Loss of p27Kip1 function results in increased proliferative capacity of oligodendrocyte progenitors but unaltered timing of differentiation

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SUMMARY

In many tissues, progenitor cells permanently withdraw from the cell cycle prior to commitment towards a differentiated phenotype. In the oligodendrocyte lineage a counting mechanism has been proposed, linking the number of cell divisions to growth arrest and differentiation. A direct prediction of this model is that an increase in the number of cell divisions would result in a delayed onset of differentiation. Since the cell cycle inhibitor p27Kip1 is an essential component of the machinery leading to oligodendrocyte progenitor growth arrest, we examined the temporal relationship between cell cycle withdrawal and expression of late differentiation markers in vivo, in mice carrying a targeted deletion in the p27Kip1 gene. Using bromodeoxyuridine to label proliferating cells, quaking (QKI) to identify embryonic glial progenitors, NG2 to identify neonatal oligodendrocyte progenitors, and myelin basic protein to label differentiated oligodendrocytes, we found an increased number of proliferating QKI- and NG2-positive cells in germinal zones of p27Kip1−/− mice at the peak of gliogenesis. However, no delay was observed in these mice in the appearance of the late differentiation marker myelin basic protein in the developing corpus callosum and cerebellum. Significantly, a decrease in cyclin E levels was observed in the brain of p27Kip1 null mice coincident with oligodendrocyte growth arrest. We conclude that two distinct modalities of growth arrest occur in the oligodendrocyte lineage: a p27Kip1-dependent mechanism of growth arrest affecting proliferation in early phases of gliogenesis, and a p27Kip1-independent event leading to withdrawal from the cell cycle and differentiation.

Key words: Development, CNS, Cell cycle, p27Kip1, Oligodendrocyte, Mouse

INTRODUCTION

A crucial issue in the development of the central nervous system is the appropriate spatial and temporal generation of the correct number of neuronal and glial cells. This is presumably achieved by a mechanism of regulation linking cell division to initiation of differentiation (Zavitz and Zipursky, 1997). For instance, the number of cell cycles a progenitor cell undergoes prior to growth arrest is a crucial parameter in determining cell number (Raff, 1996). Progenitor pool size may be regulated by cell intrinsic mechanisms modulated by secreted factors and contact with other cells and with the extracellular matrix (Edgar and Pfeiffer, 1985; Wang et al., 1998; Wood and Bunge, 1986). Signalling pathways converge at the restriction point of the G1/S transition of the cell cycle, thereby affecting the decision of a progenitor cell to proliferate or arrest growth. A key mechanism to account for this decision is the ability of cell cycle inhibitory proteins to regulate the activity of cyclin-dependent kinases (Sherr and Roberts, 1995).

The relationship between cell cycle control and differentiation in neural progenitor cells is not completely understood although studies on cells of the oligodendrocyte lineage have begun to address these questions. Oligodendrocytes arise from multipotential progenitors of the proliferative ventricular zones of the nascent brain and spinal cord, beginning in mid-late gestation (Hardy, 1997; Miller, 1996; Nornes and Carry, 1978; Richardson et al., 1997; Timsit et al., 1995). These cells continue to divide as they migrate (Hardy and Reynolds, 1991; Reynolds and Wilkin, 1991) and begin to acquire distinct antigenic patterns characteristic of well defined developmental stages (Gard and Pfeiffer, 1990). Differentiation proceeds within a defined temporal window and is characterized by expression of specific markers, such as myelin basic protein (MBP) (Blaschuck and ffrench-Constant, 1998).
In vitro experiments on oligodendrocyte progenitors isolated from the optic nerve and cultured in the presence of mitogens have indicated that the timing of differentiation is determined by a defined number of cell divisions (Temple and Raff, 1986). These experiments have led to the hypothesis that a counting mechanism determines cell cycle withdrawal and initiation of differentiation after a fixed number of cell divisions.

Several recent studies have indicated that such an intrinsic mechanism is dependent upon the cell cycle inhibitor p27Kip1 (Durand et al., 1997; Gao and Raff, 1997), a member of the Kip that includes p21waf1 and p57Kip2. The main role of these molecules is to modulate entry into S-phase, a decision which is positively regulated by cyclins, the regulatory subunits of the cyclin dependent kinases (CDKs). In particular, p27Kip1 acts at the G1/S transition by stoichiometrically inhibiting the enzymatic activity of cyclinE/CDK2 complexes (Sherr and Roberts, 1995). Consistent with this mechanism, targeted deletion of p27Kip1 results in increased proliferation and multiorgan hyperplasia, including brain (Fero et al., 1996; Kiyokawa et al., 1994; Nakayama et al., 1996). In order to address the effects of loss of p27Kip1 in oligodendrocyte development, we previously conducted in vitro experiments on cortical progenitors isolated from p27Kip1 null mice and showed marked misregulation of growth arrest (Casaccia-Bonnefil et al., 1997). Similar observations were obtained in oligodendrocyte progenitors isolated from the optic nerve (Durand et al., 1998).

