The formation of the myelin sheath is a crucial step during development because it enables fast and efficient propagation of signals within the limited space of the mammalian central nervous system (CNS). During the process of myelination, oligodendrocytes actively interact with the extracellular matrix (ECM). These interactions are considered crucial for proper and timely completion of the myelin sheath. However, the exact regulatory circuits involved in the signaling events that occur between the ECM and oligodendrocytes are currently not fully understood. Therefore, in the present study we investigated the role of a known integrator of cell–ECM signaling, namely, focal adhesion kinase (FAK), in CNS myelination via the use of conditional (oligodendrocyte-specific) and inducible FAK-knockout mice (FAKflox/flox, PLP/CreER<sup>T</sup> mice). When inducing FAK knockout just prior to and during active myelination of the optic nerve, we observed a significant reduction in the number of myelinated fibers on postnatal day 14. In addition, our data revealed a decreased number of primary processes extending from oligodendrocyte cell bodies at this postnatal age and on induction of FAK knockout. In contrast, myelination appeared normal on postnatal day 28. Thus, our data suggest that FAK controls the efficiency and timing of CNS myelination during its initial stages, at least in part, by regulating oligodendrocyte process outgrowth and/or remodeling. © 2009 Wiley-Liss, Inc.

Key words: oligodendrocyte; myelination; FAK; ECM molecules; process outgrowth

During development of the central nervous system (CNS), differentiation of oligodendrocytes, the myelinating cells, and the process of active myelination itself are regulated by complex interactions of the oligodendrocytes’ cell surfaces with their extracellular environment (Lubetzki-Korn et al., 1983; Fridman et al., 1985; Notterpek and Rome, 1994; Oh and Yong, 1996; Buttery and ffrench-Constant, 1999; Szuchet et al., 2000; Siskova et al., 2006; Colognato et al., 2007). These interactions are mediated to a large extent by the integrin class of extracellular matrix (ECM) receptors (Malek-Hedayat and Rome, 1994; Milner and ffrench-Constant, 1994; Frost et al., 1999; Relvas et al., 2001; Colognato et al., 2002, 2004; Gudz et al., 2002; Baron et al., 2005; Olsen and ffrench-Constant, 2005; Benninger et al., 2006; Lee et al., 2006). In support of integrin–ECM signaling playing a pivotal role in the regulation of oligodendrocyte differentiation and CNS myelination, signaling molecules that are effectors in integrin-mediated signaling cascades have also been implicated in these processes (Chun et al., 2003; Fox et al., 2004; Liang et al., 2004; Hoshina et al., 2007; Sloane and Vartanian, 2007).

One of the main regulators of integrin–ECM signaling is focal adhesion kinase (FAK). FAK, also known as protein tyrosine kinase 2, is a ubiquitously expressed nonreceptor protein tyrosine kinase that can be activated by a number of extracellular signals (Hanks et al., 1992; Schaller et al., 1992; Schlaepfer et al., 1999; Parsons, 2003; Mitra et al., 2005; Mitra and Schlaepfer, 2006). FAK has been found to be expressed in cells of the oligodendrocyte lineage and is present in myelin (Kilpatrick et al., 2000; Bacon et al., 2007). Interestingly, phosphorylation of FAK at its Tyr997 site, which represents a critical event for its activation and biological effects, has been described as occurring primarily in postmitigatory differentiating oligodendrocytes and not in migratory oligodendrocyte progenitor cells (Liang et al., 2004). Furthermore, additional phosphorylation events of FAK tyrosine residues regulate its overall function (Schlaepfer and Hunter, 1996; Hanks and Polte, 1997; Cohen and Guan, 2005). Phosphorylation of one of these residues, namely, the Tyr925 residue, has been found to be
Ubiquitous FAK knockout is early embryonically lethal because of general mesodermal defects (Furuta et al., 1995; Ilic et al., 1995a, 1995b). Therefore, to investigate the potential role of FAK in the regulation of oligodendrocyte maturation and/or CNS myelination, we generated oligodendrocyte-specific and inducible FAK-knockout mice using the Cre-loxP system (Fak\textsuperscript{loxP/loxP}; PLP/Cre\textsuperscript{ERT\textsuperscript{T}} mice). Our results revealed that when inducing FAK knockout in these mice just prior to and during the initial stages of myelination of the optic nerve, myelination was reduced on postnatal day 14 (P14). In addition, our data showed that the induction of FAK knockout resulted in a reduced number of primary oligodendrocyte processes at this developmental age. However, this phenotype appears to be transient because the number of myelinated fibers on postnatal day 28 (P28) was comparable under both control and knockout conditions. Taken together, these data demonstrate that FAK is involved in regulating the efficiency and timing of myelination in its initial stages and suggest that this regulatory role may involve the control of oligodendrocyte process outgrowth and/or remodeling.

