Schwann Cell Myelination Requires Timely and Precise Targeting of P₀ Protein

X. Yin,* G.J. Kidd,* L. Wrabetz,† M.L. Feltri,‡ A. Messing,§ and B.D. Trapp*

*Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195; †Department of Neurology and Department of Biological and Technological Research (DIBIT), San Raffaele Scientific Institute, 20132 Milano, Italy; and §Waisman Center and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Madison-Wisconsin, Madison, Wisconsin 53705

Abstract. This report investigated mechanisms responsible for failed Schwann cell myelination in mice that overexpress P₀ (P₀tg), the major structural protein of PNS myelin. Quantitative ultrastructural immunocytochemistry established that P₀ protein was mistargeted to abaxonal, periaxonal, and mesaxon membranes in P₀tg Schwann cells with arrested myelination. The extracellular leaflets of P₀-containing mesaxon membranes were closely apposed with periodicities of compact myelin. The myelin-associated glycoprotein was appropriately sorted in the Golgi apparatus and targeted to periaxonal membranes. In adult mice, occasional Schwann cells myelinated axons possibly with the aid of endocytic removal of mistargeted P₀. These results indicate that P₀ gene multiplication causes P₀ mistargeting to mesaxon membranes, and through obligate P₀ homophilic adhesion, renders these dynamic membranes inert and halts myelination.

Key words: PNS myelination • homophilic adhesion • dysmyelination • cell polarity • myelin protein gene dosage

Introduction

Myelinating Schwann cells polarize their surface membranes into functionally and molecularly distinct membrane domains (Trapp et al., 1995). Initially, there are two principle domains. The abaxonal plasma membrane is exposed to and interacts with the external environment or endoneurial fluid. The adaxonal or periaxonal membrane is in direct contact with the axon. Myelination is initiated by the spiral wrapping of the Schwann cell mesaxon membranes around the axon. Mesaxon membranes connect the periaxonal and abaxonal membranes and are ultrastructurally characterized by a 12–14-nm gap between their extracellular leaflets and Schwann cell cytoplasm separating their cytoplasmic leaflets (Trapp and Quarles, 1982). Once the mesaxon spirals upon itself two to six times, the periodicity of most mesaxonal membrane converts to compact myelin; extracellular leaflets are separated by a 2.5-nm gap and the cytoplasmic leaflets appear fused (Peters et al., 1991).

Concomitant with the changes in periodicity of mesaxon membranes to compact myelin are molecular changes in the membranes. Periaxonal and mesaxon membranes are enriched in the myelin-associated glycoprotein (MAG), a type I transmembrane glycoprotein with five immunoglobulin-like domains and a molecular weight of ~100 kD. MAG is not detected in compact myelin or the abaxonal membranes of myelinating Schwann cells (Trapp and Quarles, 1982). P₀ protein, the major structural protein of compact PNS myelin (Trapp et al., 1981) is another type I glycoprotein with a single immunoglobulin-like domain and molecular weight of 30 kD. While precise mechanisms by which mesaxon membranes convert to compact myelin are unknown, membrane insertion of P₀ and its subsequent homophilic binding in both trans and cis orientations may exclude MAG and result in compact myelin formation (Haight et al., 1991; Shapiro et al., 1996). MAG and P₀ are sorted into separate carrier vesicles as they exit the trans-Golgi network (Trapp et al., 1995). These vesicles are transported along the myelin internode in a microtubule (MT)-dependent manner, and then inserted directly into the appropriate membrane domain. This site-specific

Address correspondence to Dr. Bruce D. Trapp, Department of Neurosciences, NC30, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195. Tel.: (216) 444 7177. Fax: (216) 444 7927. E-mail: trappb@ccf.org

Abbreviations used in this paper: CMT1A, Charcot-Marie-Tooth disease type 1A; CMT1B, Charcot-Marie-Tooth disease type 1B; CNP, 2',3' cyclic nucleotide 3'-phosphodiesterase; MAG, myelin-associated glycoprotein; MDL, major dense line; MT, microtubule; P₀tg, TgN22Mes transgenic mice overexpressing P₀ proteins; PLP, proteolipid protein; PMP22, peripheral myelin protein 22 kD; WT, wild-type.
targeting of P0 and MAG plays an important role in establishing the polarity and expansion of Schwann cell membranes (Heath et al., 1991; Trapp et al., 1995). Myelinating Schwann cells can synthesize several square millimeters of surface membranes. This requires high-level transcription of myelin protein genes and precisely regulated doses of translated proteins. Natural and induced myelin protein gene duplications cause dysmyelination and often more severe phenotypes than null mutations in the same gene, indicating the functional importance of appropriate myelin protein gene dosage during myelination. Proteolipid protein (PLP), an integral membrane protein with five membrane-spanning domains, is the major structural protein of CNS myelin (Lees and Brostoff, 1984). Duplications in PLP cause Pelizaeus-Merzbacher disease (Hodes and Dlouhy, 1996), an often fatal dysmyelinating condition of humans. PLP overexpression in transgenic mice also causes dysmyelination (Kagawa et al., 1994; Readhead et al., 1994). Peripheral myelin protein of 22 kD (PM22) has four membrane-spanning domains and is enriched in PNS myelin (Snipes et al., 1992). Reciprocal unequal crossovers of a 1.5 megabase region of chromosome 17p11.2 causes allelic duplication or deletion of the PM22 gene (Chance et al., 1994). A lethal duplication of 17p11.2 causes Charcot-Marie-Tooth disease type 1A (CMT1A), a human peripheral neuropathy characterized by hypomyelination, demyelination/remyelination, onion bulb formation, and axonal atrophy (Lupsiki et al., 1991; Matsunami et al., 1992). Hereditary neuropathy with liability to pressure palsy (HNPP) is associated with allelic deletion of 17p11.2 (Chance et al., 1993). A mutation in the PM22 gene dosage in rodents also causes peripheral neuropathies (Magyar et al., 1992; Sereda et al., 1996) and supports altered PM22 gene dosage as the causative factor in CMT1A and HNPP.

