

# Morphological Differentiation of Oligodendrocytes Requires Activation of Fyn Tyrosine Kinase

Donna J. Osterhout,\* Amy Wolven,<sup>‡§</sup> Rebecca M. Wolf,<sup>§</sup> Marilyn D. Resh,<sup>§</sup> and Moses V. Chao\*

\*Molecular Neurobiology Program, Skirball Institute, New York University Medical Center, New York 10016; <sup>‡</sup>Molecular Biology Program, Cornell University Medical College, New York; and <sup>§</sup>Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York 10021

**Abstract.** In the central nervous system, myelination of axons occurs when oligodendrocyte progenitors undergo terminal differentiation and initiate process formation and axonal ensheathment. Although it is hypothesized that neuron-oligodendrocyte contact initiates this process, the molecular signals are not known. Here we find that Fyn tyrosine kinase activity is upregulated very early during oligodendrocyte progenitor cell differentiation. Concomitant with this increase is the appearance of several tyrosine phosphorylated proteins present only in differentiated cells. The increased tyrosine kinase activity is specific to Fyn, as other Src family members are not active in oligodendrocytes. To investigate the function of Fyn activation on differenti-

ation, we used Src family tyrosine kinase inhibitors, PP1 and PP2, in cultures of differentiating oligodendrocyte progenitors. Treatment of progenitors with these compounds prevented activation of Fyn and reduced process extension and myelin membrane formation. This inhibition was reversible and not observed with related inactive analogues. A similar effect was observed when a dominant negative Fyn was introduced in progenitor cells. These findings strongly suggest that activation of Fyn is an essential signaling component for the morphological differentiation of oligodendrocytes.

**Key words:** oligodendrocyte • myelin • Fyn • differentiation • tyrosine phosphorylation

**O**LIGODENDROCYTES are the myelin-forming cells of the central nervous system. Early oligodendrocyte progenitors arise from neuroepithelium in the ventral spinal cord and migrate throughout the brain parenchyma (Levison et al., 1993; Price 1994). Upon terminal differentiation, oligodendrocytes extend several processes that ensheath and myelinate nerve axons. In vitro, mature oligodendrocytes extend a complex network of processes, and extensive myelin-like membrane sheets in the absence of axons (Dyer and Benjamins, 1988). Differentiation of progenitors into the mature phenotype is characterized by both morphological changes and the appearance of stage-specific cell surface markers (Pfeiffer et al., 1993), but the signals that trigger this program, both in vitro and in vivo, are not known.

Fyn is a member of the Src family of cytoplasmic nonreceptor tyrosine kinases (reviewed by Thomas and Brugge,

1997). There are nine members of this family, some of which (Src, Lyn, and Fyn) are widely expressed in many tissues, and others which are more restricted in their expression. These protein kinases can be activated by a variety of extracellular signals, and interact with multiple intracellular substrates, including focal adhesion kinase (FAK)<sup>1</sup>, p130Cas, structural proteins such as cortactin, and connexin 43, and stimulate other signaling pathways within the cell. Src family kinases have been linked to cellular events such as cell adhesion, cytoskeletal rearrangements, cell migration, regulation of the cell cycle, apoptosis, and differentiation (Boschek et al., 1981; Hecker et al., 1991; Twamley-Stein et al., 1993; Kaplan et al., 1995; Rodier et al., 1995; Huang et al., 1997).

Several lines of evidence suggest that Fyn has a role in myelination. Fyn is expressed at high levels in the brain and is localized to neurons and glial cells (Umemori et al., 1992; Bare et al., 1993). The myelin content in brains from *fyn*-deficient transgenic mice is significantly reduced, as demonstrated by Western analysis of myelin basic protein

The first two authors contributed equally to this manuscript.

Address correspondence to Moses V. Chao, Molecular Neurobiology Program, Skirball Institute, NYU Medical Center, 540 First Ave., New York, NY 10016. Tel.: (212) 263-0721. Fax: (212) 263-8214. E-mail: chao@saturn.med.nyu.edu

1. *Abbreviations used in this paper:* FAK, focal adhesion kinase; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; NPTII, neomycin phosphotransferase II; PLL, polylysine; RT, room temperature.

(MBP) and by immunohistochemical analysis of MBP expression in brain sections (Umemori et al., 1994). Fyn can also be coimmunoprecipitated from brain lysates with antibodies to myelin-associated glycoprotein (MAG) (Umemori et al., 1994). Since MAG is one of the earliest myelin proteins to be expressed, these findings suggest that Fyn is involved in the myelination process. However, the precise role of Fyn in oligodendrocytes and the myelination process has not yet been defined. This study reveals that activation of Fyn is one of the earliest events triggered as oligodendrocyte progenitor cells differentiate, and that Fyn tyrosine kinase regulates process extension and myelin sheet formation *in vitro*.

## Materials and Methods

### Cell Culture

Primary cultures of oligodendrocyte progenitor cells were isolated from P2 neonatal rat brain (Sprague Dawley; Taconic Farms) according to the procedure of Osterhout et al. (1997) with slight modifications. In brief, mixed dissociated cultures of neonatal brain were allowed to reach confluence, after which the oligodendrocyte progenitor population was removed by shaking. The progenitor cells were further purified by immunopanning with A2B5 (Osterhout et al., 1997), and plated onto polylysine (PLL)-coated dishes. Progenitors were lysed 24 h after plating and analyzed for Fyn protein expression and kinase activity (see below). Differentiation of progenitor cells was initiated by switching the media to a defined media lacking growth factors (Osterhout et al., 1997). To maintain the cells as progenitors, and prevent differentiation, PDGF and FGF were included in the differentiation media at concentrations that stimulate the maximal proliferation of the cells (10 ng/ml and 20 ng/ml, respectively; Osterhout et al., 1997). The mature oligodendrocytes were analyzed for Fyn expression and activity after 4–6 d in culture. The differentiation state of the cells, either progenitor or mature oligodendrocyte, was determined by immunocytochemical analysis of stage-specific markers such as A2B5 for progenitor cells, and O1, MAG, and MBP for differentiated oligodendrocytes (Pfeiffer et al., 1993). For the inhibitor studies, PP1, PP2, PP3, and PP4 (Calbiochem Novabiochem) were resuspended in DMSO, and included in the culture media at specified concentrations.

### Immunocytochemistry and Morphological Analysis

Cells were grown on PLL-coated 10-mm glass coverslips (Dynalab) for all immunocytochemistry. For oligodendrocyte lineage markers such as A2B5 and O1, the coverslips were rinsed in PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl) and incubated in antibody diluted in PBS for 30 min at room temperature (RT). The coverslips were then fixed in 4% paraformaldehyde for 30 min. For Fyn localization, the cells were permeabilized with 0.2% Triton X-100, then incubated with a rabbit antibody to Fyn (Santa Cruz Biotechnology) overnight at 4°C. Fluorescein- and rhodamine-conjugated secondary antibodies (Vector Labs) were diluted 1:50 in PBS and left on the coverslips for 30 min. For each immunostain, one coverslip was incubated in the secondary antibody alone as a negative control for background immunofluorescence. The coverslips were rinsed in PBS and mounted on glass slides using Vectashield (Vector Labs). In all staining procedures, labeled cells were visualized using a Nikon Optiphot microscope or a Leica confocal microscope.