While these in vitro studies characterized an experimental system for the study of the mechanisms of proliferation and growth arrest, they precluded the analysis of the complex environmental regulation which affects oligodendrocyte progenitors in vivo. This is of particular importance since many studies have shown that neuronal influences, including neurotransmitters and electrical activity and contact with the extracellular matrix play a crucial role in oligodendrocyte development (Wood and Bunge, 1986; Barres and Raff, 1993; Demerens et al., 1996; Gallo and Russel, 1995; Hardy and Reynolds, 1993; Jacques et al., 1998; Yuan et al., 1998; Ghiani et al., 1999).

In this study we have analyzed the in vivo proliferative potential and timing of differentiation of early glial progenitors, oligodendrocyte progenitors and mature oligodendrocytes in mice lacking functional p27Kip1 (Kiyokawa et al., 1996). A major aim of this study was to determine whether the absence of functional p27Kip1 leads to inhibition of growth arrest in oligodendrocyte progenitors in vivo and whether increased proliferative capacity affects oligodendrocyte differentiation. If growth arrest is the driving force for developmental maturation of oligodendrocyte progenitors in vivo, as suggested by the in vitro counting clock model, then inhibition of cell cycle withdrawal would result in delayed oligodendrocyte differentiation. The results presented here indicate that functional ablation of p27Kip1 results in expansion of the oligodendrocyte progenitor pool due to increased proliferation in early stages of gliogenesis. However, downregulation of cyclin E levels in the brain of p27Kip1 null mice may be responsible for resetting the proliferative potential to wild-type levels. This suggests that in the central nervous system, oligodendrocyte progenitor proliferation, final growth arrest and differentiation are independently regulated events.

**MATERIALS AND METHODS**

**Antibodies**

Antibodies against CDK2 (SC-163), CDK4 (SC-601), p16 (SC-468), p19 (SC-1063), p57 (SC-1039), cyclin D1 (SC-6281), cyclin D2 (SC-593), cyclin E (SC-481) were all from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against p21 was purchased from Pharmingen (San Diego, CA). Rabbit anti-QKI anti-serum was generated against a GST fusion protein consisting of the C-terminal portion of the QKI proteins common to all 3 known isoforms (Hardy et al., 1996). Rabbit anti-Ng2 anti-serum was raised against the chondroitin sulfate proteoglycan NG2 and affinity purified (Levine and Stallcup, 1987). Mouse anti-BrdU and mouse anti-MBP monoclonal antibodies were purchased from Dako (Carpinteria, CA) and Boehringer Mannheim (GmbH, Germany) respectively. Rabbit anti-MBP anti-serum was a gift from Dr. D. Colman (Mount Sinai Medical Center, New York, NY). Biotin- or fluorochrome-conjugated secondary antibodies were purchased from Amersham (Arlington Heights, IL), Jackson ImmunoResearch Laboratories, Inc.(West Grove, PA), and Southern Biotechnology Associates, Inc.(Birmingham, AL).

**Mice genotyping**

Null and wild-type mice were obtained from heterozygous p27Kip1 breeding pairs (C57black/SJL) (Kiyokawa et al., 1996). The genotype was determined by PCR analysis of genomic tail DNA. Briefly, tails were digested in lysis buffer (100 mM NaCI, 10 mM Tris, pH 8.0, 0.5% SDS, 25 mM EDTA, 148 μg/ml protease K stock) for 14-18 hours at 55°C in a water bath. Genomic DNA was isolated and amplified by PCR using the following primers: 5'-CGCCCCGACCTGACTCTGC-3' and 5'-TCAACGTCAGTCAGTCAT-3' directed against the p27Kip1 gene and 5'-AGGCCTTATGATTCTGAAAGTCG-3' corresponding to the neo sequence. After a 35 cycle reaction (denaturation at 94°C for 45 seconds, annealing at 62°C for 1 minute, extension at 72°C for 1 minute) the wild-type allele yielded a 210 bp PCR product, and the null allele a longer 290 bp product, due to neo insertion in the first exon.

