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Changes in the Activity of cdk2 and cdk5 Accompany Differentiation of Rat Primary Oligodendrocytes

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Abstract Oligodendrocytes, the myelinating cells of the central nervous system, are terminally differentiated cells that originate through asynchronous waves of proliferation and differentiation of precursors present at birth. Withdrawal from cell cycle and onset of differentiation are tightly linked and depend on an intrinsic program modulated by the action of growth factors. p27 plays a central and obligatory role in the initiation of oligodendrocyte differentiation and cessation of proliferation. In this paper, we have characterized the role of modulation of cdk2 and cdk5 kinase activity during the process of oligodendrocyte precursor differentiation. As rat primary oligodendrocytes differentiate in culture there is a fall in cdk2 activity and a rise in cdk5 activity as well as an increase in the cdk inhibitor, p27 protein. The decline in cdk2 activity is not accompanied by a drop in cdk2 protein level, suggesting that it results from inhibition of cdk2 activation rather than decreased protein expression. Taken together, these data suggest that oligodendrocytes may withdraw from the cell cycle at G1-S transition through inactivation of cdk2 activity, possibly initiated by increasing amount of p27, and that cdk5 may have a role until now unrecognized in the differentiation of oligodendrocytes. J. Cell. Biochem. 68:128–137, 1998.

Key words: oligodendrocytes; cell cycle; differentiation; cyclin-dependent kinases; cdk5; cdk2

Oligodendrocytes, the myelinating cells of the central nervous system, arise from a progenitor cell population present in the developing neuroectoderm. At birth, oligodendrocyte precursors reside in the subventricular zone (SVZ), are bipolar, highly proliferative, motile cells that migrate from the SVZ to populate the entire brain [LeVine and Goldman, 1988]. Once they reach their final destination, they are postmitotic, they extend multiple processes that ensheath several axons, and they produce myelin. Withdrawal from the cell cycle is intimately coupled with the onset of differentiation and regulation of cell proliferation is an important step in the oligodendrocyte differentiation program [McKinnon et al., 1990]. Oligodendrocyte proliferation is relatively cell autonomous and depends on an intrinsic program modulated by the influence of several growth factors [McMorris and McKinnon, 1996; Raff et al., 1983; Barres et al., 1993; Pringle et al., 1992], including aa-PDGF (Platelet-Derived Growth Factor), IGF (Insulin-like Growth Factor), and possibly b-FGF (basic-Fibroblast Growth Factor).

Precursors grown in vitro in chemically defined media containing insulin and PDGF recapitulate their program of division and differentiation according to a schedule similar to that in vivo [Collarini et al., 1992]. After a number of cell divisions, the precursors identified by the A2B5 monoclonal antibody [Eisenbarth et al., 1979] withdraw from the cell cycle, lose reactivity to A2B5, express galactocerebroside (GC), and begin to activate myelin-specific genes [Raff et al., 1978]. In primary cultures each individual stage of development is clearly defined, and mitotic cells are identified with A2B5, postmitotic cells with anti-GC antibody [Raff et al., 1978; Pfeiffer et al., 1993]. Addition of FGF in the presence of PDGF maintains the precursors in the cell cycle and reversibly blocks differentiation, allowing the expansion of the pool of precursors [McKinnon et al., 1990; Collarini et al., 1992]. After withdrawal of growth factors, the precursors differentiate into postmitotic differentiated oligodendrocytes [Collarini et al., 1992].

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Cell division is controlled by a complex network of biochemical signals that regulate specific transitions in the cell cycle [MacLachlan et al., 1995]. Cyclin-dependent kinases are key regulators and have functional specificities at discrete points of cell cycle progression through their interaction with specific cyclins [Morgan, 1995; Morgan, 1996; Van Den Heuvel and Harlow, 1993]. Their activity depends on the balance of activation by cyclins and inhibition by cdks inhibitors [Sherr and Roberts, 1995] as well as inhibitory and activating phosphorylations [Morgan, 1995, 1996]. cdk2 is involved in the G1-S transition; cdk4, in association with D-type cyclins and in response to growth factors is involved in G1 progression; cdc2 is involved in the G2-M transition [Morgan, 1995].

Not all cdks are cell cycle regulators. cdk5, a cdc2-like kinase [Lew and Wang, 1995], is not involved in cell cycle progression, and although expressed in many cell types, is found to be active only in postmitotic neurons [Tsai et al., 1993], where it is essential for both differentiation [Nikolic et al., 1996] and survival at the time of cell cycle withdrawal [Ohshima et al., 1996]. Recently, cdk5 activity was shown to be associated with the developing rat lens [Gao et al., 1997] and with apoptosis in some developmental programs [Zhang et al., 1997], suggesting that this kinase has a broad spectrum of functions.