For analysis of process outgrowth, the distance from the center of the cell body to the tip of the longest process was measured, which in mature oligodendrocytes corresponds to the radius of the extensive process network that surrounds the cell body. For PP1-inhibited cells, this corresponds to the length of the longest process extending from the cell. If a process was not longer than the cell body diameter, it was not measured. For each condition, process outgrowth was measured for 200 individual cells from control and inhibitor-treated cells.

### Immunoprecipitation, In Vitro Kinase Assays, and Western Blotting

Cells were washed twice with cold STE (100 mM NaCl, 10 mM Tris, pH

7.4, 1 mM EDTA) and lysed in 1 ml of NP-40 lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 2.5 mM EDTA, 10 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 10  $\mu\text{g/ml}$  aprotinin, 10  $\mu\text{g/ml}$  leupeptin, 1 mM PMSF). Cell lysates were then processed for immunoprecipitation as described (Wolven et al., 1997). *In vitro* kinase assays, enolase phosphorylation assays, and Western blotting have been described previously (Wan et al., 1997; Wolven et al., 1997). Quantitation of band intensity in both the Western analysis and kinase assays was accomplished using a Molecular Dynamics PhosphorImager.

### Antibodies

Antibodies raised against amino acids 1–148 of Fyn for immunoprecipitation have been previously described (Wolven et al., 1997). Rabbit polyclonal antibodies to Src, Fyn, Lck, and Lyn were purchased from Santa Cruz Biotechnology. Antiphosphotyrosine antibodies 4G10 and PY20 were purchased from UBI and Transduction Labs. MAG antibodies were a generous gift from Dr. Marie Filbin (Hunter College, New York). O1 antibodies were a generous gift from Drs. Steven Pfeiffer and Rashmi Bansal (University of Connecticut, Farmington, CT). Antibodies to neomycin phosphotransferase II (NPTII) were purchased from 5'→3'.

### Cellular Expression of a Dominant Negative Fyn

The K299M Fyn construct contains a point mutation in the ATP binding pocket (lysine 299 was changed to methionine), rendering the kinase inactive (Twamley-Stein et al., 1993). The K299M Fyn cDNA was generated in SP65 using wild-type Fyn as a substrate, and subsequently cloned into the retroviral pLJ vector (Wolven et al., 1997). The mutation was verified by DNA sequencing.  $\psi$ 2 cells were transfected with K299M or pLJ plasmids, and cell supernatants containing the retrovirus were collected and used to infect oligodendrocyte progenitor cultures.

