Progression of eukaryotic cells through the cell cycle is governed by the sequential activation, inactivation, and subsequent inactivation of a series of cyclin-dependent kinase (Cdk) complexes. p27Kip1 (p27) is a Cdk inhibitor that blocks, in vitro, the activity of cyclin D-Cdk4, cyclin D-Cdk6, cyclin E-Cdk2 as well as cyclin A-Cdk2, a complex active during S phase. The level of p27 protein expression, usually high in G0/G1 resting cells, declines as cells progress toward S phase and enforced expression of p27 in fibroblasts causes G1 arrest. This situation prevails in CCL39, a Chinese hamster lung fibroblast cell line (this report). However, in addition to p27, several other Cdk inhibitors known to alter G1 progression coexist in most mammalian cells. To investigate the specific contribution of p27 in the control of the mitogen-sensitive G0/G1 arrest, we specifically reduced its expression by expressing a full-length p27 antisense cDNA in CCL39 cells. Interestingly, reduction of up to 90% of p27 protein expression increased both basal and serum-stimulated gene transcription of cyclin D1, cyclin A, dihydrofolate reductase, and DNA synthesis reinitiation. Moreover, overexpression of this antisense allows cells to grow for several generations in a serum-free medium supplemented with insulin and transferrin only, thus suggesting that p27-depleted cells cannot exit the cell cycle. These effects were fully reversed by coexpression of a plasmid encoding p27 sense. We conclude that p27, by setting the level of growth factor requirement, plays a pivotal role in controlling cell cycle exit, a fundamental step in growth control.

Cell cycle progression is controlled by cyclin-dependent kinases (Cdks) counterbalanced by Cdk inhibitors (CKIs) (1). Two gene families of mammalian Cdk inhibitors have been identified. One family includes p16INK4A (2), p15INK4B (3), p18, and related proteins of 15–20 kDa (4–6), all containing a characteristic 4-fold repeated ankyrin-like sequence. The INK4 proteins specifically inhibit Cdk4 and Cdk6 (2–5) and their overexpression blocks cell proliferation (4, 5). The p15 and p16 genes are adjacent on human chromosome 9p21, at or near a familial melanoma predisposition locus (7, 8). p16 is mutated at high rates in certain malignancies (9, 10), supporting the idea that this is a tumor suppressor gene. A second family of CKIs include p21Cip1 (11–15), p27Kip1 (16, 17), and p57Kip2 (18, 19), three proteins structurally unrelated to the INK4 proteins. In vitro, p21, p27, and p57 have broad specificity, inhibiting the kinase activity exhibited by the G1 cyclin-dependent kinases cyclin D-Cdk4 and cyclin E-Cdk2, the complex cyclin A-Cdk2, and, to a lesser extent, the mitotic complex cyclin B-Cdk1 (12, 13, 15–17). When overexpressed in cells, p21 and p27 cause only a G1 arrest, suggesting that, despite their ability to inhibit the mitotic cyclin B-Cdk1 complex in vitro, they do not act on mitotic Cdks in vivo (11, 13, 16, 17).

p21 and p27 participate in numerous regulatory responses. The G1 arrest that follows radiation-induced DNA damage, thus allowing DNA repair, is mediated by the tumor suppressor p53, which elicits an increase in p21 levels leading to Cdk inhibition (11, 20). Mitogen-induced emergence from quiescence occurs with induction of p21 expression, suggesting that cycling cells may need p21 as a regulatory device (14, 21–24). In addition, p21 but not p27 has the unique ability to bind directly to proliferating cell nuclear antigen, a DNA polymerase δ accessory factor, and thus could regulate the balance between DNA replication and DNA repair (25, 26).

p27 expression is elevated in contact-inhibited or mitogen-deprived cells (23, 27, 28), and, in contrast to p21, expression often declines upon mitogen-induced cell cycle re-entry. Various anti-mitogens including transforming growth factor-β in mammalian cells (27), rapamycin in T-lymphocytes (23), and cAMP in macrophages (28), prevent mitogen-induced p27 down-regulation, thus preventing Cdk activation and G1 progression. However, it has not been formally demonstrated whether these high levels of p27 are the cause or the consequence of a G1 arrest, since p27 protein half-life is dramatically increased in G0-arrested cells (29). Moreover, due to the coexpression of multiple CKIs in a given cell, the precise contribution of p27 on the mitogenic response remains to be answered. In this report, exploiting the use of p27 antisense to deplete cells specifically of this CKI, we demonstrate that p27 is required for the maintenance of cell quiescence.