**MATERIALS AND METHODS**

**Animals and Induction of FAK Knockout**

Mice in which the second kinase domain exon of Fak is flanked by loxP sites (Fak\textsuperscript{loxP/loxP} mice) were bred to mice that express, under the control of the proteolipid protein (PLP) promoter, Cre recombinase fused to a tamoxifen-inducible mutated ligand-binding domain of the human estrogen receptor (PLP/Cre\textsuperscript{ERT\textsuperscript{T}} mice), kindly provided by B. Popko, University of Chicago; Beggs et al., 2003; Doerflinger et al., 2003). Both strains are on a C56BL/6 genetic background. Litters used for the present study were derived from males homozygous for the floxed Fak locus and heterozygous for the PLP/Cre\textsuperscript{ERT\textsuperscript{T}} locus and females homozygous for the floxed Fak locus but negative for the PLP/Cre\textsuperscript{ERT\textsuperscript{T}} locus. Thus, knockout and control mice were derived from the same breeding pairs. To induce FAK knockout, 300-μL intraperitoneal injections of 3 mg of tamoxifen or vehicle (sunflower oil) were administered daily into lactating mothers from postnatal day 2 (P2) through P12, with P0 referring to the day of birth (Leone et al., 2003). Animals were analyzed at P14 and P28. To confirm successful Cre-mediated recombination under the above-described conditions, PLP/Cre\textsuperscript{ERT\textsuperscript{T}} mice were additionally bred to Gt(ROSA)26Sor\textsuperscript{tm1(EYFP)Cos}; PLP/Cre\textsuperscript{ERT\textsuperscript{T}} mice, which were treated as described above and analyzed at P14. All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

**PCR Analysis**

For genotype analysis, DNA was extracted from tail clips using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). For analysis of recombination efficiency upon tamoxifen treatment, optic nerves were dissected from P14 animals, and DNA was extracted as described above. Polymerase chain reaction (PCR) was performed using a Taq PCR Core Kit (Qiagen, Valencia, CA) and a PTC-200 DNA Engine Cycler (MJ Research, Waltham, MA). The following primer pairs were used at the indicated annealing temperatures: Cre\textsuperscript{ERT\textsuperscript{T}} forward (5’-GATGTAAGCCAGCAGCATGTC-3’) and Cre\textsuperscript{ERT\textsuperscript{R}} reverse (5’-ACTATACCTGAACCTTGAT-3’) at 50°C and FAK-flox-P1 (5’-GCCCTCAACTTCTCATTTCCTC-3’) and FAK-flox-P2 (5’-GAATGCTACAGGAACAAAATAC-3’) at 55°C. Custom oligonucleotides were obtained from MWG-Biotech (Huntsville, AL). The PCR cycling conditions were: 2 min at 94°C, followed by 35 cycles of amplification (45 sec at 94°C; 1 min, 30 sec at the respective annealing temperature; and 45 sec at 72°C) and 4 min of extension at 72°C. Amplified DNA was analyzed using agarose gel electrophoresis and the VersaDoc 4000 imaging system (Bio-Rad, Hercules, CA).

**Immunohistochemistry**

Immunohistochemistry was performed on longitudinal sections of optic nerves dissected from tamoxifen-treated P14 Gt(ROSA)26Sor\textsuperscript{tm1(EYFP)Cos}; PLP/Cre\textsuperscript{ERT\textsuperscript{T}} mice. Mice were deeply anesthetized, transcardially perfused with 4% paraformaldehyde in 0.1M Millonig’s buffer (150 mM sodium phosphate monobasic/100mM sodium hydroxide) and postfixed for 24 hr. Optic nerves were removed, cryoprotected in 30% sucrose/PBS, embedded in Tissue-Tek on dry ice, and 10-μm sections were then cut on a Shandon SME Cryotome (Thermo Scientific, Philadelphia, PA). Sections were immunolabeled after a permeabilization step in ice-cold acetone (Dupree et al., 1999) using the following antibodies: mouse monoclonal anti-APC/CC1 (EMD/Caibiochem, Gibbstown, NJ), rabbit polyclonal anti-GFP/YFP (Millipore, Temecula, CA), and secondary Alexa Fluor 594–conjugated donkey anti-mouse IgG and Alexa Fluor 488–conjugated goat anti-rabbit IgG (Invitrogen/Molecular Probes, Carlsbad, CA). Sections were analyzed by confocal microscopy using a Leica TCS SP2 AOBS System (Leica Microsystems Inc., Bannockburn, IL).