P0 gene duplication has not been associated with human peripheral neuropathies. P0 missense mutations, however, cause a variety of clinically defined human peripheral neuropathies including CMT1B, Dejerine-Sottas syndrome, and congenital hypomyelination (Warner et al., 1996). P0 null mutations in mice also cause dysmyelination (Giese et al., 1992). To investigate the potential consequence of increased P0 gene dosage, several lines of transgenic mice with extra copies of the mouse P0 gene were generated as described in Wrabetz et al. (2000, this issue). In this report, we have investigated the ultrastructural changes and mechanisms of dysmyelination in the line with highest P0 mRNA overexpression. Our studies indicate that P0 accumulates in inappropriate domains of the plasma membrane, blocking spiral mesaxon growth and preventing myelin formation.

Materials and Methods

Generation of Transgenic Mice Overexpressing P0 Protein Gene

The mP0TOT vector consists of the whole mouse P0 gene, including 6 kb of the promoter, all the exons and introns, and the natural polyadenylation signal. This vector directs appropriate cell-specific and developmentally regulated expression of a lacZ reporter gene (Felti et al., 1999). Generation of transgenic mice carrying the mP0TOT is described in detail in Wrabetz et al. (2000, this issue). In brief, eight founders were produced that expressed the transgene in peripheral nerve, all displaying varying levels of expression and evidence of peripheral neuropathy including weakness, tremors, and paralysis. Lines were established from three of these founders. This report focuses on the line with the highest level of expression and the most severe neuropathy, TgN22M mice (also referred to as line TG0802 in Wrabetz et al., 2000, this issue). For simplicity, throughout the rest of this report the TgN22M mice are referred to as P0/0.

Light and Electron Microscopic Analysis

Mice overexpressing P0 protein genes and littermate controls were examined at 2, 5, 14, 42, and 90 d of age. They were anesthetized with M etofane and perfused with 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.08 M Sorenson’s buffer. Sciatic nerves and lumbar ventral roots were removed, postfixed in osmium tetroxide, and embedded in Epon. For light microscopy, 1-μm thick sections of sciatic nerve were stained with toluidine blue and photographed with a Zeiss Axiophot microscope. For serial section analysis, series of 1-μm thick sections of lumbar ventral roots were mounted in order on slides and stained with toluidine blue. A reas of interest were digitally photographed using a Leica DMLB microscope fitted with an Optronics video camera and image acquisition system. Images were placed in order and aligned on separate layers in A dobe Photoshop 5 software. Profiles of interest were marked and followed by stepping through the layers.

For confocal microscopy, paraformaldehyde-fixed sciatic nerves from 25-d-old mice were stained and immunostained as described (K id et al., 1996). The teased fibers were imaged using a Leica TCS-NT confocal microscope.

For EM, thin sections were stained with uranyl acetate and lead citrate, and examined in a Philips CM100 electron microscope.

Electron Microscopic Immunocytochemistry

P0/0 and age-matched wild-type (WT) mice at 5, 14, 42, and 90 d postnatal (three mice per time point) were anesthetized with M etofane and perfused with 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.08 M Sorenson’s buffer. The sciatic nerves and L4 ventral roots were removed, infiltrated with 2.3 M sucrose and 30% polyvinylpyrrolidone, placed on specimen stubs, and were then frozen in liquid nitrogen. Ultrathin cryosections (~120-nm thick) were cut on glass knives in an A eichart Utracut 5 ultracryomicrotome (Leica Instruments) maintained at −110°C. The sections were placed on carbon- and Formvar-coated grids and immunostained by previously described immunogold procedures (Trapp et al., 1995).

Antibodies

A nibodies used in these studies are well characterized and include: polyclonal directed against P0 protein (Trapp et al., 1979, 1981), and monoclonals directed against MAG (D oberson et al., 1985; Trapp et al., 1989, 1995), neurofilaments (SMI 31 & 32; Sternberger Monoclonals Inc.), and acetylated α-tubulin (Sigma Chemical Co.). For quantitative immunogold labeling experiments, sections from WT and P0/0 mice were stained in parallel.

Quantitative Analysis of Immunogold Labeling

Ultrathin cryosections were examined and photographed in a Philips CM 100 electron microscope. Digital images were captured from negatives. Gold particles over structures of interest were counted on screen, and areas and distances measured in NIH Image or Photoshop 5 software. Statistical analysis was performed by t test. Data are expressed as mean ± SE. For P0 labeling of Schwann cell surface membranes, an average of 24 fibers was quantified at each age in both control and P0/0 mice. These fibers were obtained from two control nerves and three P0/0 nerves at each age. This analysis included 76 μm of periaxonal membrane (only 2 d analyzed) and 340 μm of axobonal membrane in control, and 340 μm of periaxonal membrane and 476 μm of axobonal membrane in P0/0 cells. A n average of 17 endosomes was analyzed at each age in P0/0 mice. In control, 20 and 5 endosomes were analyzed at 5 and 14 d, respectively. Endosomes were not analyzed at 42 and 90 d in control nerves. 36 (control) and 27 (P0/0) Golgi apparatus were analyzed at 5 d, and 18 (control) and 35 (P0/0) Golgi apparatus at 42 d. The density of P0 staining over compact myelin was determined in 25 fibers from 42 d control and P0/0 nerves. These fibers were obtained from three control and three P0/0 nerves.
Results

