cells to proliferate in adverse growth conditions is inversely proportional to the amount of $p27^{kip1}$ in the cells.

To further define the conditions that modulate the growth-suppressive actions of $p27^{kip1}$, we prepared fibroblast cell lines that inducibly express this CKI. Our data show that ectopic expression of $p27^{kip1}$ effectively blocks the cell cycle traverse of dense cultures, whereas sparse cultures are surprisingly refractory to $p27^{kip1}$-mediated growth inhibition. Regardless of cell density, $p27^{kip1}$ overexpression significantly repressed cyclin/cdk activity and p130/E2F4 dissociation. We also found that infection of cells with SV40, which prevents E2F interaction with Rb and p130, allowed cells at high density to traverse the cell cycle in conditions in which $p27^{kip1}$ levels were elevated. These findings demonstrate that the capacity of $p27^{kip1}$ to inhibit cell proliferation is a function of culture density and suggest that disruption of Rb- and p130-containing complexes overcomes the growth-inhibitory actions of $p27^{kip1}$ on cells at high density.

MATERIALS AND METHODS

Cell Culture

Stock cultures of all cell lines were maintained in Dulbecco's modified essential medium supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, and 50 U/ml penicillin at 37°C in a humidified atmosphere containing 5% CO_2. LAP-31 cells also received 200 μg/ml G418, and p27-30 and p27-47 cells received 200 μg/ml G418 and 25 μg/ml hygromycin B. For growth curves, cells were seeded at a density of 700 cells/cm^2 in 60-mm culture dishes, and cell number was determined electronically on a Coulter Counter (Coulter Electronics, Hialeah, FL). Experiments using sparse cells were performed 2–3 d after plating. For experiments on density-arrested cells, cells were seeded at a density of 6000 cells/cm^2 in 100-mm culture dishes and were refed with fresh medium 3 d after plating and used 5–6 d later. Density-arrested cells were stimulated to reenter the cell cycle by the addition of fresh medium containing 10–20 ng/ml platelet-derived growth factor (PDGF) and either 10% fetal calf serum or 10% platelet-poor plasma.

Establishment of Cell Lines with Conditional $p27^{kip1}$ Expression

BALB/c-3T3-derived cell lines that expressed $p27^{kip1}$ under isopropyl β-*o*-thiogalactopyranoside (IPTG) regulation were isolated by a two-step procedure as described previously (Labow *et al.*, 1990; Pestov and Lau, 1994). Cells were first transfected with a plasmid containing a neomycin resistance gene and the LAP267 gene, which encodes a chimeric protein containing the inducer-binding domain of the lac repressor inserted upstream of the VP16 transcriptional activation domain. Colonies were selected in 600 μg/ml G418 and expanded. To identify cell lines expressing tightly regulated repressor activity, exponentially growing cultures were transiently transfected with pX12-luc, which contains the luciferase reporter gene

positioned downstream of a multimerized lac operator sequence and a minimal mouse mammary tumor virus promoter. One day after transfection, cells were treated for 24 h with or without 1 mM IPTG; IPTG binds to and inactivates the lac repressor and consequently allows transcription. A cell line (designated LAP-31) that exhibited high levels of luciferase activity in the presence of IPTG and basal levels in the absence of IPTG was selected as the parent cell line. In the second step of the procedure, LAP-31 cells were cotransected with a plasmid containing a hygromycin resistance gene and pX12 containing the entire mouse p27kip1 coding sequence (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). Colonies were selected in medium containing 100 μg/ml hygromycin, expanded, and screened for IPTG-inducible p27kip1 expression by Western blotting.

Western Blotting

Cells were rinsed twice in PBS, harvested by scraping, and collected by centrifugation. The pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1 mM NaF, 0.1 mM vanadate, 0.1 mM PMSF, 2.5 μg/ml leupeptin, and 1 mM dithiothreitol) and incubated on ice for 30 min. Insoluble material was removed by centrifugation, and protein concentrations were determined. Proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in PBS plus 0.1% Tween 20 containing 5% instant milk and incubated with antibody in PBS plus 0.1% Tween 20 for 1 h at room temperature. Proteins recognized by the antibody were detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody as specified by the manufacturer.

Cyclin-dependent Kinase Assay

Cell extracts (80 μg in 350 μl of lysis buffer) were incubated with cyclin antibody for 2 h at 4°C and subsequently with protein A-agarose beads for 2 h at 4°C. Immune complexes were collected by centrifugation and washed twice with lysis buffer and once with kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 10 mM dithiothreitol). Washed complexes were incubated for 30 min at 30°C in 8 μl of kinase buffer containing 10 μCi of [γ^{32}P]ATP, 10 μM ATP, and either 1 μg of glutathione *S*-transferase (GST)-Rb for cyclin D-associated kinase assays or 1 μg of histone H1 for cyclin A- and cyclin E-associated kinase assays. Reactions were stopped by heating in loading buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, and 2% β-mercaptoethanol), and proteins were resolved on 8% SDS-polyacrylamide gels. Radiolabeled substrates were visualized by autoradiography.

Immunocytochemistry

Cells were seeded in chamber slides (Nalge Nunc, Naperville, IL) at ~5 × 10^3 cells per chamber. After the appropriate treatments, cells were then rinsed with PBS and fixed in 4% formaldehyde. Cells were stained for p27kip1 using the avidin–biotin complex technique (Vectastatin Elite ABC kit; Vector Laboratories, Burlingame, CA) at a 1:100 dilution without antigen retrieval. Positive controls and nonimmune protein-negative controls were performed at the same time. For p27kip1/bromodeoxyuridine (BrdU) double immunostaining, cells were treated with 10 μM BrdU for 20 h, rinsed with PBS, and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 20 min at 4°C. Cells were washed with PBS containing 1% glycine and permeabilized for 24 h in PBS containing 1% glycine, and 0.5% Triton X-100. DNA was denatured by treatment of cells with 2 M HCl for 1 h at 37°C, and the acid was neutralized with 0.1 M borate. Cells were rinsed with PBS and blocked in PBS containing 10% goat serum and 1% bovine serum albumin (BSA) for 2 h at room temperature. Cells were then incubated with fluorescein-conjugated BrdU mouse monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) and p27kip1 rabbit polyclonal antibody in PBS containing 0.1% NP-40, and 1% BSA for 1 h at room temperature. To detect the primary polyclonal antibody, cells were incubated with rhodamine-conjugated anti-rabbit IgG (TRITC; Sigma, St. Louis, MO) in PBS containing 0.1% NP-40, and 1% BSA for 25 min at room temperature. Cells were rinsed several times with PBS containing 0.1% NP-40 and dried, and coverslips were applied using Vectashield antifade mounting media (Vector Laboratories). Immunofluorescence was observed with a Leitz (Wetzlar, Germany) Orthoplan 2 microscope, and images were captured with a charge-coupled device camera with the Smart Capture program (Vysis, Downers Grove, IL).

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were done as described previously (Dong *et al.*, 1998b; Flores *et al.*, 1998). Whole-cell extracts (20 μg in lysis buffer) were incubated for 30 min at room temperature in binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1% NP-40, 30 mg/ml BSA, and 500 ng/ml sonicated salmon sperm DNA) containing 0.1 ng of $^{32}PO_4$-labeled DNA fragment corresponding to residues −103 to −23 of the dihydrofolate reductase promoter in a final volume of 50 μl. The resulting complexes were separated by electrophoresis using a 5% polyacrylamide gel (75:1 acrylamide:bisacrylamide) in Tris-borate-EDTA containing 5%

