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The role of β1 Integrin in Myelination and Remyelination
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The role of β1 integrin in myelination and remyelination

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for a Ph.D. degree in Cellular and Molecular Medicine

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Abstract

Myelination is a process of cellular specialization, where oligodendrocytes in the central nervous system (CNS) undergo maturation to form the myelin sheath. Proper formation of the myelin sheath is an important process as it is responsible for rapid saltatory conduction along nerves and is required for the survival of the axons. Multiple Sclerosis (MS) is a disease that can be characterized by the degeneration of the myelin sheath within the CNS and the inability of the myelin sheath to reform. The damage or loss of myelin results in the disruption of impulses along nerve axons and ultimately leads to neurological symptoms. Therefore, it is critical to determine how oligodendrocytes develop and myelinate axons, and it is important we learn how to use this information to repair the lost or damaged myelin. Research is advancing our understanding of the various factors involved in oligodendrocyte development. In particular, much research has shown the importance of β1 integrin in oligodendrocyte biology and in myelin membrane formation.

We are the first to demonstrate *in vivo* that β1 integrin is important in myelination and remyelination. With the use of the proteolipid protein promoter (PLP), we generated a transgenic mouse model that expresses the β1 integrin subunit minus the cytoplasmic domain. This dominant-negative β1 integrin mouse model is characterized by myelin defects in the optic nerve and spinal cord. Under the cuprizone-induced demyelination/remyelination model, the dominant-negative β1 integrin mouse has delayed or impaired remyelination. We also show that the mitogen-activated protein kinase (MAPK) pathway appears to be important in β1 integrin signaling during myelination and remyelination.

Further analysis of the dominant-negative mouse model permitted us to get a better understanding of the role of β1 integrin in oligodendrocyte development during remyelination. Our work demonstrates that β1 integrin is important in the survival of oligodendrocytes acting primarily through the MAPK signaling pathway.

Through the use of the dominant-negative β1 integrin mouse model, we demonstrated *in vivo* the importance of β1 integrin in myelination and remyelination, we furthered our understanding of β1 integrin's role in the survival of oligodendrocyte development, and lastly we determined downstream signaling pathways that may contribute to the function of β1 integrin in myelination and remyelination.

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List of abbreviations

β1 integrin ΔC: β1 integrin lacking the cytoplasmic domain

BDNF: brain derived neurotrophic factor

BrdU: 5-bromo-2-deoxyuridine

BSA: bovine serum albumin

CC-1: adenomatous polyposis coli

Cdk: cyclin-dependent kinase

CG4: central glia

CNP: 2'-3'-cyclin nucleotide 3'-phosphohydrolase

CNTF: ciliary-neurotrophic factor

CNS: central nervous system

DG: dystroglycan

DMEM: Dulbecco's Modification Eagle's Medium

dy/dy: *dystrophia muscularis*

ECM: extracellular matrix

FAK: focal adhesion kinase

FGF-2: fibroblast growth factor-2

FL: full length

GalC: galactocerebroside C

Id4: inhibitor of differentiation protein 4

IGF: insulin growth factor

ILK: integrin-linked kinase

MAG: myelin associated glycoprotein

MBP: myelin basic protein

MOG: myelin oligodendrocyte glycoprotein

MS: Multiple Sclerosis

NRG: neuregulins

NT-3: neutrophin-3

OPC: oligodendrocyte precursor cell

OSP: oligodendrocyte specific protein

PFA: paraformaldehyde

PLP: proteolipid protein

PDGF: platelet-derived growth factor

PNS: peripheral nervous system

POA: proligodendroblast antigen

RRMS: Relapsing Remitting Multiple Sclerosis

RT-PCR: reverse-transcriptase polymerase chain reaction

TH: thyroid hormone

TUNEL: terminal deoxynucleotidyl transferase

WAVE1: Wiskott-Aldrich syndrome protein 1

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Chapter 1. General Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory demyelinating disease that affects 1 in 500 Canadians, with approximately 3 people being diagnosed daily. MS can occur at any age, however in the majority of cases, the disease is diagnosed between the ages of 15-40, during the prime years of an individual. The disease is characterized by the degeneration of the myelin sheath within the central nervous system (CNS) and the failure in the formation of new myelin sheaths. The damage and loss of myelin results in the disruption of nerve impulses along nerve axons and ultimately leads to neurological symptoms such as visual disturbance, extreme fatigue, loss of coordination and the possibility of partial or complete paralysis.

There a several types of MS which they can be categorized into 3 groups depending on the ability to recover from the disease. Clinically isolated syndrome is the earliest form of MS and consists of a single attack of neurological symptoms. Relapsing-Remitting MS (RRMS), the most common form of MS, is characterized by clearly defined attacks followed by partial recovery (Figure1). Benign MS, a subtype of RRMS, is similar to RRMS except that there is almost complete recovery after an attack, therefore, those who have benign MS have minimal disability. The last type of MS is progressive MS. It is characterized by a slow but increasing disability with or without recovery. Progressive MS can be further subdivided into primary progressive, where from the time of onset the disease continuously worsens and secondary progressive, where after an initial RRMS phase the disease will progress with or without recovery (Figure 1).

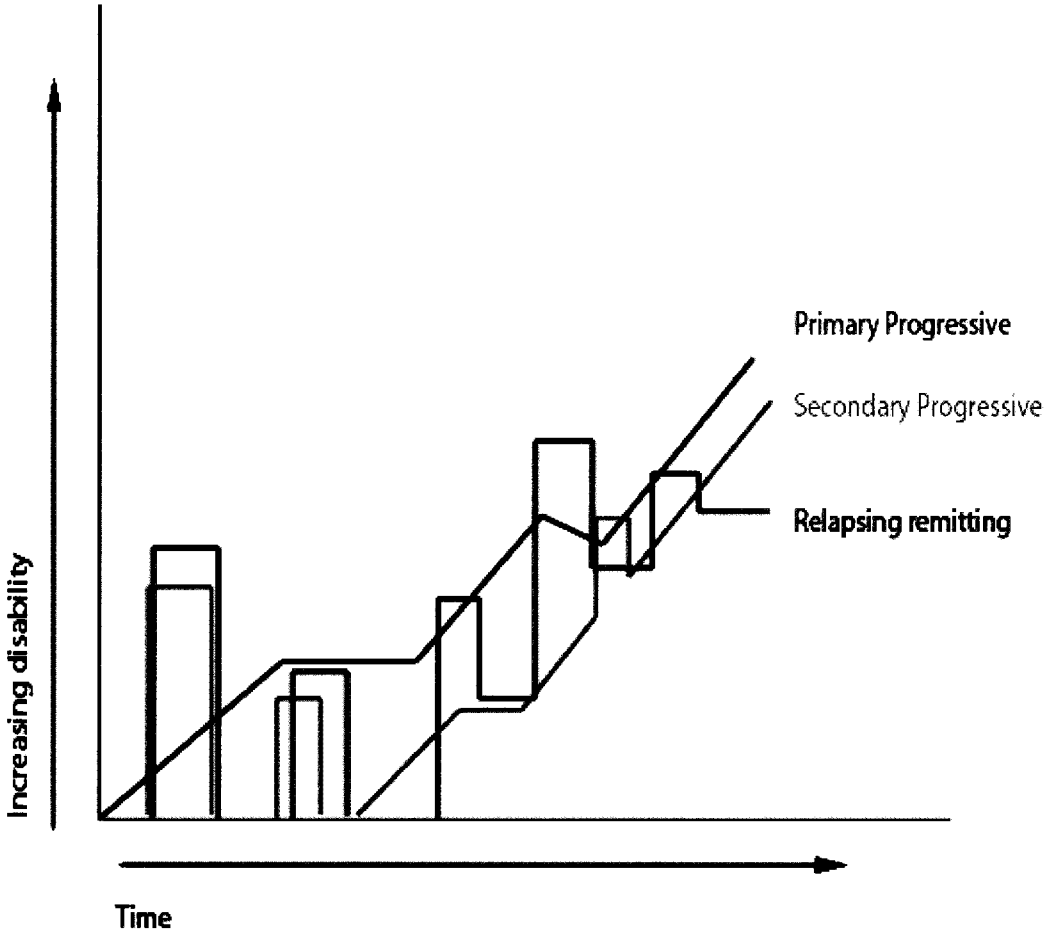

Figure 1. The disability intensity of the 3 types of MS. Relapsing Remitting MS (RRMS) is characterized by a defined attacks with partial recovery. Primary progressive MS is characterized by increasing disability over time. Secondary progressive MS is characterized by an intial phase similar to RRMS, then followed by increasing diability with or without recovery. Adapted from the www.mssociety.ca.

MS is typically categorized as a disease that affects young adults. However, over the past few years more attention has been brought to pediatric MS. Studies are emerging that identify 2.7-5% of individuals with MS are diagnosed prior to their eighteenth birthday. Children as young as three years of age have been diagnosed with MS. A number of adults that suffer from RRMS reveal that they may have experienced similar symptoms early in childhood or adolescence, indicating that MS may manifest earlier than diagnosed. Therefore, the window of diagnosing children with MS may be missed until the disease further reveals itself in adulthood. However, with further awareness of pediatric MS, there will most likely be an increase in the number of children diagnosed with MS.

The cause of MS is still unknown. It is speculated that individuals may have a genetic disposition to MS and that a common virus may trigger the disease. However, there has yet to be any evidence indicating that MS is a genetically inherited disease. It is noted that MS is prevalent in areas further away from the equator, therefore suggesting that environmental factors may also play a role in causing MS. The recent suggestions of environmental contribution to MS have brought to light the role of vitamin D in prevention of MS, however that is an area that has to be further explored (Freedman et al. 2000; Kurtzke 2000; Raghuwanshi et al. 2008). Currently, there are several treatments for MS that are available but they only slow disease progression and are by no means a cure for MS. Therefore, in the absence of a cure for MS, there are two important areas that must be further understood to obtain effective treatments: inhibition of further degradation of myelin, and regeneration of myelin in damaged areas. To facilitate this,

we must garner a better understanding of the molecular mechanisms that initiate and regulate myelination.

1.2 Myelin

The ability of the vertebrate nervous system to communicate and integrate function requires rapid nerve impulse conduction. This is accomplished by the myelin forming glial cells. In the CNS, myelin is produced by oligodendrocytes while in the peripheral nervous system (PNS) myelin is produced by Schwann cells. The myelin sheath is arranged in concentric bimolecular layers of lipids encircled between protein layers, with a composition of 30% protein and 70% lipid that has a high concentration of cholesterol and phospholipids (Pfeiffer et al. 1993). The PNS and CNS are dependent on the myelin forming at regular, discontinuous sites along the axons, leaving areas known as Nodes of Ranvier that are not myelinated. The internodal insulating presence of myelin along the axons facilitates the rapid firing of saltatory conduction from node to node.

In the PNS, each Schwann cell myelinates a single axon. This is in direct contrast to the CNS, where oligodendrocytes are multipolar cells that have the ability to myelinate multiple axons. Although the myelin of the CNS and PNS may have similar function, their myelin sheaths have distinct differences. During neural tube formation, the CNS undergoes regionalization, while neural crest cells differentiated from the neural tube have already migrated to specific regions and begun the formation of the PNS (Liu, 2004)(Le Douarin, 1986). In the PNS of rodents, myelination occurs immediately following birth, while in the CNS the onset of myelination varies between embryogenesis and 2 weeks postnatal, depending on the region of CNS (Schwab and Schnell 1989).

Structurally the CNS and PNS are similar in their myelin spiraling around axons in multiple layers. However, in the CNS the myelin is composed of the tight junction protein, claudin-11/oligodendroycte-specific protein (OSP), whereas the Schwann cells in the PNS are composed of a basal lamina and microvilli (Arroyo and Scherer 2000). Neuregulins (NRG) play a central role in the development and survival of Schwann cells at various stages (Meyer and Birchmeier 1995; Taveggia et al. 2005). NRG have also been demonstrated to play a role in oligodendrocyte development, however in the CNS there are many other growth factors that also play a role (Vartanian et al. 1999). Therefore, the question lies in what factors are responsible for the formation of myelin in the CNS?

1.3 CNS Myelination

Oligodendrocyte development can be defined as a two stage process. The first stage, oligodendrogenesis, involves the proliferation, migration and commencement of differentiation of the oligodendrocyte precursor cells (OPCs), while the second stage involves survival, differentiation and myelin membrane formation. The specification of OPCs occurs during late embryonic development from neuroepithelial cells. Following commitment, OPCs undergo extensive proliferation as they migrate away from the ventricular and/or subventricular zones towards axons. As OPCs begin to mature, there is a commencement of morphological change and expression of various antigens depending on the stage of oligodendrocyte maturity. Early OPCs have a bipolar morphology and express cell surface antigens, galactosulfatide and proligodendroblast antigen (POA), which are recognized by the O4 antibody (Bansal and Pfeiffer 1992; Pfeiffer et al. 1993).

Late OPCs are less motile, have a multiprocess morphology, and can be identified through their expression of NG2 proteogylcan antigen (Figure 2)(Fok-Seang and Miller 1994; Nishiyama et al. 1996).

1.3.1 The requirement of growth factors for proliferation of OPCs

The ability of OPCs to develop into mature oligodendrocytes is dependent on growth factors. Many growth factors and trophic factors have been implicated in promoting OPC survival and proliferation, such as platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), insulin growth factor (IGF), neutrophin-3 (NT-3) and brain derived neurotrophic factor (BDNF) (Figure 2)(Barres et al. 1993; Beck et al. 1995; Carson et al. 1993; McTigue et al. 1998). The astrocyte derived PDGF is one of the best characterized mitogens that is required for the stimulation of OPC proliferation and also has a role as a survival factor (Noble and Murray 1984; Noble et al. 1988; Richardson et al. 1988). The importance of PDGF was explored *in vivo*, where two opposing mouse models have identified the requirement of PDGF in the survival of oligodendrocytes. The overexpression of PDGF in transgenic mice results in the hyperproliferation of OPCs. However, upon maturation of oligodendrocytes, the numbers of oligodendrocytes are reduced to normal numbers in oligodendrogenesis (Calver et al. 1998). In contrast, the PDGF-A knock out mice exhibit defective oligodendrocyte development that results in severe hypomyelination due to a reduction in the number of OPCs (Fruttiger et al. 1999). In addition, the mitogenic effect of PDGF in OPC proliferation is especially potent when combined with FGF-2, together they are capable of prolonging the proliferative state of OPCs (Bogler et al. 1990; McKinnon et al. 1990).

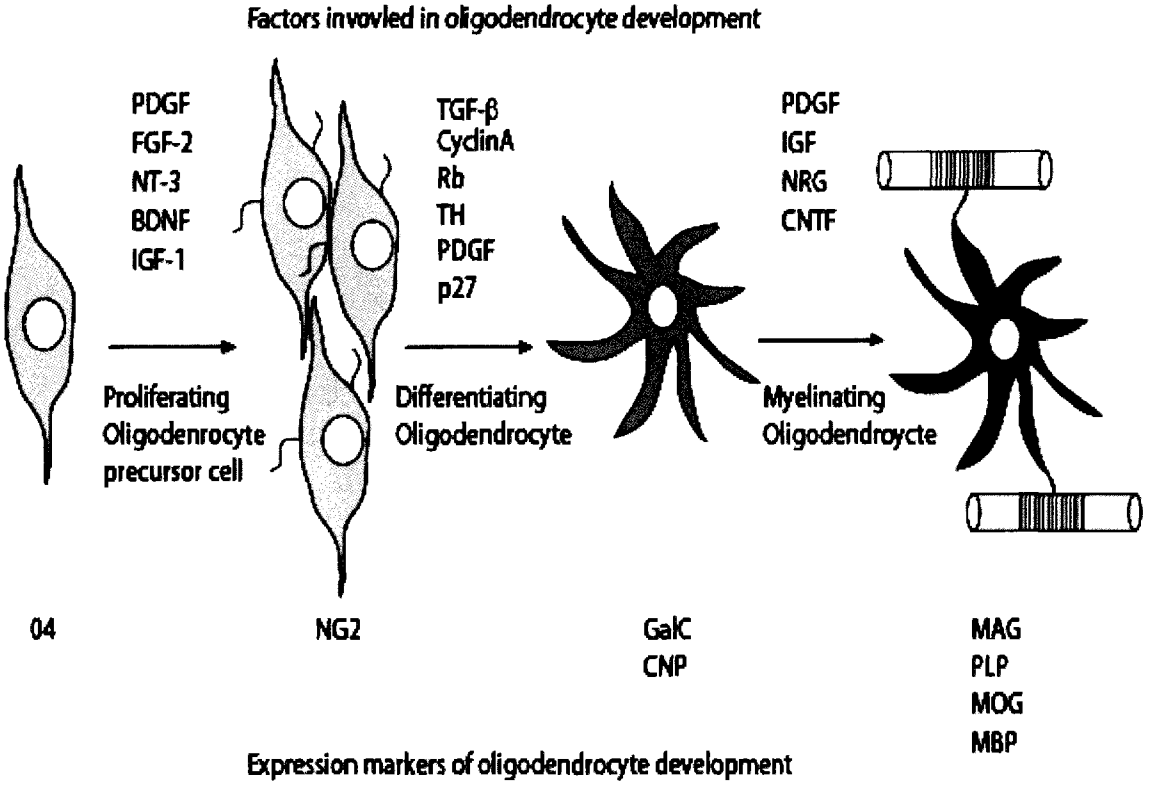

Figure 2. Schematic representation of oligodendrocyte development with expression markers of specifc stages and factors known to be involved. Early proliferative OPC have a bipolar morpholgy and have proliferative response to growth factors such as PDGF. Late proliferative OPC begin to have a multiprocess morpholgy and is recognized by NG2 antibody. Differentiation of oligodendrocyte is initiated by the accumulation of p27 and acquires an extensive network of branched processes. Myelin membrane formation is further prompted by the size of axons and the various growth factors.

The ability of oligodendrocytes to temporally control their cellular response to growth factor signaling is attributed to developmental changes in the expression of their receptors. For instance, PDGF has a single receptor expressed throughout oligodendrocyte development, but this PDGF receptor is subject to changes in expression. In particular, the PDGF receptor is downregulated at the onset of terminal differentiation, which may be attributed to the ability of FGF-2 to regulate the expression of PDGF-α receptors on early OPCs (Hart et al. 1989; McKinnon et al. 1991). Meanwhile, there are 3 forms of the FGF receptors that are expressed at various times throughout oligodendrocyte development (Bansal et al. 1996). The expression of FGF receptor-1 increases over the course of lineage maturation, FGF receptor-2 is mainly expressed by terminally differentiated oligodendrocytes, while FGF receptor-3 expression climaxes in late OPC development and declines upon further differentiation (Bansal et al. 1996). Although the FGF receptors are all responsive to the ligand FGF, the developmental regulation of their expression may allow for differences in their downstream signaling responses and therefore promote various stages of oligodendrocyte development (Bansal et al. 1996).

1.3.2 The importance of the intrinsic cell timer in differentiation

The second stage of oligodendrocyte development can be termed myelination. Upon exit from the cell cycle, OPCs become terminally differentiated and commence the formation of a network of branched processes and the synthesis of myelin lipids such as galactocerebroside C (GalC) and the expression of early myelin specific proteins, 2'-3'-cyclic nucleotide 3'-phosphohydrolase (CNP) (Figure 2). The switch from proliferating

OPC to differentiated oligodendrocyte is an important step in oligodendrocyte development. Various factors have been identified to be involved in the exit of OPCs from the cell cycle towards a differentiation pathway. Such factors include TGFβ, cyclin A and Rb (Figure 2)(McKinnon et al. 1993; Nakatsuji and Miller 2001). However, one of the intriguing aspects that have come to light is the ability of oligodendrocytes to orchestrate the switch from proliferating OPC to differentiated oligodendrocyte via an intrinsic cell clock. *In vitro* analysis demonstrated that single OPCs isolated from P7 rat optic nerve grown in individual microwells were able to divide a maximum of 8 times (Temple and Raff 1986). Interestingly, the isolated single OPCs all ceased proliferation and differentiated at the same time (Temple and Raff 1986)(Temple and Raff, 1986). Furthermore, it was determined that this timing mechanism was reliant on thyroid hormone (TH) and PDGF (Figure 2)(Barres et al. 1994). In the absence of PDGF, OPCs prematurely stopped proliferation and differentiated, while OPCs in the presence of PDGF alone would continue proliferation and failed to differentiate (Barres et al. 1994). However, with the addition of TH, OPCs would cease to proliferate and proceed to differentiate (Barres et al. 1994). These studies demonstrated the importance of TH in the transition of OPCs from the cell cycle to differentiation as dictated by the intrinsic cell clock.

The importance of the mechanisms of the cell intrinsic clock is unclear, as environmental factors have been shown to override the activity of the cell intrinsic clock (Gao et al. 1997). However, the crucial link between cell proliferation and differentiation was further elucidated with the discovery that OPCs proliferating in the presence of PDGF gradually accumulated nuclear expression of cyclin-dependent kinase (Cdk)

inhibitor p27 prior to differentiation (Figure 2)(Casaccia-Bonnefil et al. 1997; Durand et al. 1997). Interestingly, proliferation ceased when the amount of p27 expression reached a peak and at a time when TH was present, however, in the absence of TH, the OPCs would continue to proliferate even with the accumulation of p27 (Durand et al. 1997). Furthermore, in the presence of both PDGF and TH, OPCs deficient in p27 are more susceptible to mitogenic effects than normal OPCs prior to differentiation, although their cell cycle times were the same (Durand et al. 1998). Therefore, this work showed the importance of p27 as part of the intrinsic timer in cell cycle withdrawal and onset of differentiation. Currently, there are other proteins that have been identified to be components of the OPC intrinsic timer, such as p18 and inhibitor of differentiation protein 4 (Id4) (Kondo and Raff 2000; Tokumoto et al. 2002). Although, there has been progress in identifying components of the OPC intrinsic timer, how the system is controlled remains to be understood.

1.3.3 Maintenance/survival of oligodendrocytes

The final number of oligodendrocytes in the various CNS regions is subject to the survival and differentiation of OPCs. Previous studies have suggested that a density-dependent mechanism could allow for oligodendrocytes to inhibit the expansion of OPCs (Ueda et al. 1999; Zhang and Miller 1996). However, it appears that the key process to control oligodendrocyte numbers is the ability of oligodendrocytes to control cell death. PDGF and IGF are survival factors that rescue newly differentiated oligodendrocytes from cell death; the addition of PDGF diminished cell death in the postnatal optic nerve (Figure 2)(Barres et al. 1992). In the majority of CNS regions, cell death of

oligodendrocytes is typical with over 50% of the newly differentiated oligodendrocytes dying during development due to competition for limiting amounts of PDGF, IGF and ciliary-neurotrophic factor (CNTF) (Figure 2)(Barres et al. 1992; Barres et al. 1993). The oligodendrocyte competition for growth factors would ensure that the number of oligodendrocytes in the CNS is directly matched to the number of axons that are in need of myelination (Barres and Raff 1999). Not only do the axons play a role in the final number of oligodendrocytes, they also provide soluble and cell mediated signals that are required in the promotion of myelinating cell development such as NRG (Vartanian et al. 1999). Once axonal contact occurs, apoptosis is prevented. In the rat optic nerve, axonal derived NRG is required to promote oligodendrocyte survival and therefore allows for the maturation of oligodendrocyte producing myelin to proceed with further morphological changes, involving myelin sheath and spiral formation around axons, and compaction of the membrane layers (Fernandez et al. 2000). The formation of the complex structure of myelin requires the co-ordination of myelin specific components such as myelin associated glycoprotein (MAG), myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) (Figure 2)(Campagnoni 1988; Dubois-Dalcq et al. 1986; Lemke 1988). The relationship between myelin and axon is an intriguing one and requires precise co-ordination for myelin formation and there is still much to be understood about their interactions.

