Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation

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To assess the functions of the retinoblastoma protein (RB) during normal development, we have analyzed mouse embryos that lack a functional copy of the *retinoblastoma* gene (genotype: *Rb-1^{Δ20}/Rb-1^{Δ20}*). Our findings demonstrate that RB plays an important role in the regulation of the neuronal cell cycle. In mutant embryos, dividing cells are found well outside of the normal neurogenic regions in both the central and peripheral nervous systems. In addition to abnormal cell cycle regulation, however, the mutant embryos show two less expected phenotypes. First, many of the ectopically dividing cells die by apoptosis shortly after their entrance into S phase. In sensory ganglia, most nerve cells die by this process, beginning at about the same time as normal target-related neuronal death. Second, although the expression of certain differentiation markers such as N-CAM and Brn-3.0 appears to be near normal, nerve cells, especially in sensory ganglia, do not mature properly. Their morphology is stunted and expression of neuronal β III tubulin is greatly reduced. Preferential reduction in the expression of TrkA, TrkB, and the low-affinity neurotrophin receptor p75^{LNGFR} may be relevant to neuronal cell death and lack of neuronal differentiation seen in the mutant embryos. Primary cultures of dorsal root and trigeminal ganglion cells from later stage mutant embryos reveal a decrease in neuronal cell survival and in neurite outgrowth even in the presence of the appropriate neurotrophins. Taken together, these results suggest that the p110^{RB} protein not only regulates progression through the cell cycle but is also important for cell survival and differentiation.

[Key Words: Retinoblastoma gene; cell cycle control; apoptosis; neuronal differentiation]

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The differentiation of a nerve cell is a process that begins early in embryonic development and continues through subsequent cell divisions, migrations, and maturations. It culminates in the acquisition of the form and function that define each specific adult cell type. One important characteristic that applies to all mature nerve cells is that they never divide. Most central nervous system (CNS) neurons undergo their final cell division during embryogenesis and remain locked in a G₀ cell cycle state for the remainder of the life of the organism. This cell cycle arrest is one of the earliest events in the neuronal maturation process, although the expression of pattern formation genes (e.g., *Buolfone* et al. 1993) as well as certain differentiation markers (Ma et al. 1992) can precede it (see also Rothman et al. 1980).

A complete description of the cellular and molecular mechanisms that regulate the neuronal differentiation

process, including cell cycle arrest, is currently far from complete. One important constituent of the process, however, is the tumor suppressor gene *retinoblastoma*. The *retinoblastoma* gene product, p110^{RB}, is a nuclear protein that is expressed throughout the developing organism (Lee et al. 1987; Bernards et al. 1989; Szekely et al. 1992). At least five different phosphorylated forms of the protein are found in cycling cells (Shew et al. 1989; Ludlow et al. 1990), and the relative balance among these forms varies during the four phases of the cell cycle (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989, 1992). Hypophosphorylated retinoblastoma (RB) protein predominates in G₁. As cells enter S phase, phosphorylated forms become more abundant, and these forms persist through S, G₂, and M phases. Multiple serine and threonine residues are phosphorylated (Lin et al. 1991; Hu et al. 1992), and sequential phosphorylation of specific sites has been reported (DeCaprio et al. 1992). As cells enter anaphase, RB dephosphorylation begins and continues, stepwise, until G₁ when RB is once again

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hypophosphorylated (Ludlow et al. 1993). RB phosphorylation not only correlates with the phase of the cell cycle, but several studies have suggested that RB is part of the mechanism that regulates the cell cycle. Cells into which RB has been introduced by retroviral infection (Huang et al. 1988), microinjection (Goodrich et al. 1991), or transfection (Qin et al. 1992) show a reduced growth rate attributable to inhibition of cell cycle progression from G₁ to S phase. The mechanism by which unphosphorylated RB inhibits this progression is likely to involve the RB-associated transcription factor E2F-1 (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992). Finally, in addition to a major role in the G₁-to-S transition, RB may act at other stages in the cell cycle. For example, it has been shown recently that RB may result in G₂ arrest if it is overexpressed during S phase of the cell cycle (Karantza et al. 1993).

Mice with null alleles at the *Rb-1* locus have been generated independently by three laboratories (Clarke et al. 1992; Jacks et al. 1992; E.Y.-H.P. Lee et al. 1992). Lee et al. (1992) mutated the RB gene at exon 20 resulting in a carboxy-terminal truncated protein. This mutation (designated *Rb-1*^{Δ20}) destroys the regions in the RB protein that are required for binding to many cellular proteins, including E2F-1. Heterozygous, *Rb-1*^{Δ20/+}, mice develop pituitary tumors of intermediate lobe origin with nearly 100% penetrance (Hu et al. 1994). Analysis of DNA from the cells of these tumors inevitably reveals loss of the wild-type *Rb-1* allele (Jacks et al. 1992; Hu et al. 1994). This is consistent with the behavior of the gene in human retinoblastoma tumors and with the proposed role of *Rb-1* as a tumor suppressor gene. Homozygous *Rb-1*^{Δ20/Rb-1}^{Δ20} mutant embryos die between days 12.5 and 16.0 of gestation (E12.5–E16). Although most organs appear to develop properly, there are major defects in the nervous and hematopoietic systems. One of these defects is the presence of ectopic mitotic figures in the developing nervous system located at some distance from the normal neurogenic regions. Such cells are not observed in normal mice and their presence in the RB-deficient mutants is in keeping with the proposed role of RB as a cell cycle regulator (E.Y.-H.P. Lee et al. 1992). Some of the phenotypes of homozygous embryos, however, are somewhat paradoxical. For example, massive amounts of cell death are found in the same regions as the dividing cells. Dying cells can be found in all regions of the CNS and peripheral nervous system (PNS), but they are especially prevalent in hindbrain, spinal cord, and sensory ganglia.

