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Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation

Béatrice Durand¹, Fen-Biao Gao and Martin Raff

Medical Research Council Developmental Neurobiology Programme, MRC Laboratory for Molecular Cell Biology and the Biology Department, University College London, London WC1E 6BT, UK

¹Corresponding author

Many types of vertebrate precursor cells divide a limited number of times before they stop and terminally differentiate. In no case is it known what causes them to stop dividing. We have been studying this problem in the proliferating precursor cells that give rise to post-mitotic oligodendrocytes, the cells that make myelin in the central nervous system. We show here that two components of the cell cycle control system, cyclin D1 and the Cdc2 kinase, are present in the proliferating precursor cells but not in differentiated oligodendrocytes, suggesting that the control system is dismantled in the oligodendrocytes. More importantly, we show that the cyclin-dependent kinase (Cdk) inhibitor p27 progressively accumulates in the precursor cells as they proliferate and is present at high levels in oligodendrocytes. Our findings are consistent with the possibility that the accumulation of p27 is part of both the intrinsic counting mechanism that determines when precursor cell proliferation stops and differentiation begins and the effector mechanism that arrests the cell cycle when the counting mechanism indicates it is time. The recent findings of others that p27-deficient mice have an increased number of cells in all of the organs examined suggest that this function of p27 is not restricted to the oligodendrocyte cell lineage.

Keywords: Cdk inhibitor/cell cycle/Kip1/oligodendrocytes/p27

Introduction

In many vertebrate cell lineages, precursor cells divide a limited number of times before they stop dividing and terminally differentiate into post-mitotic cells. The cell divisions are driven by extracellular signals (mitogens), but it is not known what limits the proliferation and causes the cells to stop dividing when they do. The stopping mechanisms are important as they play crucial roles in both controlling cell numbers (and thereby the size of organs and organisms) and timing cell differentiation.

We have been studying these mechanisms in the oligodendrocyte cell lineage in the developing rat optic nerve (reviewed in Barres and Raff, 1994). The oligodendrocytes are post-mitotic cells, which form an insulating myelin sheath around the axons in the nerve. They develop from dividing precursor cells (Temple and Raff, 1986)

that migrate into the developing nerve from the brain, beginning about a week before birth (Small *et al.*, 1987). Oligodendrocytes first appear in the nerve around the day of birth and then increase in number for the next 6 weeks (Barres *et al.*, 1992).

Clonal analyses of either single (Temple and Raff, 1986) or purified (Barres *et al.*, 1994) oligodendrocyte precursor cells isolated from the developing optic nerve suggest that both a cell-intrinsic programme and extracellular signals play important parts in determining when the precursor cells stop dividing and differentiate. When precursor cells are stimulated to proliferate in culture by either astrocytes or a combination of growth factors, including platelet-derived growth-factor (PDGF), neurotrophin-3 (NT-3) and insulin-like growth factor-1 (IGF-1), they divide a maximum of eight times before they stop and differentiate (Temple and Raff, 1986; Barres *et al.*, 1994). The progeny of an individual precursor cell tend to stop dividing and differentiate at about the same time (Temple and Raff, 1986). Moreover, if the two daughter cells of an individual precursor cell are separated and cultured on astrocyte monolayers in separate microwells, they tend to divide the same number of times before they differentiate, suggesting that an intrinsic clock mechanism limits the number of times a precursor cell normally divides (Temple and Raff, 1986).

The clock mechanism, however, requires at least two kinds of extracellular signalling molecules to operate normally: mitogens such as PDGF and hydrophobic signals such as thyroid hormone (TH) (Barres *et al.*, 1994). The need for mitogens is indicated by the finding that precursor cells cultured in the absence of mitogens immediately stop dividing and differentiate into oligodendrocytes within 2 days, whether or not hydrophobic signals are present (Temple and Raff, 1985; Barres *et al.*, 1994). The need for hydrophobic signals is indicated by the finding that precursor cells cultured in the presence of mitogens but in the absence of hydrophobic signals tend to keep dividing and not to differentiate (Barres *et al.*, 1994). If TH is added to such cultures after 8 days, however, the precursor cells stop dividing and rapidly differentiate, suggesting that the counting mechanism continues to operate in the absence of hydrophobic signals (Barres *et al.*, 1994). It seems, therefore, that the clock mechanism consists of at least two components—a counting component that counts time or cell divisions and an effector component that stops cell proliferation (Barres and Raff, 1994). The hydrophobic signals apparently are required for the effector component to stop cell proliferation when mitogens are present and the counting component indicates it is time. Böglér and Noble (1994) independently provided evidence for separate counting and effector components of the clock mechanism, using a combination of basic fibroblast growth factor (bFGF) and PDGF, rather than PDGF and the absence of

TH, to keep oligodendrocyte precursor cells dividing beyond the time that they normally would have stopped: when bFGF was removed after this time, the cells rapidly stopped dividing and differentiated (Bögler and Noble, 1994).

The molecular nature of the counting mechanism is unknown. It is also not clear whether the mechanism primarily controls the onset of differentiation, with the cessation of proliferation following, or whether it primarily controls the cessation of proliferation, with differentiation following. As the precursor cells differentiate prematurely when deprived of mitogens (Temple and Raff, 1985; Raff *et al.*, 1988), however, and the hydrophobic signals are only required for the cells to stop dividing and differentiate in the presence of mitogens but not in their absence (Barres *et al.*, 1994), we suspect that the counting mechanism primarily controls cell proliferation.

How might the effector mechanism stop cell division in the presence of mitogens once time is reached? One possibility is that it down-regulates the cell's mitogen receptors (Itoh *et al.*, 1996). PDGF is probably the most important mitogen for oligodendrocyte precursor cells in the optic nerve (Raff *et al.*, 1988; Richardson *et al.*, 1990). Although the precursor cells eventually lose their PDGF receptors, they seem to do so only after they differentiate into oligodendrocytes (Hart *et al.*, 1989b). Moreover, although the PDGF receptors on newly formed oligodendrocytes can no longer stimulate the cells to divide, they can still be activated by PDGF to induce an increase in both Ca^{2+} in the cytosol (Hart *et al.*, 1989a) and Fos and Jun proteins in the nucleus (Hart *et al.*, 1992), as well as to promote cell survival (Barres *et al.*, 1992, 1993). Taken together, these findings suggest that the effector mechanism may stop cell division by altering events downstream from the PDGF receptors, and perhaps even downstream from the immediate early genes.

Whatever the nature of the clock mechanism, at some point it must interact with the control system that regulates progress through the cell division cycle. The eukaryotic cell cycle control system consists mainly of a family of cyclin-dependent protein kinases (Cdks) and various proteins that regulate them (Sherr, 1994; Lees, 1995; Morgan, 1995). The regulatory proteins include the cyclins, which activate the Cdks and help direct them to their substrates, the kinases and phosphatases that either activate or inhibit the Cdks by phosphorylating or dephosphorylating them (Lees, 1995; Morgan, 1995) and the Cdk inhibitors, which can inhibit the assembly or activity of cyclin-Cdk complexes (Sherr and Roberts, 1995). In principle, the effector mechanism in precursor cells could stop cell division by removing one or more components of the cyclin-Cdk complexes, inhibiting the complexes or both. Similarly, the counting mechanism could involve the progressive loss of one or more of the activating components of the cell cycle control system or the progressive accumulation of one or more of the inhibitory components, or both.