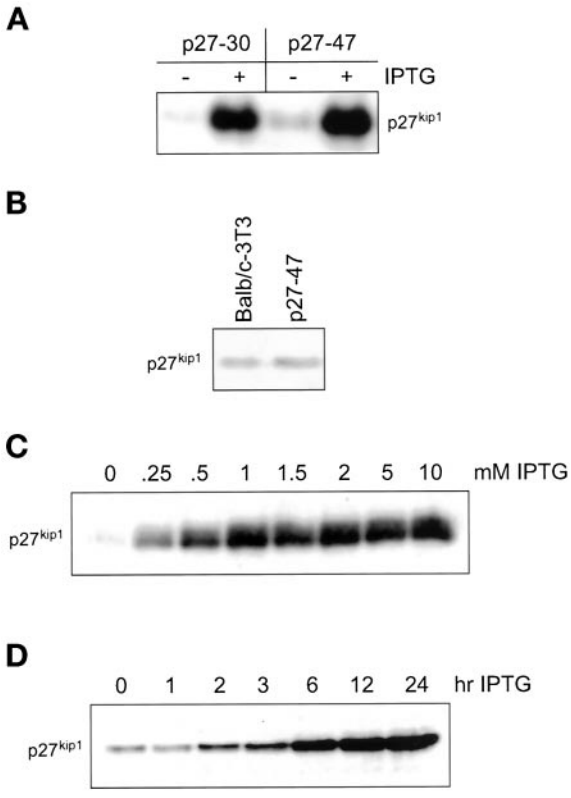


Figure 1. Inducible expression of p27kip1 in BALB/c-3T3-derived cell lines. (A) p27-30 and p27-47 cells were grown to 20–30% confluency and treated with 1 mM IPTG for 18 h. (B) Uninduced parental BALB/c-3T3 cells and p27-27 cells were compared for p27kip1 expression. (C) p27-47 cells received the indicated concentrations of IPTG for 24 h. (D) p27-47 cells were exposed to 1 mM IPTG for the indicated times. (A–D) Cell extracts (40 μg) were immunoblotted with antibody to p27kip1.

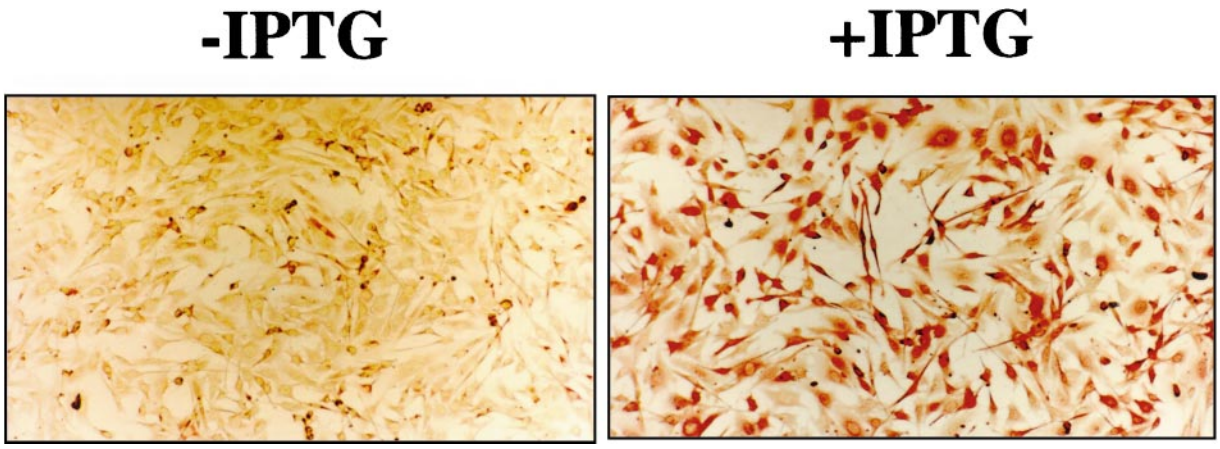


Figure 2. Nuclear localization of ectopically expressed $p27^{kip1}$ in p27-47 cells. Twenty-four hours after seeding, p27-47 cells received either no addition or 1 mM IPTG and were incubated for an additional 24 h. Cells were then rinsed with PBS and fixed in 4% formaldehyde. Cells were immunostained with $p27^{kip1}$ antibody using the avidin–biotin complex technique.

glycerol and were visualized by autoradiography. For supershifts, extracts were incubated with antibodies for 30 min before the addition of the labeled DNA probe.

Flow Cytometry

Cells were removed from the plates with 0.125% trypsin and 0.5 mM EDTA in PBS; an equal volume of medium containing 10% serum was added to neutralize the trypsin. Cells were pelleted and resuspended in PBS (1 ml), and 95% ethanol (4 ml) was added slowly. After incubation at 4°C for a minimum of 16 h, cells were pelleted and resuspended in PBS containing 0.1% Tween 20, 0.05% BSA, 10 μg/ml RNase A, and 50 μg/ml propidium iodide. After a further incubation at 4°C for at least 4 h, cell cycle distribution was determined with a Becton Dickinson (San Jose, CA) FACScan.

Autoradiography

Cells were radiolabeled with 5 μCi/ml [^{3}H]thymidine for 24–48 h, rinsed with PBS, and fixed with methanol. Fixed cultures were coated with photographic emulsion and after a 2-d exposure at room temperature were sequentially rinsed with developer (10 min), water, and fixer (10 min). To visualize unlabeled cells, cultures were incubated in Giemsa stain for 30 min.

Materials

PDGF (the BB isoform) was purchased from Biosource (Camarillo, CA). Antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA), PharMingen (San Diego, CA), and Transduction Laboratories (Lexington, KY). Roscovitine and PD98059 were obtained from Calbiochem (La Jolla, CA) and New England Biolabs (Beverly, MA), respectively.

RESULTS

Conditional Expression of $p27^{kip1}$ in BALB/c-3T3 Fibroblasts

To express high levels of the cell cycle inhibitor $p27^{kip1}$ in BALB/c-3T3 fibroblasts, we used the IPTG-inducible mammalian expression system described by Labow *et al.* (1990) and Pestov and Lau (1994). After selection in the appropriate antibiotics, resistant colonies were expanded and screened for $p27^{kip1}$ expression. Cultures were treated with or without 1 mM IPTG for 18 h, and cell extracts were immunoblotted with antibody to $p27^{kip1}$. As shown in Figure 1A, two clones, designated p27-30 and p27-47, exhibited substantially increased levels of $p27^{kip1}$ when exposed to IPTG. In the absence of IPTG, levels of $p27^{kip1}$ in both cell lines were similar to those of parental BALB/c-3T3 cells (Figure 1B; our unpublished results). IPTG dose–response curves demonstrated that IPTG concentrations as low as 0.25 mM induced $p27^{kip1}$ expression and that maximal expression occurred at 1 mM (Figure 1B). Increases in $p27^{kip1}$ levels were evident within 2 h of IPTG addition to cells and maximal at 12–24 h (Figure 1C). Collectively, the data in Figure 1 show that ectopic $p27^{kip1}$ expression in the p27-30 and p27-47 cell lines is effectively repressed in the absence of IPTG and is rapidly and dramatically elevated in response to IPTG.

We also determined the intracellular location of $p27^{kip1}$ by immunohistochemistry and found that $p27^{kip1}$ was present in both the nuclei and cytosol of IPTG-treated cells (Figure 2). A similar observation was made for NIH-3T3 cells ectopically expressing $p27^{kip1}$ and for endogenous $p27^{kip1}$ in a variety of cell types (Wang *et al.*, 1999). In addition, and in accord with the Western blots shown in Figure 1, levels of $p27^{kip1}$ immunostaining were substantially higher in IPTG-treated compared with untreated cells.