Our current understanding of the p110^{RB} protein function offers no means of explaining the phenotype of the *Rb-1*^{Δ20/Rb-1}^{Δ20} embryos. In this study we address the function of RB in both the regulation of the cell cycle and in differentiation. We report that cells in the nervous system of the mutant embryos fail to become postmitotic and die by apoptosis. In addition, the levels of expression of several early neuronal differentiation markers are reduced, leading to the suggestion that p110^{RB} serves a complex developmental function that transcends a single role in cell cycle regulation.

Results

Post-translational modification of the RB protein during CNS development

Abnormalities in the CNS of *Rb-1*^{Δ20/Rb-1}^{Δ20} embryos appear as early as embryonic day 10 (E10). To assess the correlation of these abnormalities with the activity of RB protein during development, we analyzed RB expression in the CNS from embryos of different ages. RB protein is detectable by Western blot at E9.5 (Fig. 1); significantly, at this early age, most of the protein is found as one of the phosphorylated isoforms. At E10.5, the level of RB has increased, and the fraction of the protein that is in the unphosphorylated state is substantially greater. This trend continues through E12.5, and by E14.5 the phosphorylated forms of RB are nearly undetectable. This developmental shift in RB can be contrasted with the Gβ-like protein (Shan et al. 1992) and the RB-associated protein RbAp48 (Qian et al. 1993). Western blot analysis of these two proteins reveals that their levels remain nearly constant during the same developmental time period. Appearance of unphosphorylated RB protein correlates with the times at which most neurons in the midbrain and hindbrain have left the cell cycle (Taber-Pierce 1973), consistent with the role of unphosphorylated RB in inhibiting cell cycle progression from G₁ to S phase (Goodrich et al. 1991). Significantly, the timing of the appearance of the nervous system defects seen in the *Rb-1*^{Δ20/Rb-1}^{Δ20} embryos correlates with the increasing predominance of unphosphorylated forms of RB.

Cells in RB-deficient embryos continue to transit the cell cycle

Because RB protein inhibits cell cycle progression from G₁ to S, we used a fluorescence-activated cell sorter

Figure 1. RB protein expression during embryonic development. About 600 μg of protein extract from E9.5, E10.5, and E11.5 embryonic heads, and E12.5, E13.5, E14.5, and E16.5 embryonic brains were separated on a 6–15% gradient gel, and areas containing proteins of interest were analyzed by Western blotting. RbAp48, an RB-associated protein, and Gβ-like protein served as controls for the quantities of protein loaded. The former was revealed with a monoclonal antibody; the latter was revealed with a polyclonal antiserum. The bracket indicates phosphorylated RB protein.

(FACS) analysis to study cell cycle distribution in the RB-deficient mutants. Cells were harvested from the hindbrain region of mutant and control embryos at E13.5. Cells from wild-type embryos had a fluorescence pattern that was consistent with 4% of the population being in S phase (Fig. 2a). In contrast, cells from the mutant had double this number (Fig. 2b). Similar results were found when cells were harvested from the cervical spinal cord (Figs. 2c,d).

To pursue these observations in situ, we used bromodeoxyuridine (BrdU) to label dividing cells. Following injection into a pregnant female, embryonic cells that are in S phase will take up BrdU from the circulation and incorporate it into DNA. A single injection delivers a pulse of S-phase label to the developing fetuses. The cells that were synthesizing DNA during this pulse can then be revealed by immunocytochemistry using antibodies against BrdU (Nowakowski et al. 1989). In normal embryos, the ventricular zone of the CNS is a highly organized germinative center. The distance of a cell's nucleus from the lumen of the neural tube is essentially proportional to its distance from M phase of the cell cycle. Thus, mitotic figures are only found at the luminal surface. As cells enter G₁, their nucleus ascends and cells enter S phase at the outermost regions of the ventricular zone. During G₂, the nucleus descends to the lumen to enter M phase once more at the surface of the lumen. In keeping with this pattern, if E12.5 embryos are sacrificed 1 hr after injection, BrdU-positive nuclei are found only in the synthetic region of the ventricular zone of the neural tube (Fig. 3a) and in cells in the sensory ganglia. If sacrifice and fixation of the embryos are

delayed until 3 hr after BrdU injection, labeled nuclei in the wild-type embryos are now found at the luminal surface (Fig. 3c). In E12.5 *Rb-1*^{Δ20}/*Rb-1*^{Δ20} mutants, proliferative cells are present in the synthetic regions of the ventricular zone, but many labeled nuclei are also found in regions normally reserved for migrating and maturing neurons (Fig. 3b). Descent of labeled nuclei is seen in mutant embryos if survival time after BrdU injection is lengthened (Fig. 3d). Even though the cells in the mutant ventricular zone appear to cycle properly, however, proliferative cells are found throughout the CNS in zones where normally only postmitotic cells reside.

