The EMBO Journal Vol. 19 No. 9 pp. 1998–2007, 2000

The Id4 HLH protein and the timing of oligodendrocyte differentiation

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An intracellular timer is thought to help control the timing of oligodendrocyte differentiation. We show here that the expression of the helix–loop–helix gene *Id4* in oligodendrocyte precursor cells decreases *in vivo* and *in vitro* with a time course expected if Id4 is part of the timer. We also show that *Id4* expression decreases prematurely when the precursor cells are induced to differentiate by mitogen withdrawal. Both Id4 mRNA and protein decrease together under all of these conditions, suggesting that the control of Id4 expression is transcriptional. Finally, we show that enforced expression of *Id4* stimulates cell proliferation and blocks differentiation induced by either mitogen withdrawal or treatment with thyroid hormone. These findings suggest that a progressive fall in *Id4* transcription is part of the intracellular timer that helps determine when oligodendrocyte precursor cells withdraw from the cell cycle and differentiate.

Keywords: HLH/Id4/oligodendrocyte/timer

Introduction

In many vertebrate cell lineages, precursor cells divide a limited number of times before they stop and terminally differentiate into post-mitotic cells. It is unknown what limits cell proliferation and causes the cells to stop dividing and differentiate when they do.

We have been studying the mechanisms that stop precursor cell division and initiate differentiation in the oligodendrocyte cell lineage in the rodent optic nerve (reviewed in Barres and Raff, 1994). The normal timing of oligodendrocyte development can be reconstituted in cultures of dissociated, perinatal, rat optic nerve cells: as long as the oligodendrocyte precursor cells are stimulated to proliferate by either astrocytes (Raff *et al*., 1985) or platelet-derived growth factor (PDGF) (Raff *et al*., 1988), oligodendrocytes begin to appear at the equivalent of the day of birth, just as they do *in vivo* (Miller *et al*., 1985). Clonal analyses of either single (Temple and Raff, 1986) or purified (Barres *et al*., 1994) precursor cells isolated from postnatal day 7–8 (P7–8) 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. In the presence of appropriate signalling molecules, such precursor cells divide up to eight times before they stop and differentiate, and the progeny of an individual precursor cell tend to stop dividing and differentiate at about the same time (Temple and Raff, 1986; Barres *et al*., 1994). Moreover, when the two daughter cells of an individual precursor cell are separated and cultured on astrocyte monolayers in separate microwells, they tend to differentiate more or less synchronously, suggesting that an intrinsic mechanism operates in the precursor cells to cause them to withdraw from the cell cycle and differentiate after a certain period of time or number of cell divisions (Temple and Raff, 1986). When precursor cells are cultured at 33°C rather than 37°C, they divide more slowly but differentiate sooner, after fewer cell divisions, suggesting that the intrinsic mechanism does not operate simply by counting cell divisions but instead measures elapsed time in some other way (Gao *et al*., 1997). We therefore refer to this intracellular mechanism as a timer.

Although the timer is cell intrinsic, it depends on extracellular PDGF and hydrophobic signals such as thyroid hormone (TH) in order to function normally: when cultured in the absence of PDGF, the precursor cells immediately stop dividing and differentiate (Noble and Murray, 1984; Temple and Raff, 1985); in the absence of TH (and the presence of PDGF), the cells keep dividing, without differentiating, for much longer than normal (Barres *et al*., 1994). If TH is added after a week or more to P7–8 precursor cells growing in PDGF, most of the cells stop dividing and differentiate within 4 days, suggesting that the timer consists of a timing component that measures elapsed time independently of TH and a TH-regulated effector component that stops proliferation and initiates differentiation when the timer indicates that it is time (Barres *et al*., 1994; Bögler and Noble, 1994). TH is also required for the normal timing of oligodendrocyte differentiation *in vivo* (Ibarrola *et al*., 1996; Ahlgren *et al*., 1997; Knipper *et al*., 1998).

The cyclin-dependent kinase (Cdk) inhibitor p27/Kip1 (p27) is apparently one element of the timer and seems to play a part in both the timing and effector components, as both are perturbed in p27-deficient precursor cells (Casaccia-Bonnefil *et al*., 1997; Durand *et al*., 1997, 1998). Moreover, p27 protein levels progressively increase as the precursor cells proliferate *in vivo* and *in vitro* (Durand *et al*., 1997) and do so more quickly when the cells are cultured at 33°C rather than at 37°C (Gao *et al*., 1997). As cell numbers are increased in all organs that have been examined in p27-deficient mice (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996), it seems likely that p27 normally plays a similar role in limiting cell proliferation in many cell lineages. Oligodendrocyte precursor cells in p27-deficient mice, however, still stop dividing and differentiate, although belatedly (Durand *et al*., 1998), suggesting that p27 is only one part of a multicomponent mechanism that is respon-

sible for stopping the cell cycle and initiating differentiation at the appropriate time in development.

Id proteins are helix–loop–helix (HLH) proteins that lack the basic DNA-binding domain of basic HLH (bHLH) proteins. They form heterodimers with bHLH proteins that regulate cell type-specific gene expression during cell commitment and differentiation and block the ability of the bHLH proteins to bind to DNA and activate gene transcription (reviewed in Norton *et al*., 1998). In this way, the Id proteins inhibit the commitment or differentiation that the bHLH proteins promote. Thus, overexpression of *Id* genes, for example, can inhibit the differentiation of B lymphocytes (Sun, 1994), muscle cells (Jen *et al*., 1992), mammary epithelial cells (Desprez *et al*., 1995), myeloid cells (Kreider *et al*., 1992) and erythroid cells (Lister *et al*., 1995). In addition, Id proteins can also stimulate cell proliferation: antisense oligonucleotides that inhibit *Id* gene expression, for instance, delay the serum-stimulated re-entry of growth-arrested 3T3 cells into the cell cycle (Barone *et al*., 1994; Hara *et al*., 1994), whereas overexpression of *Id1*, *Id2* or *Id3* in rat embryo fibroblasts promotes progression into S phase (Norton and Atherton, 1998). Moreover, *Id* expression declines as cultured human fibroblasts undergo replicative senescence and lose their proliferative capacity (Hara *et al*., 1994).

A role for Id proteins in oligodendrocyte development has not yet been demonstrated, although there is a preliminary report that *Id2* is expressed in oligodendrocyte precursor cells and that *Id2* overexpression inhibits oligodendrocyte differentiation (Sdrulla *et al*., 1999). In the present study, we show that all four known mammalian *Id* genes are expressed in rat oligodendrocyte precursor cells and that Id4 mRNA and protein, which are only expressed in the nervous system (Riechmann *et al*., 1994; Jen *et al*., 1996), progressively decrease as the precursor cells proliferate *in vitro* and *in vivo* and do so more quickly when the cells are cultured at 33°C compared with at 37°C. Moreover, Id4 mRNA and protein levels fall prematurely when the precursor cells are induced to differentiate prematurely by PDGF withdrawal. We also show that enforced expression of *Id4* in purified precursor cells stimulates cell proliferation and blocks differentiation induced by either PDGF withdrawal or TH addition. Together, these findings suggest that the progressive decrease in *Id4* transcription may be part of the cell-intrinsic timer that helps determine when oligodendrocyte precursor cells withdraw from the cell cycle and differentiate.

