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p21^{cip1} is required for the differentiation of oligodendrocytes independently of cell cycle withdrawal

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Differentiation of most cell types requires both establishment of G_1 arrest and the induction of a program related to achieving quiescence. We have chosen to study the differentiation of oligodendrocyte cells to determine the role of p27 and p21 in this process. Here we report that both p27 and p21 are required for the appropriate differentiation of these cells. p27 is required for proper withdrawal from the cell cycle, p21 is not. Instead, p21 is required for the establishment of the differentiation program following growth arrest. Similar observations were made *in vivo*. We show that *p21*$^{-/-}$ cells withdraw from the cell cycle similar to wild-type cells; however, early in animal life, the brain is hypomyelinated, inferring that the loss of p21 delayed myelination in the cerebellum. We found that we could complement or bypass the differentiation failure in *p21*$^{-/-}$ cells with either PD98059, an inhibitor of Mek1, or by transducing them with a tat–p16^{Ink4a} protein. We concluded that the two cdk inhibitors serve non-redundant roles in this program of differentiation, with p27 being responsible for arrest and p21 having a function in differentiation independent of its ability to control exit from the cell cycle.

INTRODUCTION

The development of a tissue requires the integration of cell cycle exit with morphological changes culminating in the specialized nature of a differentiated cell. Proliferation is controlled by the activation of cyclin-dependent kinases (cdk), which involves a cascade of structural changes controlled by interactions with other proteins and post-translational phosphorylation events (Morgan, 1995, 1996). Together, cyclin D and E associated kinase activities are required to drive cells through the G_1 phase of the cell cycle (Sherr, 1995; Roberts, 1999). There are two cdk-inhibitor (cki) families: the Ink family, which targets cdk4 complexes specifically, but can also affects the cdk2 activities due to a 'ping-pong' effect on Cip/Kip members; and the Cip/Kip family, which inhibits the cdk2-containing complexes (Sherr and Roberts, 1995, 1999). The inhibitors overlap in many tissues. The biochemical activities and their expression in the most differentiated cells, both in mouse development and *in vitro*, implicate these proteins as effectors of cell cycle exit and differentiation. However, it has not been clear whether they cooperate to mediate cell cycle arrest or whether they have other functions in the differentiation program. To address this experimentally, we have compared the role of p21 and p27 during the differentiation of oligodendrocyte progenitor cells (OPC).

Oligodendrocytes are myelinating cells of the central nervous system (CNS). They originate from multipotential precursors in the proliferative ventricular zones of the brain and spinal cord that generate specialized progenitor cells that proliferate and migrate through the CNS. Primary cultures of progenitors can be isolated from many regions of the CNS, including the optic nerve and cortex. These progenitor cells can be induced to differentiate following withdrawal of serum (McCarthy and de Vellis, 1980; Temple and Raff, 1985). Not all OPC are equivalent and the locations from which the cell is isolated and the developmental age of the animal alter the response to differentiation-inducing conditions (Ibarrola *et al.*, 1996; Gao *et al.*, 1997). The

