p21 Is a Critical CDK2 Regulator Essential for Proliferation Control in Rb-deficient Cells

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Abstract. Proliferation in mammalian cells is controlled primarily in the G1-phase of the cell cycle through the action of the G1 cyclin–dependent kinases, CDK4 and CDK2. To explore the mechanism of cellular response to extrinsic factors, specific loss of function mutations were generated in two negative regulators of G1 progression, p21 and pRB. Individually, these mutations were shown to have significant effects in G1 regulation, and when combined, Rb and p21 mutations caused more profound defects in G1. Moreover, cells deficient for pRB and p21 were uniquely capable of anchorage-independent growth. In contrast, combined absence of pRB and p21 function was not sufficient to overcome contact inhibition of growth nor for tumor formation in nude mice. Finally, animals with the genotype Rb+/−:p21−/− succumbed to tumors more rapidly than Rb+/− mice, suggesting that in certain contexts mutations in these two cell cycle regulators can cooperate in tumor development.

The hallmark of cancer is uncontrolled cell proliferation (for review see Sherr, 1996). Proliferation control, manifested by the integration and coordination of extracellular signals with the cell cycle machinery, is lost in tumor cells. Tumor cells proliferate autonomously; they are less dependent on growth-promoting signals and also less responsive to growth inhibitory signals. Numerous tissue culture assays have been developed that underscore these aspects of cellular transformation. For example, tumor cells grow at concentrations of growth factors insufficient for normal cell proliferation (Holley and Kiernan, 1968; Dulbecco, 1970; Jainchill and Todaro, 1970). They are also anchorage-independent for growth (MacPherson and Montagnier, 1964; Sanders and Burford, 1964) and less susceptible to contact inhibition than wild-type cells (Temin and Rubin, 1958; Todaro et al., 1963; Vogt and Dulbecco, 1963; Dulbecco, 1970).

The enzymes that regulate cell cycle progression, the cyclin-dependent kinases (CDKs) are candidates to integrate growth control signals with the cell cycle machinery (for review see Sherr, 1996). These enzymes, which are composed of a catalytic kinase subunit and a regulatory subunit called cyclin, are regulated by multiple mechanisms, including the rate of synthesis, subcellular localization and degradation rate of the cyclin subunit (for review see Morgan, 1995). CDK activity is also regulated by stimulatory and inhibitory phosphorylation events (for review see Morgan, 1995) as well as by the binding of cyclin-dependent kinase inhibitors (CKIs; for review see Peter, 1994). There are two families of CKIs, the Cip/Kip family and the InK4 family (for review see Sherr and Roberts, 1995). The Cip/Kip family consists of p21, p27, and p57 and is characterized by a conserved NH2-terminal CDK-binding domain, exclusive binding to heterodimeric complexes and affinity for multiple cyclin/CDK complexes. The InK4 family, consisting of p15, p16, p18, and p19, is distinguished by loosely conserved ankyrin motifs, binding to the catalytic subunit as well as the heterodimeric enzyme and exclusive association with CDK4 (or the similarly acting CDK6).

In mammalian cells, proliferation control is primarily achieved in the G1-phase of the cell cycle (Nilausen and Green, 1965; Todaro et al., 1965; Stoker et al., 1968; Otsuka and Moskowitz, 1975; Matsushita and Mori, 1981). After G1, cells become largely independent of extracellular signals and progress automatically through subsequent cell cycle phases to the next G1 (Todaro et al., 1965; for review see Pardee, 1989). Hence, the G1 CDKs are likely to play a particularly important role in the integration of growth control signals with the cell cycle machinery. G1 progression is catalyzed by two enzymes: cyclin D (D1, D2, or D3)/CDK4 and cyclin E/CDK2 (for review see...
functional pRB, inhibition of cyclin D/CDK4 activity arrests cells with properties of the retinoblastoma protein, pRB. Although progression is the inactivation of the growth suppressive action (Brugarolas et al., 1995).

The only essential function of cyclin D/CDK4 for G1 progression is the inactivation of the growth suppressive properties of the retinoblastoma protein, pRB. Although inhibition of cyclin D/CDK4 activity arrests cells with functional pRB, Rb-deficient cells are not arrested by such treatments (Lukas et al., 1994, 1995). In its unphosphorylated form, pRB binds and inhibits the transcription factor E2F/DP (Chellappan et al., 1991). Upon phosphorylation, pRB releases E2F/DP, which then activates the expression of genes important for G1 completion and DNA replication (for review see Weinberg, 1995). Furthermore, overexpression of E2F1 is sufficient to drive quiescent cells into S-phase (Johnson et al., 1993). Although other proteins are known to associate with pRB in its unphosphorylated state, their significance in G1 progression is not clear (for review see Weinberg, 1995). The lack of requirement of Rb<sup>−/−</sup> cells for cyclin D/CDK4 suggests that these cells might not be responsive to growth control signals that act through cyclin D/CDK4. For example, exposing wild-type cells to low concentrations of growth factors results in the decreased expression of D-type cyclins (Matsumise et al., 1991), and consequently decreased CDK4 activity, leading to G1 arrest (for review see Sherr, 1995). In contrast, Rb<sup>−/−</sup> cells are capable of growth in somewhat reduced concentrations of growth factors (Lukas et al., 1995). However, Rb<sup>−/−</sup> cells are not tumorigenic indicating that they have not lost all the mechanisms of growth control (Brugarolas, J., and T. Jacks, unpublished data). One candidate to regulate proliferation in Rb<sup>−/−</sup> cells is cyclin E/CDK2. In fact, inhibition of cyclin E/CDK2 arrests cells irrespective of Rb status (Ohtsubo et al., 1995). Furthermore, in Drosophila, the ability of ectopically expressed E2F to drive cells into S-phase is dependent on cyclin E (Duronio et al., 1995). Thus, Rb<sup>−/−</sup> cells are likely to be susceptible to growth control signals that modulate CDK2 activity.