By E14.5, cell proliferation is nearly complete and the ventricular zone contains only a few cycling cells (most likely non-neuronal) that are labeled with BrdU (Fig. 3e). The spinal ganglia still contain labeled cells, but their numbers have decreased. In contrast, in mutant embryos, many cells continue to enter the cell cycle throughout the CNS (Fig. 3f). In the PNS, the number of labeled cells in mutant embryos far exceeds that found in the wild type (cf. Fig. 3, e and f). The BrdU injections illustrate that the cells of RB-deficient embryos enter S phase even in zones of the neural tube that are normally reserved for postmitotic cells. Furthermore, proliferation of the cells continues, nearly unabated, until the ages at which the mutant embryos die. In contrast to these findings in the nervous system, little difference is found in the patterns of cell division in other tissues. For example, in the embryonic tongue where muscle fibers and dividing myoblasts can be identified, the patterns of BrdU incorporation in mutant and wild-type embryos are nearly indistinguishable (Fig. 3g,h). The results suggest

Figure 2. DNA fluorescence flow cytometric profiles of propidium iodide-stained neuronal cells. Cell suspensions from hindbrain and cervical spinal cord regions of E13.5 embryos were prepared, stained with propidium iodide, and analyzed by FACScanning. Numbers indicate percent of cells in S phase. (a) Hindbrain of wild-type embryos; (b) hindbrain of *Rb-1*^{Δ20}/*Rb-1*^{Δ20} embryos; (c) cervical spinal cord of wild-type embryos; (d) cervical spinal cord of *Rb-1*^{Δ20}/*Rb-1*^{Δ20}.

Figure 3. DNA synthesis in the spinal cord and dorsal root ganglia of wild-type and *Rb-1 $\Delta 20$ /Rb-1 $\Delta 20$* embryos. (a–f) Transverse sections of the embryonic spinal cord and associated ganglia were immunostained with an anti-BrdU monoclonal antibody. (Left) Wild-type embryos; (right) mutant embryos. (a,b) E12.5 embryos were sacrificed 1 hr after BrdU injection. (c,d) E13.5 embryos were sacrificed 3 hr after BrdU injection. (e,f) E14.5 embryos were sacrificed 1 hr after BrdU injection. Note that in mutant embryos cells in the intermediate zone as well as the ventricular zone undergo DNA synthesis. (g,h) Longitudinal sections of the embryonic tongue. E14.5 embryos were sacrificed 1 hr after BrdU injection to show the pattern of dividing cells in control (g) and mutant (h) tongue. (dg) Dorsal root ganglion; (i) intermediate zone; (v) ventricular zone; (sc) spinal cord; (t) tongue; (arrow) muscle fibers in tongue; (Dor) dorsal side. Scale bar, 100 μ m.

that without RB protein, cell cycle arrest during neurogenesis is incomplete.

Apoptosis in *RB*-deficient embryos

Although we observed the spatial correlation of ectopic mitoses and cell death previously (E.Y.-H.P. Lee et al. 1992), there was no direct proof that the dying cells were the ones that had divided recently. The BrdU injection studies allow us to address this question. Following a short pulse of BrdU, immunostained pycnotic remnants can be identified (Fig. 4b, arrows). This cellular debris must be removed soon after the cells enter S phase because the numbers of pycnotic, BrdU-positive cells decrease if the embryos are labeled for 3 hr (Fig. 3; data not shown). Thus, it appears that many of the dead cells are cells that have recently entered the cell cycle and begun to synthesize DNA.

We asked whether the cell death associated with the

failure of the *Rb-1 $\Delta 20$ /Rb-1 $\Delta 20$* cells to exit the cell cycle was apoptotic programmed cell death. Genomic DNA was extracted from E13.5 dorsal root and trigeminal ganglia, where pycnotic cells are most prominent. The DNA was end-labeled using [α - 32 P]ddATP and separated on an agarose gel (Tilly and Hsueh 1993). The characteristic ladder of DNA fragments, a hallmark of apoptosis, was prominent in mutant and, to a much lesser degree, in wild-type embryos (Fig. 5a). No evidence of fragmentation could be found in DNA from tails of adult mice. This suggests that the cell death seen in the mutant embryonic nervous system is apoptotic in nature.

To confirm this in situ, we applied the TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling] technique to histological sections of embryonic tissue (Gavrieli et al. 1992). By labeling DNA on sections using terminal deoxynucleotidyl transferase, any cell undergoing DNA fragmentation will expose numerous “ends” for dUTP-biotin

Figure 4. Consequences of ectopic mitosis. A higher magnification view of the spinal cord (sc) and dorsal root ganglia (dg) of E14.5 wild-type embryo (a) and mutant embryo (b), following a 1-hr pulse-label of BrdU. Note the condensed and fragmented BrdU-positive nuclei of apoptotic cells in the mutant embryos. Abbreviations as in Fig. 3. Scale bar, 20 μ m.

addition. In wild-type embryos, TUNEL staining is observed throughout the nervous system during development. In the PNS of both mutant and wild-type embryos, the first structure that shows positive cells is the trigeminal ganglion. At E11.5, however, a two- to threefold elevation in the density of apoptotic cells is seen in RB-deficient embryos compared with wild type (Fig. 5b,c). Twenty-four hours later, the same phenomenon is repeated in the dorsal root ganglia (Fig. 5d,e). In the spinal cord of the mutant embryos, apoptotic cell clusters are seen in the outer half of the ventricular zone as well as in the more superficial nerve cell regions. The time of appearance and spatial distribution of the apoptotic cells is unique in each region and is highly reproducible from one mutant embryo to the next.

