

Focal adhesion kinase regulates laminin-induced oligodendroglial process outgrowth

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In the central nervous system (CNS), myelination of axons occurs when oligodendrocyte progenitor cells undergo terminal differentiation, and initiate process formation and axonal ensheathment. Although Fyn, a member of the Src-family kinases (SFKs), plays an important role in this differentiation process, the substrates of Fyn in oligodendrocytes are largely unknown. Using mass spectrometric analysis, we identified focal adhesion kinase (FAK) as a tyrosine-phosphorylated protein in the rat-derived CG4 oligodendrocyte cell line. Tyrosine phosphorylation of FAK was enhanced during differentiation of CG4 cells in a Fyn-dependent manner. In addition, phosphorylation of FAK was stimulated by laminin, one of the ligands for integrin. Knockdown of FAK expression in CG4 cells suppressed process outgrowth on laminin. Rac1 and Cdc42 activities, which are required for oligodendrocyte process formation, were down-regulated in FAK-knockdown cells. Expression of wild-type (WT) FAK in FAK-knockdown CG4 cells restored outgrowth of processes, but the Y397F mutant lacking the autophosphorylation site did not. These results suggest that FAK/Fyn-mediated activation of Rac1 and Cdc42 is critical for laminin-induced outgrowth of oligodendrocyte processes.

Introduction

Myelin is a multilayered membranous sheath surrounding nerve axons that functions as an insulator facilitating the conduction of electrical impulses. Sufficient myelin production under precise developmental control is essential for the structure and function of the central nervous system (CNS). Disruption of the myelin sheath causes severe neurological diseases including multiple sclerosis. In the mammalian CNS, myelin is synthesized soon after birth by oligodendrocytes. Upon terminal differentiation, oligodendrocytes extend several processes that recognize, ensheath and myelinate nerve axons (Sherman & Brophy 2005). Therefore, clarification of the signaling pathways that underlie oligodendrocyte morphological differentiation is crucial to understanding the mechanism of myelination.

Fyn, a member of the Src-family of non-receptor tyrosine kinases (SFKs), is expressed at high levels in brain

and is activated during the initial stages of myelination (Umemori *et al.* 1992, 1994). Fyn-knockout mice display severe hypomyelination, characterized by a decrease in total myelin content and a reduced number of myelin lamellae on axons (Umemori *et al.* 1999; Goto *et al.* 2004). Fyn is essential for the morphological differentiation and survival of oligodendrocytes (Osterhout *et al.* 1999; Colognato *et al.* 2004). Fyn kinase activity increases upon differentiation of primary oligodendrocytes (Osterhout *et al.* 1999). Hence, Fyn functions as a critical mediator in the oligodendroglial signaling pathway to control CNS myelinogenesis. Recent studies have identified several transmembrane proteins that activate Fyn in oligodendrocytes, including FcR γ , CD45 and integrin $\alpha 6\beta 1$ (Nakahara *et al.* 2003, 2005; Colognato *et al.* 2004; Liang *et al.* 2004). However, Fyn substrates in oligodendrocytes have not been studied in detail, although p190RhoGAP, p250GAP and QKI have been implicated as downstream targets of Fyn in these cells (Wolf *et al.* 2001; Taniguchi *et al.* 2003; Lu *et al.* 2005).

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is a known substrate of SFKs. Integrins and

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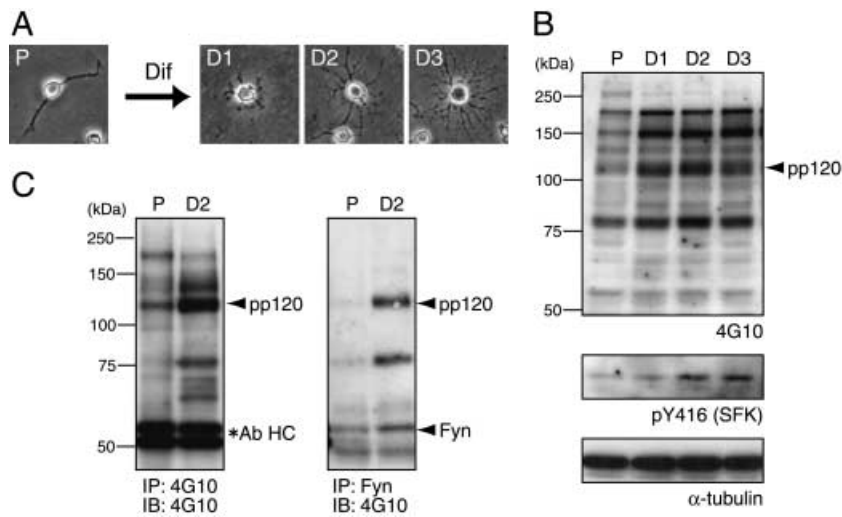