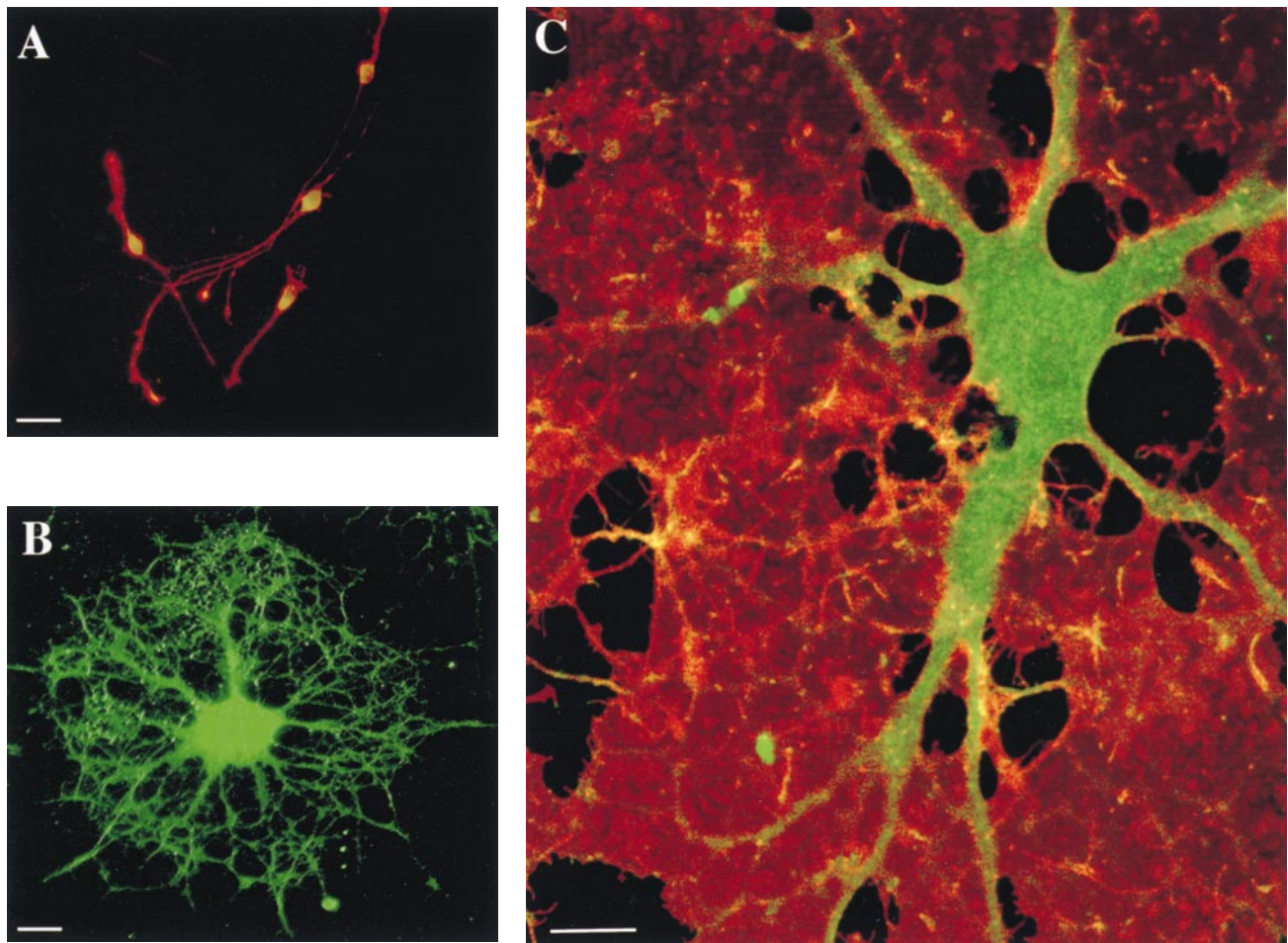
## Results

### Intracellular Localization of Fyn

Primary cultures of oligodendrocyte progenitors typically contain bipolar cells that are characterized by the expression of the surface markers A2B5 (80–90%) and O4 (10–20%). These progenitors can be induced to differentiate by switching to a serum-free differentiation media 24 h after plating (see Materials and Methods). After several days in culture, mature cells are characterized by marked process outgrowth, with an increase in the number of processes emanating from the cell body and extensive branching of these processes. The result is an intricate lacework of processes that surrounds the oligodendrocyte cell body. The morphological changes are accompanied by the appearance of the cell surface galactocerebroside O1, as well as myelin proteins such as MAG and MBP. At later stages, the extensive process outgrowth gives rise to the formation of myelin-like membrane sheets, which can be visualized by staining for O1 (Sommer and Schachner, 1981). Fyn is expressed in both the progenitors and differentiated cell populations (Figs. 1 and 2, immunocytochemistry and Western analysis, respectively). In progenitors, Fyn is localized primarily to the cell body and found along the length of the processes. (Fig. 1 A). Likewise in the mature oligodendrocyte, Fyn is observed in the cell body and throughout the processes (Fig. 1 B). Staining for O1 and Fyn also confirmed that the majority of Fyn immunoreactivity can be visualized in the cell body and processes of the mature cell (Fig. 1 C).

### Fyn Upregulation in Oligodendrocytes

To investigate whether Fyn protein levels changed during

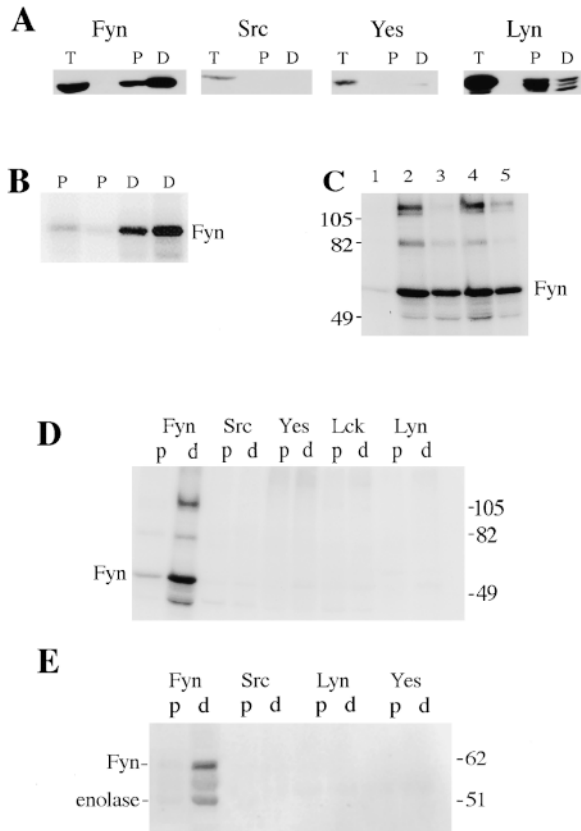


**Figure 1.** Fyn localization in oligodendrocytes. (A) Primary cultures of oligodendrocyte progenitors contain simple bipolar cells, which are A2B5 positive (rhodamine fluorescence). Fyn (fluorescein fluorescence) is localized to the cell body and processes. (B) After 4 d in culture, oligodendrocytes show extensive process outgrowth, a characteristic of differentiation. Fyn is localized to the cell body and throughout the processes (fluorescein fluorescence). (C) At later stages of differentiation, oligodendrocytes will extend large myelin-like membrane sheets in culture, which can be visualized by immunostaining for the galactocerebroside O1 (rhodamine fluorescence). Fyn (fluorescein fluorescence) is expressed at high levels in the cell body, and can also be seen in small veins throughout the myelin sheet. Bars: (A–C) 20  $\mu$ m.

differentiation, Western analysis of oligodendrocyte cell populations was performed. The bipolar progenitors were harvested 24 h after plating. Mature oligodendrocytes were harvested after allowing progenitors to differentiate for 5–6 d. Fyn protein is expressed at levels two- to three-fold higher in the mature cells compared with progenitors (Fig. 2 A). Moreover, Fyn tyrosine kinase activity in mature oligodendrocytes is 10–30-fold higher than that of the progenitors (Fig. 2, B, D, and E), as measured by autokinase activity and enolase phosphorylation. This suggests that the specific activity of Fyn is increased 3–15-fold per unit of Fyn protein in the mature cells. Since oligodendrocyte differentiation occurs over 5–6 d *in vitro*, Fyn activity was examined each day after the plating of oligodendrocyte progenitors to establish the time course of Fyn upregulation during differentiation. Fyn autokinase activity was used as an indication of Fyn activation, as it mimics the results obtained by measuring enolase phosphorylation (Fig. 2, D and E). The observed increase in Fyn activity occurs

within 24 h after switching to differentiation media, which corresponds to 48 h after plating (Fig. 2 C). Remarkably, cells at this time point are indistinguishable from those progenitors harvested at 24 h after plating, both in morphology and in the expression of stage specific markers. Since Fyn activation precedes any morphological changes that accompany differentiation, these observations suggest that the upregulation of Fyn is a very early event in the maturation of oligodendrocyte progenitors.

Several members of the Src family of tyrosine kinases are expressed in the neonatal brain, including Src, Fyn, Lyn, and Yes (Fig. 2 A, lanes denoted T). However, analysis of oligodendrocyte cell lysates (Fig. 2, D and E) revealed that Fyn is the only member of the Src family with significant kinase activity in either cultured oligodendrocytes or progenitor cells. Src is not expressed or catalytically active in these cells; Yes protein is barely detectable in differentiated cells, but is not active (Fig. 2, A, D, and E). Lyn protein is expressed in both the progenitors and



**Figure 2.** Fyn is the only Src family member upregulated during oligodendrocyte differentiation. (A) Western blot of Fyn and other Src family members immunoprecipitated from progenitor cells (P) or differentiated oligodendrocytes (D). As a control, equal amounts of total brain lysate (T) were analyzed in parallel for protein expression. (B) In vitro autokinase assay of Fyn in progenitor cells (P) and differentiated oligodendrocytes (D). Two independent cell preparations were used for these experiments. (C) Activation of Fyn occurs very early during oligodendrocyte differentiation. Fyn autokinase activity was assayed every day after plating (lanes 1–5); the upregulation is observed 2 d after plating (lane 2), which is 24 h after introducing media to initiate differentiation (see Materials and Methods). Lane 1 corresponds to progenitor cells, harvested 24 h after plating. (D) Specificity of the Src family member tyrosine kinase activity in oligodendrocytes. Fyn, Src, Yes, Lck, and Lyn were immunoprecipitated from equal amounts of cell lysates (100  $\mu$ g protein) from progenitor cells (P) or differentiated oligodendrocytes (D) and autokinase assays were performed. (E) Fyn is the only Src family kinase active in oligodendrocytes. Src, Fyn, Yes, or Lyn were immunoprecipitated from equal amounts of cell lysate (100  $\mu$ g protein) and both autokinase and enolase phosphorylation was measured. Note that Fyn kinase activity is significantly increased as compared with other Src kinases in both the autokinase and enolase phosphorylation.