In the present study, we have examined the changes in a number of proteins of the cell cycle control system that occur during oligodendrocyte development. We show that two components of the control system, cyclin D1 and Cdc2 kinase, which are required for cell cycle progression, are lost when the precursor cells differentiate, suggesting

that the control system is dismantled in oligodendrocytes. Most importantly, we show that the Cdk inhibitor p27/Kip1 (p27) protein progressively increases in the precursor cells as they proliferate and is high when the cells stop dividing and differentiate. The time course of this increase is consistent with the possibility that p27 accumulation is part of both the counting mechanism that limits precursor cell proliferation and the effector mechanism that stops precursor cell division. We also show that the accumulation of p27 is not sufficient on its own to stop the cell cycle, or even to slow it down in the absence of hydrophobic signals such as TH. The recent reports that p27/Kip1-deficient mice are abnormally large and have increased numbers of cells in many organs (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996) suggest that p27 plays an essential part in limiting cell proliferation in many mammalian cell lineages.

Results

Loss of Cdc2 kinase and cyclin D1 in oligodendrocytes

We studied the expression of Cdc2 and cyclin D1 in cultures of postnatal day 7 (P7) rat optic nerve cells stimulated with PDGF. These cultures contained a variety of cell types, including meningeal cells, astrocytes, oligodendrocytes (including those that differentiated in the optic nerve and those that differentiated in culture) and oligodendrocyte precursor cells, some of which were still proliferating in response to the PDGF and some of which had stopped dividing and begun to differentiate. After 48 h in culture, the cells were stained on their surface with either the A2B5 monoclonal antibody or monoclonal anti-galactocerebroside (GC) antibody and then stained intracellularly with antibodies against either Cdc2 or cyclin D1. It has been shown previously that oligodendrocytes and their precursors can be identified readily in such cultures by their morphology and antigenic phenotype: oligodendrocytes are GC^+ , have multiple branched processes, and lose A2B5 as they mature; oligodendrocyte precursor cells are A2B5^+ and GC^- , have fewer processes than oligodendrocytes, and are often bipolar. As in the past, we used GC expression as the defining characteristic of an oligodendrocyte (Raff *et al.*, 1984).

Because Cdc2 staining was generally weak and both cytoplasmic and nuclear, it was easier to assess oligodendrocyte lineage cells for Cdc2 if they were not also stained on their surface. In cells that were GC^- and had the characteristic morphology of oligodendrocyte precursor cells, Cdc2 staining varied from moderate (Figure 1A and B) to weak (Figure 1C and D). By contrast, in A2B5^- cells with the characteristic morphology of oligodendrocytes, Cdc2 staining was never above background levels (Figure 1E and F). Some fibroblast-like cells, which were presumably mainly astrocytes and meningeal cells, were also stained by the anti-Cdc2 antibody (not shown).

Anti-cyclin D1 staining of optic nerve cells was nuclear, with non-specific staining of the Golgi apparatus. Cells that were A2B5^+ and had the characteristic morphology of oligodendrocyte precursor cells showed variable nuclear cyclin D1 staining, presumably reflecting differences in the stage of the cell cycle: some stained intensely (Figure

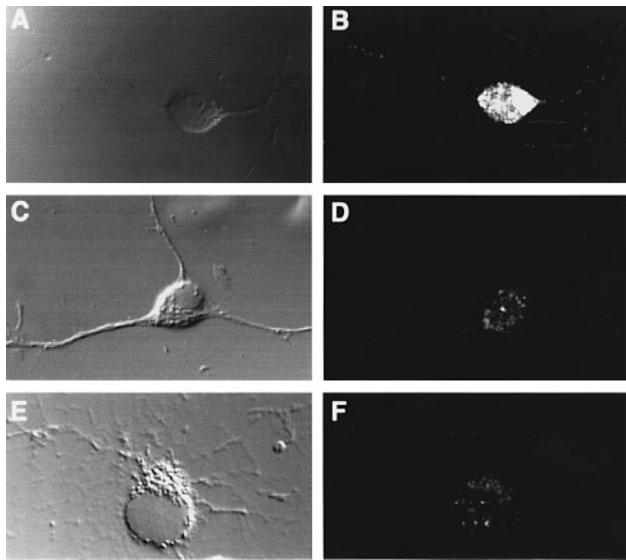


Fig. 1. Confocal fluorescence micrographs of Cdc2 staining. P7 rat optic nerves were cultured for 2 days in PDGF and then stained for Cdc2 by immunofluorescence. Oligodendrocyte precursor cells are shown in (A–D) and an oligodendrocyte in (E) and (F), using interference contrast (A, C and E) or fluorescence (B, D and F) optics. Note that the staining is both cytoplasmic and nuclear. The staining in (F) is not above background.

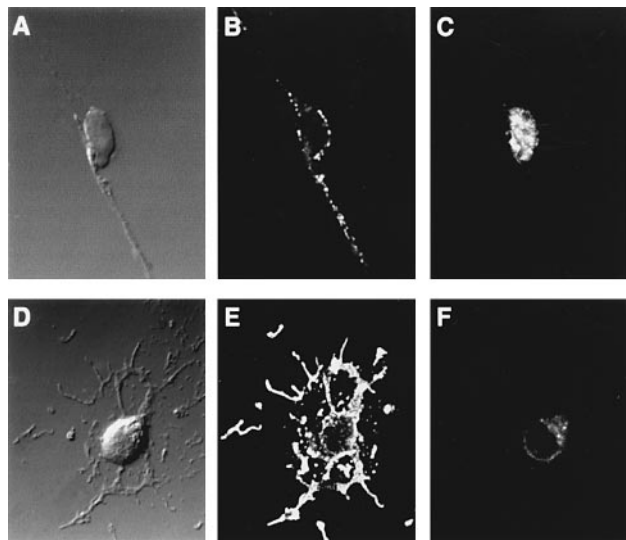


Fig. 2. Confocal fluorescence micrographs of cyclin D1 staining. P7 optic nerve cells were cultured for 2 days in PDGF and then stained for either A2B5 (B) or GC (E), and then for cyclin D1 (C and F). An A2B5⁺ oligodendrocyte precursor cell is shown in (A–C) and a GC⁺ oligodendrocyte is shown in (D–F), using differential interference (A and D) or fluorescence (B, C, E and F) optics. The cyclin D1 staining is nuclear; the staining of the Golgi apparatus by the anti-cyclin D1 serum is non-specific.

2A–C), while others were not stained above background levels (not shown). By contrast, most of the GC⁺ oligodendrocytes were not stained above background (Figure 2D–F). A small proportion of GC⁺ oligodendrocytes showed weak cyclin D1 staining, suggesting that the disappearance of cyclin D1 occurs after the precursor cells stop dividing. As for Cdc2, some fibroblast-like cells also expressed cyclin D1 (not shown).

In frozen sections of the adult rat optic nerve, Cdc2 staining was restricted to a small subpopulation of cells,

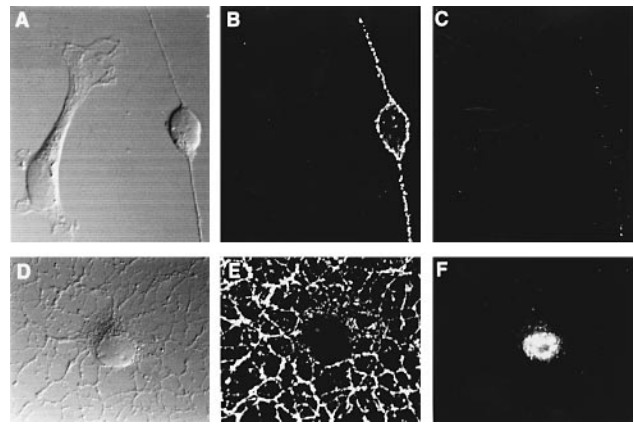


Fig. 3. Confocal fluorescence micrographs of p27 staining. P7 optic nerve cells were cultured for 2 days in PDGF and then stained for either A2B5 (B) or GC (E), and then for p27 (C and F). An A2B5⁺ precursor cell is shown in (A–C) and a GC⁺ oligodendrocyte in (D–F), using optics as in Figure 2. Note that the p27 staining is nuclear; the weak surface staining in (C) and (F) is breakthrough from the A2B5 and GC staining.

possibly adult oligodendrocyte precursor cells (French Constant and Raff, 1986; Wolswijk and Noble, 1989; Fulton *et al.*, 1992), while there was no appreciable cyclin D1 staining (not shown).