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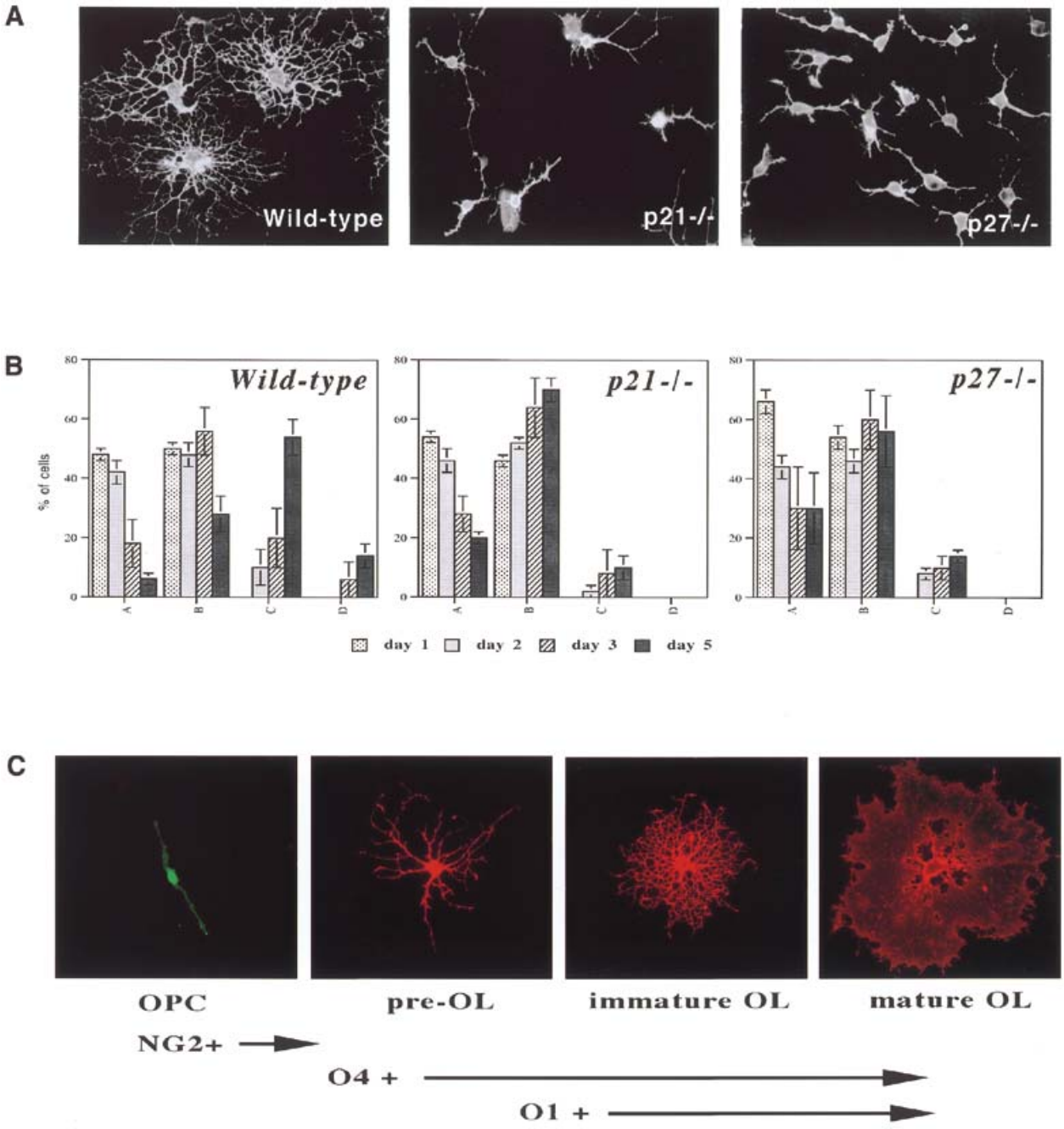

Fig. 1. p21 and p27 participate in the differentiation of mouse oligodendrocytes. (**A**) Impaired differentiation of *p21*$^{-/-}$ oligodendrocytes *in vitro*. Purified OPC from wild-type, *p21*$^{-/-}$ and *p27*$^{-/-}$ animals were plated at equal density and cultured in DM + TH. After 5 days in this medium cells were stained for the O4 surface marker. This was repeated five times with cells from each genotype. (**B**) Kinetic analysis of the differentiation program in the cki-deficient OPC. Following the switch to DM + TH, cells were harvested at days 1, 2, 3 and 5, and the numbers of cells at each differentiation stage, as shown in (C), were scored by morphology and reactivity to NG2, O4, Rantsch and O1 antigens. (**C**) Stages of oligodendrocyte differentiation: oligodendrocyte precursor cell (OPC), pre-oligodendrocyte (pre-OL), immature oligodendrocyte (OL) and mature OL as per Pfeiffer *et al.* (1993).

differentiation of OPC correlates with an induction of p21 and p27 expression, while the levels of p16 expression are maintained throughout different developmental stages (Casaccia-Bonnefil *et al.*, 1997; Tang *et al.*, 1998; Ghiani *et al.*, 1999). Thus, this cell type provides an experimental avenue through which to gain insight into the respective roles of cdk inhibitors during differentiation of an unaltered primary cell. In this study, we show that p27 and p21 carry out separate and distinct functions, p27 participating in cell cycle withdrawal and p21 in differentiation of the non-proliferating cell.

