A Critical Temporal Requirement for the Retinoblastoma Protein Family During Neuronal Determination

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Abstract. In this report, we have examined the requirement for the retinoblastoma (Rb) gene family in neuronal determination with a focus on the developing neocortex. To determine whether pRb is required for neuronal determination in vivo, we crossed the Rb−/− mice with transgenic mice expressing β-galactosidase from the early, panneuronal Tø1 α-tubulin promoter (Tø1:nlacZ). In E12.5 Rb−/− embryos, the Tø1:nlacZ transgene was robustly expressed throughout the developing nervous system. However, by E14.5, there were perturbations in Tø1:nlacZ expression throughout the nervous system, including deficits in the forebrain and retina. To more precisely define the temporal requirement for pRb in neuronal determination, we functionally ablated the pRb family in wild-type cortical progenitor cells that undergo the transition to postmitotic neurons in vitro by expression of a mutant adenovirus E1A protein. These studies revealed that induction of Tø1:nlacZ did not require proteins of the pRb family. However, in their absence, determined, Tø1:nlacZ-positive cortical neurons underwent apoptosis, presumably as a consequence of “mixed signals” deriving from their inability to undergo terminal mitosis. In contrast, when the pRb family was ablated in postmitotic cortical neurons, there was no effect on neuronal survival, nor did it cause the postmitotic neurons to reenter the cell cycle. Together, these studies define a critical temporal window of requirement for the pRb family; these proteins are not required for induction of neuronal gene expression or for the maintenance of postmitotic neurons, but are essential for determined neurons to exit the cell cycle and survive.

During embryogenesis cycling neural progenitor cells in the ventricular zones of the nervous system commit to a neuronal fate, and as a consequence of that decision, undergo terminal mitosis and adopt a neuronal phenotype. These newly born neurons migrate through a complex environment, extend axons that pathfind their way to targets, and ultimately form synaptic connections. A key developmental step in this process is the decision to undergo terminal mitosis, an event that is essential for appropriate neuronal differentiation and survival. One key regulator of the cell cycle, the tumor suppressor protein pRb (reviewed in Slack and Miller, 1996a), has recently been implicated in terminal mitosis and neuronal differentiation.

The retinoblastoma (Rb) gene was the first tumor suppressor gene to be cloned and is thought to function as a regulator of cell cycle by controlling the G1/S phase transition (Cobrinik et al., 1992; Hamel et al., 1992; Sherr et al., 1993). Through its A/B pocket domain the hypophosphorylated form of pRb binds and inactivates a number of cellular factors associated with cell proliferation (Picksley et al., 1994). Reports indicate that the E2F/DP1 family of transcription factors is the primary target for pRb-mediated cell cycle regulation. Genes that contain the recognition sequences for E2F in their promoters include p34cdc2, cyclin A, c-myc, n-myc, c-myb, and E2F-1 itself (Blake, et al., 1989; Thalmeier et al., 1989; Pearson et al., 1991; Dalton et al., 1992; Farnham, et al., 1993; Dalton et al., 1992; Farnham, et al., 1993; Lam et al., 1993; Hsiao et al., 1994; Johnson et al., 1994). Other members of the pRb gene family have been identified; these proteins, p107 and p130, contain similar pocket domains and are also believed to regulate cell growth (Hannon et al., 1993; Li et al., 1993; Zhu et al., 1993).

A role for pRb in neurogenesis was first indicated by the fact that pRb is highly upregulated during neuronal develop-

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1. Abbreviations used in this paper: MOI, multiplicity of infection; Rb, retinoblastoma.
development both in vitro and in vivo (Szekely et al., 1992; Slack et al., 1993). The appearance of the activated, hypophosphorylated species of pRb coincides with the time differentiating P19 cells become postmitotic and express a neuronal phenotype (Slack et al., 1993). The inactivation of the pRb gene by homologous recombination clearly demonstrated that pRb is essential for the development of the nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Since many cells appear to cycle normally in the absence of pRb, the observed phenotype is consistent with a role for pRb in terminal differentiation. Rb−/− mice die by E15 from hematopoietic and neurological defects attributed to failure to permanently withdraw from the cell cycle (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Previous reports indicate that by E12.5 onward, ectopic mitoses and massive cell death are observed throughout the developing nervous system, particularly in sensory ganglia and the hindbrain. The expression of a number of neural genes, such as TrkA and B, was found to be significantly decreased (Lee et al., 1994). Thus, pRb has been hypothesized to play a role in the developing nervous system both in the regulation of terminal mitosis and differentiation, two processes that are intimately coupled.

At what developmental timepoint is pRb required in neuronal development? Studies in pRb-deficient mice suggest that pRb is necessary either during or shortly after neuronal determination since mitotic figures and pyknotic cells were found in the intermediate zones, regions through which newly born neurons migrate (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In P19 cells induced to neuroectoderm, inactivation of the pRb gene family by deletion mutants of E1A resulted in apoptosis shortly after induction of the early neuronal commitment marker, munc 18 (Slack et al., 1995). Taken together, these studies suggested that pRb family proteins are essential for the development of neurons, and that, in their absence, neurons and/or progenitor cells undergo apoptosis (reviewed in Slack and Miller, 1996a).

In this paper, we examined the temporal requirement for pRb relative to the time when neurons exit the cell cycle and express neuronal genes using two approaches. First, we generated Rb−/− mice expressing a panneuronal Tα1-α-tubulin:nlacZ transgene that is induced as newly differentiating neurons exit the cell cycle (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In P19 cells induced to neuroectoderm, inactivation of the pRb gene family by deletion mutants of E1A resulted in apoptosis shortly after induction of the early neuronal commitment marker, munc 18 (Slack et al., 1995). Taken together, these studies suggested that pRb family proteins are essential for the development of neurons, and that, in their absence, neurons and/or progenitor cells undergo apoptosis (reviewed in Slack and Miller, 1996a).