Sensory neurons in RB-deficient embryos undergo incomplete differentiation

Several morphological observations have prompted us to ask whether RB might function in yet another capacity during neuronal maturation. This issue is well illustrated by the cells of the spinal ganglia. In wild-type embryos, by E14.5, most neurons in the trigeminal ganglion are well differentiated with large nuclei and well developed Nissl substance (Fig. 6a). In contrast, those trigem-

inal ganglion cells that survive in the *Rb-1^{Δ20}/Rb-1^{Δ20}* mutant appear much less differentiated (Fig. 6b). They have smaller cell bodies and scant cytoplasm with little or no dark Nissl substance. The nuclei of even the largest mutant cells are dramatically smaller than their wild-type counterparts, although the nucleoplasm is clear and one or two nucleoli are visible. Similar observations are made in dorsal root ganglia from comparable axial levels at E14.5 (Fig. 4a,b), and in the CNS (data not shown). Curiously, this poor cytological development does not seem to be correlated with problems in axonal outgrowth. Analysis of the cranial and spinal nerves was performed by whole-mount immunostaining of E10.5 and E11.5 embryos using an antibody (2H3) directed against the 155-kD component of neurofilament. The central and peripheral nerves of the trigeminal, petrosal, and nodose ganglia are all formed in the *Rb-1^{Δ20}/Rb-1^{Δ20}* mutant (Fig. 7, cf. b with the +/+ embryo in a). Thus, neither the absence of RB protein nor the poor cytological state of the ganglion cell bodies prevented proper axonal outgrowth and guidance.

Expression of specific genes in RB-deficient embryos

These morphological observations led us to examine the expression of several genes known to be active during early nervous system development. Many POU domain

Figure 5. Programmed cell death in the trigeminal and dorsal root ganglia of *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos. (a) DNA ladder of fragmented genomic DNA. (Lane 1) Five hundred nanograms of genomic DNA from adult mouse tail. (Lanes 2–4) DNA from E13.5 dorsal root ganglia: (lane 2) wild type; (lane 3) heterozygous, and (lane 4) mutant. (b–e) TUNEL staining of apoptotic nuclei. Coronal sections showing dorsal root ganglia from E11.5 (b,c) and E12.5 embryos (d,e) were processed for the TUNEL technique to identify cells undergoing apoptotic, programmed cell death. (b,d) Wild-type embryo; (c,e) *Rb-1^{Δ20}/Rb-1^{Δ20}* embryo. (dg) Dorsal root ganglia; (tg) trigeminal ganglia; (Dor) dorsal side. Scale bar, 100 μ m (b,c) and 80 μ m (d,e).

Figure 6. Incomplete differentiation of trigeminal ganglia neurons of *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos. (a,b) Trigeminal ganglia in the coronal sections of E14.5 wild-type (a) and mutant (b) embryos. Note the presence of large neuronal nuclei and prominent Nissl substance in wild-type but not mutant embryos. (c–f) Expression of β II tubulin mRNA as detected by in situ hybridization. Coronal sections from E13.5 wild-type (c,e) and mutant (d,f) embryos are shown. (Arrow) trigeminal ganglia; (arrow-head), dorsal root ganglia; (m) Motor horn. Scale bar, 12.5 μ m (a,b).

genes have been shown to be involved in neuronal differentiation and cell type specification (He and Rosenfeld 1991). One member of the POU family, Brn-3.0, is expressed in sensory ganglion starting at E9.5, with peak expression by E12.5 (Gerrero et al. 1993). In situ hybridization using a specific oligonucleotide shows that the expression patterns of Brn-3.0 are similar in mutant (Fig. 7d,f) and wild-type embryos (Fig. 7c,e). Likewise, the expression of message for the neural cell adhesion molecule, NCAM, appears to be unchanged in the mutant (not shown). Thus, in addition to axonal growth, the expression of some developmentally regulated genes is not affected by the *Rb-1^{Δ20}* mutation.

As with the morphology, however, many molecular features of neuronal differentiation are either retarded or blocked in the mutant embryos. The β II isoform of tubulin is neuron specific and its expression normally increases dramatically during the early differentiation of most nerve cells. At E11.5 and E13.5, β II tubulin is expressed in the wild-type brain and spinal cord, as well as in the trigeminal and dorsal root ganglia (Fig. 6c,e). In the CNS of the mutant embryos, expression of β II tubulin was not obviously different from wild type. In trigeminal and dorsal root ganglia, however, levels of β II tubulin message decreased to nearly background levels (Fig. 6d,f).