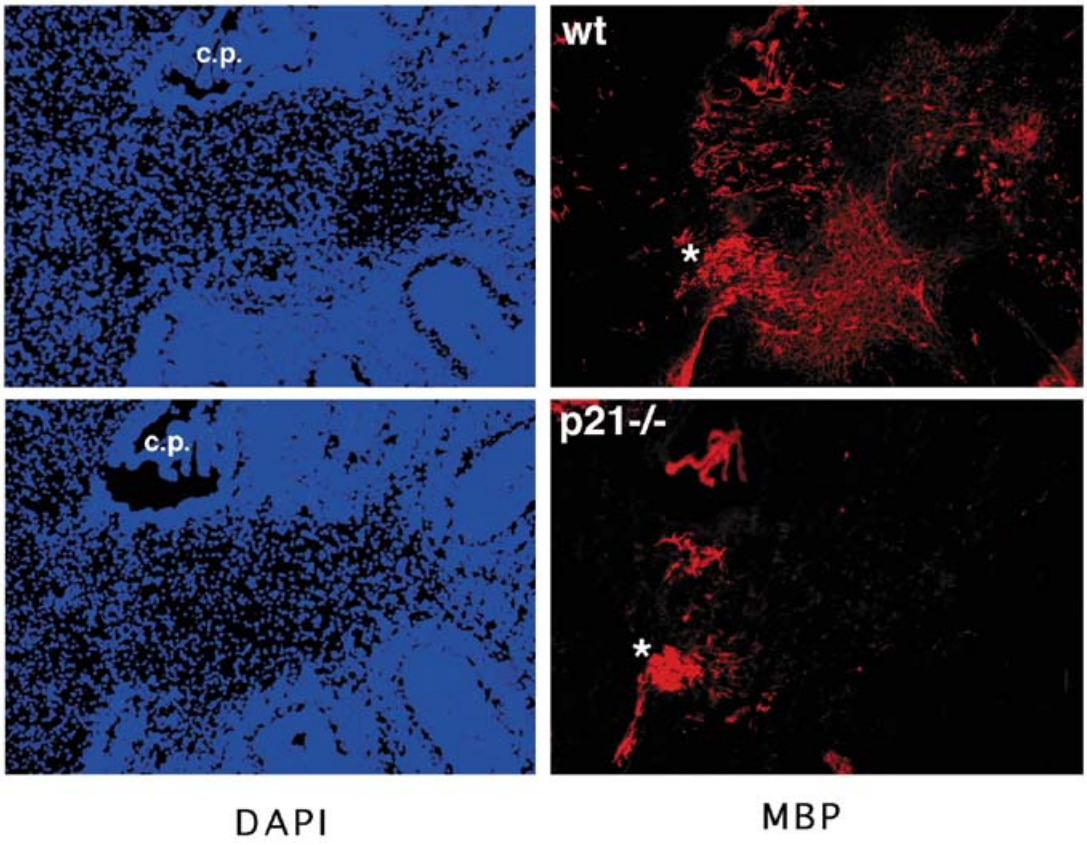

Fig. 2. Decreased myelination of cerebellar regions of $p21^{-/-}$ mouse brain. Sections were generated from wild-type (top panels) and $p21^{-/-}$ mice (bottom panels), and placed on the same slide. Slides were then doubly stained for MBP (right) and DNA (DAPI). MBP reactivity is more apparent throughout the cerebellum in the wild-type mice. Myelinating oligodendrocytes and newly myelinated fibers in the cerebellar peduncles are indicated by the asterisks. This was repeated four times on four different animals of each genotype with similar results. c.p., choroid plexus.

RESULTS

p21 and p27 are both required for proper OPC differentiation *in vitro*

Several CDK inhibitor proteins are expressed in oligodendrocytes, including p21 and p27 (Casaccia-Bonnefil *et al.*, 1997). We therefore examined the differentiation process (defined by Pfeiffer *et al.*, 1993) of cells isolated from either $p21^{-/-}$ (Brugarolas *et al.*, 1995) or $p27^{-/-}$ (Kiyokawa et al., 1996) mice cultured in differentiation medium containing thyroid hormones (DM + TH) for 5 days. Within 3 days, ~60% of wild-type cells develop the morphological characteristics of pro-oligodendrocytes with ~20% progressing into immature oligodendrocytes (Figure 1A). By day 5, ~60% have an immature oligodendrocyte morphology, with ~15% appearing as mature oligodendrocytes producing myelin sheets. Similar results were observed using O4 staining as a marker of the less mature pro-oligodendrocytes, and O1 and Rantsch staining as markers of more differentiated immature and mature oligodendrocytes (Pfeiffer *et al.*, 1993) (data not shown). In contrast, the majority of $p21^{-/-}$ and $p27^{-/-}$ cells did not progress past the pro-oligodendrocyte stage during the differentiation period (Figure 1). The genetic background *vis-à-vis* the absence of the cdk inhibitor genes does not prevent differentiation: these cells ultimately differentiate, it just takes longer than the 5 day period. From the seventh to tenth day, more cells have differentiated (P. Casaccia-Bonnefil and J. Zezula, unpublished data); however, an extensive amount of cell death prevents any conclusions about the extent of the delay. We concluded, using the criteria of cell morphology and surface marker expression, that both p21 and p27 play a role in the differentiation of oligodendrocytes.