1.4 Remyelination

Demyelination results in the loss of myelin within the CNS. The process of remyelination can form a new myelin sheath around the demyelinated axons and restore saltatory conduction. However, the process of remyelination is notably absent or delayed in diseases such as MS. Therefore, understanding the process of remyelination is an important step in finding treatments or a cure for diseases of demyelination. During oligodendrocyte development, there are a number of OPCs that do not undergo differentiation and remain as OPCs. This endogenous pool of adult OPCs remains as an evenly distributed population within the adult CNS and makes up the major dividing population in the adult CNS (Wolswijk and Noble 1989; Wolswijk et al. 1991). The adult rodent OPCs express cell surface markers like NG2 proteoglycan and PDGF-α receptor (Dawson et al. 2000; Dawson et al. 2003; Nishiyama et al. 1996). For some time, the data remained questionable whether adult OPCs are the source of oligodendrocytes for remyelination or whether mature oligodendrocytes that survive myelin insult are responsible for remyelination. Immunocytochemical examination of fresh lesions in patients with MS identified a large number of cells with oligodendrocyte-like morphology throughout the lesions (Prineas et al. 1989). This observation prompted the idea that perhaps mature oligodendrocytes had the capacity to remyelinate demyelinated areas. A number of studies have provided evidence that in areas of demyelination mature oligodendrocytes were incorporating thymidine, therefore, indicating proliferation of these cells (Ludwin and Bakker 1988). However, studies are now emerging pointing away from mature oligodendrocytes as the supply of cells for remyelination due to their apparent nondividing nature around lesions and the inability to form new oligodendrocytes for the formation of a myelin sheath (Blakemore and Keirstead 1999).

Therefore, the issue of which cells are responsible for remyelination is turning in the favor of adult OPCs. During the process of demyelination or at sites of active MS lesions, cells with early glia like morphology have been identified (Ludwin 1978). It has been demonstrated that in response to demyelination, NG2 positive cells undergo changes that involve increased immunoreactivity, changes in morphology and cell division (Levine 1994; Levine and Reynolds 1999). This indicates that adult OPCs may indeed be the source of oligodendrocytes for remyelination in demyelinated areas. This is further supported by the work of Gensert and Goldman, where cycling cells in a demyelinated area within the CNS were retrovirally labeled and traced. The traced cells differentiated into mature oligodendrocytes and remyelinated the demyelinated axons (Gensert and Goldman 1997). At the same time, much work has been done in determining the ability of endogenous adult OPCs to migrate to areas of demyelination. It has been demonstrated that three days after the onset of demyelination, the density of NG2-positive cells increases (Blakemore and Keirstead 1999). Furthermore, it was shown that this increased density of NG2-positive cells was a local event, with the recruitment of remyelinating NG2-positive cells being no greater than 2 mm distance away from the site of demyelination (Blakemore and Keirstead 1999). This suggests that demyelination induces mitogenic and differentiation signaling factors that may only be allowed to function at a short distance from the area of demyelination (Blakemore and Keirstead 1999).

With increasing evidence that adult OPCs are the source of cells for remyelination, the question emerging is whether adult OPCs are recruited to the to the area of demyelination. Elegant studies performed by Ludwin et al. take advantage of the cuprizone demyelination model (Ludwin 1980). The cuprizone toxin ablates mature

oligodendrocytes in the corpus callosum of test rodents and when the toxin is removed, almost complete remyelination can occur. Ludwin et al., demonstrated that repetitive demyelination and remyelination or long term demyelination resulted in a significant decrease in the amount of remyelination, which may be linked to a depletion of the adult OPC pool that may typically be involved in remyelination (Johnson and Ludwin 1981; Ludwin 1980). However, work done by Chari et al. has demonstrated that adult OPCs are capable of repopulating areas of demyelination, while maintaining OPC levels in the surrounding areas (Chari and Blakemore 2002). This indicates that adult OPCs may have the ability to replenish their reserves following an episode of demyelination and remyelination. If this is true, could this repopulation step malfunction in those who suffer from MS or is it that those who suffer from MS are unable to remyelinate due to an increased susceptibility to myelin insult? Further understanding of the remyelination process will hopefully lead us to a better understanding of how to treat those that suffer from MS.

1.5 Extracellular Matrix

The extracellular matrix (ECM) is a complex assembly of many proteins that include collagen, fibronectin, vitronectin, elastin and laminin. The proteins of the ECM are involved in the maintenance of cytoarchitecture and within the CNS, the ECM is able to act as a scaffold for cells such as neurons and glial cells (Bandtlow and Zimmermann 2000). At the same time, the ECM regulates many aspects of cell behavior, such as cell proliferation, survival, morphology, migration and differentiation. Laminin is the most abundant protein of the ECM, with a heterotrimer composition of α, β and γ chains

(Miner and Yurchenco 2004). In the CNS, laminin expression is localized to the surface of axons (Powell et al. 1998). The importance of laminin in myelination was first identified *in vitro*, where oligodendrocytes cultured in the presence of laminins displayed enhanced myelin membrane formation when compared to oligodendrocytes grown in the presence of other ECM constituents (Buttery and ffrench-Constant 1999). Furthermore, *in vivo*, laminin-2-deficient mice exhibit CNS region specific dysmyelination and laminin deficiency in humans exhibit white matter alterations and CNS dysmyelination (Chun et al. 2003; Farina et al. 1998). Other ECM proteins have also been identified to be involved in oligodendrocyte development. Both fibronectin and vitronectin are expressed in the white matter tracts of the CNS with each ECM component involved in oligodendrocyte migration and oligodendrocyte proliferation respectively (Baron et al. 2002; Gudz et al. 2006; Neugebauer et al. 1991; Sheppard et al. 1991). Although it is appreciated that the ECM plays a critical role in myelin membrane formation, it is through integrins that these ligands transmit their signals intracellularly.

1.6 Integrins

Integrins comprise a large family of cell surface receptors that are able to integrate the interactions between the cell's exterior and interior environments. Integrins are heterodimers composed of an α and β subunit. There are approximately 18 α and 8 β subunits; together they are able to form 24 distinct heterodimers (Hynes 2002). The various αβ combinations allow for binding specificity and signaling properties. One of the unique properties of integrins is that they are able to mediate inside-out and outside-in signaling, and this is accomplished through their structural domains. Integrins consist of a

large extracellular domain, a single transmembrane domain and a small cytoplasmic domain of approximately 25-50 amino acids, with the exception of the β4 integrin subunit which has the largest cytoplasmic domain of 1088 amino acids (Figure 3).

Outside-in signaling is comprised of the α and β subunit integrin heterodimer forming a receptor to allow for ligand binding. Most integrins are able to recognize several ECM components such as fibronectin, laminin, collagen and vitronectin (Figure 3)(Liu et al. 2000). Once integrins are bound to the ECM there is an induction of an intracellular signaling cascade (Giancotti 1997). Most of the signaling occurs on the β subunit, with numerous proteins interacting at the cytoplasmic domain. Focal adhesion kinase (FAK) is the most prominent kinase that interacts with the β subunit (Schaller et al. 1995). Once FAK is activated, it can lead to the autophosphorylation and recruitment of adapter protein Shc/Grb2 and the initiation of the RAS/ERK pathway. The activation of FAK also triggers numerous pathways, which eventually induce cell motility, cytoskeleton changes, cell spreading, proliferation and survival (Figure 3).

The second half of integrin's characteristic behavior is inside-out signaling where it has been suggested that the binding of the cytoplasmic protein talin induces an integrin conformation change that allows for signals from within the cell to propagate through integrins and regulate integrin ligand binding affinity and cell adhesion (Figure 3)(Qin et al. 2004; Vinogradova et al. 2004). The two well-known integrin-cytoplasmic protein

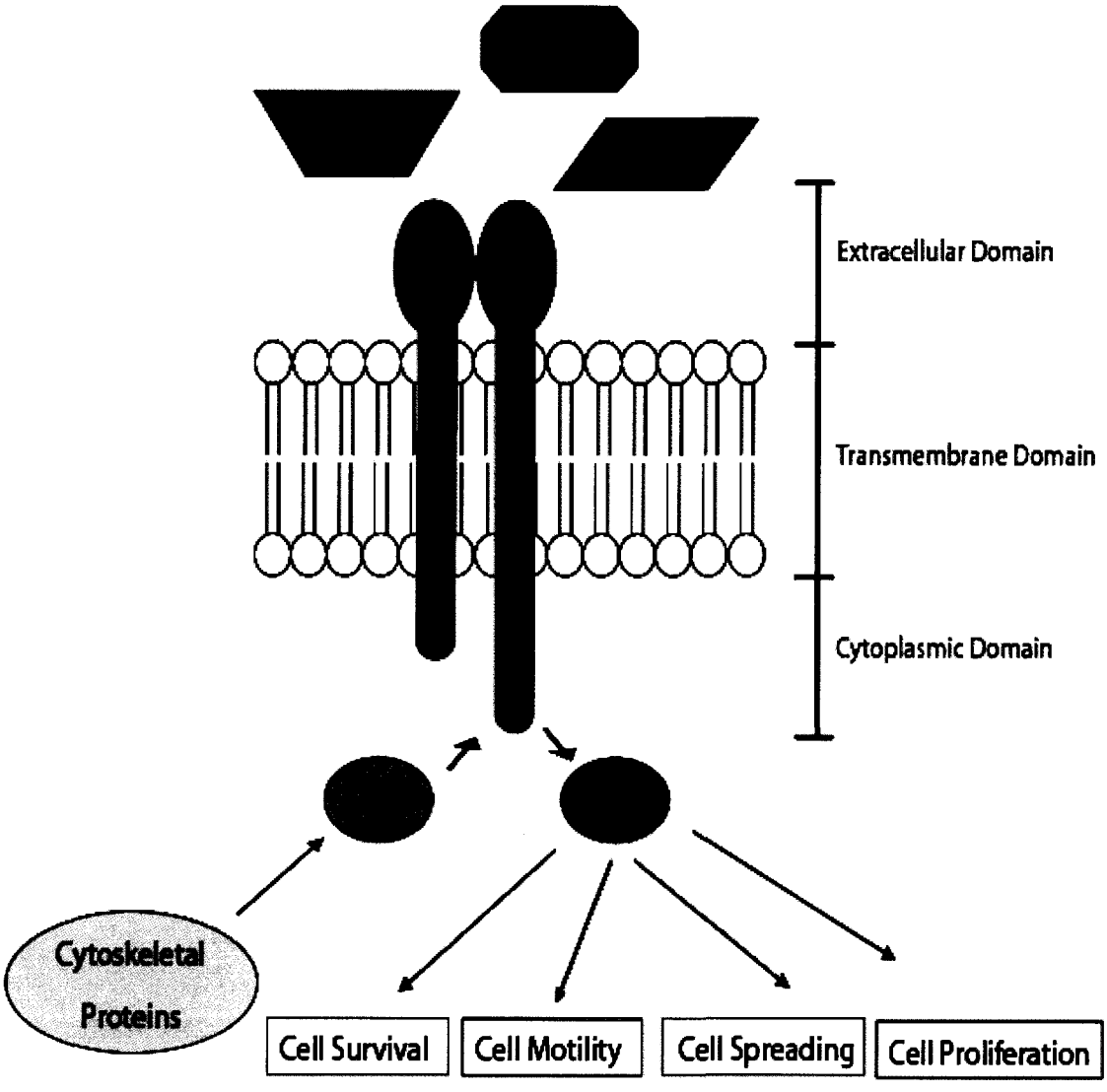

Figure 3. Schematic representation of integrin and the inside-out and outside-in signaling. Integrin is composed of an α and β subunit. Integrins bind to various ECM such as laminin,vitronectin and collagen. Once bound they are able to relay information from the outside into the interior of the cell, which allows for the phosphorylation of kinase proteins such as FAK and initiate downstream signaling pathways that leads to various cell functions. Inside-out singaling involves the binding of talin to the β subunit which alters the binding of the integrin subunits and ultimately change the ECM binding.

complexes are focal adhesions and hemidesmosomes Focal adhesions mediate adhesion to the ECM and are sites of actin filament anchoring (Burridge et al. 1990). Hemidesmosomes are sites of intermediate filament anchoring (Fuchs et al. 1997; Green and Jones 1996). The cytoplasmic domain of the integrin not only plays a structural role in anchoring within the integrin-cytoplasmic protein complexes but can also be considered as transducers since they are able to interpret information from the cell's basal environment (Chou et al. 1997).

1.6.1 Integrin function in oligodendrocytes

Recent literature has brought to light the role integrins play in oligodendrocyte behavior. OPCs and oligodendrocytes have been demonstrated to express a set of integrin heterodimers, αvβ1, αvβ3, αvβ5, αvβ8 and α6β1 throughout oligodendrocyte development (Milner and Ffrench-Constant 1994; Milner et al. 1997). The expression of αvβ1, αvβ3 and αvβ5 are sequentially upregulated and downregulated during differentiation of OPCs, while αvβ8 and α6β1 are expressed throughout oligodendrocyte development (Figure 4)(Milner and Ffrench-Constant 1994; Milner et al. 1997). The differential expression of the various integrins in oligodendrocyte development has brought forth the possibility of their varying roles in this process.

In vitro, it has been demonstrated that αvβ1 regulates oligodendrocyte migration, αvβ3 is involved in oligodendrocyte proliferation and αvβ5 regulates oligodendrocyte differentiation. Through antibody blocking experiments, the fibronectin receptor αvβ1, has been shown *in vitro* to be vital in migration (Milner et al. 1996). Interestingly, the

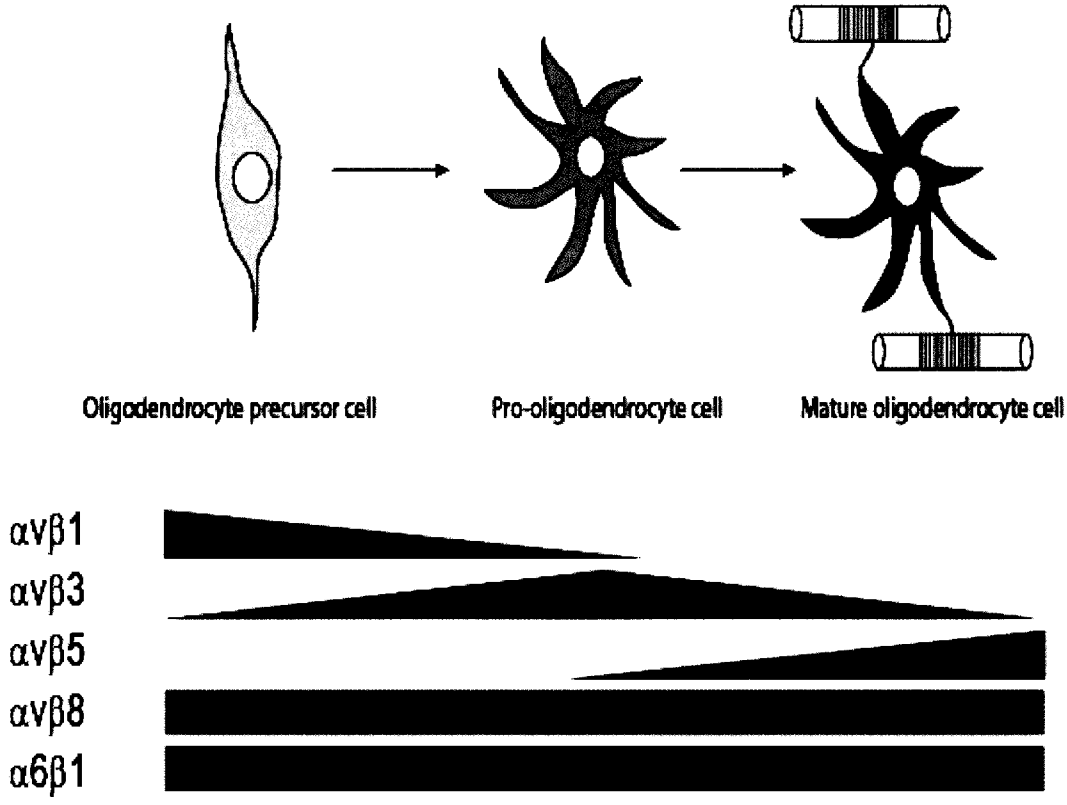

Figure 4. Integrin expression in oligodendrocyte development. αvβ1 is upregulated early in oligodendrocyte development and down regulated during differentiation. αvβ3 is upregulated during differentiation and down regulated when the oligodendrocytes begin forming myelin membrane. αvβ5 is upregulated later in oligodendrocyte development. αvβ8 and α6β1 are expressed throughout oligodendrocyte development.

OPCs retain their characteristic bipolar morphology when under the migration block, however, upon removal of the blockade the OPCs resume migration and ultimately differentiate (Milner et al. 1996). Therefore, these experiments brought forth the notion that migration and differentiation are regulated by separate integrin signaling pathways. Consequently the other integrins expressed in oligodendrocytes were then explored to determine their involvement in oligodendrocyte development. During oligodendrocyte development, αvβ3 is upregulated prior to expression of αvβ5 and is immediately downregulated with the onset of differentiation (Milner and Ffrench-Constant 1994). This suggested that the role of αvβ3 and αvβ5 was in proliferation and differentiation of oligodendrocytes respectively. Blaschuk et al. have demonstrated *in vitro* that overexpression of the β3 subunit resulted in enhanced proliferation and inhibition of differentiation (Blaschuk et al. 2000). In addition, blocking antibodies of αvβ5 exhibited inhibition of differentiation (Blaschuk et al. 2000). *In vivo* and *in vitro* studies also corroborated the role of αvβ3 in proliferation. The knockout of tenasin-C, which is a ligand of αvβ3, resulted in a reduction of OPC proliferation in mice (Garcion et al. 2001). Moreover, *in vitro* the reduction of OPC proliferation phenotype could be rescued with exogenous tenasin-C (Garcion et al. 2001). The αvβ8 integrin is the most abundant integrin and is expressed throughout oligodendrocyte development. However, its role in oligodendrocyte biology has yet to be determined. The cytoplasmic domain of β8 has a unique amino acid composition when compared to the other integrin cytoplasmic domains, therefore, suggesting a unique role for αvβ8 in oligodendrocyte development (Moyle et al. 1991). Recently, it has been demonstrated in epithelial cells that αvβ8

regulates the function of TGFβ and is involved in proliferation (Fjellbirkeland et al. 2003). The role of αvβ8 in oligodendrocyte differentiation still needs to be explored.

The other integrin that is expressed throughout oligodendrocyte development is α6β1. Previous work has demonstrated that α6β1 may be important in the later stages of oligodendrocyte development, in particular, in the survival of oligodendrocytes and in the process of myelination. α6β1 was first suggested to be involved in oligodendrocyte survival by Colognato et al. Transgenic mice lacking the α6 integrin subunit demonstrated a decrease in the number of MBP positive cells and an increase in the number of apoptotic cells in the brain stem, implicating the α6 integrin in the promotion of oligodendrocyte survival (Colognato et al. 2002).

In vitro, it was first demonstrated that laminin is able to enhance myelin membrane formation (Buttery and ffrench-Constant 1999). The ability of oligodendrocytes to extend their process and form myelin membrane was tested on various ECMs. The oligodendrocytes grown on laminin had greater extension of processes and myelin membrane formation when compared to the oligodendrocytes grown on vitronectin, fibronectin and poly-D-lysine (Buttery and ffrench-Constant 1999). To determine if integrins were involved in relaying the enhancing capabilities of laminin, oligodendrocytes were matured on laminin in the presence of various integrin blockers. In the presence of GRGDSP peptide, cyclic RGD, anti-β3 and anti-β5 antibody, the oligodendrocytes showed enhanced myelin membrane formation similar to oligodendrocytes grown on laminin alone (Buttery and ffrench-Constant 1999). However, oligodendrocytes grown in the presence of anti-β1 integrin antibody exhibited a reduction in the proportion of cells that had mature myelin membrane (Buttery and ffrench-

Constant 1999). Therefore, laminin enhancement of the oligodendrocyte myelin membrane formation appears to be mediated through β1 integrin. *Ex vivo* experiments further promoted the importance of β1 integrin in myelination (Relvas et al. 2001). Rats with demyelinated spinal cords were transplanted with oligodendrocytes expressing a dominant-negative β1 integrin construct that consisted of an interleukin-2 extracellular domain and a β1 integrin cytoplasmic domain (Relvas et al. 2001). The rats that were transplanted with oligodendrocytes expressing a dominant-negative β1 integrin showed impaired remyelination when compared to rats that were transplanted with oligodendrocytes expressing endogenous β1 integrin (Relvas et al. 2001).

Recently, two different strategies were used to generate β1 integrin mouse models to further the understanding of β1 integrin's role in myelination *in vivo*. One mouse model was produced by a Cre/lox strategy, the β1 integrin gene was ablated in premyelinating oligodendrocytes during the development of the CNS (Benninger et al. 2006). The conditional β1 integrin knock-out mice surprisingly does not have defects in myelin. The mice had phenotypically normal myelination and remyelination (Benninger et al. 2006)(Benninger et al., 2006). In contrast, a transgenic mouse model expressing a dominant-negative β1 integrin specifically in oligodendrocytes demonstrates defects in myelination and remyelination (Lee et al. 2006). The optic nerve and spinal cord displayed hypomyelination, while the corpus callosum exhibited a delay or inhibition in remyelination after an episode of demyelination (Lee et al. 2006). These two β1 integrin mouse models vary in their results for determining the role of β1 integrin in myelination *in vivo*. Further work is required to resolve the differences and to establish the role of β1 integrin in myelination.

1.6.2 Integrin's downstream signaling pathways in myelination

Although the role of integrins in myelination has been a primary focus in myelin research, the past few years has brought to light other proteins that play a role in myelination, specifically some of the proteins that are either interactors of integrins or are involved in known downstream signaling pathways of integrins. Integrin-linked kinase (ILK) is a well known interactor of integrin that has recently been shown to be involved in myelin membrane formation (Chun et al. 2003). *In vitro*, it was determined that laminin enhanced oligodendrocyte cell spreading was inhibited in the presence of a dominant-negative ILK (Chun et al. 2003). This demonstrated the importance of ILK in myelin membrane formation. Furthermore, it was also determined that laminin enhanced oligodendrocyte cell spreading was promoted through the PI3-kinase pathway. Interestingly, in our own work with the dominant-negative β1-integrin mice, it was shown that in the tissues of the CNS where there were defects in myelination and remyelination, the MAP-kinase activity was decreased when compared to control mice (Lee et al. 2006). Hence, it appears that the MAP-kinase pathway is also important during myelination and remyelination.

There have also been other signaling pathways that are known to be involved in oligodendrocyte development and are downstream of the integrins. *In vitro*, it was reported that the Src family kinases Fyn and Lyn regulate each of the distinct stages of integrin driven oligodendrocyte development (Colognato et al. 2004). It was determined that in oligodendrocytes Lyn associated with αvβ3 while Fyn associated with α6β1. It is reported that in the early oligodendrocyte lineage, Lyn is required to drive the αvβ3 directed progenitor proliferation. However, later in oligodendrocyte development when

the OPCs have achieved axonal contact and access to axonal laminin, α6β1 associated Fyn comes in to play and is required for laminin enhanced differentiation in myelin membrane formation and survival (Colognato et al. 2004). *In vivo*, the importance of Fyn in myelination has also been supported with the transgenic mice lacking Fyn activity displaying hypomyelination in specific tissues of the CNS (Sperber et al. 2001).

There are currently many other proteins downstream of or interactors of integrins that are proving to be involved in various stages of myelination and remyelination. However, further work is required to determine the mechanisms of integrin signaling pathways in myelination for potential therapeutic approaches in finding a treatment or cure for MS.

1.7 Research Plan and Hypothesis

Our hypothesis is that β1 integrin and its downstream signals in oligodendrocytes play a role in myelination within the CNS. Determining *in vivo* the role of β1 integrin and its downstream signaling in myelination and remyelination will lead us to a better understanding of its function in myelin formation, thus potentially leading to therapeutics for those who suffer from MS. To study this, we have proposed the following aims:

Aim 1: Characterization of the dominant-negative β1 integrin transgenic mouse

Previous *in vitro* studies have demonstrated the role of β1 integrin in oligodendrocyte maturation. Similar studies *in vivo* have been difficult due to the embryonic and perinatal lethality of null mutations in integrin subunits. To overcome lethality, we have generated a transgenic mouse model that expresses a dominant-

negative β1 integrin construct under the control of the PLP promoter. The generation of a dominant-negative β1 integrin transgenic mouse model will allow for a better appreciation of the role of β1 integrin in myelination and remyelination in CNS tissues.