Effect of Increased Expression of $p27^{kip1}$ on the Growth of Sparse Cells

Previous studies have shown that levels of $p27^{kip1}$ increase in logarithmically growing BALB/c-3T3 cells deprived of serum and suggest that this increase is responsible for the subsequent exit of cells from the cell cycle (Coats *et al.*, 1996). This supposition implies that enforced expression of $p27^{kip1}$ in cycling cells should result in premature growth arrest, and to test this, the capacity of p27-47 cells to proliferate in the presence and absence of IPTG was determined. p27-47 cells were plated at low density and were refed 24 h later with fresh serum-containing medium supplemented with or

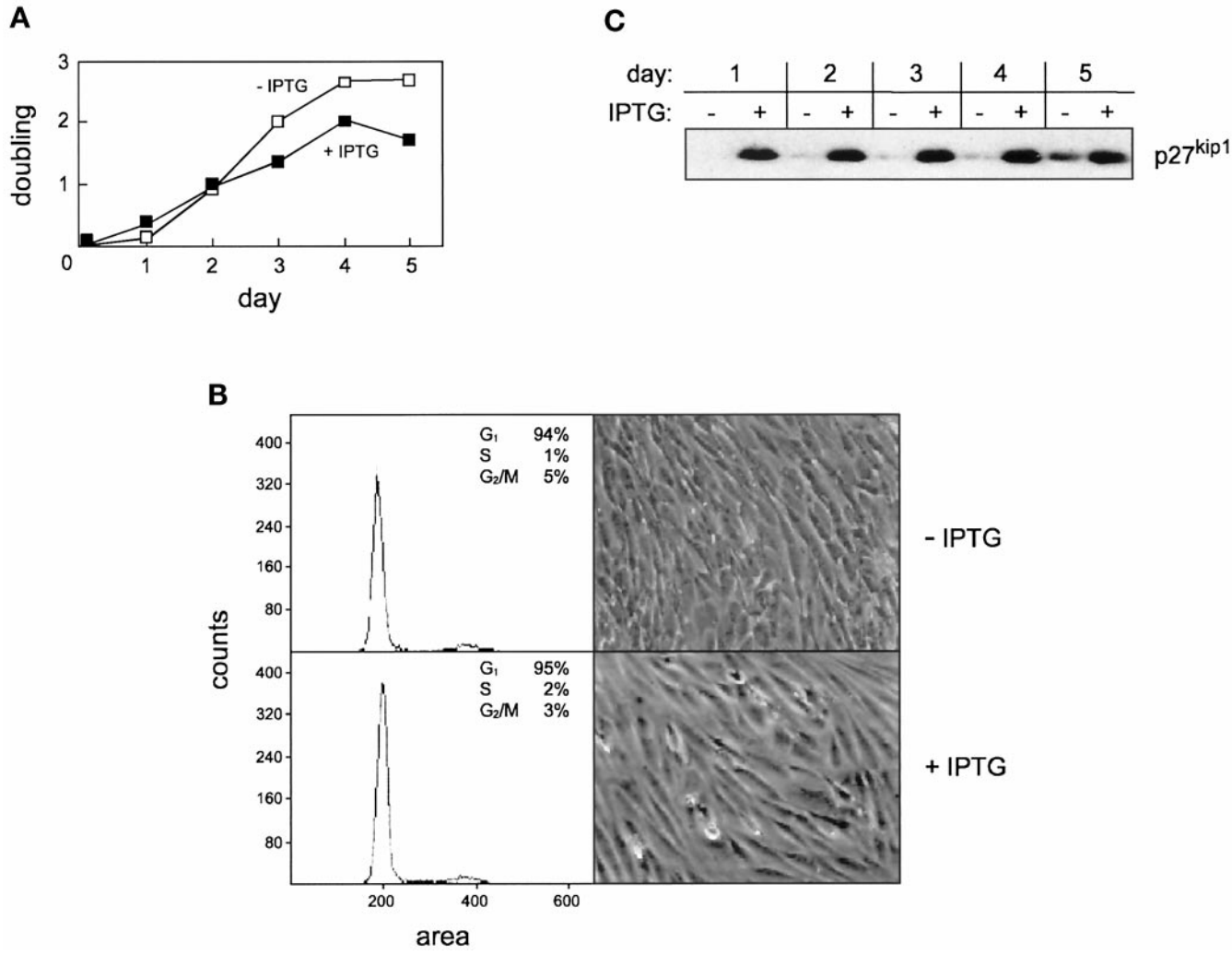
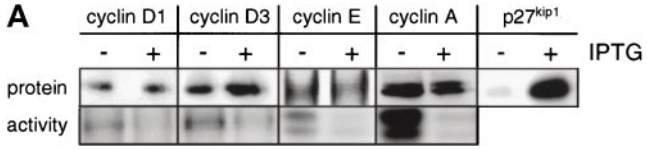


Figure 3. Continued proliferation of low-density cells expressing high levels of $p27^{kip1}$. p27-47 cells were seeded at a density of 700 cell/cm^2 in 60-mm culture dishes and 24 h after plating were refed with fresh medium containing 10% serum and either no addition (open squares) or 1 mM IPTG (closed squares; designated day 0). (A) At the indicated times, triplicate plates were harvested, and cell number was determined on a Coulter Counter. Data are expressed as number of population doublings per day. (B) Left panels, day 5 cultures were stained with propidium iodide, and the position of cells in the cell cycle was determined by flow cytometry. Right panels, day 5 cultures were photographed at a magnification of 100× (right panels). (C) Cell extracts were prepared at the indicated times and immunoblotted with antibody to $p27^{kip1}$.

without 1 mM IPTG (designated day 0). The number of cells per plate was determined daily for 5 d. As shown in Figure 3A, growth rates in the presence and absence of IPTG were not significantly different on days 1 and 2. After day 2, the IPTG-treated cells proliferated more slowly and reached a lower saturation density than did the untreated cells. However, both populations ceased proliferation at the same time (days 4 and 5), and both formed confluent monolayers (Figure 3B). Because confluent cultures of IPTG-treated cells were less dense than those of untreated cells, IPTG-treated cells were larger when density-arrested than were untreated cells. In the presence and absence of IPTG, cultures underwent two and nearly three population doublings, respectively, and it is noted that similar results were obtained using p27-30 cells. The ability of p27-47 cells to grow in the presence of IPTG was not due to a failure to maintain elevated levels of $p27^{kip1}$ for extended times; as shown in Figure 3C, amounts of $p27^{kip1}$ that exceeded those of control cells were present in IPTG-treated cells throughout the experimental period. The data in Figure 3C also show that levels of endogenous $p27^{kip1}$ increased progressively throughout the time course, and that the highest levels were observed on day 5, at which time cells were quiescent (Figure 3B). Collectively, these results indicate that increased $p27^{kip1}$ expression, while perhaps necessary for growth arrest (Coats *et al.*, 1996; Rivard *et al.*, 1996), is not sufficient.

To ensure that ectopically expressed $p27^{kip1}$ was functional, its capacity to inhibit cyclin/cdk activity in sparse