The defects in the *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos are found throughout the length of the neuraxis, but they are most dramatic in the neurons of the peripheral ganglia. As these PNS centers are critically dependent on several of

the known neurotrophin molecules for survival, we sought to characterize the effects of the *Rb-1^{Δ20}* mutation on the expression of several neurotrophic factors and their receptors. The message levels for the growth factors themselves—nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3)—are not altered significantly in mutants (by in situ hybridization, not shown). On the other hand, expression of both the low-affinity ($p75^{\text{LNGFR}}$) and high-affinity (TrkA) NGF receptor are greatly reduced in sensory ganglia. At E12.5, $p75^{\text{LNGFR}}$ can be detected in the ventral horn of the spinal cord of both mutant (Fig. 8b,d) and wild-type (Fig. 8a,c) embryos. In the *Rb-1^{Δ20}/Rb-1^{Δ20}* mutants, however, specific hybridization is conspicuously missing in both trigeminal and dorsal root ganglia (Fig. 8 a–d), and this reduced expression persists through E13.5 (Fig. 8e,f) and E 15.5 (not shown). TrkA expression is also significantly reduced in *Rb-1^{Δ20}/Rb-1^{Δ20}* sensory ganglia at E13.5 and E14.5 (Fig. 9b,d) compared with the control embryos (Fig. 9a,c). For unknown reasons, significant TrkA hybridization is observed in the ventral horn of the spinal cord of the mutant (Fig. 9d), but not wild-type (Fig. 9c), embryos. The level of TrkB expression (the receptor for BDNF/NT-4) is also reduced in the mutant although the difference is not as dramatic as with TrkA and $p75^{\text{LNGFR}}$ (not shown). RNase protection assays of mRNA from the RB-deficient dorsal root ganglia confirmed the in situ hybridization results for TrkA, TrkB and $p75^{\text{LNGFR}}$ (not shown).

Figure 7. Axon extension from central and peripheral ganglia, and expression of Brn-3.0. *a,b*) Whole-mount immunohistochemistry of wild-type (*a*) and mutant, *Rb-1^{Δ20}/Rb-1^{Δ20}* embryo (*b*), using mAb 2H3 against the neurofilament 155-kD subunit. Photographs of the mutant embryo are made at two different focal planes. (dg) dorsal root ganglia; (h) hindbrain; (m) midbrain; (t) telencephalon. (*c,f*) Expression of Brn-3.0 in *Rb-1^{Δ20}/Rb-1^{Δ20}* and control embryos. Bright-field (*c,d*) and dark-field (*e,f*) photographs of sagittal sections from E12.5 embryos showing in situ hybridization with Brn-3.0 probe. (*c,e*) Wild-type embryos; (*d,f*) *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos. (g) Geniculate and acoustic-facialis ganglia; (n) nodose ganglia; (p) petrose ganglia; (t) trigeminal ganglia.

Survival and neurite formation of *Rb-1^{Δ20}/Rb-1^{Δ20}* neuronal cells in vitro

Normal development of a neuron depends on the proper expression of endogenous genes as well as the proper response to exogenous environmental factors. Using sensory ganglia as a model system, we addressed the question of whether death of the ganglionic neurons is a function of their *Rb-1^{Δ20}/Rb-1^{Δ20}* genotype or occurs instead as an indirect consequence of the failing microenvironment in advanced stage mutant embryos. Dissociated single-cell neuronal cultures were established as described in Materials and methods. Trigeminal ganglia cells, harvested at E11.5, survived well in culture for 24 hr in the presence of NGF. When harvested at E12.5 and afterwards, however, the trigeminal neurons showed much poorer survival (Fig. 10a) and a striking reduction in neurite formation (Fig. 10b). A similar picture was obtained with dorsal root ganglia cells. If E12.5 dorsal root ganglia cells were used as the source of the cell suspension, the neurons survived well for 24 hr (Fig. 10c), and their ability to grow neurites was indistinguishable from controls (Fig. 10d). However, if the cells were harvested 1 day later (E13.5), survival was reduced by 30% and the ability of the surviving cells to grow neurites was decreased by 70%. These results suggest two conclusions. First, until E12.5, the differentiation program of these ganglionic neurons is unaffected by the *Rb-1^{Δ20}/Rb-1^{Δ20}* mutation; this includes the ability of the neurons to extend axons. Second, after E13.5, not even the supportive environment of an NGF-supplemented culture can rescue the neurons or stimulate their maturation (neurite outgrowth). Thus, whereas early differentiation events seem to be unaffected, later events are either retarded or blocked.

Discussion

The *Rb-1* gene is usually ascribed a role in cell cycle regulation. Specifically, hypophosphorylated RB negatively regulates cell growth by blocking the cell cycle late in G_1 (for review, see Riley et al. 1994). Our analysis of mouse embryos lacking an intact copy of the *Rb-1* gene further substantiates this function of the protein. Yet, consideration of the entire syndrome of *Rb-1^{Δ20}/Rb-1^{Δ20}* mice suggests that RB may serve additional functions in the developing nervous system beyond its role in cell cycle arrest.

RB in cell cycle progression and cell cycle exit

The data described above support the conclusion that RB plays a major role in establishing the postmitotic state of many neuronal cells. BrdU labeling of *Rb-1^{Δ20}/Rb-1^{Δ20}* mutants shows that RB-deficient cells continue to enter S phase even if they are positioned outside of the ventricular zone. Whereas theoretically this could be attributable to premature migration of proliferative RB-deficient cells prior to their normal birthdays, we find this unlikely for three reasons. First, expression of other ner-

Figure 8. Lack of p75^{LNGFR} expression in sensory ganglia of *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos. In situ hybridization of p75^{LNGFR} in coronal sections of wild-type (a,c,e) and mutant (b,d,f) embryos taken from comparable axial levels. Arrows indicate trigeminal ganglia and arrowheads point to dorsal root ganglia. (dg) Dorsal root ganglia; (m) motor horn; (tg) trigeminal ganglia; (Dor) dorsal side.

