

Review

Redefining the Lipophilin Family of Proteolipid Proteins

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The past few years have seen a dramatic increase in our understanding, in molecular terms, of the involvement of the central nervous system proteolipid protein in myelinogenesis and X-linked genetic diseases. In addition, we have expanded our knowledge of the proteins that have been recruited into the vertebrate myelin membrane over the past 400 million years with the molecular cloning of several cDNAs encoding proteins which are homologous to the proteolipid protein gene. In searching for a name to distinguish these proteins from other “proteolipid” proteins of nonneural origin I propose that we resurrect the term “lipophilins” which describes a small family of unusually hydrophobic integral membrane proteins exhibiting identical topologies and similar physical properties. Two subgroups are distinguishable among the lipophilins based on the patterns of expression during development and the presence or absence of a small motif that is exposed to the extracellular space. *J. Neurosci. Res.* 50:659–664, 1997. © 1997 Wiley-Liss, Inc.

Key words: myelin; PLP; DM-20; M6; DM family; evolution; protein motifs; review

INTRODUCTION

The proteolipid protein (PLP) of central nervous system (CNS) myelin (Fig. 1) has a prominent history in the neurosciences. This is not simply because of the abundance of the protein in compact myelin or that it was probably the first protein to be identified with a lipid-like solubility in chloroform:methanol (Folch and Lees, 1951) but also because of the important role that PLP appears to play in the pathogenesis of several genetic hypomyelinating and autoimmune demyelinating diseases such as Pelizaeus-Merzbacher disease, spastic paraplegia and multiple sclerosis. With this in mind, it is somewhat surprising that many of the hypothesized functions of PLP have yet to be conclusively demonstrated. Furthermore, the *PLP* gene gives rise to two alternatively-spliced transcripts (Nave et al., 1987) that encode PLP and a

smaller isoform, DM-20 (Fig. 1), but until recently very few studies have examined the activities of both proteins.

Many studies conducted using reconstituted systems have revealed a range of potentially important properties of PLP that have been rather difficult to confirm in vivo. For example, PLP appears to facilitate the transport of Na⁺, K⁺, and Ca²⁺ across purified lipid bilayers (de Cozar et al., 1987; Diaz et al., 1990) but these studies have not yet been repeated in vivo with an intact myelin membrane or in other in vivo paradigms such as expression in *Xenopus* oocytes. Detergent-purified PLP has been shown to bind inositol hexakisphosphate (IP₆) with nanomolar affinity at physiological ionic strength (Yamaguchi et al., 1996); however, in situ overlay experiments with radiolabeled IP₆ on rat and human sagittal brain sections reveal substantial binding of these ligands in the gray matter but negligible binding in myelinated tracts where PLP is almost exclusively localized (Parent and Quirion, 1994).

In contrast to in vitro experiments, the ablation of *Plp* gene expression in mice by homologous recombination in embryonic stem cells (PLP-KO mice) has had a significant impact on our understanding of the function of PLP during myelinogenesis. Using this technology, two groups have independently generated *Plp* null alleles and have shown that mutant animals exhibit a minimal behavioral phenotype (Boison and Stoffel, 1994; Klugmann et al., 1997). However, compact myelin in the absence of DM-20 and PLP appears to exhibit reduced stability, which was inferred from the unusual susceptibility to fixation artifacts as compared with sheaths from wild type littermates (Klugmann et al., 1997; Rosenbluth et al., 1996) and the 50% reduction in conduction velocities along optic nerves of mutant mice compared to

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Received 20 May 1997; Revised 28 July 1997; Accepted 28 July 1997

normal animals (Gutierrez et al., 1995). Importantly, the absence of DM-20/PLP does not appear to trigger compensation by other myelin-specific genes (Klugmann et al., 1997). Thus, these studies convincingly establish a major function of DM-20/PLP that has had strong indirect support for many years (Duncan, 1990), that these proteins stabilize close appositions of the extracellular bilayer surfaces in compact CNS myelin. At the same time, the PLP-KO mice have cast some doubt on speculation that the embryonic expression of DM-20 is crucial for oligodendrocyte lineage cell survival and that DM-20/PLP are required for oligodendrocyte differentiation as well as myelin membrane synthesis and compaction.

Although we have a satisfying view of the function of DM-20/PLP as a structural element in myelin, the molecular interactions with lipids and other proteins through which DM-20/PLP effect membrane stabilization are less clear. Central to this issue has been the characterization of protein topology to ascertain which protein domains are accessible at the cytoplasmic and extracellular membrane surfaces. Early studies of topology yielded conflicting topologies (Popot et al., 1991); however over the last few years a consensus for PLP has been reached using a variety of independent techniques (Gow et al., 1997; Greer et al., 1996; Weimbs and Stoffel, 1992). PLP possesses four transmembrane domains with both the amino- and carboxyl-termini exposed to the cytoplasm (Fig. 1). Importantly, DM-20 also adopts this topology *in vitro* and *in vivo* (Gow et al., 1997) which, *a priori*, was not an inevitable outcome. In this regard, one of the major determinants of the cytoplasmic versus extracellular disposition of hydrophilic domains in integral membrane proteins is net charge, with the cytoplasmic domains of many proteins being positively charged (Hartmann et al., 1989). The net charge of the *b-c* loop domain of DM-20 (the hydrophilic loop between the transmembrane domains labeled *b* and *c* in Fig. 1) is very low at +1 which, conceivably, is insufficient to confer a cytoplasmic orientation. On the other hand, the presence of the PLP-specific segment with a net charge of +6 substantially increases the charge of this domain.