Several lines of transgenic mice were generated which carried additional copies of the mouse P₀ gene, under control of the native P₀ promoter region. Molecular and morphological features and neurological phenotypes of these mice are described in Wrabetz et al. (2000, this issue). Histologically, the most prominent feature of P₀-overexpressing mice was hypomyelination of peripheral nerves; CNS tissues, which express little or no P₀, appeared unaffected. The degree of neurological impairment correlated with transgene expression and dysmyelination was observed in several lines, indicating that the phenotype was due to overexpression of the P₀ gene and not insertional mutagenesis. The present study investigated the cellular and molecular phenotypes in the line of mice with highest P₀ transgene expression (P₀tg). These mice displayed two consistent phenotypes, failed axonal sorting and arrested myelination.

P₀ Distribution in P₀tg Nerves

Most Schwann cells in P₀tg nerves surrounded single axons or bundles of large diameter axons. To determine if these Schwann cells expressed P₀, teased nerve fibers from 25 d P₀tg nerves were stained with P₀ antibodies and examined by confocal microscopy. A myelinating Schwann cells surrounding single large diameter axons expressed abundant P₀, had small cell bodies, and were closely spaced along individual axons (Fig. 1 A). P₀ was detected in abaxonal and periaxonal regions of these cells and was concentrated in perikaryal cytoplasm. P₀tg Schwann cells that surrounded bundles of axons also expressed abundant P₀ (Fig. 1 B). This observation suggests that P₀ overexpression impedes the sorting of axons into individual ensheathments.

To investigate whether the same axon/Schwann cell relationships were maintained for the length of individual axons, serial 1-μm sections from 42 d ventral roots were digitally photographed, and individual axons followed.

Figure 1. P₀tg Schwann cells express P₀, fail to grow longitudinally, and develop asynchronously along single axons. Confocal micrographs of teased fibers from 25 d P₀tg nerves (A and B) immunostained with P₀ (green) and tubulin (red). Schwann cells associated with single (A and B, arrows) or bundled (B, arrowheads) axons express P₀ protein, but do not myelinate. C and D are identical fields from a set of 50 serial sections (1-μm thick) from a 42 d P₀tg nerve. E is a schematic reconstruction of axonal profiles in the serial sections. Two adjacent axons (C and D, arrowheads) diverge and attain one-to-one contacts with Schwann cells. Others (C and D, arrows) leave one-to-one ensheathments and join large bundles; the reverse also occurs (C and D, paired arrows). One axon exits a bundle, attains a one-to-one relationship with Schwann cells, and then becomes myelinated by another Schwann cell (E, m). Bars, 25 μm.
through 50–100 μm. Axon bundles split into smaller arrangements or combined to form larger bundles (Fig. 1, C, D, and E). Axons were observed to leave a bundle, achieve one-to-one contact with a Schwann cell and then join another (or the same) bundle. No obvious changes in axon diameter marked the transition from large bundles to smaller bundles or to one-to-one ensheathment. The diameters of myelinated and nonmyelinated segments of the same axon did not appear to differ significantly. There was no obvious proximal-to-distal preference in Schwann cell–axon associations (Fig. 1 E). In unmyelinated fibers, P₀tg Schwann cell nuclei were often spaced 1–3 μm apart. In contrast, in WT nerves, nuclei of nonmyelinating (Remak) Schwann cells were 50 μm or more apart, and the nuclei of myelinated Schwann cells were 200–1,000 μm apart.

EM analysis confirmed the phenotypes of failed axonal sorting and arrest of myelination in P₀tg nerves (Fig. 2). At early stages of nerve development (five days), axons that failed to sort in one-to-one relationships were tightly bundled and totally or partially surrounded by Schwann cells (Fig. 2 A). A basal lamina often surrounded the outer perimeter of unenveloped regions of axon bundles, indicating former Schwann cell ensheathment. Myelin was not detected around axons in one-to-one relationships with Schwann cells, and occasionally individual Schwann cells were associated with two axons (Fig. 2 B). These phenotypes predominated in 90 d P₀tg nerves (Fig. 2, C and D). Many axons that failed to sort in one-to-one Schwann cell relationships had diameters in excess of 1 μm, and therefore should have been myelinated.

At all ages analyzed by EM, perinuclear cytoplasm of P₀tg Schwann cells contained the usual complement of organelles and did not display substantial lipid vacuoles, swollen ER, abnormal appearing Golgi apparatus, or apoptotic changes that would indicate that the Schwann cells were dying. Myelin degeneration, debris-laden macrophages, and onion bulbs were not observed in P₀tg or WT nerves.

P₀ Is Mistargeted in P₀tg Schwann Cells

The correlation between extra copies of the P₀ gene and arrest of myelination suggests that overexpression of P₀ protein is responsible for the phenotype. To investigate this and elucidate the possible mechanism, the ultrastructural distribution of P₀ protein was compared in ultrathin cryosections of control and P₀tg nerves. In 2-d WT nerves, P₀ antibodies labeled compact myelin, but not Schwann cell periaxonal, mesaxonal, or abaxonal membranes (Fig. 3 A). In P₀tg nerves, however, intense P₀ labeling of Schwann cell periaxonal, mesaxon, and abaxonal membranes was detected (Fig. 3 B). P₀ labeling of axons and Schwann cell nuclei was rare in both control and P₀tg nerves.

