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SYMPOSIUM: Pathobiology of the Oligodendrocyte

The Pathobiology of Myelin Mutants Reveal Novel Biological Functions of the MBP and PLP Genes

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Substantial biological data indicate that the myelin basic protein (MBP) and myelin proteolipid protein (PLP/DM20) genes produce products with functions beyond that of serving as myelin structural proteins. Much of this evidence comes from studies on naturally-occurring and man-made mutations of these genes in mice and other species. This review focuses upon recent evidence showing the existence of other products of these genes that may account for some of these other functions, and recent studies providing evidence for alternative biological functions of PLP/DM20. The MBP and PLP/DM20 genes each encode the classic MBP and PLP isoforms, as well as a second family of proteins that are not involved in myelin structure. The biological roles of these other products of the genes are becoming clarified. The non-classic MBP gene products appear to be components of transcriptional complexes in the nucleus, and they also may be involved in signaling pathways in T-cells and in neural cells. The non-classic PLP/DM20 gene products appear to be components of intracellular transport vesicles in oligodendrocytes. There is evidence for other functions of the classic PLP/DM20 proteins, including a role in neural cell death mechanisms, autocrine and paracrine regulation of oligodendrocytes and neurons, intracellular transport and oligodendrocyte migration.

Introduction

The literature on the major myelin protein genes, their products, expression and genetic mutants is quite large and several excellent reviews have appeared recently (40, 43, 45, 46, 56, 81, 91). For this reason, this review focuses upon the discovery of newly identified gene transcripts encoded by the myelin protein genes and their possible biological functions in normal and pathological tissues.

The myelin basic proteins (MBP) and myelin proteolipid proteins (PLPs) are among the most abundant proteins in the central nervous system (30, 95). Consequently, it is not surprising that the structures of the genes encoding these proteins were among the first to be elucidated in the central nervous system (26, 55, 87, 113, 117, 122, 125, 138). These studies established that the size of the MBP and PLP genes were ~35 Kb and ~17 Kb, that they mapped to chromosome 18 and the X chromosome, respectively, in mouse and man. They also showed that the multiple mRNAs encoding MBP and PLP isoforms were derived through alternative splicing of the genes(25, 62, 94, 115, 122, 125, 138, 147). As discussed further in this article, considerable cell biological and morphological data performed in the 1970's and 1980's suggested the existence of other products and functions of these two genes, but the molecular corroboration of these findings took place in the 1990's. New exons and previously unidentified products have now been determined, and the recently revised structures of the MBP and PLP genes are shown in Figures 1 and 2.

The myelin basic protein gene and its products.

The MBP gene is very large, numbering among a relatively small number of neurobiologically-important genes of greater than 100Kb in length (22, 108). The gene contains three transcription start sites, generating three sets of mRNAs that encode two families of proteins. The classic MBPs are the products of transcription start sites 2 and 3 (Figure 1), and are well known as

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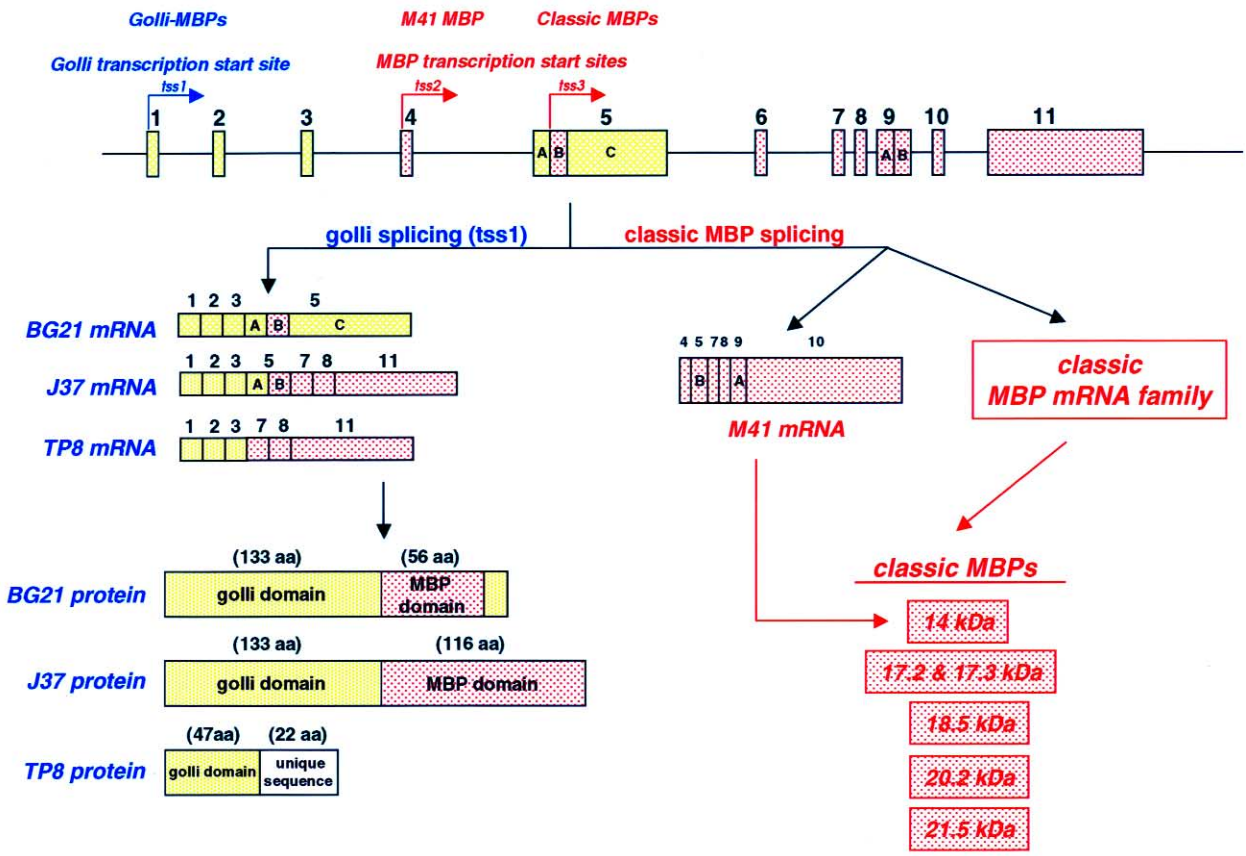

Figure 1. Intron-exon structure of the mouse myelin basic protein gene. The MBP gene consists of 11 exons with three transcription start sites. The most downstream start site (tss3) is the strongest and it is most active in myelin forming cells in the nervous system. The second start site (tss2) is responsible for producing the M41-MBP mRNAs. It is active in myelin forming cells at levels far below that of tss3, but it also is active at low levels in some cells of the immune system. Tss2 and tss3 are responsible for producing the class MBP isoforms. A second family of proteins, called the golli proteins, is produced from tss1, the most upstream promoter of the MBP gene. The golli proteins share in common at least 47 amino acids of the golli domain and the most abundant of them also contain MBP sequences. Tss1 is active in the immune system, oligodendrocytes and neurons. On the basis of RNase protection assays, tss1 appears to about as active in the immune system as it is in the brain.

major protein constituents of the myelin membrane. The classic MBPs appear to be expressed in greatest abundance, and almost exclusively, in myelin-forming cells. The second family of proteins encoded by the MBP gene is the golli (-mbp) proteins. These proteins are generated from the first transcription start site of the gene. In brain the steady-state levels of golli mRNAs are significantly lower than those of the classic MBP mRNAs (22). Because of this, cDNAs for the golli mRNAs went largely undetected in early studies to identify the MBP gene products from cDNA libraries.

