Combinatorial roles for pRB, p107, and p130 in E2F-mediated cell cycle control

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Numerous studies have implicated the pRB family of nuclear proteins in the control of cell cycle progression. Although overexpression experiments have revealed that each of these proteins, pRB, p107, and p130, can induce a G1 cell cycle arrest, mouse knockouts demonstrated distinct developmental requirements for these proteins, as well as partial functional redundancy between family members. To study the mechanism by which the closely related pRB family proteins contribute to cell cycle progression, we generated 3T3 fibroblasts derived from embryos that lack one or more of these proteins (pRB−/−, p107−/−, p130−/−, pRB−/−p107−/−, pRB−/−p130−/−, and p107−/−p130−/−). By comparing the growth and cell cycle characteristics of these cells, we have observed clear differences in the manner in which they transit through the G1 and S phases as well as exit from the cell cycle. Deletion of Rb, or more than one of the family members, results in a shortening of G1 and a lengthening of S phase, as well as a reduction in growth factor requirements. In addition, the individual cell lines showed differential regulation of a subset of E2F-dependent gene promoters, as well as differences in cell cycle-dependent kinase activity. Taken together, these observations suggest that the closely related pRB family proteins affect cell cycle progression through distinct biochemical mechanisms and that their coordinated action may contribute to their diverse functions in various physiological settings.

The generation and characterization of mice deficient in pRB, p107, and/or p130 proteins revealed both unique and redundant functions for these proteins during development. Mice deficient in pRB die as embryos between days 13 and 15 of gestation, exhibiting defects in both differentiation and proliferation. Mice deficient in either p107 or p130 develop normally and exhibit no obvious adult phenotypes (13, 14), although genetic background differences have recently been shown to play a major role in determining the consequences of genetic loss of p107 and p130 (15, 16). Furthermore, intercrossing these mouse strains or generating chimeric animals that eliminate more than one protein has revealed significant functional overlap within this gene family (13, 17–19). The phenotypes seen in these mice potentially reflect cell cycle changes mediated by deregulation of E2F target genes, but could also be due to changes in other transcriptional programs involved in proliferation or differentiation control. Indeed, pRB has been shown to interact with and regulate the activity of a large number of diverse transcription factors.

To clarify the role of the individual pRB family members in the regulation of the cell cycle and E2F-dependent transcription, we sought to establish a genetically defined cellular system. We generated immortalized 3T3 fibroblast cell lines from mouse embryos deficient in various combinations of pRB, p107, and p130 and examined the cell cycle characteristics of these cells. Our experiments indicate that the combined activities of the pRB family members affect the mechanism by which cells transit through and exit the cell cycle. Moreover, the differences in the cell cycle properties observed among the various genotypes correlate with the regulation of E2F-sensitive promoters, suggesting that these proteins may function coordinately to couple gene transcription to cell cycle progression.

Materials and Methods

Cell Culture, Establishment of Cells, and Differentiation Protocol. The 3T3 cells were established by using the protocol described by Todaro and Green (20). In short, cells were counted and seeded in DMEM supplemented with 10% calf serum (CS) at a density of 105 cells per 10-cm dish for 24 h (Fig. 1), confluent, as well as cells maintained at confluence or under serum-deprived conditions were labeled with 10 μM BrdUrd cell labeling reagent (Amersham Pharmacia) for 30 min, harvested, and analyzed with a fluorescence-activated cell sorter (FACS) according to

Abbreviations: RB, retinoblastoma; cdk, cyclin-dependent kinase; FSC-H, forward scatter height; wt, wild type; MEF, mouse embryo fibroblast.

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the manufacturer (Becton Dickinson). In short, cells were fixed in 80% ethanol, the DNA was denatured by treatment with 2 M HCl/0.5% Triton X-100 at room temperature for 30 min followed by a neutralization step in 0.1 M Na$_2$B$_4$O$_7$, pH 8.5. For immunofluorescence staining, anti-BrdUrd antibodies (Becton Dickinson) and FITC-conjugated horse anti-mouse antibodies (Vector Laboratories) were used. The immunofluorescence step is followed by an incubation in propidium iodide and RNase prior to two-dimensional FACS analysis using CELLQUEST software (Becton Dickinson). The number of gated cells in G$_1$ or G$_2$/M is presented as %, and the number of gated cells incorporating BrdUrd at the time of the analysis is presented as % cells in S phase. To measure the relative cell size of the different cell lines, single cells with a DNA content ranging from 2N to 4N were gated, and forward scatter height (FSC-H) histograms were plotted. The mean FSC-H was calculated by using CELLQUEST software (Becton Dickinson).