Cryosections from 2, 5, 14, 42, and 90 d control and P₀tg nerves were stained with P₀ antibodies and gold particle densities were quantified per linear length of the periax-
Yin et al.  

**Overexpression Arrests Myelination**

In control nerves, P₀ gold particles could only be reliably quantified over periaxonal membranes in Schwann cells that had not formed compact myelin at two days. In Schwann cells from control nerves, the labeling indices of two day periaxonal membranes and 2, 5, 14, 42, and 90 d abaxonal plasma membrane ranged from 0.17–0.33 gold particles/µm² (not statistically different). The data are consistent with previous reports describing low or undetectable levels of P₀ in these membranes during normal myelination (Trapp et al., 1981). In contrast, the periaxonal and abaxonal membrane of P₀ tg Schwann cells that attained one-to-one relationships with axons but failed to myelinate, contained 10–20 times more P₀ labeling than WT fibers (Fig. 3 C). The similarity between the labeling indices of periaxonal and abaxonal membrane suggests constitutive insertion of P₀ into all surface membranes with arrested myelination in P₀ tg mice.

The possibility that Golgi membrane-associated P₀ protein is increased in P₀ tg Schwann cells was investigated by quantifying the number of gold particles associated with Golgi membranes and their associated vesicles in 5 and 42 d control and P₀ tg Schwann cells with arrested myelination (Fig. 3 E). Gold particles were expressed per micrometer squared of Golgi apparatus area. At five days, there was a threefold increase in P₀ gold particles (30.4 vs. 91.6) in P₀ tg Schwann cell Golgi membranes, when compared with five-day WT Golgi membranes. This difference was statistically significant (P < 0.0001). At 42 d, P₀ labeling of control Schwann cell Golgi apparatus (35.1 gold par-

---

**Figure 3.** P₀ is mistargeted in P₀ tg Schwann cells with arrested myelination. Immunogold labeling in WT Schwann cells at 2 d detected P₀ in compact myelin (A, m), but not in periaxonal membranes (A, arrows), mesaxons, or abaxonal membranes (A, arrowheads). In P₀ tg Schwann cells with arrested myelination, P₀ labeling is abundant in the periaxonal (B, arrows), mesaxonal, and abaxonal (B, arrowheads) membranes. Gold particles were quantified and expressed per linear micrometer of abaxonal (C) and periaxonal membrane (D), and per micrometer squared of Golgi apparatus (E) at several ages (mean ± SEM). t tests indicated that the density of P₀ was significantly increased (P < 0.0001) in P₀ tg membranes at all ages (C, D, and E). In addition, P₀ labeling of 5 d P₀ tg periaxonal membranes was significantly greater than P₀ tg periaxonal membranes at 14, 42, and 90 d (C, P < 0.0001). Periaxonal P₀ labeling cannot be scored after compact myelin forms, so WT values were only obtained at 2 d (D). Bar, 0.25 µm.
Figure 4. $P₀$ is correctly targeted in myelinating Schwann cells from 45 d $P₀^{tg}$ nerve. $P₀$ immunogold labeling of myelinating Schwann cells from WT (A) and $P₀^{tg}$ (B) ventral roots. $P₀$ is concentrated in compact myelin (A and B, m), but little $P₀$ is detected on the abaxonal plasma membrane (A and B, arrowheads). In contrast, myelination-arrested Schwann cells have abundant $P₀$ staining of the abaxonal membrane (C, arrowheads). $P₀$ labeling of myelination-arrested $P₀^{tg}$ Schwann cell abaxonal membranes was significantly increased ($P < 0.0001$, t test) compared with abaxonal membranes in myelinating $P₀^{tg}$ and WT Schwann cells (F). Compact myelin appears ultrastructurally similar in WT (D) and $P₀^{tg}$ internodes (E). $P₀$ labeling of compact myelin was significantly increased ($P < 0.0001$) in $P₀^{tg}$ nerves (F). Bars, 1 µm.

Some Schwann cells in $P₀^{tg}$ nerves escaped the inhibition of myelination and formed compact myelin, suggesting that these cells may have established normal targeting pathways for $P₀$. $P₀$ labeling indices for the abaxonal membrane of these $P₀^{tg}$ myelinating Schwann cells were identi-
cal (0.2 particles/μm) to age-matched WT fibers (Fig. 4, A and B) and tenfold less than those detected in nonmyelinating P0tg Schwann cells in the same sections (Figs. 3, C and D, 4 C). When P0 labeling over compact myelin was compared in controls (Fig. 4 D) and P0tg mice (Fig. 4 E), the labeling index was ~40% higher for P0tg internodes (358 vs. 251 gold particles/μm²). This observation argues against the complete silencing of the P0 transgene in Schwann cells that myelinate in P0tg nerves.

**MAG Sorting and Targeting Is Unaffected by P0 Overexpression**

Overexpression and mistargeting of P0 may also affect the sorting and targeting of MAG, which is enriched in periaxonal and mesaxon membranes and excluded from compact myelin and the Schwann cell abaxonal membrane (Trapp and Quarles, 1982; Trapp et al., 1995). Immunogold experiments were performed to determine if MAG was appropriately targeted in P0tg Schwann cells. As in WT myelinating cells, MAG antibodies labeled Golgi membranes and cytoplasmic vesicles of myelination-arrested P0tg Schwann cells (Fig. 5A), indicating that the protein was synthesized and processed in these cells. When MAG labeling indices were compared in five day WT and P0tg nerves (Fig. 5 B), abaxonal membrane MAG staining was low (<0.2 particles/μm) in both control and P0tg nerves (Fig. 5 C). In contrast, the periaxonal membrane of control and P0tg fibers contained 1.3 and 1.0 gold particles/μm (Fig. 5 C). Whereas P0 overexpression slightly reduced the amount of periaxonal MAG, it had no apparent effect on the targeting of MAG to the periaxonal membrane. MAG labeling was rare in Schwann cell nuclei or axons.