Terminally differentiated cells withdraw from the cell cycle in G1 phase either at mid-G1 or G1-S transition [Halevy et al., 1995; Guo-zai and Ziff, 1995]. In the case of the oligodendrocytes, cell cycle withdrawal and the mechanisms that regulate it are not completely elucidated. The cyclin dependent kinase inhibitor, p27, appears to play a central role in the cessation of cell proliferation and initiation of differentiation of oligodendrocytes, suggesting that withdrawal from the cell cycle may occur at the G1-S transition [Casaccia-Bonnefil et al., 1997]. However, p27 accumulation precedes cell cycle withdrawal and occurs in actively dividing precursors grown in the presence of PDGF and FGF [Durand et al., 1997]. In addition, differentiation of p27 null mutant oligodendrocytes proceeds normally in vivo, although a greater number of oligodendrocytes is generated [Casaccia-Bonnefil et al., 1997; Kiyokawa et al., 1996] suggesting that other mechanisms in addition to p27 may play a role.

Regulation of cdks activities plays a critical function in both control of cell cycle progression as well as initiation and maintenance of the differentiated phenotype [Van Den Heuvel and Harlow, 1993; Ohshima et al., 1996]. To characterize the functional role of cell cycle related cdks and of the postmitotic kinase, cdk5, in the process of oligodendrocyte differentiation we have determined changes of their activities as oligodendrocyte precursors differentiate. For these experiments we have employed primary immunoselected oligodendrocyte precursors grown in the presence of PDGF and FGF, and postmitotic oligodendrocytes generated by removal of growth factors.

Here, we report that differentiation of oligodendrocytes is accompanied by a fall in cdk2 activity as well as a rise in cdk5 kinase activity and p27 protein level. The fall in cdk2 activity is not accompanied by a drop in cdk2 protein level, thus suggesting that inhibition of cdk2 activity is the initial step leading to G1-S arrest, in keeping with the obligatory function of p27 in cell cycle arrest. cdk5 activity, although present in both proliferating precursors and postmitotic oligodendrocytes, is higher in postmitotic oligodendrocytes, suggesting a role of cdk5 in the process of oligodendrocyte differentiation. To our knowledge, this is the first report which points out that cdk5 is an active kinase in oligodendrocytes and suggests that cdk5 may play a role in their process of differentiation.

METHODS

Rat-Mixed Primary Brain Cultures

Primary cortical glial cultures were established from 2-day-old Sprague-Dawley rat brain after carefully removing the meninges, as previously described [Behar et al., 1988]. Cultures were maintained in DMEM (4.5 g/L D-glucose) supplemented with gentamycin 25 µg/ml, insulin 5 µg/ml, and 10% FCS for 7–10 days in incubator at 37°C and 10% CO_2. Oligodendrocyte precursors were isolated from mixed cultures by shaking on a rotary platform (250 rpm) overnight at 37°C followed by immunoselection to enrich for the precursor cells.

Immunoselection

Cell suspensions were plated on uncoated Petri dishes for 3 h at 37°C to remove microglia and macrophages. The loosely attached precursors were flushed gently and contaminating

astrocytes and differentiated oligodendrocytes were removed by sequential immunopanning. Petri dishes, 100 mm in diameter, were incubated overnight at 4°C with anti-Ig antibody solution at 5 μg/ml in 50 mM Tris pH 9.5. The dishes were washed with Ca- and Mg-free PBS and incubated for 2 h with the appropriate antibody for selection. Single-cell suspension in MEM containing 0.5% FCS was passed over one dish coated with 1:10 dilution of Ran-2 hybridoma (a kind gift of Dr. McMorris) supernatant to remove astrocytes and meningeal cells and over one dish coated with 1:10 of O1 (a monoclonal antibody that recognizes GC) hybridoma (a kind gift of Dr. McMorris) supernatant to remove differentiated oligodendrocytes. Each selection step was carried out for 30 min at room temperature (RT) with occasional gentle swirling. Precursors were removed by gentle flushing, spun down, and resuspended in DMEM supplemented with insulin, gentamycin, and 10% FCS. Precursors were allowed to attach O/N to 6 well plates (approximately 5×10^5 cells per well) coated with poly-D-ornithine and containing poly-D-ornithine coated glass coverslips (Bellco). Cells were grown in chemically defined media [Collarini et al., 1992] containing 30 nM T3, 5 μg/ml insulin, 10 ng/ml biotin, and 10 ng/ml each of bovine bFGF and human aa-PDGF (R&D) for 3 days. To generate postmitotic oligodendrocytes, growth factors were withdrawn and cells were maintained for 3 days in chemically defined media supplemented with 0.5% of FCS to prevent apoptosis.