mature oligodendrocytes (Fig. 2 A), but Lyn tyrosine kinase activity was not detected in either population of cells (Fig. 2, D and E). Analysis of kinase activity was performed using two different substrates, including autokinase as well as enolase phosphorylation for all Src family members (Fig. 2, D and E). These findings distinguish oligodendrocytes as one of the few cell systems in which Fyn is the major active Src family member. These cells there-

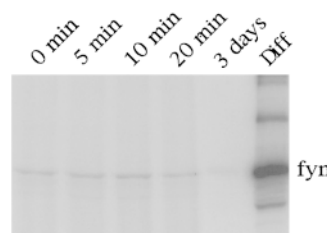
fore can provide a useful system in which to study Fyn function.

### Lack of Growth Factor Modulation of Fyn

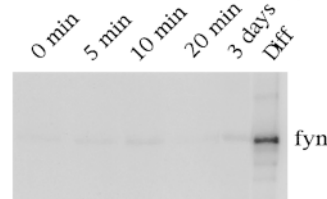
Growth factors such as PDGF and FGF stimulate progenitor cell proliferation, regulate cell migration and modulate differentiation in the oligodendrocyte lineage (Noble et al., 1988; Besnard et al., 1989; Osterhout et al., 1997). In vitro, progenitors maintained in the presence of these growth factors fail to differentiate; withdrawal from the cell cycle and terminal maturation is triggered by removal of the growth factors. In the protocol used for this study, progenitors are harvested soon after plating; under these conditions, the progenitors have a low basal level of Fyn kinase activity (Fig. 2, D and E). Since PDGF and FGF can activate Src and Fyn in other cell types (Twamley-Stein et al., 1993; Langren et al., 1995), we examined the possibility that PDGF and FGF are required to activate Fyn in the progenitor cell population. Fyn kinase activity was examined after acute treatment with FGF (20 ng/ml) and PDGF (10 ng/ml) for 5, 10, and 20 min. There was no increase in Fyn activity above basal levels (Fig. 3, A and B). Since differentiation can be prevented by maintaining progenitors in the presence of growth factors (McKinnon et al., 1990), we also examined the effects of chronic treatment with growth factors. After 3 d in culture with either PDGF (10 ng/ml) or FGF (20 ng/ml), Fyn activity remained at basal levels (Fig. 3, A and B). Fyn activity was not upregulated until the growth factors were withdrawn, which triggered differentiation.

It is also possible that the increase in Fyn activity could be influenced by specific media components. Fyn kinase activity was assayed in cells cultured under several different media formulations, each of which will allow oligodendrocyte differentiation, but vary in their composition (Barres et al., 1993; Osterhout et al., 1997; Yoon et al.,

### A FGF Treatment



### B PDGF Treatment



**Figure 3.** Lack of growth factor stimulation of Fyn kinase activity. Progenitor cells were treated with either FGF (A) or PDGF (B) for various times, then harvested and assayed for Fyn kinase activity. Neither growth factor could elicit an increase in activity, after acute or chronic treatments. The only increase in Fyn tyrosine kinase activity was measured after progenitors were induced to differentiate by withdrawing the growth factors.

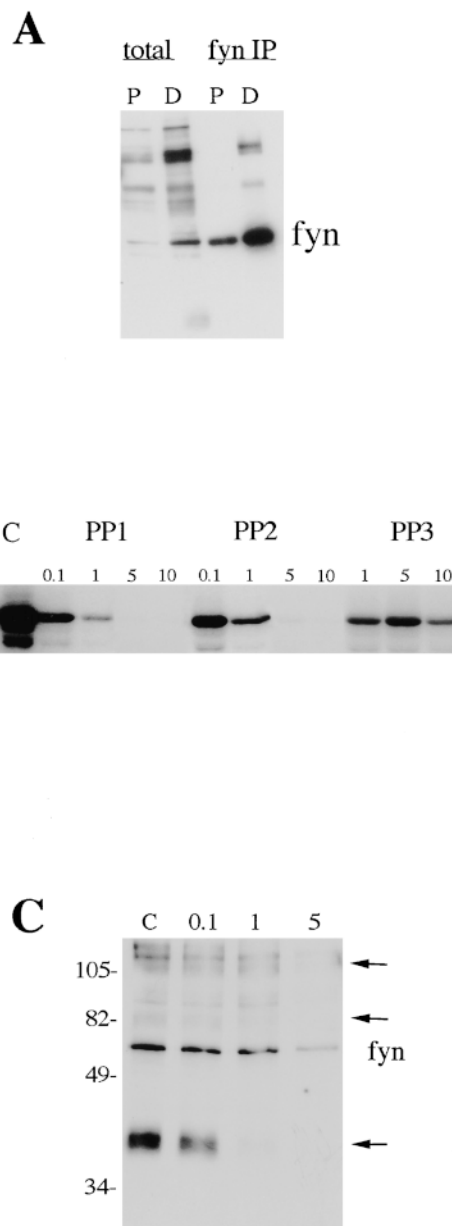
1998). There were no differences in the levels of Fyn kinase activity in the mature oligodendrocytes under these different culture conditions (data not shown). These data indicate that the activation of Fyn is closely associated with the start of progenitor cell differentiation and not stimulated by FGF or PDGF. The lack of growth factor regulation suggests that activation of Fyn in oligodendrocytes is achieved via a novel regulatory mechanism, which is an early event in the terminal differentiation of oligodendrocytes.