P0 and MAG are sorted into separate carrier vesicles as
they exit the trans-Golgi network (Trapp et al., 1995). To determine if P₀ overexpression alters P₀ or MAG sorting, cryosections from five day mice were labeled with both P₀ and MAG antibodies, and gold particles associated with vesicles in the area of the trans-Golgi network (Fig. 5 B) were quantified (Fig. 5 C) as MAG-positive, P₀-positive, or MAG-P₀-positive. As described previously in control nerves (Trapp et al., 1995), ~80% of vesicles in P₀⁰⁰ Schwann cells contained only P₀ gold particles (5 nm), 19% contained only MAG gold particles (10 nm), and 1–2% contained both MAG and P₀ (5- and 10-nm gold particles). In both control and P₀⁰⁰ Schwann cells, ~65% of the P₀-labeled vesicles contained more than one gold particle. These data indicate that MAG and P₀ are appropriately sorted into separate carrier vesicles as they exit the trans-Golgi network of P₀⁰⁰ Schwann cells with arrested myelination.

**P₀ Is Abnormally Enriched in Endosomes in P₀⁰⁰ Schwann Cells**

A abundant P₀ labeling of transport vesicles and normal appearance of RER and Golgi membranes suggested that P₀ protein was appropriately synthesized and processed in P₀⁰⁰ Schwann cells with arrested myelination. However, P₀ labeling indices of Schwann cell surface membranes remained relatively constant between 14 and 42 d (Fig. 3, C and D). These observations suggest a rapid turnover of P₀ from P₀⁰⁰ Schwann cell surface membranes, possibly by endocytosis and P₀ degradation through the endosomal/lysosomal system. This hypothesis was tested by comparing the density of gold particles over endosomes in P₀⁰⁰-stained cryosection from control and P₀⁰⁰ Schwann cells at 5, 14, 42, and 90 d (Fig. 6). At five days, endosomes were prominent in P₀⁰⁰ Schwann cells with arrested myelination (Fig. 6 B). Endosomes were less conspicuous in control Schwann cells (Fig. 6 A). Quantification of P₀ labeling detected 10 gold particles/μm² of endosomal area in control Schwann cells. In contrast, P₀⁰⁰ Schwann cells contain 191 gold particles/μm² of endosome area. At 14 d, endosomes in P₀⁰⁰ Schwann cells contained 129 gold particles/μm², compared with 12 gold particles/μm² in control Schwann cells. At 42 and 90 d, endosomes in P₀⁰⁰ Schwann cells contain 152 and 101 gold particles/μm². Endosomes were rare in control Schwann cells of the same ages and, when present, contained >1 gold particle/μm². These data are consistent with continuous removal of mistargeted P₀ in P₀⁰⁰ Schwann cells with arrested myelination.

**Mistargeted P₀ Disrupts the Mesaxon**

Mesaxon membranes spiral upon themselves during initial stages of myelination. They initially form 3–6 spiral wraps, which contain detectable levels of MAG, but not P₀ protein (Trapp, 1988). The periodicity of portions of these mesaxon membranes then changes to that of compact myelin: extracellular leaflets are separated by a 2.5-nm gap and cytoplasmic leaflets appear as a fused major dense line (MDL). At this stage, P₀ protein is detectable in single MAG-negative compact myelin lamellae (Trapp, 1988). These observations are consistent with the hypothesis that mesaxon membranes are dynamic migratory membranes and that the insertion of P₀ protein into these mesaxon membranes excludes MAG and favors homophilic binding between the extracellular domains of P₀ in both cis and trans orientations, resulting in the 2.5-nm periodicity of compact myelin. A nalysis of the periodicity of P₀⁰⁰ and control Schwann cell periaxonal and mesaxonal membranes provides an opportunity to test this hypothesis. The normal 12–14 nm spacing between the extracellular leaflets of periaxonal membrane and axolemma, and between apposing mesaxon membranes are shown in a control fiber (Fig. 7, A and B). In P₀⁰⁰ Schwann cells (Fig. 7, C and D), the periaxonal space is similar to that in control fibers (12–14 nm). However, the extracellular leaflets (Fig. 7 D) of the mesaxon wraps are separated by a smaller gap resembling that of compact myelin. This compact myelin-like separation of the extracellular leaflets of this mesaxon was documented in most P₀⁰⁰ Schwann cells with arrested myelination. The length of these P₀-containing mesaxon membranes varies from short outer mesaxon-like structures that connect periaxonal and plasma membranes (Fig.
Similarly, where the abaxonal membrane of two adjacent P0\textsuperscript{tg} Schwann cells were closely apposed, the basal lamina was excluded and the extracellular leaflets were separated by distances reminiscent of the periodicity of compact myelin (Fig. 7 F, arrowheads). The mesaxon membranes with closely apposed extracellular leaflets were stained by P0\textsuperscript{tg} antibodies (Fig. 7 G).