Immunofluorescence Analysis

Purity of the primary cultures was established by indirect immunofluorescence using the following antibodies: (1) a rat monoclonal antibody to glial fibrillary acidic protein (GFAP), an intermediate filament specific for astrocytes and the stage-specific antibodies; (2) monoclonal antibody A2B5 [Eisenbarth et al., 1979], which labels a subset of gangliosides at the surface of the progenitor cells [Raff et al., 1978]; and (3) the Rancht monoclonal antibody [Ranscht et al., 1982], which recognizes an unidentified antigen in addition to galactocerebroside [Pfeiffer et al., 1993] on the surface of postmitotic oligodendrocytes. Cells grown on poly-D-ornithine-coated coverslips were washed in PBS and fixed in 2% paraformaldehyde for 15 min at RT. After rinsing with PBS, coverslips were incubated with A2B5 and Rancht monoclonals at a dilution 1:2 for 30 min at RT; and after washing with PBS were incubated with the secondary rhodamine-conjugated anti-IgM and FITC-conjugated anti-IgG 1:50 diluted in PBS for 30 min at RT. For GFAP staining, coverslips were fixed in 2% paraformaldheyde, permeabilized with 0.1% Triton X-100 in PBS, and reacted with anti-GFAP antibody (a kind gift of Dr. McMorris) at 1:10 dilution and with an FITC-conjugated anti-IgG2a secondary antibody. Coverslips were mounted on glass slides under Elvanol. Labeled cells were viewed using Zeiss fluorescent microscope.

Cell Cycle Status and DNA Synthesis of Precursors and In Vitro Differentiated Oligodendrocytes

DNA synthesis was assessed by using the 5-bromo-2-deoxyuridine (BrdUdr) incorporation and labeling kit (Boehringher-Mannheim, Indianapolis, MN). Precursors and mature oligodendrocytes were cultured as described on poly-D-ornithine-coated glass coverslips and BrdUdr was added (10 μmol) for the last 18 h of culture and cells were stained according to the manufacturer's protocol. Positive cells were counted in 10 different fields. Cell cycle analysis was carried out by staining with propidium iodide using the DNA test plus kit (Becton-Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, cells were suspended in a buffer containing 250 mM sucrose, 40 mM trisodium citrate, and DMSO, kept frozen until staining with propidium iodide, then analyzed by flow cytometry.

Cell Extracts and Immunoprecipitation

Cell extracts were obtained by lysing cells in lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA (pH 8), 0.1% NP-40, 5 mM DTT, 1mM PMSF, 10 mM NaF, 1 mM NaVO4, 20 mM NaP2O7, 10 μg/ml each of leupeptin, aprotinin, and pepstatin) for 30 min on ice [Tsai et al., 1993]. Cell lysates were recovered after trituration and spun at 12,000 rpm for 5 min in an Eppendorf microfuge at 4°C. Protein concentration of each samples was determined by using the BioRad protein assay (BioRad).

50 μg protein were incubated with rabbit IgG anti-cdc2 and anti-cdk2 (kind gifts of Dr. J. Th'ng), and anti-cdk5 (Santa Cruz Biotechnology, CA) for 1h at 4°C, followed by precipitation with Protein A-agarose (Santa Cruz Biotechnology) for 1 h at 4°C. The agarose beads were

washed three times with lysis buffer and equilibrated with two washes with kinase reaction buffer (50mM Hepes pH 7.9, 5 mM MgCl, 1mM DTT). Competition of cdk5 antibody with cdk5-specific peptide was carried out by incubating the antibody with 10 µg of the peptide at RT for 2 h prior to immunoprecipitation.

Cyclin-Dependent Kinase Assay

To assay cdc2, cdk2, and cdk5, the agarose beads were incubated for 30 min at 30°C in kinase reaction buffer containing ATP 20 µM, with histone H1 2 µg (Upstate Biotechnology, Lake Placid, NY) as substrate and 2 µCi of gamma-^{32} P-ATP (New England Nuclear, DuPont NEN, Boston, MA) per sample. The reactions were terminated by adding 2X Laemmli sample buffer and boiled for 3 min prior to loading on the gel. Phosphorylated histone H1 was separated by 10% SDS-PAGE, transferred to PVDF membranes (Immobilon, Millipore), visualized, and quantitated by phosphorimager (Molecular Dynamics).

Western Blot Analysis of Inhibitors and Cdks

To determine steady-state levels of cdks, and cyclins inhibitors, 50 µg of cell extracts of precursors and postmitotic oligodendrocytes were separated in 10% SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore). Membranes were blocked at RT for 1 h with 5% dry milk in Tris-HCl 25 mM pH 7.6, NaCl 137 mM, 0.1% Tween-20 (TBS-T), incubated for 2 h at RT with antibodies to cdk5, cdc2, cdk2, p27, and p21 (Upstate Biotechnology) at a dilution 1:1,000 in 3% dry milk in TBS-T and reacted with peroxidase-conjugated secondary antibody at a dilution 1:10,000 in 3% milk in TBS-T for 1 h at RT. The reactions were developed with enhanced chemiluminescence (ECL, Amersham). Quantitation was carried out by densitometry and data were analyzed by Kodack Digital Science 1D Image Analysis Software (Eastman–Kodak, Rochester, NY).

