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A Role for the Helix-Loop-Helix Protein Id2 in the Control of Oligodendrocyte Development

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Summary

Compared to neurons, the intracellular mechanisms that control glial differentiation are still poorly understood. We show here that oligodendrocyte lineage cells express the helix-loop-helix proteins Mash1 and Id2. Although Mash1 has been found to regulate neuronal development, we found that in the absence of Mash1 oligodendrocyte differentiation occurs normally. In contrast, we found that overexpression of Id2 powerfully inhibits oligodendrocyte differentiation, that Id2 normally translocates out of the nucleus at the onset of differentiation, and that absence of Id2 induces premature oligodendrocyte differentiation in vitro. These findings demonstrate that Id2 is a component of the intracellular mechanism that times oligodendrocyte differentiation and point to the existence of an as yet unidentified MyoD-like bHLH protein necessary for oligodendrocyte differentiation.

Introduction

Compared to neurons, little is known about the intracellular mechanisms that control glial development. Whereas many of the genes that control vertebrate neuronal development have been identified in genetic analyses of invertebrate model systems, the genes that control vertebrate glial development so far appear poorly conserved, if at all, and thus we still know relatively little about them (Granderath and Klambt, 1999). We have been focusing on the development of oligodendrocytes in the rat optic nerve. How do developing oligodendrocytes know when to divide and when to differentiate? As is the case for myoblasts (Gu et al., 1993), there is an obligate relationship between proliferation and differentiation in the oligodendrocyte lineage (Temple and Raff, 1986; Barres and Raff, 1994). Dividing oligodendrocyte precursor cells (OPCs) cannot myelinate; myelinating oligodendrocytes cannot divide. The molecular basis of this relationship is poorly understood. Interestingly, oligodendrocyte precursor cells in vitro cannot divide forever in response to their mitogens such as platelet-derived growth factor (PDGF). After a maximum of about eight divisions, they stop dividing and differentiate into oligodendrocytes. The progeny of an individual precursor cell tends to stop dividing and differentiate at around the same time, suggesting that an intrinsic timing mechanism limits the number of times a precursor cell can divide (Temple and Raff, 1986). When OPCs are cultured in medium containing mitogens but no thyroid hormone, the OPCs divide indefinitely and fail to differentiate into oligodendrocytes (Barres et al., 1994). These studies indicated that the developmental timer consists of two components: a counting component that measures time or number of cell divisions, and an effector component, which is signaled by extrinsic hydrophobic signals including thyroid hormone. The molecular mechanism of this developmental timer is unknown and is of interest, as it may provide insight into how proliferation and differentiation are coupled.

In this study, we have focused on the potential role of helix-loop-helix proteins (HLH) in controlling oligodendrocyte development. The basic HLH transcription factor family regulates the development of many cells by regulating cell-type-specific gene expression (Weintraub, 1993; Guillemot, 1999). Genes of the basic HLH (bHLH) class, such as the *Achaete-Scute* (AS-C) complex in *Drosophila* and its mammalian homolog Mash1, control the determination and differentiation of neurons (Johnson et al., 1990; Jan and Jan, 1993; Guillemot, 1999). It is not yet known whether there are bHLH proteins required for glial differentiation, but two oligodendrocyte lineage-specific bHLH proteins have been identified recently (Lu et al., 2000; Zhou et al., 2000).

Tissue-specific class B bHLH proteins control cell-type specification and differentiation by dimerizing with more ubiquitously expressed E2A, class A, bHLH proteins such as E12 and E47. The function of tissue-specific bHLH proteins is blocked by Id (inhibitor of DNA binding) proteins that are structurally similar to bHLH proteins except that they lack the basic DNA binding sequence (Benezra et al., 1990). Ids block differentiation in diverse cell types, including neural cells, by acting as dominant-negative blockers of the function of bHLH proteins. Id proteins bind to class A bHLH proteins, preventing their dimerization with class B bHLH proteins and activation of gene transcription (Norton, 2000). Id proteins are ideal candidates to help couple proliferation to differentiation, as in many cell types they are upregulated by mitogens and help to promote proliferation as well as to inhibit differentiation by interacting with Rb and other bHLH proteins responsible for differentiation.

In the present study, we have taken advantage of our ability to highly purify and culture oligodendrocyte precursor cells (OPCs) to begin to examine whether they express HLH proteins that help to control their development. We show that OPCs express Mash1 and Id2 mRNA and protein. We show that both the ability of oligoden-

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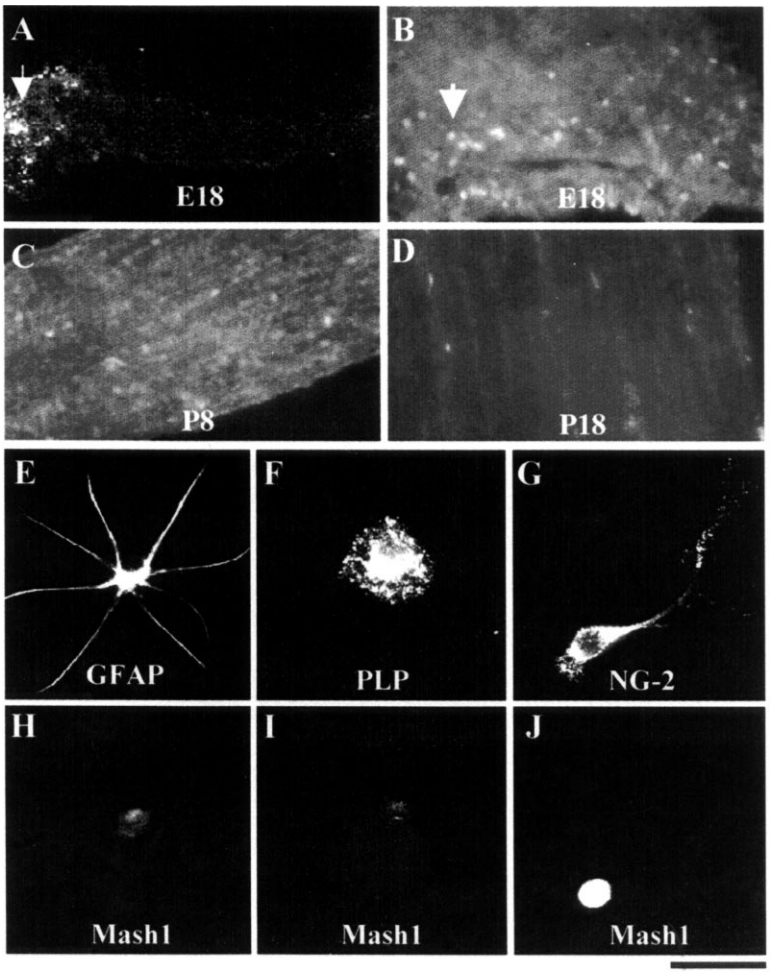

Figure 1. Mash1 Immunoreactivity in the Developing Rat Optic Nerve and in Acutely Isolated Rat Optic Nerve Glial Cell Types

(A–D) Cryosections of developing rat optic nerves were stained with mouse anti-Mash1 antibody, followed by a biotinylated anti-mouse IgG antibody and a FITC-conjugated Streptavidin. (E–J) Acutely isolated P4 optic nerve cells were cultured in a serum-free medium in the presence of PDGF for 2 hr. They were double labeled with mouse anti-Mash1 antibody (H, I, and J) and a rabbit antiserum (E, F, and G) for cell-type identification, followed by a biotinylated anti-mouse IgG antibody. Signals were detected by a FITC-conjugated Streptavidin and a Cy3-conjugated anti-rabbit antibody. In developing rat optic nerve, Mash1 immunoreactivity was detected primarily in a subset of cells at the chiasm end (arrows) of E18 (A and B) optic nerves but became more evenly distributed in a subset of cells along the entire course of the nerve by P8 (C) and P18 (D). In acutely isolated rat optic nerve glial cells, Mash1 immunoreactivity was expressed by NG-2-positive OPCs (G and J), where it was localized to the nucleus. Mash1 immunoreactivity was not detected in GFAP-positive astrocytes (E and H) or by PLP-positive oligodendrocytes (F and I). Scale bar: (A) 300 μm, (B–D) 50 μm, (E–J) 20 μm.

drocytes to differentiate and the timing of oligodendrocyte differentiation are unaffected in the absence of Mash. In contrast, we find that Id2 overexpression blocks oligodendrocyte differentiation and enhances proliferation, that Id2 normally translocates out of the nucleus at the onset of differentiation, and that absence of Id2 slows proliferation and causes premature differentiation of OPCs in vitro. These findings demonstrate a role for Id2 in controlling the timing of oligodendrocyte differentiation in vitro and point to the existence of an as yet unidentified bHLH protein necessary for oligodendrocyte development.