Taken together, these results suggest that both Cdc2 kinase and cyclin D1 are lost rapidly when oligodendrocyte precursor cells differentiate into oligodendrocytes.

High p27 in oligodendrocytes

Although the rabbit anti-p21 antiserum stained the nucleus of C2 muscle cells, it did not stain any cells in cultures of P7 optic nerve cells stimulated with PDGF for 48 h. This is not surprising, as previous studies found little p21/Cip1/Waf1 mRNA by *in situ* hybridization in developing mouse brain (Parker *et al.*, 1995).

By contrast, the anti-p27 antiserum (Toyoshima and Hunter, 1994) stained the nucleus of many cells in such cultures. The staining of A2B5⁺ oligodendrocyte precursor cells was variable—from undetectable (Figure 3A–C) to intense (not shown). By contrast, all GC⁺ oligodendrocytes were stained intensely (Figure 3D–F). Some fibroblast-like cells were also stained strongly (not shown). Similar results were obtained with affinity-purified anti-p27 antibodies, and all of this staining was removed by absorption with the immunizing peptide (not shown).

In frozen sections of adult optic nerve, no p21 staining was seen, but most of the glial cell nuclei showed strong p27 staining (not shown). As most of the glial cells in the adult optic nerve are oligodendrocytes, it is likely that most oligodendrocytes in the nerve have high levels of p27.

Taken together, these results suggest that the level of p27, but not of p21, increases in oligodendrocyte lineage cells as they differentiate, and that the level remains high in mature oligodendrocytes in the adult optic nerve.

Increase in p27 in relation to oligodendrocyte differentiation

The finding that p27 staining was high in some oligodendrocyte precursor cells raised the possibility that p27 begins to accumulate before the precursor cells stop dividing and begin to differentiate. To investigate this

Table I. Intensity of p27 staining in oligodendrocyte lineage cells in cultures with PDGF and TH

Cell type	p27 staining [average (range) in pixels]
Bipolar precursor cells	45 000 (20 000–80 000)
Multiprocessed precursor cells	110 000 (70 000–130 000)
Early oligodendrocytes	130 000 (80 000–180 000)
Late oligodendrocytes	130 000 (120 000–180 000)

P7 optic nerve cells were cultured for 48 h in the presence of PDGF and TH and were then stained by immunofluorescence for either A2B5 or GC and then p27. The brightness of p27 staining was measured using a confocal microscope, as described in Materials and methods. The average fluorescence intensity was selectively measured in the cell nucleus and converted into a numerical reading of arbitrary value (pixels). Between 50 and 100 cells were assessed for each cell type.

possibility further, we took advantage of previous findings that optic nerve oligodendrocyte lineage cells in culture can be classified by their morphology and surface antigenic phenotype into four categories representing successive stages of maturation (Noble and Murray, 1984; Raff *et al.*, 1985; Temple and Raff, 1986): (i) bipolar precursor cells, which are bipolar, A2B5⁺ and GC⁻; (ii) multiprocessed precursor cells, which have multiple processes but are still A2B5⁺ and GC⁻; (iii) early oligodendrocytes, which have multiple processes and are weakly A2B5⁺ and weakly GC⁺; and (iv) late oligodendrocytes, which have multiple processes and are A2B5⁻ and strongly GC⁺. Oligodendrocyte precursor cells normally withdraw from the cell division cycle while in category (ii), and GC expression begins ~24 h later (Noble and Murray, 1984; Raff *et al.*, 1985; Temple and Raff, 1986).

We double-labelled the cells in P7 optic nerve cultures after 48 h in PDGF for either A2B5 or GC, and then for p27. We then quantified the nuclear p27 staining by confocal fluorescence microscopy. As the A2B5 and anti-GC antibodies were not used together, we identified category (i) cells as A2B5⁺ and bipolar, category (ii) cells as GC⁻ and multiprocessed, category (iii) cells as weakly GC⁺ and multiprocessed, and category (iv) cells as strongly GC⁺ and multiprocessed. As can be seen in Table I, although there was some overlap in the intensity of p27 staining between categories, there was a progressive increase in p27 staining from category (i) through category (iv), with a 4- to 10-fold increase between (i) and (iv).

The finding that p27 begins to accumulate in oligodendrocyte precursor cells before they acquire GC to become oligodendrocytes raised the possibility that this accumulation is, in part at least, responsible for arresting the cell cycle, thereby initiating differentiation. To study further the relationship between the accumulation of p27 and the withdrawal from the cell cycle, we cultured P7 optic nerve cells in the absence of added PDGF. It was shown previously that most oligodendrocyte precursor cells in such cultures rapidly stop dividing and differentiate prematurely into post-mitotic, GC⁺ oligodendrocytes: by 24 h, ~10% of the cells become GC⁺ and by 3 days 85% do so (Raff *et al.*, 1984). We began by studying how quickly the precursor cells withdraw permanently from the cell cycle when deprived of PDGF.

Timing of cell cycle withdrawal upon PDGF removal

To determine how long it takes for an oligodendrocyte precursor cell to exit permanently from the cell cycle when deprived of mitogens, we cultured P3 optic nerve cells with or without added PDGF. After 6, 12, 18 or 24 h, PDGF and bromodeoxyuridine (BrdU) were added, to see how many precursor cells could still be induced to synthesize DNA. After a further 48 h, the cells were fixed and immunostained for either A2B5 and BrdU, or A2B5 and GC. For each time point, we counted both the percentage of A2B5⁺ cells that were BrdU⁺, and the percentage of oligodendrocyte lineage cells that were GC⁺. As shown in Table II, 25% of the oligodendrocyte lineage cells were GC⁺ oligodendrocytes in control cultures maintained for 66 h in the continuous presence of PDGF. As only 10% of the cells were GC⁺ at the start of the culture, the majority of these oligodendrocytes developed in culture, presumably through their normal intrinsic clock mechanism that operates in the presence of mitogens and hydrophobic signals (such as thyroid hormone, which was present in the culture medium). In control cultures maintained for 66 h without PDGF, 89% of the oligodendrocyte lineage cells were GC⁺ oligodendrocytes at the end of the culture period. When cells were cultured for the same time, but without PDGF for the first 18 h, 75% were GC⁺ by 66 h, suggesting that most of the precursor cells permanently withdrew from the cell cycle during the 18 h deprivation period, even though PDGF was present for the final 48 h. The results of the PDGF deprivation experiments are plotted in Figure 4 (lower curve).

Also shown in Table II are the results of cultures maintained for a total of 54, 60 or 72 h. When cells were maintained in culture for 54 h, there was only a small difference in the proportion of GC⁺ oligodendrocytes in cultures maintained in PDGF for the entire period and the proportion in cultures deprived of PDGF for the first 6 h. Thus a 6 h deprivation period was insufficient to cause most of the precursor cells to withdraw permanently from the cell cycle and differentiate, although it was enough to cause a few to do so. A 12 h deprivation induced about half of the precursor cells to withdraw from the cell cycle and differentiate (Table II and Figure 4), and a 24 h deprivation period induced 89% of them to do so.