Here, we analyze the functions of p21 and pRB in the regulation of G1 and in the coupling of extracellular signals with the cell cycle. We have studied the role of p21 and pRB using mouse embryo fibroblasts (MEFs) that are deficient for these genes. The analysis of MEFs from knockout mice allows us to study the function of a specific gene, or subset of genes, in a very physiological context, using a primary cell population that has not accumulated other mutations, and with the rigor of tissue culture assays. For the experiments described here, we have generated p21<sup>−/−</sup>, Rb<sup>−/−</sup>, Rb<sup>−/−</sup>;p21<sup>−/−</sup>, and wild-type MEFs from littermate embryos. The analysis of these cell types has revealed critical roles for p21 and pRB in G1 and in the regulation of pathways that are critical for the integration of growth control signals with the cell cycle machinery.

Materials and Methods

MEF Generation and Cell Culture

MEFs were generated from 12.5 days post-coitum mouse embryos. Embryos were harvested, the brain and internal organs were removed and the carcasses were minced and incubated with trypsin for 30–45 min at 37°C. Tissue culture media (DME supplemented with 10% heat-inactivated FCS [IF], 5 mM glutamine, and penicillin/streptomycin) was added to the cell suspension and the cells were further disaggregated. Homogeneous cell suspensions were added to T75 flasks containing 20 ml of media. After 3–4 d in an incubator at 37°C, confluent cultures were trypsinized and re-plated onto two T175 flasks containing 50 ml of tissue culture media. After 3 d cells were frozen at 2–3 × 10<sup>6</sup> cells per vial in DME supplemented with 10% FBS and 10% DMSO. Cells were frozen at −80°C and stored at −150°C. Vials were subsequently thawed and cells expanded every 3 d. Cells were seeded at 1.5–2 × 10<sup>6</sup> cells per 10-cm dish or equivalent. Early passage (P<sub>≤</sub> 6) MEFs were used for all the experiments.

Genotyping

PCR to detect Rb status was performed as described previously (Jacks et al., 1992). The following primers were used to determine the p21 genotype: 5′ AAG CCT TGA TTC TGA TGT GGC C 3′ (for both the wild-type and the mutant allele), 5′ TGA CGA AGT CAA AGT TCC ACC C 3′ (specific to the wild-type allele) and 5′ GCT ATC AGG ACA TAG CGT TGG C 3′ (specific to the mutant allele). 10× PCR buffer: 500 mM KCl, 100 mM Tris (pH 8.3), 15 mM MgCl<sub>2</sub>, 1 mg/ml BSA, 2 mM dNTPs. Thermocycling: step 1, 4 min at 94°C, step 2, 40 cycles of 1 min at 72°C, 1 min at 64°C and 3 min at 72°C, step 3, 7 min at 72°C. Polyacrylamides were separated in a 2% agarose gel with the wild-type being ~900 bp and the mutant band being ~750 bp.

G0 Synchronization

1.5–2 × 10<sup>6</sup> MEFs were plated in 10-cm dishes and grown to confluency...
for 4 d in media supplemented with 10% IFS. Fibroblasts were washed with PBSA and incubated for an additional 4 d in media supplemented with 0.1% IFS.

**Cell Cycle and Cell Size Analysis**

Asynchronously growing cells were washed with PBSA, trypsinized, and fixed in 70% methanol at −20°C for several hours. Cells were centrifuged at 2,000 rpm and resuspended in PBS containing RNase A (Sigma Chemical Co., St. Louis, MO) at 0.1 mg/ml. Samples were incubated at 37°C for 15–30 min and propidium iodide (PI; Sigma Chemical Co.) was added to final concentration of 0.2 mg/ml. After 6 h at 4°C, samples were processed by a FACScan® (Becton Dickinson, Mountain View, CA). Data was analyzed by ModFit LT software (Becton Dickinson). Cell size analysis was performed on 2n and 4n DNA containing populations gated from a forward light scatter height (FSC-H)/FL2-A dot plot and represented in a FSC-H histogram. To analyze the cell cycle distribution, cells were pulsed for 5 h with 0.3 μg/ml 5-bromodeoxiuridine (BrdU; Sigma Chemical Co.) and 0.03 μg/ml 5-fluoro-5-deoxiouridine (Sigma Chemical Co.). Samples were processed as described in Brugaras et al. (1995).

**Soft Agar Assays**

50,000 cells synchronized in G0 were resuspended in 0.34% low melting point agarose (LMP agarose; GibCO BRL, Gaithersburg, MD) in DME supplemented with 20% IFS. Cells were plated onto 6-cm dishes coated with 3% LMP agarose in DME plus 20% IFS. Cultures were maintained in an incubator at 37°C and were supplemented with 2 ml of 0.34% LMP agarose in DME containing 20% IFS every 2 d. Samples were analyzed for colony formation at 3 wk.

