Cellular/Molecular

Signaling from Integrins to Fyn to Rho Family GTPases Regulates Morphologic Differentiation of Oligodendrocytes

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Differentiation of oligodendrocyte progenitor cells requires activation of the Src family kinase Fyn. The signals that are upstream and downstream of Fyn in oligodendrocytes remain essentially unknown. Here we show that extracellular matrix engagement regulates the morphology of oligodendrocytes and activates Fyn. Infection of primary oligodendrocyte cultures with recombinant adenovirus revealed that expression of Fyn or its downstream target p190RhoGAP induced process extension. This phenotypic change was not observed when kinase-inactive Fyn or GAP-defective p190 mutants were expressed. Because Rho family proteins are regulated by p190, we monitored the effects of introducing dominant-negative (DN) or constitutively activated (CA) versions of Rho, Rac1, or Cdc42 into primary oligodendrocyte cultures. Expression of DN Rho, CA Rac1, or CA Cdc42 induced outgrowth of oligodendrocyte processes, whereas introduction of CA Rho, DN Rac1, or DN cdc42 inhibited oligodendrocyte differentiation, indicating that Rho and Cdc42–Rac1 exert opposing effects on oligodendrocyte differentiation. Direct measurement of Rho family activity revealed that RhoA was downregulated, and Cdc42 and Rac1 were upregulated during differentiation of primary oligodendrocytes. Moreover, inhibition of integrin engagement or of Fyn activation blocked activation of Rac1 and cdc42 as well as myelin basic protein expression. Taken together, these results suggest a linear signal transduction pathway of integrin–Fyn–Rho family GTPases that controls morphologic differentiation of oligodendrocytes.

Key words: kinase; oligodendrocyte; RhoA; Fyn; signaling; myelination; integrins

Introduction

Myelin formation in the CNS occurs when oligodendrocyte precursor cells (OPCs) proliferate, migrate throughout the CNS, and differentiate into mature oligodendrocytes. The Src family kinase Fyn plays an important role in oligodendrocyte differentiation. In Fyn−/− mice, the myelin sheath is thinner and more irregular than in wild-type (wt) mice. The content of myelin basic protein (MBP) in Fyn−/− mice is ~50% of that in wild-type mice, and transactivation of the MBP gene by Fyn regulates myelination (Umemori et al., 1999; Sperber et al., 2001). Moreover, Fyn is the only Src family kinase that is upregulated during oligodendrocyte differentiation, and treatment of oligodendrocytes with Src family kinase inhibitors blocks oligodendrocyte differentiation (Osterhout et al., 1999).

One potential mechanism to activate Fyn is via integrin engagement. Oligodendrocytes express integrins $\alpha6\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, and $\alpha v\beta5$ (Milner et al., 1994). Integrin expression is regulated during differentiation, with expression of $\alpha v\beta1$ downregulated and $\alpha v\beta5$ upregulated (Milner and ffrench-Constant, 1994). Proliferation and differentiation of OPCs are regulated by

αv integrins (Blaschuk et al., 2000). Disruption of oligodendrocyte adhesion to a glial-derived matrix decreases MBP and proteolipid protein mRNA levels (Malek-Hedayat and Rome, 1994), suggesting that integrin signaling induces myelin gene expression and oligodendrocyte differentiation.

Integrin engagement triggers multiple signaling cascades, including activation of tyrosine kinases and Rho family GTPases (Price et al., 1998; Ren et al., 1999; del Pozo et al., 2000; Arthur and Burridge, 2001; Berrier et al., 2002). Rho family GTPases act as molecular switches by cycling between GTP-bound (on) and GDP-bound (off) states. Guanine nucleotide exchange factors (GEFs) stimulate exchange of GDP for GTP to generate the active form, whereas GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity to inactivate the switch. Several mechanisms link integrin engagement with activation of the Rho family protein Rac, including signaling by focal adhesion kinase (Schlaepfer et al., 1997; Schaller et al., 1999; Das et al., 2000), p130Cas (Klemke et al., 1998; Cheresh et al., 1999), and Vav family GEFs (Schwartz, 2001).

Rho family GTPases play an essential role in the control of cellular morphology. In fibroblasts, Rho activation promotes stress fiber formation, whereas activation of Cdc42 and Rac induces lamellipodia and filopodia, respectively (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). It is likely that Rho, Cdc42, and/or Rac regulate the cell shape changes associated with oligodendrocyte differentiation. Indeed, our laboratory demonstrated that overexpression of dominant-negative RhoA causes hyperextension of oligodendrocyte processes (Wolf et al., 2001).

We set out to identify the upstream regulators and downstream effectors of Fyn and study their function in oligodendrocyte differentiation. Here we show that integrin engagement activates Fyn. Fyn phosphorylates and activates p190RhoGAP, which in turn inactivates Rho and causes alterations in oligodendrocyte morphology. In addition, Fyn activates Cdc42 and Rac, which also triggers morphologic differentiation of oligodendrocytes. Thus, Rho and Cdc42–Rac exhibit opposing effects on oligodendrocyte morphologic complexity. These results provide evidence for a linear signal transduction pathway of integrin–Fyn–Rho family GTPases that regulates morphologic differentiation of oligodendrocytes.

Materials and Methods

Materials. Poly-D-lysine (PDL), bovine fibronectin (FN), vitronectin, insulin, transferrin, progesterone, putrescine, and sodium selenite were purchased from Sigma-Aldrich (St. Louis, MO). PP3 and PP2 were obtained from Calbiochem (La Jolla, CA). Laminin-2 (merosin) was purchased from Invitrogen (Carlsbad, CA). Recombinant human PDGF-AA and bFGF were purchased from PeproTech Inc. (Rocky Hill, NJ). The following antibodies were used for immunoprecipitation, Western blotting, or immunofluorescence: mouse anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-RhoA, rabbit Cdc42 (Santa Cruz Biotechnology), mouse anti-MBP (Chemicon, Temecula, CA), mouse anti-Rac, mouse anti-Fak (Upstate Biotechnology, Lake Placid, NY), mouse anti-Fyn, and hamster anti-rat CD29 (integrin β-1) (BD Biosciences, Franklin Lakes, NJ) antibodies. The rabbit polyclonal antiserum to Fyn used for immunoprecipitation was described previously (van't Hof and Resh, 1997).

Plasmids. pAdeasy-1 and pShuttle-CMV were generously provided by Dr. James R. Bamburg (Colorado State University, Fort Collins, CO). pFB-neo and pShuttle-IRES-hrGFP-2 were purchased from Stratagene (La Jolla, CA). pEGFP-N1 was purchased from Clontech. The EGFP insert was subcloned into pShuttle-CMV at the *Bgl*II–*Not*I site. Full-length HA-tagged wild-type p190RhoGAP and GAP-defective (R1283A)p190RhoGAP mutants were prepared as described (Wolf et al., 2001). To clone wild-type and mutants of p190RhoGAP into pShuttle vectors, p190 was first inserted into pFB-neo vectors at *Eco*RI sites using PCR and was then subcloned into pShuttle vectors at Sal–*Not*I sites.

To prepare full-length FynEGFP fusion constructs, PCR products of wild-type, G2A, or K299M Fyn mutant with the stop codon deleted were inserted into pEGFP-N1 at *Eco*RI–*Bam*HI sites. The fragment containing FynEGFP was subcloned into pFB-neo at *Eco*RI–*Not*I sites. Finally, the FynEGFP fragment was subcloned into pShuttle-CMV at Sal–*Not*I sites.