In this paper, we examined the temporal requirement for pRb relative to the time when neurons exit the cell cycle and express neuronal genes using two approaches. First, we generated Rb−/− mice expressing a panneuronal Tα1-α-tubulin:nlacZ transgene that is induced as newly determined neurons exit the cell cycle and express a neuronal phenotype (Gloser et al., 1994; Gloster A., H. El-Bizri, S.X. Bamji, and F.D. Miller, manuscript submitted for publication; reviewed in Miller et al., 1996). Second, using a mutant E1A adenovirus, we functionally ablated the pRb gene family in cortical progenitor cells that undergo the transition to postmitotic neurons in vitro, as well as in postmitotic cortical neurons. Together, these two approaches define a critical temporal window of requirement for the pRb family. These cell cycle regulators are not required for induction of neuronal gene expression or for maintenance of postmitotic cortical neurons, but are essential for determined neurons to exit the cell cycle and survive.

Materials and Methods

Generation and Genotyping of Transgenic Mice

The pRb-deficient transgenic mice originally generated by Jacks et al. (1992) were purchased from the Jackson Laboratories (Bar Harbor, ME). The transgenic mice carrying the Tα1-α-tubulin promoter driving expression of a murine nlacZ reporter (Tα1:nlacZ, line K6) have been previously characterized (Gloser et al., 1994; Bamji and Miller, 1996; Wu et al., 1997; Gloster A., H. El-Bizri, S.X. Bamji, and F.D. Miller, manuscript submitted for publication). To generate pRb+/−; Tα1:nlacZ+/− mice, we crossed the pRb+/− mice in a C3H background with Tα1:nlacZ+/− mice in a CD1 background. Animals were outbred for at least seven successive generations in order to maintain uniformity in the genetic background. For developmental studies, transgenic mice were bred and the time of plug identification was counted as day 0.5. On the specified day whole embryos were fixed and stained as described below. Transgenic mice were genotyped by PCR, as previously described (Jacks et al., 1992) using DNA extracted from tails for adults, or from embryos for the tissue culture studies.

Histological Analysis of Embryos

For β-galactosidase staining, embryos were fixed for 60 min at 4°C in 4% paraformaldehyde and 0.1 M Na2HPO4, pH 7.3. After three rinses for 30 min each in a wash containing 0.1 M Na2HPO4, pH 7.3, 2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% NP-40, embryos were stained in a solution containing all the components of the rinse buffer with 1 mg/ml X-gal, 5 mM K3Fe(CN)6, and 5 mM K4Fe(CN)6. Staining was allowed to proceed for 6 h at 37°C after which time they were postfixed for 24 h in 4% paraformaldehyde. For histology, embryos were cryoprotected in graded sucrose solutions (12, 16, and 18%) and tissue was sectioned at 14 μm. Slides were counter stained with eosin, dehydrated in ascending concentrations of ethanol, followed by xylene, and then coverslips were mounted with permount.

Primary Cultures of Cortical Progenitors and Neurons

The preparation of cortical progenitors from mouse embryos was based on the method described by Ghosh et al. (1995) for rat cultures and modified corresponding to Brewer et al. (1993). Cortices were collected from E12-13 mouse embryos, triturated, and then plated at a density of 104 per well of a 4-well tissue culture plate. The culture medium consisted of Neurobasal medium (GIBCO BRL, Gaithersburg, MD), 0.5 mM glutamine, penicillin-streptomycin, 1% N2 supplement (GIBCO BRL), and bFGF (40 ng/ml; Collaborative Research, Inc., Waltham, MA). After 48 h, medium was replaced with the same medium except 1% N2 supplement was now replaced with 2% B27 (GIBCO BRL). The cortical neurons generated from these progenitor cells could be maintained for at least 3 wk under these conditions. When cells were cultured from transgenic mice, tissue from each embryo was removed, triturated, and plated separately before genotyping. Mature postmitotic neurons were prepared from E17-18 embryos, from which cortices were collected, triturated in culture media (Neurobasal with 0.5 mM glutamine, penicillin-streptomycin, 0.5% N2, 1% B27 supplements), and plated at a density of 0.5 × 106/ml.

Recombinant Adenovirus Vectors

The adenovirus vectors carrying the p300-binding mutant (Adl101) of E1A were on a 12S lacking E1B (Jelsma et al., 1988). The control vector carrying the Escherichia coli lacZ expression cassette, Ad5CA17lacZ (AdlacZ), was constructed by Christina Addison in the laboratory of Dr. Frank Graham (McMaster University, Hamilton, Ontario). Cortical progenitor cells were infected at the time of plating and cortical neurons were infected 8 day after plating to ensure that the vast majority of neurons in the cultures were postmitotic. For virus infection, cells were plated in 4-well tissue culture dishes in 400 μl medium with the addition of another 400 μl of medium containing the appropriate titers of virus. 18 h after infection, a complete media change was carried out. The multiplicity of infection (MOI) indicates the number of plaque-forming units added per cell. Cell survival assays were carried out after 72 h infection with cortical progenitor cells and 4 d for cortical neurons.