It appears that the primary cell biological function of the classic MBPs is to maintain the structure of the myelin sheath. There is evidence, however, that one classic MBP isoform alone is capable of fulfilling this function in the absence of the other isoforms (64, 69), making it is unclear why the multiple isoforms are needed to maintain myelin structure. It has been observed that the classic MBP isoforms containing the sequence encoded by exon 6 (old exon 2) may be transported to the nucleus *in vitro* and *in vivo* during early stages of classic MBP expression (49, 103). This translocation appears to occur by active transport (103). It is not yet clear what role the classic MBPs may play in the nucleus although it has been speculated that it may have some regulatory function in implementing the myelination program (103).

About 10 years ago cDNA products of the MBP gene were isolated that suggested the existence of additional exons upstream of what was thought to be the beginning of the gene (70) and several years later the gene structure and the array of products (Figure 1) were elucidat-

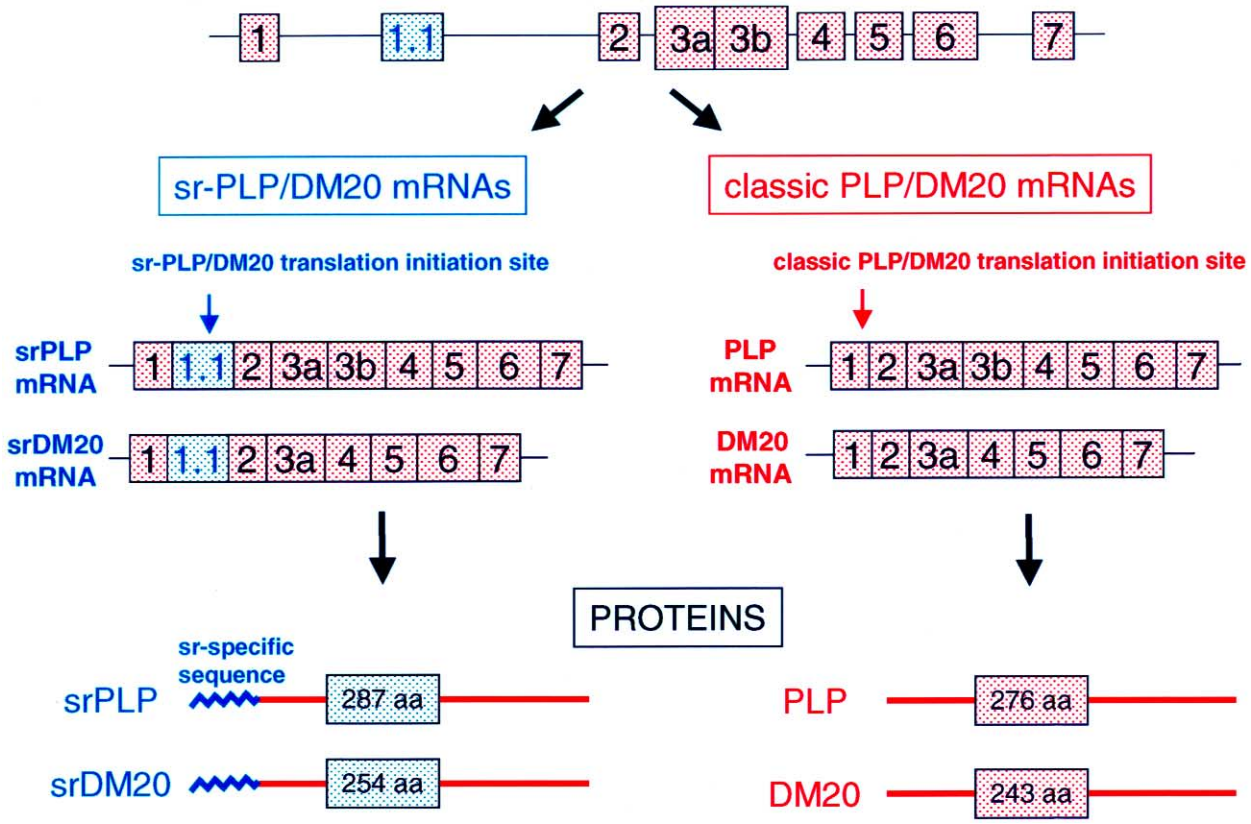

Figure 2. Intron-exon structure of the mouse PLP/DM20 gene. Alternative splicing of the PLP gene yields four products — the classic PLP and DM20 proteolipids, and the more recently described proteolipids, srPLP and srDM20. The classic PLP and DM20 are the major products produced by the gene and they are major components of the myelin sheath. The srPLP/DM20 proteolipids are not components of the myelin sheath and are localized in the cytoplasm of oligodendrocytes and neurons, probably in transport vesicles. Both the classic and sr products of the gene are expressed in the thymus. Exon 3b, and the protein sequence it encodes, is unique to the PLP isoforms. The sr-PLP/DM20 contain exon 1.1, thereby creating a preferred downstream translation intiation site. Utilization of this translation initiation site produced PLP and DM20 proteins with an additional sequence at their N-termini. This sequence is likely to be a targeting sequence, which sorts these proteolipids to the endoplasmic reticulum and vesicular structures rather that to the myelin sheath.

ed in mouse and man (22, 108). Since then the golli products of the MBP gene have been found in both neurons and oligodendrocytes (OLs) in the nervous system as well as T-cells and macrophages in the thymus of several species (40). The findings that MBP-related proteins are expressed in the immune system have begun to lead to a new understanding of the mechanisms of EAE induction as well as tolerance development to the MBPs, which are autoimmune-inducing agents in the nervous system (51, 53).

In the central nervous system, the golli proteins are expressed at high levels in the embryonic brain and peripheral nervous system, and expression declines somewhat after birth. Golli proteins are expressed in neurons in a number of important regions of the brain involved in the formation of the cerebral cortex (subplate neurons) and in secondary neurogenesis (*e.g.* the rostral migratory stream, cerebellar granule cells, and the hippocampus) (59). Some golli immunoreactivity can be detected in the myelin sheath (107); however, the majority of golli staining is associated with the nucleus and cell somas in OLs, *in vivo* and *in vitro* (Figure 3) (40, 107).

An unusual feature of the golli proteins is that they appear to be localized in two subcellular compartments, the cytoplasm and the nucleus, and that this localization is developmentally sensitive in a number of neuronal populations (79). Recently, it has been found that the site responsible for the translocation of the golli proteins into the nucleus resides within the MBP domain of the golli proteins (112).