**Experimental Procedures**

Materials

[methyl-3H]Thymidine and the enhanced chemiluminescence (ECL) immunodetection system were obtained from Amersham Corp. (Paris, France). CCL39 is an established cell line of Chinese hamster lung fibroblasts (ATCC), and PS200 is a mutant derivative lacking Na[+]-3H-exchange activity (30). p27 antibody (DSC-72) is a mouse monoclonal antibody of IgG isotype produced by immunizing BALB/c mice with recombinant full-length mouse p27 protein purified from bacteria. It recognizes rodent as well as human p27 and does not cross-react with p21 or any of the members of the p16 family of Cdk inhibitors. The
EB4 polyclonal antibody, which recognizes preferentially p42 and also p44 MAP kinases, was provided by Dr. F. R. McKenzie (31). The polyclonal antibody against p21 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody 12CA5, raised to a peptide from influenza hemagglutinin HA1 protein (32) was purchased from Babco (Emeryville, CA). All other materials were obtained from Sigma unless otherwise stated.

Methods

Cell Culture—CCL39 and its derivative PS200 were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) containing 7.5% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Constructions—The full-length mouse p27(Kip1) (LexIlog(+), Novagen), provided by J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY), was subcloned into the expression vectors pCE or pCMV5. A 1-kilobase pair restriction fragment (EcoRI-HindII) from pECE was inserted in the reverse orientation in the pCMV expression vector in order to obtain a full-length antisense p27(Kip1). The cDNAs coding for the NHE1 and NHE3 isoforms of the Na+ /H+ antiporter genes have previously been subcloned into the pECE vector (35).

Assays were performed in either duplicate or triplicate. The data presented are from representative experiments performed at least twice.

RESULTS AND DISCUSSION

Growth factor deprivation rapidly terminates cyclin D1 synthesis. Moreover, immune complexes prepared with antisera to cyclin D1 or CdK4 lacked kinase activity able to phosphorylate the retinoblastoma protein (pRb). It has been proposed that this Cdk inactivation results from an excess of p21 and p27. In accordance with this model, we show that in CCL39 cells, a decrease in the level of p27 expression is evident as early as 4 h (data not shown) after the addition of acid (5%). Cells were then harvested with 0.1N NaOH and the radioactivity incorporated was measured.

Growth suppression was then measured according to the Promega protocol.

Transient Transfection and DNA Synthesis Reinitiation—CCL39 cells were seeded at density of 500,000 cells/well in a six-well plate and cotransfected by the calcium phosphate precipitation technique with 1 μg of the selection vector pEAP (NHE1 CDNA) and 0.25 μg of pCE, p27, or the antisense constructs. Forty-eight hours after transfection, cells were subjected to an acid-load selection that killed nontransfected cells (usually >90% of the cell population). Cultures were subsequently changed to complete growth medium for 12 h and thereafter deprived of growth factors for 24 h in a 1:1 mixture of DMEM and Ham’s/F-12 medium. Cells were then stimulated with different concentrations of serum in fresh DMEM/F-12 medium containing 10% fetal bovine serum and 10% horse serum. After 24 h of incubation, the cells were fixed and washed three times with ice-cold trichloracetic acid (5%). Cells were then harvested with 0.1 N NaOH and the radioactivity incorporated was measured.

Transient Transfection and Colony Formation—PS200 cells were seeded at a density of 500,000 cells/well in a six-well plate and cotransfected by the calcium phosphate precipitation technique with 1 μg of the selection vector pEAP (NHE1 CDNA) and 18 μg of pECE, p27, or the antisense constructs. Forty-eight hours after transfection, cells were subjected to an acid-load selection. Cultures were subsequently changed to complete growth medium and allowed to proliferate for 36 h before repeating one cycle of acid-load selection. Cells were then put into complete growth medium (DMEM with 7.5% FCS) or ITS medium (DMEM supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium). Acid-load-resistant populations or independent clones were isolated and counted after 1 week.

Data Presentation—Assays were performed in either duplicate or triplicate. The data presented are from representative experiments performed at least twice.

p27Kip1 Is Required for Cell Quiescence

p27

MAPK

FCS (hours)