Brdu Incorporation and Immunocytochemistry of Cultured Cells

For BrdU-labeling, cells were incubated in media containing 10 μM BrdU for 12-18 h before staining. After a 30-min fixation in 70% ethanol, cells were air dried, treated with 2 N HCl for 10 min to denature DNA, and then treated with 0.1 M NaB4O7, pH 8.5, for another 10 min. Samples were washed three times with PBS containing 0.5% Tween 20 and 1% BSA before
an overnight incubation with the primary anti-BrdU antibody (Becton Dickinson, Mountain View, CA) diluted at 1:10. After three 5-min washes samples were incubated in a goat anti-mouse CY3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature and washed three times in PBS before examination.

For immunocytochemical detection of MAP2A/B (MAP2; Sigma Chemical Co., St. Louis, MO), cells were fixed in acetone/methanol (1:1) for 5 min. After air-drying for 5 min, cells were incubated in the primary antibody for MAP2A/B (1:1,000). After three washes in PBS, samples were incubated in a goat anti-mouse CY3-conjugated secondary antibody for 1 h at room temperature. To quantitate the number of immunoreactive cells within the total population, cells were counterstained with Hoechst stain (ICN Biomedicals, Costa Mesa, CA) at a final concentration of 2 μg/ml in PBS. Cells were examined after three washes in PBS using a fluorescent microscope (Zeiss Axiovert 100; Carl Zeiss Inc., Thornwood, NY).

To detect β-galactosidase expression, cells were fixed in cold acetone (pH 7.4) for 15 min at 4 °C. After two washes with PBS, cells were incubated for 18 h in X-gal stain (2 mM MgCl₂, 1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆ in PBS)

**Cell Survival Assays**

To assay cell survival three different assays were used including Live/Dead staining, TUNEL labeling, and a quantitative MTT assay. For the Live/Dead staining the Live/Dead viability/cytotoxicity Kit ( Molecular Probes, Inc., Eugene, OR) was used according to the manufacturer’s instructions. In brief, two reagents, calcine-AM and ethidium bromide, were added to cultures in their usual media. As an indicator of cell viability the calcine-AM is metabolically converted by intracellular esterase activity resulting in the green fluorescent product, calcine. Ethidium bromide is excluded from live cells but is readily taken up by dead cells and stains the DNA. Cells are incubated in these reagents for 10–15 min at 37 °C after which time they are examined and photographed immediately due to toxicity of these reagents.

To assay apoptosis, terminal transferase was used to visualize fragmented DNA (TUNEL labeling). Parallel cultures were infected with Ad1101 or AdlacZ at 25 MOI. After 72 h, cells were fixed in acetone/methanol (1:1) for 10 min at −20 °C. 50 μl of a cocktail consisting of 1.0 μl of 10 mM biotin dUTP, 1.5 μl terminal transferase, 20 μl of 5× TdT buffer, and 78 μl distilled water was added to each coverslip. After a 1-h incubation at 37 °C, samples were washed three times in PBS, pH 7.4, and once in TBS, pH 8.0, to stop the reaction. Samples were incubated with a streptavidin-CY3 dilituted at 1:2,000 for 30 min. After three 5-min washes in PBS, samples were examined with an inverted fluorescent microscope.

For a quantitative measure of cell survival, the MTT survival assay (Cell Titer Kit; Promega Corp., Madison, WI) was used as previously described (Slack et al., 1996). This assay measures the mitochondrial conversion of the tetrazolium salt to a blue formazan salt, the accumulation of which can be measured colorimetrically.

**Western Blot Analysis**

Cells were harvested in lysis buffer (Slack et al., 1995). Protein was separated on a 12% acrylamide gel and transferred to a nitrocellulose membrane. After blocking for 2 h in 5% skim milk, filters were incubated with different antibodies overnight at 4 °C. After five 5-min washes in TBST (0.5 N NaCl, 20 mM Tris, pH 8.0, 0.1% Tween-20), filters were incubated for 1 h at 25 °C in a goat anti-mouse (for monoclonal antibodies) or goat anti-rabbit (for polyclonal antibodies) secondary antibody conjugated to HRP. Blots were developed by the Enhance chemiluminescence system (ECL, Amersham Corp., Arlington Heights, IL) according to the manufacturer’s instructions. Antibodies used were mouse anti-E1A (antibody M73 used at dilution 1:1,000; gift from Dr. Phil Branton; McGill University, Montreal, Quebec), mouse anti-Rb (1:500; Oncogene Science Inc., Manhasset, NY), rabbit anti-p107 (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-p130 (dilution 1:1,000; Santa Cruz Biotechnology), and anti-phospho-tubulin (dilution 1:20, 10 μg protein per lane; gift from Dr. M.W. McBurney).

**Results**

**Analysis of Tα1 α-tubulin:nlacZ Expression in Rb−/− Mice**

To more precisely determine the developmental deficit in the nervous system of Rb−/− mice, we crossed Rb+/− mice with transgenic mice carrying a nuclear β-galactosidase reporter gene downstream of the early, neuron-specific Tα1 α-tubulin promoter (Tα1:nlacZ, line K6; Gloster et al., 1994). This transgene is neuron specific, is expressed panneuronally (Gloster et al., 1993; Bammhi and Miller, 1996), and is induced shortly after neuronal terminal mitosis (Gloster A., H. El-Bizri, S.X. Bammhi, and F.D. Miller, manuscript submitted for publication; Miller et al., 1996).

Expression of the Tα1:nlacZ transgene was monitored by X-gal staining at embryonic days E12.5, E13.5, and E14.5; this developmental window encompasses a major period of neurogenesis and neuronal differentiation up to the point when Rb−/− embryos die at E15. Transgene expression was directly compared in whole mount embryos that were Rb−/−, Tα1:nlacZ+/- versus their Tα1:nlacZ+/- littermates.