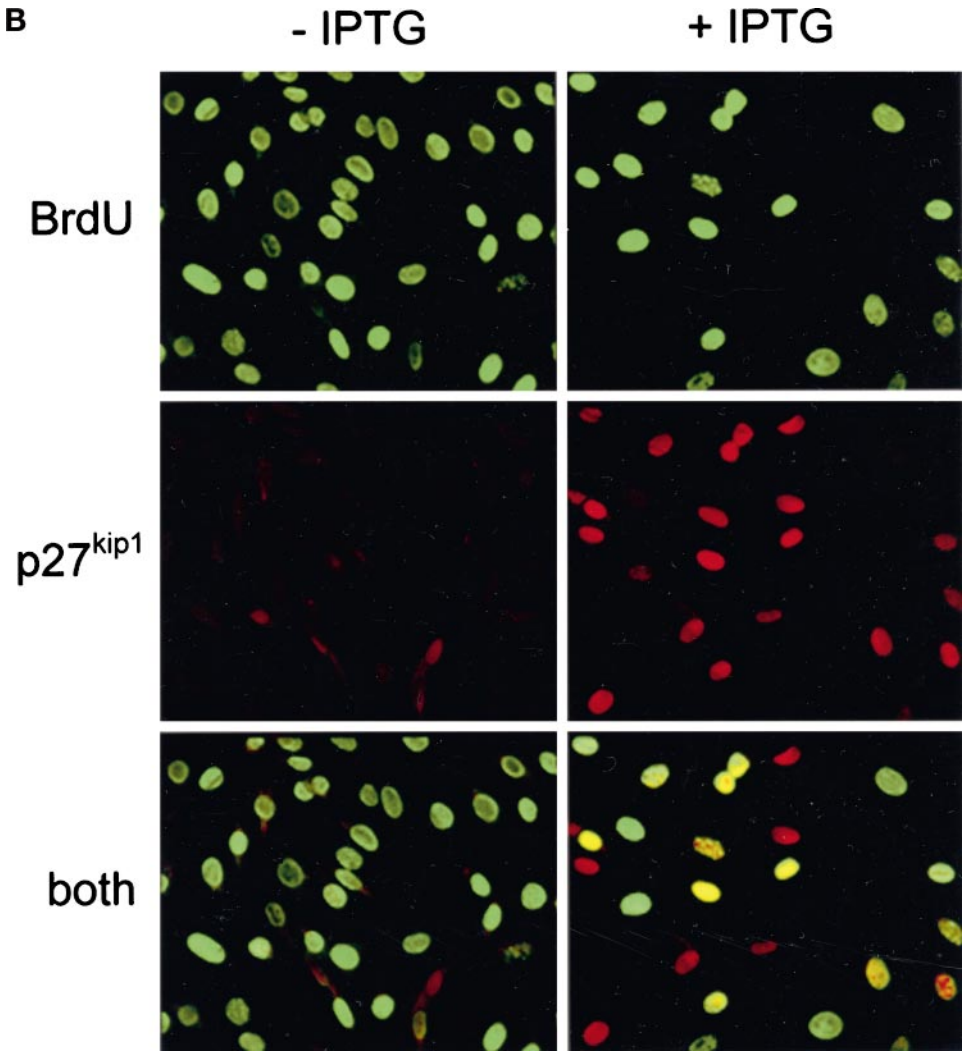


Figure 4. Inhibition of cyclin/cdk activity by ectopically expressed $p27^{kip1}$. (A) IPTG (1 mM final concentration) was added to p27-47 cells 24 h after plating, and cells were harvested 2 d later. Top panels, cell extracts were immunoblotted with the antibodies to the indicated proteins. Bottom panels, cell extracts were immunoprecipitated with antibodies to the indicated proteins, and immune complexes were assayed for kinase activity using GST-Rb (cyclins D1 and D3) or histone H1 (cyclins E and A) as substrate. (B) Logarithmically growing p27-47 cells were treated with (right panels) or without (left panels) 1 mM IPTG for 18 h. BrdU was added to a final concentration of 10 μM, and cells were incubated for an additional 20 h. Cells were harvested and immunostained for both $p27^{kip1}$ and BrdU as described in MATERIALS AND METHODS. Top panels, BrdU staining. Middle panels, $p27^{kip1}$ staining. Bottom panels, BrdU and $p27^{kip1}$ staining were superimposed.

cultures was determined. p27-47 cells received 1 mM IPTG 24 h after plating and were harvested 2 d later, at which time they were actively proliferating (our unpublished results). Cell extracts were immunoprecipitated with antibody to cyclin D1, D3, E, or A, and cyclin-dependent cdk activity was determined in immune complexes by in vitro kinase assay using Rb (cyclin D1 and D3) or histone H1 (cyclin E and A) as substrate. As shown in Figure 4A, treatment of p27-47 cells with IPTG substantially reduced the kinase activities associated with all four cyclins; as noted above, cyclins E and A associate with cdk2, and in BALB/c-3T3 cells, the D cyclins complex predominantly with cdk4. The data in Figure 4A also show that IPTG effectively induced the expression of $p27^{kip1}$ in this experiment, and that the levels of cyclins D1, D3, E, and A were similar in IPTG-treated and untreated cultures. Thus, the decrease in kinase activity in the treated cells resulted from $p27^{kip1}$-mediated inhibition and not from lack of cyclin expression.

To exclude the possibility that the proliferation of p27-47 cells in IPTG-containing medium reflected a segment of the population that did not express high levels of $p27^{kip1}$, we examined $p27^{kip1}$ expression and DNA replicative capacity in individual cells. In these experiments, low-density logarithmically growing p27-47 cultures were treated with or

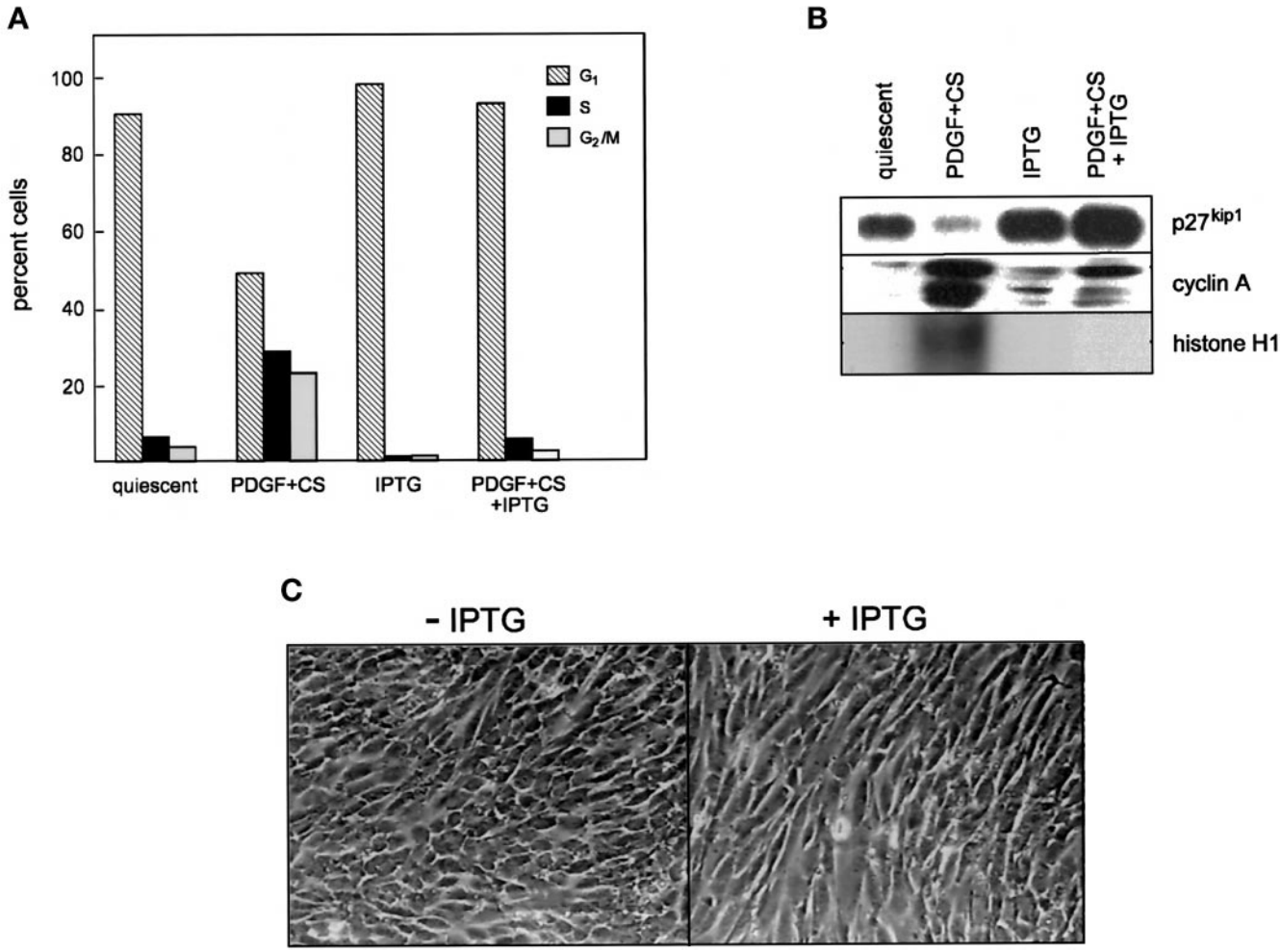


Figure 5. Growth inhibition of dense cultures by high levels of $p27^{kip1}$. p27-47 cells were grown to confluency in medium containing 10% serum and incubated overnight in the presence or absence of 1 mM IPTG. (A) Cells were either harvested at this time (quiescent, IPTG) or refed with medium containing 10 ng/ml PDGF and 10% calf serum (CS); cultures pretreated with IPTG received fresh IPTG. Cells were harvested 24 h later for analysis of cell cycle position by flow cytometry. (B) Cultures were treated as in A. Cell extracts were immunoblotted with antibody to $p27^{kip1}$ (top panel) or cyclin A (middle panel) or were immunoprecipitated with antibody to cyclin A for determination of cyclin A/cdk2 activity using histone H1 as substrate (bottom panel). (C) Confluent cultures were pretreated with IPTG overnight and stimulated with PDGF and serum for 24 h. Cultures were trypsinized and replated at a density of 700 cells/cm^2 in medium containing 10% serum. Cells were cultured for 7 d in the presence or absence of 1 mM IPTG and were photographed at a magnification of 100×.

without IPTG for 18 h and subsequently with 10 μM BrdU for an additional 20 h. Cells were harvested and immunostained for both BrdU and $p27^{kip1}$. The IPTG-treated population was much more intensely stained than was the control population, and many of the highly stained cells in this population incorporated BrdU into DNA (Figure 4B). These findings indicate that most of the cells in the IPTG-treated culture express elevated amounts of $p27^{kip1}$, and that such cells are capable of traversing S phase. Cells exhibiting increased levels of $p27^{kip1}$ staining but not BrdU staining were also detected in cultures exposed to IPTG; these nonreplicating cells presumably contribute to the slowdown in proliferation that occurs as these cultures approach confluency. Cultures receiving IPTG also contained BrdU-stained cells that lacked detectable $p27^{kip1}$ staining; however, the number of these cells was less than that of double-stained cells. Thus, although cells expressing low (or endogenous) levels of $p27^{kip1}$ account for a portion of the proliferative response exhibited by sparse cultures in IPTG-supplemented medium, the bulk of this response can be attributed to cells expressing high amounts of $p27^{kip1}$.