In cultures deprived of PDGF for either 6 or 18 h, the proportions of A2B5⁺ oligodendrocyte precursor cells that were labelled with BrdU were not significantly different from the proportions labelled with BrdU in cultures maintained in PDGF for the entire culture period (Table II). This result suggests that all of the precursor cells that did not withdraw permanently from the cell cycle during the PDGF deprivation period were able to recover from the deprivation and re-enter S phase during the 48 h period in PDGF and BrdU.

Taken together, these findings indicate that, whereas 6 h of mitogen deprivation is enough to induce some oligodendrocyte precursor cells to stop dividing and differentiate, 18–24 h of deprivation is required to induce almost all of the cells to do so.

Timing of p27 accumulation upon PDGF removal

To help determine if p27 accumulation could be responsible for causing the oligodendrocyte precursor cells to

Table II. Commitment to oligodendrocyte differentiation upon PDGF deprivation for various times

Total culture period (h)	PDGF present (h)	Period of PDGF deprivation (h)	Percentage oligo lineage cells that are GC ⁺	Percentage A2B5 ⁺ cells that are BrdU ⁺
54	54	0	20 ± 1	62 ± 2
54	last 48	6	28 ± 5	60 ± 4
54	0	54	75 ± 2	28 ± 4
60	60	0	20 ± 3	ND
60	last 48	12	48 ± 4	ND
60	0	60	91 ± 3	ND
66	66	0	25 ± 2	77 ± 2
66	last 48	18	75 ± 2	83 ± 3
66	0	66	89 ± 3	2 ± 1
72	72	0	27 ± 3	ND
72	last 48	24	89 ± 4	ND
72	0	72	91 ± 3	ND

P3 rat optic nerve cells were cultured for up to 3 days, and PDGF was added at various times or not at all. BrdU was added for the last 48 h. The cells were stained for either A2B5 and BrdU or A2B5 and GC. The results are expressed as mean ± SEM of triplicate cultures. The experiment was repeated twice with similar results.

withdraw permanently from the cell cycle in response to PDGF deprivation, we cultured P3 optic nerve cells for 6, 12 or 24 h in the presence or absence of PDGF and immunostained them for A2B5 and p27. We assessed the intensity of p27 staining in A2B5⁺ cells by eye and categorized the staining as weak (corresponding to 20 000–30 000 pixels on the confocal microscope) or strong (corresponding to >60 000 pixels on the confocal microscope). The results are plotted in Figure 4 (upper curve).

As shown in Table III, at both time 0 and after 24 h in PDGF, 60–65% of the A2B5⁺ cells were stained weakly for p27, and 5–8% were stained strongly; the remainder were intermediate in staining intensity. The results were similar after 6 and 12 h in PDGF (not shown). After 6 h in the absence of PDGF, however, the proportion of A2B5⁺ cells that stained strongly for p27 had increased to ~20%, and after 24 h without PDGF the proportion had increased to ~70%; in both cases there was a corresponding decrease in A2B5⁺ cells that were stained weakly for p27 (Table III and Figure 4). Thus, an increase in p27 staining could be seen in some cells within 6 h of PDGF deprivation.

p27 accumulation in continuously proliferating precursor cells

To help determine if the accumulation of p27 could be part of the counting mechanism that measures time or counts cell divisions, we purified oligodendrocyte precursor cells from P7 optic nerve by sequential immunopanning and cultured them at low density in the presence of PDGF, NT-3 and forskolin, but in the absence of hydrophobic signals, as previously described (Barres *et al.*, 1994). In these conditions, precursor cells continue dividing even after the counting mechanism indicates that it is time to stop (Barres *et al.*, 1994). We stained the cells for p27 after 2 or 10 days and assessed the level of staining using a confocal microscope. At both time points, almost all of the cells were bipolar precursor cells, although a few cells with an oligodendrocyte morphology were present after 10 days. As shown in Table IV, the intensity of p27 staining in bipolar precursor cells increased from an average of 27 500 pixels (range 10 000–60 000) at 2 days (Figure 5A) to an average of 98 000 (range 65 000–150 000) at 10 days (Figure 5B). Even after 10 days, most

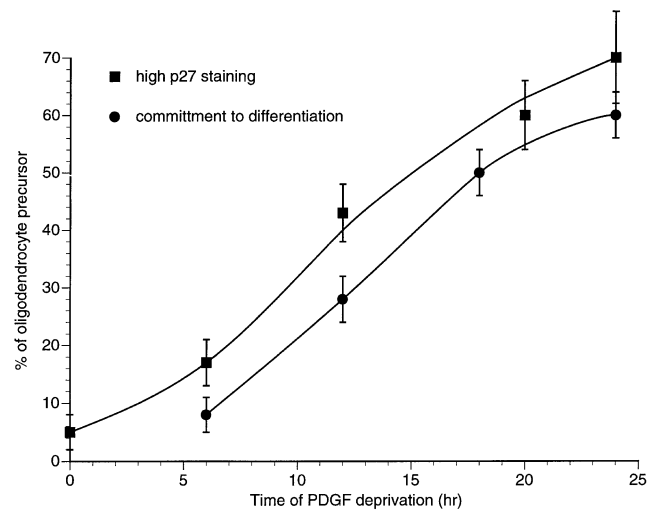


Fig. 4. Commitment to oligodendrocyte differentiation and increased p27 staining upon PDGF deprivation for various periods. P3 optic nerve cells were cultured for 3 days in PDGF and then deprived of PDGF for the time indicated. The data are taken from Tables II, except for the 24 h control point of the lower curve, which is taken from Raff *et al.* (1984).

Table III. Increase in p27 staining in oligodendrocyte precursor cells deprived of PDGF for various times in culture

Culture period (h)	PDGF	Weak p27 (%) (20 000–30 000 pixels)	Strong p27 (%) (≥60 000 pixels)
0	–	65 ± 10	5 ± 3
6	–	47 ± 5	17 ± 3
12	–	23 ± 7	43 ± 7
24	–	20 ± 7	70 ± 10
24	+	60 ± 10	8 ± 3

P3 optic nerve cells were cultured for up to 24 h and then labelled for A2B5 and p27. The brightness of p27 nuclear immunofluorescence was measured using a confocal microscope. About 100 cells were counted for each time point. The results are expressed as means ± SEM of triplicate cultures. The experiment was repeated twice with similar results.

of the cells were not in contact with other cells, suggesting that the increase in p27 did not depend on the cells reaching confluence. Similar results were obtained with both anti-p27 reagents.

Table IV. Increase in p27 staining in continuously dividing purified oligodendrocyte precursor cells

	Time in culture (days)	Additives	p27 staining [average (range) in pixels]
Expt 1	2	PDGF	27 500 (10 000–60 000)
	10	PDGF	98 000 (65 000–150 000)
Expt 2	2	PDGF	85 000 (45 000–140 000)
	10	PDGF	210 000 (130 000–320 000)
	2	PDGF + bFGF + TH	85 000 (45 000–140 000)
	10	PDGF + bFGF + TH	190 000 (110 000–280 000)

Purified P7 precursor cells were cultured for 2 or 10 days with the additives as indicated. All cultures contained NT-3 and forskolin. Cells were stained for p27 with either the anti-p27 antiserum (experiment 1) or the affinity purified anti-p27 antibodies (experiment 2). About 100 cells were measured for each point.

