P₀ Protein Is Required for and Can Induce Formation of Schmidt-Lantermann Incisures in Myelin Internodes

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Axons in the PNS and CNS are ensheathed by multiple layers of tightly compacted myelin membranes. A series of cytoplasmic channels connect outer and inner margins of PNS, but not CNS, myelin internodes. Membranes of these Schmidt-Lantermann (S-L) incisures contain the myelin-associated glycoprotein (MAG) but not P₀ or proteolipid protein (PLP), the structural proteins of compact PNS (P₀) and CNS (PLP) myelin. We show here that incisures are present in MAG-null and absent from P₀-null PNS internodes. To test the possibility that P₀ regulates incisure formation, we replaced PLP with P₀ in CNS myelin. S-L incisures formed in P₀-CNS myelin internodes. Furthermore, axoplasm ensheathed by 65% of the CNS incisures examined by electron microscopy had focal accumulations of organelles, indicating that these CNS incisures disrupt axonal transport. These data support the hypotheses that P₀ protein is required for and can induce S-L incisures and that P₀-induced CNS incisures can be detrimental to axonal function.

Key words: myelin; P₀ protein; proteolipid protein; myelin-associated glycoprotein; axon; axonal transport

Introduction

Rapid communication between nerve cells can be enhanced by increasing the diameter of axons or by insulating axons with myelin. During evolution, myelination was naturally selected over larger axons to conserve space. Myelin first appeared in fish (Waehneldt, 1990; Yoshida and Colman, 1996), in which two myelin-forming cells evolved: Schwann cells in the PNS and oligodendrocytes in the CNS. Although PNS and CNS myelin serve similar functions, their structure and molecular composition differ (for review, see Trapp and Kidd, 2004). Schwann cells form single myelin internodes and contain a series of cytoplasmic channels that are continuous with Schwann cell cytoplasm at the outer and inner margins of the myelin internode (Blakemore, 1969; McDonald and Ohlrich, 1971). These Schmidt-Lantermann (S-L) incisures facilitate transport of nutrients and molecules to myelin and the periaxonal region of the internode (Balice-Gordon et al., 1998). Oligodendrocytes form multiple myelin internodes and contain few if any incisures. The major structural proteins of mammalian PNS and CNS also differ. P₀ protein, a type I integral membrane glycoprotein, comprises 70% of PNS myelin proteins (Lemke and Axel, 1985), whereas proteolipid protein (PLP), a tetraspan protein, comprises >50% of CNS myelin proteins (Milner et al., 1985). The myelin-associated glycoprotein (MAG), which comprises <1% of myelin proteins (Quarles et al., 1983), is absent from compact myelin and located in periaxonal membranes in PNS and CNS myelin and in inci-

sural and paranodal myelin loop membranes in PNS myelin internodes (Trapp et al., 1989). Although connexin-32 and E-cadherin are also present in PNS incisures, neither appears essential for incisure formation (Scherer et al., 1998). PNS incisures are increased in myelin basic protein- and sulfatide-deficient mice (Gould et al., 1995; Hoshi et al., 2007) but have not been detected in the CNS of these mutants.

Myelinating cells also provide trophic support that is essential for long-term axonal survival. Several myelin proteins play essential roles in facilitating this support. Mice null for MAG (Yin et al., 1998) or CNP (Lappe-Siefke et al., 2003) myelinate normally but have primary axonal phenotypes that result in axonal degeneration. PLP-null mice have altered myelin compaction and significant axonal degeneration (Griffiths et al., 1998). The myelin compaction phenotype in PLP-null mice was rescued by P₀ protein, but the axonal degeneration was enhanced (Yin et al., 2006). Thus, PLP also appears to provide trophic support to axons by mechanisms independent of its function in stabilizing myelin compaction. It should be noted that PLP replaced P₀ as the major structural protein of CNS myelin in terrestrial vertebrates (Yoshida and Colman, 1996). The P₀ to PLP switch, therefore, bestowed a neuroprotective function to CNS myelin (Yin et al., 2006). The mechanisms by which MAG, PLP, and CNP affect axonal function and stability are unknown and most likely indirect (for review, see Nave and Trapp, 2008). It is also possible that inefficient saltatory conduction and reduced trophic factor expression by myelinating cells contribute to the axonal changes.