Aim 2: Characterization of oligodendrocyte behavior of the dominant-negative β1 integrin transgenic mouse

The dominant-negative β1 integrin mouse model demonstrates regional defects in myelination and remyelination, therefore indicating an important role for β1 integrin in those processes. To garner a further understanding of β1 integrin in oligodendrocyte behavior during remyelination, we take advantage of the cuprizone model. Analysis of the corpus callosum of the dominant-negative β1 integrin mice under the demyelination and remyeliantion model will lead us to a better understanding of the specific stages and mechanisms that β1 integrin plays in oligodendrocyte development.

In parallel to these aims, we sought out to identify differences in ILK interacting protein partners during proliferation and differentiation

Recent work has demonstrated the importance of ILK in CNS myelination (Chun et al. 2003). ILK is a key component of the integrin signaling pathway that functions in concert with multiple partners to transmit cues from the ECM to the cytoskeleton. Although it is clear that ILK performs this role in multiple cell types, it is not clear whether it associates with the same set of binding partners in different cells or whether unique partners exist in specific cell types. Therefore, we sought to determine if ILK interacting partners change during proliferation and differentiation in an oligodendrocyte

cell line, central glia (CG4). Characterization of various protein partner of ILK during proliferation and differentiation will highlight the potential of other potential proteins involved in myelination.

Chapter 2. Dominant-negative β1 integrin mice have region-specific myelin defects accompanied by alterations in MAPK activity

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Dominant-negative β1 integrin mice have region-specific myelin defects accompanied by alterations in MAPK activity

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Running title: Beta 1 integrin and CNS myelination

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Abstract

Recent studies have demonstrated the importance of β1 integrin in oligodendrocyte maturation *in vitro*. Similar studies *in vivo* have been difficult due to the embryonic and perinatal lethality of null mutations in integrin subunits. Here, we have generated transgenic mouse models that overexpress full length β1 integrin or express a dominant-negative β1 integrin ΔC (lacking the C-terminal tail) under the control of the proteolipid protein (PLP) promoter. We demonstrate that these transgenes are expressed predominantly in CNS tissues and more specifically in oligodendrocytes. Further analysis reveals that the dominant-negative β1 integrin ΔC transgenic mice, but not the full length β1 integrin mice, have hypomyelinated axons in spinal cords and optic nerves. In addition, there is a significant increase in the number of unmyelinated axons within the spinal cords and optic nerves of the β1 integrin ΔC mice. In contrast, the corpus callosum from these mice did not show similar myelin defects. To assess if remyelination would be affected in the corpus callosum, mice were subjected to a cuprizone-induced demyelination. Interestingly, the dominant-negative mice recovered from this insult in a manner similar to the wild type littermates. Axons within the corpus callosum that were remyelinated had normal g-ratios, however, the actual percentage of myelinated axons was significantly reduced compared with wild type mice. We also show that the defects observed in the dominant-negative β1 integrin ΔC mice are accompanied by disruption of the MAP-kinase signaling pathway. Our work highlights the importance of β1 integrin-mediated signaling in CNS myelination *in vivo*.

Introduction

The first stage of myelination involves differentiation of proliferating oligodendrocyte precursor cells into post-mitotic oligodendrocytes, whereas the second stage involves a morphological maturation that results in myelin sheath assembly (Gard and Pfeiffer 1989; Knapp et al. 1987; Raff et al. 1978). These processes are dependent on cell-axon and cell-extracellular matrix (ECM) interactions. Cell culture studies demonstrate that oligodendrocytes rely on bi-directional signaling between the extracellular milieu and the intracellular cytoskeleton for the promotion of extensive membrane production. This signaling is mediated by the membrane-spanning proteins called integrins.

Integrins comprise a family of cell surface receptors (Hynes 2002) consisting of heterodimers of α and β subunits that recognize several ECM components (Liu et al. 2000). Integrins have a large extracellular domain, a single transmembrane domain and a small cytoplasmic domain of approximately 25-50 amino acids. Once integrins are bound to the ECM, an intracellular signaling cascade is induced (Giancotti 1997). Most of the signaling occurs through the β subunit, with the cytoplasmic domain playing a structural role as well as acting as a transducer (Chou et al. 1997) by associating with actin binding proteins α-actinin, talin and filamins, and with focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (Schaller et al. 1995; Wu and Dedhar 2001).

Oligodendrocyte precursor cells express αvβ1, αvβ3, and αvβ5 integrins differentially during maturation, whereas αvβ8 and α6β1 integrins are expressed throughout development (Milner and Ffrench-Constant 1994; Milner et al. 1997). Interaction of β1 integrin with the ECM is crucial for the promotion of myelination

(Buttery and ffrench-Constant 1999). In cell culture experiments, oligodendrocytes grown on various ECM substrates differentiated normally, but there was a significant difference in the morphology of the cells depending on the nature of the substrate, with laminin-2 being the best at enhancing myelin membrane formation (Buttery and ffrench-Constant 1999). This latter effect was mediated through β1 integrin. Other experiments have shown that oligodendrocytes expressing a dominant-negative β1 integrin, when transplanted into a demyelinated area of a rat spinal cord, were unable to contribute to remyelination (Relvas et al. 2001). Furthermore, signal transduction cascades involving laminin-2, β1 integrin, PI3-kinase and ILK are required in myelin membrane formation (Chun et al. 2003). Finally, myelin-forming oligodendrocytes undergo an integrin-regulated switch from PI3-kinase pathway dependence to a MAP-kinase pathway dependence upon contact with axonal laminins (Colognato et al. 2002).

Null mutations of integrin subunits result in embryonic or perinatal lethality and therefore have not been a suitable approach for assessing myelination *in vivo* (Fassler et al. 1996). In the present report, we describe the generation and characterization of transgenic mice expressing full length β1 integrin or a dominant-negative version of β1 integrin (β1 integrin ΔC) under the control of the proteolipid protein (PLP) promoter. These mice have been used to study myelination in different central nervous system (CNS) regions.

Materials and Methods

Generation of Transgenic mice

The cDNA sequence coding for β1 integrin was originally from R. Fassler and provided by E. Roos. The full length (FL) β1 integrin cDNA was further modified by an addition of a stop codon at position 695 by oligonucleotide site-directed mutagenesis. This allowed for the removal of the region encoding amino acids 696-742 and to generate a cDNA lacking the coding information for the cytoplasmic domain (β1 integrin ΔC). Both the FL β1 integrin and β1 integrin ΔC cDNAs were inserted into the PLP promoter cassette (Fuss et al. 2000). The resulting transgenes (Fig. 1A) were microinjected into one-cell mouse embryos. Positive founders were identified by PCR genotyping and confirmed by Southern blot hybridization. Although several founder mice were obtained, two for each transgene were selected for further breeding with C57BL6/C3H F1 mice to establish transgenic lines.

RT-PCR Analysis

For reverse-transcriptase polymerase chain reaction (RT-PCR), RNA was isolated from different tissues of 3 week old mice. Equal amounts of total RNA were reverse-transcribed with MuLV reverse transcriptase (Invitrogen). PCR was performed using 30 cycles with primer pairs sense 5'-GGTCCATGTCTAGCGTG-3' and antisense 5'-CCTCATATACTTCGGATTGAC-3' to detect endogenous β1 integrin transcripts. In addition, a primer set that can amplify the transgene product but not the endogenous β1 integrin transcript was selected: sense 5'-CTTCTCTGCTGTGCTGAGC-3' and antisense 5'-GGCATCAGGGATGGTGAGG-3'. This primer set specifically amplifies from exon

1 of the PLP promoter cassette (sense) and from within the β1 integrin transgene (antisense). Actin primers were used as positive controls.

Western Blot Analysis

Different tissues from 1 month old mice were homogenized in RIPA buffer (40 mM Tris-HCl, pH 8.0, 276 mM NaCl, 20% glycerol, and 2% NP-40) containing 1 mM PMSF, 0.01 mg/ml aprotonin, 0.01 mg/ml pepstatin, 0.01 mg/ml leupeptin, 10 mM NaF and 10 mM Na_3VO_4 on ice. Non reduced protein samples were analyzed by SDS-PAGE on standard 8% polyacrylamide gels and transferred semi-dry onto nitrocellulose membrane (Amersham Pharmacia Biotech, NJ). Membranes were blocked with Odyssey blocking buffer (Li-Cor Biosciences, NE) and incubated for 1 hour in β1 integrin antibody (a gift from B. Chan, University of Western Ontario) diluted 1:100 in the Odyssey blocking buffer. Bound β1 integrin antibody was detected using AlexaFluor 680 anti-rat secondary antibody (Molecular Probes, OR). Blots were scanned and quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences, NE). For all other western blot analysis, reduced protein samples were resolved by SDS-PAGE on standard 10% gels and transferred semi-dry onto PVDF membrane. Membranes were blocked in 5% skim milk powder or 5% bovine serum albumin in TBS-T (1 M Tris-HCl, pH 7.5, 5 M NaCl, and 0.1% Tween-20). Blots were incubated with antibodies to AKT, phospho-Akt (Ser473), p44/42 MAP kinase and phospho-p44/42 MAP kinase (Cell Signaling; MA) for 1 hour. Bound primary antibodies were detected using horseradish peroxidase conjugated rabbit-anti-rat, goat-anti-rabbit or goat-anti-mouse secondary antibody (Jackson

Immunoresearch, PA). Protein bands were detected by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc., NJ).

Immunocytochemistry

Paraformaldehyde (PFA) fixed brains were embedded in 30% sucrose and OCT (Sakura, CA), and coronal cryostat sections of 10 micrometer thickness at the fornix region of the corpus callosum were obtained. Frozen sections were fixed in 70% ethanol and blocked with TBLS (0.05% 1 M Tris pH 7.4, 0.0085% NaCl, 0.01% BSA, 0.009% L-Lysine, and 10% sodium azide) with 30% goat serum and 0.3% Triton-X-100. Sections were incubated overnight at 4°C in primary antibodies diluted in TBLS at a dilution of 1:50 for β1 integrin and 1:10 for CC-1 (Abcam, MA). Sections were incubated with secondary antibodies diluted 1:200. Slides were mounted and viewed using a Zeiss Axioplan epifluorescence microscope.

Electron Microscopy

Mice were anesthetized and perfused with PBS followed by Karnovsky's Fix (8% PFA, pH 7.0, 5% glutaraldehyde, and 0.2 M sodium cacodylate). Optic nerves, spinal cord and corpus callosum were sliced into 1 mm sections. Thin sections were cut, stained with uranyl acetate and lead citrate, and analyzed by electron microscopy. The g-ratio was calculated as the diameter of the axon divided by the diameter of the axon and the myelin sheath. For each time point and strain, a total of 3 mice were analyzed and, where possible, a minimum of 100 fibers were measured.

Induction of demyelination and remyelination

To induce demyelination, 6 week old mice were fed Harlan Teklad mouse chow containing 0.2% cuprizone (Sigma, MS) for a period of 6 weeks. Cuprizone was incorporated within a pelleted chow. Remyelination was induced by returning the mice to a normal diet. A total of 3 mice per time point were analyzed at different times of recovery.

Results

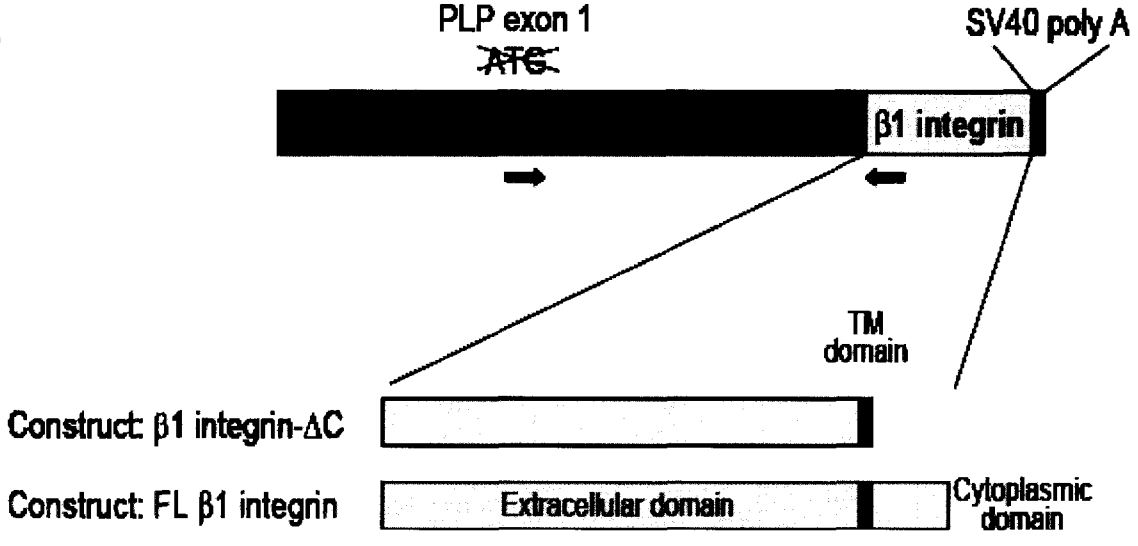
Generation of β1 integrin transgenic mice

cDNAs encoding full length and truncated versions of the β1 integrin protein were placed under the regulation of the PLP promoter (Fig. 1A), which directs oligodendrocyte-specific expression (Wight et al. 1993). For the full length β1 integrin transgene, 13 out of the 78 potential founder mice were positive. For the dominant-negative β1 integrin ΔC, 7 of the 22 potential founder mice were positive (summarized in Fig. 1B). For both transgenic constructs, two founders were selected for further breeding to establish independent lines (FL mice: lines 3880 and 3853; ΔC mice: lines 5603 and 315).

The PLP-β1 integrin transgenic mice display CNS-specific expression

We demonstrate that the endogenous β1 integrin gene is expressed in all tissues examined (Fig. 2A). To assess transgene expression, we designed primers that were specific to the PLP exon 1 (sense) and the β1 integrin cDNA (antisense). Note that since the primers are on either side of an intron, the pre-mRNA will be too large to be amplified and therefore only the processed mRNA will be amplified. Several of the founder mice were sacrificed at three weeks of age to examine their expression profile. Strikingly all the mice showed expression of the transgene predominantly in CNS tissues (data not shown). We have restricted our more detailed analysis to the two strains of mice established for each of the two transgene constructs. For the PLP-β1 integrin transgenic lines, analysis of RNA from several tissues by RT-PCR revealed predominant expression in CNS tissues including optic nerve, spinal cord, cortex, and cerebellum (Fig. 2B). In

A



B

Transgene name	Mice born	Number of founder mice	Transgenic strains established
FL β1 integrin	78	13	2
β1 integrin ΔC	22	7	2

Figure 1. Schematic representation of the PLP-full length β1 integrin and PLP-β1 integrin ΔC transgenes. **A.** The transgenes are under the regulation of the mouse PLP promoter. The initiating ATG in exon 1 has been mutated to make it an untranslated first exon. (TM = transmembrane domain). Arrows indicate primers used for RT-PCR analysis of transgene derived transcripts. **B.** Summary of transgenic mice production.

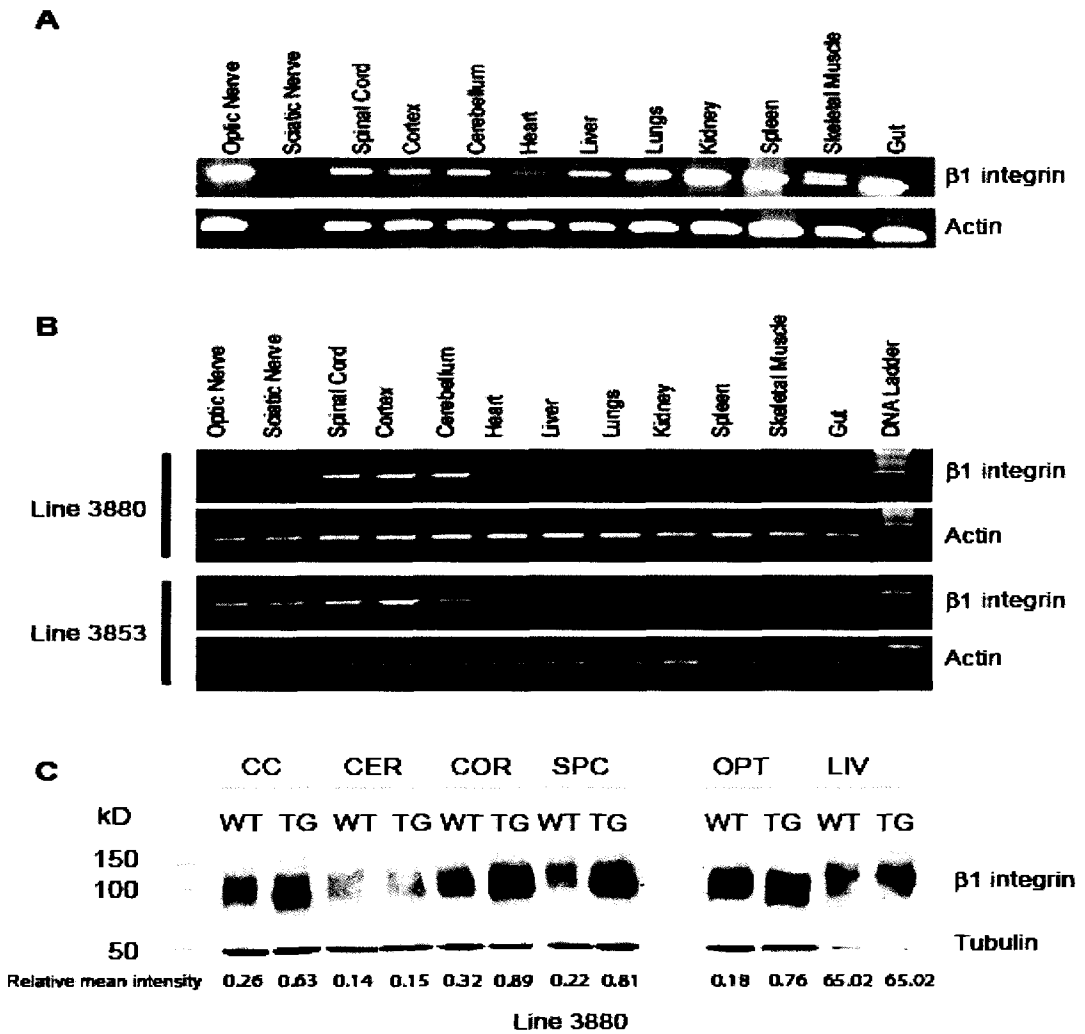


Figure 2. The expression profile of endogenous β1 integrin and full length β1 integrin transgene. A. RT-PCR analysis. Endogenous β1 integrin transcript is detected in all tissues. Actin primers were used as control. B. RT-PCR analysis of RNA from tissues of 3 week old full length β1 integrin transgenic mice. Results from two different lines are shown. Predominant transgene expression is in CNS tissues. Low level transgene RNA is detected in other tissues of line 3853. Actin primers were used as control. C. Western blot analysis of proteins (50 μg) from 1 month old full length β1 integrin transgenic mice. The antibody used in this assay recognizes the endogenous as well as the transgene derived β1 integrin. An increase in the total level of β1 integrin compared to their non-transgenic littermates is observed in some CNS tissues. Tubulin is used as loading control and the relative mean intensities determined from an analysis of a minimum of two mice. CC = corpus callosum; CER = cerebellum; COR = cortex; SPC = spinal cord; OPT = optic nerve; LIV = liver.

line 3853, transcripts were also detected in other tissues, albeit at a lower level, whereas in line 3880 expression of the transgene was reduced in the sciatic nerve. These latter changes likely reflect a position-effect of transgene integration. Nevertheless, the primary site of transgene expression is in CNS tissues. Using an antibody that recognizes mouse β1 integrin, we performed western blot analysis on proteins from several tissues of 1 month old mice from line 3880 (Fig. 2C). Since this antibody cannot distinguish the endogenous β1 integrin from the transgene product, we have to rely on differences in absolute amounts of the protein. We have quantified the amount of total β1 integrin using an infrared imaging system and normalized the value to tubulin levels. β1 integrin levels were increased in CNS tissues and there is no change in other tissues compared to the non-transgenic littermates (Fig. 2C).

Similarly, expression studies were performed on the two PLP-β1 integrin ΔC transgenic mouse lines. Both lines display a tight CNS-specific expression pattern (Fig. 3A). The CNS tissues from line 5603 were also analyzed by western blot analysis using the same antibody used above. The truncated β1 integrin ΔC protein was readily distinguishable from the endogenous protein in the corpus callosum, cerebellum and spinal cord as a slightly faster migrating species that was not present in the other tissues examined (Fig. 3B). Furthermore, quantification of total β1 integrin indicates that there is an increase in the transgenic mice when compared to the non-transgenic mice.

To determine the onset of transgene expression in various lines, we have analyzed spinal cords from P2 to P35 stage mice. The earliest time at which we detected transgene product was at P5 (data not shown).

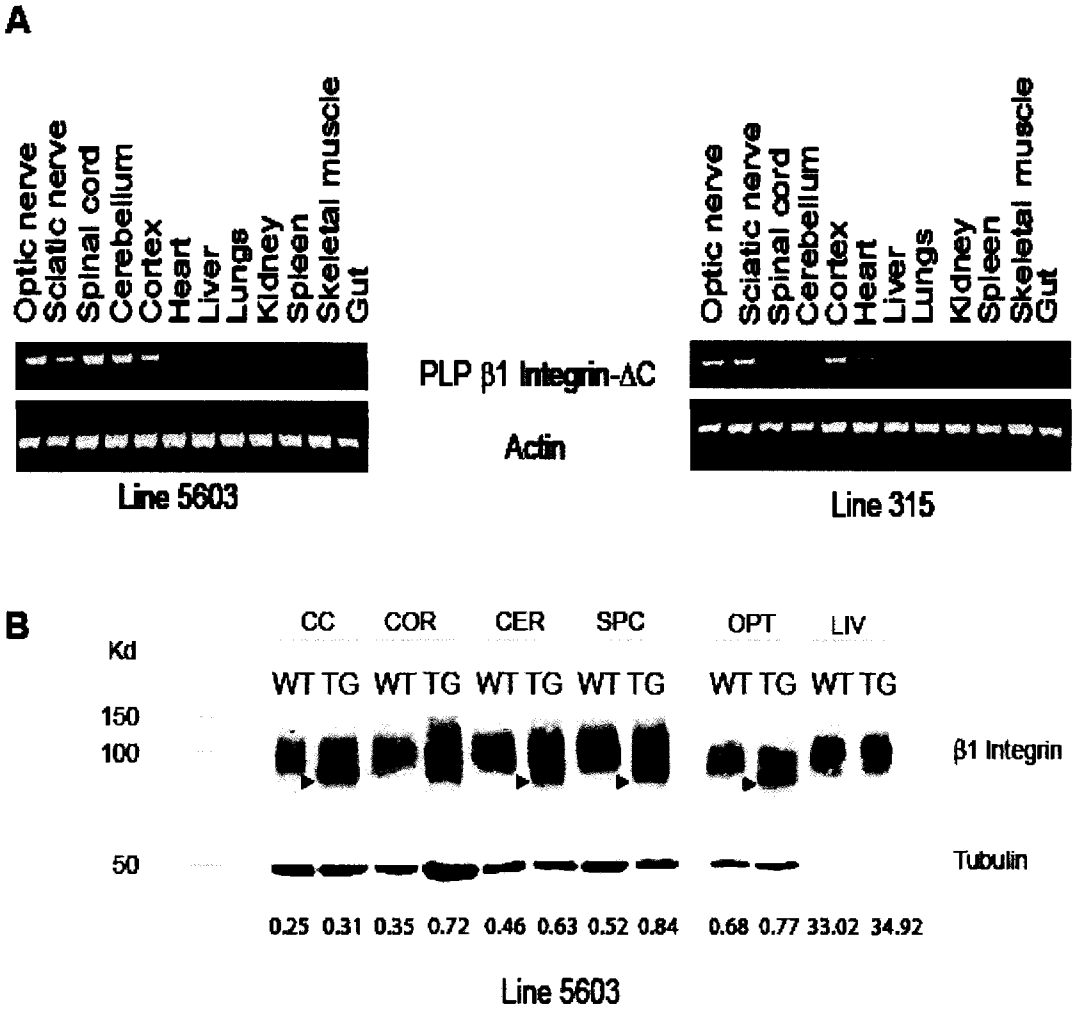


Figure 3. The expression profile of the β1 integrin ΔC transgene. **A.** RT-PCR analysis of RNA from tissues of PLP-β1 integrin ΔC transgenic mice. Results from two different lines are shown. Predominant transgene expression is in CNS tissues. **B.** Western blot analysis of proteins (30 μg) from 1 month old β1 integrin ΔC transgenic mice. Truncated β1 integrin protein is indicated with arrowheads. Tubulin is used as loading control and the relative mean intensities determined from an analysis of a minimum of two mice. CC = corpus callosum; COR = cortex; CER = cerebellum; SPC = spinal cord; OPT = optic nerve; LIV = liver.