N-terminal 3× HA-tagged CDC42(G12V), CDC42(T17N), RhoA(G14V), RhoA(T19N), Rac1(G12V), and Rac1(T17N) in pcDNA3.1 were generously provided by the Guthrie cDNA Resource Center (Sayre, PA). Rac1(G12V), and Rac1(T17N) were subcloned into pShuttle-IRES-hrGFP at the *Nhe*I and *Xho*I sites. RhoA(G14V) and RhoA(T19N) were subcloned into pShuttle-CMV at the *Kpn*I and *Xho*I sites and then subcloned into pShuttle-IRES-hrGFP at the *Bgl*II and *Xho*I sites. CDC42(G12V) and CDC42(T17N) were subcloned into pShuttle-IRES-hrGFP at the *Not*I and *Sal*I sites using PCR. The shuttle vectors were linearized with *Pme*I and were cotransformed with the pAdeasy-1 vector into BJ5183 cells (Stratagene) to generate recombinant Adeasy plasmid DNA.

Cell culture. Isolation of oligodendrocyte precursor cells (OPCs) was previously described (Wolf et al., 2001). Briefly, primary oligodendrocyte precursors were grown for ~9 d in DMEM medium supplemented with 15% FBS at 37°C and 5% CO_2. Flasks were preshaken at 300 rpm for 10 min to remove microglia from the culture, replaced with fresh medium, and incubated for 5–6 hr at 37°C and 5% CO_2 before being shaken at 190 rpm overnight to dislodge the loosely attached oligodendrocyte precursors. OPCs were further purified from astrocytes by plating them in uncoated tissue culture dishes for ~40 min. More than 80% of the isolated OPCs were A2B5-positive (data not shown). Purified OPCs were cultured on PDL- or FN-coated dishes for 6–16 hr. Cells were lysed or induced to differentiate for 4–6 d by switching to a defined differentia-

tion DMEM medium [high glucose, 0.5% FBS, 50 μg/ml human apotransferrin (Sigma), 30 nM sodium selenite, 30 nM 3,3,5 tri-iodo-L-thryronine (Sigma), and 50 ng/ml insulin]. For biochemical studies, differentiated cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, 0.5 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin. To expand oligodendrocyte progenitor cells, cells were cultured in OPC proliferation DMEM medium containing 5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 10 ng/ml PDGF-AA, and 10 ng/ml bFGF.

The O2A myc oligodendrocyte cell line (Barnett and Crouch, 1995) was grown on PDL-coated dishes in DMEM high glucose supplemented with 4.5 gm/l glucose, 1 mM pyruvate, 2 mM L-glutamine, 100 μg/ml penicillin–streptomycin, 0.1 mg/ml bovine serum albumin, 60 μg/ml N-acetyl-L-cysteine, 100 μM putrecine, 5 μg/ml insulin, 6.28 ng/ml progesterone, 100 μg/ml transferrin, 5.2 ng/ml sodium selenite, 20 ng/ml bFGF, and 10 ng/ml PDGF. For differentiation, growth factors were removed, and the medium was supplemented with 20.2 ng/ml tri-iodo-thyronine.

Adenovirus preparation and infection. To generate adenovirus, recombinant Adeasy plasmid DNA (3 μg) was linearized with *Pac*I and transfected into a 60 mm plate of 293 cells using Lipofectamine 2000 (Invitrogen). The progress of infection was monitored by green fluorescence microscopy. Cells were cultured 7–10 d, and primary viral stock was prepared with four rounds of freezing and thawing. Amplification of a primary viral stock was achieved by infection of fresh 293 cells. For adenoviral infection of oligodendrocytes, freshly isolated progenitors were incubated with diluted adenoviral stocks (10–50 multiplicities of infection) overnight in DMEM medium supplemented with 15% FBS or in DMEM proliferation medium. Progenitors were then induced to differentiate for 2 d by changing to the defined differentiation medium.

Immune complex kinase assays. Cell lysates from progenitors or differentiated oligodendrocytes were immunoprecipitated with polyclonal anti-Fyn antibody. Immune complexes were washed with lysis buffer and incubated with 30 μl of kinase buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 10 μCi of $[\gamma\text{-}^{32}P]$ATP at 30°C for 20 min. Reactions were terminated by the addition of SDS sample buffer. The $[\gamma\text{-}^{32}P]$-labeled proteins were analyzed by phosphorimaging.

Rho family GTPase activities. GST–Rhotekin-binding domain (RBD) and GST–PAK binding domain (PBD) were kindly provided by Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). Small GTPase activities were measured similarly as described (Ren et al., 1999). Briefly, progenitor and differentiated oligodendrocytes were lysed in 300 μl of 50 mM Tris, pH 7.4, containing 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 500 mM NaCl, and 0.5 mM $MgCl_2$. Cell lysates (200–500 μg) were clarified at $100,000 \times g$ for 15 min and incubated for 40 min with 20 μg of GST fusion proteins containing the Rhotekin-binding domain (for RhoA assay) or the PAK-binding domain (for Rac1 and cdc42 assays) bound to glutathione-Sepharose beads (Amersham Biosciences, Uppsala, Sweden). Samples were washed with lysis buffer and then immunoblotted with anti-RhoA, Rac1, or cdc42 antibodies. Whole-cell lysates were directly immunoblotted to determine the total amount of Rho, Rac, or Cdc42 proteins. To determine the effect of anti-β1 antibody on Cdc42–Rac activities, oligodendrocyte progenitor cells were preincubated with control serum or anti-β1 antibody (20 μg/ml) for 1 hr in differentiation medium and were then plated in differentiation medium in the presence of control serum or anti-integrin β1 antibody on FN-coated dishes for 5 d. Activities of Cdc42–Rac were measured as described above. To determine the effect of Src family kinase inhibitor on Cdc42–Rac activities, oligodendrocyte progenitor cells were induced to differentiate for 2 d in the presence of 3 μM PP2 or the nonfunctional analog PP3, and activities of Cdc42–Rac were determined.