Figure 1 Tyrosine phosphorylation in differentiating CG4 cells. (A) Morphological changes during differentiation (Dif) of the rat-derived CG4 oligodendrocyte cell line. Oligodendrocyte progenitor cells (P) and oligodendrocytes after 1–3 days of differentiation (D1–D3) are shown. (B) Enhanced tyrosine phosphorylation during CG4 differentiation. The lysates of progenitor CG4 and differentiating CG4 cells were subjected to immunoblotting with anti-phosphotyrosine mAb (4G10) and anti-active SFKs [pY416 (SFK)]. Anti- α -tubulin was used as a loading control. (C) Enhanced tyrosine phosphorylation in phosphotyrosine and Fyn immunoprecipitates (IP) of differentiating CG4 cells. Phosphotyrosine and Fyn immunoprecipitates from lysates of progenitor cells and differentiating oligodendrocytes were probed (IB) with 4G10. Ab HC, antibody heavy chain.

other extracellular signals activate FAK, which in turn leads to multiple cellular processes including cell adhesion, migration and survival (Mitra *et al.* 2005). In response to integrin stimulation, FAK is autophosphorylated at Tyr397, generating a binding site for the Src homology 2 (SH2) domain of SFKs such as Fyn (Cary *et al.* 1996). The recruited SFKs phosphorylate several additional FAK tyrosine residues to ensure full activation (Calalb *et al.* 1995). FAK-knockout mice exhibit embryonic lethality with defects in mesoderm development (Ilic *et al.* 1995). Neuron-specific FAK-knockout mice show abnormalities in axonal and dendritic morphology and synapse formation, indicating that FAK is critical for neuronal development *in vivo* (Beggs *et al.* 2003; Rico *et al.* 2004). FAK regulates neurite outgrowth in response to growth factors, integrin stimulation and neuronal guidance cues (Ivankovic-Dikic *et al.* 2000; Liu *et al.* 2004; Robles & Gomez 2006). However, the roles of FAK in the oligodendrocytic cell lineage are poorly understood, although FAK is expressed in primary oligodendrocytes (Kilpatrick *et al.* 2000).

Affinity purification with anti-phosphotyrosine antibody followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis is a powerful tool to identify tyrosine-phosphorylated proteins (Blagoev *et al.* 2004; Kratchmarova *et al.* 2005). In the present study, we attempted to identify tyrosine-phosphorylated proteins in differentiating oligodendrocytes using mass spectrometric analysis. We identified FAK as a substrate of Fyn in the rat CG4 oligodendrocyte cell line. Furthermore, we found that FAK was critical for outgrowth of processes from CG4 cells.

Results

Identification of FAK as a tyrosine-phosphorylated protein in differentiating CG4 cells

To identify tyrosine-phosphorylated proteins in oligodendrocytes, we used the rat-derived CG4 oligodendrocyte cell line. As shown in Fig. 1A, CG4 cells underwent differentiation from bipolar oligodendrocyte progenitor cells into immature oligodendrocytes extending multiple branched processes in glial-defined medium (GDM) (Louis *et al.* 1992). CG4 cells were induced to differentiate for 1, 2 or 3 days on poly-L-lysine (PLL)-coated dishes. Lysates of these cells were subjected to SDS-PAGE, and tyrosine-phosphorylated proteins were then visualized by immunoblotting with 4G10, an anti-phosphotyrosine monoclonal antibody (mAb) (Fig. 1B). We confirmed that tyrosine phosphorylation levels in CG4 cells were increased upon differentiation. The levels of activated SFKs also increased as monitored by anti-active SFKs [pY416 (SFK)] antibody (Bjorge *et al.* 2000). In addition, tyrosine phosphorylation in both anti-phosphotyrosine and anti-Fyn immunoprecipitates was enhanced during differentiation of CG4 cells (Fig. 1C). Within the latter immunoprecipitate a protein band that migrated with an approximate mass of 120 kDa (pp120) was prominent, and we speculated that pp120 might be a major substrate of Fyn.

For purification of the tyrosine-phosphorylated proteins, we cultured progenitor CG4 cells and differentiating CG4 cells on PLL-coated dishes. Both cell lysates

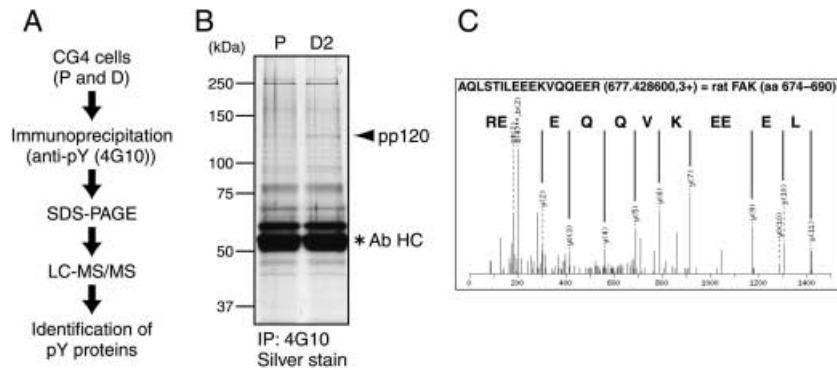


Figure 2 Identification of FAK as a tyrosine-phosphorylated (pY) protein in CG4 cells. (A) Diagram of the experimental process. (B) Visualization of pp120 by silver staining. The lysates of progenitor CG4 cells (P) and differentiating CG4 cells (D2) were immunoprecipitated with 4G10. Purified proteins were resolved by SDS-PAGE and visualized by silver staining. (C) MS/MS spectrum from one of the pp120 peptides. The sequence was derived from the mass difference of the nested set of peptide fragments. The positions to assigned series of Y ions are marked. Database search with the Mascot engine identified an amino acid sequence (aa 674–690) from rat FAK.