Results

Id4 expression decreases as oligodendrocyte precursor proliferates in vitro and in vivo

To determine whether *Id* genes might play a part in timing oligodendrocyte development, we examined the expression of the four known mammalian *Id* genes, *Id1* (Benezra *et al*., 1990), *Id2* (Sun *et al*., 1991), *Id3* (Christy *et al*., 1991) and *Id4* (Riechmann *et al*., 1994), in purified oligodendrocyte precursor cells using semi-quantitative RT–PCR. As shown in Figure 1A, all four *Id* genes were expressed in precursor cells purified from newborn (P0) rat optic nerve. The level of *Id4* mRNA gradually decreased when the cells were cultured in PDGF in the absence of TH

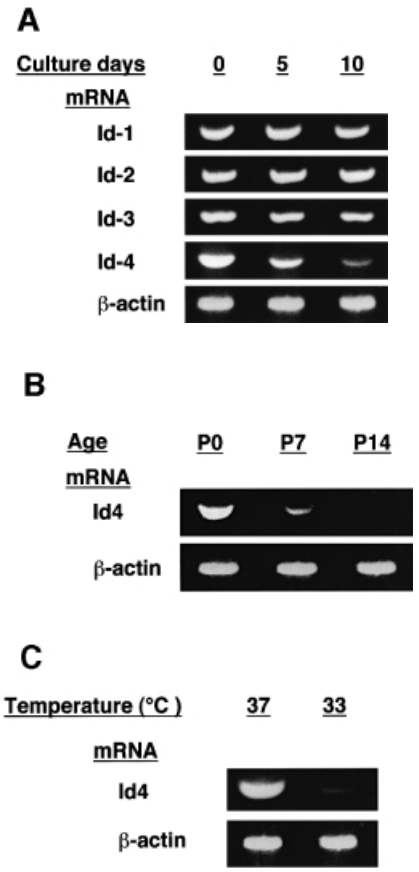

Fig. 1. The level of *Id4* mRNA in oligodendrocyte precursor cells decreases with time *in vitro* and *in vivo* and decreases more quickly *in vitro* at 33°C than at 37°C. The levels of mRNA were assessed by RT–PCR, and β-actin mRNA was analysed as a control. (**A**) Purified P0 precursor cells were studied either immediately after purification or after they were cultured for 5 or 10 days in the presence of PDGF and the absence of TH. (**B**) Precursor cells were purified from P0, P7 and P14 rats and analysed immediately after purification. (**C**) Purified P0 precursor cells were cultured for 1 day at 37°C and then for a further 4 days at either 33 or 37°C.

to stimulate proliferation and prevent differentiation (Barres *et al*., 1994), whereas the levels of *Id1*, *Id2* and *Id3* mRNA did not change under these conditions (Figure 1A).

To examine the expression of Id4 protein, we used rabbit anti-Id4 antibodies in indirect immunofluorescence assays. As shown in Figure 2, we could readily detect Id4 protein, which was mainly nuclear, in P0 precursor cells cultured for 1 (Figure 2A) or 5 days (Figure 2B). In cells cultured for 10 days, however, the cells were stained only weakly or not at all (Figure 2C). When we quantified the staining intensity by confocal microscopy, the average level of staining in the precursor cells cultured for 5 days was ~3-fold higher than that in cells cultured for 10 days (Figure 2F). The expression of Id1, Id2 and Id3 proteins did not change under these conditions (not shown). Thus, both Id4 mRNA and protein decrease as precursor cells proliferate in culture, whereas the levels of the other Id mRNA and proteins do not change detectably.

To determine whether *Id4* expression also decreases as the precursor cells proliferate *in vivo*, we studied freshly purified precursors cells from P0, P7 and P14 optic nerve.