This analysis revealed that, at E12.5, the Tα1:nlacZ neuronal marker gene was robustly expressed in the Rb−/− embryos in a pattern that was similar to that seen in their wild-type littermates (Fig. 1, a, c, and e), indicating that determined neurons can initiate neuronal gene expression in spite of the absence of pRb. However, closer examination revealed a decrease in the level of transgene expression in the dorsal root ganglia (Fig. 1 c), consistent with the reported loss of neurons from these structures (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994). The hindbrain and midbrain were also somewhat smaller in the E12.5 Rb−/− embryos, as previously reported (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994), although the Tα1:nlacZ transgene was robustly expressed (Fig. 1, a and c). In contrast, the pattern of X-gal staining in later developing structures such as the retina, neocortex and olfactory epithelium was indistinguishable from wild-type littermates at E12.5 (Fig. 1 e).

Perturbations in neural development were much more evident by E14.5. Tα1:nlacZ expression was almost undetectable in the dorsal root ganglia of E14.5 Rb−/−, Tα1:nlacZ+/- embryos (Fig. 1, b and d), consistent with an almost complete loss of neurons between E12.5 and E14.5. Similarly, although there was still robust transgene expression throughout the midbrain, hindbrain, and spinal cord, these structures were smaller than in their wild-type littermates (Fig. 1, b and d). Dramatic differences in forebrain structure were also observed at this age. The neocortex was significantly smaller in the Rb−/− mice than in wild-type embryos (Fig. 1, b and f), and transgene expression was greatly reduced (Fig. 1 f). Moreover, transgene expression in the olfactory epithelium, which was indistinguishable between Rb−/− and Rb+/- animals at E12.5 (Fig. 1 e), was greatly reduced in E14.5 Rb−/− embryos (Fig. 1 f).

To more precisely characterize these previously unreported forebrain deficits, we sectioned E14.5 embryos and examined the neocortex. In Rb+/- Tα1:nlacZ embryos, transgene expression was highest in the cortical plate, the region where newly born neurons halt and differentiate after migration from the ventricular zone (Fig. 2. A and C). Lightly stained transgene-positive cells were also observed in the intermediate zone, which contains newly born, migrating neurons (Fig. 2 C). In contrast, in the Rb−/− cortex, few transgene-positive cells were observed in the cor-
tical plate (Fig. 2, B and D), and the most obvious staining derived from lightly stained cells in the upper portion of the intermediate zone (Fig. 2 D). Histologically, the deficit in transgene-positive cells was coincident with a thinner telencephalic roofplate (Fig. 2). These data suggest that newly determined cortical neurons can initiate Tα1:nlacZ expression in the absence of pRb, but die shortly before or after they reach the cortical plate.

Perturbations in transgene expression were also observed in the E14.5 olfactory epithelium and retina. In the wild-type retina the first appearance of Tα1:nlacZ expression occurs at E12.0–E12.5, coincident with birth of the first neurons, and by E14.5, there is robust transgene expression in presumptive retinal ganglion cells (Gloster et al., 1994). Examination of the retina of Rb+/-, Tα1:nlacZ embryos confirmed this pattern of expression (Fig. 3 A). In contrast, in the Rb-/-, Tα1:nlacZ+/- embryos, Tα1:nlacZ-positive cells still appeared at E12.5, but by E14.5, there were fewer transgene-positive cells than in controls (Fig. 3, A and B). Similarly, transgene expression was indistinguishable in the E12.5 olfactory epithelia of Rb-/- and Rb+/- animals (Fig. 1 e), whereas at E14.5, there were many fewer Tα1:nlacZ-positive cells in the Rb-/- embryos relative to their control littermates (Fig. 3, C and D). Therefore, these data suggest that throughout the nervous system, determined neurons express early neuronal genes in the absence of pRb, but then apoptose, potentially because they are unable to undergo terminal mitosis.

**The pRb Family Is Essential for Survival of Cortical Progenitors, but Not for Induction of Neuronal Gene Expression**

To more precisely define the requirement for neuronal determination, we turned to cultured cortical progenitor cells that will undergo the transition to postmitotic neurons in vitro (Ghosh et al., 1995). These progenitor cells have pre-

Figure 1. Whole mount X-gal staining of Rb-/- and wild-type embryos carrying the Tα1:nlacZ reporter gene. (a) At E12.5 Rb-/- embryos (indicated by -/-) appear similar to wild-type littermates (indicated by +/-). The arrows indicate the hindbrain and the arrowheads the trigeminal ganglia. (b) A side view of E14.5 embryos reveals gross cortical deficits in Rb-/- versus wild-type embryos. The arrows indicate the midbrain whereas the arrowheads indicate the developing neocortex. (c) A dorsal view of E12.5 of Rb-/- versus control embryos demonstrates that while there are many transgene-positive cells in the dorsal root ganglia of both embryos (arrows), there is a decrease in the relative amount of staining in the Rb-/- embryo. Arrowheads indicate the spinal cord. (d) A dorsal view of E14.5 embryos demonstrates that by this stage, the transgene-positive dorsal root ganglia have virtually disappeared in the absence of pRb (arrows). (e) A ventral view of the heads of E12.5 embryos indicates that the gross morphology and transgene staining in these later-developing structures is similar in the presence and absence of pRb. Arrows indicate the developing neocortex and the arrowheads the olfactory epithelia. (f) A similar ventral view at E14.5 demonstrates the dramatic deficits in forebrain structures in the Rb-/- versus control embryos. Arrows indicate the developing neocortex and the arrowheads the olfactory epithelia.
Previously been isolated from rats. To derive mouse cortical progenitor cells, we dissected cortices from E12.5 mouse embryos and characterized the resultant progenitors with regards to cell division and the expression of neuronal markers. To characterize cell division, cortical progenitors were incubated for 18 h with BrdU after 1 d in vitro. Staining with anti-BrdU indicated that the majority of cells (~90%) incorporate BrdU at this stage (Fig. 4, a and b).