**BrdU labelling and immunofluorescence**

Heterozygous pregnant females (E12, E13, E15, E17, E19) or neonatal p27kip1+/− and wild-type littersmates (P1, P6, P16) received two injections of BrdU (100 μg/g body weight) at 1-hour intervals. One hour after the last injection, animals were anesthetized with avertin and perfused with 4% paraformaldehyde in phosphate buffer. For quantitation of the number of MBP+ cells additional time points (P1, P4, P6, P8, P10, P16) were evaluated. Upon overnight post-fixation at 4°C in the same fixative, the tissue was incubated for 24 hours in 30% sucrose in 0.1 M phosphate buffer (pH 7.5) and then embedded in OCT and frozen in cryomolds in a 8-methylbutane bath on dry ice. Cryosections (10-15 μm thickness) were collected on Superfast glass slides and processed for immunofluorescence. Frozen sections were thawed and incubated for 1 hour in blocking solution (PGBA: 0.1 M phosphate buffer, 0.1% gelatin, 1% bovine serum albumin, 0.002% sodium azide) with 10% normal goat serum (NGS, from Vector). For MBP staining, sections were permeabilized in ice cold methanol for 10 minutes. Primary antibody was typically incubated overnight (NG2 1:1000 in PGBA with 10% NGS: QKI 1:500; MBP rabbit polyclonal 1:1000; MBP monoclonal IgG1 1:100 in PGBA containing 10% NGS and 0.3% Triton X-100), followed by biotinylated secondary antibody for 1 hour at room temperature. For immunolabelling with anti-BrdU antibodies, sections were then treated with 2 N HCl for 10 minutes at room temperature, in order to denature DNA, followed by 0.1 M sodium borate, pH 8.6 for 10 minutes. Primary antibody anti-BrdU was used at 1:100 dilution in 0.1 M carbonate buffer, 0.1% gelatin, 1% bovine serum albumin, 0.002% sodium azide, 0.1% Triton X-100 for at least 3 hours at room temperature, followed by fluorochrome-conjugated secondary
antiserum. Sections were counterstained with DAPI (Molecular Probes, Eugene, OR), in order to visualize cell nuclei. Photomicrographs were obtained using a Nikon Epson fluorescence microscope or analyzed with a Leica TCS confocal microscope. Double labelling was confirmed by scanning individual cells along the z-axis.

Quantitation

Quantitation of immunolabelled cells in the subcortical white matter, cerebellum and spinal cord was performed using a 40× immersion oil objective in a Leica TCS confocal microscope and a 40× lens in the Nikon Epson microscope. For quantitative analysis of glial progenitors in the embryonic spinal cord, QKI immunoreactive cells migrating from the ventricular zone were counted. Proliferating glial progenitors were identified by QKI+/BrdU+ double labelling. The number of double labelled cells was normalized to the total number of migratory QKI+ cells, which are identified as glial progenitors migrating from the ventricular zone. For quantitative analysis of proliferating oligodendrocyte progenitors in the neonatal cerebellum, the number of cells displaying NG2 labelling and BrdU nuclear immunoreactivity within a fixed area within each cerebellar folium was counted and then normalized to the total number of NG2-labelled cells. The total number of NG2-positive cells in the cerebellum was quantitated per 0.3 mm² of surface area using a 20× objective. For MBP+ cells in the cerebellum, the quantitation was performed by analyzing the same region of the cerebellum in wild-type and knockout mice at different time points. No staining was observed at postnatal day 1. Since the localization of MBP+ cell bodies varies with age in the developing animal, we counted the number of cells in deep cerebellar regions at postnatal day 4, in the folia at postnatal day 6 and at the end of the cerebellar folia at postnatal day 8. At postnatal day 10 and 16 it was difficult to identify any MBP+ cell bodies since the immunoreactivity was totally localized to the myelinated fibers. MBP+ cells were also quantitated in the forebrain at postnatal day 6, by analyzing the number of immunoreactive somas in a 1.8 mm² surface area close to the fimbria. Experiments were performed on sections obtained from wild-type and p27Kip1 null littermates, from at least two different pregnant heterozygous females. On average, 3-9 sections per animal were quantitated and 3 mice from each genotype were assayed at each time point. A two-tailed t-test was used to evaluate statistical significance.

Tissue extracts for biochemical analysis

Mice were killed by cervical dislocation and the brains dissected in PBS. After careful removal of the meninges, cortices and cerebella were isolated. The tissues were snap frozen in liquid nitrogen and stored at −80°C. Tissue lysates were obtained by direct sonication, on ice, of the frozen samples in mild lysis buffer (120 mM NaCl, 50 mM Tris pH 7.4, 0.5% NP-40) containing protease inhibitors (1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mM PMSF) and phosphatase inhibitors (1 mM vanadate, 20 mM sodium fluoride, 1 mM sodium pyrophosphate). Equal amounts (100 µg) of lysates were then analyzed by western blot analysis. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies followed by chemiluminescence. The blots were scanned and the relative decrease of cyclin E protein levels was quantitated by Image Quant (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Early glial progenitors display enhanced proliferation in the absence of p27Kip1

Glia cells arise during late embryogenesis and early postnatal development from proliferating neural progenitors residing in the ventricular zone of the nascent central nervous system. In the spinal cord, oligodendrocyte progenitors are generated from specific subdomains of the ventricular zone located in the ventral portion of the cord (Hardy, 1997; Pringle et al., 1996, 1998). Cells destined for a glial fate migrate away from the ventricular zone into the surrounding

Fig. 1. Increased number of migratory QKI proliferating cells in the embryonic spinal cord of p27Kip1 null mice. (A) Confocal images of QKI/BrdU double immunoreactivity in the embryonic day 15 (E15) spinal cord of wild-type (+/+) and p27Kip1 null (−/−) mice. Green immunofluorescence indicates immunoreactivity to QKI antiserum and red immunofluorescence labels the nuclei of proliferating cells incorporating bromodeoxyuridine. Note that in the p27Kip1 null mouse there are several more proliferating cells within the ventricular zone and in the region immediately adjacent to it. Scale bar, 70 µm. (B) Quantitation of the percentage of proliferating migratory QKI+ cells in the ventral spinal cord. QKI+/BrdU+ double labelled cells in the ventral spinal cord were from three animals from different litters for each genotype. The error bars represent the s.e.m. The difference in number of migratory QKI+ cells between wild-type and knockout mice begins at E13 and persists until day E17 (P=0.002).
parenchyma, where they continue to divide under the influence of multiple extracellular signals.