Because golli proteins are expressed more promiscu-

ously than the classic MBPs and because they are found in different subcellular locations from the classic MBPs, the two families of proteins are likely to have different functions. Their function, however, has been unclear until very recently. *In vitro* transfection studies have shown that overexpression of golli proteins, or indeed the golli domain alone, in OL cell lines can induce these cells to elaborate extensive processes and membrane sheets, adopting a morphology similar to OLs in culture (112). Similarly, overexpression of golli in neuronal cell lines results in significant neurite extension, and in PC12 cells induces neurite growth reminiscent of that induced by NGF. These results complement the known expression of these products *in vivo* in a number of neuronal populations and in OL precursors during process extension and migration (78-80, 107). The results combine to suggest a role for golli proteins in process extension and possibly myelin elaboration.

A molecular partner for golli proteins, called GIP (golli-interacting protein) has been isolated by a yeast two-hybrid approach which suggests that, in the nucleus, golli may be part of a transcriptional regulatory complex (32). GIP is a nuclear protein found in all cells in which golli has been found. It has high homology to NLI-interacting factor (NLI-IF); and, like NLI-IF, can bind to NLI (nuclear LIM interactor), which is known to be associated with LIM-containing complexes that are involved in regulating transcription of some genes (61, 65). Thus, in the nucleus, golli is likely to be involved in the modulation of genes regulated by LIM.

Campagnoni and coworkers have generated a golli knock out mouse in which the golli products of the MBP gene have been selectively ablated (20, 101). Interestingly, in proliferation assays using T-cells isolated from golli knock out mice, it appears that proliferation of T cells via the PKC or MAP kinase pathways is altered. *In vitro* assays show that the major golli protein, BG21, is capable of being phosphorylated by PKC, and that golli protein J37 can be phosphorylated by MAP kinase (J. M. Feng, A. Fernandes and A. Campagnoni, unpublished results). The classic MBPs have served as *in vitro* substrates for PKC and MAP kinase and these results suggest that the golli proteins may be natural substrates for these kinases, and may play a role in regulating gene activity via their role in a second messenger pathway.

The myelin proteolipid protein gene and its products.

The myelin proteolipid proteins, PLP and DM-20, are transmembrane proteins that constitute about 50% of

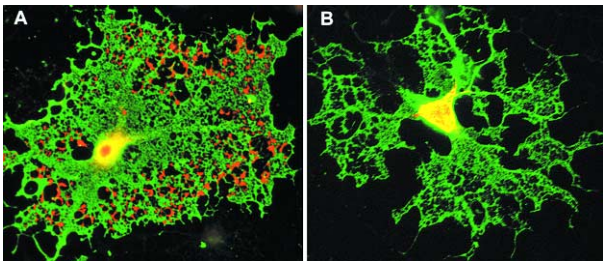

Figure 3. Differential localization of the golli and MBP products of the MBP gene in oligodendrocytes in vitro. (**A**) OL immunostained for galactocerebrosides (green) and MBP (red). Note the localization of the MBPs in the myelin-like membranes emanating from the OL cell body. (**B**) OL immunostained for galactocerebrosides (green) and golli (red). Note the localization of golli to the OL cell body and proximal regions of thick processes, and the lack of labeling of the myelin-like membranes. (Courtesy of Dr. M. Irene Givogri)

the protein in the myelin sheath and, as such, are among the most abundant proteins in the CNS (30). The amino acid sequences of the PLPs have been deduced from their corresponding cDNAs in many species, and their strong conservation among species has been noted by many investigators (See reviews 52, 84, 93). The PLP/DM20 gene, located on the X chromosome in mouse, rat and man, is ~17 kb in length and consists of 7 exons (Figure 2). An eighth exon, encoding a different 3′ untranslated sequence, has been suggested from sequence analysis of cDNA clones isolated from a rat sciatic nerve library (63) but its existence has not been investigated further. PLP was thought to be the only proteolipid in myelin until the early 1970's when a second proteolipid, called DM20, was identified and characterized (1). Peptide mapping of the purified polypeptides indicated that DM-20 differed from PLP by an internal deletion (132) and molecular biological studies established the location of a 35 amino acid deletion in the protein, encoded by exon 3B of the gene (Figures 2, 4) (92).

The PLP/DM20 gene is alternatively spliced to produce two major mRNA products that encode 30 kDa (PLP) and 25 kDa (DM20) polypeptides. The gene also possesses three polyadenylation signals within the last exon, the utilization of which generates a family of mRNAs of ~1.6 Kb, ~2.4 Kb and ~3.2 Kb. The proportions of these size classes of mRNAs varies considerably among species (21) and all three size classes appear to encode both PLP and DM20 polypeptides (82). Vouyioklis *et al*. (134) have recently isolated and determined the structure of a relatively stable PLP pre-mRNA containing an intact intron 3 and localized in the nucleus. These results explain earlier findings of an