vous system genes such as the neuronal adhesion molecule (N-CAM) and the POU family gene (*Brn-3.0*) appears to be normal in the mutant. Second, the number of proliferating neuroblasts (as revealed by BrdU labeling) is comparable in the ventricular zones of mutant and wild-type embryos. If there were premature emigration of proliferating neuroblasts in the mutant, this could deplete the precursor population. Third, at the stage when most spinal neurogenesis ceases in wild-type embryos, cell proliferation continues in mutant embryos not only in the intermediate zone but also in the ventricular zone (Fig. 3, cf. c and f). These observations argue strongly against the premature emigration of neuroblasts in the mutant. Taken together, our results suggest that in the absence of functional RB protein, cells in both the CNS and PNS continue to enter S phase temporally and spatially where they should not. Therefore, the demonstrated role of RB in cell cycle arrest *in vitro* also appears to apply *in vivo*.

Although the mechanisms of cell cycle regulation are similar in various organisms, the effect of RB deficiency during normal mammalian embryogenesis appears to be

unique to the nervous and hematopoietic systems. It is possible that defects in other tissues would become evident if the embryos were able to survive longer. However, no obvious abnormalities have been found in organs whose development precedes the nervous system. For example, the complex morphogenetic events that form the limbs and sculpt the digits on the paws are not disturbed in the mutants. Thus, it is plausible that RB protein is uniquely important to cell cycle regulation in the nervous system.

RB and neuronal differentiation

It has been demonstrated that elevated levels of RB protein corresponds to neuroectoderm but not endoderm differentiation in embryonic carcinoma cells (Slack et al. 1993). Similarly, *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos demonstrate the specific role of RB in neuronal differentiation. Based on morphological, biochemical, and developmental genetic criteria, the maturation of several neuronal cell types is either deficient or blocked. We can use the trigeminal ganglia of the PNS as an example. These ganglia

Figure 9. Lack of TrkA expression in sensory ganglia of *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos. In situ hybridization of *trkA* in the corresponding coronal sections of wild-type (*a,c*), and mutant (*b,d*) embryos. Abbreviations as in Fig. 8.

are some of the first neuronal structures to differentiate in the vertebrate nervous system. The neurons send out axons that arrive in their peripheral target fields between E9.5 and E13 (Davies and Lumsden 1984). Two waves of neurogenesis have been described, one before E9 and one between E10 and E12. At the end of the second wave, the ganglion has attained its maximum cell number of ~40,000. This is followed by a period of naturally occurring cell death during which the number of trigeminal neurons is reduced by approximately one-half (Davies and Lumsden 1984). Cells in RB-deficient embryos appear to successfully complete much of this differentiation program. Ganglion cells are formed and migrate properly to form a normal sized ganglion. Monoclonal antibodies reveal the expected presence of both central and peripheral nerve branches, and (if harvested before E11.5) neurons in single cell cultures are able to survive and extend neurites. At about this stage, however, development in the mutant appears to deteriorate. Cytological maturation is blocked; the cells remain small and the Nissl substance remains rudimentary in the mutant. In addition, several neuronal markers such as β II-tubulin are poorly expressed. N-CAM and Brn-3.0 expression is not altered significantly, and so the lack of differentiation may reflect the aberrant expression of a particular subset of genes.

Expression of neurotrophins and their receptors in RB-deficient embryos

The altered expression patterns of the neurotrophin receptors may provide a partial explanation for the RB mutant phenotypes. The neurotrophins are a family of closely related proteins that play important roles in the development and maintenance of the nervous system. There are characteristic distributions of neurotrophins and their receptors, and the interactions among them appear to be involved in proliferation (Cattaneo and

McKay 1990; Klein et al. 1991a,b), neuronal maturation (Wyatt et al. 1990; Wright et al. 1992; Ip et al. 1993), and maintaining neuronal survival (Barde 1989). These are precisely the phenotypes that are deficient in the *Rb-1^{Δ20}/Rb-1^{Δ20}* cells.

NGF, the first neurotrophin to be discovered, has been shown to be vital for growth, differentiation, and survival of sympathetic and neural crest-derived sensory neurons (Levi-Montalcini 1987). The activities of neurotrophins are mediated by cell-surface receptors (Chao 1992; Meakin and Shooter 1992; Barbacid 1993). The TrkA gene product is the high-affinity receptor for NGF (Kaplan et al. 1991; Klein et al. 1991a) and TrkB receptor for BDNF and NT-4/5 (Klein et al. 1991b). Function of the low-affinity NGF receptor, p75^{NGFR}, and its role in transduction of the neurotrophin-mediated signal cascade is less clear (Johnson et al. 1986; Radeke et al. 1987). Despite its name, its binding properties make it a poor discriminator among the neurotrophins, and, in contrast to other neurotrophin receptors, it contains no cytoplasmic tyrosine-kinase domain. Nonetheless, in p75^{NGFR} null mice, there are deficiencies in sensory responses (K.F. Lee et al. 1992). Optimal growth of p75^{NGFR}-deficient neurons requires a higher concentration of NGF (Davis et al. 1993; Lee et al. 1994).