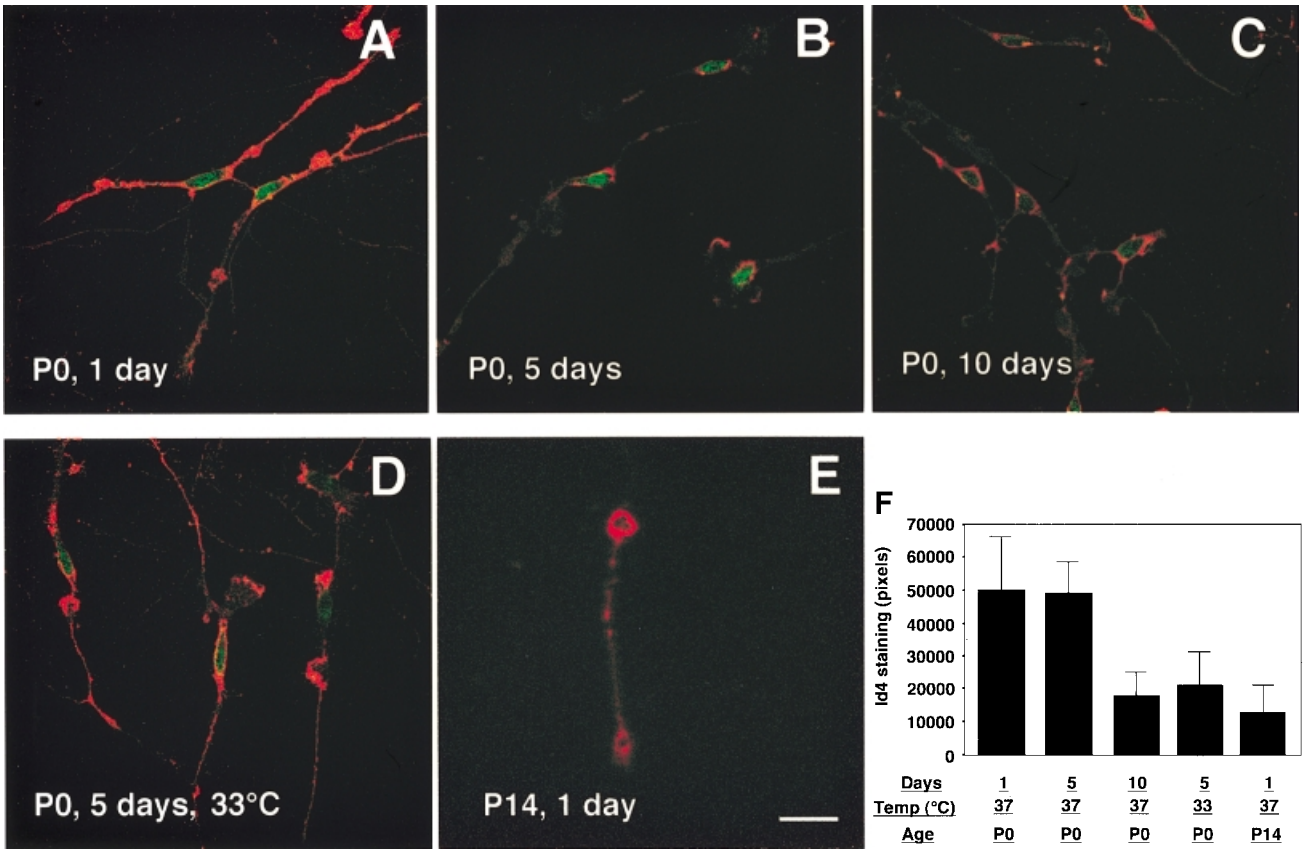

Fig. 2. Immunofluorescence staining of Id4 protein in purified oligodendrocyte precursor cells. The cells were stained for A2B5 (red), to label the precursor cells, and for Id4 (green) and then examined and photographed in a confocal microscope. P0 cells were cultured for 1 (**A**), 5 (**B**) or 10 days (**C**) at 37°C or for 1 day at 37°C and a further 4 days at 33°C (**D**). In (**E**), purified P14 precursor cells were cultured for 1 day. Quantification of the intensity of nuclear Id4 staining is shown in (**F**) as the mean ± SD of 50 cells. Scale bar, 25 μm.

The expression of *Id4* gradually decreased with age, and both Id4 mRNA (Figure 1B) and protein (Figure 2E) were undetectable in P14 precursor cells.

The intrinsic timer in the precursors runs more quickly at 33°C than at 37°C (Gao *et al*., 1997). To determine if *Id4* expression decreases more quickly at 33°C than at 37°C, we examined both Id4 mRNA and protein in cells cultured for 4–5 days at the two temperatures. Both Id4 mRNA (Figure 1C) and protein (Figure 2D) decreased more quickly at the lower temperature. The average level of Id4 protein in the precursor cells cultured at 37°C for 5 days was ~3-fold higher than that in cells cultured at 33°C for the same period (Figure 2F).

Id4 protein decreases prematurely when precursor cells are induced to differentiate prematurely by PDGF withdrawal

To determine the relationship between the level of Id4 protein and oligodendrocyte differentiation, we purified P0 precursor cells and cultured them for 1 day in PDGF and then for another 4 days without PDGF to induce them to differentiate prematurely (Noble and Murray, 1984; Temple and Raff, 1985). Using anti-Id4 antibodies and indirect immunofluorescence, we could detect Id4 protein readily in P0 precursor cells that were cultured for either 2 days without PDGF (Figure 3A) or 4 days with PDGF (Figure 3D), but the level of Id4 was greatly decreased after 3 days without PDGF (Figure 3B) and was almost undetectable after 4 days without PDGF (Figure 3C), when ~80% of the cells had differentiated into galactocerebroside $(GC)^+$ oligodendrocytes (Figures 3E and 4B).

To follow differentiation in these cells, we triple stained them with anti-GC antibody to identify oligodendrocytes (Raff *et al*., 1978), anti-Id4 antibodies, and with bisbenzimide to identify all nuclei, and quantified the intensity of Id4 staining by confocal microscopy. As shown in Figure 4, as the level of Id4 progressively decreased in cells cultured in the absence of PDGF (Figure 4A), the proportion of GC^+ oligodendrocytes increased correspondingly (Figure 4B). These findings are consistent with the possibility that Id4 normally negatively regulates oligodendrocyte differentiation.

Overexpression of Id4 inhibits oligodendrocyte differentiation induced by either PDGF withdrawal or TH addition

To determine whether Id4 can inhibit oligodendrocyte differentiation, we infected purified P6 precursor cells overnight with either the BabeG-Id retroviral vectors, which encode green fluorescent protein (GFP) and either Id1 or Id4 protein, or the BabeG control retroviral vector, which encodes GFP only. We examined the level of Id1 or