Although neuroprotection may be a major reason for the evolutionary switch in CNS myelin protein composition, it remains unclear why S-L-incisures are only found in PNS myelin internodes. We show here that incisures do not form in P₀-null PNS myelin internodes but form when P₀ replaces PLP as the structural protein of compact CNS myelin. Furthermore, these CNS

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myelin incisures disrupt axonal transport. These data support the hypotheses that P_0 protein induces incisure formation and that incisures are detrimental to functions of CNS axons.

Materials and Methods

Wild-type, P_0 -CNS, and P_0 /PLP-CNS transgenic lines. We generated transgenic mice that expressed the mouse P_0 cDNA (Lemke and Axel, 1985) ligated to a 9.1 kb region of the mouse MBP promoter (Forghani et al., 2001). These mice were maintained in the animal colony as homozygous animals. This line has equal amounts of both P_0 and PLP in CNS myelin and is called the P_0 /PLP mouse line. By crossing PLP-null mice (Klugmann et al., 1997) with P_0 /PLP mice, we obtained mice that were PLP-null and homozygous for the MBP- P_0 transgene (P_0 -CNS), as determined by genomic DNA analysis and outbreeding (Yin et al., 2006). Mice deficient for MAG at the mRNA and protein levels were generated by gene targeting and have been described in detail previously (Li et al., 1994; Yin et al., 1998). Genotypes ($MAG^{-/-}$ or $MAG^{+/+}$) were determined by tail DNA analysis by Southern blottings (data not shown) (Li et al., 1994). Mice homozygous-null for P_0 ($P_0^{-/-}$) were genotyped from tail clips using a 457 bp *HindIII*-*XbaI* DNA fragment of the P_0 gene as a probe (Menichella et al., 2001).

Morphological analysis. Three wild-type (WT), MAG-null, P_0 -null, P_0 /PLP, PLP-null, and P_0 -CNS adult mice were perfused with 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.08 M Sorensen's phosphate buffer. Three P_0 -CNS mice were also obtained at postnatal day 7 (P7). Optic nerves and/or sciatic nerves were removed and processed to Epon 812. One-micrometer-thick sections were stained with toluidine blue and examined by light microscopy (LM). Transmission EM was performed on selected blocks. Sections were cut with a diamond knife and examined in a Philips CM100 electron microscope. Optic nerve sections were obtained from WT, PLP-null, P_0 /PLP, and P_0 -CNS mouse lines. Sciatic nerve sections were obtained from WT, MAG-null, PLP-null, and P_0 -null mouse lines. The structure and density of S-L incisures was determined in optic nerve by EM and in sciatic nerves by EM and LM using established criteria. Incisure densities in WT, MAG-null, PLP-null, and P_0 -null sciatic nerves were measured by LM in three mice each (59, 57, 59, and 55 internodes counted, respectively). S-L incisure densities in three optic nerves from WT, PLP-null, P_0 /PLP, and P_0 -CNS were analyzed by EM (111, 116, 103, and 108 internodes, respectively). Data were analyzed using the Student's *t* test. To visualize the radial component, optic nerve from 3-month-old mice were fixed as above, postfixed in osmium tetroxide, and embedded in Durcupan resin according to the method of Tabira et al. (1978).

Electron microscopic immunocytochemistry. Segments of optic nerve from WT and adult P_0 -CNS mice were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.08 M Sorensen's phosphate buffer, infiltrated with 2.3 M sucrose and 30% polyvinylpyrrolidone, placed on specimen stubs, and frozen in liquid nitrogen. Ultrathin cryosections (~120-nm-thick) were cut on glass knives in an ultracyromicrotome (Ultracut S; Reichert Scientific Instruments) maintained at -110°C . Sections were immunostained by previously described immunogold procedures (Trapp et al., 1989; Yin et al., 2000). Immunostained sections were examined in a Philips CM100 electron microscope. Primary antibodies include anti-MAGB11F7 monoclonal antibody (1:500 dilution) and anti- P_0 polyclonal antibody (1:500 dilution). Secondary antibodies include donkey anti-mouse and anti-rabbit conjugated to 12 nm gold particles.