Similar experiments with BrdU labeling at later timepoints revealed that most cells continued to proliferate until 3 d in vitro, after which timepoint the number of BrdU-labeled cells dropped to ~50%. Coincident with this decrease in cycling cells was an increase in the number of neurons generated in these cultures, as monitored by induction of the neuronal marker protein MAP2. Immunostaining revealed that there were few or no MAP2-positive neurons...
for the first 2 d in culture, with the first reproducible MAP2 immunoreactivity occurring at 3 d (Fig. 4, c and d). After 3 d in vitro, increasing numbers of cells displayed the morphological characteristics of neurons and expressed high levels of MAP2 (Fig. 4, e and f); by 6 d, ~50% of the cells were MAP2 positive. A similar time course was observed by immunostaining for a second, neuron-specific marker, the 200-kD neurofilament protein (data not shown).

To determine how pRb influenced the differentiation of cortical progenitors to postmitotic neurons, progenitor cells were cultured from individual E12.5 Rb<sup>−/−</sup> embryos and allowed to differentiate in vitro. These studies revealed that for at least 4 d in vitro pRb-deficient and wild-type cortical progenitor cells were indistinguishable. Incubation of cortical progenitors in the presence of BrdU for 18 h revealed that the majority of cells were cycling in both the Rb<sup>+/+</sup> and Rb<sup>−/−</sup> cultures. Moreover, neurons were able to differentiate in the absence of pRb, as indicated by cellular morphology and expression of MAP2 (Fig. 5, a and b). In contrast, when progenitor cells were isolated one day later, at E13.5, the cells attached to the substratum and flattened as usual, but then rounded up and died within 24 h (data not shown). Sister cultures of Rb<sup>+/+</sup> embryos derived from the same litters survived and differentiated to become neurons. Thus, progenitor cells isolated at E12.5, but not E13.5, can differentiate into neurons in vitro in spite of the absence of pRb.

One explanation for the observation that E12.5 cortical progenitors undergo neuronal differentiation in the absence of pRb is that other members of the pRb family can functionally compensate under some conditions (Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993; Zhu et al., 1993). Therefore, we examined the expression of two other members of the pRb family, p130 and p107, in the brains of E12.5 Rb<sup>−/−</sup> embryos. Western blot analysis using antibodies directed towards p130 and p107 revealed no change in the level of p107, but an increase in the level of p130 in Rb<sup>−/−</sup> brains as compared with those from their
control littermates (Fig. 6). Interestingly, an increase in p107 in Rb−/− muscle cells has been previously documented (Schneider et al., 1994), and has been postulated to compensate for the pRb deficiency in these cells. A similar phenomenon might occur here, with p130 compensating for the pRb deficiency in these cells. A similar phenomenon might occur here, with p130 compensating for the pRb deficiency in these cells.

To further characterize the role of this family of proteins in neuronal determination, we chose to functionally ablate the entire pRb family in wild-type cortical progenitor cells and to determine how this affected survival and neuronal differentiation. To perform these experiments, we took advantage of an E1A deletion mutant (1101) that sequesters and inhibits pRb family members, but not p300 (Jelsma et al., 1988). To introduce this protein into cortical progenitor cells, we used the recombinant, replication-defective adenovirus Ad1101 (Jelsma et al., 1988).

We have previously used recombinant adenovirus to manipulate postmitotic neurons with no demonstrable cytotoxicity as assayed physiologically, biochemically, or morphologically (Slack et al., 1996; Slack and Miller, 1996b).

To establish the parameters for adenoviral infection of cortical progenitors, we first used an adenoviral vector expressing E. coli β-galactosidase (AdS5CA17lacZ, AdlacZ). Infection of newly plated cortical progenitors with AdlacZ at 25 or 100 MOI resulted in the transduction of ~30 and 90% of the progenitors, respectively, 2 d after infection (Fig. 7a). To ensure that this infection did not result in cytotoxicity, we performed quantitative MTT assays that measured mitochondrial function and cell survival 3 d after infection (Fig. 8a). This analysis revealed that at titers up to and including 100 MOI there was no significant perturbation of cell survival. On the basis of these experiments, we expressed the mutant E1A in cortical progenitor cells by infecting with Ad1101 at the time of plating. Western blot analysis 2 d after infection revealed that, as predicted, the mutant E1A was expressed in Ad1101-infected cells, but not in cells infected with AdlacZ (Fig. 7c). Immunocytochemistry confirmed this conclusion and demonstrated that at an MOI of 25 many of the cortical progenitors were immunopositive for E1A (data not shown).