Results

PCR Analysis of Expression of HLH mRNAs by Developing Oligodendrocytes

To determine whether oligodendrocyte precursor cells (OPCs) express helix-loop-helix proteins (bHLH), we designed degenerate primers from known families of HLH proteins and performed RT–PCR on mRNA extracted from OPCs undergoing differentiation in the presence of thyroid hormone (see Experimental Procedures). Automatic sequence analysis of the PCR fragments identified three different HLH mRNAs: an Id protein, the class A bHLH protein E47, and the class B bHLH protein Mash1.

Northern blots were performed on mRNA isolated from OPCs purified from P5 rat brain to confirm the presence of full-length mRNAs at the expected size of Mash1 and Id2. Using a P^{32}-labeled cDNA probe for Mash1, a 2.5–2.8 kb transcript was detected, which is the same size as full-length Mash1 mRNA (Johnson et al., 1990). Similarly a full-length Id mRNA was detected, although homology between Id family members precluded a definite identification of Id2 mRNA. To identify the specific Id family members expressed by OPCs, we performed RT–PCR analysis of OPC mRNA using primers designed to identify specific Id family members (see Experimental Procedures). mRNAs for Id1, 2, or 3 were identified; we did not examine the OPCs for Id4 mRNA (see Discussion).

Immunocytochemical Localization of Mash1 and Id Proteins in Oligodendrocyte Precursor Cells In Vitro and In Vivo

To determine whether Mash1 protein was present in developing glial cells in the rat optic nerve, we performed immunostaining on optic nerve cryosections using a monoclonal anti-Mash1-specific antibody kindly pro-

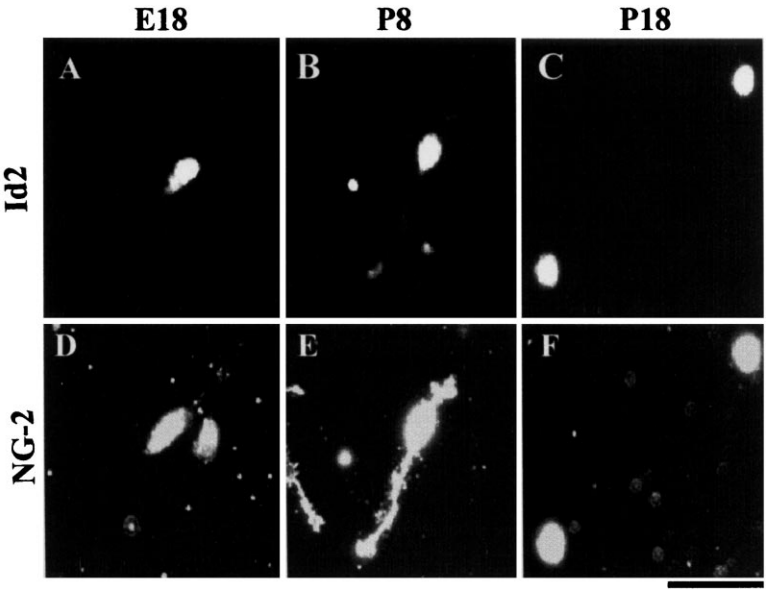

Figure 2. Id2 Immunoreactivity in Acutely Isolated Rat OPCs

OPCs were purified from E18 (A and D), P8 (B and E), and P18 (C and F) optic nerves, cultured in a serum-free medium in PDGF for 2 hr, and then double labeled by a mouse anti-Id2 monoclonal antibody and a rabbit NG-2 antiserum, followed by a FITC-conjugated anti-mouse IgG antibody and a Cy3-conjugated anti-rabbit antibody. At all three ages, nearly all OPCs were NG-2 positive and brightly Id2 positive. Id2 staining was localized to the nucleus. Scale bar: 20 μm.

vided by David Anderson (Lo et al., 1991). In sections of embryonic day 18 (E18) optic nerve, bright nuclear staining was present in a subset of optic nerve glial cells. These cells were present primarily in clusters at the chiasm side nerve (Figures 1A and 1B). Mash1-positive cells became more uniformly distributed along the nerve by postnatal day 8 (P8) (Figure 1C). The intensity of Mash1 labeling progressively decreased by P18 (Figures 1C and 1D). This pattern of staining, with a chiasm to retina gradient at E18 that disappears by P8, reflects the previously reported distribution of OPCs, and not that of oligodendrocytes or astrocyte lineage cells.

To identify the Mash1-expressing cell type, we performed double immunostaining on acutely isolated optic nerve cells (see Experimental Procedures). OPCs were labeled using an NG2 rabbit antiserum (Levine and Nishiyama, 1996), astrocytes were labeled using a GFAP rabbit antiserum, and oligodendrocytes were labeled using a proteolipid protein (PLP) antiserum (see Experimental Procedures). At P4, greater than 95% of NG-2-positive cells were Mash1 positive (Figures 1G and 1J), a commonly used marker for OPCs. None of the GFAP-positive type-1 astrocytes (Figures 1E and 1H) or PLP-positive oligodendrocytes (Figures 1F and 1I) expressed detectable Mash1. Thus, OPCs specifically express Mash1.

To determine whether OPCs express Id proteins, we first did a preliminary experiment in which we immunostained OPCs in culture with various Id-specific antisera (see Experimental Procedures). We found that antibodies to Id1 and Id3 all stained OPCs diffusely, but that Id2 staining was specifically localized to the nucleus; furthermore, two different Id2 antibodies produced an identical pattern of staining (see below). For the rest of this study, we decided to focus on Id2 because of its preferential nuclear localization.

We next stained cryosections with an Id2 monoclonal antibody (see Experimental Procedures). The specificity of this antibody for Id2 was confirmed by Western blotting (data not shown). As expected, the majority of cells in cryosections prepared from developing optic nerves were Id2 positive. To confirm that OPCs express Id2, we double labeled acutely isolated optic nerve glial cells from E18, P8, and P18 rat optic nerves, using the monoclonal anti-Id2 antibody and a polyclonal NG2 antiserum. Greater than 95% of NG2-positive OPCs were Id2 positive (Figure 2). These results show that embryonic and postnatal OPCs express both Mash1 and Id2 proteins.

Effect of Time of Culture and Thyroid Hormone on Levels of Mash1 and Id2 Immunoreactivity in Oligodendrocyte Precursor Cells

If Mash1 or Id2 regulate the timing of OPC differentiation, their levels might increase or decrease over time or successive divisions in culture. We therefore investigated whether the levels of Mash1 or Id2 immunmoreactivity in OPCs changed during 7 days of culture. We purified OPCs from P1 optic nerves, cultured them at clonal density, and then stained them after 1, 3, and 7 days of culture in serum-free medium containing survival factors and mitogens but lacking thyroid hormone. At all three of these time points, bright Mash1 and Id2 immunoreactivity was present in nearly all of the OPCs and localized to their nuclei (Figure 3). Even after 7 days of proliferation in culture, there was no obvious change in the levels of immunoreactivity of Mash1 or Id2 (Figure 3).