Purified oligodendrocyte precursor cells grown in the presence of PDGF, TH and bFGF behave similarly to precursor cells cultured in PDGF without TH: the counting mechanism operates but the effector mechanism does not, and the cells keep dividing and do not differentiate (Bögler and Noble, 1994). To determine if p27 also accumulates under these conditions, we cultured purified oligodendrocyte precursor cells at low density in the presence of PDGF, TH and bFGF. After 2 and 10 days, the cells were stained for p27. In parallel experiments, purified precursor cells were cultured in PDGF without TH. In these experiments, affinity-purified anti-p27 antibodies were used for staining, which gave higher levels of staining than the anti-p27 antiserum. As shown in Table IV, a similar increase in p27 staining was seen between day 2 and day 10 in both culture conditions.

These results suggest that p27 protein accumulates as precursor cells proliferate, but even the high levels of p27 found after 10 days in culture are insufficient to stop the cell cycle in the absence of hydrophobic signals or in the presence of both PDGF and FGF.

Time course of p27 accumulation in continuously proliferating precursor cells

To determine the time course of p27 accumulation in proliferating precursor cells, we cultured purified P7 precursor cells at clonal density in the presence of PDGF, NT-3 and forskolin, but in the absence of TH. After 2, 5, 8, 10 and 12 days, we stained the cells for p27 and assessed the intensity of staining by confocal microscopy. As shown in Figure 6A, p27 levels progressively increased between 2 and 10 days and then reached a plateau. To determine if p27 levels rise even further when precursor cells differentiate, we removed PDGF after 10 days so that the cells would stop dividing and differentiate, and we stained them 2 days later for p27. As shown in Figure 6A, p27 levels increased to levels that were substantially higher than the plateau level reached in proliferating precursor cells.

As expected, the levels of p27 staining varied less between cells within a clone than between cells of different clones, but even within clones the levels varied over a 2-fold range (not shown), presumably reflecting different phases of the cell cycle. Consistent with this presumption, when cells were caught in mitosis, just after nuclear division, the two daughter nuclei always displayed the same levels of p27 staining (Figure 6B) and this level was usually half that of the nucleus in cells with the highest level of staining within the same clone (not shown).

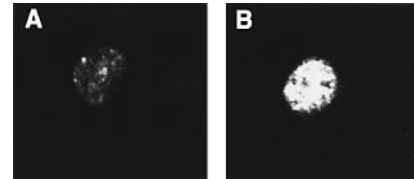


Fig. 5. Confocal fluorescence micrographs of p27 staining of purified oligodendrocyte precursor cells after 2 (A) or 10 days (B) in culture. Oligodendrocyte precursor cells were purified from P7 optic nerves and cultured in PDGF and NT-3, but without TH, and then stained for p27.

These results suggest that p27 levels plateau in proliferating precursor cells at around the time that the counting mechanism would normally indicate that it is time to stop dividing, and then the levels increase further when the cells withdraw from the cell cycle and differentiate.

Effect of p27 accumulation on cell cycle time

To determine whether the accumulation of p27 slows the cell cycle, we cultured purified P7 precursor cells at clonal density in the presence of mitogens and in the absence of hydrophobic signals, as just described. One clone at the two-cell stage was chosen to be studied by time-lapse video recording. The cell cycle time, measured between two consecutive telophases, was determined for each cell division between day 2 and day 5 in culture, and between day 9 and day 12 in culture, for cells of the same clone (Figure 7). The average cell cycle time measured in this way was 27 h between day 2 and day 5, which was similar to the doubling time (31.2 ± 2.4 h mean $\pm$ SEM, $n = 23$) during the first 4 days in culture, measured in individual clones growing at clonal density in a separate flask in an incubator. The average cell cycle time between day 9 and day 12 was ~ 30 h, which is not significantly different from the cell cycle time measured between days 2 and 5 (Figure 7). As some progenitor cells started to die or differentiate after 10 days, the doubling time of individual clones growing in a separate flask could not be assessed meaningfully. Similar results were obtained when another clone was studied in the same way in another experiment (not shown).

These findings suggest that, despite the accumulation of p27, most precursor cells continue to proliferate at about the same rate after 10 days in culture as they do at the start of the culture, as long as mitogens are present and hydrophobic signals like TH are not.

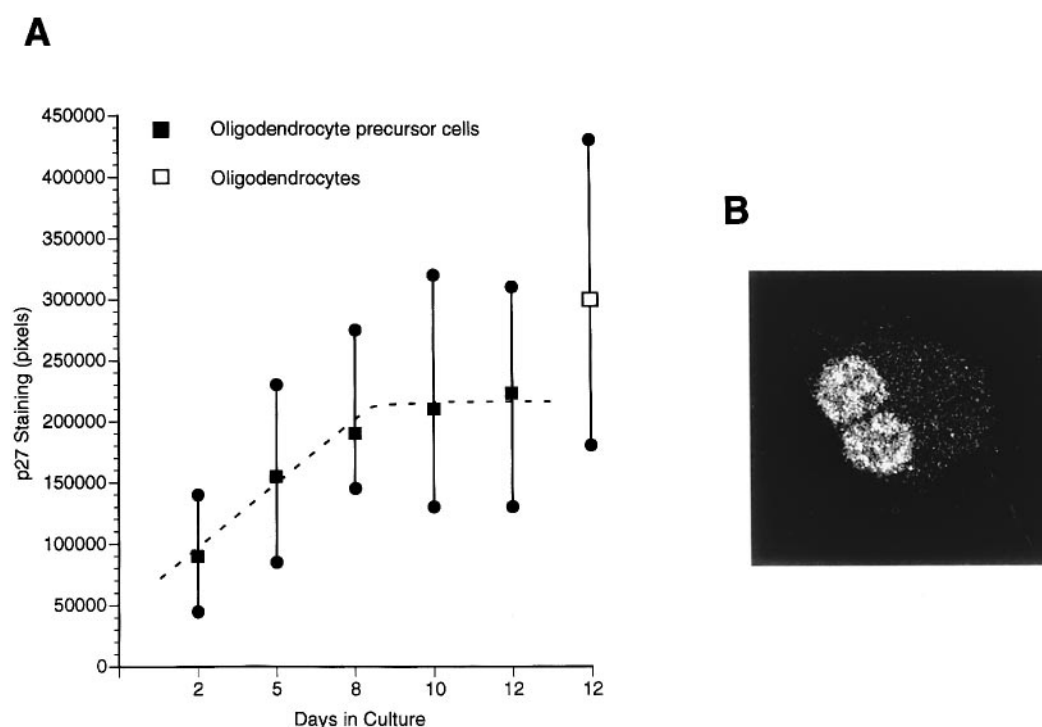


Fig. 6. Time course of p27 accumulation. (A) Purified P7 precursor cells were cultured at clonal density in PDGF and NT-3, but without TH, and were stained for p27 after various times in culture. The average intensity of staining (■) as well as the lowest and highest values (●) are shown. The experiment was done twice, and at least six clones were assessed for each point, except for 2 days where 20 clones were assessed. The oligodendrocytes were generated by removing the PDGF from the cultures after 10 days; 2 days later, the cells were stained for p27. (B) Confocal fluorescence micrograph of p27 staining in a precursor cell that had undergone nuclear division but had not yet divided.