To determine whether the expression of the mutant E1A and the resultant functional ablation of the pRb family affected cell survival, we initially performed Live/Dead staining, an assay in which the levels of cell death (red ethidium bromide staining) and cell viability (green calcine staining) can be qualitatively compared. At 3 d in vitro, the vast majority of cells in control cultures were alive (Fig. 9, a and b). Similar results were obtained when progenitors were infected with 25 MOI AdlacZ at the time

![Figure 6. Expression of Rb family proteins in the E12.5 Rb−/− brain. Western blot analysis for pRb (Rb), p107 and p130 in wild-type and Rb-deficient brains. Protein was extracted from E12.5 wild-type (lane 1) and Rb−/− (lane 2) embryonic brains, and 10 µg protein was loaded on a 10% polyacrylamide gel. After electrophoresis, protein was transferred to nitrocellulose and filters were probed with antibodies for pRb, p107, or p130. Analysis of tubulin levels in the same samples indicates that similar amounts of protein are present in the Rb+/+ and Rb−/− samples.](image)

![Figure 7. The efficacy of recombinant AdlacZ and Ad1101 infection in cortical progenitor cells and postmitotic cortical neurons. (a and b) At 0 and 8 d in vitro, respectively, cortical progenitor cells (a) and neurons (b) were infected with recombinant AdlacZ at 25 MOI. After 48 h cells were stained with X-gal to visualize β-galactosidase gene expression. (c) Cortical progenitors (CP) and neurons (CN) were infected with Ad1101 (lane 1) or AdlacZ (lane 2) at 100 MOI upon plating and protein was extracted 48 h later. E1A 1101 was detected by Western blotting with M73 antibody. Bar, 50 µm.](image)

![Figure 8. The quantitative effect of AdlacZ versus Ad1101 infection on survival of cortical progenitor cells and postmitotic neurons, as measured using MTT assays. (a) Cortical progenitor cells were infected with AdlacZ (black bars) or Ad1101 (gray bars) at 0, 10, 25, 50, or 100 MOI at the time of plating. Cell survival was assayed 3 d later using the MTT assay. A concentration-dependent decrease in cell survival was detected in cells infected with Ad1101 relative to AdlacZ infected control cells. (b) At 8 d in vitro cortical neurons were infected at the same titers as described in a and the MTT assays carried out 4 d later. No change in cell survival was detected at any viral titer tested on neuronal cultures. Results represent the mean of three different experiments ± the standard error of the mean.](image)
of plating, and examined 3 d later (Fig. 9, e and f). When progenitors were transduced with the E1A adenovirus, progenitor cultures were similar to those infected with AdlacZ at 2 d after infection, with few signs of cell death (data not shown), in spite of the robust expression of E1A at this timepoint (Fig. 7 c). However, by 3 d, the morphology of the Ad1101-infected progenitors was perturbed relative to control sister cultures, and Live/Dead staining revealed a large increase in the relative proportion of dead cells (Fig. 9, i and j). Thus, functional ablation of the pRb family led to the death of cortical progenitors over a time course coincident with the generation of MAP2-positive neurons in these cultures.

To determine whether this increased cell death was due to apoptosis, we performed TUNEL labeling. Progenitors were infected with 25 MOI of the E1A versus lacZ-expressing adenoviruses at the time of plating, and analyzed 3 d later. Cortical progenitors expressing lacZ (Fig. 10, c and d) exhibited a background level of apoptosis similar to that observed in uninfected sister cultures (Fig. 10, a and b). In contrast, progenitors infected with equivalent titers of Ad1101 displayed a notable increase in the number of TUNEL-positive cells (Fig. 10, e and f), reminiscent of the increase in dead cells detected by the Live/Dead assay. Thus, the functional ablation of the pRb family led to cellular apoptosis at 3 d in culture.

To quantitate the apoptosis observed in these two assays, we performed MTT survival assays (Fig. 8). Cortical progenitor cells were infected with the lacZ- or E1A-expressing adenoviruses at titers of 10, 25, 50, and 100 MOI. 3 d later, the MTT assays revealed a dose-dependent decrease in cell survival with the mutant E1A adenovirus, but not with the lacZ adenovirus (Fig. 8). At 25 MOI of Ad1101, the concentration at which a visible difference in cell survival was observed by Live/Dead and TUNEL staining, a 31% decrease in survival was observed. At 50 and 100 MOI, decreases of 35 and 43%, respectively, were observed relative to controls.

Together, these data indicate that cortical progenitor cells require the pRb family and that, in their absence, the cells undergo apoptosis. To determine whether this apoptosis occurred before or after neuronal determination, we examined transcription of the Ta1 α-tubulin promoter. To perform these experiments, cortical progenitors derived from E12.5 Ta1:nlacZ mice were infected with 25–100 MOI E1A, and transgene induction was monitored by X-gal staining at 1 and 2 d after infection. In control uninfected cultures, the Ta1:nlacZ transgene was induced before MAP2, with the first positive cells appearing at 1–2 d in culture (Gloster A., H. El-Bizri, S.X. Bamji, and F.D. Miller, manuscript submitted for publication). Infection with Ad1101 had no effect on this induction (Fig. 5 C), even at 100 MOI, when ~90% of the cortical progenitors were transduced and when E1A expression was robust.

Figure 9. A comparison of cell viability of cortical progenitors and neurons infected with recombinant AdlacZ and Ad1101 using Live/Dead staining. Cortical progenitors (two left columns) and cortical neurons (two right columns) were infected upon plating and at 8 d in vitro, respectively, with 25 MOI of AdlacZ (e–h), Ad1101 (i–l), or were left uninfected (a–d). Live cells were measured by the enzymatic conversion of permeant calcine-AM to fluorescent calcine (green). Dead cells were detected by the uptake of ethidium bromide into cell DNA (red). A dramatic increase in cell death accompanied by a drop in cell survival was detected in progenitor cells infected with Ad1101 (i and j) when compared with uninfected progenitor cells (a and b) or to those infected with AdlacZ (c and f). In contrast, survival of cortical neurons was unaffected by infection with either AdlacZ (g and h) or Ad1101 (k and l) relative to uninfected neurons (c and d). Bar, 50 μm.
Moreover, Ta1:nlacZ expression was detected in cells with shrunken, acentric nuclei that were apparently undergoing apoptosis (Fig. 7 d). Thus, the pRb family is not required for induction of early neuronal gene expression, at least as monitored with the Ta1:nlacZ transgene, but is required for survival of determined Ta1:nlacZ-positive neurons.