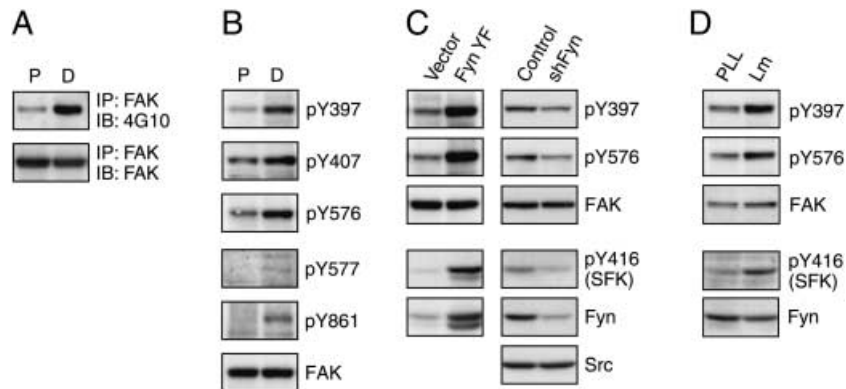


Figure 3 Tyrosine phosphorylation of FAK in CG4 cells. (A) Enhanced tyrosine phosphorylation of FAK during differentiation of CG4 cells. Anti-FAK immunoprecipitates from lysates of progenitor cells (P) and oligodendrocytes (D) were probed with 4G10 and anti-FAK. (B) Phosphorylation sites of FAK during differentiation. The lysates were probed with phosphorylation site-specific antibodies against FAK (pY397, pY407, pY576, pY577 and pY861) and anti-FAK. (C) Fyn-dependent tyrosine phosphorylation of FAK in differentiating CG4 cells. (Left) CG4 cells were infected with retrovirus expressing mock or Fyn Y531F mutant. (Right) CG4 cells were infected with retrovirus expressing control shRNA (control) or Fyn shRNA (shFyn). The infected cells were selected with puromycin. The lysates were probed with the indicated antibodies. (D) Enhanced tyrosine phosphorylation of FAK upon stimulation of differentiating CG4 cells by laminin. Lysates of differentiating CG4 cells grown on poly-L-lysine (PLL) and laminin (Lm) were probed with the indicated antibodies.

were immunoprecipitated with 4G10 (Fig. 2A). Bound proteins were subjected to SDS-PAGE and visualized by silver staining (Fig. 2B). A 120-kDa protein band was excised from the gel, subjected to in-gel trypsin digestion, and then analyzed by LC-MS/MS. Subsequent MS/MS analysis identified a peptide with the amino acid (aa) sequence AQLSTILEEEKVQQEER (Fig. 2C), corresponding to aa 674–690 of rat FAK. An additional FAK peptide, NLLDVIDQAR (aa 1036–1045), was also identified (data not shown). Identification of other

bands whose tyrosine phosphorylation is increased upon differentiation of CG4 cells are currently in progress.

Tyrosine phosphorylation of FAK in CG4 cells

We carried out immunoprecipitation with anti-FAK antibody and examined tyrosine phosphorylation of FAK during differentiation of CG4 cells. As expected, tyrosine phosphorylation of FAK was elevated 2 days after induction of differentiation (Fig. 3A). FAK is phosphorylated