**Suspension Cultures**

Suspension cultures were performed as described previously (Guadagno and Assoian, 1991), with some modifications. 3.5 × 10^6 G0 synchronized cells were resuspended in media supplemented with 20% IFS and plated onto agarose-coated plates (0.8% agarose [GIBCO BRL] in DME supplemented with 20% IFS). Cells were harvested for protein extracts after 3 d using a rubber policeman.

**Focus Formation Assay**

Cells from two different strains of Rb^−/−;p21^−/− and Rb^−/− MEFs were seeded with wild-type MEFs in 10-cm dishes at a ratio 1:100. Media was changed every 3 d, and the cultures were followed for four weeks.

**Immunoblotting**

Protein extracts were prepared as described (Zhu et al., 1996). Protein concentration was evaluated with the BioRad protein assay. 300 μg of protein were fractionated by SDS-PAGE and transferred to Immobilon-P (Millipore Corp., Waters Chromatography, Bedford, MA) or nitrocellulose (MSI, Westboro, MA). Membranes were blocked in TBS-T (10 mM Tris [pH 7.5], 150 mM NaCl, 0.03% Tween-20) containing 5% nonfat dry milk. Cyclin D1 was detected using a mouse anti-cyclin D1 antibody (HD-11; Santa Cruz Biotechnology, Santa Cruz, CA) at 0.5 μg/ml, a secondary rabbit anti–mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 0.2 μg/ml and a tertiary anti–rabbit IgG antibody conjugated to HRP (Amersham Pharmacia Biotechnology Inc., Piscataway, NJ) at a dilution 1:6,000. p27 detection was performed using a goat anti-p27 antibody (C-19-G; Santa Cruz Biotechnology) at 0.5 μg/ml, a secondary mouse anti–goat IgG antibody (Jackson ImmunoResearch Laboratories) at 0.2 μg/ml and a tertiary antibody against mouse IgG conjugated to HRP (Amersham Pharmacia Biotechnology Inc.) at 1:6,000. P21 and pRB were detected using Santa Cruz C-19-G and PharMingen G3-245 antibodies, respectively. Detection was performed by chemiluminescence.

**In Vitro Kinase Assays**

CDK2 and CDK4 in vitro kinase assays were performed as described previously (Matsushime et al., 1994) with the following modifications. Cell lysates (between 180–450 μg of protein were used for CDK2 kinase assays and between 0.8–1.3 mg of protein were used for CDK4 kinase assays) were precleared with equilibrated protein A beads (Pierce Chemical Co., Rockford, IL) and incubated with anti-CDK4 (C-22; Santa Cruz) or anti-CDK2 (kindly provided by G.J. Hannon, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) for 4 h. Immune complexes were precipitated with protein A beads (Pierce Chemical Co.) and incubated in the kinase buffer containing 4 mM ATP, 20 μM γ-³²P]ATP (NEL-Dupont, Boston, MA) and 6 μg of GST-RB (GST fusion with amino acids 792–928 from the COOH terminus of pRB) or 2 μg of histone H1 (Sigma Chemical Co.), for 30–60 min at 30°C. Quantitation was performed by phosphorimager analysis.

**Tumorigenicity Assays**

Fibroblasts were trypsinized, washed with PBSA, and resuspended at 2–10^6 cells/ml in PBSA. 2 × 10^2 cells were injected subcutaneously into two flanks of three 5-wk-old Swiss nu/nu mice (Taconic Farms Inc., Germantown, NY). Mice were monitored for 6 mo.

**Pathology Analysis**

Over 20 Rb^−/−;p21^−/− mice were examined histologically for tumors in the CNS, retina, pituitary gland, thyroid gland, salivary gland, trachea, lungs, heart, stomach, small and large intestine, liver, pancreas, spleen, testis, prostate, ovaries, uterus, mammary gland, skin, bone, kidneys, and adrenals. Blood samples were also collected for analysis.

**Results**

**p21 Is a Physiological Inhibitor of CDK2**

In an effort to characterize the cell cycle effects of mutations affecting the CDK4 and CDK2 regulatory pathways, we first determined the consequences of p21 mutation in primary cells in culture using a variety of assays. Although found in normal cells associated with multiple CDKs (Xiong et al., 1992; Harper et al., 1993), p21 does not bind all of them with equal affinity (Harper et al., 1995), suggesting differential regulation by p21. In vitro, p21 has a very high affinity for complexes containing CDK2 and CDK4 (Harper et al., 1995). To examine the role of p21 in the regulation of these G1 CDKs, we determined CDK4 and CDK2 kinase activities in exponentially growing p21^−/− and wild-type control MEFs generated from littermate embryos. Protein extracts were prepared and precleared of nonspecific kinase activity, and CDK4 and CDK2 immunoprecipitates were incubated in the presence of γ-³²P]ATP and a vast excess of GST-RB as a kinase substrate. As shown in Fig. 1 A, the catalytic activity of CDK4 was very similar in p21^−/− and wild-type cells. Therefore, in exponentially growing fibroblasts, p21 does not appear to be a major CDK4 inhibitor. In contrast, CDK2 activity in p21^−/− cells was elevated two- to fourfold compared with wild-type cells (Fig. 1, B and C). This increased activity in p21-deficient cells could not be accounted for by increased levels of cyclin E, cyclin A, or CDK2 (data not shown). Furthermore, >50% of CDK2 in wild-type MEFs was found associated with p21 (data not shown), providing support to the idea that the effect of p21 loss on CDK2 activity is direct. Thus, in exponentially growing fibroblasts, p21 is a critical inhibitor of CDK2.