**Light and Electron Microscopic Analysis**

P14 and P28 mice treated with either tamoxifen or sunflower oil were deeply anesthetized and transcardially perfused with 4% paraformaldehyde/2.5% glutaraldehyde in 0.1M Millonig’s buffer (Dupree et al., 1998; Marcus et al., 2006). Mice were postfixed for 1–2 weeks in aldehyde fixative. Optic nerves were dissected out and incubated for 1 hr in 1% osmium tetroxide/0.1M Na-cacodylate buffer (Electron Microscopy Sciences, Ft. Washington, PA). Specimens were
dehydrated with ethanol and embedded in Epon resin (Electron Microscopy Sciences, Ft. Washington, PA).

For light microscopic analysis, semithin (1-μm) transverse sections were taken every 1 mm throughout the length of the optic nerve, stained with toluidine blue, and imaged using a Nikon ECLIPSE E800M microscope equipped with a Spot RT CCD camera (Nikon Inc., Melville, NY). The number of myelinated fibers was determined in a blinded fashion for each entire transverse section. The average number for the control sections was set to 100%, and each value was calculated accordingly. Statistical significance was determined using the Student’s t test.

For electron microscopic analysis, ultrathin transverse sections (90 nm) were taken 1 mm from the lamina cribrosa and at 1-mm intervals along the length of the optic nerve. Sections were collected on Formvar-coated slotted grids and stained with uranyl acetate and lead citrate. Images were taken using a JEOL JEM1230 transmission electron microscope equipped with an Ultrascan 4000 Gatan CCD camera.

RESULTS

Administration of Tamoxifen to Lactating Females Induces Cre-Mediated Recombination in the Optic Nerve and in Cells of Oligodendrocyte Lineage in Early Postnatal Offspring Containing the PLP/CreERT<sup>+</sup> Locus

To determine the in vivo role of FAK in differentiating oligodendrocytes during the developmental period of myelination, spatially and temporally controlled transgenic FAK-knockout mice were generated using an inducible Cre–loxP system. More specifically, mice in which the second kinase domain exon of Fak is flanked by loxP sites (Fak<sup>lox/lox</sup> mice; Fig. 1B) were bred to mice that express, under the control of the PLP promoter, Cre recombinase fused to a tamoxifen-inducible mutated ligand binding domain of the human estrogen receptor (PLP/CreER<sup>T</sup> mice; Fig. 1B; Beggs et al., 2003; Doerflinger et al., 2003). Because of the breeding strategy used (see Materials and Methods section), all mice analyzed for the potential effects of FAK-knockout induction were homozygous for the floxed Fak locus and either heterozygous for the PLP/CreER<sup>T</sup> locus (Fak<sup>lox/lox</sup>:PLP/CreER<sup>T</sup> mice) or lacking the PLP/CreER<sup>T</sup> locus (Fak<sup>lox/lox</sup> littermate controls). It has been previously established that only upon administration of tamoxifen is CreER<sup>T</sup> translocated from the cytoplasm to the nucleus, where it is able to catalyze recombination at loxP sites (Leone et al., 2003). In addition, it has been shown that the PLP promoter used for the generation of the PLP/CreER<sup>T</sup> mice is well suited to direct transgene expression to differentiating oligodendrocytes (Wight et al., 1993; Fuss et al., 2000, 2001; Doerflinger et al., 2003). Thus, the use of the above-described Fak<sup>lox/lox</sup>:PLP/CreER<sup>T</sup> mice allowed for design strategies in which to induce Cre-mediated recombination and thus FAK knockout specifically in differentiating oligodendrocytes and during the developmental period of active myelination. Because of the region-specific timing of active myelination in the CNS, however, individual knockout, that is, tamoxifen injection, strategies need to be employed for specific CNS regions (Caley and Maxwell, 1968; Foran and Peterson, 1992; Leone et al., 2003; Thomson et al., 2005).