Delayed onset of myelination in $p21^{-/-}$ mice

The *in vitro* data suggested that oligodendrocyte differentiation might be impaired in the $p21^{-/-}$ mice. Previously, we examined myelin basic protein (MBP) expression in neonatal $p27^{-/-}$ mice and showed that these animals had a 'hypermyelination' phenotype in the cerebellum consistent with an increase in the number of oligodendrocytes, but in culture the progenitors had a decreased ability to arrest (Casaccia-Bonnefil *et al.*, 1997, 1999). Although paradoxical, these observations were reconciled by realizing that p27 deficiency was affecting the efficiency at which progenitors withdrew from the cell cycle. This increased

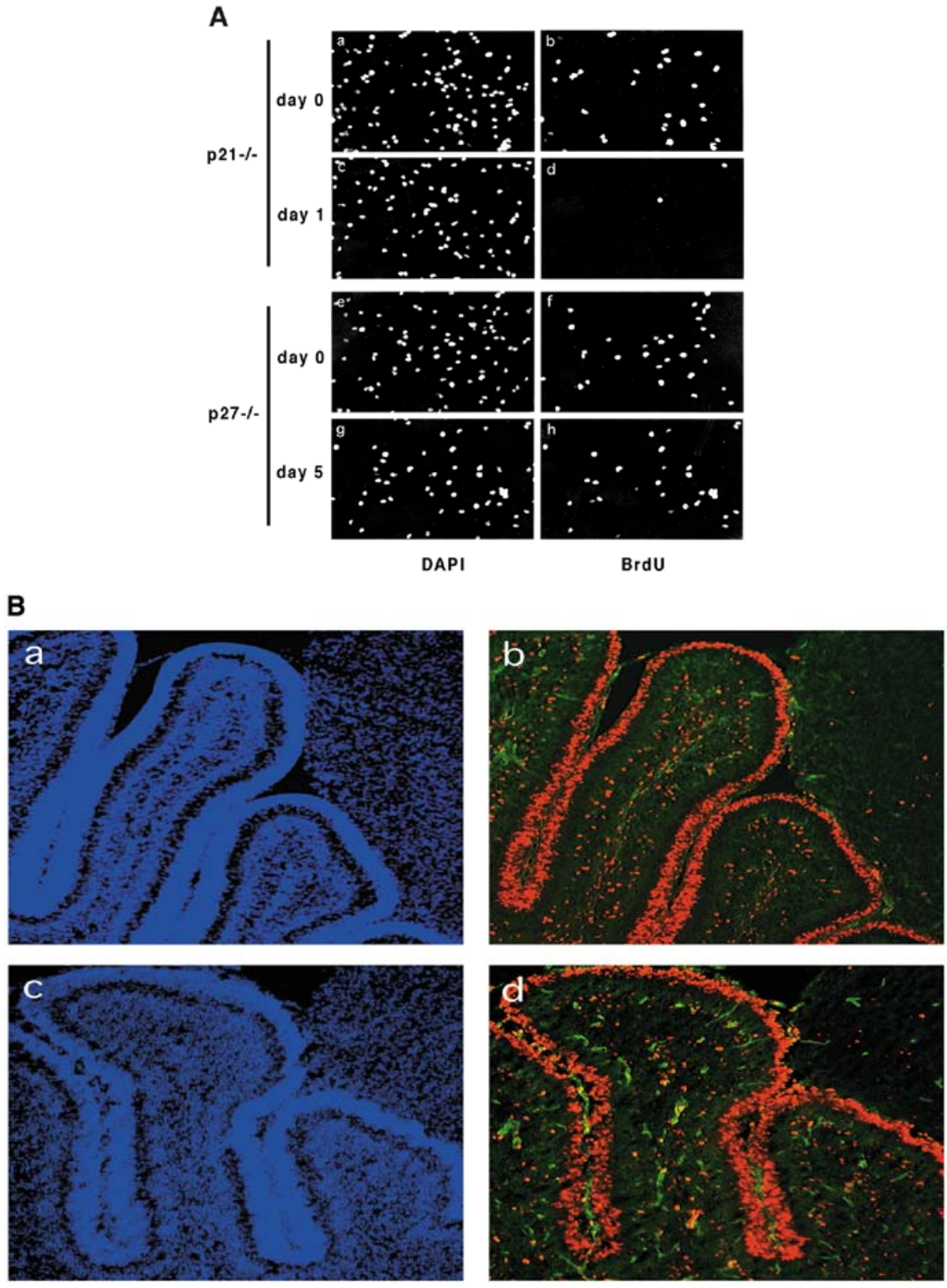

Fig. 3. p21-deficient cells withdraw from the cell cycle both *in vitro* and *in vivo*. (**A**) $p21^{-/-}$ OPC withdraw from the cell cycle whereas $p27^{-/-}$ cells continue to proliferate. Purified OPC from $p21^{-/-}$ (a–d) and $p27^{-/-}$ (e–h) animals were plated at equal densities in proliferation medium (a, b, e, f) or in DM + TH (c, d, g, h) and labeled with BrdU for 12 h. Cells were double-stained for BrdU to measure proliferation (right) and DAPI (left). This experiment was repeated 3–4 times on pooled cells obtained from different animals within a litter. (**B**) Proliferation of OPC in the cerebellum of $p21^{-/-}$ mice is similar to that observed in wild-type mice. Immunohistochemistry of cerebellar sections of postnatal day 4 wild-type (a, b) and $p21^{-/-}$ (c, d) animals. In (a and c), cerebellar structures were identified using DAPI nuclear staining. (b and d) show double immunofluorescence staining for BrdU (red) as a marker of proliferation and for NG2 (green) as a specific OPC surface marker. This was repeated twice on different animals with similar results.

the cycling in the progenitor pool, allowing an increase in the absolute number of cells that would eventually go on to differentiate. Similar effects of *p27*$^{-/-}$ on hematopoietic stem cell differentiation have now been reported (Cheng *et al.*, 2000).