RESULTS

Characterization of Purified Precursors and Oligodendrocytes

We have purified oligodendrocyte precursors from 1-week-old mixed rat primary cultures with sequential immunopanning, and cultured them in the presence of FGF and PDGF. This combination of growth factors keeps cells in the cell cycle and prevents progenitors from differentiating [McKinnon et al., 1990; Collarini et al., 1992]. Freshly immunopanned precursors in 10% FCS were also analyzed and compared to precursors cultured in the presence of the growth factors. To allow the progenitors to differentiate into postmitotic oligodendrocytes, growth factors were removed for 72 h.

TABLE I. Precursors Stop Dividing and Differentiate Rapidly After Removal of Growth Factors*

Cell type	GC$^+$ (%)	BrdUdr$^+$ (%)
Precursors in FGF/PDGF	10	47
Oligodendrocytes (24 h)	36	13
Oligodendrocytes (72 h)	61	10

*Precursors isolated as described in Materials and Methods were cultured for 72 h in PDGF and FGF and were induced to differentiate without growth factors for 24 and 72 h. DNA synthesis was evaluated by measuring the uptake of the thymidine analog BrdUdr over an 18 h period by staining with anti-BrdUdr antibody. Differentiation was determined by galactocerebroside (GC) surface expression with the Ranscht antibody. Cells were examined in the fluorescence microscope. At least 100 cells were scored on each coverslip and results are representative of multiple experiments performed.

Freshly immunopanned precursors maintained in 10% FCS/DMEM for 24 h, pulsed for 18 h with BrdUdr in the presence of 10% FCS, contained approximately 75–80% of A2B5 positive precursors, 15–20% GC positive differentiated oligodendrocytes, and about 5% contaminating astrocytes. In addition, a variable percentage of cells stained both with A2B5 and anti-GC antibody. About 30–40% of the cells incorporated BrdUdr. GC positive cells are accounted for by both oligodendrocytes that escaped the O1 panning and precursors that underwent differentiation during the culture period, since GC expression occurs within 24 h from cell cycle exit [Noble and Murray, 1984]. Precursors kept in culture for 3 days with PDGF and FGF showed an expansion of the precursors, contained 90–95% A2B5 positive precursors, and approximately 50–60% of the cells incorporated BrdUdr. These cultures, although cell cycle asynchronous, were more homogeneous than the freshly immunopanned precursors. When growth factors were removed, only 2–10% of the cells incorporated BrdUdr; and approximately 65% were GC positive (Table I and Fig. 1)

Cell cycle analysis by flow cytometry of PI-DNA content showed that 72 h after growth

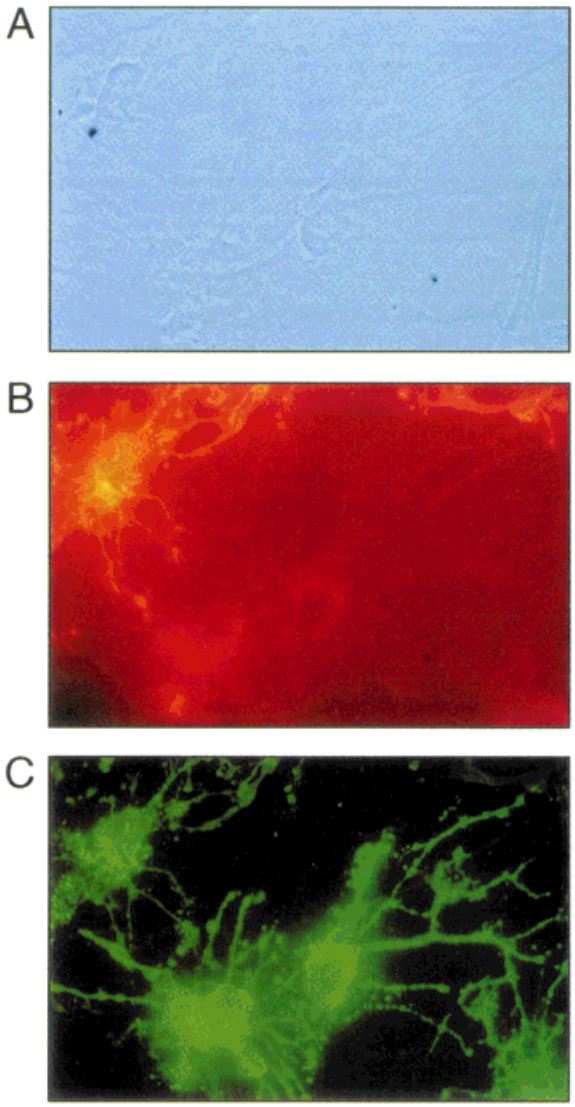

Fig. 1. Primary rat oligodendrocyte precursors differentiate into GC positive oligodendrocytes after removal of growth factors. Primary rat oligodendrocytes grown for 3 days in chemically defined media without FGF and PDGF. **A**: Phase contrast micrograph of the cell culture. **B**: Same field as in A in which oligodendrocytes are stained with A2B5 monoclonal and rhodamine-conjugated second antibody. **C**: Same field as in A in which oligodendrocytes are stained with Ranscht monoclonal and FITC-conjugated second antibody. This figure shows that the majority of oligodendrocytes express GC and are differentiated; some costain with both A2B5 and Ranscht.

factors withdrawal greater than 90% of cells are in G1, and approximately 2% are in S. In precursors grown in PDGF and FGF, 13–15% of cells were found in S (Fig. 2). Therefore, after withdrawal of growth factors, there is an increase in cells with unreplicated DNA and a decrease in cells synthesizing DNA. The greater proportion of cells that incorporate BrdUdr in the precursors likely reflects the 18-h pulse period.