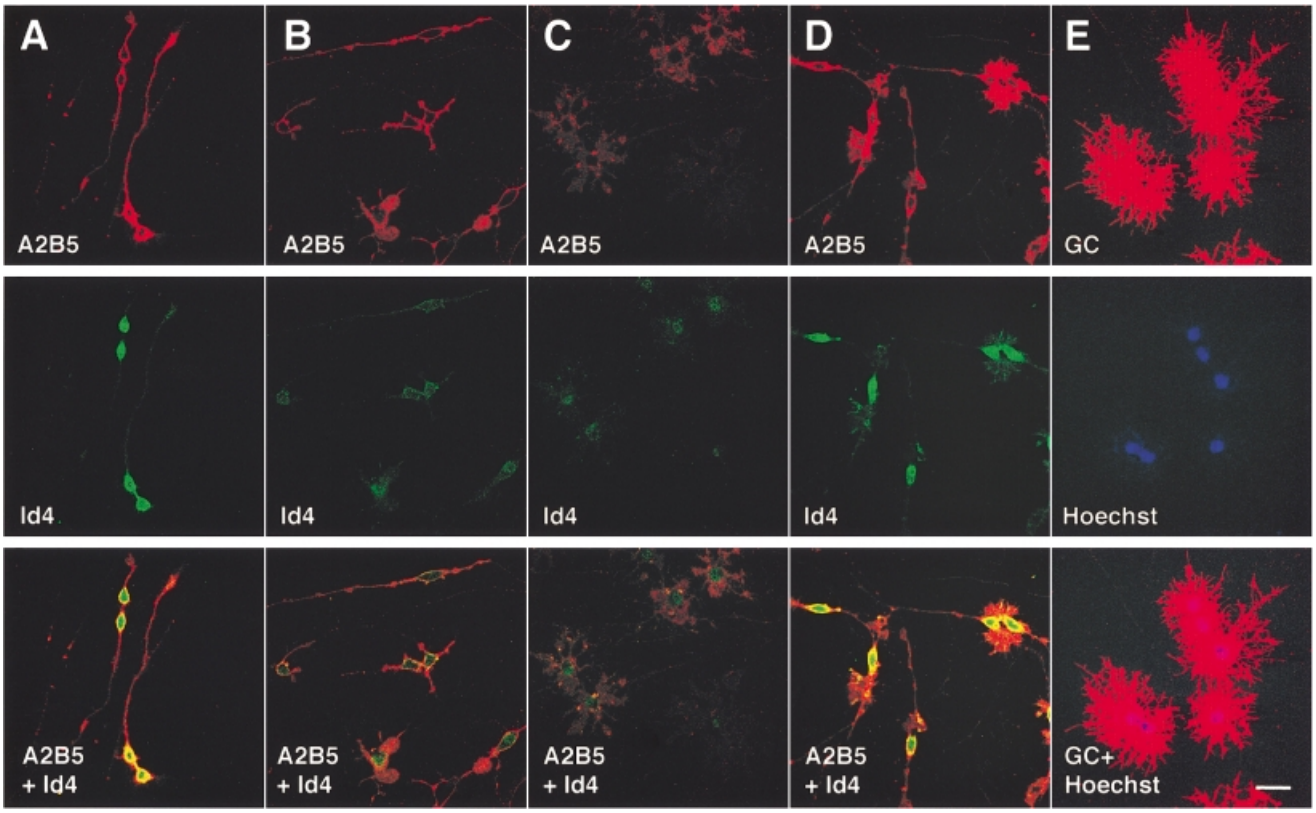

Fig. 3. Confocal immunofluorescence micrographs of purified P0 cells induced to differentiate by PDGF withdrawal. The cells were cultured for 1 day in PDGF, washed and then cultured either for 2 (**A**), 3 (**B**) or 4 days (**C** and **E**) without PDGF or for 4 days with PDGF (**D**). In (A–D), cells were immunostained for A2B5 (red, top panels) and Id4 (green, middle panels); the fused images of A2B5 and Id4 staining are shown in the bottom panels. In (E), cells were cultured for 4 days without PDGF and then stained for galactocerebroside (GC) to label oligodendrocytes (top panel) and with Hoechst 33342 to label all nuclei (middle panel); the fused image of GC and Hoechst staining is shown in the bottom panel. Scale bar, 25 μm.

Id4 protein in the infected precursor cells by immunofluorescence staining 5 days after infection. We could readily detect Id4 protein in BabeG-Id4-infected precursor cells, whereas we could barely detect the protein in BabeG-infected cells (Figure 5). When we quantified the staining intensity by confocal microscopy, the level of staining in BabeG-Id4-infected cells was at least 2-fold higher on average than in BabeG-infected cells (not shown). The level of Id1 protein in BabeG-infected cells was very high, and we could not detect a significant increase in BabeG-Id1-infected cells (not shown).

After infection, the virus-infected cells were cultured for another 2 days in PDGF without TH, and then, to induce the cells to differentiate into oligodendrocytes, they were cultured either without PDGF for 3 days or with PDGF and TH for 5 days. The cells were then stained with either anti-GC antibody to identify oligodendrocytes or A2B5 antibody to identify the precursor cells. With either oligodendrocyte-inducing treatment, most of the BabeG-Id4-infected cells remained $A2B5^+$ (Figures 6D, and 7A and B) and GC^- (Figures 6C, and 7A and B) and retained a bipolar morphology (Figure 6D) typical of precursor cells (Temple and Raff, 1986), while most of the BabeG- and BabeG-Id1-infected cells had become GC^+ (Figures 6A and B, and 7A and B) and had a multiprocess morphology typical of oligodendrocytes (Figure 6A and B). When oligodendrocyte differentiation was induced by PDGF withdrawal, >80% of BabeG-infected cells and ~50% of BabeG-Id1-infected cells differentiated into GC^+ oligodendrocytes, whereas only ~10% of the BabeG-Id4-infected cells did so (Figure 7A). When oligodendrocyte differentiation was induced by TH addition, >70% of BabeG-infected cells and >60% of BabeG-Id1-infected cells differentiated into GC^+ oligodendrocytes, whereas <4% of the BabeG-Id4-infected cells did so (Figure 7B). These results suggest that transfection with *Id4* can powerfully inhibit oligodendrocyte differentiation induced by either PDGF withdrawal or TH addition, consistent with the possibility that the progressive decrease of *Id4* expression in developing precursor cells may play a part in timing oligodendrocyte differentiation. Transfection with *Id1* had much less effect (Figure 7).

There is evidence that progressive increases in the Cdk inhibitor protein p27/Kip1 (p27) and the TH receptor protein TRβ1 are part of the intrinsic timer in oligodendrocyte precursor cells (Casaccia-Bonnefil *et al*., 1997; Durand *et al*., 1997, 1998; Gao *et al*., 1998). To determine whether the overexpression of *Id4* had any effect on the levels of p27 or TRβ1, we infected P6 precursor cells with BabeG-Id4 and BabeG and, after 7 days, stained them for p27 and TRβ1, as previously described (Durand *et al*., 1997; Gao *et al*., 1998). We quantified the staining in GFP^+ cells by confocal microscopy. The levels of p27 and TRβ1 were not significantly different in the two types of

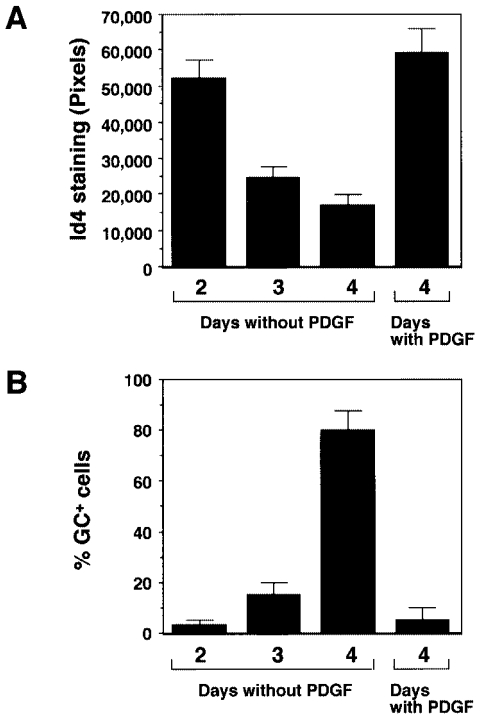

Fig. 4. Relationship between the level of Id4 protein and oligodendrocyte differentiation in cells deprived of PDGF. Purified P0 precursor cells were cultured for 1 day in PDGF, washed and then cultured for either 2, 3 or 4 days without PDGF or 4 days with PDGF. The cells were then triple stained for Id4 and GC and with Hoechst 33342. Quantification of the intensity of Id4 staining is shown in (**A**) as the mean ± SD of 50 cells. The proportions of Hoechst-stained cells that were GC^+ are shown in (**B**) as the mean ± SD of three cultures.