To examine the issue of differentiation *in vivo*, we looked at the production of MBP and proteolipid protein in the cerebellum of *p21*$^{-/-}$ mice at postnatal day 4, the earliest time at which the presence of MBP-positive cells can be detected immunohistochemically (Reynolds and Hardy, 1997). By day 4, the extent of MBP staining in the cerebellum of the *p21*$^{-/-}$ mouse was markedly less than that observed in the wild-type mouse (Figure 2). Similar observations were made at day 6 (data not shown). This correlated with the expression of proteolipid protein (PLP) and MBP determined in homogenates obtained from the same brain region (data not shown). The delay of myelination is consistent with the delayed differentiation of *p21*$^{-/-}$ OPC *in vitro* and indicates a role for p21 in maturation. The lack of an overt neurological phenotype in *p21*$^{-/-}$ adult mice suggests the presence of compensatory mechanisms responsible for appropriate myelination. Together, the phenotype of neonatal myelination in *p27*$^{-/-}$ and *p21*$^{-/-}$ mice suggests that these cdk inhibitors carry out non-redundant functions in the early differentiation of OPC.

p21 and p27 control different aspects of the differentiation program

The observations that p21- and p27-deficient cells failed to differentiate with wild-type kinetics and the mice had different myelination phenotypes were consistent with the possibility that these proteins coordinate distinct parts of the differentiation program. To ask whether *p21*$^{-/-}$ cells were capable of withdrawing from the cell cycle in an appropriate manner, we plated purified OPC from *p21*$^{-/-}$ and *p27*$^{-/-}$ mice at equal density in proliferation media or DM + TH and labeled for 12 h with 5-bromo-2′-deoxyuridine (BrdU). *p21*$^{-/-}$ cells efficiently exited the cell cycle within the first day; however, *p27*$^{-/-}$ cells continued to proliferate even after 5 days in culture (Figure 3A).