pRB is phosphorylated by CDK2 containing complexes both in vitro and in vivo (for review see Weinberg, 1995); pRB phosphorylation results in a retarded migration in SDS-PAGE. Western blot analysis of pRB from extracts of wild-type and p21^−/− cells normalized for protein content revealed an increase in the slow migrating form of pRB in p21^−/− as compared with wild-type cells (Fig. 1 D), suggesting that pRB is phosphorylated to a greater extent in p21^−/− than in wild-type cells. This increase in pRB phosphorylation is likely to reflect the increased CDK2 ac-
cells. Cells were pulsed with 5 BrdU for 5 h, fixed, stained with PI and analyzed by two-dimensional FACS® analysis. The data shows the average of four independent experiments and standard deviations of the measurements.

Table I. Cell Cycle Distribution

<table>
<thead>
<tr>
<th></th>
<th>% G1</th>
<th>% S-phase</th>
<th>% G2/M</th>
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<tr>
<td>Wt</td>
<td>47.2 ± 0.6</td>
<td>41 ± 0.9</td>
<td>11.8 ± 0.9</td>
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<tr>
<td>p21&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>42 ± 2.2</td>
<td>44.5 ± 2.1</td>
<td>12.8 ± 1.1</td>
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<tr>
<td>Rb&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>42.2 ± 4.4</td>
<td>45.5 ± 4.7</td>
<td>12.6 ± 1.1</td>
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<tr>
<td>Rb&lt;sup&gt;−/−&lt;/sup&gt;;p21&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>35.6 ± 2.8</td>
<td>50.8 ± 2.8</td>
<td>13.6 ± 0.4</td>
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Additive Effects of the Rb and p21 Mutations in the G1-Phase

The phenotype of p21<sup>−/−</sup> cells is reminiscent of the phenotype that has been previously reported for Rb<sup>−/−</sup> MEFs (Lukas et al., 1995; Herrera et al., 1996). To compare the role of p21 and pRB in the regulation of G1, we generated Rb<sup>−/−</sup> and p21<sup>−/−</sup> littermate embryos from p21<sup>−/−</sup>;Rb<sup>−/−</sup> crosses and derived MEFs from them. These cells were then analyzed in the assays described above. Rb<sup>−/−</sup> MEFs have similar levels of CDK4 and CDK2 kinase activities to wild-type MEFs (Lukas et al., 1995; Fig. 1). The analysis of the cell cycle phase distribution of Rb<sup>−/−</sup> cells indicated a pattern remarkably similar to that of p21<sup>−/−</sup> cells (Table I). Previously, Rb-deficient MEFs were shown to be smaller than wild-type MEFs (Herrera et al., 1996; Fig. 2 A); we have found that this decrease in size is due to a decrease in the size of G1 cells (Fig. 2 B). As with p21<sup>−/−</sup> cells, the size distribution of cells with a 4n DNA content was similar between Rb<sup>−/−</sup> and wild-type cells (Fig. 2 E).

Rb<sup>−/−</sup> cells contain several-fold higher levels of cyclin E compared with wild-type cells, which is thought to reflect increased E2F activity (Herrera et al., 1996). This increased cyclin E, paradoxically, does not result in a proportional increase in cyclin E–associated CDK2 activity, although the activity is elevated in Rb<sup>−/−</sup> cells in G1 (Herrera et al., 1996). We have found that Rb<sup>−/−</sup> cells also contain several-fold higher levels of p21 as compared with wild-type cells (Fig. 1 D). This increase in p21 may also reflect increased E2F transcriptional activity as E2F-1 has been shown to specifically transactivate the p21 promotor.
of p21<sup>−/−</sup>;Rb<sup>−/−</sup> mice. From these crosses, however, all resulting MEF populations were homozygous mutant for p21. Therefore, to generate wild-type, Rb<sup>−/−</sup> as well as p21<sup>−/−</sup> control embryos from the same litter as the p21<sup>−/−</sup>;Rb<sup>−/−</sup> mutants, we also intercrossed a large series of p21<sup>+/−</sup>;Rb<sup>+/−</sup> animals.

Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells (similar to p21<sup>−/−</sup> cells) had two- to fourfold higher levels of CDK2 activity compared with Rb<sup>−/−</sup> or wild-type cells (Fig. 1, B and C). Comparative analysis of the cell cycle distributions of Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells indicated a further reduction in the percentage of cells in G1; while the number of cells in G1 in p21<sup>−/−</sup> and Rb<sup>−/−</sup> cells was decreased by 5.2 and 5%, respectively, it was reduced by 11.6% in double mutant cells (Table I). Conversely, the double mutant cells showed an increase in the percentage of cells in S-phase and G2/M that was approximately twice that seen in comparing single mutant to wild-type cells (Table I). These data indicate that loss of pRB and p21 have additive effects in G1-phase regulation. Cell size analysis revealed that the cell size distribution of Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells is shifted towards the left of that of single mutant cells (Fig. 2A). This seems to be due to an increase in the proportion of smaller G1 cells (Fig. 2C). Because of the relatively small changes in cell diameter, we analyzed the size of Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells derived from two different embryos. As shown in Fig. 2D, the size of cells from these two strains was indistinguishable. As observed for the single mutant cells, the size distribution of Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells with a 4n DNA content is similar to wild-type cells (Fig. 2E).