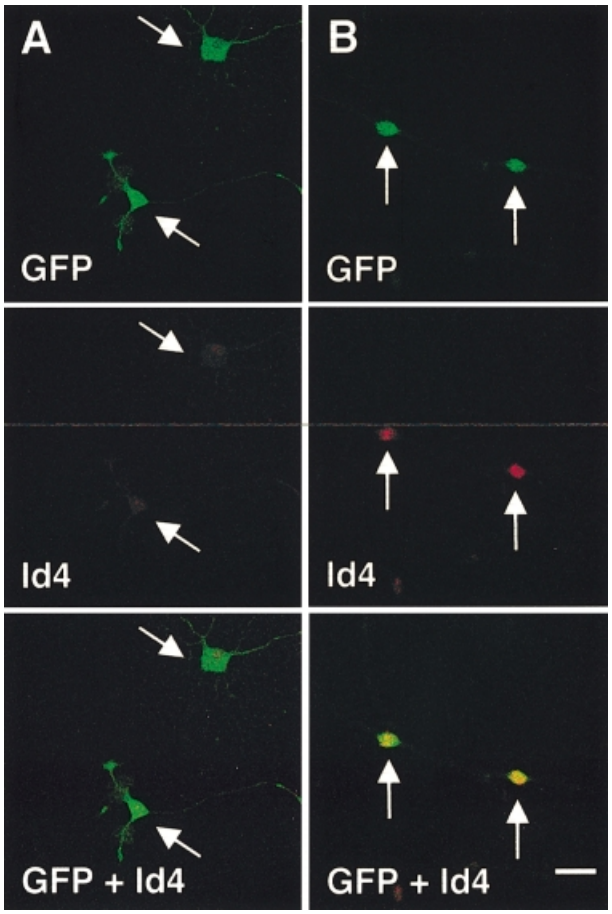

Fig. 5. Expression level of Id4 protein in BabeG-infected (**A**) and BabeG-Id4-infected (**B**) cells. Purified P6 precursor cells were infected overnight with either the BabeG control virus (A) or the BabeG-Id4 virus (B). They were then cultured in PDGF without TH for another 5 days and then immunostained for Id4 (red, middle panels). The virus-infected cells were detected by the expression of GFP (green, top panels). The fused images of the antibody staining and GFP are shown in the bottom panels. Scale bar, 25 μm.

transfected cells (not shown), suggesting that the over-expression of *Id4* did not affect the expression of either protein.

Overexpression of Id4 increases the rate of precursor cell proliferation

The cell cycle time of oligodendrocyte precursor cells increases as the cells mature (Gao and Raff, 1997) and is especially long in precursor cells found in the adult optic nerve (ffrench-Constant and Raff, 1986; Wolswik and Noble, 1989; Shi *et al*., 1998). Since the level of Id4 decreases as precursor cells proliferate *in vitro* and *in vivo*, it is possible that Id4 normally promotes cell cycle progression in these cells, as previously reported for other cell types (Barone *et al*., 1994; Hara *et al*., 1994; Peverali *et al*., 1994), and that the fall in Id4 with maturation contributes to the slowing of the cell cycle.

To examine this possibility, we infected purified P9 precursor cells with the BabeG-Id1, BabeG-Id4 or BabeG retroviral vector and cultured them at clonal density for 10 days in PDGF without TH. We then counted the number of cells in each GFP-expressing clone. As shown in Figure 8A, clones of BabeG-Id4-infected cells were ~5 times larger than clones of BabeG-infected cells and ~3 times larger than clones of BabeG-Id1-infected cells. The deduced doubling times of BabeG-, BabeG-Id1- and BabeG-Id4-infected cells were ~53, 49 and 34 h, respectively. As there was no significant difference in the number of dead cells in the three types of clone (on average, ~10% of the cells were dead in all cases), the differences in clone size seemed to reflect differences in the rates of cell division. To test this directly, we cultured infected P9 precursor cells for 10 days, pulsed them with bromodeoxyuridine (BrdU) for 8 h and stained them with anti-BrdU antibody. As shown in Figure 8B, the percentages of BabeG-, BabeG-Id1- and BabeG-Id4-infected cells that were $BrdU^+$ were 33, 36 and 53%, respectively, suggesting that *Id4* overexpression enhanced the rate of cell cycle progression.

Discussion

We show here that the expression of the neural-specific *Id* gene, *Id4*, decreases over time as oligodendrocyte precursor cells proliferate *in vivo. Id4* expression also decreases as purified precursors proliferate *in vitro*, suggesting that the decrease is an intrinsic property of the precursors themselves. We show that overexpression of *Id4* in these cells in culture inhibits their differentiation into oligodendrocytes in response to either PDGF withdrawal or TH addition and promotes cell cycle progression when the