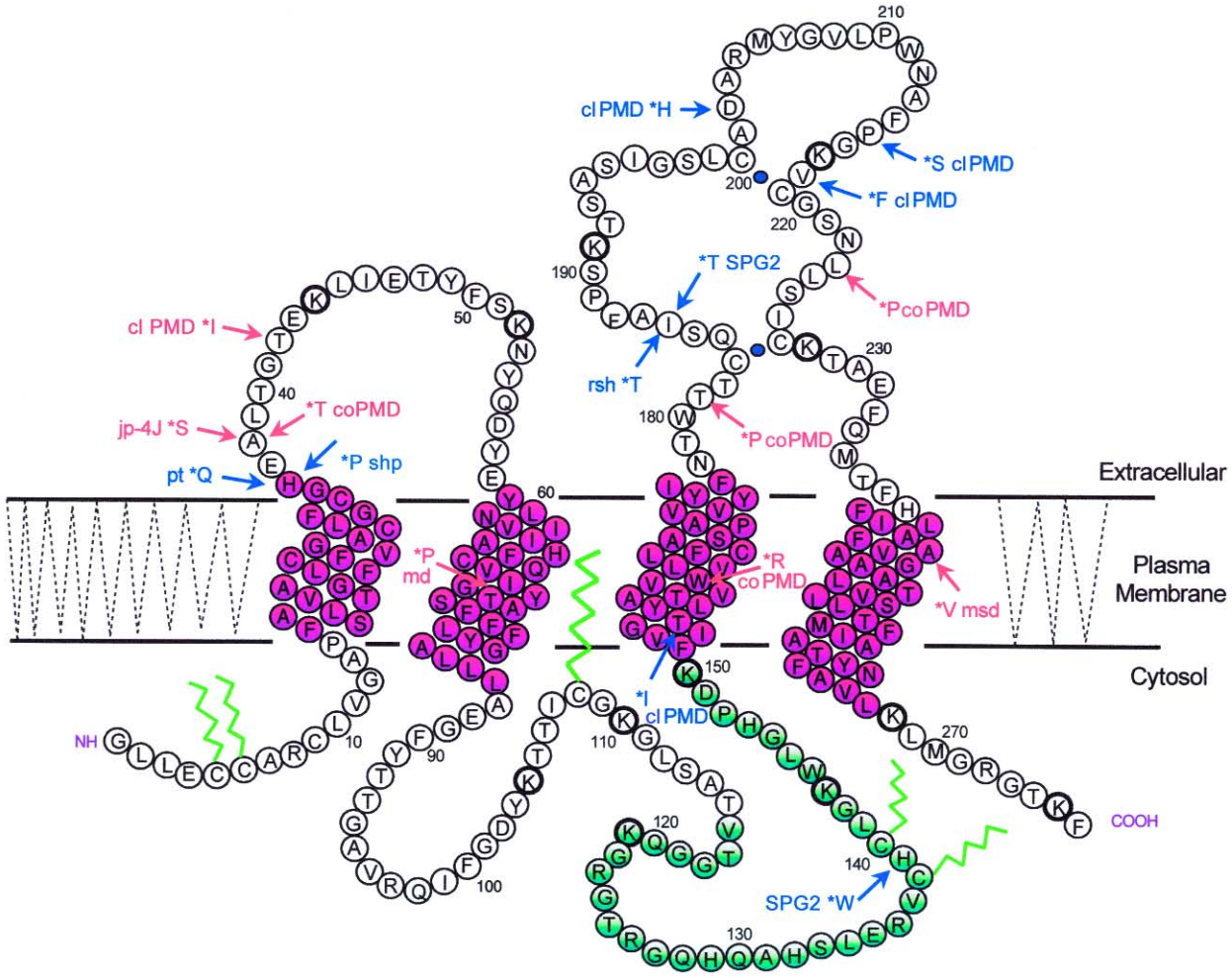

Figure 4. Proteolipid protein sequence in its likely conformation in the plasma membrane. The alternatively spliced DM20 sequence is represented by blue-green circles. Two extracellular disulfide bonds Cys183-Cys227 and Cys200-Cys219 are shown. One confirmed and 4 putative palmitoylation sites are shown (green lines). Selected animal and human mutations are shown (red arrows = severe phenotype, blue arrows = mild phenotype). PMD, Pelizaeus-Merzbacher Disease; SPG2, spastic paraplegia, type 2. Animal mutations are listed in Table 1. Figure courtesy of Dr. L. Carlock, Wayne State University Medical School.

unidentified 4.6 kb PLP mRNA related band observed in nuclear fractions of the brain by others (121)

Recently, an additional exon, lying between exons 1 and 2 of the gene, has been identified (*i.e.* exon 1.1) that gives rise to at least two additional mRNAs of ~3.4 kb, and which encode polypeptides that contain an additional 12 amino acids at the N-terminus of PLP and DM20 in the mouse (18). These products are also derived from alternative splicing of the gene (Figure 2). These proteolipids (called sr-PLP and sr-DM20) contain exon 1.1 of the gene, which is absent in the classic PLP and DM20 isoforms. The inclusion of exon 1.1 in the mRNA alters the translation start site, such that the resulting protein products are identical to the classic PLP and DM20 except that they contain a 12 amino acid "leader" at their N-termini. Because immunohistochemical results showed that antibodies against the sr-proteolipids stained cell soma and not myelin sheaths, they were named sr-PLP/DM20 for "somal-restricted".

In addition to OLs, sr-PLP/DM20 proteins are also expressed by a number of classes of neurons. Postnatally they are expressed in neuronal populations undergoing secondary neurogenesis and in some instances overlap expression of the golli proteins (59, 60). Interestingly, they are also expressed in cells emerging from both the posterior and anterior subventricular zone from birth to adults, which presumably are glial and neuronal precursors, respectively (59). Thus,

like the golli proteins they may play a role in the migration/differentiation and/or process extension of neurons and glia.

Transcriptional regulation of myelin protein genes.

Many different transcription factors in myelinating cells have been identified (88, 135, 136, 148), and current information about the regulatory regions of MBP and PLP genes have been thoroughly reviewed (136).

Several transcription factors in OLs that appear to be directly involved in OL differentiation and/or myelin gene expression have been examined. The zinc finger binding domain transcription factor, MyT1, is expressed more abundantly in OL progenitors than in mature OLs(7). Developmentally, MyT1 expression in OL precursors precedes transcription of the PLP/DM20 gene, but it temporally overlaps with PLP/DM20 gene expression. The PLP/DM20 gene has binding sites for MyT1 protein (67) suggesting that one function of MyT1 might be regulation of PLP transcription. Two homeodomain binding proteins SCIP and Gtx may be involved in OL differentiation. SCIP may be involved in the developmental switch from proliferation to a postmitotic status. Levels of Gtx parallel levels of myelin gene expression suggesting it regulates myelin mRNAs (8).

The nuclear hormone receptor superfamily deserves attention because the hormones that bind to these receptors have profound effects upon OL differentiation and myelin gene expression. Indeed, binding elements for retinoic acid, glucocorticoids, androgens, estrogens and PPARs have been identified in the golli promoter of the MBP gene, which is expressed significantly earlier in development than the classic MBP promoter; and a thyroid-binding element has been identified in the latter promoter.

The peroxisome proliferator activated receptors (PPARs) are another family within the nuclear hormone receptor superfamily that has recently been shown to regulate OL differentiation (42). PPARs have been described as a family of master genes that regulate differentiation of specific cell types. The biological function of PPARs relevant to OL differentiation is the observation that hydrophobic ligands such as T3 and retinoic acid promote high affinity interactions between their receptors and members of the PPAR family (72, 111), and they are major inducers of OL differentiation (4, 10). One of the three major PPARs (PPARδ) has been identified in OLs by nuclease protection assays and *in situ* hybridization studies (42). Moreover, bromopalmitate, a modified 16-carbon chain fatty acid known to activate PPARδ, causes a swift induction of OL differentiation in culture.

Drugs that are selective for the different PPARs have been developed (12), and application of a PPARδ-specific ligand to primary glial cultures and enriched OL cultures cause a massive increase in the size of OL membrane sheets within 1 day of application of the drugs (Saluja and Skoff, unpublished). MBP and PLP/DM20 mRNAs are also upregulated in these OLs, and one report describes a putative PPRE in the PLP/DM20 promoter (16). PPARδ null mice have a smaller corpus callosum than controls. However, it is unclear whether the absence of PPARδ affects primarily OLs and myelin formation or whether the effects are secondary due to neuronal abnormalities since the brains of the null mice are smaller (104).

Transcription factors including MyT1, PPARδ and CREB are detected in OL progenitors in tissue culture. However, it is not yet known if these transcription factors are expressed in neural progenitors during embryonic development or what their functional role would be in cells in the OL lineage *in vivo*.

Myelin PLP/DM20 gene expression.

In the nervous system, the splicing of the PLP/DM20 gene primary transcript appears to be developmentally regulated. The developmental appearance of classic DM20 precedes that of the classic PLP in several species (38, 57, 76, 82, 118, 130). Prior to and during the early stages of myelination, the expression of classic DM20 predominates and it gradually declines with development while classic PLP increases until it predominates. Interestingly, the same pattern of expression of spliced products also is found in the developmental expression of the srDM20 and srPLP isoforms (18). For many years it was thought that the PLP/DM20 gene was expressed only in myelin forming cells, but in recent years expression of the gene has been observed in the PNS, embryonic CNS, heart, spleen, thymus and lymph nodes (23, 57, 63, 106, 109, 110, 129, 130, 132). It appears that both the classic PLP/DM20 and srPLP/DM20 isoforms may be expressed outside the nervous system. Although in most cases the DM20 isoform is expressed in non-myelinating cells, the expression of PLP and DM20 has been observed at both the mRNA and protein levels in the human immune system (109), and srDM20 cDNA was first isolated from the thymus (18).