Immunofluorescence staining. Purified OPCs were plated onto PDL-coated glass coverslips, cultured for ~4–6 hr, and then were infected overnight with various adenoviruses. The progenitor cells were washed with PBS and cultured in a defined differentiation medium for 2 d and then examined live. Alternatively, cells were fixed in 3.7% paraformaldehyde for 15 min, washed with PBS, and then permeabilized with 0.2%
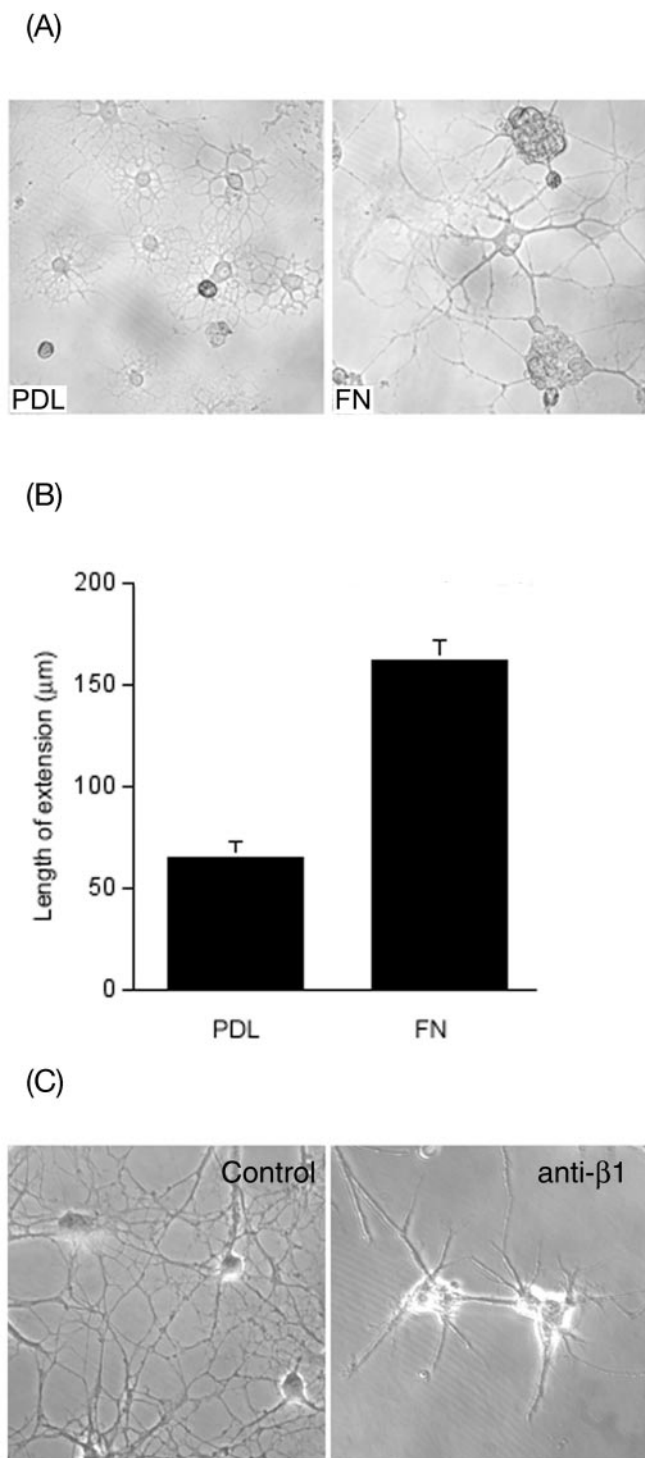
Figure 1. Effect of extracellular matrix on oligodendrocyte differentiation. *A*, OPCs plated on PDL-coated or FN-coated dishes were induced to differentiate for 5 d in differentiation medium. The morphology of the cells was visualized by phase-contrast microscopy. *B*, Quantitation of the lengths of processes using MetaMorph Imaging. *C*, Freshly isolated OPCs were preincubated with control serum or anti-β1 antibody (20 μg/ml) for 1 hr in differentiation medium and were then plated on FN-coated dishes for 5 d in the presence of control serum or anti-integrin β1 antibody. Cell morphology was examined using phase-contrast microscopy.

Figure 2. Fyn is regulated by integrins and is required for OPC differentiation. *A*, Oligodendrocyte progenitor cells (P) grown on FN-coated dishes were induced to differentiate for 5 d into mature oligodendrocytes (D). Cell lysates were immunoprecipitated using anti-Fyn antibody, and immunocomplexes were assayed for autophosphorylation activities in the presence of [γ-^{32}P]ATP. To examine the effect of anti-integrin β1 antibody on Fyn activation, OPCs were preincubated with control serum or anti-integrin β1 antibody and then induced to differentiate for 5 d in the presence of control serum or anti-integrin β1 antibody. *B*, OPCs were infected overnight with adenoviruses expressing EGFP, FynEGFP, (G2A)FynEGFP, or (K299M)FynEGFP and were then induced to differentiate for 2 d in defined differentiation medium. Live cells grown on PDL-coated coverslips were examined using confocal microscopy. Pictures shown are representative of five independent experiments.

Triton X-100 in PBS for 5 min. After washing with PBS, cells were incubated with 4% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS for 1 hr to block nonspecific binding sites, and then incubated for 1.5 hr with primary antibodies in 4% donkey serum. After extensive washing with P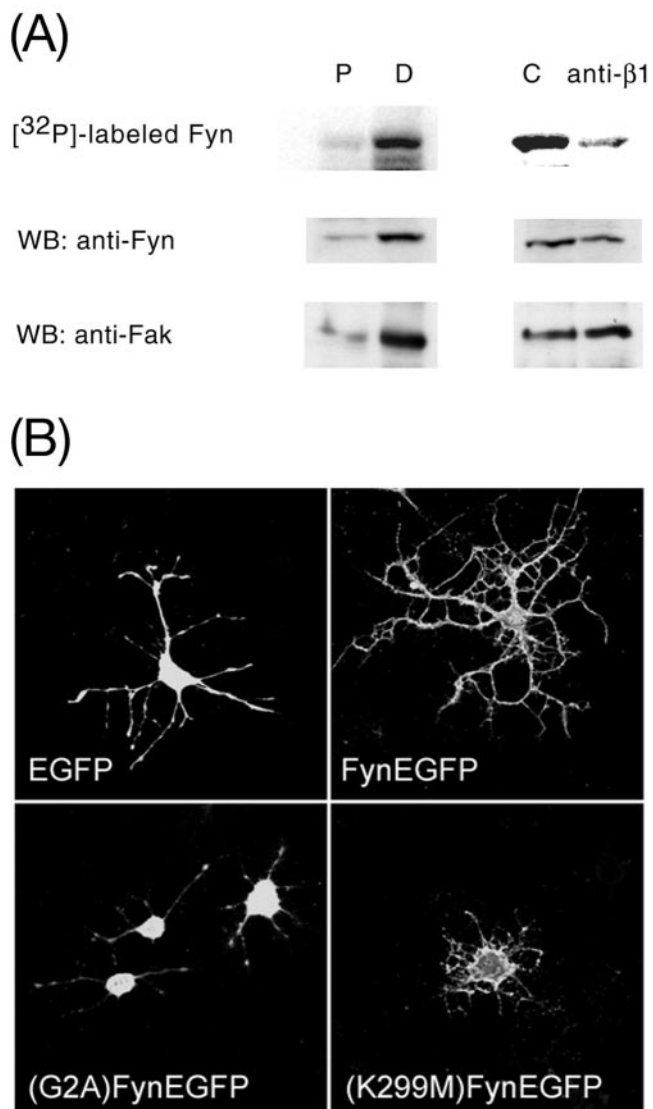BS, cells were incubated for 1 hr with Alexa Red conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) at a dilution of 1:500 in 4% donkey serum. After extensive washing with PBS, coverslips were mounted on slides using Prolong Antifade mounting media (Molecular Probes). The cells were viewed using a Zeiss (Oberkochen, Germany) LSM510 confocal microscope.