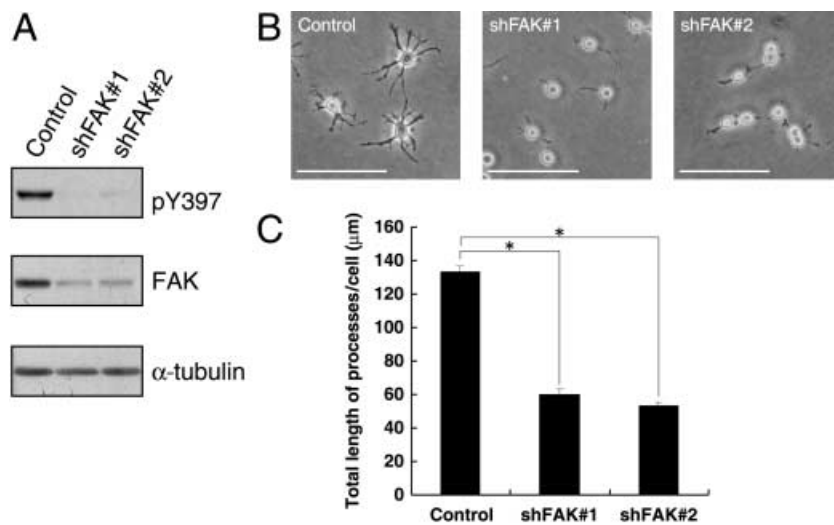


Figure 4 Inhibition of laminin-induced process outgrowth in FAK-knockdown CG4 cells. (A) Knockdown of FAK expression in CG4 cells. CG4 cells were infected with retrovirus expressing control shRNA (control) or FAK shRNA#1–2 (shFAK#1–2). The infected cells were selected with puromycin. Lysates of the selected cells were probed with the indicated antibodies. (B) Suppression of laminin-induced process outgrowth by FAK knockdown. These CG4 cells were induced to differentiate on laminin for 1 day. Scale bars, 100 μm. (C) Quantification of process length in CG4 cells. The sum total process length of individual cells was calculated for each group based upon measurements of approximately 100 cells each. Data obtained from three independent experiments are expressed as the mean ± SD. * $P < 0.0005$, Student's *t*-test.

at multiple tyrosine residues including Tyr397, 407, 576, 577 and 861 upon activation in other cell types (Mitra *et al.* 2005). Tyr397 undergoes autophosphorylation in response to extracellular stimuli such as integrin. Tyr576 and Tyr577 are phosphorylated by SFKs, and phosphorylation of both tyrosine residues increases the catalytic activity of FAK. To determine which tyrosine phosphorylation sites are regulated during differentiation of CG4 cells, we used several phosphorylation site-specific antibodies against FAK. Phosphorylation of all tyrosine residues that we examined was enhanced upon differentiation (Fig. 3B).

We next examined whether tyrosine phosphorylation of FAK is dependent on Fyn in differentiating CG4 cells. We used a retrovirus system to express a constitutively active Fyn (FynY531F) exogenously in CG4 cells. Infected cells were selected with puromycin and then differentiated for 2 days. Expression of FynY531F enhanced phosphorylation of FAK at Tyr397 and Tyr576 (Fig. 3C). Next, we knocked down Fyn expression with a retroviral vector expressing a short hairpin RNA (shRNA) against Fyn. The expression level of Fyn was reduced by approximately half in the infected CG4 cells (Fig. 3C). The level of Src remained unchanged, showing that the shRNA targeted Fyn specifically. Phosphorylation levels of FAK at Tyr397 and Tyr576 were reduced by Fyn-knockdown in CG4 cells (Fig. 3C). Therefore, Fyn is critical for tyrosine phosphorylation of FAK in differentiating CG4 cells.

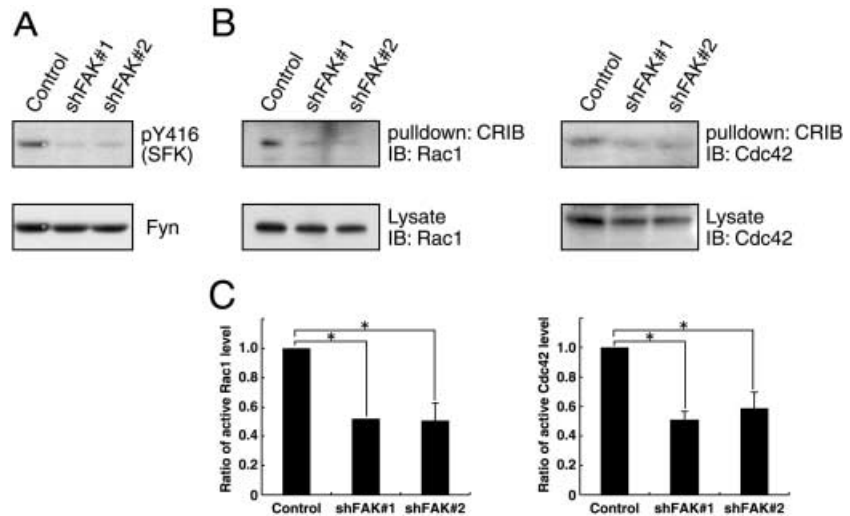
Extracellular matrix components such as laminin activate SFKs through integrins on oligodendrocytes (Colognato *et al.* 2004; Liang *et al.* 2004). Because FAK is critical for integrin signaling, we tested whether

laminin enhances tyrosine phosphorylation of FAK in CG4 cells. CG4 cells were induced to differentiate for 1 day on PLL- or laminin-coated dishes. We confirmed SFK activation in CG4 cells grown on laminin. Tyrosine phosphorylation of FAK was also enhanced in the presence of laminin (Fig. 3D), suggesting that the laminin-integrin pathway activates FAK during oligodendrocyte differentiation.