The classic PLP and DM20 proteins are expressed in a more diverse set of cells within and without the nervous system than the classic MBPs, and the sr-

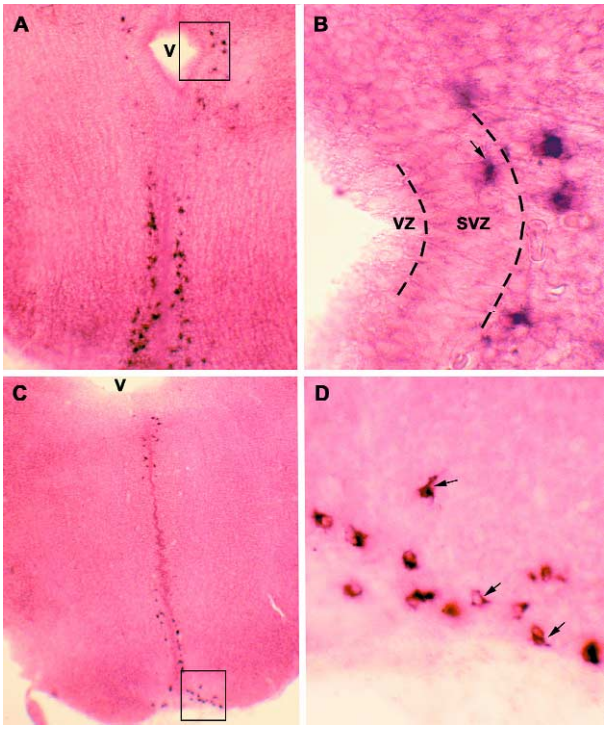

Figure 5. *In situ* hybridation of lower brainstem sections with PLP/DM20 (**A**, **B**) and MBP (**C**, **D**) digoxigenin labeled probes in 14 (**A**, **B**) and 16 (**C**, **D**) day mouse embryos. **A**. PLP/DM20 mRNA+ cells are located around the ventricle (V) and alongside the midline. As early 14 embryonic days, these PLP^+ cells have reached the ventral floor of the rhombencephalon. **B**. Higher magnification around ventricle shows a cell (arrow) located in the subventricular zone (SVZ) immediately adjacent to the ventricular zone (VZ). Other PLP^+ cells are migrating away from the SVZ. **C**. Oblique section through the rhombencephalon shows MBP $mRNA^+$ cells streaming away from the ventricle (V). **D**. Inset of figure C shows many MBP $mRNA^+$ cells located adjacent to the pia mater. Many of them have already elaborated short processes that contain MBP transcripts.

PLP/DM20 proteins appear to be expressed in a greater array of cells within the postnatal brain than the classic PLPs (18, 59, 60). Antibodies against the srPLP/DM20 immunostain OL cell bodies *in vivo* and *in vitro*, thereby providing one of the few reagents available for marking OL cell bodies. Within OLs, srPLP and srDM20 are not targeted to the myelin membrane, remaining within the cell bodies and proximal processes (18). The proteins appear to be associated with vesicles in the vesicular transport system within the cells (E. Bongarzone and A. Campagnoni, unpublished data). The sr-isoforms co-localize with syntaxin 6, a marker for vesicles transiting from trans-Golgi structures towards the plasma membrane. Co-localization of sr-proteolipids also was observed with clathrin, a specific marker for recycled vesicles from the plasma membrane. The data indicate that while both sets of isoforms may use the same trafficking pathways in OLs, the sr-PLP/DM20 remain associated with the vesicular system; and, unlike the classic isoforms, are not delivered to the myelin membrane.

Expression of the PLP gene begins as early as E9, nearly a week before the first myelinated fibers are detected in the brainstem. *In situ* hybridization with PLP probes or PLP promoter elements linked to reporter genes driving lacZ or green fluorescent protein analogues (36, 127, 129, 137, 145) have been used to study the location and earliest expression in the CNS. It has been reported that the specification of OL progenitors begins as early as embryonic day 9 in the mouse brain using PLP/DM20 regulatory sequences to drive lacZ expression (127). PLP/DM20 gene expression is restricted to specific loci in the ventricular layer along the rostro-caudal axis of the neural tube. Generally, the location of PLP/DM20 precursors overlaps with PDGFαR+ cells along the rostro-caudal axis; however, the two genes do not appear to be localized in the same cells during development. Whether distinct subpopulations of OL precursors exist in the brain, some of which are PDGFαR+/ PLP- and others PDGFαR-/PLP+; or whether the PDGFαR-/PLP+ cells are in a non-OL lineage is a point of discussion (123, 127) and further work will need to be done to sort this out. One explanation for the interpretation of these results is the possibility that srPLP/DM20 isoforms, which are known to be expressed in neurons and in OLs (18, 60), are being detected with the classical PLP/DM20 probes and that some of the $PLP/DM20^+$ cells might be neuronal precursors.

The expression of PLP/DM20 transcripts in mid-embryonic development suggests that products of the PLP/DM20 gene may facilitate migration of OL progenitors from the ventricular zone into future white and grey matter zones of brain and spinal cord. OL progenitors migrate long distances from their origins in the ventricular zone to their terminal field of differentiation during late embryonic and early postnatal development and all subfamilies of PLP/DM20 and MBP mRNAs are abundantly expressed in the perikaryon and processes of these cells (Figure 5).

During perinatal and early postnatal development, srPLP/DM20 can be detected with a sr-specific antibody to label strings of cells emerging from both the anterior and posterior sub-ventricular zone of the mouse (59). During this period, first neuronal precursors, and later, OL precursors migrate out of the subventricular zone. At

later stages, *i.e.* during early post-natal development, at least some, if not all, of these srPLP/DM20+ cells are undoubtedly OL precursors. This suggests a potential role for the srPLP/DM20 products of the gene in OL precursor migration.

In postnatal development, OLs continue to proliferate and establish their myelinating territories. Regulation of the number of OLs generated during postnatal development and the number of OLs needed for internodal formation does not appear to be tightly regulated. In the optic nerve, it is estimated that 50% of oligodendrocyte progenitors die (9) and in the cerebral cortex, 20% are estimated to die (131). OL death may be due to competition for growth factors as increasing the levels of PDGF reduce the percentage of dying OLs. The timing of OL death in the cerebral cortex correlates with expression of high levels of classic PLP/DM20 suggesting they might be involved in modulating OL death in normal development as well as in transgenic animals that overexpress PLP (see below). Supporting this notion is the finding that suppression of native PLP/DM20 gene products with an antisense technology can increase the number of OLs surviving several-fold in tissue culture (143).

Autocrine and paracrine functions of the PLP gene.