In order to determine the effect of p21 deficiency on neonatal OPC proliferation *in vivo*, we co-stained for BrdU and NG2 in the cerebellum (Levine and Stallcup, 1987). We found that the percentages of NG2 cells that incorporated BrdU in the brains of *p21*$^{-/-}$ mice compared with their wild-type controls were no different in the cerebellum at postnatal days 1, 4 and 6 (Figure 3B; Table I). Together, these data indicate that p21 is not required for cell cycle arrest in developing OPC, a role apparently fulfilled by p27.

PD98059 and TAT-p16^{Ink4a} complement the defect in *p21*$^{-/-}$ cells

In addition to the well described function for p21 as a cdk inhibitor, there are additional biochemical activities reported for this protein (Waga *et al.*, 1994; Shim *et al.*, 1996). However, due to the inherent inability to obtain a sufficient number of genetically defined cerebellar mouse oligodendrocytes, we are unable to assess directly either *in vitro* kinase activity or carry out experiments to monitor protein association. Nevertheless, we reasoned that if we could complement or bypass the differentiation defect in p21-deficient cells, we might gain some insight into the role of p21 in this process.

Table I. *In vivo* proliferation of NG2+ cells

	Wild type	*p21*$^{-/-}$
Postnatal day 1	24.7 ± 4.1	19.3 ± 5.3
Postnatal day 4	15.7 ± 5.0	17.7 ± 2.0
Postnatal day 6	8.7 ± 2.6	8.6 ± 2.5

Numbers represent the mean ± SE from three sections obtained from two animals stained with NG2 and BrdU reactive antibody. The numbers represent the percentage of NG2 cells that were also BrdU positive.

We first asked whether roscovitine, a well-characterized inhibitor of cdks, would complement the deficiency (Krucher *et al.*, 1997; Meijer *et al.*, 1997; Schutte *et al.*, 1997). In order to confirm that roscovitine was effective at this concentration, we made use of the reported observation that cdk2 inhibition was strongly correlated with a failure to enter S phase and incorporate BrdU. At the concentration used, roscovitine prevented proliferation of *p27*$^{-/-}$ cells (Figure 4A), but when added 36 h after *p21*$^{-/-}$ cells were switched into DM + TH, it could not complement the differentiation defect (Figure 4B).

As there were no pharmacological mechanisms to inhibit cdk4/6 kinase activities, we turned to the transduction of a highly specific inhibitor: tat–p16 (Ezhevsky *et al.*, 1997; Nagahara *et al.*, 1998; Schwarze *et al.*, 1999, 2000; Lissy *et al.*, 2000). We transduced growth-arrested *p21*$^{-/-}$ cells with the tat–p16 fusion protein or a control fusion protein, tat–green fluorescent protein (GFP), and measured their affect. The differentiation defect of *p21*$^{-/-}$ cells was completely complemented by transduction of p16 (Figure 4C), suggesting that cyclin D–cdk4 complexes might be interfering with the elaboration of the differentiation program. To test this further, we inhibited Mek1 activity by treating the arrested cells with PD98059. Roussel and her colleagues had shown that the formation of cyclin D1–cdk4 complex was dependent on Mek1 activity (Cheng *et al.*, 1998). At the concentration used, PD98059 would not prevent continued proliferation of wild-type, *p21*$^{-/-}$ or *p27*$^{-/-}$ OPC (Figure 4A and data not shown); however, when added 36 h after *p21*$^{-/-}$ cells were switched into DM + TH, it complemented the differentiation defect of these cells (Figure 4B).

DISCUSSION

Two decisions are made during differentiation: the decision to stop proliferating in G_1 phase (i.e. inhibition of the G_1–S transition) and the decision to initiate changes in gene expression that lead a G_1 cell to the quiescent state (i.e. the G_1–G_0 transition). The data presented here demonstrate that p21 and p27 have non-overlapping functions during the differentiation of oligodendrocytes: p27 for G_1 arrest, and p21 for another function independent of G_1 arrest.

The effects of p21 expression on differentiation are complex. While overexpression accelerates differentiation in PC12 cells (Erhardt and Pittman, 1998) and C2C12 myoblasts (Skapek *et al.*, 1995), it inhibits the differentiation of mouse keratinocytes (Di Cunto *et al.*, 1998). However, keratinocytes lacking p21 undergo growth arrest, but fail to elaborate aspects of the transcriptional program of differentiation (Missero *et al.*, 1996).