**Discussion**

This report describes arrest of myelination and failed axon sorting due to overexpression of P0 protein. Our data established that P0 protein is overexpressed in mesaxon membranes where its obligate homophilic adhesion arrests myelination. While molecular mechanisms responsible for failed axonal sorting were not investigated in detail, Schwann cells that fail to sort axons express excessive levels of P0. Early, high level P0 expression in developing Schwann cells inhibits the polarization of Schwann cell membranes into appropriate functional domains and the dynamic axonal interaction and Schwann cell membrane expansion required for appropriate axonal sorting and myelination.

**Specificity of Phenotype**

Axon-Schwann cell interactions and signaling are bidirectional and mediated by physical contact. Unidentified axonal signals initiate a myelinating genome in Schwann cells and axonal contact is essential for myelin formation (Aguayo et al., 1976; Weinberg and Spencer, 1976). Schwann cells in turn provide an extrinsic trophic effect for maturation of the axon (Windebank et al., 1985; deWaegh et al., 1992; Hsieh et al., 1994; Sanchez et al., 1996; Yin et al., 1998). Individual axons in P0\textsuperscript{tg} mice can be myelinated, surrounded by a Schwann cell with arrested myelination, or part of an axonal bundle (Fig. 1 E). These

---

*Figure 7. P0 mistargeting alters the spacing between Schwann cell membranes. A–E compare early mesaxon formation in control (A and B) and P0\textsuperscript{tg} (C, D, and E) fibers. The spacing between apposing Schwann cell mesaxon membranes (B and D, arrowheads) averages 12–14 nm in control (B, arrowheads) and resembles that of compact myelin in P0\textsuperscript{tg} (D, arrowheads). Spacing between Schwann cell peri-axonal membranes and the axons were similar in control and P0\textsuperscript{tg} fibers (B and D, arrows). Mesaxon membranes in P0\textsuperscript{tg} nerves rarely en-circled >50% of the axon (E). A baxonal membranes of adjacent P0\textsuperscript{tg} Schwann cells occasionally excluded their basal lamina and were closely apposed (F, arrowheads). Closely apposed mesaxon and abaxonal membranes were labeled by P0 antibodies (G, arrowheads). Ax, Axon; Nu, Schwann cell nuclei. Bars: (A, C, E, and G) 0.2 μm; (B and D) 0.1 μm; (F) 0.5 μm.*
observations and the rescue of the P0\textsuperscript{tg} phenotype through breeding to P0 null mice (Wrabetz et al., 2000, this issue) are most consistent with a primary Schwann cell defect in P0\textsuperscript{tg} mice. While extensive analysis of axonal diameters has yet to be performed, axonal diameters increase in the P0\textsuperscript{tg} nerves regardless of the Schwann cell state of differentiation. Schwann cells in one-to-one associations with axons contained MAG, a molecule previously demonstrated to influence axonal maturation by increasing the phosphorylation state and spacing of neurofilaments (Yin et al., 1998).

**P0 Overexpression Causes a Unique Phenotype**

Myelin protein overexpression causes a variety of phenotypes in mice. Whereas Schwann cells overexpressing P0 share some similarities with Schwann cells overexpressing other myelin proteins, the phenotype of failed axonal sorting and arrest of myelination at early mesaxon formation is unique. The P0\textsuperscript{tg} phenotype differs from that produced by the toxic accumulation of overexpressed PLP in the Golgi apparatus and RER (Kagawa et al., 1994; Readhead et al., 1994). Schwann cell death and abnormal RER and Golgi networks were not observed in P0\textsuperscript{tg} nerves. Processing and folding the four membrane-spanning PLP in the RER and Golgi apparatus may represent a greater challenge than the processing of the single membrane spanning P0 protein. Transgenic mice with extra copies of PM 22 genes display progressive neurological phenotypes and peripheral nerve pathology that correlate with copy number (Magyar et al., 1996; Sereda et al., 1996). At higher doses, Schwann cells sorted axons in one-to-one relationships, formed a normal basal lamina, rarely formed myelin, but had normal mesaxon membrane spacing. Although the function of PM 22 is unknown, the mechanisms responsible for arrest of myelination in PM 22 overexpressing mice are likely to differ significantly from those in P0\textsuperscript{tg} mice.

2′,3′ cyclic nucleotide 3′-phosphodiesterase (CNP) is an extrinsic membrane protein that associates with the cytoplasmic side of oligodendrocyte and Schwann cell surface membranes, but is normally excluded from compact myelin (Yin et al., 1997; Braun et al., 1988; Trapp et al., 1988). In transgenic mice with a sixfold increase in CNP levels (Gravel et al., 1996; Yin et al., 1997), oligodendrocytes spirally wrapped axons, but often failed to form MDL. CNP was mistargeted to the cytoplasmic leaflet of these myelin membranes and prevented normal accumulation of myelin basic protein (Yin et al., 1997), a molecular requirement of MDL formation in the CNS (Privat et al., 1979; Rouch et al., 1983). These observations provide another example of mistargeting of a myelin-related protein due to overexpression and indicate protein-specific phenotypes as a result of the molecular properties of the overexpressed proteins.

**Mechanism of P0 Targeting**

Much of what is known about mechanisms of protein sorting and targeting has been obtained from in vitro studies of epithelial cells that polarize their surfaces into apical and basolateral domains (Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990; Mostov et al., 1992; Weimbs et al., 1997). Proteins are targeted to appropriate domains by direct or indirect pathways (Rindler et al., 1984; Hubbard and Steiger, 1989). Previous studies support direct delivery of P0 protein to compact myelin in an MT-dependent manner (Trapp et al., 1995; Krid et al., 1996). In the present study, P0 protein was detected on all surface membranes of P0\textsuperscript{tg} Schwann cells, indicating that P0 is synthesized and transported, but not appropriately targeted. The distributions of Golgi apparatus, intermediate filaments, RER, and smooth ER are maintained by MTs (Trapp et al., 1995) and appeared unaltered in P0\textsuperscript{tg} Schwann cells. Based on these observations, it is unlikely that P0 mistargeting results from abnormal MT distribution or function.