We have further characterized G1 progression in Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells by analyzing the cell cycle profiles of G0 synchronized double mutant and wild-type cells as they progress to S-phase. As shown in Fig. 3, A–F, double mutant cells enter S-phase 3–4 h earlier than wild-type cells. Interestingly, however, the reduced G1 length was not associated with a significant change in the population doubling time. When compared in standard growth curve analysis, double mutant cells accumulated with similar kinetics to Rb<sup>−/−</sup>, p21<sup>−/−</sup>, and wild-type cells (Fig. 3G). These data suggest that the decrease in G1 length does not lead to a reduction in overall cell cycle length, perhaps due to compensatory effects in the S and G2/M phases; such effects would be indicated from the cell size analysis described above.

**Decreased Serum Dependence for Proliferation of Rb<sup>−/−</sup>;p21<sup>−/−</sup> Cells**

G1 CDKs are candidates to integrate extracellular signals with the cell cycle. Thus, mutations in constitutive regulatory elements of these pathways may impair the cell’s ability to regulate the cell cycle machinery in response to extracellular signals. To test this possibility, we analyzed the effects of loss of pRB and p21 in serum responsiveness. The growth properties of Rb<sup>−/−</sup>;p21<sup>−/−</sup>, p21<sup>−/−</sup>, Rb<sup>−/−</sup>, and wild-type MEFs were characterized in the presence of limiting quantities of serum. Cells were synchronized in G0 and plated at low density in 0.1 and 0.5% FCS. Duplicate samples were collected on five consecutive days and counted. As expected, no increase in cell number occurred in wild-type cells in 0.1% FCS and only a very small increase was observed in 0.5% FCS (Fig. 4, A and B). p21<sup>−/−</sup> cells were capable of some proliferation at both serum

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**Figure 2.** Cell size analysis of wild-type, p21<sup>−/−</sup>, Rb<sup>−/−</sup>, and Rb<sup>−/−</sup>; p21<sup>−/−</sup> MEFs. Asynchronously growing cells were harvested, fixed, stained with PI and analyzed by FACS<sup>α</sup>. 60,000 events were collected for each sample. 2n and 4n DNA containing populations were gated for cell size analysis in a FSC-H by FL2-A dot plot and scored in a FSC-H histogram. (A) Cell size analysis of wild-type, p21<sup>−/−</sup>, Rb<sup>−/−</sup>, and Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells. (B and C) Cell size analysis of wild-type, p21<sup>−/−</sup>; Rb<sup>−/−</sup>, and Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells with a 2n DNA content. (D) Size analysis of two strains of Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells with a 2n DNA content. These strains were derived from two different embryos. (E) Cell size analysis of wild-type, p21<sup>−/−</sup>, Rb<sup>−/−</sup>, and Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells with a 4n DNA content.

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(Hiayma et al., 1997). Thus, increased p21 levels may result in the downregulation of CDK2 activity and could explain why cyclin E associated CDK2 activity does not increase proportionally to cyclin E levels.

Next we examined whether combined dysregulation of CDK2 (through mutation of p21) with disruption of CDK4 (through Rb mutation) pathways would cause additional G1 phase defects. Constitutive activation of these two pathways through these mutations might also be expected to limit the ability of cells to stop the cell cycle machinery in response to extracellular growth inhibitory signals. To test these possibilities, we generated embryos deficient in both genes and isolated MEFs from them. p21<sup>−/−</sup>;Rb<sup>−/−</sup> embryos were generated from intercrosses...
concentrations, as were \( Rb^{-/-} \) cells (Fig. 4, A and B). Strikingly, \( Rb^{-/-};p21^{-/-} \) cells had a significant growth advantage over either single mutant strain, as well as wild-type cells (Fig. 4, A and B). Similar results were obtained in 0.05 and 0.3% FCS (data not shown). However, \( Rb^{-/-};p21^{-/-} \) cells still exhibited a significant dependence on serum for proliferation as observed by comparing the growth rate of these cells in 0.5 and 5% FCS (Fig. 4, C). It is important to note that these data do not demonstrate that p21 and pRB are direct regulators of the serum response pathway, only that mutations in constitutive regulators of G1 CDK pathways affect the ability of cells to arrest in the presence of concentrations of growth factors insufficient for normal cell proliferation.

Loss of pRB and p21 Is Sufficient for Anchorage-independent Growth

Fibroblast cell proliferation is contingent upon adhesion and spreading onto an adequate substratum (Green and Nil Hansen, 1962; for review see Assoian, 1997). Plating cells in semisolid medium results in a G0/G1 arrest (Otsuka and Moskowitz, 1975; Matsushisa and Mori, 1981; Guadagno and Assoian, 1991) that is thought to be mediated through the downregulation of CDK4 and CDK2 activities (for review see Assoian, 1997). Because \( Rb^{-/-};p21^{-/-} \) cells should not require cyclin D/CDK4 activity for proliferation and exhibit two- to fourfold higher levels of CDK2 activity than wild-type cells, we investigated whether these cells retain the ability to arrest in nonadherent conditions. \( Rb^{-/-};p21^{-/-} \) MEFs from two different embryos were assayed for anchorage-independent growth in soft agar. \( p21^{-/-}, Rb^{-/-}, \) and wild-type MEFs derived from littermate embryos were used as controls. Cells of each genotype were plated onto dishes coated with 3% LMP agarose in media containing 20% FCS and allowed to grow for 3 wk. Cultures were supplemented with 0.3% LMP agarose in media containing 20% FCS every 3 d. Although the different control cells failed to grow under these conditions (Fig. 5, A–C and E–G), \( Rb^{-/-};p21^{-/-} \) were capable of forming colonies in the presence of limiting concentrations of growth factors.
(Fig. 5, D and H). Colony size varied but some colonies were quite large (Fig. 5 H). These data demonstrate that loss of both pRB and p21 is sufficient to allow anchorage-independent growth, whereas the presence of either inhibitor singly still allows growth arrest.