In summary, we have generated homogeneous populations of either proliferating precursors or postmitotic oligodendrocytes to be used for biochemical studies of cdks activities during differentiation.

Loss of cdk2 and cdc2 Activities Is Associated With Differentiation of Oligodendrocytes

Kinase activity of cdk2 and cdc2 were assayed on histone H1 after immunoprecipitation with specific antibodies of cell lysates of precursors grown in the presence of FGF and PDGF for 3 days and postmitotic oligodendrocytes maintained in culture without growth factors for 72 h. Precursors grown in FGF and PDGF displayed high cdk2 and cdc2 activities, the highest being cdk2 (Figs. 3 and 4), while postmitotic oligodendrocytes showed at least 5–6-fold decrease in cdk2 and cdc2 activities (Fig. 4). To ascertain that the pattern of kinase activity identified in precursors grown in the presence of FGF and PDGF was not the result of a specific action of FGF, we have assayed cdk2 and cdc2 in freshly immunopanned precursors not exposed to FGF. These precursors displayed a profile of cdk2 and cdc2 activities similar to that observed in precursors expanded in FGF (Fig. 3).

To determine whether the fall in kinase activity was accompanied by a parallel decline in protein expression, steady-state levels of cdk2 and cdc2 were determined by Western blot analysis of lysates of precursors and postmitotic oligodendrocytes. cdk2 protein level remained the same (Fig. 5), suggesting that at least in the initial stages of oligodendrocyte differentiation loss of cdk2 activity results from inactivation of kinase activity rather than decreased expression. We could detect a very faint signal for cdc2 by Western in either precursors or oligodendrocytes (data not shown), despite the presence of high activity in precursors. Given the very low abundance of the protein in precursors and oligodendrocytes, no detectable differences in the steady-state levels were observed in the two cell populations.

To determine whether an increase in the cdks inhibitors occurs in the postmitotic oligodendrocytes, blots of lysates of precursors and oligodendrocytes were probed with p27 and p21 antibodies. p27 steady-state levels determined by

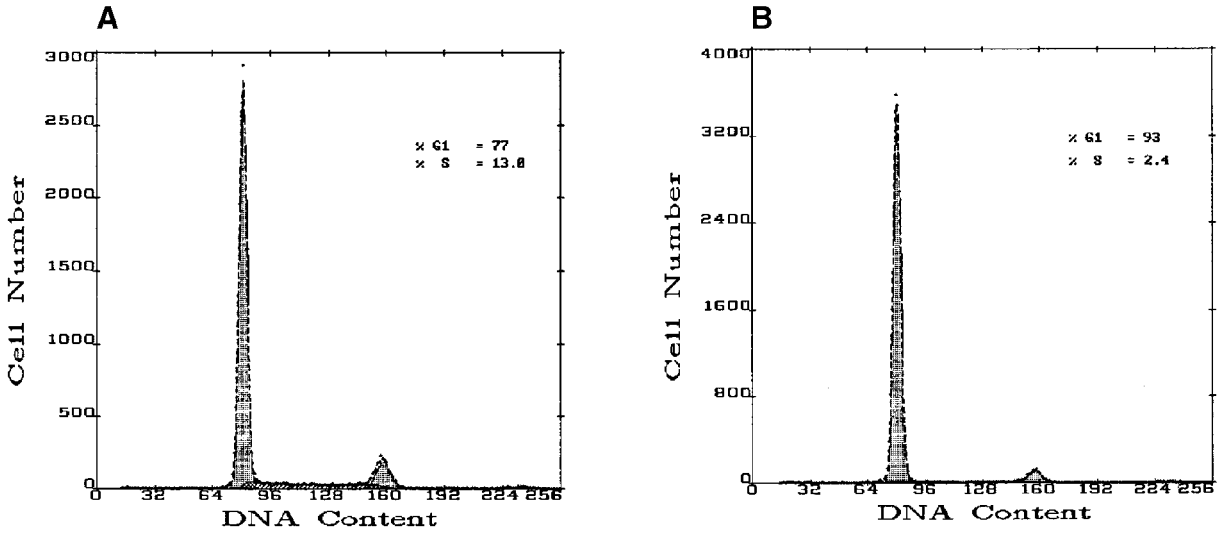

Fig. 2. Cell cycle analysis of precursors and oligodendrocytes. **A**: Precursors grown in PDGF and FGF for 72 h. **B**: Differentiated oligodendrocytes 72 h after removal of growth factors. DNA was stained with propidium iodide and analyzed by flow cytometry in Coulter flow cytometer, cell debris, and doublets were excluded using a conventional scatter gating program.