P0\textsuperscript{tg} Schwann cells in one-to-one relationship with axons appropriately ensheathe axons, target MAG to periaxonal membranes, and form a basal lamina on their abaxonal membrane. Thus, a significant degree of membrane polarization and appropriate protein targeting occurs in the presence of P0 overexpression. Site-specific targeting of membrane proteins occurs via a variety of mechanisms including sorting into specific transport vesicles in the Golgi apparatus, site-specific intracellular vesicular transport, site-specific vesicle docking or fusion, inhibition of vesicle docking or fusion, and stabilization of proteins within discrete membrane domains (Weimbs et al., 1997; Allen and Balch, 1999). Whereas our data indicated normal sorting of P0 and MAG in the trans-Golgi network, we cannot rule out the possibility that excessive P0 is missorted into vesicles destined for membranes other than compact myelin. P0 labeling of Golgi membranes was increased in P0\textsuperscript{tg} Schwann cells. P0 did not accumulate in the Golgi apparatus with age, however, and Golgi membranes appeared normal in electron micrographs. These observations support normal synthesis and processing of P0 to the carrier vesicle stage. In addition, when carrier vesicle transport is halted in myelinating Schwann cells by MT disruption, P0-enriched carrier vesicles accumulated in perinuclear cytoplasm and fused to form compact myelin-like membranes (Trapp et al., 1995). Such membranes were not abundant in P0\textsuperscript{tg} Schwann cells cytoplasm, supporting targeting of P0 to surface membranes.

Whereas our data supports interpretations regarding the synthesis, sorting, and targeting of P0, they are based on static images of P0 distribution. Pulse–chase experiments of P0 Synthesis and degradation would be essential to establish the role of the endosomal system in P0 targeting (Gu and Gruenberg, 1999; Marsh and McMahon, 1999). While the interpretation that mistargeted P0 is removed from membranes by endocytosis is supported by the close proximity of P0-labeled endosomes to abaxonal membranes, it is possible that overexpressed P0 is also directly targeted to the endosomal/lysosomal system. In either case, the presence of significant amounts of P0 in endosomes during normal myelination supports the general conclusion that P0 overexpression results in P0 accumulation in endosomes.

**P0 Homophilic Binding Inhibits Mesaxon Expansion**

During normal myelination, P0 has been detected in compact myelin, but not mesaxon membranes. As mesaxon...
membranes convert to compact myelin, however, P₀ is likely to be transiently present in MAG-positive mesaxon membranes at levels not detected by current techniques. In P₀\textsuperscript{tg} mesaxon membranes, levels of P₀ reached the threshold for detection and trans adhesion during their initial wrap. A rest of myelination may result from simple overexpression of P₀ in mesaxon membranes, where it is normally expressed at low undetectable levels on its way to the expanding myelin sheath, or by early expression and mistargeting to mesaxon membranes before establishment of migratory machinery. P₀-mediated trans adhesion at 2.5 nm also requires exclusion of molecules with large extracellular domains. MAG (Fig. 5, A and C) and P₀ (Fig. 3, B and D) were detected in the periaxinial membrane of P₀\textsuperscript{tg} Schwann cells that apposed the P₀-negative axolemma by 12–14 nm (Fig. 7). The abaxonal membrane of most P₀\textsuperscript{tg} Schwann cells contained significant levels of P₀ and a normal appearing basal lamina. The presence of P₀ in a single membrane, therefore, does not exclude other proteins with large extracellular domains. However, when P₀-positive mesaxon or abaxonal membranes apposed each other, MAG and the basal lamina were excluded, resulting in close apposition (2.5 nm; Fig. 7). In addition, P₀ transfects the membrane in nonadherent cells in vitro induced obligate adhesion of apposing plasma membranes, reorganization of submembranous cytoskeleton, and junctional complexes at the transition between adherent and nonadherent membrane domains (D’Urs o et al., 1990; Filbin et al., 1990).

Liquid crystallography at 1.9 Å resolution supports ema-

nation of the extracellular domain of P₀ as cis-linked tetramer that binds to P₀ tetramers in opposite apposing on the apoplastic membrane surface (Shapiro et al., 1996). Collectively, these data support the possibility that arrest of myelination in P₀\textsuperscript{tg} mice is caused by early trans-P₀ tetramer binding of the initial mesaxon wrap. This trans-P₀ binding induces cis-P₀ tetramer binding, which then excludes molecules responsible for spiral wrapping of me-

schon membranes.

We wish to thank Heide Peickert and D enice Springer for technical as-

sistance, and Vikki Pickett for typing the manuscript.

This work was supported by the National Institutes of Health grants NS-38186 (to B.D. Trapp) and NS-23375 (to A. M essing), by Telethon Italy (L. Wrabetz and M. L. Feltri), E uropean Community Biomed Program (L. Wrabetz), and F ondazione Giovanni Armenise-Harvard (L. Wrabetz and M. L. Feltri).

Submitted: 19 October 1999

Revised: 12 January 2000

Accepted: 24 January 2000

References

Aguayo, A. J., J. Epps, L. Charron, and G. M. Bray. 1976. Multipotentiality of Schwann cells in cross anastomosed and grafted myelinated and unmey-

elinated nerves: quantitative microscopy and radioautography. Brain Res. 104:1-20.