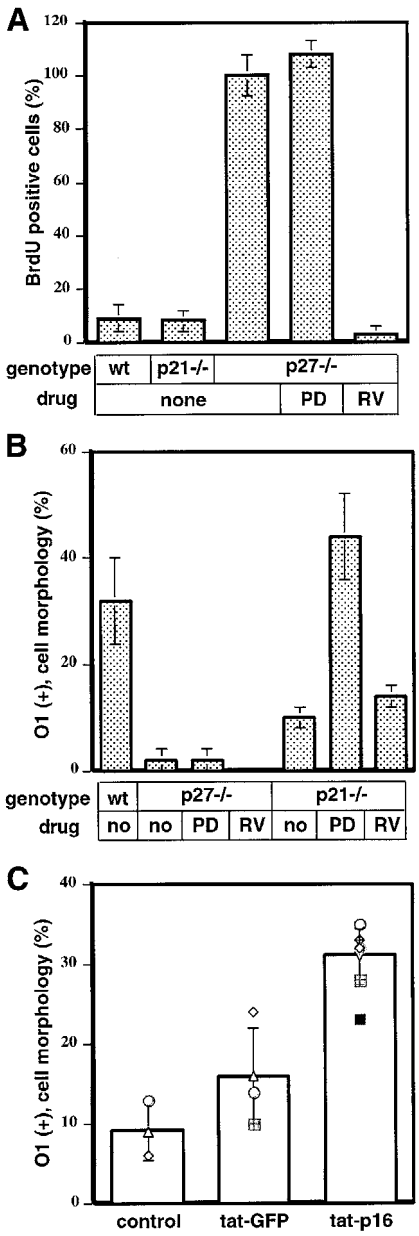

Fig. 4. p21 deficiency can be complemented by PD98059 and tat–Ink4a. (**A**) CDK2 inhibitors but not PD98059 inhibit proliferation of *p27*$^{-/-}$ OPC in differentiation medium. *p27*$^{-/-}$ cells in DM + TH were treated with either PD98059 (PD) or roscovitine (RV) and proliferation scored as the percent of OPC incorporating BrdU during a 12 h pulse, 48 h later. (**B**) PD98059 but not the cdk2 inhibitors complements the differentiation defect in *p21*$^{-/-}$ cells. Thirty-six hours after cells were switched into DM + TH, they were exposed to the drugs and incubated for a further 2 days. Cells were subsequently stained and differentiation assessed by the morphological and staining characteristics as described in the legend to Figure 1. These experiments were repeated 3–4 times on pooled cells obtained from different animals within a litter. (**C**) tat–p16 complemented the differentiation defect of *p21*$^{-/-}$ cells. As in (B), except that the cells were treated with 100 nM tat–Ink4a or a tat–GFP fusion protein following the switch to DM + TH. Transduction efficiency was determined by GFP fluorescence or immunoreactivity and was equivalent in the samples. The mean percentage of differentiated immature and mature oligodendrocytes is noted. Hatch marks represent values from individual experiments. We counted between 100 and 300 cells for each condition.

While p21 is not a transcriptional regulator itself, it is able to affect transcription indirectly through a number of mechanisms. One of the consequences of cdk inhibition by p21 might be to affect Rb differentiation-promoting functions (Kaelin, 1997). Cyclin D–cdk4 complexes phosphorylate Ser780 of Rb (Kitagawa et al., 1996), which is not sufficient to promote cell proliferation but disrupts HDAC association (Zhang *et al.*, 2000). Thus, cyclin D–cdk4 phosphorylation of Rb might directly affect the differentiation-promoting function of Rb independently of the cooperation with cyclin E–cdk2, which is required for its ability to affect cell proliferation. On the other hand, p21 can inhibit E2F activity independently of Rb (Dimri *et al.*, 1996). Additionally, p21 might stimulate gene expression as with NF-κB, perhaps through interactions with p300 and CBP (Perkins *et al.*, 1997; Snowden *et al.*, 2000). Alternatively, p21 interaction with either the JNK kinases (Shim *et al.*, 1996), gadd45 (Kearsey *et al.*, 1995), or STAT3 (Coqueret and Gascan, 2000), could impact differentiation programs. By which of these mechanisms p21 promotes oligodendrocyte differentiation presents a technical challenge for the future. Thus, we conclude that p21 has a role regulating the G_1–G_0 transcriptional program in oligodendrocytes, independent of any role in regulating cell cycle exit.