0 6 10 15 20 24 30

FIG. 1. Time course of p27 protein expression in CCL39 cells. CCL39 cells were serum-starved for 24 h and then stimulated with 10% FCS for the indicated period of time (hours). Equal amounts of whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis (10% gels), and proteins were analyzed by Western blotting and then probed with monoclonal p27 antibody.
sion in human 293 cells (Fig. 2A). We then used this antisense approach to analyze the consequences of the specific loss of p27 on growth factor–induced gene transcription and reinitiation of DNA synthesis in CCL39 cells. We first compared the effect of either enforced or reduced p27 expression on the transcriptional activity of a range of promoters such as the cyclin D1 promoter, the activation of which represents one of the earliest cell cycle–related events occurring during the G0/G1 to S phase transition. We also studied the cyclin A promoter and a plasmid construction containing the E2F–responsive element of the dihydrofolate reductase promoter (E2F-SV40), which are transcribed at the G1/S transition (41). As shown in Fig. 3, transcriptional activities of cyclin D1, cyclin A, and E2F-SV40 promoters were significantly stimulated by serum in transiently transfected CCL39 cells. Ectopic expression of p27 almost totally blocked such effects on cyclin A and E2F-SV40 promoters, but only partially inhibited cyclin D1 transcription. The dramatic effect of p27 on cyclin A and E2F-SV40 promoter–dependent transcription may be due to the previously noted p27–induced inhibition of the activity of cyclin D– and cyclin E–dependent kinases, which are rate–limiting for G1 progression and S phase onset (1, 42, 43). One of the targets for these G1 cyclin–Cdk complexes is the retinoblastoma tumor suppressor gene product Rb, a negative regulator of cell growth that specifically associates in its hypophosphorylated form with the transcription factor E2F (44). The hyperphosphorylation of Rb by the G1 cyclin–dependent kinases appears to abolish its growth suppressor activity by releasing members of the E2F family of transcription factors. These factors are then able to activate the transcription of a group of genes that are strongly induced during late G1/S phase, such as those encoding cyclin E, dihydrofolate reductase, DNA polymerase α, and thymidine

Fig. 2. Transfection of p27 antisense inhibits expression of p27 but not p21. A, 293 cells were transiently cotransfected with either 5 μg of the empty vector pCMV5 (lane 6), 5 μg of either p27 (top) or p21-HA constructs (bottom) (lanes 1–5). In each case, different concentrations of p27 antisense vector were cotransfected (lanes 1–5). Two days later, cell lysates were analyzed by Western blotting and probed with anti–p27 and anti–HA antibodies as described under “Experimental Procedures.” B, endogenous expression of p21 and p27 proteins was examined in PS200 cells cotransfected with 5 μg of the selection vector pEAP (Na+/H+ antiporter cDNA), together with either 5 or 10 μg of p27 antisense construct as indicated. Two days following transfection, cells were submitted to an acid-load selection. Cells were then allowed to proliferate in a complete medium (lanes 3–5) or allowed to recover for at least 4 h before serum starvation (lanes 1 and 2). Cells were lysed 3 days following transfection, and the expression of p21 and p27 was analyzed by immunoblotting.

Fig. 3. Effect of transient expression of p27 and antisense p27 on the cyclin D1, cyclin A, and E2F-SV40 promoter activities. CCL39 cells were cotransfected with 0.25 μg of reporter (cyclin D1, cyclin A, or E2F-SV40 promoters cloned upstream of the luciferase gene) and with 1 μg of the expression vector pECE containing p27 cDNA in either the sense or antisense orientation. One day following transfection, cells were rendered quiescent by medium aspiration, followed by two rinses in phosphate-buffered saline and 24-h serum starvation. Cells were then stimulated with 20% FCS for 20 h, lysed, and luciferase activity assessed according to the Promega protocol. All measurements are the mean ± S.E. of at least three experiments. Fold increase in luciferase activity was calculated relative to the basal expression level for each reporter, which was set to 1 unit, and was corrected for empty vector effects. * significantly different from control at p < 0.05 (Student’s t test).
kinase (45). Inhibition of G1 cyclin-dependent kinases by p27 prevents the release of E2F from Rb, thus resulting in inhibition of the E2F transcriptional activity.

In G0-arrested CCL39 cells, cyclin D1 protein expression is undetectable. However, serum stimulation of these cells led to a dramatic and rapid accumulation of cyclin D1 protein, which became readily detectable at 6 h post-stimulation and increased until cells entered S phase. We have recently demonstrated that p42/p44 MAP kinase signal transduction pathway plays a positive role in the induction of cyclin D1 expression in CCL39 cells. Here, we show that p27 overexpression downregulated that p42/p44 MAP kinase signal transduction pathway, shown). As we established that p27 overexpression does not modify MAP kinase activation, we concluded that p27-mediated inhibition of cyclin D1 production is triggered by the titration of G1 cyclin-Cdk complexes. Therefore two distinct pathways appear to regulate cyclin D1 expression. The first pathway, insensitive to p27, is driven by the MAP kinase cascade; the second pathway, responsible for the long term induction of cyclin D1 expression, involves the p27-inhibitable Cdk5, an action presumably mediated via the release of E2F transcription factors. Interestingly, expression of the p27 antisense construct significantly increased basal and serum-stimulated transcriptional activity of the cyclin D1 and A promoters, and strongly increased basal E2F-SV40 gene expression without a significant effect on the c-fos gene (data not shown). These results suggest a role for p27 in the control of genes induced during G1 progression.