Allan, B. B., and W. E. Balch. 1999. Protein sorting by directed maturation of Golgi compartments. Science. 285:63-66.

Braun, P. E., F. Sandillon, A. Edwards, J.-M. Matthieu, and A. Privat. 1988. Immunocytochemical localization by electron microscopy of 2',3'-cyclic nu-

cleotide 3'-phosphodiesterase in developing oligodendrocytes of normal and mutant brain. J. Neurosci. 8:3057-3066.

Chance, P. F., M. A. Adelson, K. A. Lepig, M. W. Lensch, N. Matsunami, B. Smith, P.D. Swanston, S. Odellberg, C. M. Disteche, and T.D. Bird. 1993. DNA deletion associated with hereditary neuropathy with liability to pres-

sure palsies. Cell. 72:143-151.

Chance, P. F., N. A. babas, M. W. Lensch, L. Pentao, B. B. R oa, J. P. Patel, and J. R.
accumulation of neurofilaments during development independently of myelin formation. J. Neurosci. 16:5095–5105.
Sereda, M., I. Griffiths, A. Pühlhofer, H. Stewart, M. J. Rossner, F. Zimmermann, J. P. Magyar, A. Schnieder, E. Hund, H.-M. Meinck, et al. 1996. A transgenic rat model of Charcot-Marie-Tooth disease. Neuron. 16:1049–1060.
Shapiro, L., J. P. Doyle, P. Hensley, D. R. Colman, and W. A. Hendrickson. 1996. Crystal structure of the extracellular domain from P0, the major structural protein of peripheral nerve myelin. Neuron. 17:435–449.
Simons, K., and A. Wandinger-Ness. 1990. Polarized sorting in epithelia. Cell. 62:207–210.
Snipes, G. J., U. Suter, A. A. Welcher, and E. M. Shooter. 1992. Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). J. Cell Biol. 117:225–238.
Trapp, B. D. 1988. Distribution of the myelin-associated glycoprotein and P0 protein during myelin compaction in Quaking mouse peripheral nerve. J. Cell Biol. 107:675–685.
Trapp, B. D., and R. H. Quarles. 1982. Presence of the myelin-associated glycoprotein correlates with alterations in the periodicity of peripheral myelin. J. Cell Biol. 92:877–882.
Trapp, B. D., L. McIntyre, R. H. Quarles, N. H. Sternberger, and H. d. Webster. 1979. Immunocytochemical localization of rat peripheral nervous system myelin proteins: P2 protein is not a component of all peripheral nervous system myelin sheaths. Proc. Natl. Acad. Sci. USA. 76:3552–3556.
Trapp, B. D., Y. Itoyama, N. H. Sternberger, R. H. Quarles, and H. d. Webster. 1981. Immunocytochemical localization of P2 protein in Golgi complex membranes and myelin of developing rat Schwann cells. J. Cell Biol. 90:1–6.
Trapp, B. D., L. Bernier, S. B. Andrews, and D. R. Colman. 1988. Cellular and subcellular distribution of 2',3' cyclic nucleotide 3' phosphodiesterase and its mRNA in the rat nervous system. J. Neurochem. 51:859–868.
Trapp, B. D., S. B. Andrews, C. Cootauco, and R. H. Quarles. 1989. The myelin-associated glycoprotein is enriched in multivesicular bodies and periaxonal membranes of actively myelinating oligodendrocytes. J. Cell Biol. 109:2417–2426.
Trapp, B. D., G. J. Kidd, P. E. Hauer, E. Mulrenin, C. Haney, and S. B. Andrews. 1995. Polarization of myelinating Schwann cell surface membranes: role of microtubules and the trans-Golgi network. J. Neurosci. 15:1797–1802.
Warner, L. E., M. J. Hitz, S. H. Appel, J. M. Killian, E. H. Koldobny, G. Karpati, S. Carpenter, G. V. Watters, D. Witt, et al. 1996. Clinical phenotypes of different MPZ (P0) mutations may include Charcot-Marie-Tooth type 1B, Demyerin-Sottas, and congenital hypomyelination. Neuron. 17:451–460.
Weimbs, T., S. H. Low, S. J. Chapin, and K. E. Mostov. 1997. A pical targeting in polarized epithelial cells: there’s more afloat than rafts. Trends Cell Biol. 7:393–399.
Weinberg, H. J., and P. S. Spencer. 1976. Studies on the control of myelogenesis. II. Evidence for neuronal regulation of myelin production. Brain Res. 113:363–378.
Windham, A. J., P. Word, R. P. Bunge, and P. J. Dyck. 1985. Myelination determines the caliber of dorsal root ganglion neurons in culture. J. Neurosci. 5:1563–1567.
Wrabetz, L., M. L. Feldri, A. Quattrini, D. Imperiale, S. Previtali, M. D’Antonio, R. Martin, X. Yin, B. D. Trapp, L. Xhou, et al. 2000. P0 overexpression causes congenital hypomyelination of peripheral nerves. J. Cell Biol. 148:1021–1033.
Yin, X., J. Peterson, M. Gravel, P. E. Braun, and B. D. Trapp. 1997. CNP overexpression induces aberrant oligodendrocyte membranes and inhibits MBP accumulation and myelin compaction. J. Neurosci. Res. 50:238–247.
Yin, X., T. O. Crawford, J. W. Griffin, P. H. Tu, V. M. Y. Lee, C. Li, J. Roder, and B. D. Trapp. 1998. Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. J. Neurosci. 18:1953–1962.