If either Mash1 or Id2 mediates the thyroid hormone–controlled effector component of the timing mechanism, then their levels might be strongly regulated by thyroid hormone. To determine whether thyroid hormone (T3) regulates the expression of Mash1 or Id2, we purified P1 OPCs and cultured them for 1 day in the presence of both survival factors and mitogens, in the presence or absence of T3. Bright nuclear Id2 immunoreactivity was present in 93% ± 2% of the OPCs after 1 day of culture regardless of the presence or absence of T3 (Figure 3). In contrast, however, 96% ± 3% of OPCs had rapidly lost Mash1 expression after only 1 day of culture. (Figure 3). Thus, T3 has no effect on Id2 levels but strongly regulates Mash1 levels negatively.

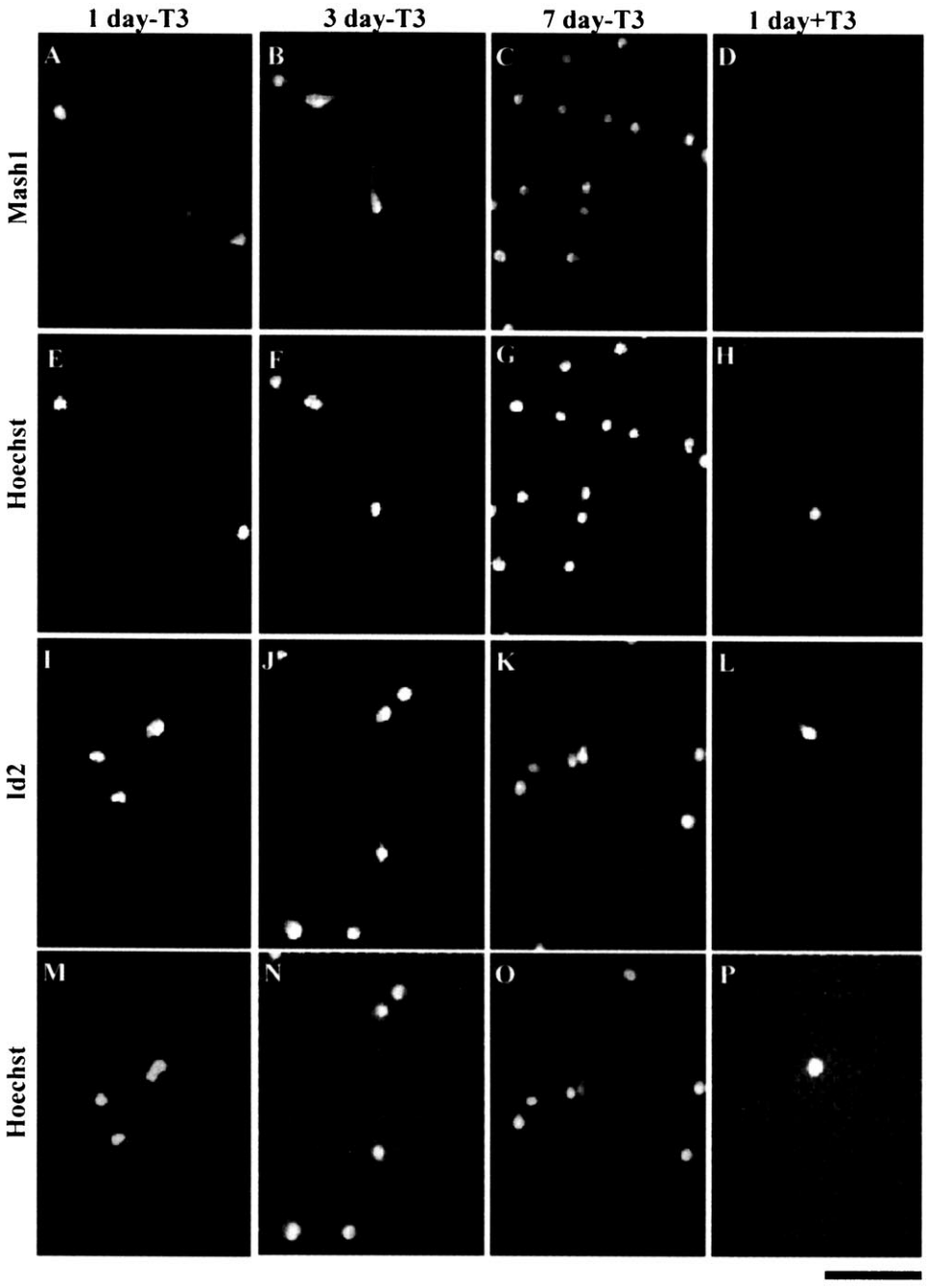

Figure 3. Mash1 and Id2 Immunoreactivity in Proliferating OPCs In Vitro

OPCs were purified from P1 rat optic nerves and cultured in a serum-free medium in the presence of PDGF for 1, 3, or 7 days without T3 (A–C, E–G, I–K, and M–O) or in the presence of both PDGF and T3 for 1 day (D, H, L, and P) prior to immunolabeling. Mash1 immunoreactivity is shown in (A)–(D), and Id2 immunoreactivity is shown in (I)–(L). All cells were visualized using a nuclear fluorescent Hoechst counterstain (E–H and M–P). Mash1 immunoreactivity was easily detected throughout the culture period in the absence of T3 but was lost by 24 hr after the addition of T3, while Id2 immunoreactivity was easily detected throughout the culture period regardless of the presence or absence of T3. Scale bar: 50 μm.

Although oligodendrocytes within the optic nerve and immediately after isolation did not express Mash1 immunoreactivity, we noticed that in culture nearly all oligodendrocytes, but not astrocytes or meningeal cells, expressed bright nuclear Mash1 immunoreactivity. This expression of Mash1 in cultured oligodendrocytes was also dependent upon thyroid hormone and was observed only in the absence of T3.

Effects of Oligodendrocyte Differentiation on Mash1 and Id2 Expression and Localization

We next investigated whether Id2 or Mash1 expression or localization is altered when OPCs differentiate into oligodendrocytes. We purified OPCs from P1 rat optic nerves and cultured them in serum-free medium, lacking thyroid hormone, but containing survival factors and mitogens to stimulate proliferation (see Experimental

Procedures). In some cultures, we removed the mitogen PDGF to trigger oligodendrocyte differentiation. Four days after removal of PDGF, nearly all of the OPCs had differentiated into GC-positive, highly process-bearing oligodendrocytes, as expected. The OPC cultures (which still contained PDGF) and the oligodendrocyte cultures (which lacked PDGF) were stained using the Mash1 monoclonal antibody. Nearly all of the OPCs (Figure 4A) as well as the oligodendrocytes (Figure 4B) were Mash1 positive. Both in OPCs and oligodendrocytes, Mash1 was localized to the nucleus. Thus, despite the continued presence of nuclear Mash1, oligodendrocyte differentiation occurred normally.

In contrast, when we stained OPC and oligodendrocyte cultures prepared identically to those described above, we found that the majority of OPCs and oligodendrocytes were Id2 positive (Figures 4C and 4D). However, the localization of the Id2 immunoreactivity had dramatically changed during differentiation. Id2 was in all cases localized to the nucleus of OPCs (Figure 4C) but to the cytoplasm of oligodendrocytes (Figure 4D). Id2 nuclear immunoreactivity was never detected in GC-positive oligodendrocytes.

We next investigated whether the translocation of Id2 to the cytoplasm occurs before or after differentiation. If Id2 plays a role in controlling differentiation, it should translocate prior to differentiation, as it can only inhibit differentiation by binding to bHLH proteins in the nucleus where they regulate gene expression. We measured the time course of Id2 translocation by staining the purified OPC cultures at various time points after oligodendrocyte differentiation was triggered by removal of PDGF from the culture medium. In the same cultures, we followed the appearance of oligodendrocytes by staining with an anti-galactocerebroside (GC) monoclonal antibody. As shown in Figure 4E, we found that by 48 hr after removal of PDGF, Id2 had translocated to the cytoplasm in 90% of the OPCs, but little oligodendrocyte differentiation had occurred until after another 2 days of culture. These observations show that the translocation of Id2 out of the nucleus precedes differentiation by about 2 days, which is consistent with the possibility that Id2 plays a role in controlling the timing or ability of OPCs to differentiate into oligodendrocytes.