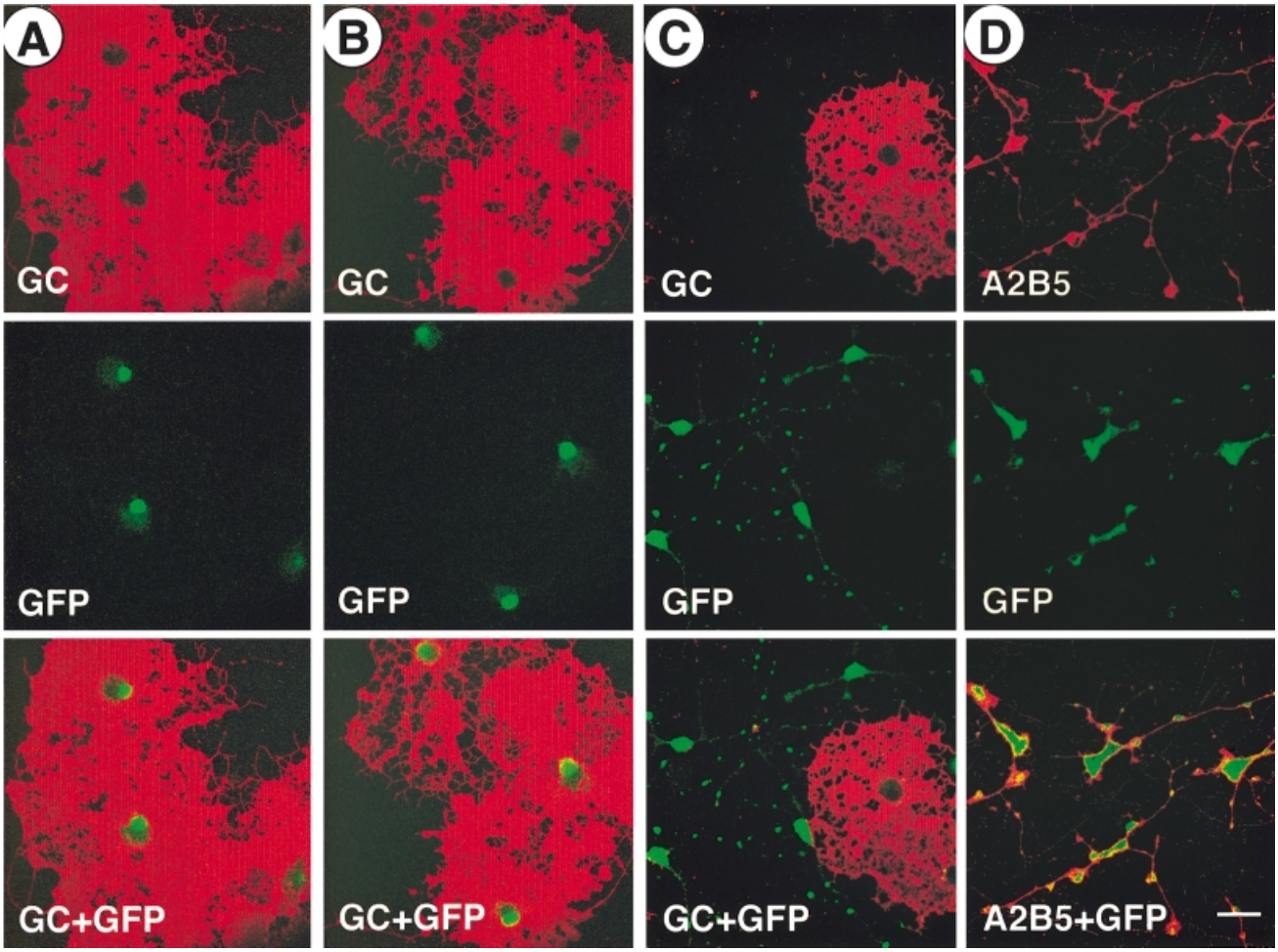

Fig. 6. Fluorescence micrographs illustrating the effects of *Id1* and *Id4* transfection on oligodendrocyte differentiation induced by PDGF withdrawal. Purified P6 precursor cells were infected overnight with either the BabeG control virus (**A**), the BabeG-Id1 virus (**B**) or the BabeG-Id4 virus (**C** and **D**). They were then cultured in PDGF without TH for another 2 days and then in the absence of PDGF for a further 3 days to induce the cells to differentiate into oligodendrocytes. The cells were immunostained for GC (A, B and C) or A2B5 (D). The antibody staining is shown in red. The virus-infected cells were detected by the expression of GFP (green, middle panels of A–C). The fused images of the antibody staining and GFP are shown in the bottom panels in (A–C). In (C), the oligodendrocyte morphology and GC^+ phenotype of the uninfected cell on the right provide a striking contrast to the BabeG-Id4-infected cells, which are GC negative and look like precursor cells. Scale bar, 25 μm.

cells are stimulated by PDGF. These findings suggest that the progressive decrease in Id4 may be part of the cell-intrinsic timing mechanism that helps to determine when the precursor cells withdraw from the cell cycle and differentiate (Temple and Raff, 1986; Barres *et al*., 1994; Gao *et al*., 1997).

Ids are expressed in various types of proliferating precursor cells. Their expression decreases when the cells withdraw from the cell cycle and differentiate (Benezra *et al*., 1990; Sun *et al*., 1991; Kreider *et al*., 1992), and their overexpression promotes proliferation and inhibits differentiation (Jen *et al*., 1992; Kreider *et al*., 1992; Sun, 1994; Desprez *et al*., 1995; Lister *et al*., 1995). Our findings are consistent with these previous findings but, in addition, provide evidence that Id proteins might be part of an intracellular timing mechanism that determines when precursor cells differentiate. Although all four known mammalian *Ids* are expressed in oligodendrocyte precursors, only Id4 mRNA and protein decrease with the appropriate time course *in vivo* and *in vitro* expected of a protein that is part of the timing mechanism in these cells.

It was shown previously that the timer in oligodendrocyte precursor cells runs faster when the cells are cultured at 33°C rather than at 37°C (Gao *et al*., 1997), and we show here that both Id4 mRNA and protein decrease more quickly at the lower temperature, consistent with Id4 being part of the timer. As overexpression of *Id4* inhibits the differentiation of oligodendrocyte precursor cells and decreases their cell cycle time, it is possible that normal levels of Id4 protein function in this way in these precursor cells and that the progressive decrease in Id4 protein with developmental time plays a part in the normal progressive slowing of the cell cycle, which was demonstrated previously (Gao *et al*., 1998), and in the timing of cell cycle withdrawal and differentiation. It will be important in the future to test this possibility by studying the consequences of inactivating *Id4* expression in these cells. It may be necessary to inhibit more than one *Id* gene to see an effect, as was required in studies of neuronal differentiation: the inactivation of both *Id1* and *Id3* caused neuronal precursors to withdraw prematurely from the cell cycle and to differentiate, whereas the inactivation of

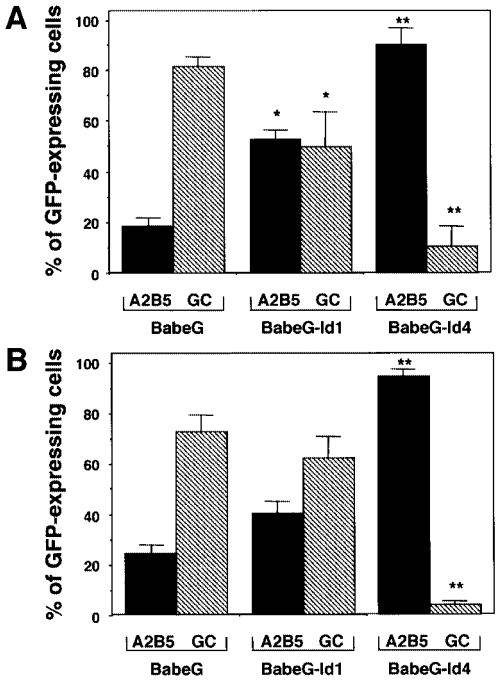

Fig. 7. Quantification of the effects of *Id1* and *Id4* transfection on oligodendrocyte differentiation induced by either PDGF withdrawal or TH addition. Purified P6 oligodendrocyte precursor cells were infected overnight with the BabeG control virus, the BabeG-Id1 virus or the BabeG-Id4 virus. They were then cultured in PDGF without TH for another 2 days. To induce oligodendrocyte differentiation, the cells were cultured either for a further 3 days in the absence of PDGF (**A**) or for a further 5 days in PDGF with TH (**B**). The proportions of GFP-expressing cells that were stained by A2B5 or anti-GC antibodies are shown as the mean ± SD of three cultures. **P* <0.05 in comparison with the result of BabeG-infected cells, and ***P* <0.001 in comparison with the result of either BabeG- or BabeG-Id1-infected cells, when analysed by Student's *t*-test.

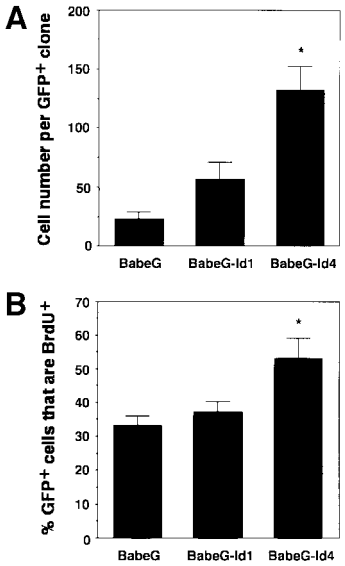

Fig. 8. Effects of *Id1* and *Id4* transfection on cell proliferation. Purified P9 precursor cells were infected with the BabeG control virus, the BabeG-Id1 virus or the BabeG-Id4 virus. They were then cultured at clonal density in PDGF without TH. (**A**) The number of cells in each GFP-expressing clone was counted after 10 days and is shown as the mean ± SD of 50 clones assessed for each virus. (**B**) Ten days after infection, the cells were incubated with 20 μM BrdU for 8 h and then fixed and immunostained for BrdU. The proportion of GFP-expressing cells that were $BrdU^+$ is shown as the mean ± SD of three cultures; * indicates a significant difference (A, P <0.001; B, P <0.01) from the result with either BabeG or BabeG-Id1, when analysed by Student's *t*-test.