In order to identify glial progenitors, we used the quaking gene product (QKI) as a marker. The QKI family of proteins are expressed in multipotent progenitors residing in the ventricular zone and also in newly generated glial cells as they migrate away from specific subdomains of the embryonic spinal cord. In contrast, newly generated neurons dramatically down-regulate QKI expression as they leave the ventricular zone (Hardy, 1998a). In order to determine the effect of functional loss of p27Kip1 on early glial progenitor cells, we have studied the proliferative capacity of QKI+ cells as they emerge from the ventral ventricular zone of the spinal cord in wild-type and p27Kip1−/− mice. Pregnant mice at different stages of gestation (E12, E13, E15, E17, E19) were injected with bromodeoxyuridine (BrdU) to label embryonic cells undergoing S-phase. Wild-type and p27Kip1−/− mice embryos were harvested and processed for immunolabelling 2 hours later. The total number of QKI+/BrdU+ cells within the ventricular zone and the proportion of QKI+/BrdU+ migratory cells were immediately determined (Fig. 1A and B).

At E12, QKI+ cells are restricted to the ventricular zone and at E13 very few QKI+ glial progenitors have begun to emerge from the ventral ventricular zone of the spinal cord (Hardy, 1998a). At this stage, an increased proportion of double labelled QKI+/BrdU+ was observed in the ventricular zone of p27Kip1−/− mice compared to littermates controls (Fig. 1A). Time course analysis indicated that between E13 to E17, the highest difference between wild-type and p27Kip1−/− mice was observed at E17 (Fig. 1B). The latter observation is consistent with the apparent slow down of the cell cycle previously described in oligodendrocyte progenitors isolated from the embryonic spinal cord (Calver et al., 1998). By E19, however the percentage of proliferating QKI+ cells in p27Kip1−/− mice had returned to wild-type levels, suggesting the existence of compensatory mechanisms operational between E17 and E19 (Fig. 1B).

Consistent with the increased percentage of QKI+/BrdU+ cells from E13 to E17, a significantly higher number of QKI+ migratory cells was found at E15, E17 and E19 in p27Kip1−/− ventral spinal cord compared to wild type (Fig. 2).

These data indicate that p27Kip1 regulates proliferation of glial progenitors in the ventral spinal cord during early stages of gliogenesis and suggest that a p27Kip1-independent event can functionally compensate for loss of p27Kip1 and eventually lead to growth arrest during the later stages of gliogenesis, preceding differentiation.

**Oligodendrocyte progenitor cells display enhanced proliferation in the absence of p27Kip1**

As loss of function of p27Kip1 leads to increased proliferation of early embryonic glial progenitors, we were interested to determine whether neonatal oligodendrocyte progenitors were also affected by the absence of p27Kip1. During early postnatal development, forebrain oligodendrocytes are generated from progenitor cells in the subventricular zone surrounding the lateral ventricles (Goldman et al., 1997; Levison et al., 1993; Levison and Goldman, 1993; Luskin and McDermott, 1994). Oligodendrocytes in the cerebellum are also generated postnatally from the neural progenitors that surround the fourth

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**Fig. 2.** Increased number of QKI+ cells in the embryonic spinal cord of p27Kip1 null mice. Quantitation of the total number of QKI+ cells in the spinal cord at different stages of development. Increased in number of QKI+ cells begins to be observed at E15 and progressively increases up to E19. Values represent the mean of at least four experiments from two animals of each genotype. The error bars represent the s.e.m. The difference in total number of QKI+ cells between wild-type and knockout mice is highly significant at E17 (P<0.0001), and day E19 (P<0.0001).

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**Fig. 3.** Increased number of proliferating NG2 cells in the subcortical white matter. Double immunohistochemistry of the subventricular zone of neonatal wild-type (A,B) and p27Kip1−/− (C,D) mice. Green immunofluorescence labels NG2-positive progenitors, while red immunofluorescence indicates BrdU labelling. B and D are high power views of the fields indicated by an arrow in A and C. An example of double labelled BrdU/NG2+ cell is shown in D. (v, lateral ventricle; cc, corpus callosum). Scale bar, 25 μm in A and C and 10 μm in B and D.
ventricle (Reynolds and Wilkin, 1988). These cells migrate away from the germinal zones into the subcortical white matter and cortex (Levison et al., 1993; Zerlin et al., 1995), or into the cerebellar folia via the superior medullary velum and the cerebellar peduncles (Curtis et al., 1988; Reynolds and Wilkin, 1988; Zhang and Goldman, 1996). These neonatal oligodendrocyte progenitors can be specifically identified in sections of mouse brain using an antibody which recognizes the surface chondroitin sulfate proteoglycan NG2 (Levine and Stallcup, 1987; Levine et al., 1993).