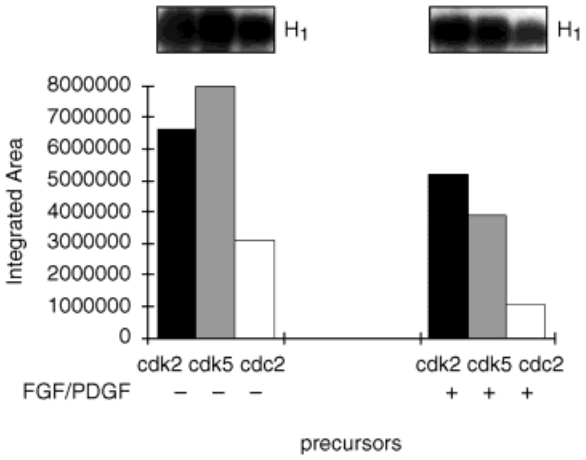

Fig. 3. Profile of cdk2, cdk5, and cdc2 kinase activities in precursors grown in PDGF and FGF and in asynchronously differentiating precursors without growth factors. Precursors grown in PDGF and FGF for 3 days and freshly immunopanned precursors grown in 10% FCS for 24 h were lysed and immunoprecipitated with anti-cdk5, anti-cdk2, and anti-cdc2 antibodies. Immune complexes were incubated in kinase buffer containing gamma-^{32} P-ATP and histone H1. Phosphorylated histone was separated in denaturing gels and bands were visualized and quantitated by phosphorimager. Data are expressed as integrated area and are representative of three separate experiments. The mean percent increase of cdk5 activity in differentiating precursors versus precursors maintained with growth factors is 172%=60 (n=3).

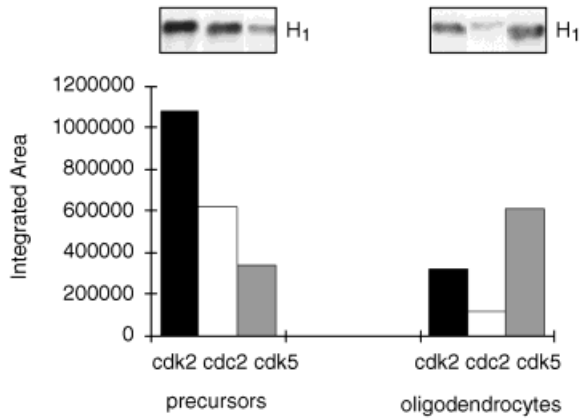

Fig. 4. Profile of cdk2, cdc2, and cdk5 kinase activities in cycling precursors and differentiated oligodendrocytes. Precursors grown in PDGF and FGF for 3 days and differentiated oligodendrocytes 72 h after removal of growth factors were lysed and immunoprecipitated with anti-cdk5, anti-cdk2, and anti-cdc2 antibodies. Immune complexes were incubated in kinase buffer containing gamma-^{32} P-ATP and histone H1. Phosphorylated histone was separated in denaturing gels and bands were visualized and quantitated by phosphorimager. Data are expressed as integrated area and are representative of three separate experiments. The mean percent increase of cdk5 activity in differentiated oligodendrocytes versus precursors grown in growth factors is 167%=26 (n=3).

densitometry of Western blots were found to be 50% higher in oligodendrocytes than in precursors (Fig. 4), suggesting that p27 may be involved in the inactivation of cdk2 activity. p21 was not detected in either precursors or oligodendrocytes in our culture system (data not shown).

Taken together, these data suggest that cell cycle withdrawal of oligodendrocytes may occur at G1-S transition due to inactivation of cdk2

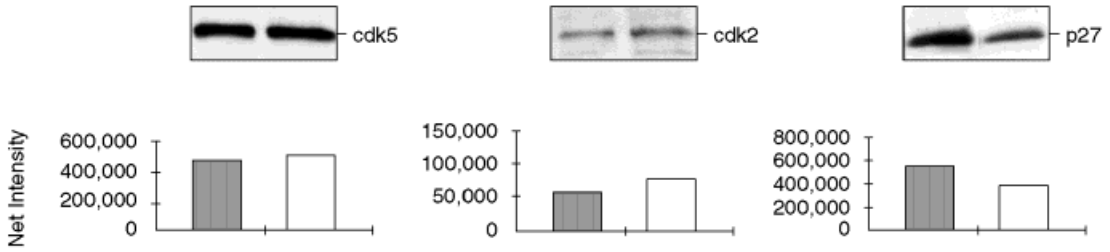

Fig. 5. Expression of cdk5, cdk2, and p27 protein in differentiated oligodendrocytes and precursors. Lysates of precursors grown in PDGF and FGF (white bars) and differentiated oligodendrocytes 72 h after removal of growth factors (gray bars) were separated in 10% SDS-PAGE and blotted. Blots were probed with anti-cdk5, anti-cdk2, and anti-p27 antibodies and developed with enhanced chemiluminescence (ECL). Bands were quantitated by densitometry and data were analyzed by Kodack Digital Science 1D Image Analysis Software. Data are expressed as net intensity and are representative of multiple experiments.

kinase in keeping with a role of p27 in controlling cessation of oligodendrocyte cell divisions.