For the study presented here, we chose the optic nerve as the anatomical region of interest because of its relatively simple morphology and the well-described chronology of oligodendrocyte differentiation and myelination (Skoff et al., 1976a, 1976b, 1980; Tennekoon et al., 1977; Hildebrand and Waxman, 1984; Hunter and Bedi, 1986; Butt and Ransom, 1993; Colello et al., 1995; Thomson et al., 2005). In the optic nerve of developing C57Bl/6 mice, that is, animals of genetic background similar to that of the Fak<sup>lox/lox</sup>:PLP/CreER<sup>T</sup> mice, both oligodendrocyte differentiation and myelination proceed from the retinal to the chiasmatic end of the nerve (Thomson et al., 2005). First axonal contact of oligodendrocyte processes can be seen at the retinal end around postnatal day 6 (P6). At this point, oligodendrocytes also begin to express the PLP isoform of PLP/DM20 and are thus considered to represent a myelinating stage of the lineage (Tennekoon et al., 1977; Trapp et al., 1997; Thomson et al., 2005). At the chiasmatic end, such initially myelinating cells are first observed at P8. Mature, that is, compacted, myelin can be found extended over the whole length of the nerve around P15 (Foran and Peterson, 1992). Thus, to induce Cre-mediated recombination in the optic nerve just prior to and during the developmental period of active myelination, tamoxifen or sunflower oil as vehicle control were administered daily to lactating mothers from 2 to 12 days postpartum, and animals were analyzed during the active stages of myelination, that is, at P14, and at a mature stage, that is, at P28 (Fig. 1A). Successful Cre-mediated recombination at the Fak locus was assessed by PCR analysis (Fig. 1C). Recombination could be observed as early as P4 (data not shown). At this point, oligodendrocytes in the optic nerve for the most part are at a progenitor stage, at which they express low levels of FAK (Liang et al., 2004). Thus, even in a case of a relatively stable FAK protein, FAK protein levels will be reduced upon FAK-knockout induction because it inhibits the normal developmental up-regulation of FAK expression. At P14, approximately 40% of the cells in the optic nerve are oligodendrocytes (Barres et al., 1992). Thus, the recombination efficiency of approximately 40% observed in our studies (Fig. 1C) suggests efficient Cre-mediated recombination in cells of the oligodendrocyte lineage, at least at this developmental age. In the other cell types of the optic nerve, which are primarily astrocytes, the PLP promoter is not operative, and thus recombination cannot be induced upon administration of tamoxifen. To further confirm successful Cre-mediated recombination in cells of the oligodendrocyte lineage under the conditions depicted in Figure 1A, PLP/CreER<sup>T</sup> mice were bred to Gt(Rosa)26Sor<sup>tm1(EYFP)Cos</sup> reporter mice, in which expression of EYFP is blocked by an upstream loxP-flanked STOP sequence and only
induced upon successful Cre-mediated recombination (Srinivas et al., 2001). As a marker for cells of the oligodendrocyte lineage, an antibody to cytoplasmic APC/CC1 was used (Bhat et al., 1996; Fuss et al., 2000). As shown in Figure 1D, EYFP expression could easily be detected in APC/CC1-positive cells of the optic nerve of a tamoxifen-treated P14 Gt(Rosa)26Sor1(EYFP)CreERTT mouse. Taken together, the above data confirm that under the conditions depicted in Figure 1A, Cre-mediated recombination at loxP sites occurs efficiently in the optic nerve and in cells of the oligodendrocyte lineage during early postnatal development (P4–P14) of offspring containing the PLP/CreERTT locus.

At P14, the Number of Myelinated Axons is Decreased in Optic Nerves of Tamoxifen-Treated Fakbox/flox:PLP/CreERTT Mice

To assess the extent to which FAK may regulate developmental myelination, we determined the number of myelinated fibers in tamoxifen-treated Fakbox/flox:PLP/CreERTT and Fakbox/flox mice at P14. Primers P1 and P2 depicted in A were used, and the amount of each amplification product was determined using the VersaDoc 4000 imaging system (Bio-Rad, Hercules, CA). Bar graph depicts means ± SEM (n = 3 per genotype); *, statistically significant as determined by Student’s t test. D: Representative confocal images of P14 optic nerve sections taken from tamoxifen-treated Gt(Rosa)26Sor1(EYFP)CreERTT, PLP-CreERTT mice after double-labeling for APC/CC1 and YFP. Nuclei were stained using Hoechst. Images depict single channel representations of a single optical section (approximately 0.2-μm x-z resolution). Scale bar = 10 μm.
microscopy (Fig. 2). To eliminate the possibility that varying levels of myelination along the length of the optic nerve affect the outcome of our analysis, we examined sections taken at 1-mm intervals along the whole length of the nerve (Skoff et al., 1980). At all levels, the number of myelinated fibers was found to be reduced in the Fak^{floxflox}:PLP/CreERT mice compared with their Fak^{floxflox} litter mates (see example in Fig. 2A). When averaging the number of myelinated fibers for all levels, these were found to be significantly decreased, by more than 30%, in Fak^{floxflox}:PLP/CreERT mice compared to Fak^{floxflox} mice (Fig. 2B,C). No such differences were noted when analyzing vehicle-treated Fak^{floxflox}:PLP/CreERT and Fak^{floxflox} mice (data not shown).