In order to determine the effect of p27Kip1 on neonatal oligodendrocyte progenitors, we studied the proliferative index of NG2+ cells in wild-type and p27Kip1−/− mice, by analyzing the number of NG2+/BrdU+ cells in both forebrain (Fig. 3) and in the cerebellum (Fig. 4A-F). We found that in the forebrain at P1, there was a dramatic increase in the proliferative index of NG2+ cells found in the subcortical white matter in p27Kip1−/− mice compared to wild-type littermates (12% vs 3%, data not shown). A confocal image representative of the increased number of NG2+/BrdU+ cells in the subcortical white matter is illustrated in Fig. 3.

Similarly, in the P1 cerebellum, we found a significant increase in the percentage of NG2+ cells which had incorporated BrdU in p27Kip1−/− mice compared to wild type (Fig. 4G). The increased proliferative potential of NG2+ cells resulted in increased number of cells by day 6 in the cerebellar folia (x=192.5±27, n=4 for wild type and x=476±56, n=4 for p27Kip−/−). The average number of NG2+cells/ 0.3 mm² in wild-type littermates was x=31±12 and x=73±24 in p27Kip1−/− mice. By postnatal day 16, however, the proliferative index of NG2+ oligodendrocyte progenitors in p27Kip1−/− mice had decreased to wild-type levels (Fig. 4G). These data suggest that loss of p27Kip1 function affects oligodendrocyte progenitor proliferation during early neonatal gliogenesis and that additional mechanisms independent of p27Kip1 function may be responsible for the final withdrawal from the cell cycle, as observed in embryonic gliogenesis.

Enhanced proliferation of oligodendrocyte progenitors does not impair differentiation

Studies on cultured oligodendrocyte progenitors isolated from cerebral cortices (Casaccia-Bonnefil et al., 1997) or optic nerves (Durand et al., 1998) of p27Kip1−/− mice, have indicated that loss of p27Kip1 alters the differentiation process in vitro. If the number of cell divisions in the oligodendrocyte lineage is tightly coupled to the initiation of the differentiation program, then enhanced proliferation, as observed in the absence of functional p27Kip1 would result in impaired timing of oligodendrocyte differentiation. In order to address this issue in vivo, we compared the time of appearance of myelin basic protein (MBP) in the developing corpus callosum (Fig. 5) and the cerebellum (Fig. 6), of postnatal wild-type and p27Kip1−/− littersmates.

Previous studies on neonatal rats have indicated that MBP+ oligodendrocytes first appear at postnatal day 7 in the subcortical white matter of the developing corpus callosum, lateral to the subventricular zone (Zerlin et al., 1995). For this reason we examined brain sections from postnatal day 6 mice for the presence of MBP immunolabelling. By postnatal day 6 however, the first MBP+ oligodendrocytes could be identified in the most lateral part of the developing corpus callosum (Fig. 5).
5) of wild-type and knockout mice consistent with the time course of appearance in rat, as previously reported (Zerlin et al., 1995).

Since studies in cultured oligodendrocytes have indicated that the first oligodendrocytes appear asynchronously (Ibarrola et al., 1996), while those generated at later times develop more synchronously (Gao et al., 1998), we analyzed the timing of appearance and the number of MBP+ cells in the developing cerebellum at several time points after birth. In control mice, MBP+ cells were not detected in the cerebellum at postnatal day 1 (Fig. 6). By postnatal day 4, however the first MBP+ cell bodies could be identified at the base of the cerebellum in a region immediately adjacent to the superior medullary velum (Fig. 7A-D). At this time, only a few MBP+ cells were observed and there was no difference in the number between wild type ($\bar{x}=4\pm0.5$, $n=3$ mice) and knockout ($\bar{x}=4\pm0.5$, $n=3$ mice) mice. In contrast, by postnatal day 6, MBP+ staining was detected in the developing white matter of the cerebellar folia. At this time, quantitation of the MBP+ cells revealed a 1.92-fold increase in number in p27Kip1−/− mice compared to wild type (Fig. 7E). The increase persisted at postnatal day 8, when MBP+ cells could be also observed at the tip of the cerebellar folia. By postnatal day 10, however, MBP immunoreactivity was lost in the soma and the staining was mainly confined to myelinated fibers. Similar findings were observed at day 16 (Fig. 6). This effect was not region specific because a similar increase could also be observed at postnatal day 6 in the forebrain and in optic nerve sections (data not shown).