### Protein Phosphorylation in Oligodendrocytes

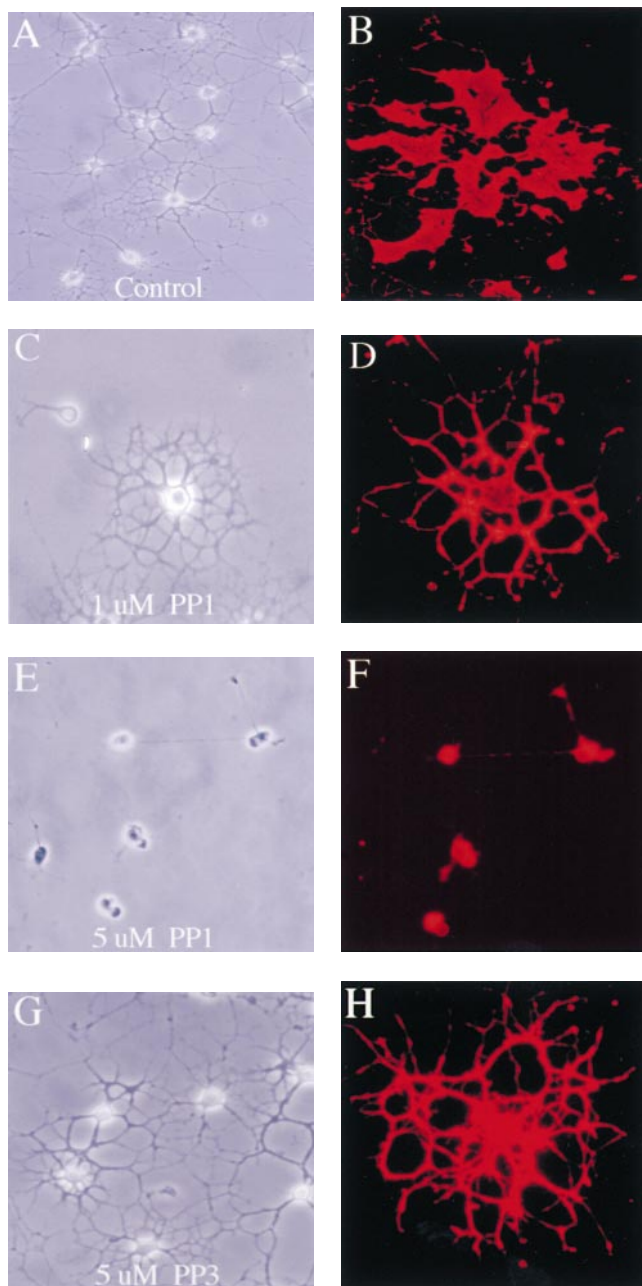
The upregulation of Fyn kinase activity during differentiation suggests that enhanced tyrosine phosphorylation of potential Fyn substrates should be evident in mature oligodendrocytes. This supposition was verified by the results depicted in Fig. 4 A, in which an increase in tyrosine phosphorylated proteins was observed in both total cell lysates and Fyn immunoprecipitates of differentiated cells. Several proteins were phosphorylated and associated with Fyn as oligodendrocytes differentiate, and these phosphorylated proteins were also observed in Fyn autokinase assays on oligodendrocyte lysates (Fig. 2, C and D). These proteins were not phosphorylated under the assay conditions required for enolase phosphorylation by Fyn (Fig. 2 E). Interestingly, these proteins were not phosphorylated in autokinase assays using progenitor cell lysates even though there is a low level of Fyn activity, which suggests that phosphorylation of these proteins is part of the maturation program. These phosphorylated bands appear to be novel and specific to oligodendrocytes. They do not correspond to any known Src or Fyn substrates, including Fak, Cbl, p130Cas, annexins, PI3 kinase, or cortactin, and the identification of these potentially novel proteins is currently under investigation.

### Inhibition of Fyn Activity and Oligodendrocyte Differentiation

To determine whether activation of Fyn was required for differentiation, we examined the fate of progenitors cultured in the presence of the tyrosine kinase inhibitors PP1 and PP2. These pyrazolopyrimidine derivatives are potent and specific inhibitors of Src family kinases in T cells and compete with ATP to prevent phosphorylation (Hanke et al., 1996). They have different affinities for each member and do not inhibit other tyrosine kinases at the concentrations effective for the Src kinase family (Hanke et al., 1996). Since Fyn is the only Src family kinase active in this cell system, Fyn should be the only Src family member affected by the inhibitors. Fyn kinase activity in lysates from mature oligodendrocytes was significantly lower in the presence of PP1 and PP2 (Fig. 4 B), and a corresponding reduction in tyrosine phosphorylation of total cellular protein was observed (Fig. 4 C). Inhibition of Fyn kinase activity by PP1 was concentration dependent, with total inhibition occurring at concentrations of 5  $\mu$ M and higher. The effects of PP1 are specific since the inactive compound PP3 could not inhibit Fyn kinase (Hanke et al., 1996). The activity levels of Fyn in oligodendrocytes in the presence of PP3 were comparable to control cells (Fig. 4 B). Tyrosine phosphorylation of proteins in the total cell lysates



**Figure 4.** Tyrosine phosphorylation in oligodendrocytes. (A) Immunoblots show an increase in tyrosine phosphorylation in total cell lysates from differentiated (D) oligodendrocytes as compared with progenitors (P). Fyn immunoprecipitates reveal several phosphorylated proteins that are associated with Fyn in the differentiated (D) oligodendrocytes but not in the progenitors (P). (B) PP1 and PP2 will inhibit Fyn kinase activity in a dose-dependent manner. Fyn kinase assays on lysates from mature oligodendrocytes were performed in the presence of 0.1, 1, 5, and 10  $\mu$ M PP1 and PP2. Fyn activity is significantly reduced at 1  $\mu$ M compared with control (C); no activity is observed at 5 and 10  $\mu$ M of either compound. PP3, an inactive analogue, has no effect on Fyn kinase activity. (C) Tyrosine phosphorylation in total cell lysates is reduced, but not abolished, in differentiated oligodendrocytes treated with increasing concentrations of PP1. Fyn autophosphorylation is reduced by 10% at 0.1  $\mu$ M PP1, and 70% at 5  $\mu$ M PP1. The bands at 100–120, 85–90, and 40 kD (arrows) are speculated to be Fyn substrates; their phosphorylation is also significantly reduced in the presence of PP1.