Results

To investigate the possible role of MAG, PLP, or P_0 in S-L incisure formation, we examined WT, MAG-null (Li et al., 1994), PLP-null (Klugmann et al., 1997), and P_0 -null (Giese et al., 1992) sciatic nerves for incisures. S-L incisures were readily detectable in 1- μm -thick plastic sections of adult WT (Fig. 1*a*), MAG-null (Fig. 1*b*), and PLP-null sciatic (Fig. 1*c*) nerves. Loss of MAG or PLP had no effect on the number of incisures, which averaged 2.17 incisures per 100 μm internode length (Fig. 1*e*). In contrast, incisures were not detected in 1- μm -thick sections of P_0 -null

Figure 1. P_0 protein is required for Schmidt-Lantermann incisure formation in PNS myelin internodes. In micrometer-thick plastic sections, Schmidt-Lantermann incisures are present in WT (*a*, arrows), MAG-null (*b*, arrows), and PLP-null (*c*, arrows) adult sciatic nerves but not detected in adult P_0 -null (*d*) sciatic nerves. The density of incisures was similar in WT, MAG-null, and PLP-null nerves, and the decrease in P_0 -null nerves was statistically significant (*e*, $p < 0.0001$). The ultrastructure of Schmidt-Lantermann incisures is similar in WT (*f*, arrows) and MAG-null (*g*, arrows) PNS myelin internodes. Myelin membranes in P_0 -null fibers (*h*) are not tightly compacted because of loss of obligate P_0 homophilic adhesions between extracellular leaflets of compact myelin. Cytoplasmic channels that traversed P_0 -null myelin internodes were never detected in electron micrographs. On rare occasions, cytoplasmic pockets were detected in outer layers of P_0 -null myelin internodes (*h*, arrows). My, Myelin; Ax, axon. Scale bars: *a*–*c*, 1 μm ; *e*–*g*, 250 nm.

(Fig. 1*d*) sciatic nerves. To extend these light microscopic observations, longitudinally oriented sections of these nerves were examined by electron microscopy. Incisures were abundant in WT (Fig. 1*f*), MAG-null (Fig. 1*g*), and PLP-null (data not shown) nerves. The absence of P_0 increased the spacing between the extracellular leaflets of compact myelin (Fig. 1*h*, My). Schmidt-Lantermann incisures were not detected in electron micrographs of P_0 -null nerves. On rare occasions, cytoplasmic pockets with microtubules were found at the outer margins of the internode (Fig. 1*h*, arrows), but they never reached the inner margin of the

Figure 2. In the absence of PLP, P₀ induces Schmidt-Lantermann incisure formation in CNS myelin internodes. Schmidt-Lantermann incisures were quantified in electron micrographs of optic nerves from adult WT mice, mice with similar amounts of PLP and P₀ (P₀/PLP mice), PLP-null mice, and mice in which P₀ replaced PLP (P₀-CNS mice) as the major structural protein of CNS myelin (**a**). Incisures were rare in WT, PLP-null, and P₀/PLP optic nerves and abundant in P₀-CNS optic nerves ($p < 0.0001$). Incisures in P₀-CNS optic nerves connect inner and outer cytoplasmic domains of the myelin internode (**b**). Cytoplasmic domains of incisures in both WT sciatic and P₀-CNS optic nerves result from splitting of the major dense line of compact myelin (**c**, **e**, arrows) and contain microtubules (**c**, **e**, arrowheads). These incisures form during myelination as they were abundant in P7 P₀-CNS optic nerves (**f**). Incisures in 7-d-old P₀-CNS optic nerves contained numerous membrane vesicles (asterisk), indicating that they serve as functional cytoplasmic channels during formation of P₀-CNS myelin internodes. Ax, Axons; My, compact myelin. Scale bars: **b**, 500 nm; **d**, 100 nm; **c**, **e**, 50 nm; **f**, 200 nm.

myelin internode. These observations raise the possibility that P₀ protein is essential for formation of S-L incisures and may explain why CNS myelin internodes do not contain S-L incisures.