Plating cells on standard agarose prevents cell spreading and allows the retrieval of cells for biochemical analysis (Guadagno et al., 1993). Fibroblasts plated under these conditions downregulate cyclin D1 (Bohmer et al., 1996; Zhu et al., 1996) the major D-type cyclin in fibroblasts (Won et al., 1992) and inhibit cyclin E/CDK2 activity (Fang et al., 1996; Zhu et al., 1996). Cyclin E/CDK2 inhibition is not caused by downregulation of cyclin E levels or by inhibitory phosphorylation of CDK2 (Fang et al., 1996), but instead is thought to be mediated by p21 and p27 (Fang et al., 1996; Zhu et al., 1996). However, p21 and p27 were only somewhat induced in anchorage-independent conditions (Fang et al., 1996; Zhu et al., 1996); increased

Figure 5. Anchorage-dependence for growth of wild-type, p21−/−, Rb−/−, and Rb−/−;p21−/− cells. G0 synchronized populations were plated in soft agar and colony formation was scored after 3 wk. Bars: (A–D and E–H) 500 μm.
levels of p21 and p27 bound to cyclin E/CDK2 may result from an increase in the pool of free inhibitors due to the decreased levels of cyclin D1/CDK4 complexes (for review see Assoian, 1997).

Similar to 3T3 fibroblasts, MEFs responded to anchorage-independent conditions with a reduction in cyclin D1 levels (Fig. 6 A) and an inhibition of CDK2 (Fig. 6, C and D). As with 3T3 cells, the levels of p21 and p27 in MEFs did not change significantly under these conditions (Fig. 6 B and data not shown), suggesting again that CDK2 inhibition may be due to a redistribution of the CKIs. In an effort to understand the molecular mechanisms that underlie the ability of Rb−/−;p21−/− cells to grow in soft agar, we characterized CDK activity biochemically. Protein extracts from Rb−/−;p21−/− and wild-type control cells in monolayers and in suspension were normalized for protein content and used for Western blot analysis and kinase assays. As shown in Fig. 6, double mutant MEFs downregulated cyclin D1 and CDK2 activity under these conditions. However, Rb−/−;p21−/− cells failed to downregulate CDK2 activity to wild-type levels (Fig. 6, C and D). In fact, the levels of CDK2 activity in nonadherent Rb−/−;p21−/− cells were very similar to the levels present in exponentially growing wild-type cells. The level of CDK2 activity observed in double mutant cells in semisolid medium can only be partially accounted for by loss of p21, however, because CDK2 activity in p21−/− cells under the same conditions was found to be two- to fourfold higher than in wild-type cells (Fig. 6 E), compared with the four- to sixfold higher levels observed in the double mutants (Fig. 6, C–E). Therefore, the remaining increase in CDK2 activity in Rb−/−;p21−/− cells could reflect the increased proportion of proliferating cells in these cultures.

Rb−/−;p21−/− Cells Are Susceptible to Contact Inhibition of Growth

Normal fibroblasts grow in monolayers and arrest with a 2n DNA content at high cell densities (Nilaulsen and Green, 1965). This arrest is thought to involve p27 (Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994), although loss of p27 does not alter the susceptibility of MEFs to contact inhibition (Nakayama et al., 1996). Thus, other molecules in addition to p21 may be involved in this response. Having shown that Rb−/−;p21−/− cells do not arrest in conditions preventing anchorage, we next tested the susceptibility of these cells to contact inhibition of growth. Rb−/−;p21−/−, wild-type, p21−/−, and Rb−/− MEFs derived from littermate embryos were plated at 2 × 104 cells per 10-cm dish and allowed to grow to confluence for 6 d. Cells were then harvested, fixed, stained with PI and evaluated by FACS® analysis. As shown in Fig. 7, all cultures exhibited a prominent G0/G1 peak indicative of a cell cycle arrest. The mutant cultures had a somewhat higher S-phase and G2/M fractions, suggesting a slightly reduced capacity for contact inhibition. Thus, in contrast to anchorage independence, loss of pRB and p21 is not sufficient to overcome growth inhibitory signals triggered by cell–cell contact.

Given the slight increase in the S-phase or G2/M fractions among the mutant cells at confluence, we examined these cells using a more stringent test for contact inhibition: the focus-formation assay. Mutant cells were plated among wild-type cells at a 1:100 ratio and cultures were scored at 4 wk for the presence of foci. Neither Rb−/− or Rb−/−;p21−/− cells were capable of forming foci under these conditions (data not shown). Thus, by this measure as well, combined elimination of p21 and pRB failed to allow escape from contact inhibition.