The expression patterns of the neurotrophins themselves are not significantly altered in the *Rb-1^{Δ20}/Rb-1^{Δ20}* mutant. In contrast, there is a significant reduction in the expression of p75^{NGFR}, the low-affinity NGF receptor, and in TrkA, the high-affinity NGF receptor. The tissue culture data complement this picture. At E12.5 there is no difference in survival and neurite formation between mutant and wild-type dorsal root ganglion cells in vitro. At E13.5, when the dependency of sensory neurons on NGF becomes apparent, there is an ~30% reduction in survival and 70% reduction in neurite formation in cultured mutant dorsal root ganglia cells. Likewise, the survival of cultured trigeminal neurons and

Figure 10. Survival and differentiation of trigeminal and dorsal root ganglia neuronal cells from wild-type and *Rb-1 Δ 20/Rb-1 Δ 20* embryos in the presence of NGF. Charts are shown of surviving wild-type neurons (hatched bars) and neurons from RB-deficient embryos (stippled bars) after 24 hr incubation with NGF (50 ng/ml). The results of typical experiments set up from E11.5–E14.5 trigeminal ganglia are shown. (a) Survival of trigeminal neurons; (b) neurite extension of trigeminal neurons. Similarly, dorsal root ganglia cells from E12.5 and E13.5 embryos were compared. (c) Survival of dorsal root ganglia cells; (d) neurite extension of dorsal root ganglia cells. The mean and standard error of the neuron survival counts and differentiating neurons in duplicate counts are represented.

their ability to grow neurites is lost at about the same ages as trigeminal neurons in vivo become sensitive to neurotrophins. Given these data, it seems possible that the lack of differentiation and the elevated neuronal cell death seen in sensory ganglia of RB-deficient embryos is at least partially, if not wholly, explained by the lack of TrkA and p75^{LNGFR} proteins. If this is true, then the RB protein might be important in the establishment of the complete neurotrophin-mediated signal transduction pathway.

RB and cell survival during nervous system development

That RB should have a role in cell survival is a curious discovery. Cell death is common during CNS development. Approximately half of all neurons generated dur-

ing development of the mammalian nervous system die (Cowan et al. 1984; for review, see Oppenheim 1991). Recently, several examples of programmed cell death have been hypothesized to result from loss of cell cycle control (Freeman et al. 1994; Meikrantz et al. 1994), and transgenic mouse experiments in which SV40 T-antigen is ectopically expressed in mature neurons of the CNS directly implicate RB in this process (al-Ubaidi et al. 1992; Feddersen et al. 1992). In the absence of RB, control of expression of a series of genes associated with the cell cycle and positively regulated by the E2F transcriptional factor would be lost; dihydrofolate reductase, thymidine kinase, thymidine synthesis, and others should be activated (Nevin 1992). Our pulse-labeling experiments demonstrate that *Rb-1 Δ 20/Rb-1 Δ 20* cells became apoptotic shortly after they enter S phase. It is conceivable that in the absence of RB protein, early events for S-phase entry are initiated and an "alternate effector

pathway" (Heintz 1993) or conflicting signals in the RB-deficient cells may then lead to cell death.

The findings presented here expand the picture of the function of RB in the developing organism. Our data support the conclusion that the RB gene product is a key player in the regulation of the cell cycle in the embryonic nervous system. The results illustrate clearly, however, that the loss of RB, in and of itself, does not lead to continuously uncontrolled cell division in any tissue. Development of various nervous structures in the mutant proceeds normally to a critical period of embryogenesis, after which further morphological development is blocked and cell death ensues. Preferential reduction in the expression of several neurotrophin receptor genes in the mutant may explain all or part of the phenotype in the peripheral nervous system. Taken together, our data support the hypothesis that RB plays multiple roles in neuronal development. It appears to be essential for the proper biochemical and morphological development of the young neurons as well as for ensuring their permanent exit from the cell cycle.

Materials and methods

Animals

Embryonic stem cells used in the targeting of *Rb-1* gene were derived from an agouti 129/SvEv mouse (E.Y.-H.P. Lee et al. 1992). Outbred progeny from crosses with C57BL/6 were used for studies described here. All embryos were acquired from the mating of heterozygous *Rb-1*^{Δ20/+} parents. Genetically wild-type or heterozygous mice were used interchangeably because our studies reveal no obvious phenotypic difference in their development.

Western blotting analysis

Timed pregnancies of wild-type mice were established. At each indicated time point, pregnant dams were sacrificed, the fetuses were removed from their amniotic sacs, and the embryonic head or brains were dissected and frozen in liquid nitrogen. Frozen tissues were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 200 U/ml 1 of aprotinin, 50 μg/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Monoclonal antibody 3C8 (Canji, La Jolla) and mAb 245 were used in Western blotting as described previously (Lee et al. 1992). RbAp48 is a cellular protein that associates with RB (Qian et al. 1993), and Gβ-like antibody recognizes a 26-kD cellular Gβ-like protein (Shan et al. 1992).

FACS analysis

Hindbrain and cervical spinal cord were removed from E13.5 embryos. Tissues were suspended, triturated in PBS, and filtered through a 53-μm nylon mesh. The 200g centrifuged cell pellet was fixed in cold 70% ethanol at 4°C for 40 min. The cells were then centrifuged, washed with PBS, treated with RNase for 5 min, followed by addition of propidium iodide according to Nicoletti et al. (1991). The propidium iodide fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA).