β1 integrin ΔC transgene expression is directed to oligodendrocytes

We performed immunocytochemistry on coronal brain sections from 1 month old mice to assess the specificity of the β1 integrin ΔC transgene expression. β1 integrin antibody was used to detect transgene product and CC-1 antibody counterstaining was used to mark mature oligodendrocytes. The transgenic mice had higher levels of β1 integrin expression in the corpus callosum when compared to equivalent regions from non-transgenic mice (Fig. 4B,F). As expected, both transgenic and non-transgenic mice labeled positive with the CC-1 antibody in the corpus callosum (Fig. 4C,G), indicating the presence of mature oligodendrocytes. Of note, the increased expression of the β1 integrin ΔC protein is in cells that co-label with CC-1 (Fig. 4H), indicating that the transgene expression is specifically targeted to oligodendrocytes.

Ultrastructural analysis of CNS tissues from the β1 integrin transgenic mice

To assess the integrity of myelin in transgenic tissues, ultrastructural analysis was performed on the optic nerve, spinal cord and brain of 1 month old mice. The electron micrographs display little variance in the myelin of the optic nerve and spinal cord of the full length β1 integrin transgenic mice when compared to their non-transgenic littermates (data not shown). In contrast, the β1 integrin ΔC transgenic mice displayed myelin abnormalities in the optic nerve and spinal cord (Fig. 5A-D). The myelin appeared thinner and several axons lacked any myelin at all. Morphometric analysis of the nerve fibers that are myelinated in the optic nerve and spinal cord revealed a significant increase in the ratio of axon diameter to fiber diameter (g-ratio) in the β1 integrin ΔC mice when compared to non-transgenic mice ($p < 0.0000001$), where a g-ratio of 1.0

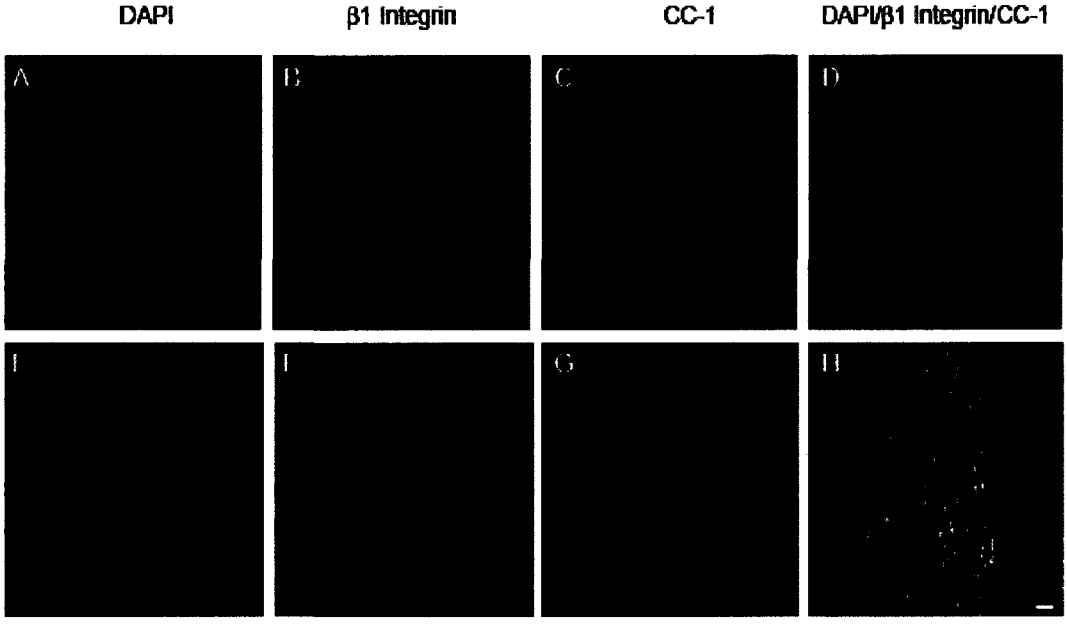
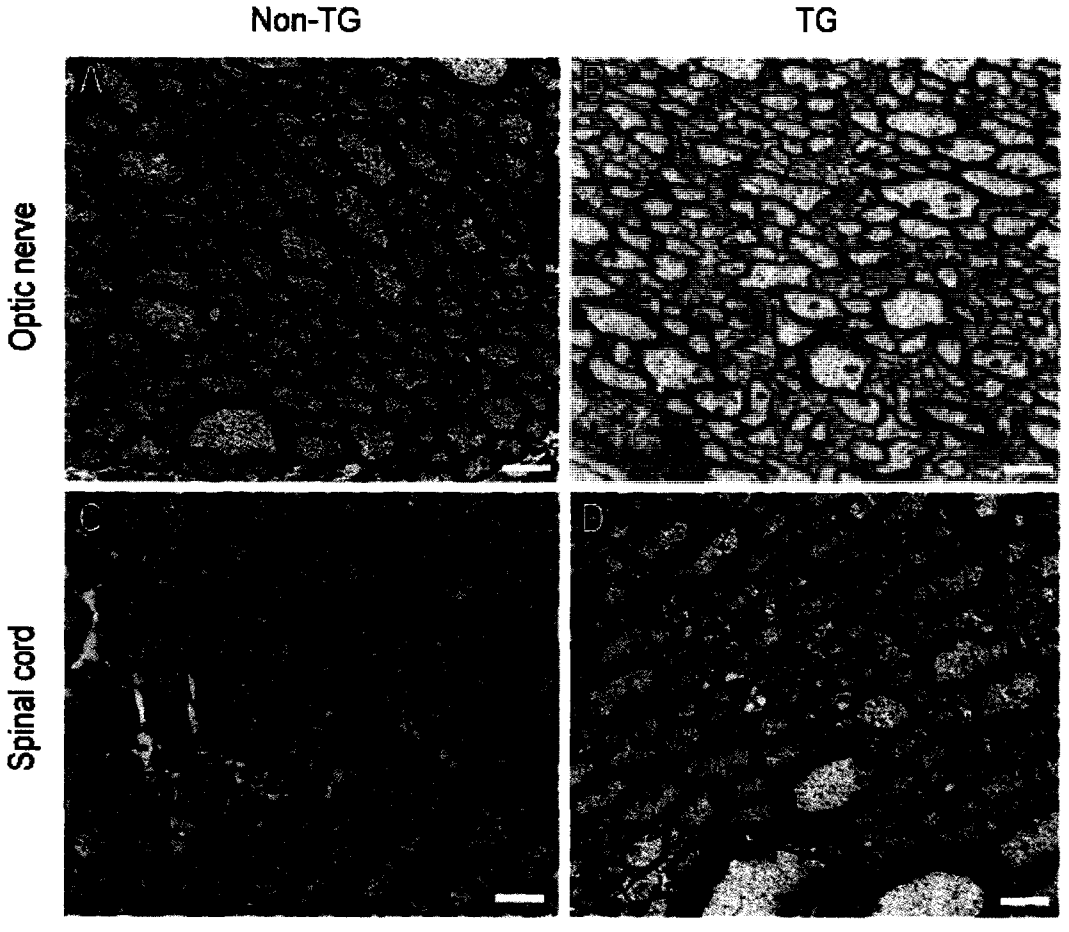


Figure 4. Expression of β1 integrin ΔC in oligodendrocytes. Immunocytochemistry of non-transgenic (A-D) and β1 integrin ΔC corpus callosum sections (E-H). Low amounts of endogenous β1 integrin protein are detected in CC-1 positive oligodendrocytes in wild type sections (B,C). By comparison, sections of the transgenic corpus callosum display greatly increased levels of total β1 integrin (endogenous plus truncated products) in CC-1 positive oligodendrocytes (F,G). Merged images in panels D and H. Scale bar = 20 μm.



E

	G-ratio	Percent unmyelinated fibers
Optic nerve		
Non-TG	0.84 ± 0.04	19.8%
TG	0.87 ± 0.05 *	45.0% **
Spinal cord		
Non-TG	0.78 ± 0.07	31.4%
TG	0.85 ± 0.05 *	62.1% **

Figure 5. Ultrastructural analysis of CNS tissues from β1 integrin ΔC transgenic mice and their non-transgenic littermates. Electron micrographs of optic nerves (**A,B**) and spinal cords (**C,D**). Scale bar = 1 μm. (**E**) Quantification of myelination defects. The g-ratios and percent unmyelinated axons were calculated and are presented as averages (n = 3 mice). Axons from the transgenic tissues are significantly hypomyelinated (* $p < 0.0000001$) and have significantly more axons that are unmyelinated (** $p < 0.05$) when compared to those from non-transgenic mice.

indicates complete demyelination (Fig. 5E). Furthermore, there was a significant increase in the number of unmyelinated axons. Of note, 45.0% or 62.1% of the axons in the optic nerve and spinal cord respectively, remained unmyelinated in the β1 integrin ΔC mice (Fig. 5E), compared to 19.8% or 31.4% for wild type mice. In the spinal cord, upon division of the axons into large axons (≥0.5 μm) and small axons (< 0.5 μm), there was a significant reduction in the number of small axons that were myelinated in the PLP-β1 ΔC mice ($p < 0.05$) (Table 1). In contrast to the optic nerve and the spinal cord, the corpus callosum from the β1 integrin ΔC mice was normal when compared to the non-transgenic mice (Fig. 6A,B). These observations suggest that there are region-specific differences in myelination in the β1 integrin ΔC mice.

To determine whether the number of mature oligodendrocytes had been affected in the β1 integrin ΔC mice, we performed CC-1 immunostaining on sections from corpus callosum, spinal cord and optic nerve of 1 month old mice. There is a significant decrease in the number of CC-1 positive cells in the optic nerve and spinal cord but not in the corpus callosum of the β1 integrin ΔC mice when compared to the non-transgenic mice (Table 2). These results are consistent with the myelination abnormalities described above.

Expression of β1 integrin ΔC affects remyelination in the corpus callosum

Our data so far indicate that expression of β1 integrin ΔC affects mature oligodendrocyte numbers and myelination, at least in the optic nerve and spinal cord. We next tested the possibility that remyelination may also be affected in our mice using the cuprizone model (Blakemore 1972; Blakemore 1973). β1 integrin ΔC transgenic mice at

	TOTAL AXONS COUNTED/DEFINED AREA	MYELINATED AXONS		UNMYELINATED AXONS	
		Large	Small	Large	Small
Non-TG	311 ± 7.3	174 ± 17.0	29 ± 4.9	33 ± 3.1	75 ± 9.3
β1 integrin ΔC	393 ± 9.2	130 ± 15.9	13 ± 1.5	41 ± 1.8	209 ± 16.2*

Table 1. The occurrence of small diameter myelinated axons is decreased in β1 integrin ΔC mice. Total numbers of large and small diameter axons that are myelinated and unmyelinated were counted (n = 3 mice for each genotype). There is a significant increase in the number of unmyelinated small axons in the β1 integrin ΔC mice when compared to non-transgenic mice (*$p < 0.05$). Large axons ≥0.5 μm and small axons <0.5 μm.

	CORPUS CALLOSUM	SPINAL CORD	OPTIC NERVE
Non-TG	49 ± 6.43	56 ± 1.49	43 ± 3.55
β1 integrin ΔC	48 ± 4.16	48 ± 2.00*	32 ± 3.37*

Table 2. The number of mature oligodendrocytes in the spinal cord and optic nerve of β1 integrin ΔC mice is reduced. Total number of CC-1 positive cells were counted in a grid of constant area in the corpus callosum, spinal cord and optic nerve (n = 3 mice per genotype).There is a significant decrease in the number of CC-1 positive cells in the spinal cord and optic nerve of the β1 integrin ΔC transgenic mice when compared to non-transgenic littermates (*$p < 0.001$).

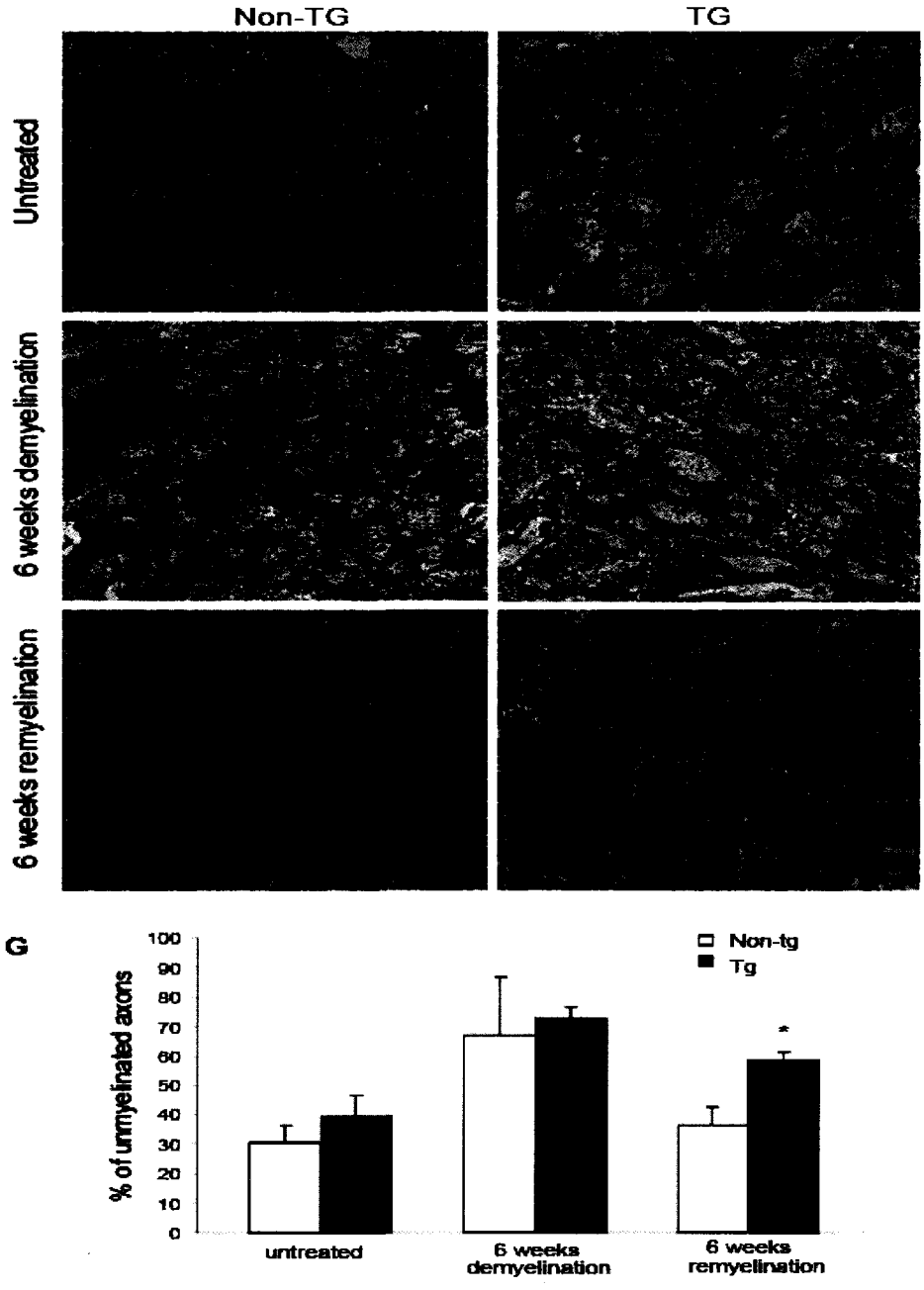


Figure 6. Remyelination is affected in the corpus callosum of β1 integrin ΔC transgenic mice. Electron microscopy was performed on cross sections of the corpus callosum from non-treated mice (A,B) and from mice fed a cuprizone diet for 6 weeks followed by recovery of 0 weeks (C,D), or 6 weeks (E,F) on regular diet. Scale bar = 0.5 μm. Non-treated non-transgenic and transgenic mice exhibited normal myelination (A,B). Cuprizone administration led to demyelination in both normal and transgenic mice (C,D). Recovery from the cuprizone-induced demyelination was apparent in both non-transgenic and transgenic mice (E,F). (G) Quantification of the number of unmyelinated fibers (3 mice per time point). β1 integrin ΔC mice have a significantly higher percentage of fibers that remained unmyelinated after 6 weeks of recovery from the cuprizone treatment (* $p < 0.01$).

6 weeks of age were fed a cuprizone diet for 6 weeks and then returned to normal diet to allow remyelination. At various times thereafter, the corpus callosum was obtained for electron microscopy.

In both the β1 integrin ΔC transgenic and non-transgenic mice, cuprizone induced almost complete demyelination in the corpus callosum (Fig. 6C,D). At the end of the 6 week recovery period, there was no difference in the g-ratio of axons within the corpus callosum of both the non-transgenic and transgenic mice (data not shown). Ultrastructural analysis of the corpus callosum of the wild type mice demonstrated that 63.5% of the axons had undergone remyelination (Fig. 6E,G), leaving only 36.5% unmyelinated. In contrast, the corpus callosum of the β1 integrin ΔC transgenic mice remained significantly more unmyelinated ($p<0.01$) with 58.5% of the axons remaining unmyelinated (Fig. 6F,G). Our results strongly indicate that expression of β1 integrin ΔC in oligodendrocytes in the corpus callosum inhibits or delays remyelination in those axons that remain unmyelinated at 6 weeks of recovery.

β1 integrin ΔC affects the MAP-kinase pathway during myelination and remyelination

We next examined both the PI3-kinase and MAP-kinase pathways to determine if one or both are involved in the phenotypes observed in the β1 integrin ΔC transgenic mice. PI3K activity was determined by measuring phosphorylation of AKT, a primary downstream substrate. Total AKT and phosphorylated AKT in optic nerve, spinal cord and corpus callosum was not significantly altered between non-transgenic and transgenic mice (Fig. 7A,B). However, activity of MAPK, as assessed by the phosphorylation of this protein, is decreased in the optic nerve and spinal cord of the β1 integrin ΔC mice when

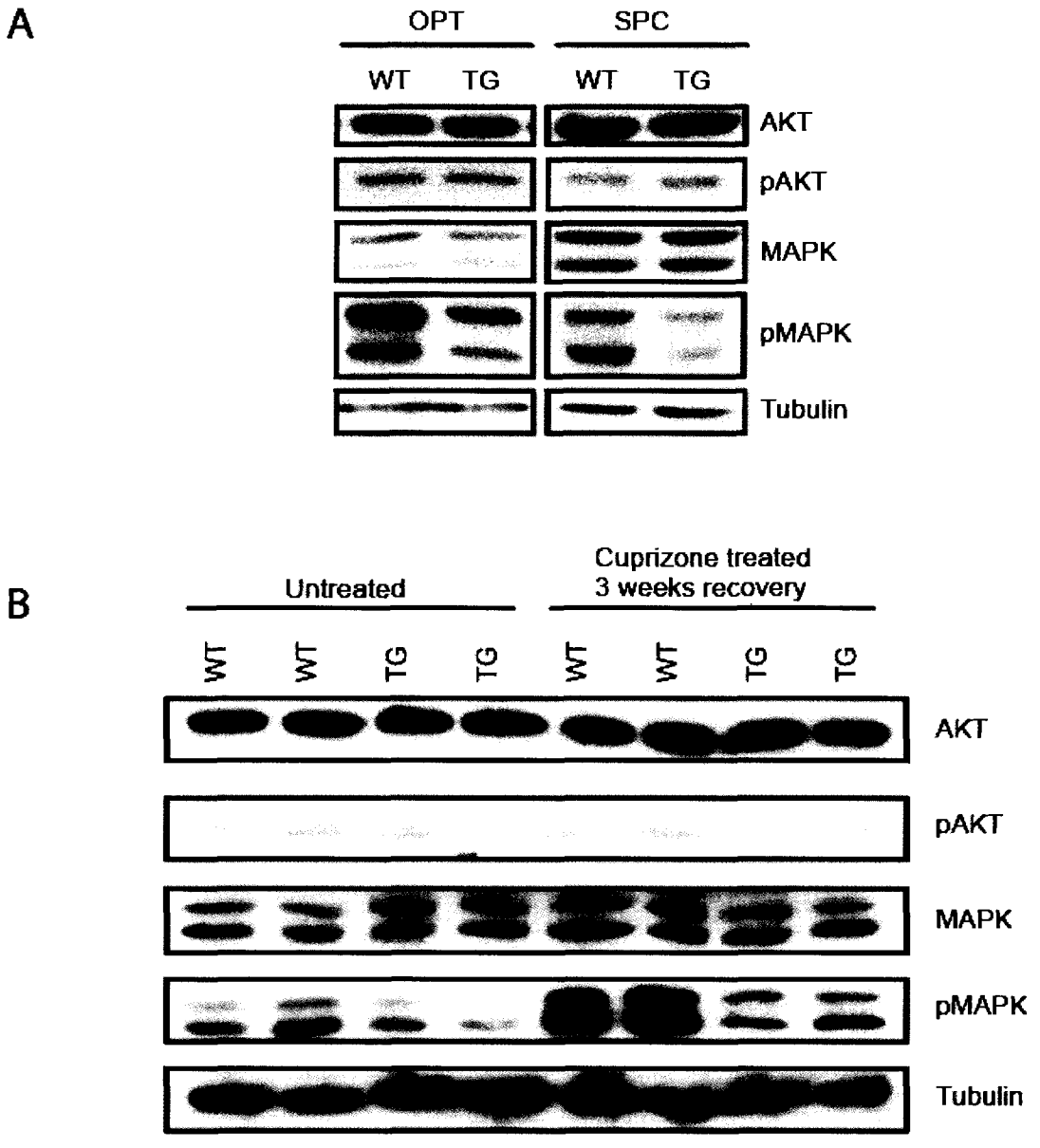


Figure 7. The MAP-kinase pathway is affected in β1 integrin ΔC mice during myelination and remyelination. Western blot analysis of protein (20 μg) from the optic nerve, spinal cord and corpus callosum of non-treated mice (A,B) and the corpus callosum of mice that were on a cuprizone diet for 6 weeks followed by 3 weeks of recovery (B). A. Total AKT, phospho-AKT and total MAPK levels were unaffected in the optic nerve and spinal cord of both the non-transgenic and transgenic mice, while phospho-MAPK levels were reduced in the optic nerve and spinal cord of the transgenic mice. B. Extracts from two independent mice are shown for each treatment. Total AKT, phospho-AKT, total MAPK levels were unaffected in the corpus callosum of untreated and cuprizone treated mice. MAPK phosphorylation was increased in the corpus callosum of non-transgenic mice recovering from a cuprizone insult. In contrast, the transgenic mice failed to display the same level of induction. Tubulin is used as a loading control. OPT = optic nerve; SPC = spinal cord.

compared to non-transgenic mice (Fig. 7A). To determine if the MAPK pathway is also affected during remyelination, mice under cuprizone treatment were allowed to recover for 3 weeks and the corpus callosum was collected for protein analysis. In the non-transgenic mice that underwent 3 weeks of remyelination, there is a noticeable upregulation of MAPK activity in the corpus callosum when compared to untreated mice (Fig. 7B). At the same time, phosphorylation of MAPK in the β1 integrin ΔC mice after 3 weeks of remyelination did not undergo a similar increase (Fig. 7B). These results suggest that the MAPK pathway, but not the PI3K pathway, is affected during the process of myelination and remyelination in the β1 integrin ΔC mice.