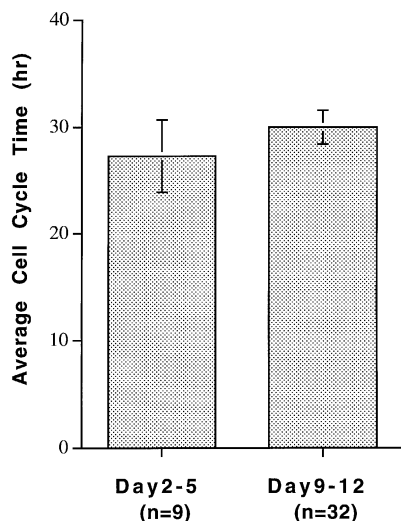


Fig. 7. Average cell cycle time of oligodendrocyte precursor cells in a single clone cultured as in Figure 5. The cell cycle times were measured by time-lapse video microscopy between days 2 and 5 in culture, and again between days 9 and 12 in culture. The results are expressed as means $\pm$ SEM.

Discussion

Much more is known about the mechanisms that stimulate cell proliferation during development than about those that stop proliferation after a limited number of cell divisions. However, the mechanisms that limit and stop cell division play crucial parts in both controlling cell number and timing cell differentiation. As these mechanisms are almost always abnormal in cell lines, they are best studied in normal (primary) cells. We have been

studying them in the cells of the oligodendrocyte cell lineage isolated from the developing rat optic nerve (Raff, 1989; Barres and Raff, 1994). In previous studies, we analysed the role of extracellular signals, mitogen receptors and intracellular signalling events (Hart *et al.*, 1989a,b; Raff, 1989; Barres *et al.*, 1992; Barres and Raff, 1994). In the present study, we focus on three components of the cell-cycle control system, Cdc2, cyclin D1 and the Cdk inhibitor p27, using immunofluorescence to detect the proteins in individual cells.

Cdc2

Cdc2 kinase drives eukaryotic cells out of G₂ into mitosis when the kinase is activated by cyclin B and appropriate phosphorylation and dephosphorylation (King *et al.*, 1994). As expected, we detect Cdc2 in oligodendrocyte precursors, which are proliferating cells, but not in oligodendrocytes, which are post-mitotic cells. The intensity of Cdc2 staining is variable in the precursor cells, suggesting that Cdc2 may start to decrease before the cells develop into GC⁺ oligodendrocytes. Because the Cdc2 staining is generally weak, we were unable to study the detailed timing of its loss as precursor cells differentiate. Given that oligodendrocyte precursor cells, like most vertebrate cells, withdraw from the cell cycle in G₁ at the start of differentiation, it seems unlikely that the loss of Cdc2 kinase, which operates at the G₂-M transition, causes the cells to stop dividing and differentiate.

Others previously have described the loss of Cdc2 when precursor cell lines (Buchkovitch and Ziff, 1994; Jahn *et al.*, 1994; Yan and Ziff, 1995) and normal neuronal precursor cells (Hayes *et al.*, 1991) stop dividing and differentiate. In all of these cases, the decrease in Cdc2

occurs late in the differentiation process, suggesting that it is not responsible for arresting the cell cycle.

Cyclin D1

Cyclin D1, like the other D cyclins, helps activate the Cdks that operate during G₁ to drive the cell past the restriction point (R) and into S phase (Hunter and Pines, 1994; Sherr, 1995). Cyclin D synthesis depends on mitogen stimulation (Sherr, 1995), and the levels of cyclin D, which increase in G₁ and then fall, help to control the length of G₁: overexpression of D cyclins, for example, shortens G₁ and reduces the ability of the cell to exit the cell cycle (Sherr, 1995).

As expected, we find that cyclin D1 is expressed in some oligodendrocyte precursor cells, presumably those in G₁, but not in oligodendrocytes, which are no longer dividing. The findings that both cyclin D1 and Cdc2 are not detectable in oligodendrocytes, either in cultures of developing optic nerve cells or in adult optic nerve, indicate that at least part of the cell cycle control system is dismantled in these cells.

Does the loss of cyclin D1 play a part in causing the precursor cells to withdraw from the cell cycle? The weak cyclin D1 staining we see in some GC⁺ oligodendrocytes suggests that the loss of the cyclin may follow rather than precede the cell cycle arrest. Previous studies on the differentiation of other cell types suggest that cyclin D1 levels can fall or rise when a cell differentiates. In the C2 mouse myoblast cell line, for example, cyclin D1 mRNA and protein levels decline during terminal cell differentiation (Jahn *et al.*, 1994; Skapek *et al.*, 1995); the decline in the mRNA, at least, probably occurs too late to be responsible for stopping cell division and initiating differentiation (Jahn *et al.*, 1994). By contrast, when the human myeloid cell line HL60 is induced to differentiate by phorbol esters, cyclin D1 mRNA and protein levels increase (Burger *et al.*, 1994; Horiguchi-Yamada *et al.*, 1994). Similarly, when PC12 cells are induced to differentiate into post-mitotic neurons by nerve growth factor (NGF), cyclin D1 protein gradually increases over 10 days (Yan and Ziff, 1995), and similar results have been reported for neuron differentiation during normal brain development (Tamaru *et al.*, 1994).

p27

p21 and p27 are Cdk inhibitors of the Kip/Cip family, which inhibit the Cdks responsible for the G₁–S transition. They bind to the cyclin–Cdk complexes and prevent the phosphorylations required for Cdk activation (Aprelikova *et al.*, 1995; Harper *et al.*, 1995). We cannot detect p21 protein in cells of the oligodendrocyte lineage, either in culture or in the adult optic nerve, which is consistent with the failure of Parker *et al.* (1995) to find p21 mRNA in the developing mouse brain. p27 mRNA, however, has been detected in both developing and adult mouse brain (Polyak *et al.*, 1994b; Lee *et al.*, 1996) and we detect the protein in oligodendrocytes both *in vitro* and *in vivo*.

The most important finding of the present study is that p27 protein levels progressively increase as oligodendrocyte precursor cells proliferate, and rise even higher when the cells stop dividing and terminally differentiate. Two previous studies suggested that the intrinsic clock mechanism that determines when oligodendrocyte precursor cells

stop dividing and differentiate consists of at least two components: a counting component that counts time or cell divisions and an effector component that stops cell proliferation when the counting component indicates it is time (Barres *et al.*, 1994; Böglér and Noble, 1994). The present findings raise the possibility that p27 accumulation is part of both components. Moreover, the finding that p27 levels are high in most glial cells in the adult optic nerve suggests that p27 may also play a role in preventing mature oligodendrocytes from dividing.

p27 and the effector mechanism that stops cell division

The finding that p27 levels are high in some oligodendrocyte precursor cells in short-term cultures of optic nerve cells stimulated by PDGF raised the possibility that p27 may help stop the precursor cells dividing, and thereby initiate differentiation when the counting component indicates it is time. To compare more accurately the timing of differentiation with the timing of p27 accumulation, we cultured optic nerve cells without PDGF in order to induce the oligodendrocyte precursor cells to stop dividing prematurely and differentiate more or less synchronously. Even after only 6 h of PDGF deprivation, there is an increase in the number of precursor cells with high p27 staining compared with control cells maintained in PDGF: whereas $5 \pm 3\%$ of the cells are strongly stained after 6 h in the presence of PDGF, $17 \pm 3\%$ are strongly stained after 6 h in its absence (Figure 4, upper curve).

To determine how quickly precursor cells irreversibly withdraw from the cell cycle in the absence of mitogen, we cultured optic nerve cells without PDGF for various times and then added PDGF for the last 48 h to see how many precursor cells had become post-mitotic GC⁺ oligodendrocytes and how many went back into cycle. We find that after 6 h of PDGF deprivation, 5–8% more precursor cells exit the cell cycle and differentiate than in control cultures maintained continuously in PDGF. As seen in Figure 4, there is a strong correlation between the percentage of precursor cells expressing high p27 staining and the percentage that commit to differentiate after the same period of PDGF deprivation, strengthening the possibility that the increase in p27 plays a part in stopping the cell cycle and initiating differentiation.