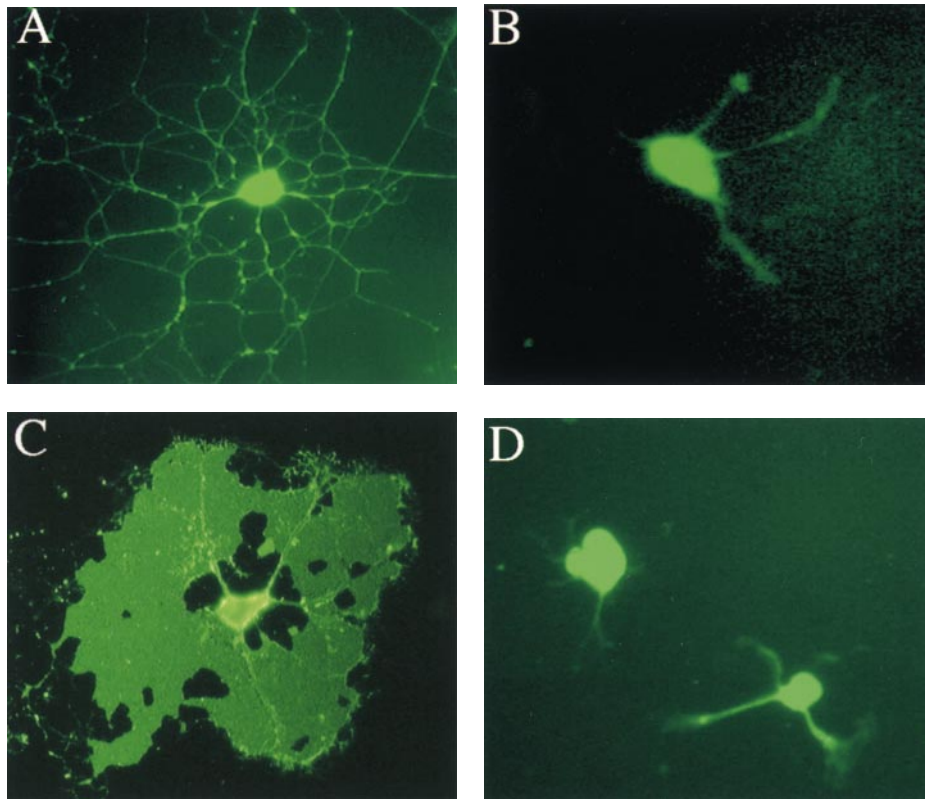
**Figure 5.** Effect of PP1, a Fyn tyrosine kinase inhibitor, on oligodendrocyte differentiation. (A and B) Oligodendrocyte progenitor cells differentiate into the mature phenotype after 4–6 d *in vitro*. Phase contrast (A) of live oligodendrocyte cultures 6 d after plating. The differentiating cells extend a complex network of branched processes. O1 immunostaining of sister cultures (B) reveal the myelin-like membrane sheets that are formed in the later stages of oligodendrocyte differentiation, which are not visible under phase contrast optics. (C and D) PP1 inhibits process outgrowth and membrane sheet formation in a dose-dependent manner. Phase contrast (C) of oligodendrocyte progenitor cells induced to differentiate in the presence of 1  $\mu$ M PP1 for 4 d. Some cells in culture with 1  $\mu$ M PP1 show a slight inhibition of process outgrowth; O1 expression is not affected (D). (E and F) At higher concentrations of PP1 where Fyn kinase activity is totally abolished (5  $\mu$ M; see Fig. 4 B), process outgrowth is significantly inhibited after 3–4 d in culture. Few cells extend short processes, and the cell bodies have rounded up and clumped together (E). However, these cells do express cell surface markers characteris-

was reduced but not eliminated in the presence of PP1 (Fig. 4 C). At low concentrations, where PP1 has limited effects on Fyn activity (Fig. 4 B), there was a slight reduction in tyrosine phosphorylation, including Fyn autophosphorylation. As the concentration of PP1 increased, the levels of tyrosine phosphorylation measurably decreased. Fyn autophosphorylation was reduced by 70% at 5  $\mu$ M PP1 (Fig. 4 C), a concentration at which Fyn kinase activity is totally inhibited (Fig. 4 B). Similar reductions in tyrosine phosphorylation were observed in immunoblots probed with anti-phosphotyrosine antibodies, including proteins at 100–120, 80–85, and 40–45 kD, which may represent Fyn substrates in oligodendrocytes. The observation that tyrosine phosphorylation was not totally eliminated in the presence of PP1 suggests that while Fyn is an important kinase in these cells, other tyrosine kinases may be active and not inhibited by the PP1 inhibitor family. One possibility is Csk, which phosphorylates Fyn at tyrosine 527 and acts as a negative regulator of Fyn (Chow et al., 1993).

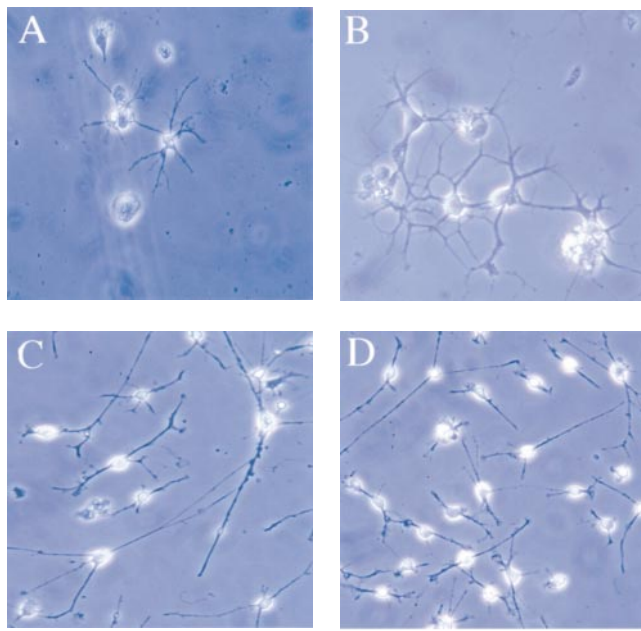
If activation of Fyn tyrosine kinase is indeed responsible for differentiation, then treatment of cells with PP1 should block this process. Progenitors induced to differentiate in the presence of PP1 retracted their processes and clumped together in the dish (Fig. 5). This retraction was evident after 1–2 d in differentiation media, just as the control cells started to extend multiple processes. This effect was dose dependent; at low concentrations (1  $\mu$ M), there was a 10% reduction in process outgrowth after 4 d, as assessed by measuring the radius of the extensive process network that surrounds the oligodendrocyte cell body. At concentrations  $<1$   $\mu$ M, there were no observable effects on cell morphology (data not shown). In the presence of 5  $\mu$ M PP1, process outgrowth was significantly inhibited ( $>95\%$ ). All of the cells extended only one or two short, unbranched processes which were generally less than one cell body diameter in length. The treated cells lacked the extensive process network that is characteristic of the differentiated phenotype, and morphologically resembled oligodendrocyte progenitor cells. None of the cells formed the myelin-like membrane sheets observed in cultures of mature oligodendrocytes. The degree of process retraction observed with increasing concentrations of the inhibitors can be correlated with the levels of Fyn kinase inhibition observed in the *in vitro* kinase assays (Fig. 4 B), which suggests that Fyn kinase activity is required for process extension during oligodendrocyte differentiation.

While the inhibition of Fyn activity clearly retarded process outgrowth, it did not affect the expression of the cell surface marker galactocerebroside O1 (Fig. 5, B, D, F, and H) and myelin proteins MAG and myelin basic protein

tic of mature oligodendrocytes, including O1 (F). (G and H) PP3 has no effect on oligodendrocyte differentiation. Oligodendrocyte progenitors will fully differentiate in the presence of PP3, which does not inhibit Fyn kinase activity (G; see Fig. 4 B). Phase contrast of differentiating oligodendrocytes 4 d after plating. Note the extensive process outgrowth evident at this timepoint. These cells have not yet reached the stage where they form myelin-like membrane sheets, but they do express markers characteristic of mature cells, including O1 (F).