Results

Effect of extracellular matrix on OPC morphologic differentiation

To examine the role of extracellular matrix–integrin interactions in oligodendrocyte differentiation, OPCs were freshly isolated from neonatal rat brain and were plated on either PDL- or FN-coated dishes for 6–16 hr. Freshly isolated OPCs did not adhere

well to merosin-coated dishes (data not shown). OPCs were then induced to differentiate in differentiation medium for 4–6 d, at which point they became O4-positive (data not shown). Process extension was examined by phase contrast microscopy, and the length of extended processes was measured using the Meta-Morph analytic program. Obvious morphological differences were observed between the cells plated on PDL-coated and FN-coated dishes (Fig. 1A). Although the processes of oligodendrocytes plated on PDL were more highly branched than those plated on FN, process extension length was almost threefold greater when cells were plated on FN compared with PDL (Fig. 1B). The morphology of oligodendrocytes plated on vitronectin was similar to that of cells plated on FN (data not shown). These data suggest that extracellular matrix regulates the morphology of oligodendrocytes. To address the function of integrins on OPC differentiation, freshly isolated OPCs were preincubated with control serum or anti-integrin β1 antibody in differentiation medium for 1 hr, and then plated on FN-coated dishes. The cells were allowed to differentiate in the presence of control serum or anti-integrin β1 antibody for 5 d, and their morphology was examined by phase-contrast microscopy. As depicted in Figure 1C, process extension of OPC was significantly inhibited in the presence of anti-integrin β1 antibody. These findings suggest that integrins regulate OPC morphologic differentiation and are consistent with the observation that integrin β1 is essential for myelination *in vivo* (Relvas et al., 2001).

Fyn is regulated by integrins and is required for OPC morphologic differentiation

We previously showed that upregulation of the Src family kinase Fyn is required for OPC differentiation (Osterhout et al., 1999). Next, we tested whether integrins were responsible for Fyn activation. Freshly isolated OPCs were preincubated with control serum or anti-integrin β1 antibody and then induced to differentiate for 4–6 d on FN-coated dishes in the presence of control serum or anti-integrin β1 blocking antibody. Cells were lysed and immunoprecipitated with anti-Fyn antibody, and immunocomplexes were assayed for Fyn autophosphorylation in the presence of [γ-^{32}P]ATP. The expression level of Fyn was determined by Western blotting using anti-Fyn antibody. As depicted in Figure 2, both the kinase activity and expression level of Fyn were upregulated during oligodendrocyte differentiation, consistent with our previous findings (Osterhout et al., 1999). Treatment of cells with anti-integrin β1 blocking antibody blocked Fyn upregulation, suggesting that integrins regulate the activation of Fyn during oligodendrocyte differentiation. To ensure that the blocking antibody was not inducing general cell toxicity, we examined the
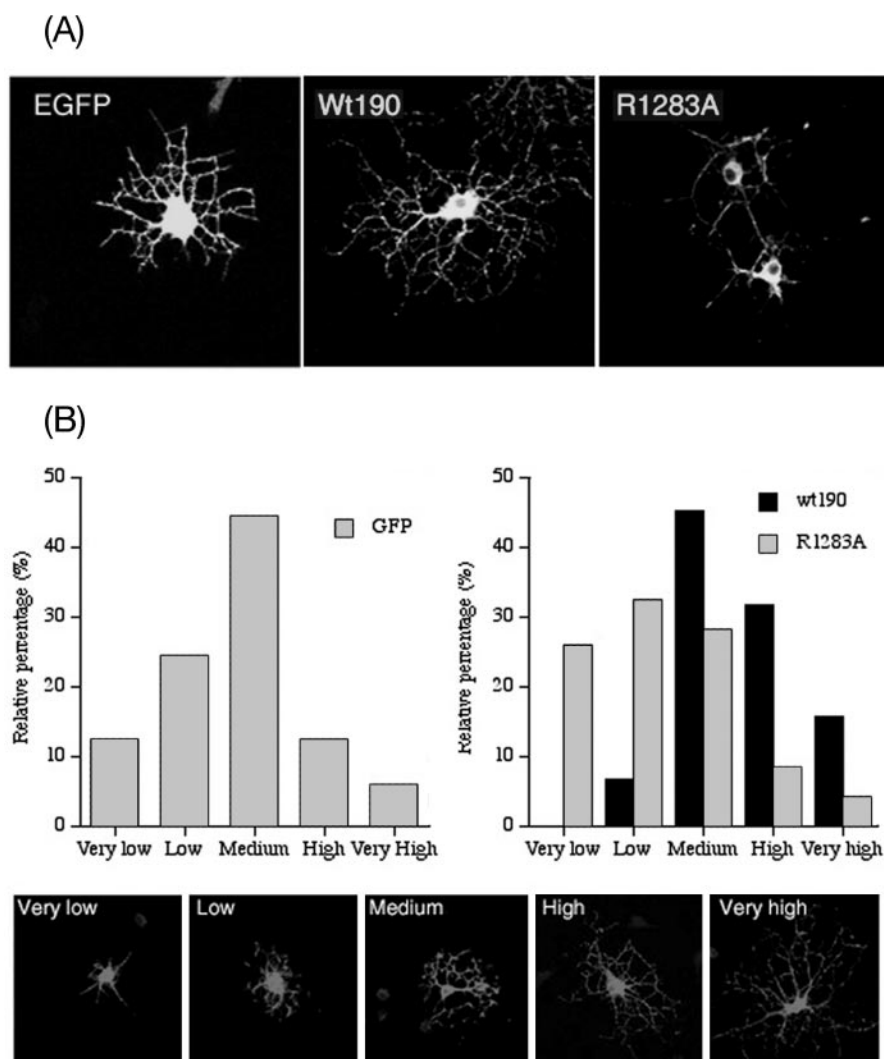

Figure 3. p190RhoGAP expression regulates oligodendrocyte differentiation. OPCs were infected overnight with adenovirus expressing HA-tagged wild-type p190RhoGAP or (R1283A)p190RhoGAP and were then induced to differentiate for 2 d in defined differentiation medium. Cells were grown on PDL-coated coverslips, fixed, and stained with anti-HA antibody and counterstained with goat Alexa Red-conjugated anti-mouse antibody. *A*, Representative morphology of oligodendrocytes expressing EGFP, wild-type p190RhoGAP, or (R1283A)p190RhoGAP. *B*, Quantitation of the morphology of oligodendrocytes based on cell complexity. More than 60 infected cells were blindly selected and assigned to one of five categories of complexity: very low, low, medium, high, and very high. Results were subjected to a paired *t* test, and the differences between wt and mutant p190RhoGAP were deemed statistically significant ($p < 0.001$).

levels of Fak, another protein that we have observed to be regulated during OPC differentiation. As depicted in Figure 2, no change in the levels of Fak was observed when cells were treated with anti-integrin β1 antibody.

Our previous studies showed that treatment of oligodendrocytes with Src family kinase inhibitors blocked differentiation of OPCs. We next examined whether direct introduction of Fyn would induce alterations in oligodendrocyte morphology. Because it is extremely difficult to transfect OPCs efficiently, we generated adenoviruses expressing full-length EGFP fusions of wild-type Fyn, (G2A)Fyn, or kinase-dead (K299M)Fyn mutants. Approximately 80% of the cells became infected with the recombinant adenoviruses. (G2A)Fyn is a Fyn mutant that is neither myristoylated nor palmitoylated and cannot bind membranes (Liang et al., 2001). OPCs were infected overnight with recombinant adenovirus in the presence of PDGF and bFGF and were then induced to differentiate for another 2 d. The morphology of

live oligodendrocytes was examined using confocal microscopy. As depicted in Figure 2, when OPCs were infected with adenovirus expressing wild-type FynEGFP, the cells extended more processes that were highly branched compared with control cells expressing EGFP. However, when OPCs were infected with adenovirus expressing (G2A)FynEGFP, morphologic differentiation of OPC was significantly inhibited, suggesting that fatty acylation of Fyn is required for inducing changes in oligodendrocyte morphology. When OPCs were infected with adenovirus expressing (K299M)FynEGFP, differentiation of OPCs was also inhibited. These results are consistent with previous studies showing a requirement for Fyn (Osterhout et al., 1999; Sperber et al., 2001) and indicate that both membrane localization and kinase activity of Fyn are essential for OPC differentiation.