SPECULATION

To begin to understand what p21 might be doing, we used a pharmacological approach to complement the deficiency. We found that either PD98059 or tat–p16, both of which are known to affect the formation of cyclin D–cdk4/6 complexes in cells cultured in the presence of serum growth factors, would complement the deficiency. Thus, we speculate that the presence of a cyclin D1–cdk4 complex in the absence of p21 might be interfering in the transcriptional program of differentiation in some manner. Findings from Ohnuma *et al.* (1999) support a role for p21 in the determination of Muller glia cells, independent of growth arrest but still dependent on its interaction with cyclin D1–cdk4 complexes. Thus, any mechanism should take into account the potential for cyclin D1–cdk4, not associated with p21, to prevent the correct induction of a differentiation program.

METHODS

Reagents. Tissue culture reagents were obtained from Gibco Life Technologies and Sigma–Aldrich. PDGF was from PreproTech. Rabbit anti-NG2 antiserum was raised against the chondroitin sulfate proteoglycan NG2 and affinity purified. Antibodies for O4 and O1 were a gift from Dr S. Pfeiffer, Rantsch from Dr A. Gow and MBP from Dr D. Coleman. Antibodies to BrdU and MBP were from Dako and Boehringer Mannheim, respectively. Biotinylated or fluorochrome-conjugated secondary antibodies were from Amersham, Jackson ImmunoResearch Laboratories, Inc. and Southern Biotechnology Associates, Inc. DAPI was from Molecular Probes.

Drugs (Calbiochem) were used as follows: 2 μM PD98059 or 10 μM roscovitine. Bacterial tat–p16 protein was purified as described previously (Ezhevsky *et al.*, 1997; Nagahara *et al.*, 1998) by sonication in 8 M urea followed by passage over a Ni-NTA column (Qiagen), desalted over a PD-10 column

(Pharmacia) into phosphate-buffered saline (PBS), then flash frozen in 10% glycerol and stored at –80°C.

Cell culture. Primary cortical cultures (postnatal day 1) of OPC were obtained by differential shaking of mixed glial cultures that were kept in NM15 media [Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal calf serum] for 7 days. Immediately after shaking, progenitors were plated on poly-lysine-coated dishes in NM medium. After 6–18 h, progenitors were differentiated in basic differentiation medium consisting of DMEM supplemented with 25 µg/ml insulin, 5 ng/ml selenium and 50 µg/ml transferrin. Tri-iodothyronine (20 ng/ml), 20 ng/ml thyroxine and 10 ng/ml PDGF were added to basic differentiation medium when indicated.

***In vivo* BrdU labeling.** Mice (day 4) received two injections of BrdU (100 µg/g body weight) at 1 h intervals. One hour after the last injection, animals were anesthetized with avertin and perfused with 4% paraformaldehyde in PBS pH 7.4. Upon overnight post-fixation at 4°C in the same fixative, the tissue was incubated for 24 h in 30% sucrose in PBS, embedded in OCT and frozen in cryomolds in 8-methylbutane on dry ice. Cryosections (10–15 µm thickness) were collected on Superfrost glass slides and processed for immunohistochemistry.

Cells were pulsed for 12 h with 10 µM BrdU, fixed in 4% paraformaldehyde in PBS overnight at 4°C and processed.

Immunocytochemistry/immunohistochemistry. Thawed tissue sections were incubated for 1 h 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) at room temperature. Cells were incubated in 3% NGS–3% bovine serum albumin–Hank's buffered salt solution for 30 min. For BrdU, sections and cells were permeabilized with 0.1% Triton X-100 and denatured with 2 N HCl for 10 min at room temperature. For MBP staining, sections were permeabilized in ice-cold methanol for 10 min. Primary antibody was typically incubated overnight at 4°C or for 1 h at room temperature (NG2 1:1000 in PGBA with 10% NGS; O4, Rantsch and O1 undiluted hybridoma supernatant; BrdU 1:100, MBP rabbit polyclonal 1:1000 in PGBA; MBP monoclonal IgG1 1:100 in PGBA with 10% NGS and 0.3% Triton X-100), followed by biotinylated and/or fluorochrome-conjugated secondary antibody for 30 min at room temperature. Sections or cells were counterstained with DAPI in order to visualize cell nuclei.

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