For the nocodazole arrest and release experiments, cells were plated at $8 \times 10^5$ cells per 10-cm dish for 24 h followed by nocodazole treatment (50 ng/ml) for 10 h, whereafter the mitotic cells were shaken off, washed, and replated at the same density. At the indicated points, the cells were labeled for 30 min with BrdUrd and analyzed as described above.

**Protein Analysis and Kinase Assays.** Protein extracts from total cells were harvested from proliferating cells of different genotypes in RIPA buffer (0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS). Aliquots of protein samples were separated by SDS/8% PAGE (pRB, p107, p130), transferred to poly(vinylidene difluoride) membranes (Immobilon-P; from Millipore), and probed with relevant antibodies. Mouse monoclonal antibodies used to detect pocket proteins were as follows: for pRB (G3-245; PharMingen), for p107 (SD9), for p130 (RB-2; Transduction Laboratories, Lexington, KY). The immunoreactive protein species were visualized by ECL detection kit. Immunoprecipitations for p107 were done in RIPA buffer, by using a mixture of antibodies (SD4, -6, -9, -15) coupled to Sepharose beads.

For the immunoprecipitation (IP)-kinase assays, two 10-cm dishes of each cell type were washed and lysed for 10 min in 2 ml Nonidet P-40 lysis buffer [50 mM Tris-HCl, pH 8.0/250 mM NaCl/1% Nonidet P-40/10% glycerol/0.4 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)/5 μg/ml leupeptin/5 μg/ml aprotinin/2 mM PMSF/20 mM β-glycerophosphate/2 mM NaF/2 mM Na$_3$VO$_4$]. For immunoprecipitations, 250 μg of total lysate was precleared with 12.5 μl of normal rabbit serum and Staphylococcus aureus Cowan (SAC) as described (35). The cleared lysates were incubated for 1 h with 10 μl of anti-CDK2 antibody (M2; Santa Cruz Biotechnology) at 4°C, 100 μl of a 10% protein A-Sepharose slurry was added, and the mixture was rotated for another 30 min. Immunoprecipitates were washed three times in lysis buffer, and 50 μl of kinase reaction mix was added to the beads (50 mM Hepes, pH 7.6/5 mM MgCl$_2$/2.5 mM MnCl$_2$/0.5 μg His$_8$-pRB/1.5 mM ATP/5 μCi of $[^32$P]ATP). The kinase reactions were incubated for 30 min at 30°C and stopped with 50 μl of 2× Laemmli sample buffer, and 50 μl of this mixture was analyzed by SDS/PAGE and autoradiography.

For p27 levels, 50 μl of the indicated lysates was run on SDS/12% PAGE, transferred to a Millipore poly(vinylidene difluoride) membrane, and probed with an anti-p27 antibody (Transduction Laboratories).

**Stable Transfections and Transcriptional Assays.** Transfection of the 3T3 cells was performed by using calcium phosphate or Fugene (Boehringer Mannheim). Reporter constructs carrying the p107 or the B-myb promoter driving luciferase were stably integrated into the various cell lines by using a cotransfected puromycin selectable marker (pBABE). The cells were selected in DMEM/
Results and Discussion

From EG & G Berthold (Wallac, Gaithersburg, MD).

Results were obtained by using a luminometer and expressed as relative activity compared with the asynchronous (asynchronous), 2 days after serum deprivation (serum transcriptional analysis. Cells were harvested the day after

Number of passages required to immortalize p130

2

stable lines, with the exception of p130 fibroblasts of all genotypes were relatively easily established as

2

acquired p53 mutations rather than p16 during the establishment of 3T3 lines (21–23) (see below for details), to simplify presentation, we will refer to the various cell lines by the

2

/ p130 P/S phases of the cell cycle in asynchronously growing populations plated at 8 x 10⁶ cells per 10-cm dish 24 h prior to harvesting was measured by performing FACS analysis and plotting FSC-H histograms. The percentage of cells in the G₁, S, and G₂/M phases of the cell cycle were determined by plating 10⁶ cells in a 10-cm dish followed by cell counting 3 days later. The values shown are mean doubling times at passage 55–60. The saturation densities were measured by plating cells at 2 x 10⁶ in 3-cm dishes, followed by cell counting each day until saturation was reached. The relative size of asynchronously growing cells plated at 7 x 10⁶ per 10-cm dish 24 h prior to serum deprivation was measured by using BrdUrd incorporation (S phase) and two-dimensional FACS analysis. The data presented are a representative example of many experiments.