To test whether P₀ protein can cause S-L incisure formation, we replaced PLP with P₀ protein in mouse CNS myelin by transgenic complementation. Details of how these mice were generated and characterized have been described previously (Yin et al., 2006). When P₀ replaced PLP in CNS myelin, the compact myelin membranes had the spacing of WT peripheral nerve myelin (Yin et al., 2006). We examined electron micrographs of adult optic nerves from WT mice, mice with equal amounts of PLP and P₀ in CNS myelin (P₀/PLP mice), and mice in which P₀ replaced PLP (P₀-CNS mice). S-L incisures were not detected in WT, PLP-null, or P₀/PLP optic nerves but were abundant in P₀-CNS optic nerves (Fig. 2*a–c*), in which they display many of the ultrastructural characteristics of incisures in WT sciatic nerve (Fig. 2*d,e*). P₀-CNS incisures traversed the entire width of the compact myelin (Fig. 2*b*), and they contain microtubules (Fig. 2*c*, arrowheads), indicating their potential to transport oligodendrocyte components between the outer and inner margins of the myelin internode. In electron micrographs, the cytoplasmic domains of P₀-CNS and WT incisures form by splitting of the major dense lines of compact myelin (Fig. 2*c,e*, arrows). Compared with WT PNS (Fig. 1*e*), there were fewer S-L incisures per 100 μm length in P₀-CNS optic nerve (Fig. 2*a*). Because S-L incisure density is related to internodal length (Hiscoe, 1947), this reduction is not surprising because P₀-CNS optic nerve internodes are significantly shorter than WT PNS internodes (Yin et al., 2006).

To determine whether the P₀-CNS optic nerve incisures formed as myelin internodes formed, we investigated their presence in early stages of myelin internode formation in P₀-CNS optic nerves. Incisures were readily detected in P7 P₀-CNS optic nerves (Fig. 2*f*). These incisures contained microtubules and a

high density of membrane vesicles (Fig. 2*f*, asterisk) that reflect the abundant myelin membrane synthesis that is occurring at this age. These observations provide additional support to the hypothesis that these S-L incisures are functional oligodendrocyte cytoplasmic conduits in P₀-CNS internodes. S-L incisures therefore appear to be an integral structure of developing P₀-CNS internodes and not part of a remodeling response during latter stages of myelination. Incisures were not detected in electron micrographs of P7 WT, P₀/PLP, or PLP-null optic nerves.

Because MAG is enriched in S-L incisure and paranodal membranes of PNS but not CNS myelin internodes, we asked whether MAG was targeted to incisure or paranodal membranes in P₀-CNS myelin internodes, by determining their ultrastructural distributions. As in WT CNS myelin (Trapp et al., 1989), MAG was enriched in periaxonal membranes of P₀-CNS myelin internodes (Fig. 3*a*, arrowheads). In contrast to WT PNS internodes, MAG was not detected in the membranes of paranodal loops (data not shown) or S-L incisures (Fig. 3*b*) in P₀-CNS internodes. Although P₀ induces formation of S-L incisures in CNS myelin that ultrastructurally resemble PNS incisures, it does not direct MAG to these incisure membranes. EM immunocytochemistry did, however, detect MAG in vesicles in P₀-CNS incisure cytoplasm (Fig. 3*b*, inset), supporting the possibility that these CNS incisures are in fact a functional conduit between inner and outer margins of the P₀-CNS myelin internodes. Because P₀ is absent from PNS incisures, we asked whether P₀ was enriched in S-L incisure membranes in P₀-CNS myelin internodes. As in WT PNS internodes, P₀ was enriched compact myelin (Fig. 3*c*) and absent from S-L incisure membranes (Fig. 3*d*) in P₀-CNS myelin internodes. Connexin-32 is present in PNS incisures (Scherer et al., 1995) but was not detected in P₀-CNS incisures. The radial component of CNS myelin (Tabira et al., 1978) was retained in P₀-CNS compact