An Increase in cdk5 Kinase Activity Accompanies Differentiation of Oligodendrocyte Precursors

cdk 5 is expressed in a variety of cells [Tsai et al., 1993], including the mitogen-expanded oligodendrocyte precursor line, CG4 cells [Tikoo et al., 1997; Tang and Cambi, unpublished data], and in primary mouse oligodendrocytes [Casaccia-Bonnefil et al., 1997]. cdk5 activity has been shown to be restricted to postmitotic neurons [Tsai et al., 1993], where it has essential cellular functions for survival and differentiation [Ohshima et al., 1996]. Recently, cdk5 activity has been associated with other developmental processes [Zhang et al., 1997; Gao et al., 1997]. In light of these data that suggest a multiplicity of functions of cdk5 and a role in differentiation, we have explored whether cdk5 may have a function in oligodendrocyte differentiation. We have characterized cdk5 activity in rat primary precursors and studied whether modulation of cdk5 activity accompanies the process of oligodendrocyte differentiation.

We have found that although cdk5 is expressed in both cycling precursors and postmitotic oligodendrocytes at the same level (Fig. 5), cdk5 kinase activity is approximately 2-fold higher in postmitotic oligodendrocytes than in precursors (Fig. 4). Preabsorption of cdk5 antibody with a competing peptide specific for cdk5 effectively abolished cdk5 kinase activity (data not shown). These data show that cdk5 is an active kinase in the oligodendrocyte lineage and that its activity is regulated as oligodendrocytes differentiate.

Interestingly, cdk5 activity assayed in extracts of freshly immunopanned precursors kept in 10% FCS/DMEM for 18–24 h was also 1.5–2-fold higher than that of precursors maintained in PDGF and FGF for 3 days (Fig. 3). Freshly immunopanned precursors contain a percentage of GC-positive cells and cells positive for both A2B5 and GC higher than in precursors grown in PDGF and FGF, thus accounting for the high cdk5 activity detected in these cells that are heterogeneous and asynchronously differentiating. This finding further supports the interpretation that an increase in cdk5 activity accompanies the differentiation of oligodendrocytes.

DISCUSSION

Differentiation of oligodendrocytes involves a coordinated sequence of events that leads to withdrawal from the cell cycle and activation of genes specific for the differentiated phenotype. The precise sequence by which these events occur has not been fully elucidated. We have determined how changes in the activity of cell cycle related kinases, cdk2 and cdc2; and of cdk5 relate to the differentiation of primary rat oligodendrocytes. In this report, we show that differentiation of oligodendrocytes is accompanied by the coordinate fall in cdk2 activity and the rise in cdk5 activity, as well as an increase in the cdk inhibitor p27 protein level. cdk2 protein level remains unchanged, suggesting that inactivation of cdk2 activity may be initially responsible for G1-S arrest, possibly through an interaction with p27. In addition, cdk5 is activated as oligodendrocytes differentiate and may play a role in their program of differentiation.

Most terminally differentiated cells withdraw from the cell cycle during G1, either at mid-G1 or in late G1, G1-S transition, before DNA synthesis ensues [Halevy et al., 1995; Guo-zai and Ziff, 1995; Sherr, 1994; Pardee,

1989]. G1-S appears to be the critical transition at which oligodendrocyte precursors arrest, since p27 accumulates in differentiated oligodendrocytes [Durand et al., 1997; Casaccia-Bonnefil et al., 1997; this report] and precursors that lack p27 are unable to withdraw from the cell cycle and differentiate [Casaccia-Bonnefil et al., 1997]. Since transition through G1-S is controlled by cdk2 activation [Van Den Heuvel and Harlow, 1993], it is conceivable that changes in cdk2 activity are involved in inducing arrest of cell cycle progression of oligodendrocyte precursors. Here, we demonstrate for the first time that loss of cdk2 activity occurs in differentiated oligodendrocytes. A previous report failed to show a drop in cdk2 kinase activity in differentiated oligodendrocytes, although cdc2 activity was found to be low [Rus et al., 1996]. We also find loss of cdc2 activity, a mitotic kinase involved later in cell cycle at the transition between G2 and M, to be a result of cell cycle withdrawal in G1. cdk2 protein level does not change in differentiated oligodendrocytes 3 days after removal of growth factors, suggesting that at least in the early stages of differentiation, the fall in cdk2 activity is the result of inhibition rather than decreased expression. Other investigators have reported a fall in cdk2 protein level, as mouse oligodendrocytes differentiate in vitro [Casaccia-Bonnefil et al., 1997]. We do not know the reason for this discrepancy; however, it may reflect differences in the culture system and the higher degree of oligodendrocyte differentiation reported to be as high as 95%.