Function of p190RhoGAP in OPC morphologic differentiation

We recently identified p190RhoGAP as a substrate of Fyn that becomes tyrosine phosphorylated after OPC differentiation (Wolf et al., 2001). To study the function of p190RhoGAP in oligodendrocyte differentiation, adenoviruses were generated that express HA-tagged wild-type p190RhoGAP or a mutant that is defective in GAP activity, (R1283A)p190RhoGAP. OPCs were infected overnight with adenoviruses and were induced to differentiate for 2 d in defined medium. The cells were then fixed, stained with anti-HA antibody, and counterstained with Alexa Red-conjugated goat anti-mouse secondary antibody. We have previously shown that p190RhoGAP colocalizes extensively with actin filaments (Wolf et al., 2001). Thus, the HA staining pattern fully elaborates the cell processes and is an accurate representation of cell morphology. To quantitate the effects of the wild-type and (R1283A)p190RhoGAP mutant on OPC differentiation, >60 infected cells were chosen blindly, analyzed by confocal microscopy, and divided into five categories according to their complexity: very low, low, medium, high, and very high. As depicted in Figure 3, expression of wild-type p190RhoGAP induced a more complex morphology, with a greater percentage of cells exhibiting longer and more highly branched processes. In contrast, cells expressing the (R1283A)p190RhoGAP mutant had shorter and less branched processes compared with control cells. It is likely that R1283Ap190RhoGAP acts as a dominant-negative mutant, by sequestering Rho-GTP or other essential signaling components. These results suggest that p190RhoGAP promotes OPC morphologic differentiation and that the GAP activity of p190RhoGAP is necessary for this effect.

Regulation of Rho activity during oligodendrocyte differentiation

The primary function of P190RhoGAP is to activate the GTPase activity of Rho, thereby promoting formation of Rho-GDP and Rho inactivation. To determine Rho activity during OPC differentiation, a Rhotekin-GST fusion was used to pull down activated Rho (Manser et al., 1994). Freshly prepared cell lysates from progenitor cells or differentiated oligodendrocytes were normalized to the same amount of total protein and were incubated with Sepharose beads conjugated to the Rhotekin-GST fusion. The amount of RhoA bound to Rhotekin as well as the amount of total RhoA in the whole-cell lysate was analyzed by Western blotting using anti-RhoA antibody (Fig. 4A). Levels of total RhoA protein were reduced twofold to threefold, and levels of Rho-GTP bound to Rhotekin-GST were reduced fourfold to fivefold in differentiated cells. Thus, downregulation of both RhoA protein and activity occurs during OPC differentiation.
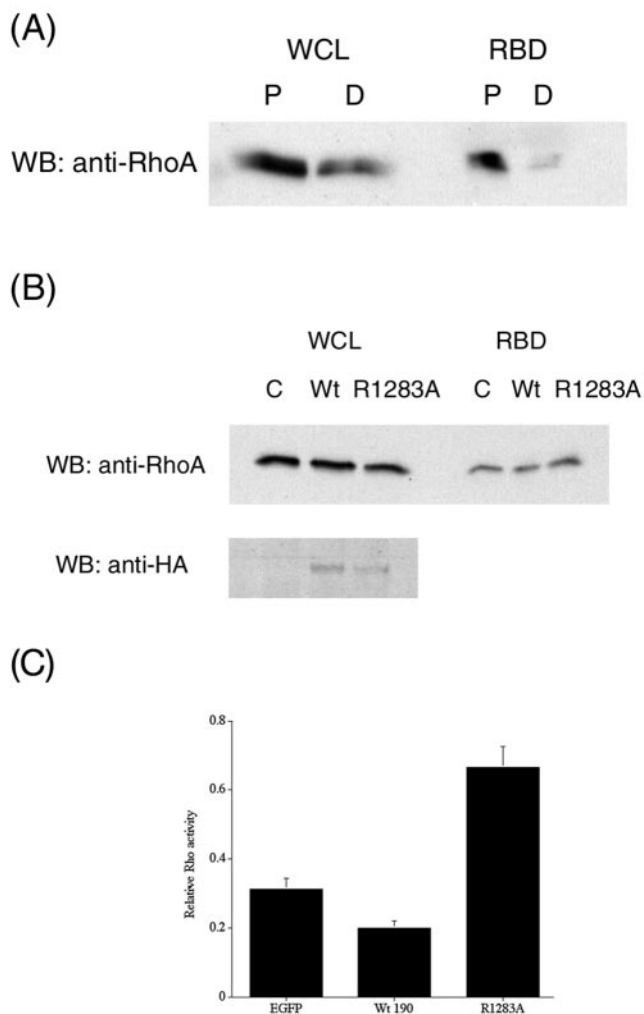

Figure 4. Regulation of RhoA activities during OPC differentiation. *A*, Cell lysates from progenitors (P) and differentiated oligodendrocytes (D) grown on PDL-coated dishes were incubated with a GST fusion of the Rhotekin-binding domain (RBD) to pulldown the GTP-bound form of RhoA. The amount of RhoA in the pulldown assay as well as in the whole-cell lysate (WCL) was determined by Western blotting with anti-RhoA antibody. *B*, *C*, Quantitation of the effect of p190RhoGAP on RhoA activity. OPCs were infected overnight with adenoviruses expressing EGFP, p190RhoGAP, or (R1283A)p190RhoGAP and were then induced to differentiate for 2 d in differentiation medium. Cells were lysed, and RhoA activity was determined as described above. The activity of RhoA was normalized to the amount of total RhoA protein in the whole-cell lysate.

We next determined the effect of expressing p190RhoGAP on RhoA activity. OPCs were infected overnight with adenoviruses expressing EGFP, 190RhoGAP, or the GAP-defective mutant (R1283A)p190RhoGAP and were induced to differentiate for 2 d. The amount of active RhoA was normalized to the amount of total RhoA in each sample. As depicted in Figure 4, *B* and *C*, expression of wild-type 190RhoGAP resulted in a decrease in RhoA-specific activity. In contrast, expression of the GAP mutant caused RhoA activity to increase more than twofold. Little change in total RhoA protein levels was noted. These results indicate that exogenous expression of 190RhoGAP can regulate RhoA-specific activity.