Effect of Increased Expression of $p27^{kip1}$ on the Reentry of Density-arrested Cells into the Cell Cycle

Although IPTG had only a limited effect on the growth rate of cycling cells at low density, the capacity of cells to grow in the presence of IPTG decreased as cells approached confluency. This finding suggests that $p27^{kip1}$ expression may have a greater impact on cells at higher densities, and for this reason,

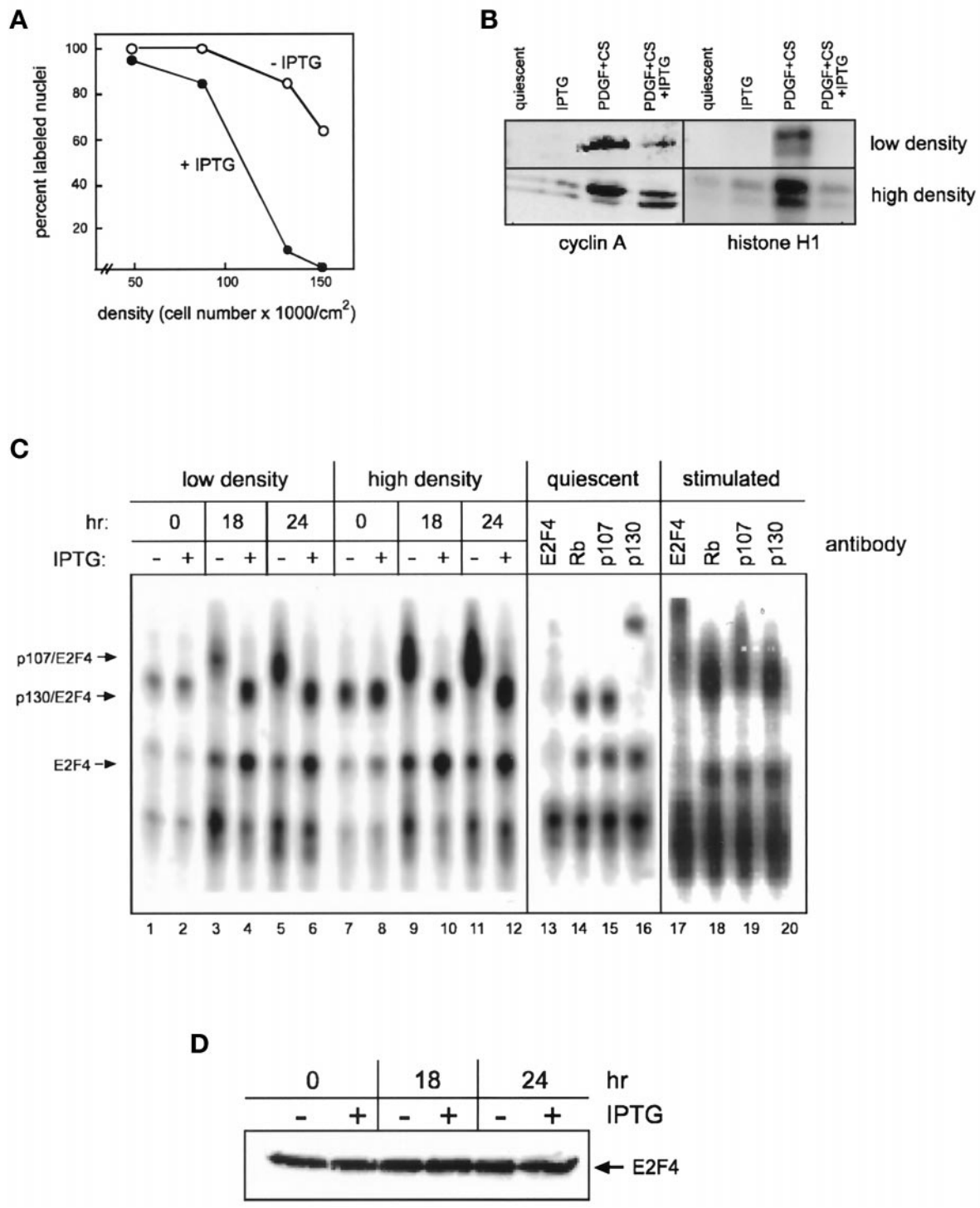


Figure 6. Effect of cell density on the growth of cells expressing elevated amounts of $p27^{kip1.}$ (A) p27-47 cells were seeded at a density of 6000 cells/cm^2 in 100-mm plates and grown to confluency in medium containing various concentrations of serum; cell number was determined. Replicate cultures were pretreated overnight with (closed circles) or without (open circles) 1 mM IPTG and subsequently refed with medium supplemented with 20 ng/ml PDGF, 10% PPP, 1 mM IPTG, and 5 μCi/ml [^{3}H]thymidine. Cells were harvested 48 h later, and the percentage of cells incorporating [^{3}H]thymidine was determined by autoradiography. (B) p27-47 cells were arrested at low or high density and pretreated overnight with 1 mM IPTG. After pretreatment, cultures either were harvested (quiescent, IPTG) or received fresh medium containing 10 ng/ml PDGF and 10% calf serum (CS) with or without IPTG for 24 h. Cell extracts were prepared and immunoblotted with cyclin A antibody

we assessed the capacity of density-arrested cells to initiate proliferation in medium containing IPTG. In these experiments, p27-47 cells grown to confluency in medium containing 10% serum were pretreated overnight with 1 mM IPTG, and control and pretreated cultures were subsequently refed with fresh medium supplemented with 10 ng/ml PDGF plus 10% serum either with or without IPTG. In the absence of IPTG, PDGF plus serum induced cell cycle traverse; at 24 h after stimulation, 28.5% of the cells were in S phase, and 22.7% were in G_2–M compared with only 6.6 and 3.5%, respectively, in unstimulated populations (Figure 5A). On the other hand, the percentages of S and G_2–M phase cells in cultures exposed to PDGF plus serum in the presence of IPTG were similar to those of unstimulated cultures. Thus, treatment of density-arrested cells with IPTG completely inhibits G_0–G_1 traverse in response to PDGF plus serum. Similar results were obtained in experiments in which proliferative capacity was assessed by [^{3}H]thymidine labeling (our unpublished results). In agreement with previous findings by us (Agrawal *et al.*, 1995, 1996; Winston *et al.*, 1996b) and others (Kato *et al.*, 1994; Nourse *et al.*, 1994; Rivard *et al.*, 1996), endogenous levels of p27^{kip1} decreased in cells stimulated in the absence of IPTG, whereas the amount of cyclin A and the activity of its associated kinase increased (Figure 5B). As expected, total (endogenous and ectopic) levels of p27^{kip1} remained high in cells receiving PDGF plus serum and IPTG, and although levels of cyclin A increased somewhat, its associated kinase was not detectably activated.

To ensure that the inability of density-arrested cells to enter S phase in the presence of IPTG was not due to a loss of cell viability, confluent cultures stimulated in the presence of IPTG for 24 h were trypsinized and replated at subconfluent densities in medium containing 10% serum supplemented with or without IPTG. Although incapable of traversing the cell cycle when incubated with IPTG and mitogens at high density, cells reinitiated proliferation and grew to confluence in both the presence and absence of IPTG when placed at lower densities in serum-containing medium (Figure 5C). Similar to the results shown in Figure 3, A and B, cells grown in the presence of IPTG arrested at a lower density than did those grown in its absence. These results demonstrate that confluent cultures are not irreversibly damaged by IPTG treatment.