Discussion

We describe transgenic mice that either over-express full length β1 integrin or express a dominant-negative β1 integrin ΔC in oligodendrocytes. Overexpression of these transgenes did not result in an overt phenotype. However, morphological analysis revealed myelination defects in the β1 integrin ΔC mice. Axons within the spinal cord and the optic nerve were hypomyelinated, and there was an increase in unmyelinated fibers. Interestingly, myelination in the corpus callosum of the β1 integrin ΔC transgenic mice or in any tissues of the full length β1 integrin transgenic mice was not affected. Coincident with the myelination abnormalities in the spinal cord and optic nerve of β1 integrin ΔC transgenic mice, we observed a decrease in the number of mature oligodendrocytes in these tissues. Although, remyelination was normal after a neurotoxicant-induced demyelination in the corpus callosum of the β1 integrin ΔC transgenic mice, there was a higher number of axons that remained unmyelinated. Finally, we have correlated these defects in myelination and remyelination with a decrease in MAP kinase phosphorylation.

The PLP promoter drives expression to adult CNS, especially in areas with high white matter (Wight et al. 1993). In addition, the PLP promoter's spatial and temporal regulation has proven to be effective in studying various transgenes in the CNS (Fuss et al. 2001). The PLP promoter turns on at P5 with expression levels increasing over the following 16 days (Wight et al. 1993). It is during these early stages that active myelination is occurring in many regions of the CNS (Barres et al. 1992; Butt et al. 1997a; Butt et al. 1997b; Miller et al. 1985; Skoff et al. 1976). In the present work, both PLP-full length β1 integrin and PLP-β1 integrin ΔC transgenes produced significant amount of the

protein in CNS tissues by one month. This expression is predominant in the oligodendrocytes suggesting that any changes observed are due to effects on this cell type.

It is remarkable that despite the timing issue discussed above, there is significant myelin abnormalities in specific CNS regions in β1 integrin ΔC mice. The tissue differences in the extent of the defects may reflect the fact that myelination is a gradual process that is initiated and completed in different CNS tissues at various times during embryogenesis and after birth. The mild phenotype observed in certain CNS regions could also be attributed to the fact that other integrin receptors are expressed throughout oligodendrocyte development (Milner and ffrench-Constant, 1994) and their involvement may be missed in our model. The variability in myelination defects may also be due to some other differences in the areas examined, such as differences in integrin or laminin redundancy.

Interestingly, other mouse models of defective myelination also have region-specific differences. Laminin deficient *dystrophia muscularis* (*dy/dy*) mice and Fyn-knockout mice exhibit regional differences in myelination (Chun et al. 2003; Sperber et al. 2001). The *dy/dy* mice demonstrate hypomyelination in the corpus callosum and optic nerve but not in the spinal cord (Chun et al. 2003). Furthermore, the *dy/dy* mice display preferential hypomyelination in small sized axons in the corpus callosum (Chun et al. 2003). In comparison, the PLP-β1 integrin ΔC mice display hypomyelination in the optic nerve and spinal cord but not in the corpus callosum. Analysis of the spinal cord demonstrated hypomyelination preferentially in small sized axons which may be attributed to the CNS's preferential myelination of large sized axons earlier than small sized axons (Schwab and Schnell 1989). As well, it has been demonstrated that

oligodendrocyte cell bodies are closely associated to large sized axons, and therefore their processes are readily accessible to myelinate those axons (Hildebrand et al. 1993). Therefore, if there is a defect in oligodendrocytes, the inability to extend processes and myelinate the small sized axons that are not in close proximity may result in a more severe hypomyelination in those axons. We also can not rule out the possibility that myelin may induce increased axon caliber, and if fewer axons are myelinated, then the lack of myelin may be directly responsible for the reduction in axonal caliber. Therefore, for our transgenic model, the question remains whether the differences that are observed are due to the timing of myelination, the timing and specificity of transgene expression, the requirement of β1 integrin in selected areas of myelination, or a combination of some or all of the above.

Development of models to assess remyelination after a demyelinating event is valuable to uncover the molecular mechanisms implicated. The cuprizone model allows for induction of demyelination and complete remyelination to occur in the corpus callosum over a 12 week period, making it an ideal model to use in mimicking Multiple Sclerosis (Arnett et al. 2001; Matsushima and Morell 2001). As expected, in our studies the non-transgenic littermates fully recovered from the demyelination in the corpus callosum after six weeks of recovery. The majority of the axons had significant remyelination and there were few unmyelinated axons remaining. Similarly, the β1 integrin ΔC mice were able to remyelinate axons within the corpus callosum. However, the number of axons that underwent remyelination in the dominant-negative mice was significantly lower than in wild type littermates. This finding indicates that the number of oligodendrocyte precursors may be reduced in the β1 integrin ΔC mice and/or that the

differentiated oligodendrocytes present may be unable to extend processes to distant axons. Thus, the finding that an inherited cause of mild hypomyelination can later exhibit more pronounced hypomyelination after toxin exposure is intriguing and supports the concept that a certain amount of Multiple Sclerosis could be the product of an underlying myelination problem that worsens when another factor injures CNS myelin.

The β1 integrin cytoplasmic domain is important in cell adhesion, spreading, migration and matrix assembly (LaFlamme et al. 1994). Expression of single-subunit chimeras containing β1 integrin intracellular and transmembrane domains but lacking the extracellular domain function in a dominant-negative manner to inhibit endogenous integrin function in cell migration, spreading, adhesion, and matrix assembly in a number of cell and organ systems (Chen et al. 1994; Faraldo et al. 1998; Faraldo et al. 2000; Faraldo et al. 2001; LaFlamme et al. 1994; Relvas et al. 2001; Zimmerman et al. 2000). Our approach of using a β1 integrin ΔC protein in which the cytoplasmic tail is absent has similarly shown the importance of β1 integrin in myelination/remyelination and that the cytoplasmic domain is playing a crucial role in this context. The absence or delayed remyelination seen in some of the axons within the corpus callosum of the cuprizone induced β1 integrin ΔC mice could be attributed to the inability of the β1 integrin to associate with various binding partners such as ILK or α-actinin with the cytoplasmic domain, thereby leading to a disruption in biochemical signaling pathways and/or a disruption in the scaffolding provided by the structural binding partners.

The β1 integrin signaling pathway has been extensively studied with various downstream effectors such as FAK and Fyn (Faraldo et al. 2001; Sperber et al. 2001). Using *dy/dy* mice, the importance of β1 integrin downstream effectors ILK and PI3K in

myelin membrane formation has been demonstrated (Chun et al. 2003). Additionally, recent work by Colognato et al. (2002) suggests a model of integrin-growth factor synergy for oligodendrocyte development. This model proposes that neuregulin alone promotes proliferation and inhibits differentiation through the PI3-kinase pathway. However, when oligodendrocytes contact axonal laminin, the combination of laminin and neuregulin activates an integrin mediated switch that promotes survival and differentiation through the MAP-kinase pathway, therefore allowing myelination to proceed. Our data shows that the β1 integrin ΔC mice have lower MAP kinase activity, as judged by a decrease in phosphorylation, during both myelination and remyelination. Therefore, in the β1 integrin ΔC mice, the truncation of the cytoplasmic domain may be preventing oligodendrocyte differentiation by inhibiting the engagement of key factors within the MAPK pathway and effectively disrupting myelination and remyelination. As well, we have demonstrated that the MAPK pathway is important during remyelination, as emphasized with the upregulation of MAPK phosphorylation within the corpus callosum of recovering non-transgenic mice.

In summary, our work has highlighted the importance of β1 integrin-mediated signaling in the process of myelination within the CNS. The transgenic models presented here will allow for further elucidation of the molecular mechanisms underlying β1 integrin's role in this process.

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Chapter 3. β1 integrin promotes the survival of oligodendrocytes during remyelination

β1 integrin promotes the survival of oligodendrocytes during remyelination

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Abstract

Myelination ultimately depends on the proper proliferation, migration, and differentiation of oligodendrocyte precursor cells into myelin producing cells within the CNS. Recent studies have demonstrated the importance of β1 integrin in oligodendrocyte maturation *in vitro*. Here, we demonstrate the importance of β1 integrin in myelination *in vivo*. We have generated a transgenic mouse line that expresses a dominant-negative β1 integrin ΔC transgene under the control of the proteolipid protein (PLP) promoter that drives expression of the transgene specifically to CNS tissues. The dominant-negative β1 integrin ΔC transgenic mice have hypomyelinated and increased numbers of unmyelinated axons in the spinal cord and the optic nerve; however the corpus callosum remains unaffected. The dominant-negative β1 integrin ΔC transgenic mice, under a demyelination and remyelination model, demonstrate a significantly reduced number of remyelinated axons within the corpus callosum when compared with wild type mice. The defects in myelination and remyelination in the dominant-negative β1 integrin ΔC mice were attributed to the disruption of the MAPK signaling pathway. Further histological and immunohistochemical analysis on tissue sections from the CNS of the dominant-negative β1 integrin ΔC transgenic mice under the demyelination and remyelination model demonstrate that there is a decrease in the number of proliferating oligodendrocytes, followed by a decrease in the number of mature oligodendrocytes. The reduction in the number of proliferating oligodendrocyte progenitors and mature oligodendrocytes may be attributed to an increase in the occurrence of apoptosis in the

dominant-negative mice. The increased apoptosis is coupled with an increase in MAPK phosphorylation. Our results highlight the importance of β1 integrin for oligodendrocyte survival *in vivo*.

Introduction

The ability of vertebrates to integrate communication to the entire central nervous system (CNS) in an effective manner relies on the presence of a healthy and functional myelin sheath. The myelin sheath plays an important role as an insulator of axons, allowing for saltatory conduction down nerves, and ultimately allowing for proper co-ordination and movement of the organism. During dysmyelination and demyelination, effective communication in the CNS is disrupted and the consequences can be quite devastating. An example of this is in Multiple Sclerosis (MS), a demyelinating disease where loss of myelin in the white matter results in impairment of the function of the CNS. When myelin is damaged, the body reacts by attempting to initiate remyelination. Unfortunately, for those who suffer from MS, there is a delay or inability to reform the lost or damaged myelin.

During oligodendrocyte development, actively proliferating oligodendrocyte progenitor cells (OPCs) migrate from the ventricular zones towards axons within the CNS (Noll and Miller 1993; Tsai and Miller 2002). Upon contact with the axons, the OPCs commence differentiation and begin the process of forming myelin around the axons (Barres and Raff 1999). However, there are many OPCs that remain as an evenly distributed undifferentiated group of cells within the CNS and are defined as adult OPCs. The adult OPCs have a distinct multipolar morphology and express cell surface markers such as the NG2 proteoglycan and the PDGFα receptor (Dawson et al. 2000; Dawson et al. 2003; Nishiyama et al. 1996). Recent work has demonstrated that the endogenous pool of adult OPCs are responsible for remyelination. It is believed that remyelination

progresses in a similar manner as the initial myelination, where the pool of endogenous adult OPCs are activated and recruited to the damaged area to reform new myelin (Zhao et al. 2005). However, the factors that are involved in this process are not well understood.

Previously, it has been shown that integrins are important in the process of myelination. *In vitro* analysis determined that oligodendrocytes express a specific set of integrins (Milner and Ffrench-Constant 1994). αvβ1, αvβ3 and αvβ5 are sequentially upregulated throughout oligodendrocyte development and downregulated during differentiation, while αvβ8 and α6β1 are expressed throughout oligodendrocyte development. The importance of each integrin heterodimer in oligodendrocyte development is still an area of investigation. However, integrin blocking experiments in cultured oligodendrocytes highlighted that β1 integrin was the subunit responsible for myelin membrane formation (Buttery and ffrench-Constant 1999).

Recent *in vivo* work has highlighted the importance of β1 integrin in the process of myelination and remyelination. We have described a line of transgenic mice (PLP-β1 integrin ΔC mice, henceforth to be called the dominant-negative β1 integrin mouse model) in which a dominant-negative version of β1 integrin is expressed predominantly in the oligodendrocytes of the CNS (Lee et al. 2006). The resulting effect is dysmyelination and demyelination in the optic nerve and spinal cord of the transgenic mouse. Furthermore, when these mice are subjected to cuprizone-induced demyelination, they display impaired or delayed remyelination in the corpus callosum (Lee et al. 2006). The dominant-negative β1 integrin mouse model demonstrates that β1 integrin is important both in myelination and remyelination *in vivo*.

In the current study, we have further examined the role of β1 integrin in oligodendrocyte development during remyelination *in vivo*. We demonstrate that β1 integrin is important in the maintenance and survival of adult OPCs, thereby ensuring proper remyelination capacity in the CNS.

Materials and Methods

Induction of demyelination and remyelination

The dominant-negative β1 integrin mouse model was generated as previously described (Lee et al. 2006). To induce demyelination, 6 week old mice were fed Harlan Teklad mouse chow containing 0.2% cuprizone (Sigma, MS) for a period of 6 weeks. Cuprizone was incorporated within the pelleted chow. Remyelination was induced by returning the mice to a normal diet. A total of 3 mice per time point were analyzed.

Bromodeoxyuridine incorporation and Immunohistochemistry

For the proliferation experiments, intraperitoneal injection of 100 mg/kg 5-bromo-2-deoxyuridine (BrdU) (Sigma) in DMEM was performed every 8 hours for 4 days prior to culling of the mice. Mice were anaesthetized and perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were further fixed overnight with 4% PFA and embedded in paraffin. Coronal sections of 5 micrometer thickness at the fornix region of the corpus callosum were obtained. Paraffin sections were deparaffinised with xylene and treated with 0.1 M citric acid buffer (0.24% citric acid, 2.5% sodium citrate, pH 6.0) for antigen unmasking. Sections were stained with NG2 antibody at a 1:50 dilution (a gift from Dr. W. Stallcup) and CC-1 antibody at a 1:10 dilution (Abcam) using the Vectastain Elite Kit (Vector Laboratories). To obtain cryosections, PFA fixed brains were incubated overnight in 4% PFA and further incubated overnight in 30% sucrose. Subsequently, brains were embedded in 30% sucrose and OCT (Sakura, CA). Coronal cryostat sections of 10 micrometer thickness at the fornix region of the corpus callosum were obtained. Frozen sections were fixed with 70% ethanol. Sections stained for BrdU, were treated

with 2 N HCl for 20 minutes at 37°C followed by a 10 minute incubation in 0.1 M Tris-HCl pH 8.8. Sections were blocked and permeabilized with TBLS (0.05% 1 M Tris pH 7.4, 0.00085% NaCl, 0.01% bovine serum albumin (BSA), 0.009% L-lysine, and 10% sodium azide) with 30% goat serum and 0.3% Tritin-X-100. Sections were incubated at 4°C in primary antibodies diluted in TBLS at 1:100 for BrdU and 1:50 for NG2. Sections were incubated with secondary rabbit antibodies diluted 1:200. The TUNEL assay was performed on frozen sections. Slides were treated with 0.1 M citric acid buffer and sections were treated as per instructions for treatment of cyropreserved tissue from In situ Cell Death Detection Kit, Fluorescein (Roche Applied Science).

Western Blot Analysis

The corpus callosum region was dissected from the brains of mice and homogenized on ice in RIPA buffer (40 mM Tris-HCl, pH 8.0, 276 mM NaCl, 20% glycerol, and 2% NP-40) containing 1 mM PMSF, 0.01 mg/ml aprotonin, 0.01 mg/ml pepstatin, 0.01 mg/ml leupeptin, 10 mM NaF and 10 mM Na_3VO_4. Reduced samples were analyzed by SDS-PAGE on standard 10% polyacrylamide gels and transferred semi-dry onto PVDF membrane. Membranes were blocked in 5% skim milk powder or 5% BSA in TBS-T (1 M Tris-HCl, pH 7.5, 5 M NaCl, and 0.1% Tween-20). Blots were incubated with antibodies to AKT, phospho-Akt (Ser473), p44/42 MAP kinase and phospho-p44/42 MAP kinase all at 1:1000 dilution (Cell Signaling; MA) for 1 hour. Bound primary antibodies were detected using horseradish peroxidase conjugated rabbit-anti-rat, goat-anti-rabbit or goat-anti-mouse secondary antibody (Jackson Immunoresearch, PA).

Protein bands were detected by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc., NJ).

Results

The number of oligodendrocyte precursor cells is decreased in the corpus callosum of dominant-negative β1 integrin mice

Previously, the dominant-negative mouse model when placed on the cuprizone diet demonstrated a delay or inhibition of remyelination within the corpus callosum when compared to non-transgenic mice. This defect implied that β1 integrin signalling in oligodendrocytes was important for initiating remyelination. However, the question remains as to when exactly β1 integrin contributes to the process of remyelination. It is possible that it could be important for oligodendrocyte survival, proliferation and/or differentiation. To assess the ability of the dominant-negative β1 integrin mice to remyelinate and to determine where a defect may lie in this process, the mice were subjected to experimental toxin-induced demyelination through the use of cuprizone in their diet. Over a course of the 12 week experiment (6 weeks on cuprizone diet followed by 6 weeks of recovery on regular diet), mice were analyzed at various stages of demyelination and remyelination. Immunocytochemistry was preformed on coronal brain sections of the brain at 3 and 4 weeks of demyelination and 2 weeks of remyelination (Figure 1A). The NG2 antibody was used to identify the number of OPCs present within the corpus callosum. Initially, as a control, we examined mice that had not been subjected to the cuprizone diet. At 6 weeks of age, in untreated mice, the dominant-negative β1 integrin mice had significantly fewer NG2 positive cells when compared to the non-transgenic wild type mice (Figure 1B). This trend of fewer NG2 positive cells in the corpus callosum was also observed in cuprizone fed transgenic mice at 3 and 4 weeks of demyelination, and at 2 weeks of remyelination when compared to non-transgenic

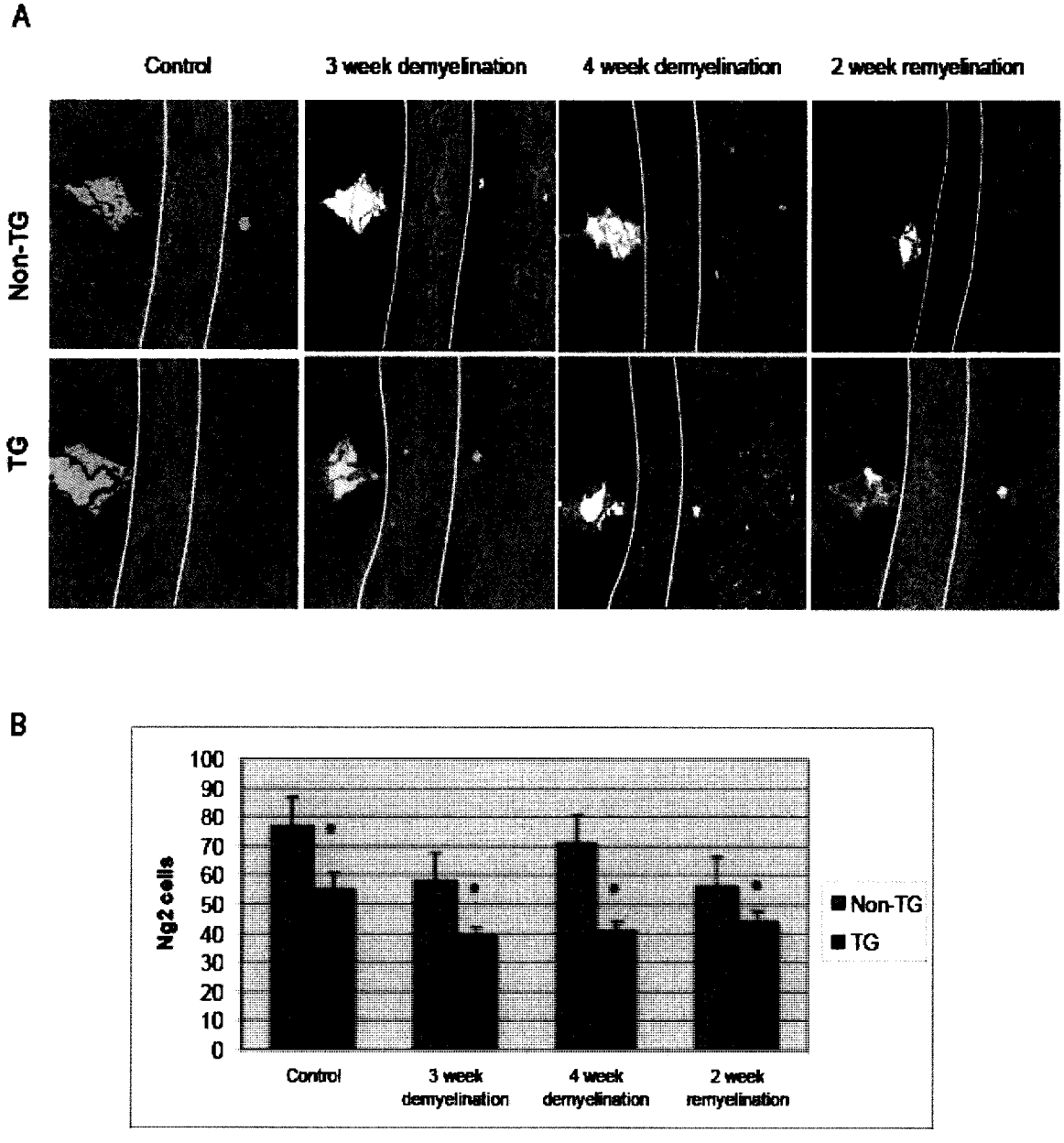


Figure 1. The number of oligodendrocyte progenitors is decreased in dominant-negative β1 integrin mice. A. Oligodendrocyte progenitors in the corpus callosum were identified by immunostaining for NG2 (pink). Various time points during demyelination and remyelination were examined. White lines delineate the region of the corpus callosum (CC). B. Total number of NG2 positive cells were quantified and there was a significant decrease in the number of oligodendrocyte progenitors in the dominant-negative β1 integrin mice (* $P < 0.05$).

mice (Figure 1B). These results suggest that β1 integrin is important for controlling the number of OPCs in the corpus callosum.

The number of proliferating oligodendrocyte precursor cells is decreased in the corpus callosum of the dominant-negative β1 integrin mice

Previous *in vitro* work has suggested that αvβ3 integrin is responsible for proliferation of oligodendrocyte precursor cells (Blaschuk et al. 2000). To assess if β1 integrin also contributes to the proliferation of oligodendrocyte progenitor cells, the dominant-negative β1 integrin mice and non-transgenic mice were injected with BrdU three days prior to analyzing the corpus callosum to identify proliferating NG2 positive cells. The mice were analyzed at 1, 2, 3, 4 and 5 weeks of cuprizone-induced demyelination (Figure 2A). At 1, 2, and 3 weeks of demyelination, the dominant-negative β1 integrin mice had significantly fewer proliferating OPCs when compared to the non-transgenic mice (Figure 2B). At 2 weeks of demyelination, there was an increase in the number of proliferating NG2 positive cells both in the transgenic and non-transgenic mice when compared to other time points throughout the demyelination regime (Figure 2B). Interestingly, at 4 and 5 weeks of demyelination both transgenic and non-transgenic mice have similar numbers of proliferating oligodendrocyte precursor cells (Figure 2B). The overall decrease in proliferating oligodendrocyte precursor cells in the dominant-negative β1 integrin mice suggests the potential involvement of β1 integrin early in oligodendrocyte development.