We detect a modest increase in p27 steady-state protein level in our cultures of differentiated oligodendrocytes, lower than that reported at the single cell level and in differentiated oligodendrocyte cultures by other investigators [Casaccia-Bonnefil et al., 1997]. This may reflect heterogeneity of individual cell contents of p27 at the single cell level [Durand et al., 1997], as well as a lower degree of oligodendrocyte differentiation in our cultures as compared to that reported by others, and a higher number of mature oligodendrocytes present in our precursor preparations [Casaccia-Bonnefil et al., 1997].

Furthermore, the absolute increase in p27 level may not be the critical determinant of cdk2 inhibition; rather, the distribution of p27 in cyclin-cdks complexes may be the important element that regulate cdk2 activity. First, p27 has been shown to accumulate in cycling oligodendrocyte precursors to a similar degree as differentiated oligodendrocytes that have stopped dividing, suggesting that an increase alone in p27 is not sufficient to stop cell cycle progression [Durand et al., 1997]. Second, in other cell types, p27 has been shown to associate with cdk4 and cdk5 complexes in dividing cells and associate with cdk2 as they differentiate without changes in the absolute amount of p27 [Reysnidottir et al., 1995; Reysnidottir and Massague, 1997]. Although we do not have direct evidence to demonstrate that association of p27 with cdk2/cyclinE complexes occurs in differentiated oligodendrocytes, it is tempting to speculate that formation of p27 complexes with cdk2/cyclinE leads to inhibition of cdk2 kinase and initiates cell cycle arrest of oligodendrocyte precursors, in light of the obligatory role of p27 in oligodendrocyte precursors differentiation.

However, other regulatory mechanisms, such as inhibitory phosphorylation of cdk2 [Gu et al., 1992] or decreased cdk2 expression may also contribute to the onset and/or the maintenance of cell cycle arrest. Indirect evidence to support that additional mechanisms may play a role comes from findings that p27 null mutant oligodendrocytes withdraw from the cell cycle and differentiate normally in vivo, despite the absence of p27 [Casaccia-Bonnefil et al., 1997]. Furthermore, p27 accumulates in cycling precursors in vitro [Durand et al., 1997] without affecting progression through the cell cycle, suggesting that accumulation of p27 may act in conjunction with other mechanisms that control cell cycle progression and/or with a simultaneous differentiating signal. The role of other cdks inhibitors, such as p21, remains unclear at present. We, as well as others, [data not shown, Durand et al., 1997], have found undetectable levels of p21 both in precursors and differentiated oligodendrocytes; while other investigators have shown a fall in p21 level in differentiated mouse oligodendrocytes [Casaccia-Bonnefil et al., 1997]. However, p21 does not seem to compensate for loss of p27 function in p27-null mutant oligodendrocytes in vitro, since these cells fail to differentiate and to arrest cell division [Casaccia-Bonnefil et al., 1997]. Whether p21 has a role in vivo in cell cycle arrest of oligodendrocytes remains to be elucidated.

We have found that cdk5 kinase activity is present in the oligodendrocyte lineage, and increases both in oligodendrocyte precursors that asynchronously differentiate and in differenti-

ated oligodendrocytes, raising the possibility that cdk5 may play a role in oligodendrocyte differentiation. The increase in cdk5 activity is reproducibly demonstrated in differentiated oligodendrocytes and precursors that are undergoing differentiation, as compared to precursors that are prevented from differentiating by FGF. The modest fold increase is likely explained by the characteristics of our precursor cultures that contain a number of GC positive cells in growing conditions and display a degree of differentiation upon removal of growth factors of 65%, reducing the degree of differences detected.

The role of cdk5 in oligodendrocytes remains to be elucidated. It is tempting to speculate that cdk5 may be a differentiation signal for oligodendrocytes, possibly in conjunction with p27, and may participate in the regulation of differentiation. In neurons, cdk5 has multiple targets, both in the nucleus, where it interacts with the retinoblastoma protein [Lee et al., 1997]; and in the cytoplasm, where it interacts with the cytoskeleton components and plays a central role in survival and differentiation of postmitotic neurons [Nikolic et al., 1996]. Unlike neurons, cdk5 is active both in cycling oligodendrocyte precursors, although at a lower level, and in differentiated oligodendrocytes. It is possible that in oligodendrocytes cdk5 resides in more than one intracellular compartment, as also shown for neurons; thus affecting the interacting molecules [Matsushita et al., 1995], the level of activity, and the specific targets resulting in diversified functions in precursors and differentiated oligodendrocytes. Since cdk5 null mutants die perinatally [Ohshima et al., 1996] and oligodendrocytes are generated in the postnatal period, a role of this kinase in oligodendrocytes is difficult to assess in these mutants and will require studies aimed at disrupting cdk5 expression and function in oligodendrocytes in vitro.

In summary, cdk2 and cdk5 activities are modulated during differentiation of oligodendrocytes, and the understanding of the relationship of the role of these kinases with that of p27 will help elucidate the mechanisms underlying oligodendrocyte differentiation.

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