An intriguing biological question is whether the PLP/DM20 molecules or their fragments are secreted into the extracellular space and have autocrine and paracrine effects. An autocrine function is suggested by tissue culture studies, which show that fragments of PLP alter OL proliferation. Secretion of PLP/DM20 gene products by transfected non-neural cell lines has been reported to increase the number of OLs several fold *in vitro* (90). The increase in the number of OLs was dependent upon the concentration of protein in the supernatant, and both classic PLP- and classic DM20-secreting cells had similar effects (90). Similar studies showed a twenty-fold increase in the number of jimpy OLs in the presence of conditioned media from NIH3T3 cells expressing either classic PLP or DM20 (75). Yamada *et al*. (142) reported that PLP peptides located at or near the carboxy terminus caused an increase in the number of OLs. The proliferative effects of the peptides were found only in the picomolar range and as the concentration increased, proliferation decreased. At the moment, it is unclear how the intracellularly located PLP carboxy terminus can be secreted into the media.

A paracrine function of classic PLP/DM20 is suggested by transgenic animal studies showing that either ablation or overexpression of these proteins affects the viability of neurons. Transgenic mice that overexpress the PLP gene exhibit neuronal degeneration and axonal disintegration (6). Paradoxically, the absence of PLP/DM20 in PLP null mice also causes axonal swellings in some neurons (44). The phenotype of the null mice is particularly interesting because virtually all axons are myelinated, and the myelin sheaths are compacted with only subtle alterations in lamellar spacing (73). An obvious conclusion is that expression of the PLP gene is tightly regulated throughout life, otherwise its absence or overabundance causes both glial and neuronal abnormalities.

It is unclear whether the axonal changes in the PLP

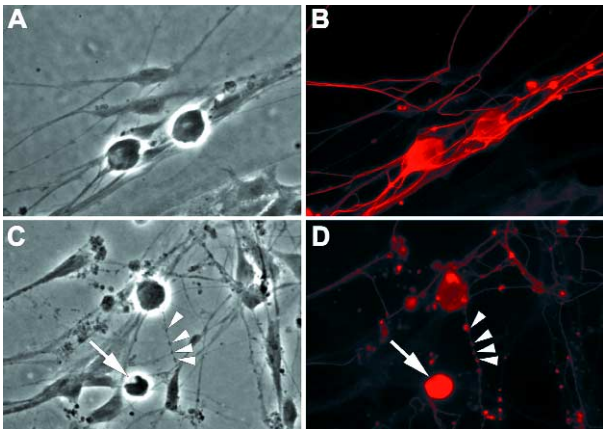

Figure 6. Morphology and class III β-tubulin immunostaining of enriched DRG neurons co-cultured with 293 or 293 cells stably transfected with a full length PLP cDNA driven by a CMV promoter. PLP cell surface expression by 293 cells was intense as visualized by EGFP coupled to 3' PLP sequence. Non-transfected or stably transfected 293 cell lines were plated onto previously plated DRG neurons and co-cultured for five days. At the end of the co-culture experiments, the density of the slower dividing 293 PLP expressing cells was typically less than 293 cells. **A**. Phase contrast micrograph of DRG neurons and other non-neuronal cells co-cultured with 293 cells. Somata of neurons are spherical and refractile while fibroblasts, nonneuronal cells, or 293 cells are flattened and less-refractile. **B**. Class III β-tubulin immunostaining of same DRG neurons in figure A. Axonal processes and neuronal somata are evenly stained. **C**. Phase contrast micrograph of DRG neurons co-cultured with 293 PLP expressing cells. Neurons display signs of somal shrinkage (arrow) and axons are degenerating as they contain condensed granules (arrowheads). **D**. Class III β-tubulin immunostaining of same DRG neurons in Figure C. Note intense class III β-tubulin immunoreaction in the somata/nucleus of one degenerated neuron. Axonal processes are weakly immunostained compared to axons in control. However, note intense and granular tubulin immunostaining along linear segments that corresponds to degenerated axons (arrowheads). Neurons co-cultured with 293 DM20 expressing cells did not exhibit degenerative changes, indicating PLP but not DM20 can be severely toxic to neurons. (Courtesy of S.E.M. Boucher).

amino acid position	substitution	name of mutation	species
36	H→Q	paralytic tremor	rabbit
36	H→P	shaking pup	dog
38	A→S	PLPjp-4J	mouse
38	A→T	classical/connatal PMD	human
42	T→I	classical PMD	human
74	T→P	myelin deficient	rat
139	H→W	SPG2	human
155	T→I	classical PMD	human
162	W→R	connatal PMD	human
181	T→P	connatal PMD	human
186	I→T	rumpshaker	mouse
186	I→T	SPG2	human
202	D→H	classic PMD	human
215	P→S	classic PMD	human
218	V→F	classic PMD	human
223	L→P	connatal PMD	human
242	A→V	jp-myelin synthesis deficient	mouse
point mutation in intron 4 splice site	exon 5 deletion,jimpy truncated COOH terminus		mouse

Table 1. List of selected point mutation in the PLP/DM20 gene. Different amino acid substitutions at the same amino acid position usually produce a similarly severe or mild phenotype. A histidine to glutamine/proline produces a moderate phenotype in rabbit and dog. An alanine to serine/threonine produces a severe phenotype in mouse and human.

overexpressers and null mice are a direct consequence of the presence or absence of classic PLP/DM20 or whether instability of the myelin sheath affects axonal integrity. A less widely held view is that the effects on neurons may be attributed to the newly described srPLP/srDM20 transcripts described in neurons. Low levels of srPLP/DM20s may be required for long-term neuronal viability. Overexpression of srPLP/DM20 transcripts may be deleterious to neurons; although recent *in vitro* data with OL cell lines suggest that the sr-isoforms are far less "toxic" to the survival of OLs than the classic isoforms (Bongarzone and Campagnoni, unpublished data).

With regard to the toxicity of classic PLP and DM20, stably transfected non-neural cell lines expressing high levels of classic PLP or DM20 were co-cultured with dorsal root ganglion cells (19). The PLP expressing — but not the DM20-expressing — cells caused a significant loss of neurons and disintegration of axons. Similarly, conditioned media from the PLP but not DM20 expressing cells caused neuronal loss (Figure 6). These studies suggest that expression of the PLP/DM20 gene directly affects neuron survival and is not a secondary effect resulting from myelin instability. Thus, a direct effect of PLP/DM20 gene expression on neurons may help explain the secondary abnormalities in astrocytes and neurons described in the PLP mutants. The abnormalities observed in neurons of the PLP null mice and the PLP overexpressers also could due to secretion of (sr) PLP related peptides or, if (sr)PLP is involved in transport, due to facilitated transport of many secreted factors.

Mutations of the proteolipid protein gene.

Point mutations, frameshifts, deletions, and duplications of the proteolipid protein gene have been described in many mammalian species and the phenotypes of different animals with PLP mutations have been the subject of extensive review (21, 45, 84, 91). The topology of the PLP and DM20 protein in the membrane is illustrated in Figure 4 and corresponding animal and human mutations are shown in Table 1.