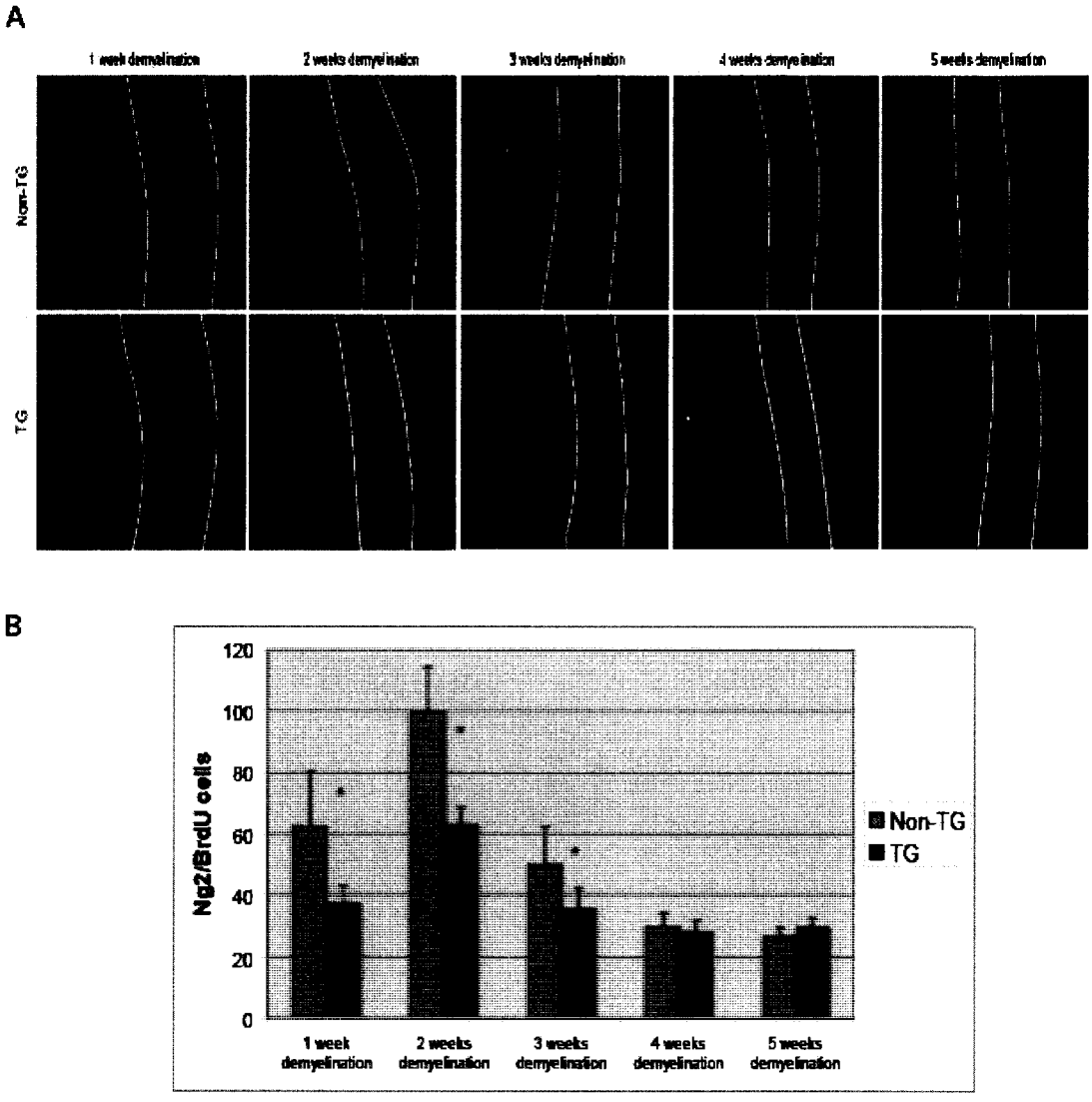
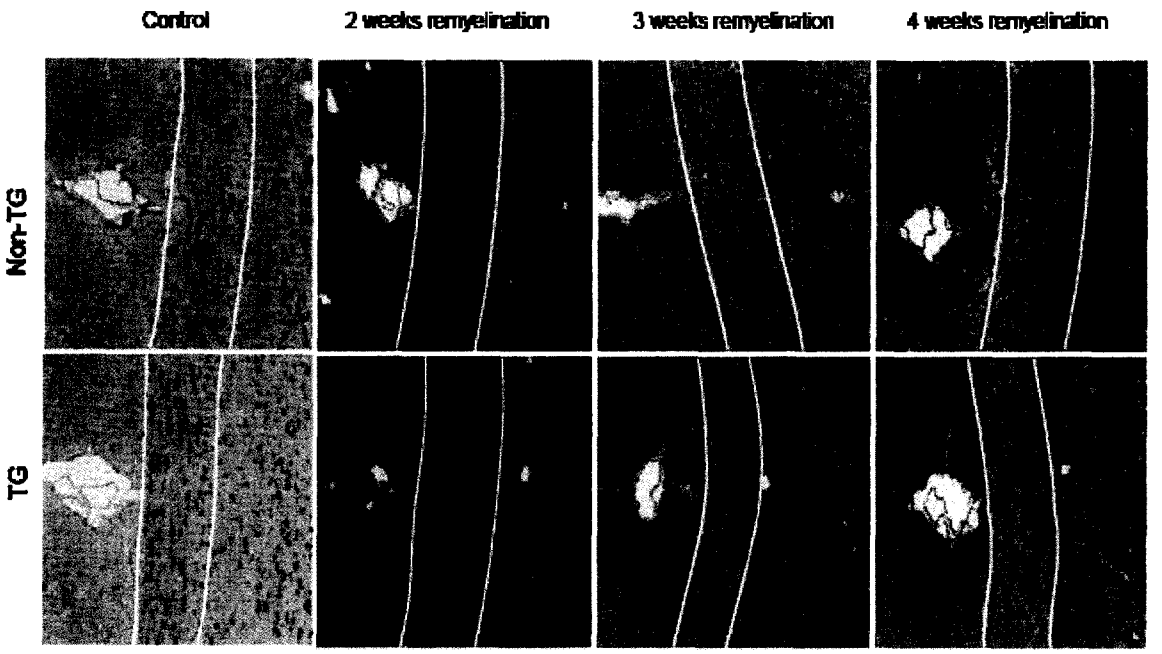


Figure 2. The number of proliferating oligodendrocyte progenitors is decreased in dominant-negative β1 integrin mice. **A.** Proliferating oligodendrocyte progenitors in the corpus callosum were identified by double immunostaining for NG2 (red) and BrdU (green) at various time points during demyelination. White lines delineate the region of the corpus callosum (CC). **B.** Total number of NG2/BrdU double-positive cells were quantified and there was a significant decrease in the number of proliferating oligodendrocyte progenitors at 1, 2 and 3 weeks of demyelination in the dominant-negative β1 integrin mice (* $P < 0.05$).

The number of mature oligodendrocytes is decreased in the corpus callosum of the dominant-negative β1 integrin mice

Previously we had demonstrated that the expression of a dominant-negative β1 integrin inhibits or delays remyelination in axons within the corpus callosum of mice that have been administered the cuprizone diet (Lee et al. 2006). Therefore, to determine if the hypomyelination observed in the dominant-negative β1 integrin mice could be attributed to a decrease in the number of mature oligodendrocytes, we quantified various time points of remyelination with a marker for mature oligodendrocytes, namely adenomatous polyposis coli (CC-1) (Figure 3A). At 6 weeks of age, the control untreated transgenic and non-transgenic mice have similar levels of CC-1 positive cells in their corpus callosum (Figure 3B). However, at 2, 3 and 4 weeks of remyelination after cuprizone-induced demyelination, the dominant-negative β1 integrin transgenic mice have significantly fewer CC-1 positive cells in the corpus callosum when compared to the non-transgenic mice (Figure 3B). Thus, the reduced number of proliferating oligodendrocytes during early demyelination correlates with the observed decrease in the number of mature oligodendrocytes during remyelination. The reduction in the number of mature oligodendrocytes is also consistent with the previously observed inhibition or delay in the ability of the dominant-negative β1 integrin mice to remyelinate after cuprizone-induced demyelination (Lee et al. 2006).

A



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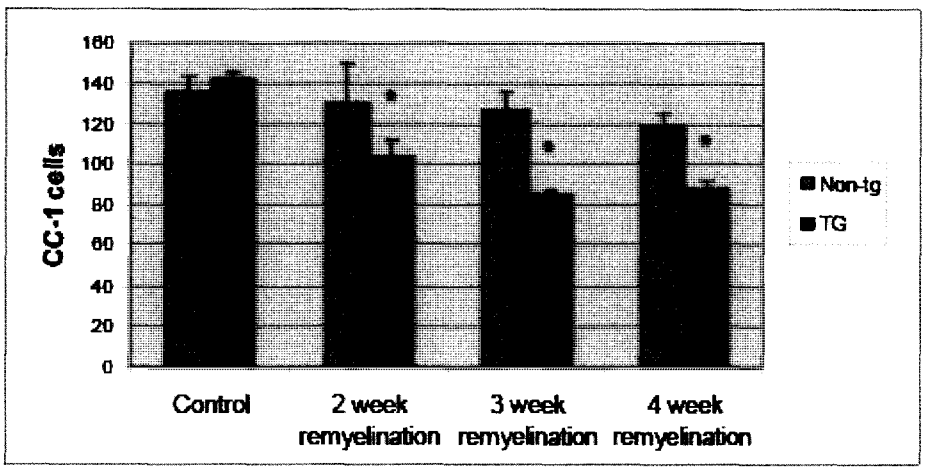

Figure 3. The number of mature oligodendrocytes is decreased in the dominant-negative β1 integrin mice. A. Mature oligodendrocytes in the corpus callosum were identified by immunostaining for CC-1 (brown) at various time points during remyelination. White lines delineate the region of the corpus callosum (CC). B. Total number of CC-1 positive cells were quantified and there was a significant decrease in the number of mature oligodendrocytes in the dominant-negative β1 mice (* $P < 0.05$).

Increased apoptosis in the corpus callosum of the dominant-negative β1 integrin mice

To address the decrease in the number of OPCs and mature oligodendrocytes in the corpus callosum of dominant-negative β1 integrin mice, we quantified the number of apoptotic TUNEL-positive cells in the same region of the corpus callosum. In the non-transgenic mice, over the course of 5 weeks of cuprizone-induced demyelination, we observe a progressive increase in the number of TUNEL-positive cells (Figure 4). However, at 1, 2 and 3 weeks of demyelination in the dominant-negative β1 integrin transgenic mice, there are significantly more TUNEL-positive cells when compared to the non-transgenic mice (Figure 4). In contrast, at 4 and 5 weeks of demyelination, the dominant-negative β1 integrin transgenic mice have significantly fewer TUNEL-positive cells compared to the non-transgenic mice (Figure 4). Interestingly, at 2 weeks of demyelination, the dominant-negative β1 integrin mice not only display a significant increase in the number of TUNEL-positive cells when compared to the non-transgenic mice, but also have significantly more TUNEL-positive cells then at all the other time points analyzed (Figure 4). The observed increase in the number of apoptotic cells in the dominant-negative β1 integrin transgenic mice at 2 weeks of demyelination correlates with the dramatic decrease in the number of proliferating OPCs observed at the same time point. Therefore, these results suggest that β1 integrin may be important in the survival of OPCs cells during demyelination.

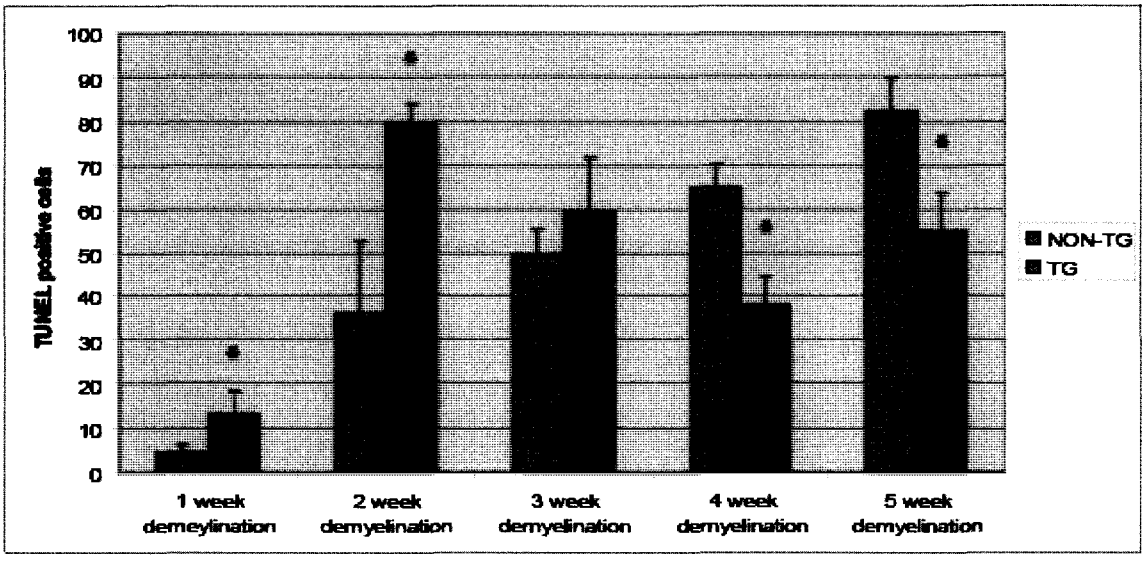

Figure 4. The number of apoptotic cells is increased in the dominant-negative β1 integrin mice early in demyelination. Apoptotic cells in the corpus callosum were identified by TUNEL staining at various time points during demyelination. Total number of apoptotic cells were quantified and there is a significant increase in the dominant-negative β1 integrin mice at 1 and 2 weeks of demyelination (* $P < 0.05$).

Downstream signalling pathways affected during remyelination

With the increase in cell death specifically at 2 weeks of demyelination in the corpus callosum of the dominant-negative β1 integrin transgenic mice, we went on to explore the signal transduction pathways that were potentially affected. Previously, we had observed a significant decrease in MAP-kinase phosphorylation in the dominant-negative β1 integrin mice after cuprizone treatment followed by 3 weeks of recovery when compared to non-transgenic mice (Lee et al. 2006). To determine if the MAP-kinase pathway is affected during the early phases of demyelination, we extracted protein from the corpus callosum of dominant-negative β1 integrin mice and non-transgenic mice. Our analysis revealed that at 2 weeks of cuprizone-induced demyelination, total MAP-kinase levels were equal between non-transgenic and dominant-negative β1 integrin mice (Figure 5). In contrast, the levels of phosphorylated MAP-kinase in the dominant-negative β1 integrin mice were increased when compared to the non-transgenic mice (Figure 5). These results suggest the importance of MAP-kinase acitivity at 2 weeks of demyelination, where the dominant-negative β1 integrin mice have increased apoptosis and increased proliferation of OPCs.

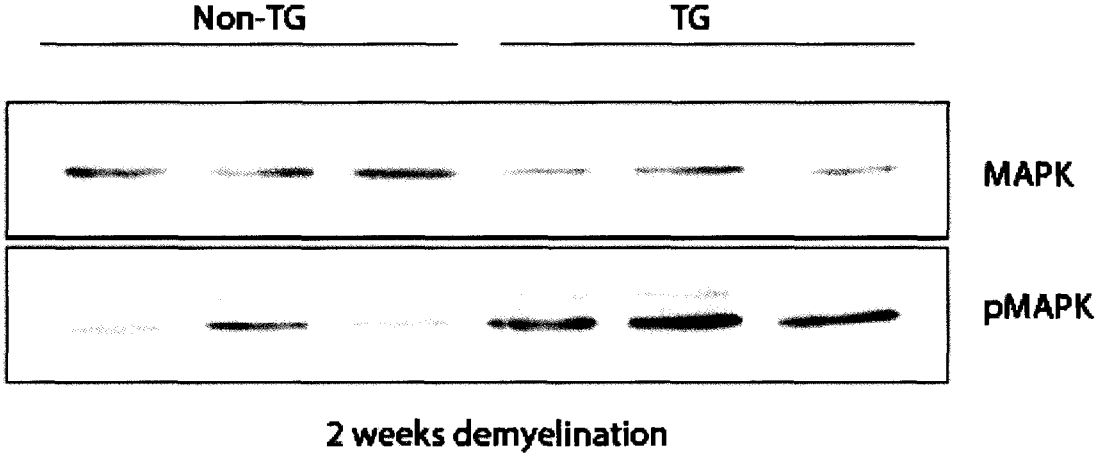

Figure 5. Increase in the levels of phosphorylated MAP-kinase in the dominant-negative β1 integrin mice at 2 weeks of demyelination. Western blot analysis of protein (20 μg) from the corpus callosum of mice under 2 weeks of cuprizone treatment. Extracts from 3 independent mice for each genotype was analyzed and the total MAPK levels were equal between the non-transgenic and dominant-negative β1 integrin mice. MAPK phosphorylation was increased in the dominant-negative β1 integrin mice when compared to the non-transgenic mice.

Discussion

Myelination and remyelination of axons in the CNS requires interplay between the oligodendrocyte, the axon, and the extracellular matrix. Here, we have focused our work on the role that one cell membrane receptor, β1 integrin, plays in the process of remyelination. To do this, we take advantage of a mouse model expressing a dominant-negative β1 integrin in an oligodendrocyte-specific manner (Lee et al. 2006). We have previously demonstrated that in this mouse model, there are a significant number of unmyelinated axons during remyelination after cuprizone-induced demyelination. This highlighted the importance of β1 integrin in remyelination *in vivo* but it was not clear as to what stage of oligodendrocyte development was being affected in these mice. We now demonstrate that β1 integrin is important in oligodendrocyte progenitor cell survival. The decreased survival of the oligodendrocyte progenitor cells in the corpus callosum of cuprizone treated dominant-negative β1 integrin mice leads to an overall decrease in the number of proliferating oligodendrocyte progenitor cells and the number of mature oligodendrocytes. This ultimately leads to an increase in the number of axons that are unmyelinated.

The cuprizone model allows for initiation of demyelination and complete remyelination over a course of 12 weeks (Matsushima and Morell 2001). This experimental demyelination model is ideal for studying the process of remyelination. Remyelination had been previously thought to be carried out by surviving oligodendrocytes, however more recent studies have demonstrated that remyelination results from a pool of adult oligodendrocyte progenitors present in the adult CNS

(Blakemore and Keirstead 1999). In the dominant-negative β1 integrin mice, at 6 weeks of age, the corpus callosum did not display any defects in myelination (Lee et al. 2006). However, when the transgenic mice were subjected to cuprizone-induced demyelination and then allowed to undergo a period of remyelination, the corpus callosum of the transgenic mice displayed a significant decrease in the number of axons that were remyelinated (Lee et al. 2006). The PLP promoter, which was used to drive the expression of the dominant-negative β1 integrin transgene, is active beginning at P5, while myelination in the brain commences prior to birth. It is therefore possible that the timing of the activation of the PLP promoter may not fully overlap with the timing of myelination in the brain. By the age of 6 weeks, the dominant-negative β1 integrin mice fully express the transgene, and therefore make possible the assessment of the role of β1 integrin in oligodendrocyte development during remyelination.

At 6 weeks of age, the dominant-negative β1 integrin mice display significantly fewer adult oligodendrocyte progenitor cells and maintain a lower than normal number of adult oligodendrocyte progenitor cells over the course of demyelination. Furthermore, this decrease in the number of adult oligodendrocyte progenitor cells is translated into a decrease in the number of proliferating oligodendrocyte progenitor cells and a decrease in the number of mature oligodendrocytes. The observed overall decrease in adult oligodendrocyte progenitor cells and mature oligodendrocytes may be attributed to an increase in apoptosis early in the course of demyelination. Taken together, these observations indicate the importance of β1 integrin in the maintenance and survival of adult oligodendrocyte progenitor cells.

Previous *in vitro* and *in vivo* work has demonstrated the importance of α6β1 integrin for oligodendrocyte survival in the developing CNS. In survival assays, newly formed oligodendrocytes grown on laminin in the presence of polyclonal antiserum against β1 integrin demonstrate reduced survival. However, increasing amounts of the growth factor PDGF in culture ameliorates the survival rate (Colognato et al. 2007; Frost et al. 1999). *In vivo*, the α6 integrin-null mice at E18.5 exhibit half the number of mature oligodendrocytes and an increase in apoptosis when compared to their wild-type littermates (Colognato et al. 2002). While the oligodendrocyte-specific β1 integrin knock-out mouse did not display a difference in the number of mature oligodendrocytes when compared to control mice, the mice did have increased apoptosis of premyelinating oligodendrocytes in the peripheral regions of the cerebellum (Benninger et al. 2006). These *in vitro* and *in vivo* models demonstrate the importance of β1 integrin in myelination during development and in the survival of oligodendrocytes during this period. Our current dominant-negative β1 integrin mouse model further provides evidence that β1 integrin is also important in the survival of adult oligodendrocyte progenitor cells. Furthermore, at 2 weeks demyelination, we observe a significant increase in the number of apoptotic cells in the corpus callosum of the dominant-negative β1 integrin mice, therefore further reinforcing the importance of β1 integrin in protecting oligodendrocytes to demyelination insults and/or on its role in maintaining the adult oligodendrocyte progenitor pool. To fully appreciate the role of β1 integrin in survival and maintenance of the oligodendrocyte progenitor pool, further work is required between the various mouse models to carefully analyze the stages of oligodendrocyte development where OPC pool development is critical.

The two mouse models that analyze the role of β1 integrin in myelination *in vivo* have resulted in contradictory observations. The approach of conditional ablation of β1 integrin demonstrated early apoptosis but normal myelination and remyelination. Our approach of generating a dominant-negative β1 integrin mouse model resulted in myelin defects in the optic nerve and spinal cord, while the corpus callosum demonstrated a delay or inhibition of remyelination. Here, we show that this dominant-negative β1 integrin mouse model also exhibits increased apoptosis during remyelination. Why the two models display differing outcomes has not been completely resolved but it has been suggested that there might be a compensatory mechanism at work in one of the models (Benninger et al. 2006; Colognato et al. 2007; Laursen and Ffrench-Constant 2007). Recently, a second laminin receptor, dystroglycan, was shown to play a role in myelination. Colognato et al. propose models of integrin and dystroglycan hierarchies, where laminin may have a preference of binding to integrins during early oligodendrocyte formation and to dystroglycan later in oligodendrocyte maturation (Colognato et al. 2007). Conversely, laminin may interact in parallel with integrin and dystroglycan, however signalling changes may alter the contribution of each receptor during various stages of oligodendrocyte development (Colognato et al. 2007). In the conditional ablation of β1 integrin in oligodendrocytes, it is possible that apoptosis is apparent early in development due to β1 integrin's role in survival of early oligodendrocytes, however, later in development myelination resumes normally due to the ability of dystroglycan to compensate for the loss of β1 integrin. In the dominant-negative β1 integrin mouse model, it is possible that expressing a β1 integrin subunit that excludes the cytoplasmic domain, allows for the extracellular domain to continue

dimerization with the α subunits and thereby continue to bind to laminin. The continued association of the β1 integrin extracellular subunits to laminin perhaps limits the availability of laminin to bind dystroglycan and therefore not allowing for any significant compensatory effects. Furthermore, the ability of the β1 integrin extracellular domain to bind to the α subunits results in activation of signalling, however with the absence of the cytoplasmic domain on the β1 integrin subunit, there is no outlet for the signals to transmit within the cell or a cytoplasmic domain for protein binding and therefore further inhibiting any downstream signalling effects of β1 integrin. This would thereby create a true dominant-negative β1 integrin effect and allows for the specific study of the role of β1 integrin in myelination and remyelination.

We have previously observed a decrease in MAP-kinase phosphorylation during myelination in the optic nerve and spinal cord, and in the corpus callosum during remyelination in our dominant-negative β1 integrin mouse model when compared to the control mice (Lee et al. 2006). Here we have assessed what signalling pathways are affected coincident with the survival of the oligodendrocytes at the earlier time point of 2 weeks demyelination, at the peak of apoptosis in the dominant-negative β1 integrin mice. Interestingly, at this time point of demyelination, the corpus callosum from the dominant-negative β1 integrin transgenic mouse has increased levels of phosphorylated MAP-kinase, indicating an increased activity of the MAP-kinase pathway in oligodendrocytes from this region. The MAPK pathway has been proposed to play a role in both survival of oligodendrocytes and in myelin membrane formation (Colognato et al. 2002). The demyelination insult of cuprizone results in cell death of mature oligodendrocytes and at the same time prompts adult OPCs to commence the process of remyelination to recover

the demyelinated lesions in the corpus callosum (Matsushima and Morell 2001). At 6 weeks of age, the dominant-negative β1 integrin mice have significantly fewer adult oligodendrocyte progenitor cells, which would suggest that there are fewer adult OPCs readily available to participate in remyelination. With the decreased number of adult OPCs, the increased MAPK phosphorylation in the corpus callosum may be an attempt to compensate for this loss. In our dominant-negative β1 integrin mice, we did not observe complete inability to remyelinate, in fact the axons that were remyelinated had normal g-ratios, indicating that there were adult OPCs that were able to remyelinate normally (Lee et al. 2006). This raises the possibility that another receptor, such as dystroglycan, is able to compensate for the inability of β1 integrin to participate in survival and myelin membrane formation (Colognato et al. 2007).