Rho family GTPases regulate OPC morphologic differentiation

Rho family GTPases regulate the polymerization of actin and thus control cellular cytoskeletal structure. We previously
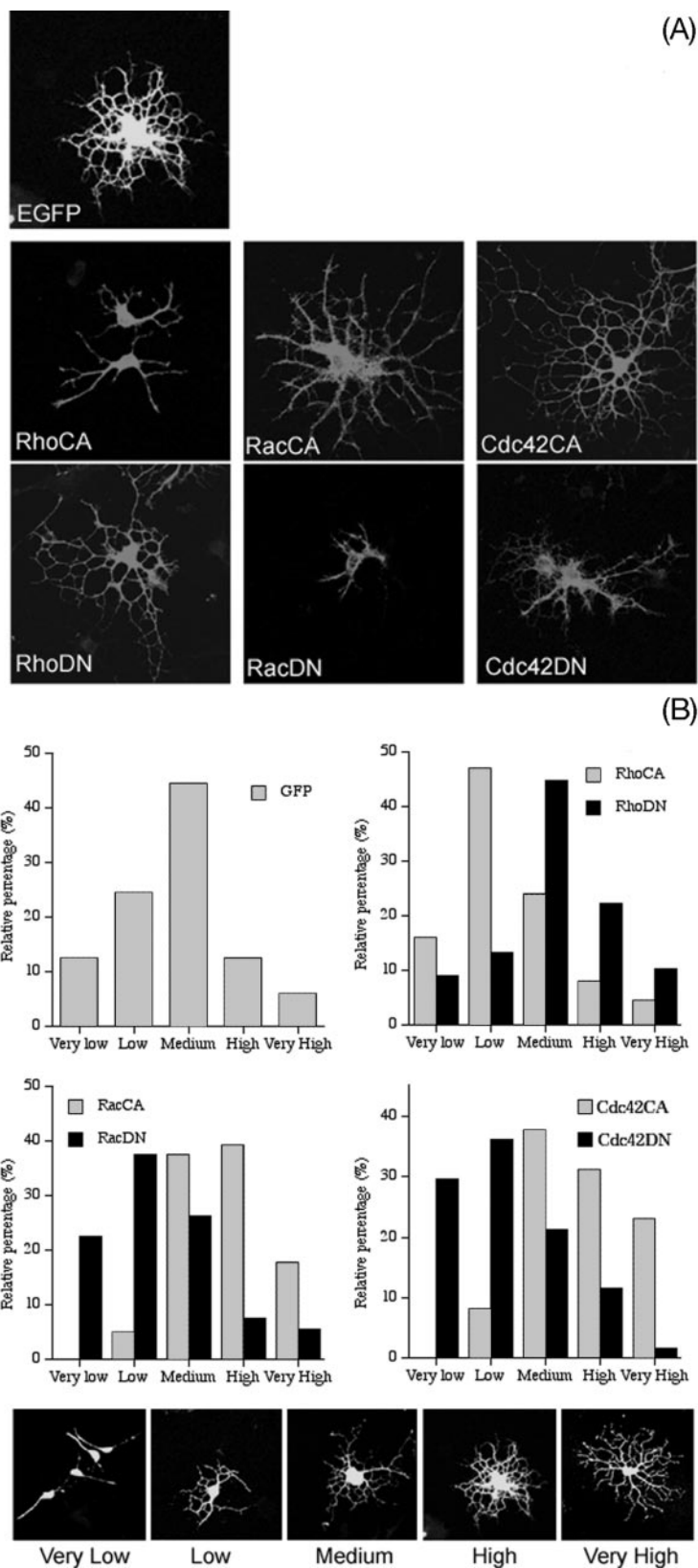
Figure 5. Constitutively active Cdc42 and Rac1 induce outgrowth of oligodendrocytes, whereas dominant-negative Cdc42 and Rac1 inhibit OPC differentiation. OPCs were infected overnight with adenoviruses expressing EGFP, CA and DN forms of RhoA, Rac1, or Cdc42, grown on PDL-coated dishes, and were then induced to differentiate for 2 d in defined differentiation medium. Cells were stained with anti-HA antibody and counterstained with Alexa Red-conjugated goat anti-mouse antibody. *A*, Representative morphology of oligodendrocytes expressing EGFP, or constitutively active and dominant-negative RhoA, Rac1, or Cdc42. *B*, Quantitation of the morphology of oligodendrocytes according to its complexity as described in Figure 3*B*. Results were subjected to a paired *t* test. The differences between wt and mutant constructs were deemed statistically significant: $p < 0.01$ for RhoA, $p < 0.005$ for Rac1, and $p < 0.02$ for Cdc42.

showed that expression of constitutively active RhoA inhibited process extension in oligodendrocytes, whereas a dominant-negative mutant of RhoA induced hyper-extension of oligodendrocyte processes (Wolf et al., 2001). To determine the contribution of the other Rho family members, Cdc42 and Rac, on morphologic differentiation of oligodendrocytes, adenoviruses were generated that express constitutively activated and dominant-negative HA-tagged RhoA, Rac1, or Cdc42. Oligodendrocyte progenitor cells were infected overnight and were then induced to differentiate for 2 d in defined medium. Cells were fixed and immunostained with anti-HA antibody and counterstained with Alexa Red-conjugated goat anti-mouse secondary antibody. Cell morphology was examined using confocal microscopy. To quantitate the effect of Rho family GTPases on OPC differentiation, we blindly selected infected cells and divided them into five categories according to their complexity, as described above. As depicted in Figure 5, *A* and *B*, expression of dominant-negative RhoA increased the percentage of oligodendrocytes with highly branched, hyperextended processes. In contrast, expression of constitutively active Cdc42 or Rac1 caused hyperextension of oligodendrocyte processes, whereas expression of dominant-negative Cdc42 or Rac1 inhibited morphologic differentiation of oligodendrocytes. These data are consistent with the notion that Rho and Cdc42–Rac are reciprocally regulated and exhibit opposite effects on process extension.

Regulation of Cdc42 and Rac1 during oligodendrocyte differentiation

To determine whether Cdc42 and/or Rac1 were being activated during oligodendrocyte differentiation, the activities of Cdc42 and Rac1 were measured biochemically. Cdc42 and Rac1 in their GTP-bound states interact specifically with PAK, p21-activated kinase (Manser et al., 1994). A GST-fusion protein containing the PBD was incubated with cell lysates from progenitors and differentiated oligodendrocytes, and the amounts of Cdc42 and Rac1 bound to the GST fusion protein, as well as the amount of total Cdc42 and Rac1 in the whole-cell lysate, were analyzed by Western blotting. As depicted in Figure 6*A*, both the activities as well as the expression levels of Cdc42 and Rac1 increased after differentiation of oligodendrocytes, with ~2.5-fold and 2.3-fold increases in specific activity for Cdc42 and Rac1, respectively.

To establish the links between integrin, Fyn, and Cdc42–Rac1 during differentia-

tion, OPCs were preincubated for 1 hr with control serum or anti-β1 antibody and were then plated on FN-coated dishes for 5 d in differentiation medium in the presence of control serum or anti-integrin β1 antibody. The activities of Cdc42 and Rac1 were then determined by the PAK–GST pulldown assay. As depicted in Figures 1C and 6B, disruption of extracellular matrix interaction by anti-β1 antibody blocked activation of Cdc42 and Rac1 as well as OPC morphologic differentiation and expression of MBP. To determine whether Fyn was responsible for activation of Cdc42 and/or Rac1, OPCs were induced to differentiate for 2 d in the presence of the Src family kinase inhibitor PP2 (3 μM) or the nonfunctional analog PP3, and the activities of Rac1 and Cdc42 were measured. As depicted in Figure 6B, treatment of OPC with PP2 inhibited activation of Rac1 and Cdc42 as well as morphologic differentiation of OPC and expression of MBP. As a control, PP2 had little effect on the amount of p190 in the cells. These data imply that integrin and Fyn activation is upstream of Cdc42–Rac1.