We have shown that the capacity of Rb−/−;p21−/− cells to sustain anchorage-independent growth correlates with failure to downregulate CDK2 activity to wild-type levels. Because Rb−/−;p21−/− cells are susceptible to contact inhibition of growth, they might be able to inhibit CDK2 under these conditions. To test this hypothesis, CDK2 kinase activity was assayed from protein extracts prepared from contact-inhibited and asynchronously exponentially growing Rb−/−;p21−/− and wild-type MEFs. As shown in Fig. 8 B and C, Rb−/−;p21−/− cells at high densities downregulated CDK2 activity to wild-type levels. Furthermore, under these conditions, we detected a several-fold increase in p27 levels in wild-type and double mutant cells (Fig. 8 A), suggesting that p27 upregulation may contribute to the inhibition of CDK2 at high cell densities in both cell types.

Figure 6. Cyclin D1 and p27 protein levels, and CDK2 kinase activity in wild-type and Rb−/−;p21−/− cells grown in monolayer (Mn.) and in suspension (Sp.). (A) Cyclin D1 protein levels assayed by Western blot analysis. (B) p27 protein levels assayed by Western blot analysis. (C) In vitro CDK2 kinase assay using GST-RB as a substrate. (D) Histogram representing quantitation analysis of the results from the CDK2 kinase assay. (E) In vitro CDK2 kinase assay of wild-type, p21−/−, and Rb−/−;p21−/− cells in suspension using GST-RB as a substrate. Lanes shown were reassembled in appropriate order from a single autoradiogram. All samples were derived from the same experiment.
Rb<sup>−/−</sup>;p21<sup>−/−</sup> Cells Do Not Form Tumors in Nude Mice

Growth in soft agar usually correlates with tumorigenic potential (Freedman and Shin, 1974; for review see Assoian, 1997). Thus, to assay the tumorigenicity of Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells, 2 x 10<sup>6</sup> MEFs were injected subcutaneously into nude mice. p53<sup>−/−</sup> cells expressing the viral oncogene E1A and an activated ras allele (T24 H-ras; Lowe et al., 1994) were used as a positive control. Whereas, mice injected with p53<sup>−/−</sup>;E1A;ras transformed cells developed tumors within 2 wk, no tumors were evident after 6 mo in mice injected with Rb<sup>−/−</sup>;p21<sup>−/−</sup> cells (data not shown).

Decreased Tumor Survival Rates of p21<sup>−/−</sup>;Rb<sup>+/−</sup> Mice

As another means to address the potential cooperative tumorigenic effects of p21 and Rb mutations, we have characterized the tumor phenotype of animals with the genotypes p21<sup>−/−</sup>;Rb<sup>+/−</sup>. Rb<sup>+/−</sup> mice have a strong tumor predisposition (Jacks et al., 1992; Williams et al., 1994), with a mean age of survival ~340 d on a mixed genetic background (C57BL/6-129/Sv; Williams et al., 1994). These mice develop intermediate lobe pituitary and medullary thyroid adenomas and adenocarcinomas (Jacks et al., 1992; Williams et al., 1994), and tumors show loss of heterozygosity at the Rb locus (Williams et al., 1994). In addition to these tumors, chimeric mice composed of wild-type and Rb<sup>−/−</sup> cells also develop pheochromocytomas, indicating that the Rb mutation can also predispose to this tumor type (Williams et al., 1994). In contrast, p21<sup>−/−</sup> mice do not develop tumors (Deng et al. 1995) by 1.5 yr of age (data not shown). Absence of p21 did not alter the tumor spectrum caused by the Rb mutation, as p21<sup>−/−</sup>;Rb<sup>+/−</sup> mice also seemed to develop exclusively pituitary tumors, medullary thyroid adenomas and adenocarcinomas and pheochromocytomas (Table II). Interestingly, the mutation of p21 did have a significant effect on the lifespan of animals heterozygous for an Rb mutation. As shown in Fig. 9 and Table II, the mean age of survival of p21<sup>−/−</sup>;Rb<sup>+/−</sup> mice was 261 d compared with 340 d for Rb<sup>−/−</sup> mice. Although subtle differences in the balance of 129/Sv versus C57BL/6 alleles in these mixed genetic background animals could contribute to this effect, it is likely that absence of p21 in-
creases the transformation potential or growth properties of Rb+/- cells in certain tissues, perhaps in a manner analogous to the effects reported here for fibroblasts in culture. Moreover, similar tumor size at necropsy between Rb+/- and p21-/-;Rb+/- mice further supports the contention that loss of p21 can accelerate tumor development in Rb+/- mice.

Discussion

In this manuscript, we describe the use of specific loss-of-function mutations in two key inhibitors of the mammalian cell cycle to explore the mechanism of cellular response to extrinsic factors that regulate cell cycle progression. Previous studies using cyclin overexpression or p27 antisense have demonstrated that pathways regulated by the G1 CDKs, CDK4(6) and CDK2 are important in this process (Ohitsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994; Coats et al., 1996; Rivard et al., 1996). Our approach has been to systematically eliminate functions that regulate or are regulated by these two pathways in an attempt to render the cell insensitive to conditions that normally elicit cell cycle arrest. From these experiments, we hope to clarify the normal cellular response to growth inhibitory conditions as well as to investigate the relationship between the loss of normal cell cycle regulation and neoplastic transformation. Taken together, our data support a model in which regulation of G1 progression can be divided into two discrete stages controlled by the activities of CDK4(6) and CDK2, such that inhibition of either stage can prevent cell cycle progression. Thus, in cells in which the CDK4(6) pathway is constitutively activated through Rb mutation and CDK2 pathway is upregulated through p21 mutation, neither growth factor limitation nor detachment from the substratum results in proper cell cycle arrest.