The increased MAPK phosphorylation observed at 2 weeks of demyelination may be due to an early promotion of remyelination. Not only is 2 weeks of demyelination the time point of increased apoptosis in the dominant-negative β1 integrin mice, but also the time of increased oligodendrocyte progenitor proliferation. It is possible that with the significant increase of cell death, the OLs are attempting to initiate remyelination earlier than in non-transgenic mice to compensate for the loss of OPCs. Correspondingly, at 3 weeks of remyelination, we observe a decrease in MAPK activity in the dominant-negative β1 integrin mice which could be attributed to an early initiation of survival and myelin membrane formation during the early demyelination time points of the cuprizone model.

In summary, we have demonstrated the importance of β1 integrin in the maintenance and survival of adult OPCs during remyelination. The transgenic model

presented here has allowed for specific studies of β1 integrin's role in myelination and in remyelination. What is clear though is that further work is required to determine how β1 integrin's role is shared with other receptors to ensure proper regulation of myelination and remyelination in the CNS.

Acknowledgments

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Chapter 4. General Discussion

4.1 The importance of β1 integrin in myelination of the CNS

Integrins are cell surface glycoproteins that integrate the signals between cells and the cell extracellular matrix (Hynes 2002). They are involved with many aspects of cellular processes including survival, proliferation, migration and differentiation. Integrins are composed of heterodimers of α and β subunits. Currently, there are 18 α subunits and 8 β subunits known, and together these subunits can form 24 different heterodimers that have their own binding specificity to various extracellular matrices which help to establish unique signaling properties. The β1 integrin subunit is expressed in a variety of cell types including neurons, glial cells, meningeal cells and endothelial cells. β1 integrin expression is regulated in a time and regions specific manner during development (Jones and Walker 1999; Pinkstaff et al. 1999). In this thesis, we focus on the β1 integrin subunit and its importance in CNS myelination.

Myelination is a process that requires precise cues from the oligodendrocyte environment for the orchestration of cell to cell interactions. It is these interactions that are required for the eventual production of myelin and the wrapping of the myelin sheath around axons. Various signals have been identified to play roles in oligodendrocyte development and CNS myelination. Oligodendrocytes were shown to express a specific set of integrins dependent on their stage of development (Milner and Ffrench-Constant 1994). The β1 integrin is the only β subunit that has been shown to be expressed at all stages of oligodendrocyte development. Through *in vitro* analysis, it has been shown that β1 integrin is important in myelin membrane formation, while the work presented here is

the first to demonstrate *in vivo* that β1 integrin is important for myelination in the optic nerve and spinal cord (Buttery and ffrench-Constant 1999; Lee et al. 2006).

4.2 Regional differences in myelination within the CNS

In the dominant-negative β1 integrin mouse that we have generated and characterized, dysmyelination and hypomyelination are apparent in the optic nerve and spinal cord but not in the corpus callosum. This regional difference in myelin defects is also observed in other mouse models. The *Wave1* null mice, the laminin deficient *dy/dy* mice and the *Fyn* null mutant mice have hypomyelination in the optic nerve and corpus callosum but not the spinal cord (Chun et al. 2003; Kim et al. 2006; Sperber et al. 2001). In contrast, the mice haploinsufficient for type III neuregulin-1 display hypomyelination in the brain but not in the optic nerve or spinal cord (Taveggia et al. 2008). These regional myelin defects highlight the potential for differences in the control of CNS myelination, with β1 integrin potentially having a greater role in the optic nerve and spinal cord. At the same time, recent work has also demonstrated that there is potential for OPC diversity (Chandran et al. 2003; Kessaris et al. 2006). During CNS development, it is presumed that the neural progenitors of the oligodendrocyte lineage originate from the ventral ventricular zone of the neural tube, however it has now been shown that OPCs can also be derived from the dorsal region of the neural tube (Cai et al. 2005; Vallstedt et al. 2005). The ability of OPCs to be derived from two different zones leads to the likelihood that they may have differences in their responses to different signals. Therefore, different signaling pathways could lead to the generation of different oligodendrocytes or differences in the way that they behave. In this case, β1 integrin may

only be important in a specific subtype of oligodendrocytes that reside in the optic nerve and spinal cord. Further work is required to determine the different origins of OPCs and how various signaling pathways such as that mediated by β1 integrin are involved in their regulation.

4.3 The teachings of two different β1 integrin mouse models

Our dominant-negative β1 integrin mouse model demonstrates regional defects in myelination. Conversely, another mouse model where β1 integrin was conditionally deleted from oligodendrocytes did not display defects in myelination (Benninger et al. 2006). Although the two different models would seem contradictory on the surface, they bring to light an understanding of how β1 integrin might function in mediating oligodendrocyte maturation and myelin formation that would not have been possible with either model alone. It must be noted that the two mouse models differ in the method of generating a non-functional β1 integrin subunit in oligodendrocytes, which may have influenced the outcome of the contrasting phenotypes. In our work, the transgenic mice overexpressed a dominant-negative β1 integrin lacking the cytoplasmic domain, while Benninger et al. conditionally ablated β1 integrin in oligodendrocytes. Based on the combined findings from the two models, it has been suggested that perhaps a compensatory mechanism is responsible for the "rescue" in the null mouse (Colognato et al. 2007; Laursen and Ffrench-Constant 2007).

The approach of ablating β1 integrin results in the removal of β1 integrin, therefore the lack of β1 integrin could potentially allow another protein to compensate for the loss of β1 integrin and correct the potential myelin defect. On the other hand, in the

dominant-negative β1 integrin mouse, a mutant protein is produced that can interfere with signaling from the wild type protein in a dominant-negative manner. The β1 integrin that is expressed in the oligodendrocytes contains the extracellular domain and the intracellular domain, which leaves the potential for the β1 integrin ΔC subunit to bind an α integrin subunit. The formation of the integrin heterodimers may still allow for the binding to laminin in the ECM, but would not allow for the transmission of the downstream signals due to the lack of the appropriate cytoplasmic domain. Even though the dominant-negative β1 integrin is unable to relay downstream signals, its ability to bind laminin may inhibit other laminin receptors to bind and potentially trigger compensatory effects.

4.4 Dystroglycan – a second laminin receptor involved in oligodendrocyte-mediated myelin formation?

Recent work by Colognato et al. demonstrates that oligodendrocytes express the laminin receptor dystroglycan (Colognato et al. 2007). *In vitro*, dystroglycan appears to play a role in myelin formation, as the absence of dystroglycan results in the inability of oligodendrocytes to differentiate and produce myelin specific proteins (Colognato et al. 2007). In addition, laminin-enhancing effects for survival are reliant on β1 integrin and not dystroglycan, therefore bringing forth a potentially interesting model of myelination with β1 integrin regulating survival of oligodendrocytes and both β1 integrin and dystroglycan regulating myelin membrane formation (Figure 1). This model could explain the contrasting phenotypes in the two β1 integrin mouse models. In the

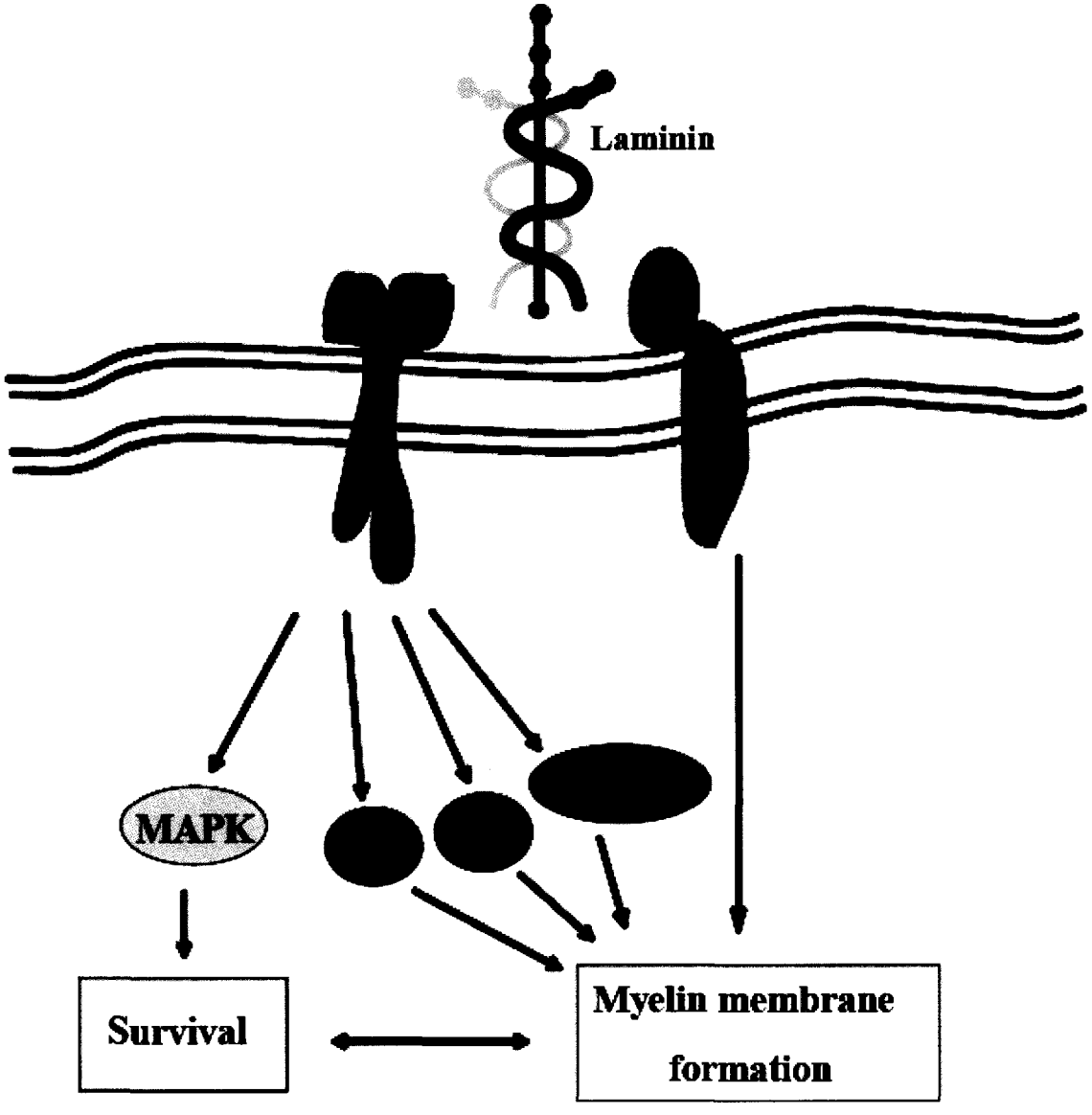

Figure 1. Model of β1 and dystroglycan interaction in myelination and remyelination. It is proposed that laminin is able to interact with both β1 integrin and dystroglycan, with β1 integrin's role focused in the survival of oligodendrocytes while dystroglycan is invovled in mylein membrane formation. It is possible that β1 integrin is invovled in myelin membrane formation through various downstream pathways that involve Fyn, ILK and WAVE1. There is also potential cross talk between the two pathways of β1 integrin and dystroglycan that can lead to compensation of failed receptor function.

conditionally ablated β1 integrin mouse model, dystroglycan signaling could rescue the myelin phenotype, while in the dominant-negative β1 integrin mouse model, dystroglycan is unable to bind laminin and therefore unable to rescue the myelin phenotype. Future *in vivo* work involving mouse models that address both β1 integrin and dystroglycan together are required to unravel the involvement of the two laminin receptors and how they may work in concert with one another in myelination.

4.5 The requirement of β1 integrin in the survival of oligodendrocytes

Remyelination is the process by which oligodendrocytes repopulate an area of the CNS that is demyelinated and initiate reformation of the myelin sheath. It is now well established that the adult CNS contains a pool of adult OPCs that generate oligodendrocytes with remyelinating capabilities. Understanding the demyelination response mechanisms that regulate the adult OPCs in their activation, proliferation, migration and differentiation for remyelination is crucial in finding better treatments or a cure for those who live with Multiple Sclerosis.

The dominant-negative β1 integrin mouse model did not reveal any myelin defect in the corpus callosum. However, with the aid of the cuprizone-induced model of demyelination/remyelination, the corpus callosum proved to be useful to study the role of β1 integrin in remyelination. Our experimental analysis using this model demonstrates that β1 integrin plays a role in remyelination. Specifically, β1 integrin is likely required for the maintenance and the survival of the adult OPCs. Several lines of evidence suggest a role of β1 integrin in the survival of oligodendrocytes (Benninger et al. 2006; Buttery and ffrench-Constant 1999; Colognato et al. 2007). It is hypothesized that the α6β1

integrin receptor is responsible for relaying downstream survival signals once the oligodendrocyte processes come in contact with laminin on the surface of axons (Colognato et al. 2002). In the conditionally ablated β1 integrin mouse, there was increased apoptosis in premyelinating oligodendrocytes within the cerebellum, even though there were no defects in myelination or remyelination. These results indicate the importance of β1 integrin in the survival of oligodendrocytes and the likely involvement of another laminin receptor, possibly dystroglycan, for myelination. In our mouse model, the number of adult OPCs is significantly reduced prior to and after the demyelination and remyelination episodes. At the same time, it appears that during demyelination, oligodendrocytes from the dominant-negative β1 integrin mouse have an increased susceptibility to cell death. Due to the dominant-negative nature of our β1 integrin mouse model and the possible inability of another laminin receptor to compensate for the loss of oligodendrocytes, a phenotype of decreased remyelination arises. Our results further confirm the role of β1 integrin in the survival of oligodendrocytes and demonstrate the importance of β1 integrin in remyelination.

4.6 The potential β1 integrin downstream signaling pathways within oligodendrocytes

Understanding the downstream signaling pathways of β1 integrin are valuable in determining what other proteins may be involved in the process of survival of oligodendrocytes and the process of myelination and remyelination. An integrin model of myelination proposed by Colognato et al. suggests that prior to the oligodendrocyte processes contacting the laminin on the surface of axons, the survival of the

oligodendrocytes is driven by growth factors such as neuregulin that activate the PI3 kinase pathway. However, once the oligodendrocytes processes contact laminin, this leads to the activation of the α6β1 integrin to trigger the MAPK downstream signaling pathway for both survival and myelin membrane formation (Colognato et al. 2002).

In our dominant-negative β1 integrin model, we extensively studied both the PI3 kinase and MAPK pathways during myelination and remyelination. The optic nerve and spinal cord contained lower levels of phosphorylated MAPK at 6 weeks of age, and at 3 weeks of remyelination the corpus callosum had significantly reduced levels of phosphorylated MAPK. This therefore infers the importance of the MAPK pathway in the survival of oligodendrocytes and/or myelination and remyelination mediated by oligodendrocytes (Figure 1). We also looked at the MAPK pathway at 2 weeks of demyelination. This particular time point had a significantly high number of apoptotic cells, therefore prompting further investigation into potential pathways involved in β1 integrin mediated survival of oligodendrocytes. Interestingly, we discovered the levels of phosphorylated MAPK in the corpus callosum were higher than in the control mice. The increased MAPK phosphorylation levels at this particular time point could be attributed to another survival factor or an attempt by dystroglycan to compensate for the increasingly high number of apoptotic cells. It is also possible that we are observing an early start of remyelination to compensate for the initially low numbers of adult OPCs. Other work has implicated the role of MAPK activity in myelination, specifically it has been shown that p38 MAPK is involved in the early stages of oligodendrocyte differentiation, however the upstream signals are still undefined (Fragoso et al. 2007)(Fragoso, 2007). Further work will be required to determine if β1 integrin is

implicated in the regulation of p38 MAPK activity and the other factors that are potentially involved in the β1 integrin survival and remyelination signaling pathways.

Oligodendrocytes have a complex cytoarchitecture that is characterized by an elaborate network of microtubules. These microtubules are important in the integrity of the cytoskeleton, which is an essential determinant of the function and survival of oligodendrocytes. Therefore, it must not be neglected that the myelination and remyelination defects observed in the dominant-negative β1 integrin mouse may be linked to the role of β1 integrin in regulating cytoskeletal proteins that are involved in migration of oligodendrocytes and in the morphological changes of oligodendrocytes, which if altered may lead to cell death. β1 integrin has previously been identified to mediate cell migration in a variety of cells (Brakebusch and Fassler 2005; Carlson et al. 2008; Hauzenberger et al. 1994). Previous work has demonstrated that the cytoplasmic domain of β1 integrin contains domains that are distinct in controlling both migration and survival of precursor oligodendrocytes (Buttery and ffrench-Constant 1999). Therefore, the disruption of β1 integrin function in our dominant-negative β1 integrin mouse could result in alterations or disruptions of the cytoskeleton, which may result in abnormal aggregates of cytoskeleton proteins that disable the OPCs to migrate and/or inhibit the OPCs to extended their processes and ultimately cause cell death and degeneration.

Numerous proteins such as ILK, Wiskott-Aldrich syndrome protein 1 (WAVE1), Fyn and Tau have been identified as important players in oligodendrocyte migration and morphogenesis (Chun et al. 2003; Gordon et al. 2008; Kim et al. 2006; Osterhout et al. 1999). New studies that allow for analysis of protein activity using the dominant-negative β1 integrin mouse model should be directed towards understanding how these proteins

may be regulated in the β1 integrin signaling pathway to further our understanding of β1 integrin's role in survival of oligodendrocytes during myelination and remyelination (Figure 1).

The dominant-negative β1 integrin mouse has proven to be a useful *in vivo* model to study the role or β1 integrin in myelination and remyelination. The dominant-negative β1 integrin mouse model has allowed for a better understanding of the specific role β1 integrin has in oligodendrocyte development, while it has also allowed for the unearthing of other potential partners in oligodendrocyte development. Our work suggests the importance of β1 integrin in the survival of oligodendrocytes and has shed more light on potential downstream targets that may be important players in initiating myelination and remyelination.

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Appendix A. The identification of ILK protein interactors during oligodendrocyte development in CG4 cells

The identification of integrin linked kinase interacting proteins in CG4 oligodendrocyte cells

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Abstract

The β1 integrin signaling pathway has proven to be an important player in oligodendrocyte-mediated production of the myelin sheath in the CNS. Integrin linked kinase (ILK) is a known binding partner of β1 integrin and recently has been shown to be important in myelin membrane production in oligodendrocytes in culture. In various cell types, ILK functions in numerous signaling pathways that ultimately impact cell behavior. However, how ILK functions at the molecular level in oligodendrocytes is not clearly defined. To begin to address this question, we have sought to identify ILK binding proteins in oligodendrocytes during proliferation and differentiation stages. Through the use of the tandem affinity purification (TAP) method, we identified known and novel ILK protein interactors. This will allow for a further understanding of the signaling pathways downstream of β1 integrin which are important in oligodendrocyte development.

Introduction

The interaction of the extracellular matrix with the integrin receptors has been a primary focus of understanding oligodendrocyte biology, specifically on the role of β1 integrin in myelination and remyelination (Buttery and ffrench-Constant 1999; Lee et al. 2006). Over the years, three important proteins have emerged as important regulators of integrin-mediated signaling. These are integrin linked kinase (ILK), and the adaptor proteins PINCH (particularly interesting Cys-His rich protein) and parvin. The three proteins form a heterotrimeric complex (Legate et al. 2006). The heterotrimeric complex, named IPP, functions as an adaptor between integrins and the actin cytoskeleton, and is involved in numerous signaling pathways as shown in cardiac myocytes, thrombocytes and, in *Caenorhabiditis elegans* (Birschmann et al. 2008; Chen et al. 2005; Feng and Walsh 2004). Since the identification of ILK as an integrin associated protein, much research has revolved around the structural role it plays in linking integrins to the cytoskeleton and its role in signal transduction for various cellular activities such as survival, migration, adhesion and cell spreading.

Recent work has demonstrated that the integrin signaling pathway is central to the regulation of oligodendrocyte-mediated myelination within the CNS (Buttery and ffrench-Constant 1999; Lee et al. 2006). We have specifically demonstrated *in vivo* that β1 integrin is important in the process of myelination and remyelination (Lee et al. 2006). At the same time, it has been shown that the MAP-kinase pathway is an important downstream signaling target of β1 integrin during both myelination and remyelination (Lee et al. 2006). Therefore, to further elucidate the signaling pathways downstream of β1 integrin involved in myelination, it is crucial to identify proteins that could be

potentially involved. ILK has been identified as the central constituent of the IPP heterotrimeric complex and links this complex to the cytoplasmic tails of β1 integrin and β3 integrin. Therefore ILK is a logical downstream protein kinase to focus on in terms of its role in CNS myelination (Legate et al. 2006).

Not only does ILK and β1 integrin play a role in CNS myelination, but recent work has also demonstrated that the ligand for integrins, extracellular matrix (ECM) components, expressed on the surface of axons are also important in myelin membrane formation. Recent *in vitro* and *in vivo* work has shown specifically that laminin-2, a ECM component, enhances myelin membrane formation in oligodendrocytes (Buttery and ffrench-Constant 1999; Chun et al. 2003). *In vitro*, laminin-2 stimulates oligodendrocytes to extend their processes through α6β1 integrin (Buttery and ffrench-Constant 1999). Moreover, recent work has demonstrated that ILK activity is required for laminin-2-induced oligodendrocyte cell spreading and myelin membrane formation (Chun et al. 2003).

ILK works in concert with multiple binding partners to transmit cues from the ECM to the cytoskeleton (Legate et al. 2006). This particular role of ILK has been identified in various cell types, however, it is uncertain if ILK associates with the same set of binding partners in different cells or if exclusive partners exists in specific cell types to provide cell-specific functions. The involvement of various proteins, such as integrins, FAK, Fyn, and Src, in different phases of oligodendrocyte development has been well reviewed (Colognato et al. 2004; Milner et al. 1997; Milner and Ffrench-Constant 1994). It has been shown that each of the proteins may vary in their roles and alter their binding partners in triggering various downstream signaling pathways for the

execution of various cell functions. Although, it has been demonstrated that ILK is involved in myelin membrane formation in cultured oligodendrocytes, the actual complex of proteins involved with ILK during this process is unknown (Chun et al. 2003)).

Here, we have used the Tandem Affinity Purification (TAP) method to identify ILK associated proteins from the oligodendrocyte cell line CG4. The tandem affinity purification method has been proven to be the most efficient and discriminatory separation technique for the retrieval of protein complexes (Puig et al. 2001). We have performed our studies on cells during the growth and differentiation phases to determine if ILK interacting partners change during this transition. We show that the ILK complex does indeed differ in CG4 cells during proliferation and differentiation. Our study has identified known binding partners of ILK such as parvin and RSU1. At the same time, we have identified novel ILK binding proteins such as MCM5. The identification of the differing ILK protein complex in oligodendrocyte development will lead to a better understanding of the signaling pathways involved during oligodendrocyte maturation.

Material and Methods

Generation of NTAP-ILK and CTAP-ILK constructs

The retroviral expression constructs pBrit-LoxP-CTAP HIS-FLAG (CTAP) and pBRIT-LoxP-NTAP FLAG-HIS (NTAP) are as described before (McKinnell et al. 2008). The mouse ILK cDNA was PCR amplified from a pNEB-WT-ILK vector (generously provided by Dr. L. Dagnino) for insertion into the NTAP construct with 5'- GGA GAA TTC GAC GAC ATT TTC ACT CAG TGC CGG -3' (forward primer) and 5'- GGA CTC GAG CTT GTC CTG CAT CGT TCT CAA GGA TAG G - 3' (reverse primer) and with 5'- GGA GAA TTC GCC GCC ATG GAC GAC ATT TTC ACT CAG TGC CGG - 3' (forward primer) and 5'- GGA CTC GAG CTT GTC CTG CAT CTT CTC AAG GAT AGG - 3' (reverse primer) for the CTAP construct. The amplified ILK cDNA was gel purified and digested with EcoR1 and Xho1. For insertion of ILK cDNA, pBrit-LoxP-CTAP HIS-FLAG and pBRIT-LoxP-NTAP FLAG-HIS vectors were digested with EcoR1 and Xho1, and were then ligated with the ILK cDNA to form the NTAP-ILK and CTAP-ILK constructs, respectively.