Suppression of laminin-induced process outgrowth by FAK knockdown in CG4 cells

To elucidate the function of FAK in oligodendrocytes, we established FAK-knockdown CG4 cells using retroviral vectors expressing an shRNA sequence against FAK. After retroviral infection, the infected cells were selected with puromycin. The expression level of FAK was significantly reduced by either of two independent shRNA constructs (Fig. 4A). To investigate whether FAK regulates process outgrowth in CG4 cells, we measured the sum total length of process on control and FAK-knockdown CG4 cells. In CG4 cells that had differentiated on PLL-coated dishes for 1 day, FAK-knockdown had no significant effect on process outgrowth (data not shown). Then, we examined process outgrowth on laminin, because laminin promotes process outgrowth of oligodendrocytes in a Fyn-dependent manner (Colognato *et al.* 2004). In CG4 cells that had differentiated on laminin-coated dishes for 1 day, the process outgrowth was significantly suppressed by FAK knockdown (Fig. 4B,C). These data were consistent with our observation that tyrosine phosphorylation of FAK in CG4 cells

Figure 5 Reduced activities of SFKs, Rac1 and Cdc42 in FAK-knockdown CG4 cells. (A) Reduced activity of SFKs in FAK-knockdown CG4 cells. Lysates of the control and FAK-knockdown CG4 cells grown on laminin were probed with the indicated antibodies. (B) Reduced activities of Rac1 and Cdc42 in FAK-knockdown CG4 cells. The lysates were pulled down with GST-CRIB immobilized on glutathione-Sepharose beads. The amounts of GTP-loaded Rac1 and Cdc42 as well as total Rac1 and Cdc42 were analyzed by immunoblotting. (C) Quantification of Rac1 and Cdc42 activities in control and FAK-knockdown CG4 cells. Data obtained from three independent experiments are expressed as the mean \pm SD. * $P < 0.005$, Student's *t*-test.



was enhanced by laminin (Fig. 3D). These results suggest that FAK is critical for laminin-induced process outgrowth in CG4 cells.

Reduced activities of SFKs, Rac1 and Cdc42 in FAK-knockdown CG4 cells

We next analyzed how FAK regulates the early biochemical events relevant to the process outgrowth in CG4 cells. Previous studies showed that SFKs are also regulated by FAK activity (Schlaepfer & Hunter 1997). Therefore, using immunoblotting with anti-pY416 (SFK) antibody, we examined whether knockdown of FAK expression alters the activity of SFKs in laminin-stimulated CG4 cells. Indeed, laminin-induced activation of SFKs was suppressed in CG4 cells expressing FAK-specific shRNA in comparison with those expressing control shRNA (Fig. 5A). The data suggested that SFKs and FAK are synergistically involved in laminin-induced process outgrowth.

Rho-family GTPases, such as RhoA, Rac1 and Cdc42, play important roles in cytoskeletal remodeling and are critical for morphological differentiation of oligodendrocytes and myelination (Liang *et al.* 2004). Rac1 and Cdc42 are activated upon differentiation as part of the integrin-Fyn pathway (Liang *et al.* 2004). To investigate whether FAK is involved in this pathway, we used Rho-family effector pull-down assays to examine the activities of Rac1 and Cdc42 in laminin-stimulated CG4 cells. GTP-loaded active Rac1 and active Cdc42 bind specifically to the CRIB domain of PAK. GST-fusion protein containing the CRIB domain was incubated with lysates of control or FAK-knockdown CG4 cells cultured on laminin for 1 day. GST-CRIB-

bound as well as total Rac1 and Cdc42 in cell lysates were analyzed by immunoblotting. By this measure Rac1 and Cdc42 were less active in FAK-knockdown than in control cells (Fig. 5B,C), showing that FAK was required for activation of Rac1 and Cdc42 in laminin-stimulated CG4 cells. These results suggest that the reduction of Rac1 and Cdc42 activities contributes to suppression of laminin-induced process outgrowth in FAK-knockdown CG4 cells.

Autophosphorylation of FAK at Tyr397 is required for laminin-induced process outgrowth in CG4 cells

Integrin clustering promotes FAK autophosphorylation at Tyr397. To examine the significance of the tyrosine phosphorylation event in process outgrowth of CG4 cells, we prepared retroviral RNAi-refractory FAK (rFAK) constructs. FAK-knockdown CG4 cells were infected with retrovirus expressing wild-type (WT) or Y397F mutant rFAK. Expression levels of rFAK WT and rFAK Y397F in FAK-knockdown cells were similar to that of FAK in the parental CG4 cells. Phosphorylation of Tyr397 occurred on rFAK WT but not rFAK Y397F (Fig. 6A). We measured the sum total length of processes on CG4 cells grown on laminin after differentiation for 1 day. Expression of rFAK WT efficiently restored process outgrowth in FAK-knockdown CG4 cells (Fig. 6B,C), confirming that suppression of process outgrowth by FAK knockdown was not due to off-target effects of the FAK shRNA. However, the rFAK Y397F mutant failed to rescue process outgrowth of CG4 cells. These results indicated that Tyr397 phosphorylation of FAK was needed for laminin-induced process outgrowth in CG4 cells.