As expression of p27 antisense stimulated cyclin D1, cyclin A, and E2F-SV40 promoter activities, we hypothesized that expression of p27 antisense would promote DNA synthesis reinitiation. We thus performed serum-induced thymidine incorporation experiments in resting PS200 cells expressing either the sense or antisense p27 construct. The enforced expression of p27 exerted a strong inhibitory effect on DNA synthesis (Fig. 4). In control cells, thymidine incorporation is stimulated 10-fold by 10% FCS, whereas the enforced expression of p27 reduced this stimulation to 3-fold. In marked contrast, the cellular depletion of p27 strongly increases basal (4–10-fold) and serum-stimulated DNA synthesis. Is is noteworthy that low serum concentration (1%) becomes remarkably mitogenic, suggesting that depletion of p27 has “relaxed” the cells for growth factor requirements, a situation often encountered in transformed cells. These results prompted us to determine the capacity of these “p27-depleted cells” to proliferate in serum-free medium supplemented with insulin, transferrin and selenium. Under these very restrictive growth conditions, only rare variants of CCL39 are usually able to form colonies. Depletion of p27 increased the number of growing colonies 4-fold (Fig. 5). This growth-promoting activity exerted by p27 antisense, although less pronounced, is also observed in serum-supplemented medium (Fig. 5). Thus, expression of the p27 antisense construct promotes cell cycling in a minimal growth medium in which insulin is the sole growth factor. In other words, p27 levels appear to set the concentration of growth factors required for cells to remain in the active cycle. Recently, Nakashima et al. (46) demonstrated that expression of p21Cip1 antisense RNA in G0-arrested human fibroblasts induced G0 exit and entry into mitosis, results very similar to those reported here for p27. At the amino acid level, p27 shows significant homology to p21; the similarity, however, is limited to a 60-amino acid segment near the N terminus that is 44% identical to the corresponding region in p21. This region of p27 is sufficient to inhibit Cdk activity (1, 16). However, experiments depicted in Fig. 2B demonstrated that p27 antisense did not significantly affect the levels of p21. In addition, cotransfection of the sense p27 fully abolished the growth-promoting effects induced by p27 antisense. These results emphasize the specificity of this antisense approach.

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In this report, by forcing the expression of p27 in CCL39 cells, we have confirmed that this CKI is a potent growth suppressor recruiting cells into the G0 resting state. The anti-mitogenic effect of cAMP suppressor recruiting cells into the G0 resting state. The anti-cells, we have confirmed that this CKI is a potent growth progression.

investigators who supplied the plasmids indicated under “Experimental might simply be a secondary consequence of G0 arrest. Paga-

rested in G0/G1. In cells arrested by growth factor deprivation mitogenic response.

activation of this pathway. 5A very likely candidate is the cyclin

ble to observe accumulation of p27 under conditions of full

p27 in the maintenance of the quiescent state, theregulation of some pathway. Interestingly, this proteolysis is reduced

diation in exponentially growing cells by the ubiquitin-protea-

no's group has demonstrated that p27 undergoes rapid degra-

fibroblasts (p15, p16, p21, p27, etc.) that could play a similar

generations. Considering the coexpression of several CKIs in

factor concentration. While insulin, acting via the insulin-like

are much more prone to re-enter the cell cycle at low growth

is a major player in growth control. Indeed, p27-depleted cells

some pathway. Interestingly, this proteolysis is reduced

this degradative process is certainly a key issue to understand

regulation of entrance into the quiescent state, it is remark-

remarkable that the depletion of p27 alone has such a pro-

pronounced effect on the loss of maintenance of the G0-resting state.

The generality of this notion must still be tested in other cell

in particular vascular endothelial cells, where cell to cell contacts appear to potently antagonize the mitogenic response.

p27 accumulates in many situations in which cells are ar-

rested in G0/G1. In cells arrested by growth factor deprivation or by high cell density, the accumulation of p27 may be the main cause of G0/G1 arrest. A similar increase in p27 levels have also been implicated in the anti-mitogenic effects of cAMP (28), transforming growth factor-β (27), and rapamycin (23). However, the accumulation of p27 in response to these agents might simply be a secondary consequence of G0 arrest. Pagano's group has demonstrated that p27 undergoes rapid degrada-

in exponentially growing cells by the ubiquitin-proteasome pathway. Interestingly, this proteolysis is reduced severalfold in resting cells (29). Considering the pivotal role of p27 in the maintenance of the quiescent state, the regulation of this degradative process is certainly a key issue to understand growth control. Which critical pathway in G1 does signal p27 degradation? Preliminary experiments indicate that the p42/p44 MAPK cascade is not directly implicated, since it is possible to observe accumulation of p27 under conditions of full activation of this pathway. 4A very likely candidate is the cyclin-D-Cdk activity, since it represents an initial sensor of G0 progression.

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