Generation of NTAP-ILK and CTAP-ILK CG4 cells

The Central Glia (CG4) cells established by Louis et al. (1992) were generously supplied by Dr. A. Peterson. CG4 cells were cultured on poly-L-ornithine (Sigma) coated dishes in growth media containing 70% DMEM (Wisent), 30% conditioned medium from B104 cells, 2% FBS, N1 supplement including insulin, and biotin (Louis et al. 1992). The cells were incubated at 37°C with 90% air and 10% $C0_2$. To obtain differentiated CG4 cells, cells were cultured in media similar to the proliferating media as described above,

however, the B104 conditioned media was not included. The retroviral packaging cell line, Phoenix-Eco was maintained in DMEM supplemented with 5% FBS, and 1% penicillin/streptomycin (Invitrogen). High titer infectious retrovirus stocks were generated by transfecting the NTAP-ILK and CTAP-ILK constructs into Phoenix-Eco cells using Lipofectamine 2000 (Invitrogen). Polybrene-enhanced retroviral infection of the NTAP-ILK and CTAP-ILK retrovirus stocks into CG4 cells was used to obtain stable lines by drug selection with 2 μg/ml puromycin (Invitrogen), and cells were subsequently maintained in the selection media to ensure the continued expression of NTAP-ILK and CTAP-ILK.

Tandem Affinity Purification

Approximately 1 g of cells was collected during proliferation and at 3 days of differentiation. The cells were snap frozen and lysed in 1.3 volume (ml/g of cell pellet) of Buffer A (10 mM Tris-HCl pH 7.9, 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.19% NP40) with the addition of protease inhibitors (2.4 μg/ml chymostatin, 1.5 μg/ml papstatin A, 88 μg/ml PMSF, 0.5 μg/ml leupeptin, 1.7 μg/ml aprotinin, 310 μg/ml benzamidine). Following lysis in Buffer A, one volume of Buffer B (50 mM Tris-HCl pH 7.9, 0.6 M NaCl, 1.5 mM MgCl2, 25% glycerol) was added and cells were further homogenized. Cells were left to rotate at 4°C for 30 minutes with 1 μl of benzonase nuclease (Novagen). The homogenate was then spun at 40,000 g for 1 hour in an ultracentrifuge. The supernatant was dialyzed with a Slide-A-Lyzer Dialysis Cassette (PIERCE) for 3 hours in dialysis buffer (10 mM Tris-HCl pH 7.9, 0.1 M NaCl, 10.1 mM EDTA, 10% glycerol). After dialysis, the extract was spun at 14,000 g for 20 minutes and the supernatant removed and placed into Low

Retention Microcentrifuge Tubes (Fisher Scientific). For the purification of NTAP-ILK, Ni-NTA agarose beads (60 µl/sample) (Qiagen) were washed twice in Ni-NTA wash buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 0.1 mM EDTA, 5 mM imidazole) and resuspended in an equal volume of Ni-NTA wash buffer. The beads were incubated with the supernatant, rotating overnight at 4°C. The Ni-NTA agarose with supernatant were spun at 25,000 g for 3 minutes and the supernatant was removed. The Ni-NTA beads were washed 3 times with Ni-NTA buffer and then eluted with Ni-NTA elution buffer with TEV (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 0.1 mM EDTA, 0.4 M imidazole). The TEV cleaved supernatant was then incubated overnight at 4°C with M2 agarose beads, which were previously washed with AFC buffer (10 mM Tris-CL pH 7.9, 100 mM NaCl, 0.1% NP40) and resuspended in an equal volume of AFC buffer. The supernatant and M2 agarose beads were spun at 25,000 g for 3 minutes, the supernatant was removed and the M2 agarose beads were washed 3 times with AFC buffer followed by resuspension with TEV buffer and 2 mg/ml Flag peptide, each time the supernatant was collected for future use.

Mass Spectrometry

The TAP-tag purification recovered protein complexes were further concentrated with the Microcon Centrifugal Filter device (Millipore). The concentrated protein complexes were resolved on a gradient SDS-PAGE gel (Bio-Rad). For non-fixing silver staining of the gel, the gel was fixed with 50% ethanol, 5% acetic acid solution for 30 minutes. The gel was washed in 50% ethanol followed by H_20. The gel was stained with chilled 0.1% silver nitrate for 30 minutes followed by H_20. The gel was briefly washed

with developer solution (0.04% formalin, 2% sodium carbonate) for a couple of minutes. Following the pre-wash with developer solution, the gel was incubated further with developer solution until bands appeared on the gel. After the appearance of bands, the developed solution was replaced with a stop solution of 5% acetic acid for 5 minutes. We chose to excise the 14 most abundant bands from both proliferation and differentiated samples. The excised bands were digested with trypsin and underwent LTQ linear ion trap mass spectrometry and analysis courtesy of Julian Vasilescu of the Ottawa Institute of Systems Biology.

Results

Generation of N-TAP-ILK expressing CG4 cells

The method of tandem affinity purification (TAP) was originally designed for use in yeast but is equally as successful in mammalian cells (Puig et al. 2001). The approach of the TAP method is similar to epitope tagging, with the main difference in the sequential utilization of two tags instead of one. The use of 2 constructs, an N-terminal and C-terminal vector, allow for the determination of ILK co-factors with tags on either terminal.

The N-terminal TAP ILK (NTAP-ILK) and C-terminal TAP ILK (CTAP-ILK) constructs were generated and expressed in CG4 cells (Figure 1). The CG4 cells that expressed NTAP-ILK or CTAP-ILK were kept under puromycin selection. The first 2 passages of both constructs resulted in continued expression of the NTAP-ILK and CTAP-ILK fusion proteins at the predicted size (Figure 2). However, upon further expansion of the CTAP-ILK CG4 cells, the CTAP-ILK protein was no longer detectable in the cells. The loss of the CTAP-ILK protein may indicate the importance of the C-terminal end of ILK for interaction with other proteins to maintain the function of ILK in CG4 cells. Such a construct may therefore have acted as a dominant-negative, and its expression not tolerated by the cells. Therefore, the remainder of the experiments were performed with the NTAP-ILK CG4 cells, which appeared to tolerate the expression of the fusion protein. The NTAP-ILK CG4 cells readily expressed the protein both during proliferation and differentiation at or above the endogenous levels (Figure 3).

Purification of ILK complexes

Both proliferating NTAP-ILK CG4 cells and 3 day differentiated NTAP-ILK CG4 cell extracts were collected and subjected to tandem affinity purification. ILK was

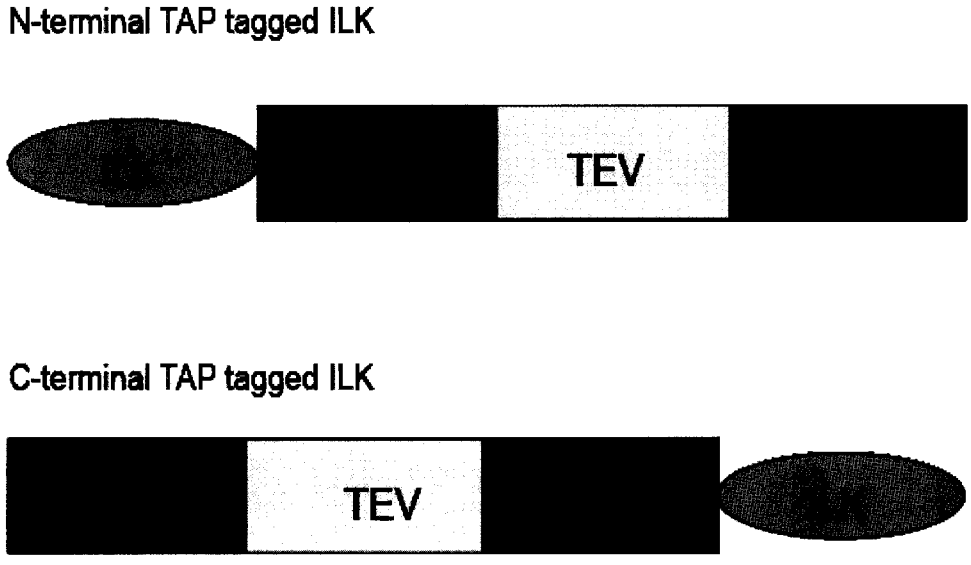

Figure 1. Schematic of NTAP-ILK and CTAP-ILK. The schematic demonstrates the difference in the sequence in the His and Flag tags when bound to the N-terminal ILK (NTAP-ILK) or C-terminal ILK (CTAP-ILK).

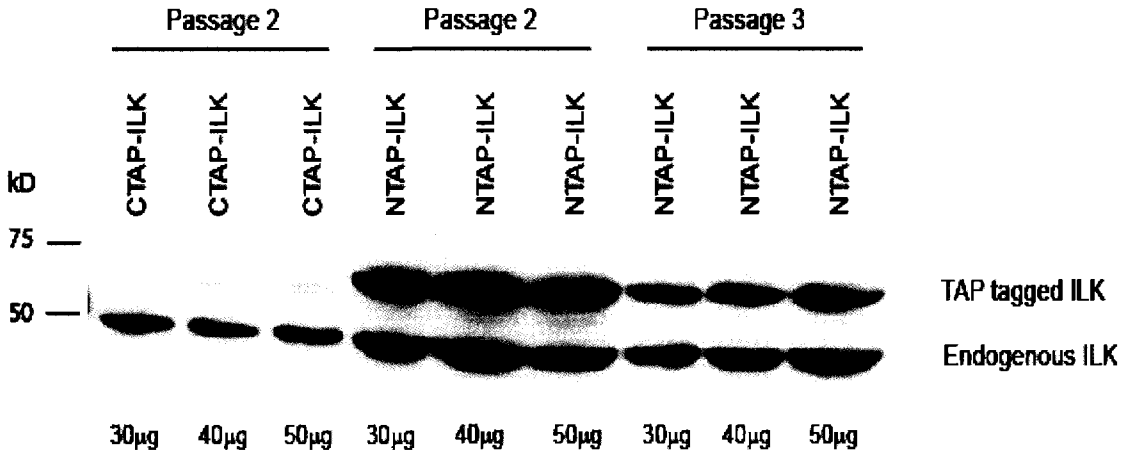

Figure 2. Expression of CTAP-ILK and NTAP-ILK in CG4 cells. Western blot analysis of ILK in CG4 cells infected with either CTAP-ILK and NTAP-ILK constructs. The endogenous ILK migrates at a faster rate than TAP tagged ILK. At passage 2, NTAP-ILK is strongly expresses in the CG4 cells, while the CTAP-ILK construct is barely detectable when compared to endogenous ILK. At passage 3, the NTAP-ILK construct is still detectable in the CG4 cells.

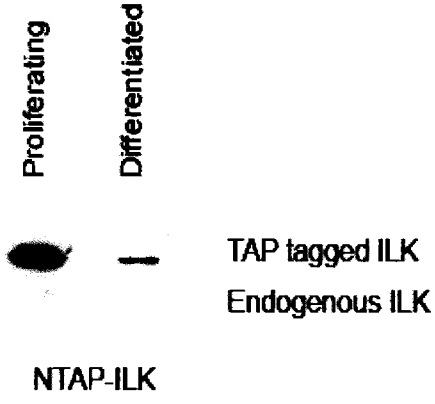

Figure 3. Expression of NTAP-ILK in CG4 cells. Western blot anlaysis of ILK in proliferating CG4 cells and 3 days differentiated CG4 (30 μg). The NTAP- ILK expression is higher when compared to endogenous ILK in both proliferating and differentiated CG4 cells.

detected in the final elute of the tandem affinity purification for the two samples. Samples were resolved by SDS-PAGE, and silver staining of the gel revealed protein banding patterns for CG4 cells undergoing proliferation or differentiation (Figure 4). The different banding pattern observed between proliferating and differentiated CG4 cells suggests that the ILK protein complex differs between the 2 stages of CG4 cell development.

Mass spectrometry reveals different ILK binding partners during proliferation and differentiation of CG4 cells

To identify ILK interacting proteins, 14 band slices were cut from each lane of the silver stained polyacrylamide gel. The excised bands were subjected to in gel digestion with trypsin and then put through an LTQ linear ion trap mass spectrometer for the identification of the resulting peptides (Table 1). In the proliferating CG4 cells, known or previously identified interactors of ILK were identified such as eukaryotic translation initiation factor 5B, splicing factor 3a and paraspeckle protein 1 (Dobreva et al. 2008). At the same time, new interacting proteins were identified such as elongation factor 1-alpha, cdc42 effector, and phosphoserine phosphatase. In the differentiated CG4 cells, known ILK partners were also detected, e.g. parvin, a member of the heterotrimeric complex. The Ras-suppressor protein (RSU-1) was also identified as an interactor of ILK. RSU-1 is a known interactor with PINCH1. In addition to known ILK partners, we identified several novel interactors of ILK (Table 1). Among these was the heterogeneous nuclear ribonucleoprotein U and DNA replication licensing factor MCM5.

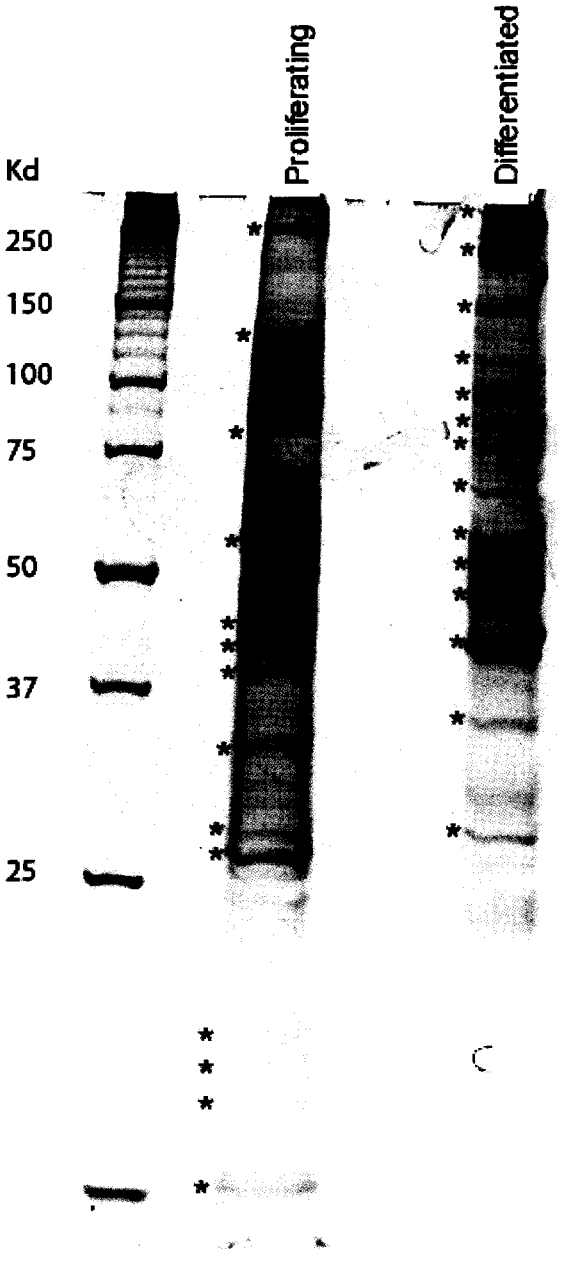

Figure 4. NTAP-ILK proliferating and differentiated complex. The NTAP-ILK proliferating and differentiated complex were resolved on a SDS-PAGE and silver stained. The NTAP-ILK proliferating complex differs from the CTAP-ILK differentiated complex. * indicates the 14 bands that were sequentially cut from the top to the bottom of the gel in each lane.

	Biological process or Molecular function	Molecular Weight	Protein Identification	Number of Peptides
Proliferation	Nuclear	75553	NonO/p54nrb homolog	32
	Nuclear	64307	Cdc42 effector short isoform	6
	Nuclear	59147	Splicing factor 3a, subunit 3	8
	Nuclear	58836	Paraspeckle protein1	5
	Nuclear	55005	Non-POU domain containing, octamer-binding	10
	Nuclear	44382	Splicing factor 3b, subunit 4	3
	Intracellular signaling	25180	Phosphoserine phosphatase	11
	Nuclear	17049	Eukaryotic translation initiation factor 5A	3

Differentiation	Cytoskeleton	227566	Myosin, heavy polypeptide 9	7
	Cytoskeleton	229793	Myosin heavy chain 10, non-muscle	45
	Nuclear	138001	Similar to Eukaryotic translation initiation factor 5B	6
	Nuclear	91159	Topoisomerase (DNA) I	4
	Nuclear	88492	Heterogeneous nuclear ribonucleoprotein U	3
	Nuclear	83222	Similar to DNA replication licensing factor MCM5 (CDC46)	8
	Nuclear	75553	NonO/p54nrb homolog	5
	Cytoskeleton	41732	Similar to parvin, beta	3
	Intracellular signaling	38717	Serine/threonine kinase receptor associated protein	3
	Intracellular signaling	31442	Ras suppressor protein 1	10
	Nuclear	29724	Spindlin	3

Table 1. Identification of proteins recovered from the NTAP-ILK in proliferating and differentiated CG4 cells. Listed are 24 proteins that have been identified to be of interest in the role of myelination and have a minimum of 3 peptides. The proteins identified have also been categorized into various biological processes and molecular function.

Discussion

ILK was first identified through a yeast-2-hybrid screen for binding partners of the cytoplasmic tail of β1 integrin (Hannigan et al. 1996). Since its initial discovery, it is clear that ILK plays a central role in integrating signals in mammalian cells (Legate et al. 2006). ILK has been demonstrated to regulate cell motility, cell survival, cytoskeleton reorganization, and tumor progression and invasion (Hannigan et al. 2005). ILK functions in a heterotrimeric complex with adaptor proteins PINCH and parvin. The biological functions of the members of the heterotrimeric complex have been examined in a variety of cell types and much of the work points to a major role at focal adhesions. However, recent studies have emerged suggesting that ILK is able to localize to the nucleus and may be considered a nuclear protein with nuclear functions (Acconcia et al. 2007; Fielding et al. 2008).

Previous work has indicated that the integrin signaling pathway plays an important role in oligodendrocyte mediated myelination of axons, however the downstream effectors of the integrin signaling pathway in this context have yet to be determined (Buttery and ffrench-Constant 1999; Lee et al. 2006). ILK is a natural downstream effector of integrin signaling, with studies demonstrating its direct interaction with the cytoplasmic tail of integrins (Hannigan et al. 1996). More importantly, for the study of myelination, it has been demonstrated that ILK is activated upon attachment of oligodendrocytes to a laminin matrix (Chun et al. 2003). Laminin, specifically laminin-2, is expressed on the surface of axons and perhaps by acting through β1 integrin, oligodendrocytes are able to extend their myelin sheaths.

Due to the still undetermined β1 integrin downstream signaling pathways during the process of myelination and the role that ILK plays, we sought to identify ILK interacting proteins in CG4 oligodendrocyte cells and whether these change when cells go from proliferation to differentiation. With the use of the TAP strategy, we have shown that the ILK protein complex differs between proliferating and differentiating CG4 cells. At the same time, we have highlighted novel ILK protein interactors that may further our understanding of the ILK signaling pathways in oligodendrocytes.

The results of our ILK TAP-tag experiments reveal banding patterns that are different when extracts from CG4 cells undergoing proliferation versus CG4 cells that are differentiated are used. The variant pattern in the ILK associated proteins could be attributed to the morphological change that occurs in oligodendrocytes when transitioning from the proliferative state to the differentiated state. During the proliferating stage, oligodendrocytes maintain a bipolar morphology with very few processes, however upon differentiation they begin to extend multiple processes and initiate an extensive branched myelin membrane network. The transition from a bipolar cell to a multipolar cell involves changes in interaction with the extracellular matrix, cell adhesion modifications, and cytoskeleton remodeling. Therefore, it is not surprising the protein interactions mediated through ILK change during this time. The morphological changes observed in CG4 cells during differentiation could impact the ILK protein complex and therefore lead to changes in ILK protein interactions.

Our analysis of the various ILK protein interactors was focused on using the N-TAP ILK fusion as the expression of C-TAP ILK was not tolerated for more than 2 passages of proliferating CG4 cells. The N-terminal domain of ILK has three ankyrin repeats and is the site which mediates protein interactions, while the C-terminal domain contains the kinase domain as

it shares sequence homology to Ser/Thr kinases (Legate et al. 2006). The addition of a TAP tag to the C-terminus may inhibit the kinase activity of ILK which may be critical in the normal function of ILK. The inability of the C-TAP ILK to function normally may in turn have been detrimental to the cells and resulted in selection of the non-expressing cells.

From our analysis of N-TAP ILK in CG4 cells, we identified well known protein interactors of ILK in differentiated CG4 cells, specifically β-parvin and myosin (Deng et al. 2001; Yamaji et al. 2001). Interestingly, we did not identify α-parvin in the ILK protein complex. However this could be attributed to the fact that we may have failed to identify known and possible new interactors of ILK as we only sampled 14 bands from each purified N-TAP ILK protein complex. At the same time, α-parvin may not have been identified since the interaction of ILK with α-parvin and β-parvin are mutually exclusive (Zhang et al. 2004). α-parvin and β-parvin have distinct roles in mammalian cells and in this particular case β-parvin may have a specific function in oligodendrocyte differentiation. In our analysis, we identified myosin heavy chain as an ILK interacting protein. However, to date it is myosin light chain that has been shown to interact with ILK (Deng et al. 2001). Nonetheless, it has been demonstrated that ILK influences the expression of myogenic proteins such as myosin heavy chain and more specifically myosin heavy chain is detectable in the main processes of oligodendrocytes rich in microtubules and microfilaments (Huang et al. 2000; Song et al. 2001). Therefore, we can not rule out the possibility that myosin heavy chain is an ILK interactor during differentiation. Another protein that was identified in our ILK protein complex analysis is RSU1. RSU1 has recently been identified as part of the heterotrimeric complex through its interaction with PINCH (Dougherty et al. 2008). In mammalian cells RSU1 works in conjunction with PINCH in the IPP complex and plays a role in promoting adhesion and inhibition of migration (Dougherty et al.

2008) . This aspect is important since oligodendrocytes rely on focal adhesion for process outgrowth (Hoshina et al. 2007; Miyamoto et al. 2007).

More recently, novel unexpected functions for ILK have been described. ILK has been localized to the centrosome and shown to regulate mitotic spindle organization (Fielding et al. 2008). Intriguingly, our analysis has identified spindlin and tubulin as being interactors of ILK in differentiated CG4 cells. These proteins were also identified by Dobreva et al. through SILIAC (stable isotope labeling with amino acids in cell culture)-based quantitative mass spectrometry in HEK293 cells. The identification of spindlin and tubulin as protein interactors of ILK in differentiated oligodendrocytes highlights the potential role of ILK in regulating mitotic spindle organization during OL differentiation.

A few of the ILK interacting proteins were identified in both proliferating and differentiating CG4 cells. These proteins include the non-POU domain and heterogeneous nuclear ribonucleoprotein U. We also identified novel and/or uncharacterized ILK interacting proteins. Many of these are classified as nuclear proteins, in particular elongation factor-1 alpha and DNA replication licensing factor MCM5. Typically ILK has been viewed as a cytoplasmic protein, however with recent work demonstrating the capability of ILK to shuttle between the cell nucleus and cytoplasm, it opens the possibility of ILK associating with other nuclear proteins and functioning as a nuclear protein (Acconcia et al. 2007). MCM5 has a well defined role as a DNA prereplication complex factor responsible for DNA replication. However, recent work has also described MCM as having a role as a regulator of the centrosome duplication cycle (Ferguson and Maller 2008). Therefore, it would be of interest to further validate the role of MCM5 in oligodendrocytes to determine how it may play a part in oligodendrocyte differentiation in conjunction with ILK.

To further our analysis, we will validate the MCM5 and ILK interaction with co-immunoprecipitation experiments in CG4 cells. It would also be of interest to see the co-localization of the two proteins in both cell culture and *in vivo* in CNS tissues. This analysis will shed light on ILK as a nuclear protein with roles in centrosomal function and assess the importance of ILK and MCM5 in oligodendrocyte differentiation.

The identification of known and unknown ILK protein interactors in CG4 cells has led us to formulate new hypotheses on the function of ILK and how its protein interactors may have a role in oligodendrocyte development.

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