To determine the resolution of our type of analysis, we compared semithin sections (light microscopy) with consecutive ultrathin sections (electron microscopy). As shown in Figure 3, such analysis revealed that visualizing transverse sections of the optic nerve using light microscopy allowed myelinated axons enwrapped with as few as four layers of myelin to be distinguished. Thus, our data demonstrate that FAK is involved in the regulation of the initial steps of myelination and/or myelin wrapping.

At P14, the Number of Primary Oligodendrocyte Processes is Reduced in Optic Nerves of Tamoxifen-Treated Fak^{floxflox}:PLP/CreERT Mice

To further investigate the above role of FAK during developmental myelination of the optic nerve, we determined the total number of cells in the semithin 1-μm transverse sections taken at 1-mm intervals (measured from the lamina cribrosa). This analysis revealed no differences between P14 Fak^{floxflox}:PLP/CreERT mice and their Fak^{floxflox} littermates (Fig. 4A), suggesting that the effect of FAK-knockout induction on myelination may not be a result of changes in the number of oligodendrocytes. However, when analyzing the number of primary processes, that is, processes that directly extend from oligodendrocyte cell bodies, a significant decrease, of approximately 30%, was observed in P14 Fak^{floxflox}:PLP/CreERT optic nerves compared to optic nerves from P14 Fak^{floxflox} littermates (Fig. 4B–D). Oligodendrocyte cell bodies can be easily distinguished from cell bodies of the major other cell type found in the optic nerve, namely, astrocytes, as astrocytes exhibit abundant glycogen granules and contain intermediate filaments. Thus, we are confident that to a large extent, our analysis was restricted to oligodendrocytes. Taken together, the above data therefore suggest that the decrease in the number of myelinated fibers seen upon induction of FAK knockout is a result, at least in part, of impaired oligodendrocyte process outgrowth and/or remodeling.

At P28, the Number of Myelinated Axons within Optic Nerves is Comparable Between Tamoxifen-Treated Fak^{floxflox} and Fak^{floxflox}:PLP/CreERT Mice

To assess the extent to which the above-observed effects of FAK-knockout induction during periods of active myelination are persistent into adulthood, we determined the number of myelinated axons at P28. At this age, the number of myelinated axons in the optic nerve was found to be comparable between Fak^{floxflox} and Fak^{floxflox}:PLP/CreERT mice.
and FafLOX/flox:PLP/CreERT mice (Fig. 5). These data suggest that the role of FAK may be of particular importance for the efficiency and timing of the initial stages of myelination.

**DISCUSSION**

In the present study, we have demonstrated that induction of FAK knockout just prior to and during active stages of myelination results in hypomyelination in the optic nerve at a time-point when normally myelinated fibers can be found extended throughout the whole length of the nerve, that is, at P14. Furthermore, our data suggest that this effect of FAK-knockout induction is a result at least in part of reduced outgrowth and/or impaired remodeling of primary oligodendrocyte processes. However, myelination appears to have reached normal levels by P28. Thus, our data suggest that in vivo in the optic nerve, FAK promotes efficient and properly timed myelination during the active phases of myelin sheath formation.
neurons and oligodendrocytes (Beggs et al., 2003; Falk 2007). However, future studies will be necessary to assess such a potentially important role of Pyk2 in myelination.

Taken together, our data suggest that FAK promotes efficient and properly timed myelination in the optic nerve, where it likely acts as an effector of integrin signaling activated by oligodendrocyte–ECM interactions. Our data further suggest that this signaling event promotes process outgrowth and potentially remodeling during the initial stages of myelination. Impairment of these steps of oligodendrocyte maturation appears to be responsible for, at least in part, the limited repair of the myelin sheath seen in lesions of patients suffering from the major demyelinating disease in humans: multiple sclerosis (Chang et al., 2002; Franklin and ffrench-Constant, 2008; Kuhlmann et al., 2008). Thus, increasing the understanding of the role of FAK in CNS myelination not only furthers our understanding of normal CNS development but may also reveal novel targets suitable to stimulate remyelination under pathological demyelinating conditions.

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