Figure 3. P_0 -CNS incisures do not contain MAG or P_0 protein. The distribution of MAG and P_0 protein was determined in ultrathin cryosections of P_0 -CNS optic nerves. As in WT CNS and PNS myelin internodes, MAG was enriched in periaxonal membranes and absent from compact myelin in P_0 -CNS internodes (**a**, **b**, arrowheads). In contrast to WT PNS incisures, MAG is not enriched in P_0 -CNS incisure membranes (**b**, asterisk). MAG immunoreactivity was detected in membrane vesicles present in P_0 -CNS incisure cytoplasm (**b**, inset), indicating that P_0 -CNS incisures can transport membrane components between (to or from) the periaxonal membrane and the outer tongue process of P_0 -CNS myelin internodes. As in WT compact PNS myelin, P_0 protein was enriched in P_0 -CNS compact myelin (**c**) and absent from incisure membranes (**d**, asterisk). Ax, Axon; My, compact myelin. Scale bars: **a**, **b**, 250 nm; **b**, inset, and **c**, **d**, 100 nm.

myelin (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Although P_0 rescued the myelin compaction defect in PLP-null mice, it accelerated the axonal pathology and degeneration. The axonal pathology in PLP-null and P_0 -CNS mice predominates at paranodal regions. We asked whether axonal pathology also occurred beneath incisures, which, like the paranodes, bring oligodendrocyte cytoplasmic channels to the axon. We analyzed electron micrographs to determine whether axonal pathology was associated with S-L incisures in P_0 -CNS optic nerve. Adult P_0 -CNS optic nerve axons had significant organelle accumulations associated with 37 of 57 (65%) incisures (Fig. 4) examined by electron microscopy. As in PLP-null paranodes (Griffiths et al., 1998; Edgar et al., 2004) and P_0 -CNS paranodes (Yin et al., 2006), organelle accumulation was more abundant at distal regions of the incisure axoplasm (Fig. 4*a,c*, asterisks). The axoplasmic organelle accumulation included mitochondria and vesicular bodies, and some mitochondria appeared ultrastructurally intact (Fig. 4*b*), whereas others were swollen with distended cisternae (Fig. 4*d*). Formation of S-L incisures, therefore, represents an additional aspect of the gain-of-function mutation that causes axonal pathology in P_0 -CNS optic nerves.

Discussion

The purpose of this study was to investigate why Schmidt-Lantermann incisures are restricted to PNS myelin internodes. We present three major findings. First, by quantifying incisures in sciatic nerve fibers null for individual myelin proteins, we establish that P_0 protein is essential for PNS incisure formation. Second, by swapping PLP for P_0 protein, we demonstrate that P_0 protein can induce incisure formation in CNS myelin internodes. Third, we show that these CNS incisures disrupt CNS axoplasm and alter axonal transport. We conclude that incisures are present in myelin internodes when P_0 protein is the major structural protein of compact myelin.

MAG is highly enriched in incisure membranes and absent from compact myelin, whereas P_0 protein is enriched in compact myelin and absent from incisures. It was somewhat surprising, therefore, that incisures were detected at the same density in MAG-null and wild-type nerves but absent from P_0 -null nerves. Although a transmembrane protein capable of adhesive events, MAG is not essential for myelination nor does it appear to play a structural role in PNS myelin internode integrity. MAG can inhibit axonal sprouting (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and provides trophic support to axons (Yin et al., 1998). In adult MAG-null sciatic nerves, axonal pathology is restricted to axonal regions directly beneath paranodes and incisures (Yin et al., 1998). Thus, the function of MAG in PNS incisures appears more related to axonal integrity than incisure integrity.