What distinguishes the precursor cells that drop out of cycle after 6 h of PDGF deprivation from those that do not? It has been shown in cultured fibroblasts that transient mitogen deprivation for an hour or more at any time after the cells have passed the restriction point (R) in G₁ results in an extension of the subsequent G₁ phase in the progeny cells (Larsson *et al.*, 1985; Okuda *et al.*, 1989). The cells record the deprivation by a concomitant decrease in *c-myc* expression (Waters *et al.*, 1991) and enter a resting G₀ phase after completing M phase; if mitogens are present, the cells re-enter the cell cycle after an 8 h delay (Zetterberg and Larsson, 1991; Zetterberg *et al.*, 1995). Assuming that oligodendrocyte precursors have a cell cycle time of ~1 day (Temple and Raff, 1985) and that the 6 h deprivation of PDGF causes the cells to enter a G₀ state after completing M phase (irrespective of where they were in the cycle at the time of deprivation), it is possible that the cells that irreversibly withdraw from the cell cycle are those that were in G₁ and not yet through R; they would

be the cells that enter G_0 earliest and therefore stay in G_0 longest. A less likely possibility is that the precursor cells respond to the deprivation according to their own counting mechanism; those that have been counting longest, for example, may be the most sensitive to mitogen deprivation.

As we discuss below, an increase in p27 is apparently insufficient on its own to stop the precursor cells dividing in the presence of mitogens. It has been shown previously that the effector mechanism responsible for stopping cell division in the presence of mitogens depends on extracellular hydrophobic signals such as thyroid hormone; in the absence of such signals, most of the precursor cells keep dividing and fail to differentiate (Barres and Raff, 1994; Barres *et al.*, 1994a). Because retinoic acid can mimic the effects of thyroid hormone, and both signalling molecules have been shown to inhibit the activity of the transcription factor AP-1 (Saatcioglu *et al.*, 1994), it has been suggested that the effector mechanism may act by inhibiting AP-1 activity (Barres *et al.*, 1994). One possibility is that both an increase in p27 and inhibition of AP-1 activity are required to stop precursor cell division. Another is that other Cdk inhibitors may co-operate with p27 to stop cell proliferation. A possible candidate is p15, which collaborates with p27 to stop proliferation when the Mv1Lu mink lung epithelial cell line is treated with transforming growth factor- β (TGF- β) (Reynisdóttir *et al.*, 1995). Another candidate is p57, which is highly expressed in the developing brain and is a potent inhibitor of Cdks (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). It is not known, however, if these, or other Cdk inhibitors, are expressed in oligodendrocyte lineage cells.

Role of Cdk inhibitors in arresting cell cycle in other cell types

Since their discovery, Cdk inhibitors have been associated with various examples of cell cycle arrest, and they have been proposed to play a major part in inducing terminal cell differentiation during development (Peter and Herskowitz, 1994; Reed *et al.*, 1994; reviewed in Sherr and Roberts, 1995). In yeast, for example, the Cdk inhibitor Far1 mediates the cell cycle arrest induced by secreted mating factor (Peter and Herskowitz, 1994). In various mammalian cell lines, an increase in p21 is associated with terminal differentiation into post-mitotic cells (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Halevy *et al.*, 1995; Macleod *et al.*, 1995; Parker *et al.*, 1995), but it has not been shown that the increased p21 expression is responsible for either arresting the cell cycle or initiating differentiation.

An increase in p27 has also been associated with cell cycle arrest in various circumstances. p27 activity increases, for example, when cell proliferation is inhibited by either cell-cell contact or treatment with TGF- β (Polyak *et al.*, 1994a; Reynisdóttir *et al.*, 1995) or cyclic AMP analogues (Kato *et al.*, 1994). Moreover, mitogens can decrease p27: interleukin-2 (IL-2), for instance, stimulates lymphocytes to enter S phase and decreases p27, and rapamycin, which blocks the mitogenic action of IL-2, prevents the decrease in p27 (Nourse *et al.*, 1994). Overexpression of p21 or p27 blocks cell cycle progression in G_1 in all cell lines that have been tested (reviewed in Hunter and Pines, 1994; Peter and Herskowitz, 1994; Reed *et al.*, 1994; Sherr and Roberts, 1995).

p27 and the counting mechanism

The most interesting finding in the present study is that p27 protein increases when purified oligodendrocyte precursor cells proliferate in culture under conditions where the counting mechanism operates but the effector mechanism does not: in one case the cells are cultured in the presence of mitogens but in the absence of the hydrophobic signals (Barres *et al.*, 1994); in the other they are cultured in the presence of PDGF, bFGF and TH (Bögler and Noble, 1994). After 10 days in culture under these conditions, only rare cells differentiate into oligodendrocytes, while the other cells continue to divide, even though many of the precursor cells have as high levels of p27 as the few oligodendrocyte-looking cells that develop in the cultures. Moreover, time-lapse video microscopy shows that the cell cycle time is not increased after 10 days in the first of these conditions. These remarkable findings indicate that the increase in p27 is insufficient on its own to stop or slow the cell cycle or to induce oligodendrocyte differentiation. This suggests that the p27 is somehow sequestered, perhaps by binding to cyclin D-Cdk4/6 complexes (Soos *et al.*, 1996). Most importantly, our findings suggest that the accumulation of p27 may be part of the intrinsic counting mechanism in the precursor cells that counts time or cell divisions and triggers the effector mechanism to stop cell division and initiate differentiation when time is reached. Our finding that p27 levels reach a plateau after P7 precursor cells have been proliferating in culture for 10 days is consistent with this suggestion, as it has been found previously that most P7 precursor cells will have stopped dividing and will have differentiated by this time in culture (Temple and Raff, 1986; Barres *et al.*, 1994). The correlation between p27 accumulation and the counting mechanism is seen in another circumstance: purified oligodendrocyte precursor cells stop dividing and differentiate sooner when cultured at 33°C than when cultured at 37°C, suggesting that the counting mechanism runs faster at the lower temperature, and p27 also increases faster at 33°C than at 37°C (F.-B.Gao, B.Durand and M.Raff, submitted).

If p27/Kip1 accumulation is part of the intrinsic counting component, it would be one of the few examples in developmental biology where the molecular basis of a timing mechanism is starting to become clearer. Normal mammalian fibroblasts in culture also divide a limited number of times before they arrest, a process called cell senescence. It has been suggested that the progressive accumulation of Cdk inhibitors may be responsible (Noda *et al.*, 1994; reviewed in Stein and Dulic, 1995), and recent evidence suggests a role for p16Ink4 (Hara *et al.*, 1996; Serrano *et al.*, 1996). In senescent fibroblasts, however, cyclin D1 increases (Dulic *et al.*, 1993; Lucibello *et al.*, 1993; Stein and Dulic, 1995), in contrast to oligodendrocytes where it disappears.

By what mechanism might p27 accumulate in proliferating oligodendrocyte precursor cells? Whereas p21 levels are often regulated transcriptionally (Sherr and Roberts, 1995), p27 levels are often regulated post-transcriptionally—by protein sequestration (Polyak *et al.*, 1994a), translational control (Hengst and Reed, 1996) or ubiquitin-dependent proteolysis (Pagano *et al.*, 1995). In some cells, p27 degradation depends on the ubiquitin-conjugating enzymes Ubx2 and Ubx3: when these cells arrest in

response to growth factor deprivation, the level of p27-ubiquitinating activity decreases, causing a marked increase in the half-life of p27 (Pagano *et al.*, 1995). It is possible, therefore, that a progressive decrease in the ubiquitin-dependent degradation of p27 is responsible for the accumulation of p27 in proliferating oligodendrocyte precursor cells.