**The pRb Family Is Dispensable for Maintenance of Postmitotic Cortical Neurons**

Two explanations can be invoked to explain these results. First, in the absence of the pRb family, determined neurons may be able to induce neuronal genes but not be able to undergo terminal mitosis, and the resultant “conflicting signals” may activate an apoptotic default pathway. Alternatively, postmitotic cortical neurons may require the pRb family for survival, and the apoptosis we observe in vivo and in vitro may reflect the death of postmitotic neurons.

Next we determined whether postmitotic cortical neurons could be effectively transduced with recombinant adenovirus by infecting them with AdlacZ. Staining with X-gal 2 d after infection revealed that 25 MOI of AdlacZ was sufficient to transduce ~90% of neurons in these cultures (Fig. 7 b). As previously observed for postmitotic sympathetic neurons (Slack et al., 1996), transduction of cortical cultures with 25–100 MOI adenovirus had no effect on neuronal survival, as monitored using MTT assays (Fig. 8).

Having established these parameters, we infected 8-d-old cortical neuron cultures with 25–100 MOI of lacZ- or E1A-expressing adenovirus and monitored cell viability. Immunocytochemistry revealed that the mutant E1A gene product was expressed in the transduced neurons for at
least 5 d (data not shown), and Western blot analysis revealed that levels of expression of the transduced E1A were similar to those observed in cultures of cortical progenitors (Fig. 7c). Live/Dead staining of cortical cultures 3 d after infection with Ad1101 demonstrated that expression of the mutant E1A had no effect on the proportion of live to dead cells relative to either control, uninfected cells or to sister cultures infected with the lacZ adenovirus (Fig. 9). TUNEL labeling confirmed this qualitative conclusion (Fig. 12); the number of apoptotic cells was similar in uninfected cultures relative to those infected 3 d previously with the lacZ- or E1A-expressing adenoviruses. Finally, to ensure that there were no quantitative differences that were missed using these qualitative approaches, we performed MTT assays (Fig. 8). At 25 MOI, the concentration of adenovirus at which an apoptotic effect of E1A was noted for progenitor cells, no significant change in cell survival was seen in lacZ-infected (100%) neurons in comparison to those infected with Ad1101 (101%). Even at 100 MOI, AdlacZ-infected cells exhibited 105% survival and Ad1101-infected cells 101%, indicating no correlation between expression of the mutant E1A and cell death. Thus, the pRb family is dispensable for postmitotic cortical neurons, at least with regards to neuronal survival.

Although these results indicate that the pRb family is not required for survival of postmitotic cortical neurons, it might be necessary to keep these cells locked out of the cell cycle. To assess this possibility, cortical cultures were infected with 25 MOI of lacZ or E1A-expressing adenoviruses and, 3 d after infection, were incubated with BrdU for 12 h to label dividing cells. Immunocytochemistry with
anti-BrdU revealed that expression of the mutant E1A had no effect on the number of dividing cells in the cortical neuron cultures (Fig. 13); from 5 to 9% of the cells were labeled with BrdU under these conditions, irrespective of the expression of E1A. Thus, the pRb family is not required for survival of postmitotic neurons, nor is it required to keep them locked out of the cell cycle.

Discussion

The results of these studies support a number of conclusions. First, using transgenic mice that express a β-galactosidase marker gene from the early panneuronal β-tubulin promoter (Tα1:nlacZ), we demonstrate that the neurological defects in Rb-deficient embryos are more widespread than previously reported, and include the forebrain, retina, and olfactory epithelium, regions previously believed to be spared. Second, we show that Tα1:nlacZ expression is initiated normally in Rb−/− mice, indicating that neuronal gene expression is induced in the absence of pRb, but that the number of cells expressing Tα1:nlacZ decline developmentally, presumably as a consequence of apoptosis. Third, we show that Rb-deficient cortical progenitor cells, when isolated at or before E12.5, can undergo neuronal differentiation in vitro, but that these same cells fail to differentiate and die when isolated at E13.5. Fourth, functional ablation of the Rb gene family with a mutant E1A (1101) demonstrates that, as seen in vivo, this family is not required for induction of neuronal gene expression, but is essential for survival at the time they would normally become postmitotic neurons. Finally, our studies reveal that once cortical neurons have exited the cell cycle, the pRb family is dispensable for neuronal survival, and is not required to keep these cells locked out of the cell cycle. Together, these studies define a critical temporal window of requirement for the pRb family; these proteins are not required for induction of neuronal gene expression or for maintenance of postmitotic neurons, but are essential for determined neurons to exit the cell cycle and survive.

For many of these studies, we have used transcription of the Tα1 α-tubulin promoter as an assay for early neuronal gene expression. The endogenous Tα1 α-tubulin gene, which encodes the vast majority of the α-tubulin required for morphological differentiation of developing neurons, is induced shortly after neurons are born, is expressed at high levels during neuronal growth, is decreased in expression as neurons mature, and is subsequently reinduced during neuronal regeneration (Miller et al., 1987, 1989). We have previously demonstrated that a 1.1-kb fragment of the Tα1 α-tubulin promoter is sufficient to drive expression of a marker gene in a similar neuron-specific fashion in transgenic mice (Tα1:nlacZ mice; Gloster et al., 1994; Bamji and Miller, 1996; Wu et al., 1997; reviewed in Miller et al., 1996), with transcription commencing within hours of neuronal terminal mitosis (Gloster A., H. El-Bizri, S.X. Bamji, and F.D. Miller, manuscript submitted for publication).