Effect of Id2 Overexpression on Differentiation of Oligodendrocyte Precursor Cells

To directly determine whether Id2 can inhibit oligodendrocyte differentiation, we investigated whether Id2 overexpression would inhibit the ability of OPCs to differentiate. We purified P8 OPCs and transfected them with either a control plasmid expression vector encoding green fluorescent protein (GFP) or a rat Id2 gene together with GFP (see Experimental Procedures). Cells overexpressing Id2 displayed strong Id2 immunoreactivity in both their cytoplasm and nucleus (data not shown); presumably, the overexpression overloaded the E2A protein–mediated nuclear translocation system (Deed et al., 1996). We cultured the test and control cells in medium lacking mitogens in order to allow them to differentiate into oligodendrocytes. After 3 days of culture, most of the OPCs that had been transfected with the control vector and were expressing GFP had differentiated into process-bearing GC-positive oligodendro-

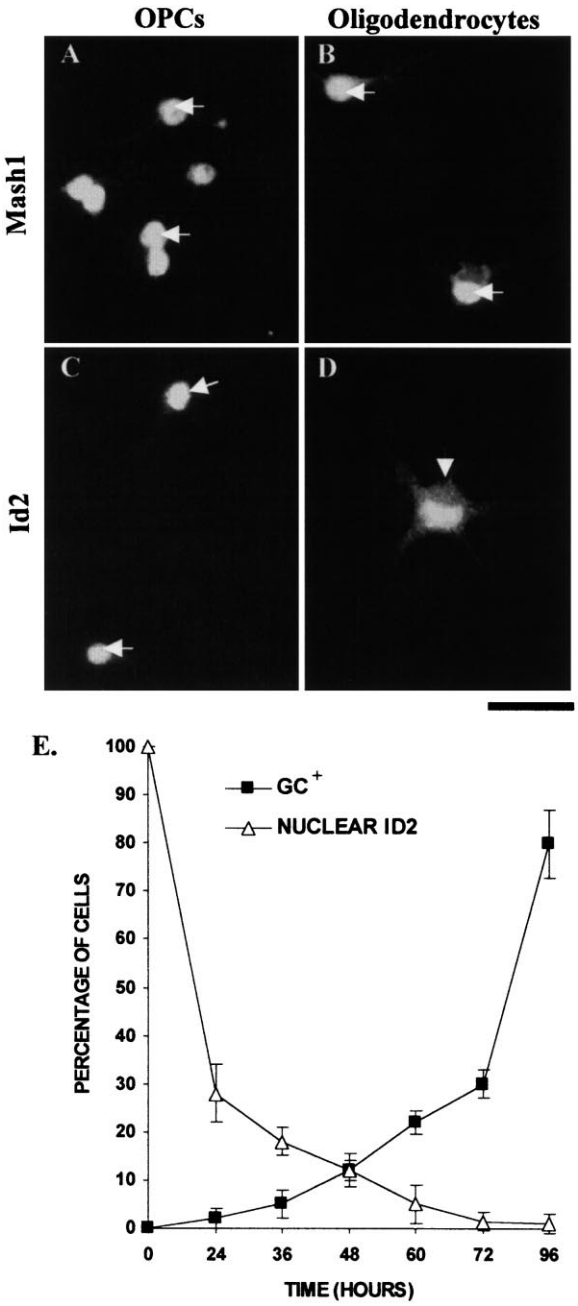

Figure 4. Localization of Mash1 and Id2 in OPCs Induced to Differentiate by PDGF Removal

Purified OPCs isolated from P1 rat optic nerves were cultured in a serum-free medium in the presence (A and C) or absence of PDGF (B and D). After 3 days, they were stained with either the mouse anti-Mash1 antibody or the Id2 antiserum. Mash1 was expressed in the nuclei of nearly all OPCs (A) and oligodendrocytes (B). Id2 protein was found in the nuclei of nearly all OPCs (C) but translocated to cytoplasm upon oligodendrocyte differentiation (D). A time course of the percentage of cells expressing nuclear Id2 immunoreactivity or GC immunoreactivity after PDGF withdrawal is shown in (E). Translocation of Id2 from the nucleus to the cytoplasm occurred 48 hr prior to morphological and antigenically detected signs of oligodendrocyte differentiation. All values are shown as mean ± SEM (n = 3). Scale bar: 20 μm.

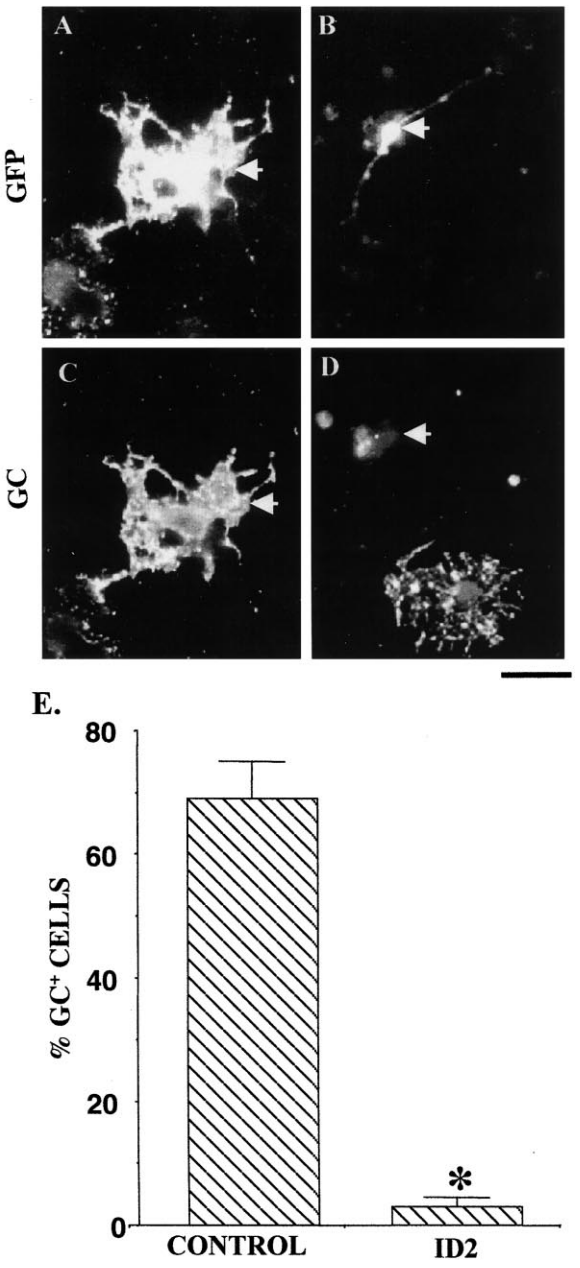

Figure 5. Effect of Id2 Overexpression on Oligodendrocyte Precursor Cell Differentiation

Purified P8 OPCs were transfected with either a control plasmid vector encoding GFP or an Id2-expressing vector that coexpresses GFP (see Experimental Procedures), and then cultured in serum-free medium lacking PDGF. After 3 days, the cultures were stained by a mouse anti-GC monoclonal antibody, followed by a FITC-conjugated anti-mouse IgG antibody. The majority of OPCs expressing exogenous Id2 (B, D, and E) failed to differentiate into oligodendrocytes, whereas most OPCs transfected by the control vector differentiated (A, C, and E). The proportions of the stained cells were shown as the mean ± SEM (asterisk, $p < 0.05$). Scale bar: 20 μm.

cytes, as expected (Figures 5A and 5C). Remarkably, the majority of the cells transfected with the Id2 vector, which were easily identified by expression of GFP vectors, failed to differentiate. These cells retained the typical simple bipolar morphology of OPCs and were GC negative (Figures 5B and 5D). Although Id2 overexpression blocked their ability to differentiate into postmitotic oligodendrocytes, it did not stimulate them to divide in the absence of mitogen based on BrdU incorporation and on average clone size. Interestingly, however, Id2 overexpression did significantly increase the rate of proliferation in response to PDGF by nearly 50% (data not shown). Over 3 days of culture, nearly 70% of the control OPCs differentiated into oligodendrocytes, whereas only about 5% of OPCs overexpressing Id2 were able to differentiate, as judged by acquisition of GC immunoreactivity (Figure 5E). These data demonstrate that Id2 overexpression blocks the differentiation of OPCs and does so without stimulating their proliferation.