We next addressed the potential cross talk between and among p190, Rho, and Rac1. Purified p190 has been reported to function in vitro as a GAP for both RhoA and Rac1 (Settleman et al., 1992; Ligeti et al., 2004). To test whether this can occur in vivo, COS-1 cells were cotransfected with p190 (wild-type or R1283A mutant) and either HA-tagged Rac1 or Cdc42. As depicted in Figure 7A, wild-type, but not GAP-defective p190 was able to decrease the amount of active Rac1. No change in Rac1 total protein levels was observed. Moreover, there was no effect of p190 expression on levels of total or active Cdc42.

To examine cross talk between Rho and Rac1–Cdc42, we performed Rhotekin pulldown assays on COS-1 cell lysates that had been transfected with dominant-negative or constitutively activated Rac1 or Cdc42. No apparent changes in either the amount of total RhoA or activated RhoA (RhoGTP) were observed (Fig. 7B). Finally, we used a differentiated oligodendrocyte cell line, O2Amyc (Barnett and Crouch, 1995), to examine whether Fyn was able to regulate Rho activity. As depicted in Figure 7C, an increase in activated Rho was observed when cells were pretreated with the Src family kinase inhibitor PP2. This is consistent with the ability of Fyn to upregulate p190 (Wolf et al., 2001). Taken together, these data suggest a signal transduction pathway of integrin–Fyn–Rho family GTPases that controls oligodendrocyte morphology and myelination.

Discussion

In this study, we have identified signaling components that operate upstream and downstream of Fyn to trigger alterations in oligodendrocyte morphology during differentiation. There is extensive evidence in the literature to indicate a central role for Fyn in myelination and oligodendrocyte differentiation (Umemori et al., 1999; Sperber et al., 2001), but few studies have examined Fyn targets in oligodendrocytes. Here we show that integrin engage-
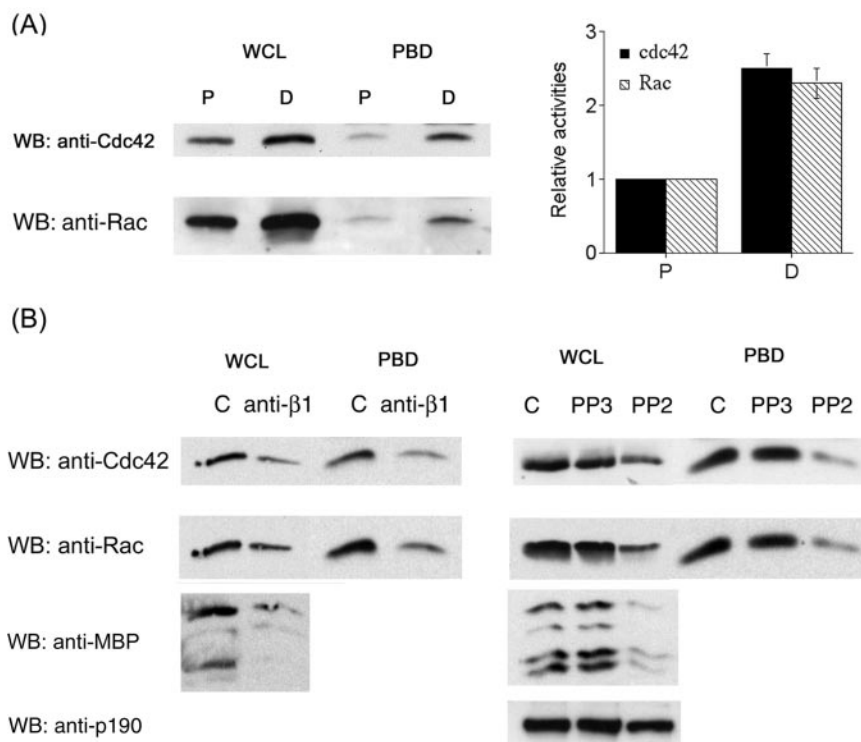

Figure 6. Regulation of Cdc42 and Rac1 activities during OPC differentiation. A, Cell lysates from progenitors (P) and differentiated oligodendrocytes (D) grown on PDL-coated dishes were incubated with a GST fusion containing the PAK-binding domain (PBD) to pulldown the GTP-bound form of Cdc42–Rac1. The amount of Cdc42 and Rac1 in the pulldown assay as well as in the whole-cell lysates was determined by Western blotting with anti-Cdc42 or anti-Rac antibodies, respectively. B, OPCs were preincubated with control serum or anti-β1 integrin antibody and were induced to differentiate on FN-coated dishes in the presence of control serum or anti-β1 integrin antibody for 5 d. Activities of Cdc42 and Rac1 were determined as described above. Alternatively, OPCs were induced to differentiate on PDL-coated dishes for 2 d in the presence of 3 μM of PP3 or PP2, and activities of Cdc42–Rac1 were determined. Expression of MBP and p190 in whole-cell lysates was determined by Western blotting with anti-MBP and anti-p190 antibodies, respectively.

ment activates Fyn in primary oligodendrocytes. Moreover, treatment of progenitor cells with an anti-β1 integrin blocking antibody inhibited both Fyn activation and morphologic differentiation. These findings are consistent with previous studies demonstrating that β1 integrin function is important for myelination in oligodendrocytes and Schwann cells (Buttery and ffrench-Constant, 1999; Relvas et al., 2001; Feltri et al., 2002). In addition, a link between integrins and Src family kinases, especially Fyn, is well established in fibroblasts and endothelial cells (Wary et al., 1998; Felsenfeld et al., 1999). The anti-integrin β1 antibody was present in the media for 5 d, the time required for full morphological differentiation of oligodendrocytes. Thus, multiple events could have occurred during the course of the treatment. However, because upregulation of Fyn is a very early event in oligodendrocyte differentiation (Osterhout et al., 1999), it is likely that effects of blocking integrin signaling also occur early during the differentiation process. It should be noted that other processes, besides integrin engagement, likely also contribute to Fyn activation in oligodendrocytes, such as Ig Fc receptor engagement (Nakahara et al., 2003) and association with GPI-anchored proteins in rafts (Kramer et al., 1999).

Several recent studies have identified downstream targets for Fyn in oligodendrocytes. These include the F3 adhesion molecule, the cytoskeletal proteins tau and α-tubulin, and two proteins that regulate Rho activity, p190RhoGAP and p250RhoGAP (Wolf et al., 2001; Klein et al., 2002; Taniguchi et al., 2003). Our laboratory previously demonstrated that Fyn phosphorylates
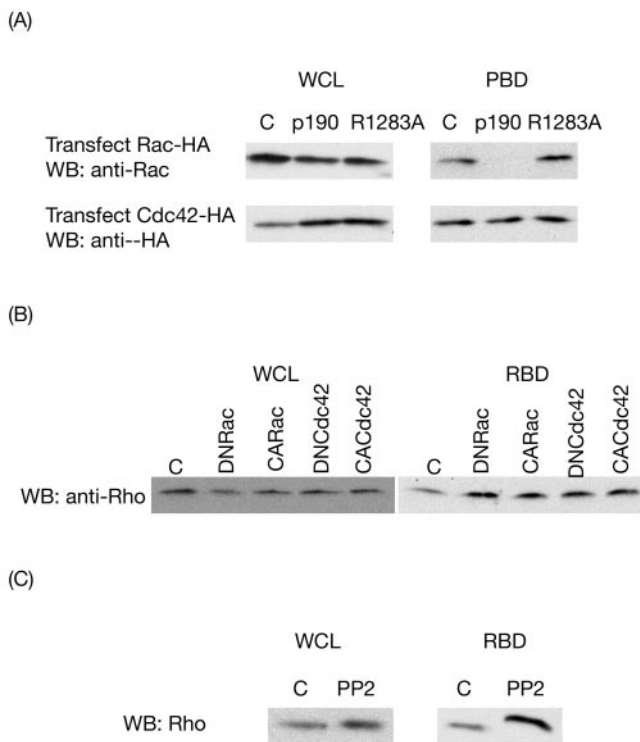
(A)