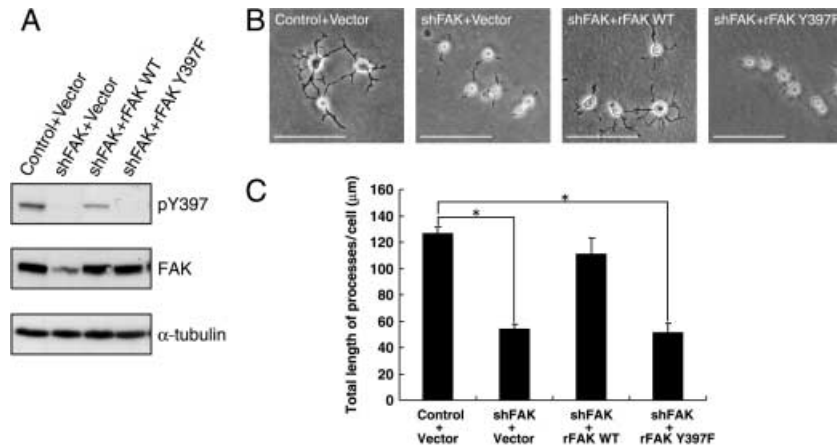


Figure 6 Requirement of FAK phosphorylation at Tyr397 for laminin-induced process outgrowth in CG4 cells. (A) CG4 cells were infected with retrovirus expressing control shRNA (control) or FAK shRNA #2 (shFAK) and selected with puromycin. The selected CG4 cells were then infected with retrovirus expressing mock (vector), RNAi-refractory wild-type FAK (rFAK WT) or FAK Y397F mutant (rFAK Y397F) and selected with G418. Lysates of these CG4 cells were probed with the indicated antibodies. (B) Rescue of laminin-induced process outgrowth by rFAK WT but not rFAK Y397F. These CG4 cells were induced to differentiate on laminin for 1 day. Scale bars, 100 μm . (C) Quantification of process length in CG4 cells. The sum total process length of individual cells was calculated for each group based upon measurements of approximately 100 cells each. Data obtained from three independent experiments are expressed as the mean \pm SD. * $P < 0.0005$, Student's *t*-test.

Discussion

Recent studies have revealed that Fyn plays important roles in oligodendrocyte differentiation and myelination. Although several proteins, including p190RhoGAP, p250GAP and QKI, have been suggested as Fyn substrates in oligodendrocytes (Wolf *et al.* 2001; Taniguchi *et al.* 2003; Lu *et al.* 2005), there is no convincing evidence that these proteins regulate oligodendrocyte differentiation via Fyn signaling. In the present study, we identified FAK as a Fyn substrate in CG4 cells (Fig. 2). We also found that FAK regulated laminin-induced process outgrowth in differentiating CG4 cells (Fig. 4).

We showed that laminin stimulation increased FAK tyrosine phosphorylation in differentiating CG4 cells (Fig. 3D). Moreover, FAK Tyr397 phosphorylation was critical for laminin-induced process outgrowth in CG4 cells (Fig. 6). In the CNS, $\alpha 2$ chain-containing laminins are found within axon tracts, and these laminins are required for oligodendroglial differentiation and survival (Colognato *et al.* 2002, 2004). In addition, $\alpha 6$ integrin-knockout mice exhibit loss of oligodendrocytes in the brain (Colognato *et al.* 2002), suggesting that signaling from extracellular matrix components such as laminin to integrins plays a critical role in oligodendrocyte development. Thus, laminin-induced FAK activation is likely to occur when an oligodendrocyte process contacts on axonal surface. In other cell types, FAK integrates growth-

factor and integrin signals to promote cell migration (Sieg *et al.* 2000). It is reported that axonal neuregulin-1 engages ErbB2 and ErbB4, members of the ErbB-family of receptor tyrosine kinases, to regulate oligodendrocyte survival and differentiation (Fernandez *et al.* 2000; Kim *et al.* 2003; Sussman *et al.* 2005). In addition, co-stimulation with laminin and neuregulin-1 promotes the survival and differentiation of oligodendrocytes (Colognato *et al.* 2002). Therefore, FAK may be required for integration of integrin-neuregulin-1 signaling in oligodendrocytes.

To elucidate roles of FAK in myelination, analysis of conditional FAK-knockout mice would be informative. Recently, it is reported that Schwann cell-specific FAK-knockout mice show impaired myelination in the peripheral nervous system due to defects in proliferation of Schwann cells and radial sorting of axonal bundles (Grove *et al.* 2007). Analyses of oligodendrocyte lineage-specific FAK-knockout mice may clarify the roles of FAK in oligodendrocyte development and myelination in the CNS.