The hallmark of the PLP mutations in animals and man is a generalized failure to establish and maintain

compact myelin sheaths. The degree of dysmyelination varies considerably among mutants. Jimpy, first described in 1954 (105), remains the best characterized of the PLP mutants. Jimpy, at one end of the spectrum, has severe dysmyelination and extensive OL death. Rumpshaker (47, 119), at the other end of the spectrum, has near normal amounts of myelin sheaths and very modest OL death. The phenotype of the other PLP mutants lies somewhere in between, except for the recently described jp-4J which has a missense mutation in exon 2 and has more severe dysmyelination than jimpy (13, 102). Efforts have been made to correlate lifespan with the extent of dysmyelination in these mutants, but recent studies indicate that these two parameters are not well correlated. In the jp-msd mutant, most of the animals have severe dysmyelination and die around the first month yet some jp-msd mice live as long as 210 days after birth (13). Albeit more myelinated fibers are found in these mice, their total number remains a fraction of the normal values. Similarly, the myelin deficient PLP mutant rat is severely dysmyelinated and has a shortened lifespan of 1 month yet some of their offspring have a longer life with no significant increase in myelin (27). Even more striking are quaking-shiverer double mutants, which have essentially no myelin sheaths but live beyond 100 days (140). The explanation for the longevity of these different mouse mutants is unknown, but their existence emphasizes the fact that still unknown factors modulate dysmyelination and longevity.

Although PLP has been detected in a few jimpy OLs by immunocytochemistry (39, 116), it has not been detected with a battery of antibodies that recognize epitopes held in common between jimpy and normal PLP/DM20 or antibodies that are specific for jimpy epitopes(11). This finding suggests that jimpy PLP is quickly degraded in the endoplasmic reticulum by sensor mechanisms. BiP (GRP-78) mRNA, which encodes a protein that binds to abnormally folded proteins in the rough endoplasmic reticulum, is upregulated about twofold in jp and jp^{msd} mice (52). Besides degradation of misfolded mutant PLP proteins, transport of mutant proteins to the cell surface appears to be affected in some *in vitro* systems. When products of the different mutant genes are examined in transfected cells, transport of mutant PLP/DM20 products to the plasma membrane is more severely hindered in those mutations with the most severe phenotype *in vivo* (41, 128). The number of secondary abnormalities caused by the jp PLP mutation is lengthy, and includes astrocytic hyperplasia, hypertrophy, intracellular pH changes, and O_2 consumption (74, 120). More recently, the chemokine GRO-1, a chemoattractant for leukocytes, has been shown to increase dramatically in jimpy astrocytes (141). Since inflammation does not occur in jimpy, its function may be regulation of OL proliferation or astrocytic gliosis.

In man, between 50 and 100 PLP mutations have been identified. A database of the human PLP mutations and a brief description of their clinical phenotype is maintained at *http://www.med.wayne.edu/neurology/plp.html*. Pelizaeus-Merzbacher disease (PMD) and X-linked spastic paraplegia type 2 (SPG2) are human diseases that are caused by point mutations, duplications, or deletions of the PLP gene. Duplications of the PLP gene account for more than 75% of the cases of PMD, point mutations account for 15-20% of the cases, and the remainder are deletions, frameshifts, and insertions. The clinical phenotype of PMD and SPG2 ranges from severe forms with death in the first decade (connatal) to mild forms with near normal longevity. Suffice it to say that the clinical phenotype of genetic mutations in humans parallels the neurologic abnormalities in animals with the same genetic mutation. For example, an $isoleucine^{186} \rightarrow threonine^{186}$ substitution produces a mild phenotype in mice (rumpshaker) and a SPG2 phenotype in humans (Figure 4, Table 1). Individuals with deletion of the PLP gene or null alleles have a mild spastic paresis, ataxia, and cognitive delay. This phenotype is similar to the null mice, which exhibit normal behavior for the first year. Interestingly, a peripheral neuropathy was discovered in the PNS from one of the null families (37). In contrast to the PLP nulls, clinical severity and brief lifespan are associated with certain duplications of the PLP gene in humans. The severity and length of lifespan appears to correlate with gene dosage in the same way that gene dosage in mice correlates with their lifespan (58).

Several PLP/DM20 null mice have been made and while there are differences in the spacing of the myelin lamellae in the different lines, myelin sheath formation and compaction is surprisingly normal (17, 73). Furthermore, the animals have a nearly normal lifespan and do not exhibit behavioral abnormalities for several months to a year. DM20 protein cannot compensate for the lack of PLP protein, since knock-in mice that lack PLP (but have DM20 protein levels comparable to PLP) exhibit neurodegenerative changes similar to PLP/DM20 null mice (124).

Effects of mutations in the MBP gene on the immune and nervous systems.

Over the past decade much less attention has been

given to the MBP mutants than to the PLP mutants by glial biologists. Certainly, many fewer naturally occurring MBP mutants have been described than the PLP mutants. This has led to some speculation that, perhaps, mutations in the MBP gene might be more lethal than the PLP mutants, although there is little evidence for this. Indeed, humans missing portions of chromosome 18q encompassing the MBP gene are well known and present interesting phenotypes which might be relevant to the function of the golli products (66, 83, 85, 114) since they bear a resemblance to mice null for the golli products of the MBP gene (20, 101), including delayed myelination and learning difficulties.

The two most well-studied mouse MBP gene mutants have been the shiverer (shi) and the myelin-deficient (shi^{mld}) mice whose molecular mutations were defined in the 1980's. The shiverer mutation is a deletion of exons 7-11 (or exons 3-7 using the old numbering) of the MBP gene (68, 89, 113).

The myelin-deficient mutation is more complex involving the 3′ portion of the gene. Essentially there is an inversion of exons 7-11 (old numbering, exons 3-7) and a tandem duplication of the portion of the gene encoding the classic MBPs (~exon 5a – exon 11) (old numbering, exons 1-7) fused in the normal direction to this inversion at its 3′ end. There is also a duplication of a region flanking the rearrangement junction in the upstream fragment and an insertion between the two gene copies of a segment of extraneous DNA not associated with the rearranged region (2, 3, 33, 98, 100). The effect of this mutation is to generate MBP hnRNA with a substantial portion in the antisense direction (33, 97). This appears to result in inefficient transcription of the gene with little or no MBP-encoding mRNAs emerging from the nucleus (2, 33, 99). The formation of antisense-sense RNA hybrids of these mRNAs (97) and/or readthrough inhibition of the upstream duplication of the transcriptional region of the classic MBPs (33) could account for the lack of functional classic MBP mRNAs in this mutant. The expression of golli-MBPs has not been investigated in the shi^{mld} mutant.

Recently, another rodent MBP mutant has been described, called the Long Evans shaker (les) rat (77). The mutation in this animal is an insertion of an endogenous retroposon into intron 7 (old intron 3) of the MBP gene (96). This mutation leads to a major decrease in the levels of classic MBP mRNAs and the appearance of improperly spliced transcripts that are unable to encode functional proteins (96).

In most cases, the effects of the MBP gene mutations on the expression of golli-MBPs, and the contribution of this to the phenotype, have not been assessed in these mutants. The discovery of the expression of golli proteins in the immune system (22, 34, 35, 48, 106, 109) has stimulated interest in the role of these MBP gene products in establishing tolerance to classic MBP epitopes and its effect on immunological mechanisms in the EAE response to MBP (51, 53, 54). From our knowledge of the organ-specific expression patterns, the results would predict that epitopes present in golli-MBPs could induce central (thymic) tolerance, but epitopes unique to classic MBPs would not be available to induce tolerance. This notion is supported by the recent finding that T-cells are the major site of golli-MBP expression in the thymus (31). For the C3H and Balb/c strains of mice this appears to be the case (53, 126, 144) but it is not the case in the B10.PL strain, which may be related to configuration of the MBP dominant epitope and stability of the appropriate peptide-MHC complexes in this strain (50, 86).