Table II. Tumor Analysis in p21-/-;Rb+/- Mice

<table>
<thead>
<tr>
<th>Tumor Analysis in p21-/-;Rb+/- Mice</th>
<th>Rb+/+</th>
<th>p21-/-;Rb+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.A.S. (Mean age of survival)</td>
<td>340 (n = 50)</td>
<td>261 (n = 57)</td>
</tr>
<tr>
<td>Pituitary tumor</td>
<td>200/200</td>
<td>27/27</td>
</tr>
<tr>
<td>Medul lary thyroid tumor</td>
<td>19/27</td>
<td>23/26</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>ND</td>
<td>7/19</td>
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</table>

Tumor analysis in p21-/-;Rb+/- mice compared to Rb+/-; mice (Williams et al., 1994). M.A.S., Mean age of survival.

Although capable of anchorage-independent growth, p21-/-;Rb+/- cells are still growth arrested at high cell densities. The difference in response to these two conditions was correlated with the ability of the double mutant cells to downregulate CDK2 activity to wild-type levels. The more complete inhibition of CDK2 activity at confluence may be explained by the observed induction of p27, which was not seen when the cells were prevented from contacting the substratum. Interestingly, p27-deficient MEFs downregulate CDK2 kinase activity to wild-type levels (Koff, A., personal communication) and arrest at confluence (Nakayama et al., 1996). Perhaps either p21 or p27 is sufficient for CDK2 regulation under these conditions. This issue can be explored further by constructing p21/p27 double-mutant MEFs.

Defects in the regulation of growth in tissue culture have been used as a measure of neoplastic transformation (for review see Ponten, 1976; Smets, 1979), with anchorage-independent growth correlating well with tumorigenicity (Freedman and Shin, 1974; reviewed by Assoian, 1997). Interestingly, despite their ability to form colonies in soft agar, p21-/-;Rb+/- cells were not tumorigenic in nude mice. Thus, the loss of these two inhibitors is not sufficient for proliferation in vivo. In contrast to our observations, the combination of ras activation and p21 mutation is sufficient for transformation of mouse skin keratinocytes, including the ability to form tumors in nude mice (Missero et al., 1996). We believe that tumor formation in nude mice by ras transformed p21-deficient keratinocytes but not Rb+/-;p21-/- MEFs reflects critical oncogenic functions of activated ras beyond its effects on pRB (Peep er et al., 1997).

As indicated by the reduced life span of p21-/-;Rb+/- mice, germline mutations in these two genes can synergize in tumor development. By analogy with our fibroblast data, this effect could be a reflection of elevated CDK2 activity potentiating the growth of Rb+/- tumor cells. It appears that the loss of Rb function dictates the tumor spectrum, because we observed the same tumor types in double mutant as in Rb+/- animals. In contrast, germline mutations in Rb and p53 produce a broader tumor spectrum than either of the single mutants (Williams et al., 1994; Harvey, 1995). Therefore, although p21 is a key downstream target of p53 in growth arrest, it appears that the major tumor suppressor function(s) of p53 do not require p21. The acceleration of tumorigenesis in p21-/-; Rb+/- mice indicates a context in which p21 function does limit tumor cell growth, and could represent another mechanism of tumor suppression by p53; this model would assume that p53 is the major regulator of p21 expression in some tumor types.

The integrity of the CDK4 and CDK2 pathways is not
only critical in the regulation of cell cycle progression in response to extracellular signals but also in response to intrinsic cues and senescence. Thus, p21 and pRB are required for the integrity of the p53-mediated G1 arrest response to DNA damage (Slebos et al., 1994; Brugarolas et al., 1995; Deng et al., 1995), and loss of p21 (Brugarolas, J., and T. Jacks, unpublished data) or INK4a (Serrano et al., 1996) greatly enhance the life span of MEFs in tissue culture. In addition, Serrano et al. (1997) have recently reported that overexpression of the ras oncogene can induce a senescence-like state, in a manner dependent on both INK4a and p53 function. Therefore, mutations in the regulatory elements of the CDK4 and CDK2 (Keyomarsi and Pardee, 1993; Kitahara et al., 1995; Porter et al., 1997) pathways may not only be sufficient to overcome extracellular growth inhibitory signals but also intracellular growth inhibitory signals and senescence, and it might be expected that regulatory elements of these pathways would be critical targets for tumorigenesis (for review see Hirama and Koeffler, 1995).

Tumor cells proliferate autonomously, independently of growth inhibitory signals, and despite the absence of growth-promoting signals. Thus, tumor cells are defective in the integration of extracellular signals with the cell cycle machinery. These defects lie at multiple levels, in cell surface receptors, signal transduction pathways, and cell cycle regulators. We have studied the role of two inhibitors of G1 progression in this process. Our data support the conclusion that proliferation control is primarily accomplished in G1 and that p21 and pRB are critical components of these regulatory pathways. We have shown that in the absence of pRB, CDK2 can act as the gatekeeper that secures some degree of proliferation control. We postulate that constitutive activation of the CDK4 and CDK2 pathways is sufficient for unrestrained proliferation and may render cells unable to execute a senescence triggered arrest. A better understanding of the mechanisms that coordinate extracellular growth control signals with the cell cycle machinery in the normal cell is of primary importance to understand how growth autonomy is achieved in cancer.