We conclude that, despite the increased proliferative ability of progenitor cells in early stages of gliogenesis, oligodendrocyte progenitors in the p27Kip1 null mice are fully competent to withdraw from the cell cycle and respond to differentiative signals from the extracellular environment.

**Absence of functional p27Kip1 does not alter the quiescent state of mature oligodendrocytes**

We have previously reported that p27Kip1 levels are elevated in fully mature, MBP+ cultured oligodendrocytes (Casaccia-Bonnefil et al., 1997) and this results in functional inhibition of CDK2 enzymatic activity (Tikoo et al., 1998). These data suggested a potential role for p27Kip1 during quiescence of mature oligodendrocytes. If p27Kip1 function is required for quiescence, then it is conceivable that fully differentiated oligodendrocytes in p27Kip1−/− mice may still be able to proliferate. In order to address this question, we determined whether mature MBP+ oligodendrocytes incorporated BrdU in P6 wild-type and p27Kip1 null mice during a 2 hour pulse. We found that none of the MBP+ cells present in the subcortical white matter at this stage were BrdU positive (Fig. 8). The lack of BrdU incorporation in p27Kip1−/− cells expressing MBP therefore suggests that in vivo the mechanism determining the maintenance of quiescence is independent of p27Kip1 function.

**Decreased levels of cyclin E in the brain of p27Kip1 mice may account for functional compensation in later stages of development**

In this study, we show that loss of p27Kip1 causes enhanced proliferation of glial progenitor during embryonic (E13–E17) and postnatal (P1–P6) gliogenesis. This increased proliferation is transient, since by E19 (in the embryonic spinal cord) and by postnatal day 16 (in the neonatal cerebellum), the proliferative rate of oligodendrocyte progenitor in the p27Kip1−/− mice equals wild-type levels (Figs 1B and 4B). Since proliferation is increased in p27Kip1−/− progenitors only during early gliogenesis, but not in later phases, compensatory mechanisms independent of p27Kip1 may be operational and induce the final growth arrest event prior to differentiation in vivo.

Studies in Drosophila have indicated that developmental growth arrest of cells of the dorsal epidermis is controlled by downregulation of cyclin E levels (Duronio and O’Farrell, 1995; Knoblich et al., 1994) Similarly, in cultured oligodendrocyte progenitors, we have previously reported

**Fig. 5.** Timely differentiation of oligodendrocyte progenitors in the developing corpus callosum. Newly generated oligodendrocytes in the developing corpus callosum of wild-type (+/+) and p27Kip1 knockout (−/−) mice were identified by myelin basic protein (MBP) immunolabelling at P6. The arrows in A and B indicate the same fields shown by the asterisks at high power in C and D. The green immunofluorescence labels MBP+ cells and the red immunofluorescence indicate BrdU labelling. Scale bar, 40 μm in A and B, 10 μm in C and D.
decreased levels of cyclins and CDKs, together with upregulation of Kip inhibitors during oligodendrocyte differentiation (Casaccia-Bonnefil et al., 1997).

In order to assess whether changes in levels of cyclins and cyclin dependent kinases may compensate for loss of p27Kip1 in late gliogenesis, we analyzed brain extracts from p27Kip1−/− mice (Fig. 9, even numbered lanes) and wild type (Fig. 9, odd numbered lanes) by western blot analysis. We compared protein levels in cortical and cerebellar extracts at three stages of development: the first postnatal week (n=3 wt and n=5 p27Kip1−/− mice) second postnatal (n=4 wt and n=4 p27Kip1−/− mice) and adulthood (n=2 wt and n=2 p27Kip1−/− mice), using tubulin as loading control (Fig. 9A and B). In both wild type and p27Kip1−/− no change in the relative levels of cyclin D1 was observed in either cortical or cerebellar extracts during these two stages of development. In contrast, cyclin D2 was predominantly expressed in the cerebellum and downregulated in adulthood, consistent with previous studies (Ross, 1996; Ross and Risken, 1994; Ross et al., 1996). A similar temporal and regional pattern of expression was observed for CDK2 and CDK4 (Fig. 9A).

Interestingly the only G1 positive regulator to show clear differences in expression levels between wild-type and p27Kip1−/− mice was cyclin E (Fig. 9A). Two main immunoreactive bands have been described for cyclin E: a 56 kDa isoform, predominantly expressed in neurons and a 51 kDa isoform expressed in astrocyte and oligodendrocytes (Miyajima et al., 1995). In the p27Kip1−/− mice, downregulation of the 51 kDa cyclin E band was observed in cortical and cerebellar protein extracts. Quantitative analysis of the western blots revealed a decrease of 64.8±15% during the first week (n=3 wild-type and n=5 p27Kip1−/− mice), 72.4%±16% during the second week (n=4 wild-type and n=4 p27Kip1−/− mice), and a 30% decrease in adult mice. These data are consistent with our previous results in vitro, which indicated a critical role of cyclin E/CDK2 complexes in regulating proliferation of oligodendrocyte progenitors (Tikoo et al., 1998).