Effects of Mash1 and Id2 Deficiency on OPC Proliferation and Differentiation

Our findings that Id2 is present in the nucleus of OPCs but translocates to the cytoplasm prior to differentiation, and that Id2 overexpression blocks OPC differentiation, suggested that Id2 might normally control oligodendrocyte differentiation. To test this possibility directly, we examined the behavior of OPCs isolated from transgenic mice that lack Id2 (Yokota et al., 1999). Because of the postnatal lethality of these mice, we could not assess whether oligodendrocyte development and myelination was altered in adult mice. However, Id2 is clearly not necessary for the generation of oligodendrocyte lineage cells, as many OPCs had developed by P5 based on immunostaining of cryosections of $Id2^{-/-}$ brains with specific markers (data not shown).

To more carefully analyze the effects of Id2 on oligodendrocyte development, we purified OPCs from P5 wild-type and mutant mouse brains. The yields of OPCs isolated from the $Id2^{-/-}$ mice tended to be slightly lower by about 10%, approximately consistent with the slightly decreased size of these mice. We compared the ability of the purified OPCs to differentiate into oligodendrocytes in serum-free medium containing T3 in the absence of the mitogen PDGF. Over 90% of the purified OPCs isolated from wild-type mice $Id2^{+/+}$ and from homozygous $Id2^{-/-}$ became GC-positive oligodendrocytes, with the typical highly process-bearing morphology of oligodendrocytes, within 3 days of culture (Figure 6E). We next examined whether the T3-dependent ability of oligodendrocytes to withdraw from the cell cycle and differentiate in the presence of mitogens was perturbed in the absence of Id2. We cultured purified $Id2^{+/+}$ and $Id2^{-/-}$ OPCs in the presence of both PDGF and T3 for 6 days, prior to measuring the percentage of cells that had differentiated into GC-positive oligodendrocytes over this culture period. There was a moderate yet significant increase in the rate of differentiation of $Id2^{-/-}$ OPCs compared to $Id2^{+/+}$ OPCs (Figure 6F). Similarly, in clonal studies performed in the presence of PDGF and T3, we repeatedly observed premature oligodendrocyte differentiation by $Id2^{-/-}$ OPCs; typically, the percentage of oligodendrocyte clones was increased by 20% after 4–6 days of culture. In contrast, Mash1 deficiency did not appreciably alter the rate of differentiation of purified OPCs (Figures 6A and 6B). These results show that Id2 is not necessary for OPCs to differentiate into oligodendrocytes but that it normally acts to slow the rate of

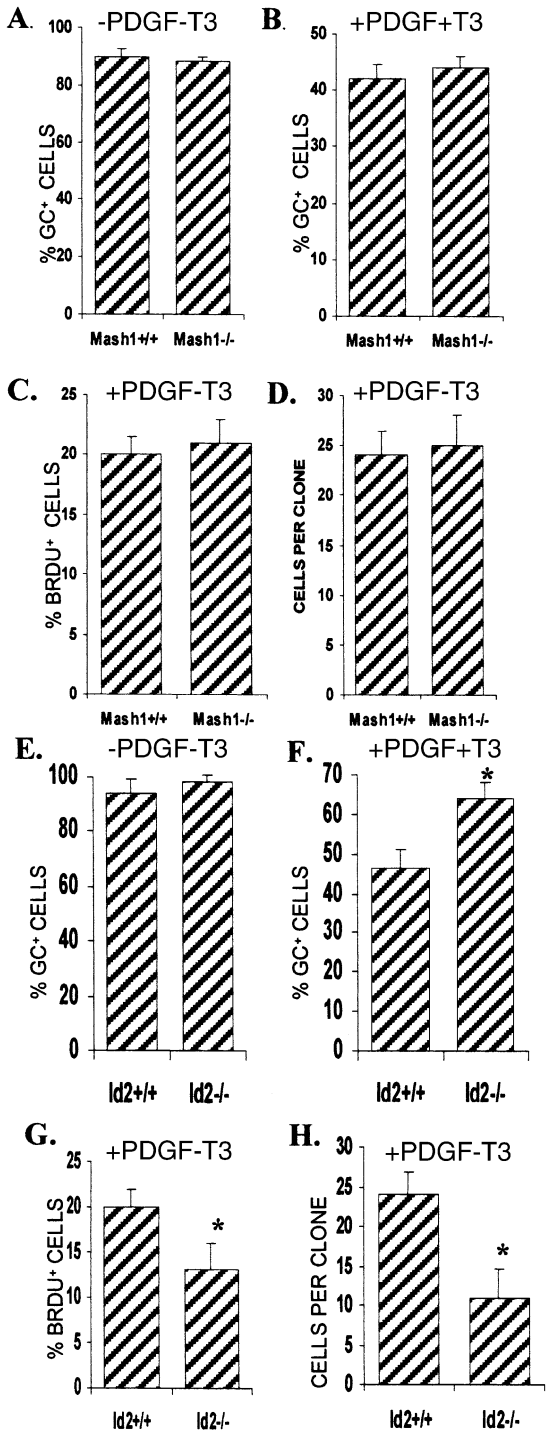

Figure 6. Effects of Mash1 and Id2 Deficiency on OPC Differentiation and Proliferation

OPCs were purified from brains of E20 Mash1$^{-/-}$ or Mash1$^{+/+}$ transgenic mice (A–D) and P5 Id2$^{-/-}$ or Id2$^{+/+}$ transgenic mice (E–H). They were cultured in serum-free medium in the absence of PDGF and T3 for 3 days (A and E) or for 6 days in the presence of PDGF (B–D and F–H) with T3 (B and F) or at clonal density without T3 (C, D, G, and H). (A, B, E, and F) The cultures were stained with a mouse anti-GC antibody, followed by a FITC-conjugated anti-mouse IgG antibody. (C and G) BrdU incorporation was measured by incubating the cultures in 10 μm BrdU for 4 hr, followed by BrdU immunostaining. There was no change in the rate of differentiation (A and B) or proliferation (C and D) of OPCs with or without Mash1. However, in the absence of mitogens, OPCs were able to differentiate normally in the absence of Id2 (E), but in the presence of mitogen they differentiated significantly faster than did wild type OPCs (F). OPCs lacking Id2 also synthesized DNA at a slower rate (G) and divided significantly slower (H). All values are means ± SEM (asterisk, $p < 0.05$).

oligodendrocyte differentiation in the presence of mitogens and T3.

To find out whether Id2 also regulates the rate of proliferation, we cultured the purified Id2$^{+/+}$ and Id2$^{-/-}$ OPCs in serum-free medium containing PDGF but not T3. After 6 days, we measured the percentage of cells that were undergoing DNA synthesis by adding BrdU for 4 hr and then measured the percentage of cells that had incorporated BrdU by immunostaining. Id2$^{-/-}$ OPCs had a significant decrease in BrdU incorporation compared to wild-type cells (Figure 6G). In addition, when we cultured the cells at clonal density under the same conditions for 6 days, the average size of clones was decreased by more than half in the absence of Id2 (Figure 6H). In contrast, Mash1 deficiency did not affect the rate of proliferation of purified OPCs in response to PDGF (Figures 6C and 6D).