Effect of Varying Cell Densities on Cell Susceptibility to p27^{kip1}-mediated Growth Inhibition

The different responses of sparse cycling and dense arrested cells to p27^{kip1} overexpression could reflect differences in cell density (sparse vs. dense) or proliferative status (growing vs. quiescent). To distinguish between these alternatives, we grew p27-47 cells in medium supplemented with various concentrations of serum for several days; as a result, cells became confluent at densities that increased as a function of serum concentration. Regardless of density, all cultures were quiescent as determined by autoradiography (our unpublished results). Cells were then stimulated with 20 ng/ml PDGF plus 10% platelet-poor plasma (PPP) in the presence of IPTG, and 48 h later, the percentage of cells that had traversed S phase was determined by autoradiography. When arrested at low density ($<10^5$ cells/cm^2), essentially all cells entered S phase when exposed to PDGF plus PPP and IPTG (Figure 6A). Thus, low-density quiescent cells were essentially refractory to p27^{kip1}-mediated growth inhibition as were sparse cycling cells. When arrested at higher densities ($>10^5$ cells/cm^2), the percentage of cells entering DNA synthesis progressively declined, eventually reaching the point at which cell cycle traverse was totally blocked by high levels of p27^{kip1}. In the absence of IPTG, entry into S phase was also a function of cell density; however, the inhibitory effects of density on proliferation were much less pronounced, with 65% of the population capable of initiating DNA synthesis at the highest density examined. These data demonstrate that the capacity of p27^{kip1} to inhibit cell cycle traverse increases as cell density increases, and because all cultures in this experiment were quiescent, that the differing effects of supraphysiological amounts of p27^{kip1} on cell cycle traverse are unrelated to growth state.

As described above, ectopic expression of p27^{kip1} inhibited both cdk2 and cdk4 activity in sparse cycling cells, and the data presented in Figure 6, B and C, demonstrate a similar effect for quiescent cells exposed to PDGF and serum. In these experiments, p27-47 cells were arrested at either high or low density and subsequently stimulated with PDGF plus serum with or without IPTG for the indicated times. Cdk2 activity was assessed by histone kinase assay of cyclin A immunoprecipitates, and the data in Figure 6B show that although cyclin A was expressed, kinase activity was not apparent in immunoprecipitates of cells stimulated in the presence of IPTG at either high or low density. As a means of assessing cdk4 activity in vivo, we monitored the DNA binding activity of p130/E2F4 complexes by EMSA using a radiolabeled DNA fragment containing an E2F binding site; we have shown previously that cdk4 phosphorylates p130 and that this event leads to the dissociation of p130 from E2F4 (Dong *et al.*, 1998a). To conclusively identify the components of the band shifts, cell extracts were incubated with antibodies to the relevant proteins before addition of the probe (Figure 6C, lanes 13–20). In the absence of IPTG, a DNA binding activity consisting of p130 and E2F4 was present in quiescent but not stimulated cells, and the loss of this activity from stimulated cells was accompanied by an increase in free E2F4 activity and the appearance of an activity containing E2F4 and p107 (Figure 6C; Rb-containing complexes and other members of the E2F family are not detected by the assay conditions used). p107, which is also a member of the Rb family, is expressed in late G_1–early S phase and is thought to bind E2F proteins released from p130 (Hauser *et al.*, 1997; Nevins, 1998). In contrast, p130/E2F4 complexes remained intact in cells stimulated in IPTG-containing medium at either high or low density, and p107/

Figure 6 (cont). (left panels) or immunoprecipitated with cyclin A antibody and assayed for kinase activity (right panels). (C) p27-47 cultures were arrested and pretreated with IPTG as in B. Lanes 1–12, cells were stimulated with 10 ng/ml PDGF and 10% calf serum plus or minus IPTG for 18 or 24 h, and cell extracts were assayed for E2F DNA binding activity by EMSA. Lanes 13–16, extracts of low-density quiescent IPTG-pretreated cells were incubated with the indicated antibodies before addition of the radiolabeled probe. Lanes 17–20, extracts of high-density cells stimulated with PDGF plus serum in the absence of IPTG were incubated with the indicated antibodies before addition of the radiolabeled probe. (D) High-density cultures were pretreated overnight with 1 mM IPTG and stimulated with 10 ng/ml PDGF and 10% calf serum with or without 1 mM IPTG for 18 or 24 h. Cell extracts (175 μg) were immunoblotted with antibody to E2F4.

E2F4 complexes were not observed. Taken together, the data in Figure 6, B and C, demonstrate that the activities of cdk2 and cdk4 are effectively repressed by ectopic $p27^{kip1}$ expression in p27-47 cells stimulated to proliferate at both low and high densities. Thus, the different capacities of these populations to initiate DNA synthesis are not due to differences in the extent of cdk inhibition. Our findings also indicate that quiescent cells at low (but not high) density require only minimal, if any, cyclin/cdk activity and p130/E2F4 dissociation for proliferation. Although cdk4 activity was inhibited, and although total levels of E2F4 protein remained constant (Figure 6D), the amount of free E2F4 activity in cells stimulated in the presence of IPTG was comparable with that of cells stimulated in its absence (Figure 6C). The reason for this is not known.

Effect of SV40 and Roscovitine on the G_0/G_1 Traverse of Cells Expressing High Levels of $p27^{kip1}$

The capacity of DNA tumor viruses such as SV40 and adenovirus to induce the proliferation of quiescent cells is well-documented (Levine, 1990) and is thought to result in part from the binding of viral oncoproteins to Rb family members in a manner that prohibits their association with E2Fs (Chellappan *et al.*, 1992). Because complexes containing the Rb and E2F proteins function in many cases as dominant-acting transcriptional repressors (Weintraub *et al.*, 1992, 1995), viral oncoproteins might also promote cell cycle traverse by removing these complexes from promoter sites and thus derepressing gene expression. Given these actions of viral oncoproteins, we asked whether infection of cells with SV40 could overcome the growth-inhibitory effects of ectopic $p27^{kip1}$ expression in cells arrested at high density. In this experiment, p27-47 cells pretreated with or without IPTG were infected with SV40 and placed in medium containing PPP and [^{3}H]thymidine for 48 h; IPTG was readded to pretreated cultures. For comparative purposes, replicate cultures were stimulated with PDGF plus PPP or PPP alone, and the percentage of cells that had synthesized DNA was determined by autoradiography. In the absence of IPTG, ~80% of the population initiated DNA synthesis in response to PDGF plus PPP, and this response was markedly inhibited by IPTG (Figure 7A). SV40, in conjunction with PPP, was as effective as PDGF in inducing entry into S phase; however, in contrast to PDGF, SV40 retained the capacity to stimulate cell cycle progression in the presence of IPTG (~78% labeled nuclei). Similar results were obtained in experiments in which cells were infected with adenovirus (our unpublished results).

Although the mechanism by which SV40 and adenovirus allow high-density p27-47 cells to proliferate in conditions in which $p27^{kip1}$ is expression is elevated is not known, it is likely that modulation of Rb and/or p130 activity plays a key role. As shown in Figure 7B, cyclin A-associated kinase activity, measured using both histone H1 and GST-Rb as substrates, was virtually undetectable in SV40-stimulated (as well as PDGF-stimulated) p27-47 cells cotreated with IPTG. Thus, SV40 does not counteract the growth-inhibitory effects of $p27^{kip1}$ overexpression by restoring cyclin/cdk activity. A similar conclusion was reached by Alevizopoulos *et al.* (1998) in studies in which the adenovirus oncoprotein E1A was used to prevent $p27^{kip1}$-mediated growth arrest in NIH-3T3 fibroblasts.