either gene alone did not (Lyden *et al*., 1999). As all four *Id* genes are expressed in oligodendrocyte precursors, it seems likely that their functions in these cells are at least partially redundant. Our failure to detect an effect of an *Id4* antisense cDNA (T.Kondo, unpublished result) is consistent with this possibility, as is the finding that overexpression of *Id2* inhibited the differentiation of oligodendrocyte precursors (Sdrulla *et al*., 1999). The functions of the four Id proteins in these cells may not be identical, however, as transfection with *Id1* (this study) or *Id3* (T.Kondo, unpublished result) has less effect on differentiation than transfection with *Id4*. The levels of Id1 and Id3 proteins in the non-transfected precursor cells are already high, however, which makes it difficult to interpret the *Id1* and *Id3* transfection experiments.

Consistent with a role for Id4 in regulating oligodendrocyte differentiation, we find that, when oligodendrocyte precursor cells are induced to differentiate prematurely by PDGF withdrawal, the rate at which Id4 protein decreases accelerates so that there is a striking correlation between the rate of Id4 decrease and the rate of oligodendrocyte differentiation.

It was shown previously that two intracellular proteins—the Cdk inhibitor p27/Kip1 (p27) and the TH receptor TRβ1—progressively increase in purified oligodendrocyte precursors as they proliferate in culture and that they reach plateau levels at around the time that the cells would normally stop dividing and begin to differentiate, suggesting that they might be part of the cell-intrinsic timer (Durand *et al*., 1997; Gao *et al*., 1998). Both of these proteins increase more quickly at 33°C than at 37°C, consistent with this possibility. Most importantly, p27-deficient cells tend to divide for longer than wild-type cells before they differentiate, suggesting that the timer is disturbed in these cells (Casaccia-Bonnefil *et al*., 1997; Durand *et al*., 1998). Together with our present findings, these observations suggest that the cell-intrinsic timing mechanism depends on both increases in intracellular proteins such as p27 and TRβ1 that inhibit cell cycle progression and promote differentiation, and decreases in intracellular proteins such as Id4 that stimulate cell cycle progression and inhibit differentiation. The decrease in Id4 protein is unlikely to play a part in the increase in either p27 or TRβ1, as overexpression of *Id4* does not seem to influence the levels of either of the two proteins.

As Id proteins function mainly as dominant-negative regulators of bHLH proteins (reviewed in Norton *et al*., 1998), the finding that the overexpression of *Id4* inhibits differentiation induced by either PDGF withdrawal or TH addition suggests that bHLH proteins are involved in oligodendrocyte differentiation. One candidate is the Mash1 proneural bHLH protein, a mammalian homologue of the *Drosophila* achaete–scute proneural proteins (Johnson *et al*., 1990), as it increases with similar kinetics to p27 and TRβ1 in oligodendrocyte precursors and increases more quickly at 33°C than at 37°C (T.Kondo, unpublished results).

Id4 might also promote cell cycle progression in oligodendrocyte precursor cells by inhibiting bHLH proteins that negatively regulate the cell cycle. The bHLH proteins, MyoD and E47, for example, arrest the cell cycle in G_1 when overexpressed in myoblasts, and Id proteins can antagonize this effect (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990; Peverali *et al*., 1994). Id proteins, however, can also associate with other types of proteins that regulate the cell cycle, including the mouse Id-associated protein 1 (MIDA1): antisense oligonucleotides that inhibit MIDA1 expression, for instance, inhibit the proliferation of mouse erythroleukaemia cells (Shoji *et al*., 1995). Thus, Id4 may accelerate the cell cycle by both inhibiting cell cycle inhibitors such as bHLH proteins and co-operating with cell cycle promoters such as MIDA1.

To understand how the intracellular timer in oligodendrocyte precursor cells works, one will have to discover how proteins such as p27 and TRβ1 increase over time and proteins such as Id4 decrease over time. Our findings that Id4 mRNA and protein decrease in parallel in dividing precursor cells and that both the mRNA and protein decrease more quickly at 33°C than at 37°C suggest that the control of Id4 protein levels in these cells is mainly transcriptional. The promoter of the *Id4* gene contains multiple E boxes, to which bHLH proteins such as Mash1 can bind. Enforced expression of bHLH genes such as *MyoD*, *E12* and *E47* stimulates *Id4* promoter activity, and mutation of the E box eliminates this activity; moreover, co-expression of *Id4* suppresses the stimulating effect of these bHLH transgenes (Pagliuca *et al*., 1998). It is possible, therefore, that bHLH proteins such as Mash1 activate the transcription of *Id4* in oligodendrocyte precursors as part of a negative feedback loop, although this would not explain why Id4 mRNA and protein levels fall at the same time that Mash1 levels rise.

In summary, we have provided evidence that Id4 may be part of the cell-intrinsic timer that helps to determine when oligodendrocyte precursor cells withdraw from the cell cycle and differentiate. It seems that multiple proteins contribute to the timing mechanism, some increasing and others decreasing over time as the oligodendrocyte precursor cells proliferate. One advantage of such a multicomponent timing mechanism is that it is robust: inactivation of individual components does not inactivate the timer but instead causes it to function inaccurately (Durand *et al*., 1998). The challenge now is to identify all of these components and determine how their levels are controlled such that they change over time.

Materials and methods

Animals and chemicals

Sprague–Dawley rats were obtained from the Animal Facility at University College London. Chemicals were purchased from Sigma, except where indicated. Recombinant human PDGF-AA and neurotrophin-3 (NT-3) were purchased from Peprotech.

Preparation of purified precursor cells

Optic nerve cells were prepared from postnatal rats, and oligodendrocyte precursor cells were purified to >99% purity from optic nerve by sequential immunopanning, as described previously (Barres *et al*., 1992). The purified cells were cultured in poly-D-lysine-coated 6-well culture dishes (Falcon) or slide flasks (Nunc) in serum-free Dulbecco's modified Eagle's medium (DMEM) containing bovine insulin (10 μg/ml), human transferrin (100 μg/ml), bovine serum albumin (100 μg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), *N*-acetylcysteine (60 μg/ml), forskolin (5 μM), PDGF-AA (10 ng/ml), NT-3 (5 ng/ml), penicillin and streptomycin (Gibco) (culture medium). In some experiments, TH (triiodothyronine, 30 ng/ml) was added as indicated. If cultures were maintained for longer than 4 days, half of the medium was replaced every 2 days.