Table 1. Characteristics of 3T3 fibroblast cell lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Doubling time, h</th>
<th>% of cells in cell cycle phases</th>
<th>Saturation density, no. of cells per 3-cm dish</th>
<th>Cell size, mean FSC-H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G₁</td>
<td>S</td>
<td>G₂/M</td>
</tr>
<tr>
<td>wt</td>
<td>43.5</td>
<td>41</td>
<td>39</td>
<td>19</td>
</tr>
<tr>
<td>p107−/−</td>
<td>41</td>
<td>41</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>p130−/−</td>
<td>45</td>
<td>40</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td>p107−/−/p130−/−</td>
<td>41</td>
<td>30</td>
<td>62</td>
<td>6</td>
</tr>
<tr>
<td>prB−/−</td>
<td>42</td>
<td>29</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>prB−/−/p107−/−</td>
<td>41</td>
<td>35</td>
<td>59</td>
<td>5</td>
</tr>
<tr>
<td>prB−/−/p130−/−</td>
<td>41</td>
<td>31</td>
<td>61</td>
<td>7</td>
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</table>

The doubling times were determined by plating 10⁶ cells containing 2 μg/ml puromycin, pooled, and used for transcriptional analysis. Cells were harvested the day after plating (asynchronous), 2 days after serum deprivation (serum starved, 0.1% FBS), or when the cells had reached saturation at confluence (confluence). Luciferase assays (Promega) were performed according to the supplier on an equal number of cells and expressed as relative activity compared with the asynchronous activity. Results were obtained by using a luminometer from EG & G Berthold (Wallac, Gaithersburg, MD).

Results and Discussion

Establishment and Cell Cycle Characterization of prB−, p107−, and/or p130-Deficient 3T3 Cells. To study the biological roles of individual prB fibroblast family members, we generated a series of 3T3 fibroblast cell lines (20) from mouse embryos lacking prB, p107, and/or p130. 3T3 cell lines, which normally express all three of these proteins, provide a relatively homogeneous and readily manipulatable system in which to dissect functional differences within this gene family. Although other mutations are known to occur during the establishment of 3T3 lines (21–23) (see below for details), to simplify presentation, we will refer to the various cell lines by the genotype of the mutant embryos from which the 3T3 lines were derived: prB−/−, p107−/−, p130−/−, p107−/−/p130−/−, prB−/−/p107−/−, prB−/−/ p130−/−, and wild type (wt). Mouse embryo fibroblasts of all genotypes were relatively easily established as stable lines, with the exception of p130−/− cells, which barely proliferated for 42 passages (Fig. 1A). The reason for the large number of passages required to immortalize p130−/− cells, after which they proliferate at rates similar to immortalized wt cells (Table 1, and data not shown), is not known and requires further investigation. Multiple isolates of each cell line behaved similarly in the assays described, and the genotypically distinct cells expressed the expected protein(s) (Fig. 1B, and data not shown).

During the establishment of the cell lines, most genotypes (wt, p130−/−, p107−/−/p130−/−, prB−/−, prB−/−/p107−/−) acquired mutations in the p53 gene but the p16/p19ARF locus seemed to be unaffected (data not shown), whereas p107−/− and the prB−/−/p130−/− cell lines lack expression of p16 and p19ARF expression (data not shown). The number of cell lines that acquired p53 mutations rather than p16/p19 deletion is similar to what has previously been published (21–23), and it is unclear at this point whether specific mutations segregate with genotype. It is interesting to note that p21−/− cells preferentially acquire p16/p19 deletions during the immortalization process (L. Stevenson, M.C., and E.H., unpublished observations), whereas wt and prB-deficient cells preferentially mutate p53.

Over-expression studies have suggested some functional overlap between prB, p107, and p130 in control of the G₁ phase of the cell cycle (1), and previous studies in mouse embryo fibroblasts (MEFs) indicated that prB−/− and p107−/−/p130−/− cells exhibit a shorter G₁ phase than wt cells in serum stimulation experiments (24). We were therefore surprised to find only minor differences in the doubling times of the 3T3 cells deficient in various combinations of prB family members, and only the p130−/− cells display slightly longer cycling time (Fig. 1A, and Table 1). However, the saturation densities of the various cell lines are quite different (Table 1). This difference correlates with differences in cell size among the various cell lines as measured by FSC-H (Fig. 1C, and Table1). Previous studies had shown that prB−/− MEFs are smaller than wt MEFs (25). Our analysis demonstrates that this size change is not unique to Rb loss, because deletion of any prB family member results in smaller fibroblast cells (Table 1, and Fig. 1C). In general, the cell lines that spend less time in G₁ are smaller. This finding is consistent with the notion that cells deficient in prB or more than one family member spend less time in G₁ (for details see below) and are subsequently smaller than wt cells (Table 1, Fig. 2). However, p107−/− and wt cells have similar cell cycle characteristics, but p107−/− cells are the smallest of all the cell lines (Fig. 1C). Therefore, the relative size of these cell lines does not strictly correlate with time spent in G₁, suggesting that other factors contribute to overall cell size.