Comparisons of the shiverer mouse with normal mouse strains has proven to be a very useful tool (146), because this mutant can express one of the golli isoforms, BG21, which contains classic MBP sequence 1-57, encoded by exon 5B (old exon 1), but none of the classic MBP isoforms. These animals appear to be tolerant to encephalitogenic epitopes found in the MBP domain of golli BG21(53).

Although studies on the shiverer mouse by glial biologists has waned in recent years, several studies on the brains of shiverer mice *in vivo* and *in vitro* are worth noting within the context of recent work related to the cellular function of the golli-MBPs. As indicated earlier, the golli-MBPs are targeted primarily to the nucleus and/or the cytoplasm of OLs, neurons and T-cells. Within the cytoplasm, immunocytochemical co-localization studies indicate that at least some golli-MBPs are associated with cytoskeletal elements (24). It is, therefore, interesting that cytoskeletal abnormalities and increased microprocesses have been described in OLs cultured from the shiverer mutant brains (28, 29). Furthermore, Billings-Gagliardi, Wolf and their colleagues described such a phenotype for shiverer OLs *in vivo* (15, 139). Although one isoform of golli-MBP is expressed in shiverer, the other two isoforms are not expressed. It is possible that these golli-MBP isoforms have some role in cytoskeletal function and/or mediators of extracellular signals in these cells (28, 29). In this regard, as indicated earlier studies on the golli null mice clearly indicate an, as yet undefined, role for these proteins in signaling pathways (J.M. Feng and A.T. Campagnoni, unpublished results).

As discussed at length in previous articles and briefly in this article, several lines of morphological and cell biological evidence strongly supported the notion that the DM20/PLP gene directed more than one function in the brain. This includes the work of Billings-Gagliardi and Wolf who created a series of double mutants containing varying combinations of shiverer and jp and their alleles, shi^{mld} and jp^{msd} (14) to examine interaction between the two MBP and PLP genes. Their studies, using morphological hallmarks, clearly suggested that *both* genes had some role in cellular function that could not be explained by their roles in myelin.

Recently, double and triple "knock-outs" of PLP-null, shiverer, and MAG-null mice were prepared and analyzed (133). The triple mutant exhibited a phenotype similar to the shiverer mutant, but which was milder and of later onset. The triple mutant also exhibited a significantly shortened lifespan compared to the PLP-null/shiverer double mutant and the authors attributed this to the presence of the MAG-null allele.

Until recently, no other products of the MBP and PLP/DM20 genes were known, and it seemed likely that the multiple functions of these gene were carried out by the classic products. While there is evidence that this is likely to be partly true for the classic DM20 and PLP, it is unlikely to be the case for the classic MBPs.

The findings of additional products of both genes expressed in OLs and neurons prove the existence of multiple roles for the genes. They do not, however, completely explain the "double mutant" results, which were difficult to assess at the time they were first published. In part, this is because we have not yet defined the other roles of the classic and non-classic myelin protein products, and in part because the finding of these other products in neurons raises the possibility of cell-cell interactions beyond simply the interaction of myelin with the neuron. Other interactions involving the survival and differentiation of the OL may be influenced by the action of these myelin protein genes in neurons in ways that are yet to be defined.

Conclusions

For about 20 years, substantial biological data have accumulated to indicate that the myelin protein genes had functions beyond that of encoding myelin structural proteins. Initial inferences were based upon morphological and cell biological analyses of the naturally occurring PLP mutants. Later, the phenotypes of a number of transgenic/knock out mice, animals bearing multiple mutations and the discovery of other products of the major myelin protein genes confirmed the suspicion of additional functions for the PLP and MBP genes.

The study of alternative functions of the two major myelin protein genes in the nervous system has had considerable relevance to work on the immunology of the EAE response to MBPs and to PLP/DM20. The discovery of the golli products of the MBP gene and their expression in the immune system has provided a new area of investigation to explain the lack of immunological tolerance of susceptible animals to EAE induced by MBPs. Similarly, the discovery of the expression of classic PLP/DM20 in the immune system is providing an explanation for lack of tolerance to PLP in susceptible mice (5, 71).

The primary cell biological function of the classic MBPs appears to be that of structural components of the myelin membrane. If this is their primary role, it is not clear why there are so many isoforms, since they all appear to be capable of serving in this capacity. Exon 6-(old exon 2)-containing isoforms can be translocated to the nucleus through active transport mechanisms but their function in the nucleus is not known.

The function of the golli proteins is becoming clarified through *in vitro* overexpression studies, targeted ablation of the proteins in knock out mice, and identification of their molecular partners. In the nucleus they are likely to be associated with transcriptional complexes involved in genes regulated by LIM. They also appear to be substrates for PKC and MAP kinase and appear to be involved in second messenger pathways at least in T-cells and probably in neural cells, as well. It remains to be determined what role the phosphorylation state of golli proteins plays in the functioning of these molecules. Important, future lines of investigation also will be the identification of the genes regulated by these proteins as well as the cell biological functions (*e.g.* process/membrane elaboration in OLs and neurons) modulated by these proteins.

Cell biological functions of the classic PLP/DM20 proteins have begun to be clarified through *in vitro* transfection, tissue culture, and transgenic animal studies. There is evidence they may be involved in OL death and survival, migration and process extension, and/or intracellular vesicle transport of molecules to the plasma membrane. The sr-PLP/DM20 gene products play a role within the cell body since these products are targeted specifically to vesicles within the cell bodies of OLs and neurons. They have been localized with both clathrin-coated and syntaxin 6-containing vesicles, establishing a localization with vesicles within the OL. They may be components of the vesicles involved in delivery of molecules (including classic PLP/DM20) to the myelin membrane as well as delivery and recycling mecha-

nisms between the Golgi and the plasma membrane. However, the molecular partners for neither the classic nor the sr-PLP/DM20 isoforms have been identified, and the molecular mechanisms by which they lead to OL and neuronal cell death, process extension, etc. are still unknown. Products of the PLP/DM20 gene appear to have autocrine and paracrine functions, especially with neurons. Particularly important to decipher is the sequence of molecular events that leads to the OL phenotype described in the PLP/DM20 overexpressers, PLP/DM20 null mice and the naturally-occurring mutants. Although OL cell death may be the final outcome for many of the animals that express the PLP/DM20 gene abnormally, the signaling pathways that lead into the apoptotic cascade may be radically different.

One of the more intriguing prospects for future experimentation lies in the roles of the myelin protein genes in neurons, which might be enlightening in defining their role in OLs. Both of the "non-classic" families of proteins of the MBP and PLP/DM20 genes are co-expressed in several classes of neurons during migration and process extension; as well as in mature neuronal populations. Indeed, the sr-PLP/DM20 proteins are expressed in glial precursor cells migrating out of the subventricular zone. Thus, the myelin protein genes have many "faces" and the challenge of the future is to determine what these are and what role they play in the cells of the nervous system and the immune system in which they are expressed.

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