RT–PCR analysis

Cells were harvested by trypsinization, and poly(A)$^+$ RNA was prepared using a QuickPrep Micro mRNA Purification kit (Pharmacia Biotech); 1.5 μg of partially purified poly(A)$^+$ RNA was reverse transcribed in 33 μl of reaction mixture, using a First-Strand cDNA Synthesis kit (Pharmacia Biotech). The RT–PCR was carried out in a 50 μl reaction mixture that contained 3 μl of cDNA as template, 1 pM of the specific oligonucleotide primer pair, 1.25 U of *Taq* DNA polymerase and 10% dimethylsulfoxide (for *Id* cDNA). Cycle parameters for *Id* cDNA were 30 s at 94°C, 30 s at 63°C and 2 min at 72°C for 35 cycles. The cycle parameters for *β-actin* cDNA were 15 s at 94°C, 30 s at 53°C and 1 min at 72°C for 25 cycles. The identity of the amplified products was checked by digestion with appropriate restriction enzymes.

The following oligonucleotide DNA primers were synthesized: for rat *Id1*, the 5′ primer was 5′-ATGAAGGTCGCCAGTAGCAGTG-3′ and the 3′ primer was 5′-TCAGCGACACAAGATGCGGTCG-3′; for rat *Id2*, the 5′ primer was 5′-ATGAAAGCCTTCAGTCCGGTGAG-3′ and the 3′ primer was 5′-TTAGCCACAGAGTACTTTGCTGTC-3′; for rat *Id3*, the 5′ primer was 5′-ATGAAGGCGCTGAGCCCGGTG-3′ and the 3′ primer was 5′-TCAGTGGCAAAAACTCCTCTTGTC-3′; for rat *Id4*, we used conserved sequences between human and mouse, the 5′ primer was 5′-TTCTCGAGATGAAGGCGGTGAGCCCGGTG-3′ and the 3′ primer was 5′-TTTCGCGATCAGCGGCACAGAATGCTGTC-3′; for rat *β-actin*, the 5′ primer was 5′-TGGAATCCTGTGGCATCC-3′ and the 3′ primer was 5′-TCGTACTCCTGCTTGCTG-3′.

Full-length rat *Id1* and mouse *Id4* cDNAs were amplified from oligodendrocyte precursor cells and NIH 3T3 cells, respectively, using RT–PCR and *Pfu* Turbo polymerase (Invitrogen), and they were cloned into a pMOSBlue vector (Amersham Pharmacia Biotech). The nucleotide sequences were determined using a BigDye terminator kit and an ABI sequencer (model 310).

Recombinant Id1 and Id4 retrovirus vector

To express *Id1* and *Id4* in oligodendrocyte precursor cells and to mark the transfected cells, we made recombinant retrovirus vectors that encode either Id1 or Id4 protein, as well as enhanced GFP. The *Id1* and *Id4* cDNAs were driven by the Moloney murine leukaemia virus (MMLV) long terminal repeat (LTR) promoter in the pBabeG vector, which is based on the pBabe vector (Morgenstern *et al*., 1990) but contains the coding sequence for GFP driven by the SV40 early promoter. Because the MMLV LTR promoter is more efficient than most internal promoters, the expression of the *Id* genes would be expected to be greater than the expression of the GFP gene in these vectors. The BabeG-Id1 and BabeG-Id4 vectors were transfected into phoenix packaging cells (Kinsella and Nolan, 1996) using LipofectAmine (Gibco-BRL), and culture supernatant was harvested 3 days after transfection. To concentrate the recombinant virus, 10 ml of culture supernatant was centrifuged at 20 000 r.p.m. for 2 h, as described in Ausubel *et al*. (1992). The virus pellet was suspended in 1 ml of culture medium, and 0.2 ml of the virus solution was used to infect purified oligodendrocyte precursor cells prepared from P6 or P9 rat optic nerve. Cells infected with the recombinant retrovirus were grown in culture medium overnight, washed and then cultured in the same medium for a further 2 days. In some cases, the cells were then cultured either without PDGF for 3–7 days or with PDGF and TH for 5 days to induce the cells to differentiate into oligodendrocytes.

To examine the proliferation of retrovirus-infected cells, the precursor cells were grown at clonal density (1000–1500 cells per slide flask) in culture medium without TH. The number of cells in GFP-expressing clones was counted after 10 days. In some cultures, BrdU (20 μM) was added after 10 days; 8 h later, the cells were fixed and stained for BrdU (see below), and the proportion of GFP$^+$ cells that were BrdU$^+$ was determined.

Immunocytochemistry

To determine whether GFP-expressing cells had differentiated into oligodendrocytes, the cells were fixed with 2% paraformaldehyde for 15 min at room temperature, treated with 50% normal goat serum and then stained with either monoclonal anti-GC antibody (Ranscht *et al*., 1982; supernatant, diluted 1:3) to detect oligodendrocytes (Raff *et al*.,

1978) or the A2B5 monoclonal antibody (Eisenbarth *et al*., 1979; ascites fluid, diluted 1:100) to detect oligodendrocyte precursors (Raff *et al*., 1983). The monoclonal antibodies were detected with Texas red-conjugated goat anti-mouse IgG or IgM, respectively (Jackson ImmunoResearch; diluted 1:100), as previously described (Gao *et al*., 1998). To examine the level of Id4 protein in precursor cells, the cells were fixed as above, treated with 50% normal goat serum and 0.1% Triton X-100, and then stained with rabbit anti-Id4 antibodies (Santa Cruz; diluted 1:100), followed by biotin-conjugated goat anti-rabbit IgG (Chemicon; diluted 1:100) and then fluorescein-conjugated streptavidin (Amersham; diluted 1:100). In some experiments, cells were triple labelled, first with anti-GC antibody to detect oligodendrocytes as above, then fixed and stained for Id4 as above, and finally stained with bisbenzimide (Hoechst 33342) to visualize all nuclei.

For BrdU staining, the cells were fixed in 2% paraformaldehyde for 10 min at room temperature, post-fixed in 100% methanol for 10 min at –20°C, incubated in 2 M HCl for 30 min to denature the DNA, followed by 0.1 M sodium borate pH 8.5 for 10 min. The cells were incubated in 50% normal goat serum and 0.1% Triton X-100 and then stained with a monoclonal anti-BrdU antibody (Maguad *et al*., 1988; culture supernatant, diluted 1:5), followed by Texas red-conjugated goat anti-mouse IgG1 (Amersham; diluted 1:100).

The stained slides were mounted in Citifluor mounting medium (CitiFluor, UK), sealed with nail varnish, and the intensity of fluorescence was quantified in a Bio-Rad MRC 1000 confocal laser-scanning fluorescence microscope as previously described (Durand *et al*., 1997).

Acknowledgements

We thank Jim Apperly for help with the construction of the recombinant retroviral vectors, and members of the Raff laboratory for advice and comments on the manuscript. T.K. is supported by a JSPS Postdoctoral Fellowship for Research Abroad. M.R. is supported by a Programme Grant from the Medical Research Council, UK.

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Received December 9, 1999; revised February 1, 2000; accepted March 6, 2000