Loss of Rb Family Proteins Shortens G₁, but Lengthens S Phase. To investigate the cell cycle characteristics of these 3T3 cell lines in more detail, we analyzed the cell cycle profiles of asynchronously growing cell populations (Table 1). The various cell lines were plated at equivalent densities 24 h before the analysis of their cell cycle profiles, which was performed by using BrdUrd incorporation and two-dimensional FACS analysis. These experiments revealed that cells deficient in prB, p107/p130, prB/p107, and prB/p130 have an increased S phase population compared with wt, p107−/−, and p130−/− cells. The larger proportion of cells in S phase could potentially be due to a shorter G₁ phase and/or a lengthening of S phase because the doubling time of these cells is approximately the same. In both yeast and mammalian cells, a shortening of one phase of the cell cycle has been shown to result in a compensatory lengthening of other phases of the cell cycle, which allows the populations to maintain a relatively constant cell size (26–28). However, the mechanism by which such compensation occurs is unknown.

To distinguish between these possibilities, we monitored the cell cycle progression of a synchronized population of cells. The cells were synchronized by nocodazole arrest, followed by a
mitotic shake-off and replating in the absence of drug. Using this protocol, we isolated uniform populations of metaphase-arrested cells that reentered the cell cycle in a synchronous manner (29, 30). In our initial experiments, we found that all of these cells undergo endo-reduplication of DNA if they are exposed to nocodazole for 24 h (Fig. 2 Upper Right). However, treatment of asynchronously growing cells (Fig. 2 Upper Left) with nocodazole for only 10 h gives rise to a uniform population of cells with a 4N DNA content and little evidence of endo-reduplication (Fig. 2 Upper Middle). Therefore, cells were treated with nocodazole for 10 h before the mitotic cells were shaken from the dish, washed, and replated. To measure the rate at which these cells entered S phase, cells were allowed to incorporate BrdUrd at various times after replating.

As shown in Fig. 2, pRB/–/–, p107/–/–/p130/–/–, pRB/–/–/p107/–/–, and pRB/–/–/p130/–/– cells spend less time in G1 relative to the wt, p107/–/–, and p130/–/– cells. Even though all of the “double-deficient” cells enter S phase prematurely, they spend a longer time period synthesizing DNA and they enter G2 with kinetics similar to wt cells (data not shown). Thus, these results indicate that the shortening of G1 is “compensated” for by a lengthening of S phase, and appears to account for the fact that all of the cell lines exhibit a similar doubling time (Fig. 1A). A potential explanation for this observation is that derepression of a subset of E2F target genes shortens the length of G1, but other factors required for efficient synthesis of DNA are still limiting, resulting in a longer S phase. Taken together, these experiments suggest that deregulation of E2F target genes, resulting from loss of pRB, p107, and/or p130 results in earlier entry into S phase, but these cells synthesize DNA more slowly because of the absence of other rate-limiting factors required for S phase, such as cdk activity.

Derepression of E2F Target Genes Can Be Separated from Cell Cycle Progression. Because each of the RB family proteins has been found to regulate E2F activity, we next wanted to examine potential differences in the regulation of E2F-sensitive promoters among cells of the various genotypes. Initially, we examined E2F target gene expression in nocodazole-arrested/released cells progressing through the G1 phase of the cell cycle. However, under these circumstances, it was difficult to distinguish between cell cycle-dependent and RB family-dependent changes in E2F-

![Fig. 2. Timing of the cell cycle in cells deficient in pRB, p107, and/or p130. Asynchronous cells were treated with nocodazole for 10 h, and mitotic cells were shaken off and replated. (Upper) Example of the direct FACS data (R3 = G1, R3 = S phase, and R4 = G2/M); these 3T3 cells enter endo-reduplication upon prolonged nocodazole treatment. (Lower) The timing of S phase entry measured by BrdUrd incorporation and two-dimensional FACS analysis. The y-axis represents % S phase cells and the x-axis, hours postrelease. A representative example of three experiments is shown.](image-url)
mediated transcriptional control. For example, we observed an earlier induction of E2F-mediated transcription in pRB/2-2 cells and all of the double-negative cells when compared with wt cells (data not shown), but these changes could simply be due to the fact that these cells enter S phase earlier. Therefore, to separate cell cycle-dependent changes in E2F activity from effects that are dependent on RB family proteins, but are cell cycle-independent, we performed studies of E2F target gene expression in the context of growth-arrested cells.