Use of these Tα1:nlacZ-transgenic mice allowed us to define those regions of the nervous system that are dependent upon pRb during development. Previous studies have demonstrated that the developing hindbrain, spinal cord, and peripheral nervous system were dramatically affected by the absence of pRb (Lee et al., 1991; Clarke et al., 1992; Jacks et al., 1992). Our studies confirm these conclusions and demonstrate that pRb is also required in the forebrain, retina, and olfactory epithelium, indicating that this cell cycle regulator is essential for neuronal development throughout the nervous system. This analysis also allowed for a more precise definition of the developmental events that require pRb in vivo. For example, the E12.5 dorsal root ganglia of Rb−/−, Tα1:nlacZ+/+ mice contained many transgene-positive cells at E12.5, but these cells were subsequently lost by E14.5. Similarly, transgene-positive cells were present in the E14.5 neocortex in the intermediate zone, which contains newly born, migrating neurons, but were largely absent from the cortical plate region, which contains somewhat older postmitotic neurons. These results are consistent with the interpretation that throughout the developing nervous system, in the absence of pRb, determined neurons induce early neuronal genes but then are lost due to apoptosis.

A specific requirement for pRb after neuronal determination but before terminal mitosis is also supported by our studies in cultured cortical progenitor cells. As seen in vivo, these progenitors induce Tα1:nlacZ expression in the absence of functional pRb family members, but subsequently undergo apoptosis around the time they would normally become postmitotic. We propose that this apoptosis is due to the conflicting signals that arise from the inability of determined neurons to exit the cell cycle. In most neuronal populations, including cortical neurons, the decision to undergo terminal mitosis is closely coupled to induction of a neuronal phenotype. For example, cells in the mitotic layer of the developing retina induce expression of a retinal ganglion cell marker within minutes of S phase of terminal mitosis (McLoon and Barnes, 1989; Wald and McLoon, 1995), and cortical neurons maintain their laminar identity in transplant experiments only if they undergo terminal mitosis before transplantation (McConnell and Kaznowski, 1991). As a consequence of this coupling, transcription of neuronal genes like Tα1 α-tubulin is only seen after terminal mitosis for the vast majority of mammalian neurons (Gloster A., H. El-Bizri, S.X. Bamji, and F.D. Miller, manuscript submitted for publication). However, results presented here indicate that, in the absence of the pRb family, induction of neuronal gene expression and terminal mitosis are aberrantly uncoupled. This is consistent with previous observations that ectopic mitoses are found throughout the intermediate zones of the developing CNS of Rb−/− embryos, regions that normally contain migrating, postmitotic neurons (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), and expression of the neuron-specific βIII-tubulin isoform is perturbed (Lee et al., 1994). We propose that it is this aberrant uncoupling of neuronal differentiation and terminal mitosis that results in the apoptosis observed both in vivo and in culture in the absence of the pRb family. Although the precise molecular mechanism whereby such conflicting signals lead to apoptosis is unclear, it likely involves the p53 tumor suppressor protein, since recent studies indicate that much of the apoptosis in the CNS of Rb−/− mice is rescued by the coincident loss of p53 (MacLeod et al., 1996).

Although our studies indicate an absolute requirement for pRb in developing cortical neurons in vivo, they also indicate that this requirement is lost if cortical progenitor
cells are cultured early. Specifically, our results indicate that E12.5 Rb−/− cortical progenitors are able to differentiate to cortical neurons, whereas E13.5 Rb−/− cortical progenitors undergo cell death. Similar results have been obtained with Rb−/− sensory neuroblasts/neurons isolated from the trigeminal and dorsal root ganglia (Lee et al., 1994); when these cells were isolated at E11.5, they survived in the presence of NGF, but when isolated at E12.5 or later, they exhibited impaired neuritogenesis and survival (Lee et al., 1994). The ability of neuronal differentiation to proceed in the absence of pRb is likely due to functional compensation by other members of the pRb family such as p107 and p130 (Hamon et al., 1993; Li et al., 1993; Mayol et al., 1993; Zhu et al., 1993), a possibility supported by the increase in p130 levels we observed in the brains of E12.5 Rb−/− embryos. A similar functional compensation by p107 has previously been proposed for myogenesis in the absence of pRb (Schneider et al., 1994). This idea is supported by the fact that functional ablation of the entire pRb family in wild-type E12.5 cortical progenitors results in a phenotype similar to that observed in vivo in the absence of pRb.

The studies reported here also indicate that, after terminal mitosis, the pRb family becomes dispensable for the maintenance of cortical neurons. Functional ablation of the pRb family did not perturb neuronal survival, nor did it cause these postmitotic cells to reenter the cell cycle. These somewhat surprising findings suggest that once neurons have exited the cell cycle, additional pRb-independent mechanisms ensure that they remain locked out of the cell cycle. The nature of these pRb-independent mechanisms is currently unknown, but it has been previously proposed that perturbation of such “fail-safe” mechanisms may well result in apoptosis of terminally differentiated, postmitotic neurons (Heintz, 1993).

In summary, our results, together with previous work on the Rb−/− mice, define a critical requirement for the pRb family during neuronal determination; these cell cycle regulators are not required for induction of neuronal gene expression, nor are they required for maintenance of postmitotic neurons, but they are instead essential for determining neurons to exit the cell cycle. In their absence, determined neurons undergo apoptosis both in vivo and in culture, presumably as a consequence of conflicting signals deriving from the inability to undergo terminal mitosis. Elucidation of the molecular mechanisms whereby pRb collaborates with transcriptional regulators of neuronal gene expression will allow fundamental insights into the coupling of terminal mitosis and induction of the neuronal phenotype.

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