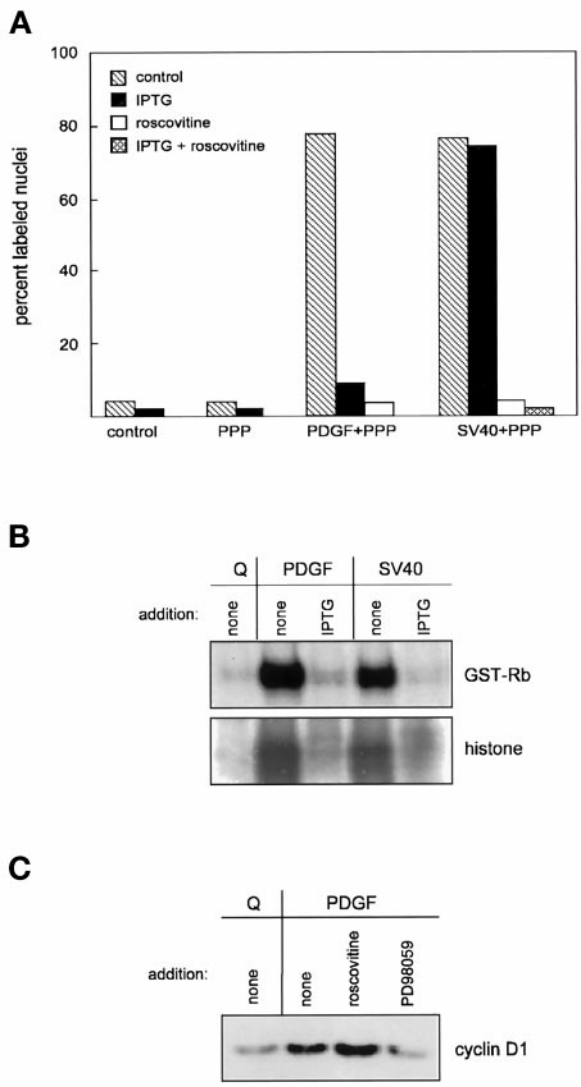


Figure 7. Growth of dense cells infected with SV40 in the presence of elevated $p27^{kip1}$ expression. (A) p27-47 cells were arrested at high density and incubated with or without 1 mM IPTG overnight. Cultures received the additions indicated at the following concentrations: PPP, 10%; PDGF, 20 ng/ml; SV40, multiplicity of infection = 100:1; IPTG, 1 mM; roscovitine, 25 μM; and [^{3}H]thymidine, 5 μCi/ml. Cells were harvested 48 h later for quantitation of percent labeled nuclei by autoradiography. (B) Cells were pretreated overnight with IPTG as in A and were stimulated with PDGF and 10% serum or SV40 and 10% serum for 24 h. Cyclin A-associated kinase activity was measured in an in vitro kinase assay using histone H1 and GST-Rb as substrates. (C) Cells were pretreated with 25 μM roscovitine or 100 μM PD98059 for 1 h and stimulated for 12 h in fresh medium containing PDGF and serum; roscovitine and PD98059 were readded to pretreated cultures. Cyclin D1 levels were determined by Western blotting.

As described above, both sparse cycling cells and low-density quiescent cells proliferate in mitogen-supplemented medium containing IPTG in the apparent absence of cyclin/cdk activity. It is possible, however, that residual levels of

cdk2 activity, barely or not detectable by in vitro kinase assays but sufficient for the growth of low-density or SV40-infected cells, remain. To test this, p27-47 cells arrested at high density were stimulated with SV40 and PPP (plus or minus IPTG) or PDGF and PPP; some cultures also received the cdk2 inhibitor roscovitine, and all cultures received [^{3}H]thymidine. Cells were harvested 48 h later, and the percentage of labeled nuclei was determined. As shown in Figure 7A, cells stimulated in medium lacking roscovitine entered S phase, whereas cells receiving this agent did not. This finding demonstrates that ablation of cyclin/cdk activity precludes cell cycle traverse and suggests that cdk2 activity is required, albeit at minimal levels, even in cells overexpressing $p27^{kip1}$. Because roscovitine has been reported to inhibit the activity of mitogen-activated protein kinase (MAPK), albeit at higher concentrations than those required to inhibit cdk2 activity (Meijer *et al.*, 1997), the effect of this agent on MAPK activation was assessed. Previous studies have shown that cyclin D1 expression is dependent on MAPK activation (Lavoie *et al.*, 1996; Winston *et al.*, 1996a), and in accord with these studies, we show that treatment of cells with PD98059, which inhibits the activity of MAPK kinases (MAPKK 1/2), ablates the increase in cyclin D1 levels induced by PDGF and serum (Figure 7C). In contrast, roscovitine had no effect on cyclin D1 induction. Thus, it is likely that at the concentration of roscovitine used, cdk2 activity is specifically targeted.

DISCUSSION

In response to environmental cues, cells enter and exit the cell cycle, and the cdk inhibitor $p27^{kip1}$ is thought to play a critical role in this process (Sherr and Roberts, 1999). Because of alterations in both synthetic rate and stability and sequestration by a large pool of cyclin D/cdk complexes, levels of free $p27^{kip1}$ fluctuate during the cell cycle. In general, $p27^{kip1}$ levels increase when conditions are suboptimal for growth, and as a result, cyclin/cdk complexes are inactivated and proliferation ceases. Conversely, after mitogenic stimulation, $p27^{kip1}$ levels decrease, cyclin/cdk complexes become active, and growth-arrested cells reenter the cell cycle.

It is possible, however, that cells react differently to a given amount of $p27^{kip1}$ in different situations, and we show here that the capacity of cells to growth arrest in response to $p27^{kip1}$ is governed by cell density. Our experiments were done on a BALB/c-3T3-derived cell line (termed p27-47) that ectopically expressed high levels of $p27^{kip1}$ in the presence of IPTG and essentially basal levels in the absence of IPTG. When added to sparse cycling cells, IPTG had little effect on log phase growth; differences in growth rate, however, became apparent as cells approached confluency, with the IPTG-treated cells doubling more slowly and reaching a lower saturation density than the untreated cells. Similarly, cells arrested at low densities entered DNA synthesis when exposed to PDGF plus serum (or PPP) and IPTG, whereas those arrested at high densities did not. Thus, cells at low density, whether cycling or quiescent, are essentially refractory to levels of $p27^{kip1}$ that markedly inhibit cyclin/cdk activity (see below), and the capacity of cells to escape $p27^{kip1}$-mediated growth inhibition decreases as cell density increases.

The different responses of low- and high-density cells to IPTG were not due to differences in $p27^{kip1}$ levels; regardless of cell density, the amount of $p27^{kip1}$ in IPTG-treated cells far exceeded that of uninduced cells. In addition, ectopic expression of $p27^{kip1}$ essentially abrogated the activities of cdk4 and cdk2 in both sparse and dense cells. Our in vivo assays of p130/E2F DNA binding activity clearly demonstrate inhibition of cdk4 activity by $p27^{kip1}$ expression in p27-47 cells, and the observation that levels of cyclins D1 and D3 were unaffected by IPTG treatment indicates that repression of this activity by $p27^{kip1}$ was a direct result of cyclin D/cdk4 interaction with this CKI and not a secondary consequence of reduced cyclin synthesis. Although studies by others (Blain *et al.*, 1997; Cheng *et al.*, 1999) propose that binding of $p27^{kip1}$ to cyclin D/cdk4 complexes at low stoichiometry does not impair cdk4 activity, numerous reports have demonstrated cdk4 inactivation by supraphysiological levels of $p27^{kip1}$ (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994; Shiyanov *et al.*, 1997; Alevizopoulos *et al.*, 1998). Similar to cyclins D1 and D3, cyclin E was present at comparable amounts in IPTG-treated and untreated p27-47 cells at both high and low density (Figure 4; our unpublished results); thus, association of cyclin E/cdk2 complexes with $p27^{kip1}$ presumably accounts for the lack of detectable cdk2 activity in cyclin E immunoprecipitates of treated cells. Inactivation of cdk4 and cdk2 in NIH-3T3 cells by high levels of $p27^{kip1}$ generated by methods similar to those used here has been described previously (Shiyanov *et al.*, 1997).