Initially, we examined the ability of 3T3 cells deficient in pRB family members to arrest in response to serum withdrawal or confluence. These experiments show that the various cell lines vary substantially in their ability to arrest in low serum (Fig. 3A). Thus, following serum deprivation, all of the double-negative cells exhibit a substantial fraction of BrdUrd-incorporating cells, with the pRB-/-/p130-/- cells having the highest fraction of cells in S phase, followed by pRB-/-, pRB-/-/p107-/-, and p107-/-/p130-/- . Cells deficient in p107 and p130 exhibit an intermediate phenotype compared with wt cells, which arrest well in response to serum deprivation. In sharp contrast, all of the cell lines efficiently arrest in G1 after contact inhibition, with barely detectable S phase populations (Fig. 3A).

Fig. 3. Cell cycle arrest and derepression of E2F target genes in pRB, p107, and/or p130 deficient cells. (A) The % of cells in S phase, as measured by two-dimensional FACS in asynchronous (solid bars), serum-starved (striped bars), and confluent cells (gray bars) in the genotypically different cells, as indicated in the figure. (B) Relative luciferase activity of pools of cells harboring an integrated B-myb reporter under serum-starved (striped bars) and confluent conditions (gray bars) as compared with asynchronous populations (arbitrarily set to 100). A representative example from three different experiments is shown. (C) Relative luciferase activity from pools of cells harboring an integrated p107 promoter construct under serum-starved (striped bars) and confluent conditions (gray bars) as compared with asynchronous populations (arbitrarily set to 100). A representative example from three different experiments is shown. (D) (Upper) cdk2 kinase activity, using a His-tagged RB protein as a substrate in confluent (C) and asynchronous (A) wt cells and confluent knockout cell lines. (Lower) The corresponding levels of p27.

Several E2F-responsive genes had been identified whose expression appear to be deregulated in cells deficient in RB family proteins (24, 31). We chose two of these, B-myb and p107, for detailed analysis. To closely mimic physiological conditions for gene regulation, constructs harboring these promoters linked to a luciferase reporter were stably integrated into each of the 3T3 cell lines. Promoter activity was then measured in asynchronously growing and confluence-arrested cells. In addition, promoter activity was measured in serum-starved cells for comparison, because these two promoters had previously been found deregulated in serum-starved p107-/-/p130-/- (B-myb promoter) and pRB-/- (p107 promoter) cells, respectively (24). As shown in Fig. 3B, expression from the integrated B-myb reporter construct is repressed in contact inhibited wt cells. However, in p107-/-/p130-/- cells, this reduction in B-myb expression is virtually absent following confluence arrest, as was previously reported for serum-starved p107-/-/p130-/- MEFS. This derepression is specific to the combined loss of p107 and p130.
It has previously been demonstrated that cdk2 kinase activity is crucial for S phase entry and that the cdk2 inhibitor p27 is up-regulated under confluent conditions (33). Furthermore, it has been shown that cyclin E, which is one of the cyclin partners for cdk2, can overcome a cell cycle block imposed by a dominant negative DP-1 construct or a nonphosphorylatable RB (34). These experiments suggest that activation of cdk2 can promote S phase entry in the absence of E2F activity. Hence, we examined the various lines for the status of p27 and the activity of cdk2 in confluence arrested cells. These experiments revealed that the level of p27 protein is increased in all of the cell lines at confluence (Fig. 3D, Lower). Moreover, the up-regulation of p27 results in a significantly lower cdk2 activity (Fig. 3D, Upper) in all of the confluent cell populations compared with wt cells, suggesting that these cells arrest because of limiting amounts of cdk2, even if E2F activity is significantly increased.

In summary, we have established a cell system in which the functions of pRB, p107, and p130 can be compared and contrasted under conditions where these proteins are not over-expressed. Our experiments suggest that loss of pRB or more than one family member results in a shortening of G1 and an associated lengthening of S phase. Furthermore, the cells deficient in pRB family members display a reduced growth factor requirement, but, independent of genotype, all of the cells arrest at confluence despite deregulation of genotype-specific E2F target genes. Taken together, these results suggest that the pRB proteins can function in a combinatorial way to regulate the expression pattern of E2F-responsive genes and the characteristics of cell cycle progression and arrest.

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