Recently three laboratories independently produced p27-deficient mice by targeted gene disruption (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). The mice are on average 36% larger than wild-type mice, apparently as a result of increased cell division in multiple organs. Moreover, heterozygous p27^{+/-} mice are intermediate in size between p27^{+/+} and p27^{-/-} mice, as might be expected if an accumulation of p27 normally helps to limit cell proliferation in various cell lineages. These results potentially generalize the significance of our findings.

Materials and methods

C2C12 cells

The C2C12 myoblast cell line was obtained from Dr J. Adams. The cells originally were derived from an adult C3H mouse (Yaffe and Saxel, 1977). They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS) and passaged when they reached ~70% confluence.

Antibodies

We used a monoclonal anti-Cdc2 antibody (Nebreda *et al.*, 1995) and a rabbit antiserum against cyclin D1 (kindly provided by G. Peters). We used two rabbit antibodies against p27, both of which gave similar results. One was an antiserum raised against full-length mouse p27 protein fused with GST (Toyoshima and Hunter, 1994); it showed no cross-reactivity with recombinant mouse p57/Kip2 protein when tested by Western blotting (R. Poon, personal communication). The other anti-p27 antibodies (sc-528, Santa-Cruz) were raised against a peptide corresponding to amino acids 181–198 at the carboxy-terminus of the human p27 protein; they were affinity purified using the same peptide. We used a rabbit antiserum against mouse p21 (a gift from W. Harper and S. Elledge). The antibodies used to detect the cell cycle proteins were first tested on the mouse skeletal muscle cell line C2, proliferating in 1% FCS. The monoclonal anti-Cdc2 antibody (Nebreda *et al.*, 1995) stained the cytoplasm and nucleus of C2 cells. The rabbit anti-cyclin D1 antiserum stained the nucleus and, non-specifically, the Golgi apparatus; only the nuclear staining could be inhibited by absorption with cyclin D1 protein. Both p27 reagents and the anti-p21 serum stained the nucleus. The staining with the affinity-purified anti-p27 antibodies was removed completely by absorption with the immunizing peptide.

Optic nerve cultures

Sprague-Dawley (SD) rats were obtained from the breeding colony of University College London. All chemicals were from Sigma, unless indicated otherwise. The optic nerves were removed from P3 or P7 rats. They were cut into fragments and dissociated with trypsin (0.05%, Boehringer Mannheim) in Earle's balanced salt solution (EBSS). Cells were dissociated by passing the nerve fragments through a 21 and then 23 gauge needle in DMEM containing 30% FCS and DNase (0.004%). The cells were plated onto poly-D-lysine (PDL)-coated glass coverslips (25 000 cells per coverslip) and grown in 5% CO₂ at 37°C in Bottenstein-Sato (B-S) medium (Bottenstein and Sato, 1979; Lillien and Raff, 1990), modified as previously described (Lillien and Raff, 1990), containing 0.5% FCS. PDGF-AA (Peprotech) was added to a final concentration of 10 ng/ml when indicated.

Cultures of purified oligodendrocyte precursor cells

Oligodendrocyte precursor cells were purified to >99% purity from P7 optic nerves by sequential immunopanning, as described previously (Barres *et al.*, 1992). About 5000 cells were cultured in a PDL-coated slide flask (Nunc) in 2 ml of B-S medium containing PDGF-AA (10 ng/ml), NT-3 (5 ng/ml), ciliary neurotrophic factor (CNTF, 10 ng/ml) and forskolin (10 µM), but in the absence of TH. All of the

factors were used at concentrations that were on the plateau of their dose-response curves. Half of the medium was changed every 2 days. Recombinant rat CNTF was a gift from M. Sendtner, and mouse NT-3 was a gift from Y.-A. Barde. They were prepared as previously described (Stockli *et al.*, 1989; Gotz *et al.*, 1992).

Immunofluorescence staining

For staining cells on their surface for A2B5 or GC, the cells were fixed in 2% paraformaldehyde for 3 min at room temperature. After washing, they were incubated for 15 min in 50% normal goat serum (NGS) to block non-specific staining. They were then incubated in either the A2B5 monoclonal antibody (Eisenbarth *et al.*, 1979, ascites fluid, diluted 1:100), followed by Texas red-coupled goat anti-mouse IgM (Accurate; diluted 1:100) or monoclonal anti-GC antibody (Ranscht *et al.*, 1982; hybridoma culture supernatant, diluted 1:5), followed by Texas red-coupled goat anti-mouse IgG3 (Nordic; diluted 1:100).

For intracellular staining for Cdc2, cyclin D1, p21 or p27, the cells were fixed in 100% methanol at -20°C for 5 min. After washing and blocking in NGS as above, the cells were stained with monoclonal anti-Cdc2 antibody (Nebreda *et al.*, 1995; ascites fluid diluted 1:100), followed by biotin-coupled goat anti-mouse immunoglobulin (Ig) (Amersham; diluted 1:100) and fluorescein-coupled streptavidin (Amersham; diluted 1:100). The other proteins were detected with rabbit antisera against cyclin D1 (gift from G. Peters, diluted 1:1000), p21 (gift from W. Harper and S. Elledge, diluted 1:500), p27 (gift from H. Toyoshima and T. Hunter, diluted 1:500) or with affinity-purified rabbit anti-p27 antibodies (sc-528; purchased from Santa-Cruz and diluted 1:30). The rabbit antibodies were visualized with biotin-coupled goat anti-rabbit Ig (Chemicon; diluted 1:100), followed by fluorescein-coupled streptavidin.

For BrdU staining, BrdU (Boehringer Mannheim) was added to the culture medium to a final concentration of 10 µM. Cells were fixed in 100% methanol at -20°C for 5 min, incubated in 2 M HCl for 10 min to denature the DNA, followed by 0.1 M sodium borate pH 8.5 for 10 min. The cells were then incubated in 50% NGS for 15 min, then monoclonal anti-BrdU antibody (Magaud *et al.*, 1988; ascites fluid, diluted 1:100) and then fluorescein-coupled goat anti-mouse IgG1 (Amersham; diluted 1:100).

All incubations were for 25 min at room temperature, and the dilutions were in Tris-buffered saline containing 1% bovine serum albumin and 10 mM L-lysine. The coverslips were mounted in Citifluor mounting medium (Citifluor UK, UK) on glass slides and sealed with nail varnish, before they were examined with a Zeiss Universal fluorescence microscope.

Quantification of fluorescence by confocal microscopy

To quantify the intensity of p27 staining, cells were viewed in a Bio-Rad MRC 1000 confocal, laser scanning fluorescence microscope. Individual cells were selected at random, and the area command was used to collect brightness readings in the nucleus of the cell. The settings were kept the same for all the measurements in all experiments. The average fluorescence intensities were converted into numerical readings of arbitrary value (pixels).

Time-lapse video recording

Purified oligodendrocyte precursor cells from P7 optic nerve were cultured at clonal density in a PDL-coated slide flask (Nunc) in B-S medium containing PDGF, NT-3 and forskolin, but no TH. After 2 days in an incubator, the flask was placed on the stage of an inverted phase-contrast microscope and maintained at 37°C. A two-cell clone was chosen for study, and time-lapse video recordings were made using a Sony CCD black and white video camera and a Sony video cassette recorder. Cell cycle times were measured for 3 days and the cells were returned to a CO₂ incubator for 4 days. The same clone was then identified and studied by time-lapse video recording for another 3 days. Cell cycle times were measured from one telophase to another.

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