This decrease in proliferation and clone size in the absence of Id2 was not accounted for by a decrease in the survival of oligodendrocyte lineage cells. After 6 days of OPC culture at clonal density in serum-free medium containing PDGF and T3, dead cells accounted for 25% ± 2% of the +/+ cells and 28% ± 1% of the −/− cells. Similarly, in medium containing PDGF but no T3, dead cells accounted for 23% ± 2% of the +/+ cells and 26% ± 3% of the −/− cells. Thus, the deficiency of Id2 did not significantly increase cell death. In summary, these findings show that Id2 is not necessary for OPC survival, proliferation, or differentiation. Endogenous levels of Id2 normally act to enhance the rate of proliferation in response to mitogens and to slow the rate of OPC differentiation, thereby preventing premature differentiation.

Discussion

Developing Oligodendrocytes Express Helix-Loop-Helix Proteins

In this study, we have focused on the potential role of helix-loop-helix proteins in oligodendrocyte development. We found that oligodendrocyte precursor cells express members of each of these 3 classes of HLH proteins. OPCs expressed Mash1, Id1, Id2, Id3, and E47. While our studies were in progress, several other labs also reported the presence of HLH proteins in developing oligodendrocytes. Kondo and Raff (2000b, 2000c) observed the expression of Mash1 and all four Id mRNAs in developing OPCs. In addition, two new class B bHLH family members, olg1/oligo2 and olg2/oligo1, were recently identified and found to be specifically expressed by oligodendrocyte lineage cells (Lu et al., 2000; Zhou et al., 2000). These are excellent candidates to control the generation of OPCs from multipotent neural stem cells, as so far they are the earliest cell-type-specific proteins to be expressed by developing oligodendro-

cyte lineage cells. Their role in oligodendrocyte development is not yet clear, however, as overexpression has so far not increased the number of oligodendrocytes that develop in culture or within the developing brain, and the effects of olg deficiency have not yet been examined. Whereas these recent olg studies have focused on the mechanism by which multipotent neural stem cells give rise to OPCs, we have focused on the mechanisms by which OPCs give rise to oligodendrocytes.

Mash1 Does Not Regulate Proliferation, Differentiation, or the Timing of Differentiation of Oligodendrocyte Lineage Cells

Mash1 regulates the development of many types of neurons in both the peripheral and central nervous systems. Mash1 is expressed by precursor cells and is downregulated after differentiation. Similarly, we found that Mash1 is expressed by OPCs but not by oligodendrocytes, astrocytes, or astrocyte precursor cells and is downregulated when the OPCs differentiate into oligodendrocytes within the developing rat optic nerve in vivo. While our studies were in progress, Kondo and Raff (2000b) independently showed that OPCs express Mash1 mRNA and protein. Interestingly, they found that Mash1 levels increased in OPCs over time in culture. Despite this increase of Mash1 in vitro over time, we found that the levels of Mash1 immunoreactivity progressively decrease in OPCs within the optic nerve during development, with high levels of immunoreactivity embryonically and much lower levels postnatally. This developmental decrease in vivo is probably caused by the steep postnatal rise in thyroid hormone, given our finding that thyroid hormone rapidly turns off Mash1 expression. Because thyroid hormone triggers oligodendrocyte development and because Kondo and Raff (2000b) proposed that Mash1 may play a crucial role in the timing mechanism that controls oligodendrocyte development, we tested the effects of Mash deficiency on oligodendrocyte development. We found that OPCs were able to differentiate normally and that the timing of their differentiation in response to thyroid hormone was normal. These findings do not support a role for Mash1 in oligodendrocyte development.

What function might Mash1 have in oligodendrocyte lineage cells if it does not control oligodendrocyte differentiation? Recently, it was discovered that OPCs cultured under certain conditions have the ability to become "reprogrammed" to multipotential neural stem cells able to generate neurons as well as glial cells (Kondo and Raff, 2000a). Our finding that T3 rapidly downregulates Mash1 raises the interesting possibility that the presence of Mash1 in OPCs might correlate with, or be necessary for, their ability to undergo neurogenesis. If so, the T3-mediated downregulation of Mash1 might explain why multipotential neural stem cells that persist with the adult mammalian brain appear unable to generate many neurons in vivo although they can in vitro.

Id2 Regulates the Timing of OPC Differentiation into Oligodendrocytes

Id proteins are expressed by developing neuronal precursor cells and regulate the timing of their generation by enhancing proliferation and inhibiting differentiation, as they do for many non-neural cell types, such as muscle. For instance, neurons are generated prematurely in transgenic mice lacking Id1 and Id3 (Lyden et al., 1999). Similarly, Id proteins are expressed by developing glial cells, where Id3 helps to control astrocyte proliferation (Tzeng and deVellis, 1997, 1998). In this study, we investigate the possible role of Id proteins in controlling oligodendrocyte differentiation. While these studies were in progress, Kondo and Raff (2000c) reported the identification of all four Id mRNAs in OPCs. They showed that Id4 overexpression enhanced proliferation and inhibited differentiation of OPCs in culture, whereas they observed little effect of Id1 (the effects of Id3 overexpression have not yet been examined). They focused on Id4 because they found that levels of Id4 mRNA and protein appreciably decreased in OPCs over time in culture. In contrast, although we identified three of these Id proteins in OPCs, we focused on Id2 because of its nuclear localization and its ability to regulate pRb, which is expressed by OPCs and has been shown to regulate the proliferation and differentiation of developing myoblasts and neurons (Iavarone et al., 1994; Lasorella et al., 1996).

In contrast to Mash1, we found many lines of evidence supporting a role for Id2 in controlling oligodendrocyte differentiation. First, we found that Id2 overexpression nearly completely blocked the differentiation of OPCs into oligodendrocytes. Second, we found that Id2 is normally localized to the nucleus of OPCs but translocates to their cytoplasm extremely early in the course of differentiation. A nuclear localization would be consistent with a dominant-negative role of Id2 in antagonizing differentiation, which would be relieved by cytoplasmic translocation. Third, and most importantly, we found that OPCs that lack Id2 were capable of differentiating normally in the absence of mitogen. However, in the presence of mitogen, compared with wild-type OPCs, the $Id2^{-/-}$ OPCs proliferated significantly more slowly and differentiated prematurely in response to T3 stimulation. These findings demonstrate that Id2 normally plays a role in the timing mechanism that regulates oligodendrocyte development by enhancing the rate of proliferation and by slowing the rate of differentiation.

Why does Id2 translocate out of the nucleus into the cytoplasm early during oligodendrocyte differentiation? The nuclear localization of Id proteins is regulated by an E protein–mediated chaperone mechanism (Deed et al., 1996). In the absence of E2A proteins, Id3 remains in the cytoplasm of COS cells. Cotransfection of E47, which has a nuclear localization signal, binds to and induces the nuclear translocation of Id3. Similarly, E47 has been shown to mediate the nuclear translocation of Id2 (Y. Y., unpublished data). The preferential localization of Id2 in the nucleus of dividing OPCs is therefore most likely explained by their normal coexpression of E47. The movement of Id2 out of the nucleus early during differentiation most likely reflects the upregulation of an as yet to be identified class B bHLH protein that preferentially binds to E47. Ultimately, Id2 mRNA is downregulated by oligodendrocytes as they mature (Tzeng and Vellis, 1998).