(B)

(C)

Figure 7. Cross talk among p190, Rho, Rac1, and Cdc42. *A*, COS-1 cells were cotransfected with empty vector (C), wt p190, or GAP-defective p190 (R1283A) and either HA-tagged Rac1 or Cdc42. Activities of Rac1 and Cdc42 were determined as described in Figure 6. *B*, COS-1 cells were transfected with DN or CA forms of Rac1 or Cdc42, and Rho activity was determined as described in Figure 4. *C*, Differentiated O2Amyc oligodendrocytes grown on PDL-coated dishes were treated with buffer or 3 μM PP2, as described in Figure 6, and Rho activity was determined.

p190RhoGAP during oligodendrocyte differentiation and thereby increases p190 GAP activity toward Rho (Wolf et al., 2001). We postulated that p190-induced generation of Rho-GDP was essential for regulating process extension during differentiation. This hypothesis was supported by our finding that transfection of primary oligodendrocytes with dominant-negative (DN) Rho, which mimics Rho in the GDP-bound state, was sufficient to promote hyperextension of cellular processes.

A major limitation of the use of primary oligodendrocytes has been the low transfection efficiency that can be achieved with these cells. We, therefore, exploited recombinant adenovirus technology to efficiently express heterologous proteins in progenitor cells and determine their effects on oligodendrocyte morphology during differentiation. In addition, we adopted an oligodendrocyte complexity scoring system (Sperber and McMorris, 2001; Sperber et al., 2001) to quantitate the extent of process extension and cell arborization in cells infected with the various recombinant adenoviruses. Our results establish that expression of wt p190RhoGAP increases the percentage of more morphologically complex cells. In contrast, expression of the R1283A GAP-defective p190 mutant inhibited these changes, with ~60% of the cells exhibiting very low or low complexity, compared with 35% of the GFP control and <10% of the wt p190-expressing cells. These data imply that GAP activity of p190 is important for its function in inducing alterations in oligodendrocyte morphology.

The scoring system that we used was designed to evaluate morphological complexity, a parameter that we and others have equated with differentiation. However, it is also possible that changes in morphology are reflecting alterations in cytoskeletal organization or myelin membrane elaboration, rather than the differentiation process per se. Although we did observe a decrease in the overall amount of MBP when cells were treated with anti-integrin β1 antibody or with PP2, quantitation of MBP levels in cellular processes has not been performed. Moreover, it is not clear whether the reduction in the number and complexity of processes that was observed in this study correlates directly with the reduction in the number of myelin lamellae observed in studies of Fyn−/− mice.

The presence of p190 is essential for normal neural development. The p190 knock-out mouse exhibits an embryonic lethal phenotype, with defects in neural tube closure, axon guidance, corpus callosum formation, and eye development (Brouns et al., 2001). p190 is the major tyrosine-phosphorylated protein in the brain, and Src and Fyn are the kinases primarily responsible for p190 phosphorylation (Brouns et al., 2000). Although glial cell morphology was not directly evaluated in the p190−/− mice, our findings suggest that defects in oligodendrocyte differentiation should be present.

Overexpression of p190 likely functions by promoting formation of Rho-GDP and thereby downregulating Rho signaling. Indeed, levels of Rho-GTP were decreased in differentiated oligodendrocytes, compared with progenitors (Fig. 4*A*). Rho-GTP levels were modestly decreased in cells expressing wt p190, compared with control cells (Fig. 4*B*). It is possible that other GAPs, or other regulatory proteins, are involved in the downregulation of Rho activity during differentiation. For example, a recent report identified another Rho GAP, p250, as a Fyn substrate in differentiated oligodendrocytes (Taniguchi et al., 2003). The mechanism whereby total levels of RhoA are downregulated during differentiation is unknown. However, it does not appear to be mediated by p190, because little to no change in RhoA protein levels was observed in cells overexpressing wild-type or mutant p190 constructs. Interestingly, overexpression of the GAP-defective p190 mutant resulted in a twofold to threefold increase in Rho-GTP levels. The R1283A p190 may function as a dominant-negative mutant, by binding essential factors through its other domains.

p190 has been shown to exhibit GAP activity toward Rac1 as well as RhoA, particularly *in vitro* (Settleman et al., 1992; Ridley et al., 1993; Ligeti et al., 2004). Indeed, when p190 was overexpressed in COS-1 cells, the amount of activated Rac1 was reduced (Fig. 7*A*). However, this level of overexpression is unlikely to occur under physiologic conditions in oligodendrocytes, and other studies have shown that p190 exhibits a preference for regulating Rho over Rac1 *in vivo* (Ridley et al., 1993). Our data reveal that Rac1 and Cdc42 are activated in differentiated cells, a finding that cannot be caused by increased p190RhoGAP activity. Moreover, constitutively activated (CA) Rac and Cdc42 promoted the formation of more highly differentiated cells with higher levels of complexity, whereas DN versions of these proteins had the opposite effect. These results imply that Rho and Cdc42–Rac play opposing roles in oligodendrocyte morphologic differentiation. A similar situation has been observed in neurons, where Rac and Cdc42 are positive regulators that promote neurite extension, whereas RhoA mediates neurite retraction (Jalink et al., 1994; Kozma et al., 1997; Gallo and Letourneau, 1998; Katoh et al., 2000).

Cross talk between Cdc42–Rac and Rho has been observed in several other systems. For example, fibroblasts have a linear pathway in which activation of Cdc42 leads to activation of Rac1 and then Rho resulting in generation of filopodia (Nobes and Hall, 1995). In neuronal cells and epithelial cells, Rac activation downregulates Rho activity (Kozma et al., 1997; Sander et al., 1999;

Tashiro et al., 2000; Wong et al., 2000; Li et al., 2002). The balance and reciprocal regulation of Rho versus Rac–Cdc42 is likely to be achieved by multiple factors, including GAPs and GEFs. Our study is the first to establish a sequential signaling pathway, from integrins to Fyn to Rho family proteins, that results in oligodendrocyte maturation and process extension (supplemental Fig. 1, available at www.jneurosci.org/cgi/content/full/24/32/7140/DC1). We propose that Fyn functions as one of the factors that reciprocally regulates Rho and Rac–Cdc42. Phosphorylation of p190 by Fyn promotes increased p190 Rho GAP activity, leading to a decrease in activated Rho, whereas at the same time, Fyn activation leads to Rac–Cdc42 activation. Additional experiments will be needed to fully elucidate all the factors that regulate cross talk between RhoA and Rac1–Cdc42. Knowledge of the pathways that promote oligodendrocyte differentiation is important furthering our understanding of the mechanisms regulating myelination in the CNS.

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