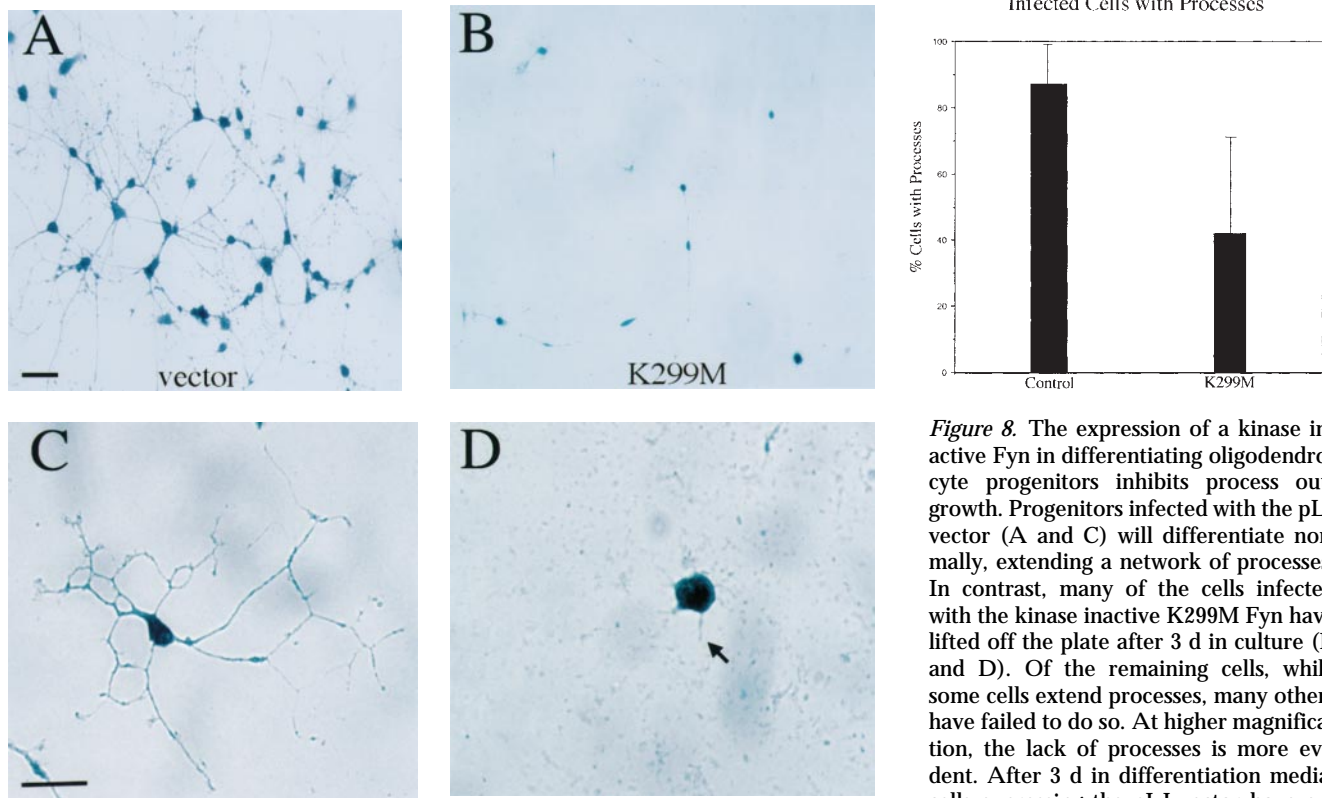
**Figure 6.** Expression of myelin proteins in oligodendrocytes. (A and B) MBP expression in differentiating oligodendrocytes (A) and in oligodendrocytes maintained in the presence of 5  $\mu$ M PP1 for 4 d (B). (C and D) MAG expression in mature oligodendrocytes (C) and in cells differentiating in the presence of 5 mM PP1 (D). Note that in the presence of PP1, myelin proteins are still expressed, despite the lack of processes or myelin-like membrane sheets.



**Figure 7.** The effects of PP1 are reversible. (A and B) Progenitors differentiated in the presence of 5 mM PP1 show little process outgrowth after 3 d (A); 24 h after removal of PP1, the cells have renewed the extensive process outgrowth characteristic of the mature oligodendrocyte (B). PP1 has no effect on non-differentiated progenitors. (C and D) Oligodendrocyte progenitors were maintained in FGF and PDGF (20 and 10 ng/ml, respectively) and 10 mM PP1 for 4 d; in contrast to the differentiating cultures, PP1 had no effect on process outgrowth after 1 d (C) or 4 d (D) of treatment. The rate of cell proliferation was identical in control and PP1-treated cells. Bar, 6  $\mu$ m.

(Fig. 6). The process retraction was reversible, as removal of PP1 resulted in renewed process extension as early as 24 h after the inhibitor removal (Fig. 7, A and B). To control for any non-specific effects, cells were treated with the related inactive analogues PP3 and PP4, which had no effect on Fyn activity (Fig. 4 B) and no effect on differentiating cells in culture (Fig. 5 G). Finally, when progenitors were maintained in FGF and PDGF, mitogens that block differentiation (Noble et al., 1988; Bogler et al., 1990; McKinnon et al., 1990), PP1 had no effect on process outgrowth or cell division (Fig. 7, C and D). Progenitor cells maintained in 10  $\mu$ M PP1, a concentration that completely inhibits Fyn kinase activity (Fig. 4 B) proliferated normally in the presence of FGF and PDGF, and maintained a bipolar morphology. These data suggest that the PP1 family of inhibitors are not toxic to the cells, but are acting solely to inhibit Fyn kinase activity. Thus, inhibition of Fyn activity appears to block morphological differentiation of progenitors into mature oligodendrocytes, without affecting the synthesis of stage-specific proteins that are characteristically expressed by mature oligodendrocytes.

To verify the requirement for Fyn in oligodendrocyte differentiation, a kinase inactive, dominant negative Fyn was introduced into cultures of differentiating oligodendrocyte progenitors. The construct contains a point mutation in the ATP-binding site in which lysine 299 was mutated to methionine (Fig. 8 A). This mutation results in a Fyn protein that cannot bind ATP and is catalytically inactive (Twamley et al., 1992; Twamley-Stein et al., 1993). The Fyn K299M mutant can still bind to substrate molecules and acts in a dominant negative fashion when expressed with wild-type Fyn in cells (Twamley et al., 1992).



**Figure 8.** The expression of a kinase inactive Fyn in differentiating oligodendrocyte progenitors inhibits process outgrowth. Progenitors infected with the pLJ vector (A and C) will differentiate normally, extending a network of processes. In contrast, many of the cells infected with the kinase inactive K299M Fyn have lifted off the plate after 3 d in culture (B and D). Of the remaining cells, while some cells extend processes, many others have failed to do so. At higher magnification, the lack of processes is more evident. After 3 d in differentiation media, cells expressing the pLJ vector have extended multiple, highly branched processes (C).