Our observations raise the question of why so many different Id proteins are expressed by developing oligodendrocytes. It is likely that these proteins have redun-

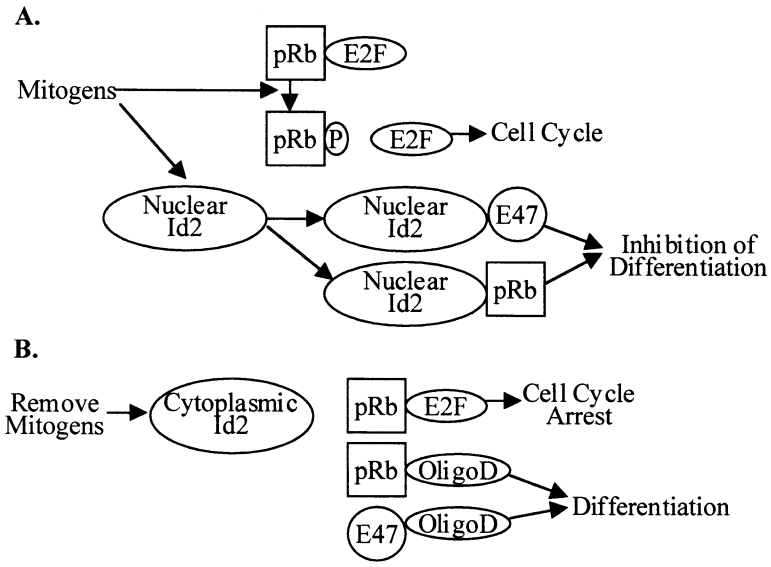

Figure 7. A Model for How Proliferation and Differentiation Are Coordinated in Developing Oligodendrocytes

(A) When mitogens are present, proliferation is stimulated by the phosphorylation of pRb, releasing E2F from sequestration, allowing it to transactivate cell cycle genes. In addition, differentiation is impeded when Id2 is phosphorylated, moves into the nucleus, and sequesters E47 and any unphosphorylated pRb, preventing activation of genes needed for differentiation by a hypothesized class B "OligoD" bHLH protein.

(B) When mitogens are withdrawn, pRB loses its phosphorylation allowing it to sequester E2F, inducing cell cycle arrest. At the same time, differentiation is promoted by Id2, which when unphosphorylated exits the nucleus freeing the class A bHLH protein E47 to heterodimerize with OligoD, which along with unphosphorylated pRB activates genes necessary for differentiation. A similar mechanism is thought to control myoblast differentiation.

dant functions. Both Id2 and Id4 overexpression inhibit OPCs from differentiating into oligodendrocytes. Although we observed premature differentiation and loss of proliferative ability in the absence of Id2, it is quite possible that compensatory mechanisms involving the remaining Id proteins prevented a far more dramatic phenotype. Id proteins might be regulated differentially; for instance, Id4 levels decrease over time in culture, whereas Id2 levels do not appear to. In addition, Notch activation profoundly upregulates Id1 and Id3 but not the other two Id proteins (S. W. and B. A. B, unpublished data). Id proteins might also have different functions, as they are differentially posttranslationally modified (Hara et al., 1997), differentially bind to Rb (Iavarone et al., 1994) and to various bHLH proteins, and have different biological activities (Lyden et al., 1999; Melnikova et al., 1999).

A Tentative Model for the Timing Mechanism that Controls Oligodendrocyte Differentiation

Our findings suggest that Id proteins, including Id2, may be at the heart of a control mechanism that links proliferation to differentiation in developing oligodendrocytes. Id upregulation would antagonize an as yet to be identified MyoD-like bHLH protein, "OligoD," that is necessary for oligodendrocyte differentiation (Figure 7). In the presence of mitogens, Id levels will be high and prevent differentiation. In the absence of mitogens, Id leaves the nucleus and differentiation ensues. Muscle cells and neurons are similar to oligodendrocytes in that they, too, have a tightly coupled obligate relationship between proliferation and differentiation. This coupling is thought to be mediated by Id proteins and also by the retinoblastoma tumor suppressor protein, Rb.

Rb, in its phosphorylated state, which is induced by mitogenic stimulation, promotes growth by unbinding from the transcription factor E2F, allowing it to activate gene expression necessary for proliferation. Unphosphorylated Rb proteins are necessary for promoting differentiation in some cell types, such as developing muscle and neurons (Gu et al., 1993; Toma et al., 2000). For instance, Rb is necessary for the MyoD-stimulated gene expression underlying muscle differentiation. MyoD acts in concert with other factors besides pRb to trigger muscle differentiation. Heterodimerization with class A bHLH proteins such as E47 is also necessary. Thus, we postulate that an as yet to be identified class B bHLH protein is upregulated during oligodendrocyte differentiation and, together with interacting proteins such as E47 and pRb, promotes oligodendrocyte-specific gene expression (Figure 7). Importantly, Id2 is unique among the Id proteins in being able to bind to and disrupt the antiproliferative effects of the tumor suppressor protein Rb when it is unphosphorylated (Iavarone et al., 1994; Lasorella et al., 1996, 2000; Norton, 2000).

Our findings provide insight into the molecular mechanism that counts and limits the maximum number of divisions an OPC can make. Id2 must directly participate in this mechanism, as it helps to control how many divisions developing OPCs can make before they differentiate. It has been previously suggested that the OPCs might count divisions by dilution of a stable protein (Temple and Raff, 1986). In fact, the levels of Id4 do decrease over time, suggesting that it may play such a role, although it is not known whether $Id4^{-/-}$ OPCs would prematurely differentiate as occurs when Id2 is absent (Kondo and Raff, 2000c). Although we did not observe any obvious change in Id2 immunoreactivity, Id4 and Id2 might well collaborate, or an Id2 binding protein might change in level over time. In addition, certain molecules that increase over time may also participate in the timer, such as the thyroid hormone receptors TRβ1 (Gao et al., 1997) and TRβ2 (Barres et al., 1994) and the cyclin-dependent kinase inhibitor p27 (Casaccia-Bonnefil et al., 1997; Durand et al., 1997, 1998; Durand and Raff, 2000).

It is clear that the molecular mechanisms that limit the number of divisions and that couple proliferation to differentiation involve complex, most likely redundant, signaling networks. The levels of many molecules, some known and many unknown, change with time in culture. How can we figure out which of these changes is primary

rather than secondary in controlling oligodendrocyte differentiation? Gene chip analyses offer the possibility of identifying the earliest genes to change, and the ability to examine highly purified populations of wild type and mutant OPCs over time promises to be a powerful future approach to understanding the molecular basis of oligodendrocyte differentiation.

Experimental Procedures

Step-by-step protocols for all procedures are available upon request (barres@stanford.edu).

Reagents

Recombinant human CNTF was generously provided by Regeneron. PDGF and NT-3 were obtained from Peprotech (NJ). Insulin was obtained from Sigma. Antibodies were kindly provided as follows: PLP antiserum (W. Stoffel), NG-2 antiserum (J. Levine), Mash1 monoclonal antibody (D. Anderson). Other antibodies were commercially obtained: CC-1 monoclonal antibody (Oncogene Science, APC-7) and GFAP antiserum (Dako); Id2 antiserum (Santa Cruz) and Id2 monoclonal antibody (PharMingen).

Purification of OPCs and Oligodendrocytes by Sequential Immunopanning

Purified cell populations from rat optic nerve were obtained essentially as previously described (Barres et al., 1988, 1992, 1993, 1994; Meyer-Franke et al., 1995; Shi et al., 1998). Briefly, neonatal optic nerves were obtained from S/D rats (Simonsen Labs, CA) and were incubated at 37°C for 30 min in a papain solution (30 units/ml, Worthington) and then purified by immunopanning as previously described.

To purify OPCs from Mash1 knockout mice, whole brains were obtained from E20 mice. To purify OPCs from Id2 knockout mice, whole brains were obtained from P5 mice. Brain cell suspensions were prepared using papain and then immunopanned. Cells were placed on the anti-Thy1.2 plate for 30 min at room temperature, after which the nonadherent cells were transferred to the anti-GC plate to deplete oligodendrocytes. The nonadherent cells were then transferred to the O4 dish to collect the OPCs.

Culture of Purified OPCs and Oligodendrocytes

Approximately 5000 purified OPCs were cultured on glass coverslips in 24-well plates (Falcon) that had been coated with PDL in a serum-free medium containing DMEM (GIBCO). The serum-free medium included bovine serum albumin (BSA), selenium, putrescine, transferrin, and progesterone, pyruvate (1 mM), glutamine (2 mM), insulin (5 μg/ml), CNTF (1 ng/ml), PDGF (10 ng/ml), and NT-3 (1 ng/ml). For in vitro differentiation of oligodendrocytes, PDGF and NT-3 were omitted from the medium.