BrdU labeling and immunohistochemistry

Timed pregnant mice at 10.5, 12.5, 13.5, and 14.5 days of gestation were injected intraperitoneally with BrdU (Sigma) at a

dose of 100 mg/kg body weight as described (Miller and Nowakowski 1988). At different time intervals (1, 2, 3, 5, 12, 24, and 48 hr) after injection, pregnant mice were sacrificed and their embryos immediately fixed in Bouin's solution for 24 hr followed by dehydration and embedding in paraffin. Coronal sections of 4–6 μm were prepared. After deparaffinization and rehydration, sections were treated with 200 ml of TBS containing 0.1% sodium azide and 0.1% H₂O₂ for 30 min, washed in TBS, and incubated with anti-BrdU antibody (1:50, Becton and Dickinson, CA) overnight at 4°C. An ABC staining procedure (1:100, ABC kit, Vector) was performed, as per the manufacturer's instructions, using a biotinylated secondary antibody (1:100). Finally, sections were counterstained with hematoxylin, dehydrated, cleared, and mounted for light microscopic analysis.

Identification of apoptotic cells in situ

The TUNEL technique essentially follows the protocol of Gavrieli et al. (1992). Tissue samples were fixed in 4% paraformaldehyde solution and embedded in paraplast, and sections were prepared for labeling.

Genomic DNA extraction and 3' end-labeling of DNA

Dorsal root and trigeminal ganglia were dissected from E13.5 mouse embryos, the constituent cells were lysed in lysis buffer (10 mM Tris at pH 8.0, 1 mM EDTA, 0.2% SDS, 0.1 M NaCl, 0.5 mg/ml of proteinase K), and genomic DNA was extracted. For 3' end-labeling, 500 ng of DNA was incubated with 20 μCi of [α -³²P]ddATP (3000 Ci/mmol; Amersham), and 24 units of terminal deoxynucleotidyl transferase (24 U/μl, IBI) in a total volume of 42 μl. The reaction was carried out at 37°C for 60 min and terminated by the addition of 5 μl of 0.25 M EDTA (Tilly and Hsueh 1993). Unincorporated radionucleotide was removed by ethanol precipitation. One-fifth of the labeled DNA sample was separated on a 1.8% agarose gel followed by transfer to a nylon membrane. An autoradiograph of the transfer was obtained by exposure to a Kodak X-ray film at –70°C for 3 hr.

In situ hybridization

Embryos at various stages were placed in ice-cold, freshly prepared 4% paraformaldehyde in PBS overnight. Paraffin sections were prepared following successive dehydration and embedding of embryos in paraffin. In situ hybridization was performed according to Cox et al. (1984). For TrkA, TrkB, TrkC, and N-CAM, single-stranded anti-sense RNA probes were generated using [³²P]UTP and T7 or SP6 RNA polymerase following the manufacturer's instructions (Promega). About 5 × 10⁷ cpm of [³²P]-UTP-labeled RNA probes of ~300 bp length were used for hybridization. Sections were covered with NB-2 photo emulsion (1:1, Kodak) and exposed for 3–6 weeks at 4°C. Sections were developed, fixed, and counterstained with toluidene blue (Cox et al. 1984). For p75^{LNGFR}, NGF, BDNF, NT-3, and neuronal βII tubulin, 50-mer oligonucleotides probes were labeled at their 3' end with [α -³²P]dATP and TdT to a specific activity of ~10⁹ cpm/μg and purified through a NENsorb column (Du Pont). The following anti-sense oligonucleotides, derived from the species and corresponding to nucleotide positions (indicated in parenthesis), were used. Rat p75^{LNGFR} (867–916; Radeke et al. 1987): 5'-ACAAGGCCACGACCACAGCAGCAGCCAAGATGG-AGCAATAGACAGGAATGAG-3'. Mouse neuronal βII tubulin (3'-untranslated region; Lewis et al. 1985): 5'-TGGAGGACAACAGAAGTTCACCTAAGGGTGCACACTGTATCTGAG-AAGTTC-3'. Mouse Brn-3.0 (1207–1257; Gerrero et al. 1993):

5'-CTTCTTCTCGCCGCCGTTGAAGAGCTCCGGCTTGTTCATTTTCTCACGTTG-3'.

For oligonucleotide probes, hybridization was carried out at 42°C overnight, followed by washing in 0.1×SSC at 40°C.

Whole-mount immunostaining of mouse embryos

Procedures essentially followed Mark et al. (1993). 2H3 is a monoclonal antibody against the 155-kD neurofilament protein and was obtained from the Developmental Studies, Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD) and the Department of Biological Sciences, University of Iowa (Iowa City, IA), under contract N01-HD-2-3144 from the National Institute of Child Health and Human Development.

Neuronal cell culture

Dorsal root and trigeminal ganglia were dissected from E11.5, E12.5, E13.5, and E14.5 mouse embryos. Ganglia were treated with 0.25% trypsin-EDTA for ~10 min, tissues were then triturated and washed. Cells were plated onto laminin (15 µg/µl, GIBCO)-coated 24-well cell culture clusters (Costar) at 4×10^4 cells/well for dorsal root ganglia and 2×10^4 cells/well for trigeminal ganglia in OPTI-MEM reduced-serum medium (GIBCO) supplemented with N-2 (GIBCO), NGF (50 ng/ml; UBI), 0.6% glucose, ampicillin, and streptomycin. Neuron survival and neurite formation were compared 6, 24, and 72 hr after plating using a phase contrast microscope.

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