In neurons, FAK regulates neurite outgrowth induced by growth factors as well as integrins (Ivankovic-Dikic *et al.* 2000; Liu *et al.* 2004; Robles & Gomez 2006). Within these cells, FAK is localized at the growth cones of axons, and tyrosine phosphorylation of FAK in response to laminin is enriched at frontal tips of growth cones, in particular at point contacts. It is suggested

that tyrosine phosphorylation of FAK at point contacts promotes neurite outgrowth by stabilizing the marginal structure on laminin (Robles & Gomez 2006). Distal tips of oligodendrocyte processes resemble growth cones of neurons (Fox *et al.* 2006), suggesting that the mechanisms underlying oligodendrocyte process outgrowth have several similarities to those of neurite outgrowth. Therefore, as in neurons, tyrosine phosphorylation of FAK may occur at the tips of the oligodendrocyte processes and lead to rapid process outgrowth.

In the present study, we showed that tyrosine phosphorylation of FAK was suppressed in Fyn-knockdown CG4 cells (Fig. 3C). Activities of SFKs were also reduced in FAK-knockdown CG4 cells (Fig. 5A). Therefore, as in other cell types (Liu *et al.* 2004; Mitra *et al.* 2005), FAK and Fyn appear to act cooperatively to evoke downstream signaling. Previous reports have suggested that Fyn regulates Rac1 and Cdc42 downstream of integrin in oligodendrocytes (Liang *et al.* 2004). In the present study, we showed FAK also to be involved in this pathway. FAK/SFK-mediated phosphorylation events activate Rac1 and Cdc42 in various cell types. Several guanine nucleotide exchange factors (GEFs) for Rac1 and Cdc42 are activated by SFK-mediated tyrosine phosphorylation. Tyrosine phosphorylation of Vav-family GEFs causes conformational changes, enhancing their GEF activities (Crespo *et al.* 1997). Tyrosine phosphorylation of FRG also elevates its GEF activity for Cdc42 (Miyamoto *et al.* 2003). In fibroblasts, FAK activates Rac1 through direct phosphorylation of β PIX (Chang *et al.* 2007). In addition, a number of GTPase-activating proteins (GAPs) for Rho-family GTPases are also regulated by SFK-mediated tyrosine phosphorylation. Fyn-mediated tyrosine phosphorylation of p190RhoGAP elevates its GAP activity for Rho and over-expression of p190RhoGAP induce process outgrowth in oligodendrocytes (Wolf *et al.* 2001; Liang *et al.* 2004). Tyrosine phosphorylation of TCGAP by Fyn decreases its GAP activity for Cdc42 (Liu *et al.* 2006). Because critical GEFs and GAPs might differ according to cell types and signaling cascades, it is important to identify the GEFs and GAPs responsible for activation of Rac1 and Cdc42 during morphological differentiation of oligodendrocytes induced by integrin-Fyn/FAK signaling.

In the present study, we identified FAK as a Fyn substrate during differentiation of CG4 cells, but other tyrosine-phosphorylated proteins remain uncharacterized. Further identification and functional analyses of tyrosine-phosphorylated proteins in oligodendrocytes may clarify the precise signaling pathways that underlie oligodendrocyte development and myelination in the CNS.

Experimental procedures

DNA constructs

Expression plasmid pME-Fyn Y531F was described previously (Liu *et al.* 2006). For retroviral expression of a constitutively active form of Fyn, the cDNA encoding Fyn Y531F was cloned into pMX-puro. The plasmid for glutathione-S-transferase-Cdc42/Rac-interacting binding domain of Pak (GST-CRIB) was provided by C. Sasakawa (University of Tokyo, Tokyo, Japan). The small interfering RNA (siRNA) sequences specific for the rat Fyn and FAK mRNAs were determined with BLOCK-iT RNAi Designer (Invitrogen, Rockville, MD). The following target sequences were used: Fyn, 5'-GGAGACCATGTCAAACAT-3'; FAK#1, 5'-GGTCCAGACCAATCACTAT-3' and FAK#2, 5'-GCAGTTTGCCAACCTTAAT-3'. A control sequence (5'-TTCTCCGAACGTGTCACGT-3') with no significant homology to any mammalian gene sequence served as a non-silencing control. The annealed nucleotides were inserted into pSIREN-RetroQ (Takara Bio, Otsu, Japan). The cDNAs encoding mouse wild-type (WT) and Y397F mutant FAK were provided by D. Schlaepfer (The Scripps Research Institute, San Diego, CA). The RNAi-refractory mutant, which contained five silent mutations within the siRNA target sequence of FAK#2, was generated by PCR-based mutagenesis. The constructs were verified by DNA sequencing. For retroviral expression of FAK, these cDNAs were cloned into pMXs-neo.