We also considered the possibility that compensatory mechanisms for loss of p27Kip1 could include upregulation of other inhibitory molecules. For this reason we analyzed levels of other members of the Kip family (p21waf1) and of the INK family of inhibitors (p16INK4a and p19INK4d) which have been shown to be expressed in the postnatal brain (Zindy et al., 1997). Levels of p57Kip2 were also analyzed using commercially available antisera, but due to lack of specificity, we were unable to obtain conclusive results. Of the inhibitors tested, however, neither p21waf1 levels nor p16INK4a and p19INK4d were upregulated in the p27Kip1 null mice (Fig. 8B).

DISCUSSION

Role of p27Kip1 in oligodendrocyte progenitor proliferation

Studies in oligodendrocyte progenitors isolated from the postnatal optic nerve have suggested a link between the number of cell divisions and timing of differentiation (Barres et al., 1994; Temple and Raff, 1986). Since the decision between proliferation and growth arrest in eukaryotic progenitor cells is mainly regulated at the G1/S transition, we have focused our analysis on p27Kip1, one of the key G1 regulators in the oligodendrocyte lineage (Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Tikoo et al., 1998). Using biochemical measurements, we previously reported a decrease in the steady state levels of cyclin E, CDK2, and a concomitant increase in inhibitor levels during oligodendrocyte differentiation. These findings have been confirmed by other studies (Durand et al., 1997; Gao and Raff, 1997; Ghiani et al., 1999; Tang, 1998). We established p27Kip1 as one of the major determinants of oligodendrocyte progenitor growth arrest by showing that the activity of cyclinE/CDK2 complexes was directly inhibited by p27Kip1 (Tikoo et al., 1998) and that cell cycle withdrawal was impaired in cultures prepared from knockout mice (Casaccia-Bonnefil et al., 1997; Durand et al., 1998). In the present study we have directly tested the role of p27Kip1 in oligodendrocyte proliferation in vivo and analyzed the relationship between growth arrest and differentiation by phenotypic analysis of the p27Kip1 knockout mouse during development.

We have used expression of the QKI family of proteins to identify glial precursor cells in the embryonic spinal cord. QKI proteins have been detected during embryogenesis in multipotent neural progenitors (Hardy, 1998a). During differentiation of neuronal progenitors, QKI protein expression is dramatically down-regulated (Hardy, 1998a), while it is
maintained at high levels in newly generated glial cells and involved in proper formation of myelin (Hardy, 1998b; Hardy et al., 1996).

We have shown that QKI+ newly generated glia show increased proliferation in the absence of functional p27Kip1, peaking at E13 when neurogenesis is completed in the ventral spinal cord (Nornes and Das, 1974). This indicates that p27Kip1 acts as a cell cycle regulator in the early phases of gliogenesis. Interestingly the effect of p27Kip1 loss of function on QKI+ cells was evident between E13-E17 (Fig. 1), coincident with the temporal window of slowing down of the cell cycle kinetics in the spinal cord (Calver et al., 1998). Since cell cycle lengthening is due to increase in the G1/S transition, it is tempting to speculate that p27Kip1 is involved in the mechanism that slows cell cycle kinetics in the spinal cord during embryonic gliogenesis (Zavitz and Zipursky, 1997).

We have also evaluated the effect of p27Kip1 loss of function on neonatal oligodendrocyte progenitors in two distinct regions, the subcortical white matter and the cerebellum, using NG2 as a marker (Levine et al., 1993). In both regions an increased number of NG2+ oligodendrocyte progenitors was observed in p27Kip1−/− mice, compared to wild type. We have thus shown for the first time that p27Kip1 is a critical regulator of cell proliferation in embryonic and neonatal oligodendrocyte progenitors in vivo. However, our data also show that defective growth arrest in the absence of functional p27Kip1 is limited to a specific temporal window of development, as the percentage of QKI+/BrdU+ cells at embryonic day 19 and that of NG2+/BrdU+ cells at postnatal day 16 returned to wild-type levels (Figs 1B and 4B). These data suggest that in vivo signals (possibly axonal contact, or neurotransmitters or extracellular matrix) can activate pathways leading to growth arrest independently of p27Kip1 function.

Recent studies on the inactivation of the C. elegans cyclin-dependent kinase inhibitor cki-1, a p27Kip1 homologue, have revealed an interesting phenotype during vulval precursor cell development (Hong et al., 1998), which parallels our observations in oligodendrocyte development in p27Kip1−/− mice. In C. elegans vulval development, loss of cki-1 function, results in precocious precursor cell divisions, without alteration of their developmental potential. It has been suggested that a major role for cki-1 is to maintain these precursor cells in G1 between two successive rounds of proliferation (Hong et al., 1998). Also in Drosophila, loss of the p27Kip1 homolog Dacapo (dap mutants) results in increased proliferation of precursor cells, without affecting the timing of permanent exit from the cell cycle (de Nooij et al., 1996; Lane et al., 1996). Similarly, in the oligodendrocyte system, the loss of p27Kip1 function results in misregulated growth arrest of progenitor cells during early gliogenesis, without affecting the timing of withdrawal from the last cell cycle. In p27Kip1 null mice higher percentages of proliferating glial progenitors were
observed during the development of the embryonic spinal cord (E13-E17) and neonatal cerebellum (P1-P6), compared to wild type, indicating an important role of the cell cycle inhibitor p27Kip1 at early stages of gliogenesis. However, by E19 in the spinal cord and by P16 in the cerebellum, proliferation in the p27Kip1 null mice had reached levels very similar to the wild type suggesting that compensatory mechanisms become operational.