When PLP is replaced by P_0 as the major structural protein of CNS myelin, incisures became a feature of the CNS myelin internode. Thus, P_0 protein can induce incisure formation. As in WT peripheral nerve, P_0 protein is not a structural component of the optic nerve incisures. The mere presence of P_0 in CNS myelin, however, does not induce CNS incisure formation, because incisures were not present in P_0 /PLP optic nerves. The extracellular domain of P_0 is larger than that of PLP, and, as a result, the spacing between extracellular leaflets of PNS myelin wraps is ~ 20 Å greater than the extracellular spacing in CNS myelin (Kirschner and Blaurock, 1992). In the presence of equal amounts of PLP, P_0 has no apparent effect on the spacing of the extracellular leaflets of compact CNS myelin (Yin et al., 2006). We show here that these internodes do not contain incisures. When P_0 replaces PLP, the spacing between extracellular leaflets of CNS myelin is ~ 20 Å greater than WT CNS myelin and similar to WT PNS myelin (Yin et al., 2006). P_0 is an obligate homophilic adhesion molecule (Filbin et al., 1990; D'Urso et al., 1990). Crystallization of the P_0 extracellular domain supports adhesion of the extracellular leaflets of PNS compact myelin membranes by *trans*-binding between *cis*-linked P_0 tetramers (Shapiro et al., 1996). We hypothesized previously that PLP may interfere with P_0 tetramer formation in *cis*-. Data from the present study extends this hypothesis and raises the possibility that *trans* P_0 adhesion in P_0 -CNS myelin is required for incisure formation. Although it remains to be determined how P_0 induces S-L incisures, the cytoplasmic tail of P_0 does contain a protein kinase C (PKC) binding domain, which can bind to activated PKC- α and RACK1 (Xu et al., 2001; Gaboreanu et al., 2007). It is possible, therefore, that downstream signaling initiated at the C-terminal of P_0 -mediated S-L incisure formation. If this is the case, such signaling would appear to require *trans*-binding between P_0 tetramers.

In contrast to WT peripheral nerve, MAG is not a structural component of P_0 -CNS incisure or paranodal membranes. MAG was detected in small vesicles within incisure cytoplasm. This

Figure 4. P₀-CNS incisures have detrimental effect on axons. Electron micrographs of adult P₀-CNS myelin internodes were examined to determine whether incisures had an effect on axons. Similar to paranodal regions of P₀-CNS axons, swollen axoplasm containing mitochondria (**b**, **d**, arrowheads), membrane vesicles, and disoriented microtubules (**a**, arrowhead) were found at the distal end (**a**, **b**, right side of incisure) of the incisures (asterisks). Axoplasmic organelles accumulated to a lesser degree immediately proximal to incisures (left side of the incisure). Scale bars: **a**, **c**, 0.5 μ m; **b**, **d**, 200 nm.

likely reflects the transport of MAG to or from the periaxonal region, the sole site of MAG enrichment in CNS myelin. The CNS incisure cytoplasm also contained microtubules. During peak periods of myelination, transport vesicles were abundant in incisure cytoplasm, supporting the concept that they are functional oligodendrocyte cytoplasmic channels.

The evolutionary switch from P₀ to PLP as the major structural protein of CNS myelin provided a positive trophic effect on myelinated CNS axon survival. When this switch was reversed, the paranodal axon pathology that occurs in the CNS of PLP-null mice was significantly increased in P₀-CNS mice (Yin et al., 2006). This gain-of-function phenotype was attributable to shorter internodes and thus more paranodal structures per length of axon. The increase in paranodal axonal pathology accelerated axonal degeneration and neurological disability and reduced the lifespan of P₀-CNS mice by 50% when compared with WT or PLP-null mice (Yin et al., 2006). CNS incisures also cause accumulations of organelles, which may reflect a disturbance of retrograde transport as documented in PLP-null mice (Edgar et al., 2004).

Myelin increases the speed of nerve transmission and provides trophic support to the axon. The insulating function of myelin was achieved early in evolution when P₀ was the major structural protein of fish CNS myelin. P₀, however, was not compatible with long-term survival of CNS axons (Yin et al., 2006). Natural selection remodeled molecular and structural aspects of myelin internodes. The molecular switch from P₀ to PLP was a dramatic evolutionary event involving the most abundantly expressed protein of a specialized organelle. This switch reduced the thickness of myelin internodes because PLP-containing CNS myelin lamella are 20 Å closer than P₀-containing CNS myelin lamella (Yin et al., 2006). This conservation of space is substantial when considering the trillions of myelin lamellae that must exist in the human brain. Replacing P₀ with PLP had additional effects related to the structure and function of myelin internodes. We provide evidence here that P₀ induces the formation of S-L incisures in CNS myelin and that these incisures are detrimental to

the long-term axonal survival. Why internodal cytoplasmic channels adversely affect CNS but not PNS axons may differentiate the role of myelin in PNS and CNS axonal survival. This is a fundamental question for developing neuroprotective strategies for treating primary diseases of CNS and PNS myelin.

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