Levels of cyclin A were similar in cycling p27-47 cells treated with or without IPTG and increased, albeit to a lesser extent, in quiescent cells stimulated in the presence compared with the absence of IPTG; like cyclins D1, D3, and E, cyclin A complexes were inactive in IPTG-treated cells. Cyclin A is encoded by an E2F target gene (Mudryj *et al.*, 1991; DeGregori *et al.*, 1995; Schulze *et al.*, 1995), and our data show that free E2F4 DNA binding activity increased to similar extents in growth-arrested cells receiving PDGF plus serum regardless of $p27^{kip1}$ levels or cell density. Whether this activity contributes to cyclin A expression in IPTG-treated cells is not known at present. The source of the E2F4 activity in cells exposed to IPTG is also unclear; E2F4 is bound to p130 in G_0-arrested cells and is released upon cdk4-mediated phosphorylation of p130 (Smith *et al.*, 1996; Dong *et al.*, 1998a). p130/E2F4 complexes, however, did not noticeably dissociate in p27-47 cells stimulated in IPTG-containing medium. Furthermore, in agreement with previous studies (Flores *et al.*, 1998), total levels of E2F4 were unaffected by mitogenic stimulation or IPTG addition. Although E2F4 also binds Rb, and thus could be derived from Rb-containing complexes, removal of E2F4 from Rb requires phosphorylation of Rb by cdk4 and/or cdk2 (Nevins, 1998; Sherr and Roberts, 1999), neither of which was appreciably active in IPTG-treated cells. Thus, these findings suggest that a cyclin/cdk-independent mechanism may be responsible for the elevated E2F4 activity in cells receiving IPTG. Alternatively or additionally, changes in the affinity of E2F4 for DNA may also be involved.

Regardless of the mechanism by which free E2F4 DNA binding activity is generated in IPTG-treated p27-47 cells, it represents a means by which low-density cells with minimal cyclin/cdk activity could traverse the cell cycle (e.g., it might allow expression of enzymes required for DNA replication; whether E2F4 was nuclear and transcriptionally active, however, remains to be determined). Increased E2F4 binding

activity was, however, insufficient for the proliferation of cells at high density. The E2F proteins modulate transcription by two mechanisms: 1) they bind E2F recognition sites in gene promoters and directly induce transcription; and 2) they tether Rb family members to DNA, thus allowing them to actively repress transcription by a mechanism thought to involve histone deacetylases (Luo *et al.*, 1998). Therefore, an increase in the activity of E2F4 (or of other E2Fs) may be insufficient for the cell cycle traverse of high-density cultures, because repressor complexes (e.g., p130/E2F4) still exist. This premise is supported by our data showing that p27-47 cells arrested at high density initiated DNA synthesis in the presence of IPTG when infected with SV40, a virus that encodes proteins known to bind Rb family members and to prevent their association with E2Fs and consequently with DNA (Chellappan *et al.*, 1992). SV40, therefore, renders high-density cells similar to low-density cells in terms of lack of responsiveness to $p27^{kip1}$-mediated growth inhibition. Although it is likely that this action of SV40 reflects its capacity to alleviate Rb- and/or p130-mediated gene repression, the ability of SV40 to modulate other cell cycle regulatory events cannot be excluded. Data by Alevizopoulos *et al.* (1998), for example, suggest that E1A prevents the $p27^{kip1}$-mediated growth arrest of rodent fibroblasts by inducing both Rb-dependent and -independent events. Although SV40 stimulated the G_0–G_1 traverse of p27-47 cells in the presence of IPTG, it did not restore cyclin/cdk activation or induce this response in cells cotreated with the potent cdk2 inhibitor roscovitine. Similarly, as shown by others (Hofmann and Livingston, 1996), SV40 large T antigen did not stimulate the proliferation of cells transfected with dominant negative cdk2. Thus, SV40 does not promote cell cycle traverse by simply bypassing the requirement of cycling cells for cdk activity but instead appears to act in conjunction with low levels of cyclin/cdk activity.

As low-density p27-47 cells entered S phase when exposed to IPTG, and as entry into S phase was blocked by the cdk2 inhibitor roscovitine, overexpression of $p27^{kip1}$ apparently inhibits but does not totally abolish cdk2 (or presumably cdk4) activity. Thus, although not necessarily detectable by in vitro kinase or other assays, cyclin/cdk activity refractory to inhibition by high levels of $p27^{kip1}$ and sufficient for the growth of low-density cells apparently persists in IPTG-treated p27-47 cells. Residual cdk activity, on the other hand, did not support the mitogen-stimulated growth of cells at high density, despite the capacity of these cells to resume proliferation in IPTG-containing medium when replated at lower densities. Cell density, therefore, apparently sets the threshold level of cdk activity required for the progression of cells through the cell cycle. At lower cell densities, less cyclin/cdk activity is required, and proliferation occurs in the presence of high amounts of $p27^{kip1}$. Conversely, it can be predicted that conditions that decrease $p27^{kip1}$ expression and/or increase cyclin/cdk activity will allow proliferation at high cell densities. In support, previous studies have shown that liver and brain cells of $p27^{kip1}$-deficient mice are more dense that those of $p27^{kip1}$-containing mice (Fero *et al.*, 1996) and that rat fibroblasts grow to higher densities when infected with cyclin E (Ohtsubo and Roberts, 1993).

The mechanism by which cell density specifies the amount of cyclin/cdk activity needed for cell cycle traverse is not known. We suggest that the proliferation of cells at high density requires a mitogenic signaling event(s) that is not required by cells at low density. The nature of this event is not known but may involve the abrogation of growth-suppressive spatial constraints imposed by cell–cell contact at high density. Such constraints may be put in place by the cadherins, a family of ubiquitous calcium-dependent transmembrane receptors that are thought to mediate density-dependent growth arrest (St. Croix and Kerbel, 1997). Accomplishment of the yet-to-be-determined signaling event allows cells arrested at high density to exit G_0 and prevents the entry of cycling cells into G_0 as they approach confluency and is dependent on a specific level of cyclin/cdk activity, which in turn is dependent at least in part on the amount of $p27^{kip1}$ in the cell (Figure 8). The capacity of agents that inactivate p130 and Rb to promote the growth of high-density cells in the presence of suboptimal amounts of cyclin/cdk activity suggests that these proteins play a role in generating the high-density proliferation-permissive signal. We speculate that induction of this signal, and consequent release of cells from spatial or other restraints, involves specific gene products, the expression of which is prevented by p130- and/or Rb-containing complexes. Thus, for cells at high density, disruption of these "pocket protein" complexes may be necessary for the traverse of cell cycle checkpoints in addition to the restriction point.

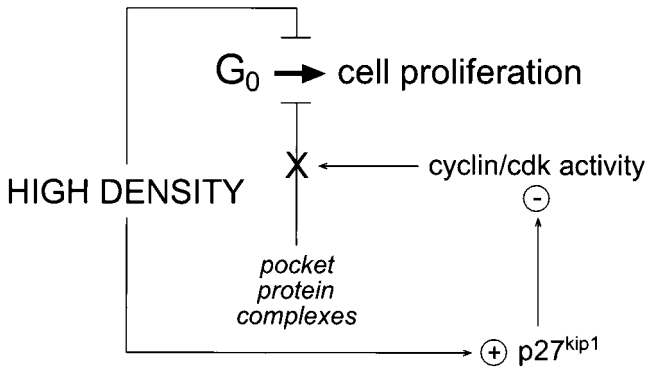


Figure 8. Model of density-dependent $p27^{kip1}$-induced growth arrest. The proliferation of cells at high density requires an event that is inhibited by pocket protein complexes containing Rb and/or p130. Growth of cultures to high density results in G_0 arrest, an increase in $p27^{kip1}$ levels (+), and a decrease in cyclin/cdk activity (−). As a consequence, Rb- and p130-containing complexes remain intact, and expression of gene products required for the proliferation of high-density cells is prevented.

In summary, our studies suggest that $p27^{kip1}$ signals and maintains cell cycle exit only when conditions for proliferation are limiting (e.g., cell density is high). This finding complements previous studies showing that cells grow in mitogen-restricted conditions when depleted of $p27^{kip1}$ (Rivard *et al.*, 1996; Tomoda *et al.*, 1999). Although fibroblasts derived from $p27^{kip1}$-deficient mice retain the capacity to growth arrest, the larger size of these mice suggests that cells remain in the proliferative cycle for extended times during development. $p27^{kip1}$ may, therefore, function as an early indicator of conditions that are suboptimal but not insufficient for cell growth, and thus allow cells to enter and exit

the cell cycle in response to subtle changes in their environment.

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