Antibodies

Affinity-purified anti-FAK antibody was generated by immunizing a rabbit with a carboxyl-terminal fragment of FAK as an antigen (Ilic *et al.* 1995). Anti-Rac1 antibody was kindly provided by T. Takenawa (University of Tokyo). The following antibodies were purchased: anti-phosphotyrosine mAb, 4G10 (Millipore, Bedford, MA); anti- α -tubulin mAb (Sigma, St. Louis, MO); rabbit polyclonal anti-Fyn and anti-Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-pY416 (SFK) (Cell Signaling, Beverly, MA); and phosphorylation site-specific antibodies against pY397, pY407, pY576, pY577 and pY861 of FAK (Biosource, Camarillo, CA).

Cell culture and retroviral infection

Rat neuroblastoma B104 cells and Plat-E cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in 5% CO₂. Rat oligodendroglial CG4 cells were maintained and differentiated on dishes coated with PLL (10 μ g/mL) as described previously (Yonemasu *et al.* 1998). Briefly, CG4 cells were cultured in a glial-defined medium [GDM, 1/1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F12 supplemented with 5 mg/L insulin, 16.1 mg/L putrescine, 50 mg/L apo-transferrin, 4.6 mg/L D-galactose and 8 mg/L sodium selenite] containing 2% fetal bovine serum for proliferation. For maintenance of oligodendrocyte progenitor cells, CG4 cells were cultured in GDM containing 30% B104-conditioned medium. For differentiation, CG4 cells were

cultured in GDM. Plat-E cells were transfected with pMX-puro, pMXs-neo or pSIREN-RetroQ plasmids with FuGENE6 Transfection Reagent (Roche, Mannheim, Germany), and the media containing the retrovirus were collected. CG4 cells were infected during the proliferating phase, and the infected cells were selected with puromycin (0.7 $\mu\text{g}/\text{mL}$) or G418 (700 $\mu\text{g}/\text{mL}$) (Liu *et al.* 2006).

Immunoprecipitation and immunoblotting

Cells were lysed for 1 h at 4 °C in a lysis buffer [TNE, 50 mM Tris-Cl (pH 7.4), 120 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM Na_3VO_4 and aprotinin at 50 units/mL] and then centrifuged for 15 min at 21 000 *g*. Protein concentrations were measured with a BCATM protein assay kit (Pierce). For immunoprecipitation, pre-cleared lysates were incubated with appropriate antibodies and protein G-sepharose (GE Healthcare, Piscataway, NJ) for 2 h. Immunoprecipitates were washed 4 times with TNE buffer. Immunoprecipitates and lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 2% bovine serum albumin and blotted with primary antibodies. Horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies and Renaissance Plus Reagent (Perkin-Elmer, Boston, MA) or CDP-Star (Tropix, Bedford, MA) were used to visualize the immunoreactive proteins.

Large-scale immunoprecipitation and mass spectrometric analysis

For purification of tyrosine-phosphorylated proteins, CG4 cells were differentiated for 2 days at 37 °C. The harvested cells were solubilized in TNE buffer. Cell lysates (2 mg/mL, 2 mL) were incubated with 40 μg of anti-phosphotyrosine antibody 4G10 and protein A-sepharose (GE Healthcare) overnight at 4 °C. Immuno-complexes were precipitated, washed 5 times with TNE buffer and then boiled with SDS. The proteins were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining and immunoblotting with 4G10. For the mass spectrometric analysis, bands visualized with Coomassie Brilliant Blue staining were excised from the gel and digested in the gel with trypsin (1 pmol/ μL) for 12 h at 37 °C. The eluted peptides were loaded on the automated nanoflow liquid chromatograph (Dina) and tandem mass spectrometer (Q-TOF2). The peptide masses obtained with the LC-MS/MS analysis were searched against the non-redundant protein sequence database of the National Center for Biotechnology Information with the Mascot search engine (Matrix Science, London, UK).

Pull-down assay for Rac1 and Cdc42

To monitor Rac1 and Cdc42 activities, we performed the Rho effector pull-down assay as described previously (Liu *et al.* 2006). Briefly, cells were lysed for 5 min with ice-cold lysis buffer [50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 2 mM MgCl_2 , 1%

Nonidet P-40, 10% glycerol with aprotinin at 50 units/mL] and the lysates were then centrifuged 5 min at 21 000 *g*. The supernatants were incubated with 20 μg of GST-CRIB for 30 min. The beads were washed with lysis buffer and the bound proteins were resolved by SDS-PAGE and subjected to immunoblotting with anti-Rac1 or anti-Cdc42 antibody. For quantification, immunoreactive protein bands were analyzed by using the IMAGE J software (National Institutes of Health). Levels of active Rac1/Cdc42 were normalized to those of total Rac1/Cdc42. Data were statistically analyzed by Student's *t*-test. The difference was considered significant when $P < 0.05$.

Analysis of process outgrowth in CG4 cells

CG4 cells (5×10^4) were plated on 6-cm dishes coated with laminin (3 $\mu\text{g}/\text{mL}$) (Asahi Techno Glass), and induced to differentiate. The sum of all of the processes lengths for approximately 100 cells 1 day after differentiation was calculated with the IMAGE J software. Data were statistically analyzed by Student's *t*-test. The difference was considered significant when $P < 0.05$.

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