Construction of a Directional Lambda Phage Oligodendrocyte Library and Isolation of HLH Proteins

Messenger RNA was extracted from highly purified differentiating oligodendrocytes. Double-stranded cDNAs were made using GIBCO Superscript II cDNA Construction Kit and ligated into Not I–Sal I phage arms. The recombinant phages were packed and delivered into proper host cells (Stratagene GigPak Gold II system).

For PCR screening, degenerate primers were made according to known bHLH sequences. mRNA extracted from differentiating oligodendrocytes was reverse transcribed into cDNA and used as template for polymerase chain reaction. After gel electrophoresis, bands with proper size were cut out and subcloned into TA vector (Invitrogen). PCR products were then sequenced and compared with known bHLH proteins for similarity through Blast search. The candidate clones were radiolabeled as probes to confirm their expression on Northern blot and also to obtain full-length cDNAs from the oligodendrocyte library. The identity of the full-length clones was then determined by sequencing. For oligonucleotide screening, degenerate oligo was end radiolabeled and used as probe to screen the phage library. The positive clones were sequenced and identified through sequencing.

MYOD-5: AA(A/G)ATIAA(T/C)GA(A/G)GCITT(T/C)GA
MYOD-3: A(A/G)IAT(T/C)TCIAC(C/T)TTIGG
E12-5: GCIAA(T/C)AA(T/C)GCI(A/C)GIGA
E12-3: TTIGT(C/T)TGIG(G/C)(T/C)TT(G/A)TC
ASCT-5: AA(T/C)(A/C)GIGTIAA(A/G)(C/T)(A/T)IGT
ASCT-3: C(G/T)IGT(A/G)TA(C/T)TCIACIGC
SPLIT-5: GCI(A/C)GIATIAA(T/C)AA(A/G)TG
SPLIT-3: TCIA(G/A)IATITCIGC(C/T)TT

RT–PCR Confirmation of HLH Proteins

Messenger RNA was purified cultured oligodendrocytes and OPCs, and was reverse transcribed using Superscript II (GIBCO) with a random hexamer (10 μM) as a primer in a 35 μl reaction containing 1× Superscript II reverse transcriptase buffer, 10 μM of each dATP, dTTP, dCTP, and dGTP, and 20 U of RNasin (GIBCO). For PCR amplification, specific oligodendrocyte primer pairs (0.5 μM each) were incubated with 1 μl of cDNA and 1 U of Taq polymerase (Perkin Elmer) in a 20 μl of reaction mixture that included 1× Taq buffer, 100 μM each dATP, dCTP, dGTP, and dTTP. Typical cycle parameters were 1 min at 94°C, 1 min at 59°C, and 3 min at 72°C for 20–30 cycles followed by a cycle at 72°C for 10 min. The whole reaction was then fractionated on 1.2% regular agarose gel, and the PCR product was visualized by ethidium bromide staining.

ID1-S: AAGGTCGCCAGTAGCAGTG
ID1-A: TCTCCACCTTGCTCACTTTG
ID2-S: AAAGCGTTGAGTCCGGTGA
ID2-A: GCTGCAGGATTTCCATCTT
ID3-S: GACATGAACCACTGCTACTC
ID3-A: ACAAGTTCCGGAGTGAGCTC
MASH1-S: CCCAACTGGTTCTGAGGAC
MASH1-A: CCCATTTGACGTAGTTGG
GAPDH-S: ATTGTCAGCAATGCATCCTGCA
GAPDH-A: AGACAACCTGGTCCTCAGTGTA

Genotyping of Transgenic Mice

Genomic DNAs were isolated from mouse tails used Qiagen DNAeasy kits. To identify the genotypes, specific oligodendrocyte primer pairs (0.5 μM each) were incubated with 1 μl of cDNA and 1 U of Taq polymerase (Perkin Elmer) in a 20 μl of reaction mixture that included 1× Taq buffer, 100 μM each dATP, dCTP, dGTP, and dTTP. Typical cycle parameters were 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C for 35 cycles followed by a cycle at 72°C for 10 min. The whole reaction was then fractionated on 1.2% regular agarose gel, and the PCR product was visualized by ethidium bromide staining. Primers for Mash1 wild type: Jim1, CCCAACTGGTTC TGAGGAC; Jim2, CCCATTTGACGTAGTTGG. Primers for Mash1 mutant: Neo1, GATCTCCTGTCATCTCACCT; Neo2, ATGGGTCACG ACGAGATCCT. Primers for Id2 wild type: YP-3, TCTGAGCTTATGT CGAATGATAGC; YP-21, CGTGTTCTCCTGGTGAAATGGCTG. Primers for Id2 mutants: NED-1, TCGTGCTTTACGGTATCGCCGCTC; YP-21, CGTGTTCTCCTGGTGAAATGGCTG.

Immunofluorescence Staining for Primary Cultures and Optic Nerve Sections

After fixation with 4% paraformaldehyde for 10 min at room temperature, cells were incubated for 30 min in a 50% goat serum solution containing 1% BSA and 100 mM l-lysine to block nonspecific binding and Triton 0.4% to permeabilize the membrane. In order to stain the surface antigens of optic nerve glial cells, cells were incubated in monoclonal A2B5 antibody, anti-GC antibody, or anti-GFAP, followed by fluorescein-coupled goat anti-mouse IgG (Jackson, 10 μg/ml), or cells were incubated with polyclonal anti-NG2, CC-1, or anti-GFAP antibodies, followed by fluorescein-coupled goat anti-rabbit IgG (Jackson, 10 μg/ml). The coverslips or slides were mounted in Citifluor on glass slides, sealed with nail varnish, and examined in a Zeiss Axioskope fluorescence microscope. In order to stain Mash1, cells were incubated in monoclonal anti-Mash1 antibody (a generous gift from Dr. David Anderson), followed by biotinylated goat anti-mouse IgG (Vector, 10 μg/ml). The nuclear staining was visualized with fluorescein-conjugated Streptavidin (Vector, 10 μg/ml).

After perfusion with 4% paraformaldehyde, optic nerves were transferred to 30% sucrose/PBS and frozen in O.C.T. compound (Tissue-Tek). Seven-micromolar cryosections were collected on gelatin (Sigma)-coated slides. For immunostaining, slides were dried at 37°C for 30 min and incubated for 1 hr in a 50% goat serum solution containing 1% BSA and 100 mM l-lysine to block nonspecific binding and Triton 0.4% to permeabilize the membrane. Sections were double stained with polyclonal antibodies against different glial cell markers and monoclonal anti-Mash1 antibody, followed by fluorescein-coupled goat anti-rabbit IgG (Jackson, 10 μg/ml) and biotinylated anti-mouse IgG, and later by fluorescein-coupled Streptavidin (Vector, 10 μg/ml). The slides were mounted in Citifluor, sealed with nail varnish, and examined with a Zeiss Axioskope fluorescence microscope.

All data in the text and figures are shown as means ± SEM (n = 3, coverslips per condition) and were repeated in three separate experiments.

Transfection of OPCs with Id2

The Id2 gene was inserted into a mammalian expression vector pIRES EGFP (Clontech) to create a plasmid overexpressing Id2, pIRES Id2-EGFP. Purified OPCs were grown in PDL-coated 60 mm culture dishes in the presence of mitogens until reaching 40% confluence. Defined Sato culture medium (100 μl) was mixed with Fugene-6 (6 μl; Boehringer Mannheim) and with (4 μg) pIRES EGFP and pIRES Id2-EGFP and incubated at room temperature for 15 min. Thirty microliters of the transfection solution was added to each well. Twenty-four hours after the start of the transfection, the medium was removed and replaced with serum-free medium minus mitogens. The differentiation of the oligodendrocytes was determined by immunostaining with an anti-GC antibody after 4 days.

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