In contrast, many of the cells expressing K299M Fyn lack processes longer than one cell body; a few short processes can be observed, but they never elongate (D; arrow). This data is quantitated above; the number of infected cells extending processes longer than one cell body in length is reduced by 50% in the cell expressing the kinase inactive Fyn. The data is summarized from three individual transient infections, with an infection rate of 10–30% per culture. Bar, 20  $\mu$ M.

The Fyn K299M cDNA was inserted into the viral pLJ vector containing an IRES sequence linked to the Neo gene, which confers resistance to geneticin, and allows for both selection and immunocytochemical detection of cells infected with the recombinant retrovirus (Fig. 8).

Oligodendrocyte progenitors were infected with a vector control or Fyn kinase inactive vector using the protocol of Wolven et al. (1997) and maintained in differentiation media. Infected cells were detected by immunostaining for neomycin phosphotransferase II (NPTII). Attempts to select for stable cell lines expressing the construct failed, even in low concentrations of G418. However, in transient infections, many cells were positive for NPTII, although the levels of staining varied considerably. Several effects were evident in the cultures infected with the Fyn K299M vector (Fig. 8, B and D). Cells infected with the pLJ vector alone proceeded to differentiate normally, extending branched processes after culture in differentiation media (Fig. 8, A and C). Cultures infected with the inactive Fyn kinase construct showed considerable inhibition of process outgrowth after 3 d (Fig. 8, B and D). During the time course allowed for differentiation, many cells rounded up and detached from the plate, and it is not known whether these cells were expressing the mutant Fyn protein. Others extended processes less than one cell diameter in length and resembled those cells treated with the PP1 inhibitors. A few NPTII positive cells extended processes similar to

the vector control. Overall, there was a 50% reduction (Fig. 8 E) in the number of cell extending processes longer than one cell body in diameter in the cells expressing the Fyn K299M virus compared with control. Since these data are derived from transient infections in which only 10–30% of the cells were infected, it was not feasible to assay the level of Fyn kinase activity in the cells expressing the mutant construct. However, the reduction in process outgrowth observed in cultures expressing the dominant negative Fyn supports the hypothesis that Fyn kinase activation is required for the formation of processes that occurs during oligodendrocyte differentiation.

### Discussion

The initial observation that Fyn-deficient mice showed reduced myelination in the central nervous system suggested that this tyrosine kinase could be one of the signals involved in regulating myelination during development (Umemori et al., 1994). However, the underlying mechanism for the failure to myelinate in these mice was unclear. The present study directly examines the potential role of Fyn in myelination using primary cultures of differentiating oligodendrocytes. This is a well-characterized cell system; differentiation proceeds along a defined pathway, marked by discrete stages that can be identified by both morphological changes and the expression of cell sur-



face markers and myelin proteins (Pfeiffer et al., 1993). Our results indicate that Fyn tyrosine kinase is activated very early after the oligodendrocyte progenitor cells are induced to differentiate (Fig. 2). This upregulation occurs at a point before the appearance of MAG in these cells, indicating that another signal triggers Fyn in these cells. Inhibition of the kinase activity during differentiation prevents the extensive process outgrowth and formation of membrane sheets normally observed in cultures of mature oligodendrocytes, suggesting that Fyn may regulate cytoskeletal rearrangements that control process extension.

The observed increase in Fyn activity is triggered as progenitor cells are induced to differentiate by maintaining them in a defined differentiation media. Activation of Src family kinases in other cell types is commonly triggered by external signals, such as growth factor-receptor binding, or integrin-substrate interactions (reviewed by Thomas and Brugge, 1997). Since cells in the oligodendrocyte lineage are responsive to growth factors, it was surprising that Fyn activation was not affected by growth factor treatment. This finding suggests that another external signal or a novel regulatory mechanism is responsible for Fyn activation. Oligodendrocytes do express integrins on their cell surface that can modulate migration of these cells (Milner et al., 1996, 1997); however it is unlikely that integrins are responsible for this response, as there are no abrupt changes in cell adhesion or extracellular substrates when Fyn is activated. Thus, it is likely that a novel regulatory mechanism is responsible for this activation, potentially involving release of a negative regulatory mechanism when the cells are placed in media lacking mitogens. It is possible that Csk, a negative regulator of Fyn, may be highly active in oligodendrocyte progenitors treated with growth factors; thus Fyn activity would be low until the growth factors are removed and Csk is downregulated. Alternatively, the withdrawal of growth factors causes the progenitors to exit the cell cycle and begin to differentiate (Casaccia-Bonnet et al., 1997; Durand et al., 1997). Signal transduction pathways that modulate differentiation may be activated once the cells leave the cell cycle, resulting in subsequent activation of Fyn. Elucidation of the upstream signal that triggers Fyn activation may ultimately reveal the specific cellular pathways involved in oligodendrocyte differentiation.

Since Fyn is the primary Src family kinase activated during oligodendrocyte differentiation, these cells provide a model system in which to study biological effects of Fyn activation. The inhibition of Fyn, by either a pharmacological inhibitor or by expression of a dominant negative Fyn protein, suggests that Fyn can regulate the cytoskeletal rearrangements that accompany oligodendrocyte differentiation. The localization of Fyn to processes further supports this hypothesis. The precise mechanism underlying this effect is unknown at present; while it is clear that several proteins are phosphorylated when Fyn is activated in these cells, the identity of these proteins is unknown. It is not clear that phosphorylation of any one specific protein is the critical event that modulates the observed process retraction. Examination of tyrosine phosphorylated proteins in the presence of the PP1 inhibitors reveals that there is a general reduction in all phosphorylated proteins, not one protein in particular. This may indicate that Fyn

activates another signal transduction pathway that ultimately contributes to the cytoskeletal rearrangement. The dominant negative studies confirm the hypothesis that Fyn is essential for oligodendrocyte process formation. Identification of Fyn substrates in these cells will be necessary to further characterize the intracellular events responsible for the observed biology reported here.

The results obtained in this study may in part explain the partial loss of myelin proteins in the Fyn-deficient transgenic mice. If Fyn directs the formation of myelin sheet formation *in vivo* as it does *in vitro*, the myelin in these animals may show a reduction in lamellar structure, or oligodendrocyte cell volume. This would lead to a subsequent reduction in the total amount of myelin proteins. It will be interesting to determine if the loss of myelin protein leads to a reduction of the myelin sheath formation in Fyn-deficient mice. The initial phenotypic characterization of the Fyn-deficient mice did not suggest a defect in myelination, as the animals exhibited normal motor function (Stein et al., 1992, 1994). However, normal nerve conduction and motor function can be realized even if myelin sheath is reduced to 25% of normal (Waxman, 1997, 1998). Further examination of the myelin content and the state of oligodendrocyte differentiation from these mice will provide more details on the role of Fyn during myelination.

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