One possibility is that inhibition of critical CDK activities is achieved by increased inhibitor levels of the Kip and INK family. Alternatively, cyclins and CDK levels may be downregulated. Downregulation of positive regulators as a mechanism of growth arrest prior to differentiation occurs during development of Drosophila epidermal cells (Knoblich et al., 1994; Neufeld et al., 1998). Similarly, downregulation of D and E cyclins is observed in oligodendrocytes cultured in vitro (Casaccia-Bonnefil et al., 1997).

In this study, we demonstrate that downregulation of cyclin E levels occurs in the postnatal brain of p27Kip1 null mice. This is of particular relevance since the enzymatic activity of cyclin E/CDK2 complexes is responsible for entry into S-phase. Interestingly, cyclin E levels can be regulated at the level of proteasome targeting and degradation by affecting its phosphorylation by cyclinE/CDK2 (Clurman et al., 1996). In wild-type mice, inhibition of cyclinE/CDK2 complexes by p27Kip1 limits proteolytic degradation of cyclin E. In contrast, in the absence of p27Kip1, cyclin E itself would be more susceptible to increased degradation. This result links the presence of p27Kip1 with cyclin E degradation and is consistent with previous studies indicating the existence of a reciprocal feedback loop between p27Kip1 and cyclin E (Clurman et al., 1996; Vlach et al., 1997).

We conclude that downregulation of cyclin E levels may play a compensatory role for loss of p27Kip function, although we cannot exclude the possibility at the present time, that other inhibitors, such as p57Kip2 or p19arf may also play a role in glial growth arrest in the developing central nervous system.

**Role of p27Kip1 in oligodendroglial lineage development**

The obligatory relationship between number of cell divisions prior to growth arrest and differentiation described in the oligodendroglial lineage would predict a delay in the initiation...
of differentiation in the absence of p27Kip1. In vitro experiments on cultured oligodendrocyte progenitors provided support for this model (Durand et al., 1997). However, in vivo, no delay in the appearance of MBP+ cells was observed in p27Kip−/− mice, although the lack of increased number of MBP cells at postnatal day 4, in the presence of increased progenitor cell number may indicate an impaired response to differentiative cues. This may be explained by alternative modes of growth arrest occurring during development or by the dominant effect of the Notch signalling, which has been shown to uncouple proliferation from timing of differentiation pathway in oligodendrocytes in the optic nerve (Wang et al., 1998).

In addition, we have previously shown that ectopic expression of p27Kip1 in oligodendrocyte progenitors using adenoviral vectors, is sufficient to induce growth arrest in vitro even in the presence of strong mitogenic stimulation, although differentiation did not occur by default (Tikoo et al., 1998). From these results and the ones presented in this study, we propose that during normal mouse brain development at least two mechanisms of growth arrest maybe operational. One mechanism, which is p27Kip1 dependent, mainly operates during gliogenesis on proliferating progenitors and regulates the number of cell divisions in the presence of mitogens. In the absence of p27Kip1, enhanced proliferation of progenitors results from increased enzymatic activity of cyclinE/CDK2. This process is autolimiting since the absence of p27Kip1 results in increased phosphorylation and degradation of cyclin. Decreased cyclin E levels may reestablish an equilibrium between positive and negative regulators which was perturbed in the p27Kip1 mice.

An additional mechanism, which is p27Kip1 independent, guarantees withdrawal from the last cell cycle prior to differentiation. Although the cellular components responsible for final growth arrest of progenitor cells are presently unknown, a likely possibility is that other inhibitors, such as p57Kip2 or p19INK may play a role in the final decision between proliferation and growth arrest and differentiation. Quiescence is maintained by downregulating the expression levels of the CDKs.

In conclusion, our results imply that p27Kip1 affects the size of the oligodendrocyte progenitor pool in vivo by affecting the number of cell divisions in early phases of gliogenesis, without affecting the timing of ultimate withdrawal from the cell cycle or their ability to respond to differentiative cues. These data support a major role for the cell cycle inhibitor p27Kip1 in the cell number regulation of multicellular organisms (de Nooij et al., 1996; Hong et al., 1998; Lane et al., 1996) which is highly conserved through evolution.

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REFERENCES
Ibarrola, N., Mayer-Proschel, M., Rodriguez-Pena, A. and Noble, M.