Attempts to understand the molecular mechanisms underlying the structural organization of compact myelin have sparked considerable interest in the evolutionary events giving rise to the myelin membrane in tetrapods. In particular, antibodies raised against a procession of proteins recruited into compact myelin have served as phylogenetic markers in western blotting analyses to refine the phylogenetic tree for higher vertebrates (Waehneledt, 1990). According to this model, living representatives of the phylogenetically earliest chordates to synthesize compact myelin, the sharks and rays, appear to utilize P₀ as the major adhesive protein in CNS and PNS myelin. PLP appears to have usurped the adhesion function of P₀

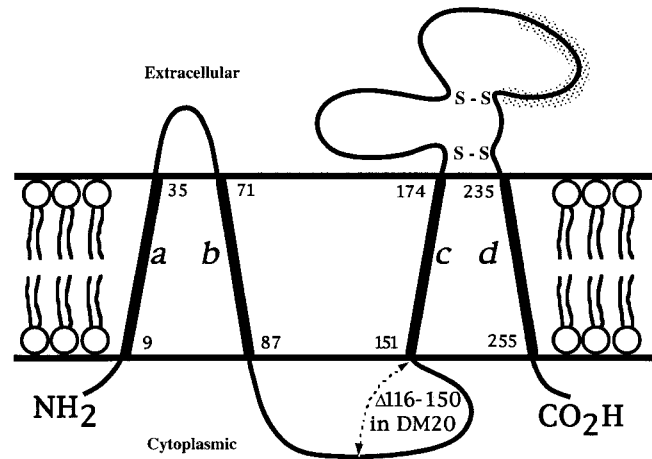


Fig. 1. Schematic representation of DM-20/PLP topology in the lipid bilayer which is likely to be common to all lipophilin family members (Kitagawa et al., 1993). DM-20 and PLP possess four transmembrane domains with both amino- and carboxyl-termini at the cytoplasmic surfaces of the membrane (Gow et al., 1997). The arrangement of disulfide bridges in the second extracellular domain (S-S) was determined empirically (Shaw et al., 1989; Weimbs and Stoffel, 1992) and the position of a motif common to group A lipophilins (see Fig. 3) is indicated by the stippled box. DM-20 and PLP differ only by the absence or presence, respectively, of a 35 amino acid peptide in the cytoplasmic loop. This PLP-specific peptide is comprised largely of polar and charged amino acids.

in the CNS of lungfish and coelacanths and the presence of DM-20 in CNS myelin coincides with the emergence of terrestrial vertebrates. However, recent work has prompted a reevaluation of this picture (Kitagawa et al., 1993; Yan et al., 1993; Yoshida and Colman, 1996). Specifically, three cDNAs cloned from the CNS of shark, DM α , DM β , and DM γ , have considerable homology to DM-20 but lack the cytoplasmic PLP-specific segment strongly inferring that DM-20 preceded PLP in evolution.

The PLP-specific peptide is encoded by a 105 base pair insertion (encoding 35 amino acids) into exon 3 of the gene (Nave et al., 1987) which must originally have encoded a DM-20-like protein. In this light, rather than being the archival member of a group of homologous proteins, PLP is probably the most recent member of a family of DM-20-like proteins that have been highly conserved over 425 million years (Kitagawa and Colman, 1994; Yoshida and Colman, 1996). Molecular studies show that the appearance of the PLP-specific peptide coincides with the emergence of amphibians and that PLP has persisted as an alternatively spliced isoform of DM-20 in all tetrapods except early amphibians in which, presumably, the splice site was lost and only PLP is synthesized in living descendants (Yoshida and Colman, 1996). Thus, although the early western blot analyses

conducted by Waehnelde and co-workers were interpreted as having identified PLP in the CNS of lungfish and coelacanth, it is now clear that the anti-PLP antibodies used in those studies cross-reacted with DM-20-like proteins in these vertebrates.

CHOOSING A FAMILY NAME

Subsequent to the cloning of the shark DM-20-like genes, two other cDNAs encoding proteins with extensive similarity to DM-20 have been cloned from mouse and human, M6a and M6b (Yan et al., 1993), which brings to seven the number of genes in this emerging family, including the *Xenopus Plp* gene. But what should this expanded gene family be called? Two solutions to this question are commonly used: the systematic renaming of all family members or the use of an umbrella term with which to refer to the related genes. Given the small number of DM-20-like genes, the latter approach seems more practical than the former at this time. To date, terms such as the “DM family proteins” and the “proteolipid protein family” have been used in the literature; however, these names neither describe the common characteristics of family members nor distinguish them from other nonneural “proteolipid” proteins. From this standpoint, other terms are worthy of consideration and I propose that the term “lipophilins” (Gagnon et al., 1971) be revived to describe the entire family of proteins, which is a term that aptly conveys the lipid-like physical properties of its members. Lipophilin was at one time used interchangeably with PLP (Folch and Lees, 1951) to describe the same protein but has fallen into disuse in the current literature. As such, the lipophilins would comprise a small family of hydrophobic integral membrane proteins expressed principally in oligodendrocytes, Schwann cells and neurons although some family members are also expressed outside the nervous system in choroid plexus, thymus, heart, kidney proximal tubules, and at vanishingly small levels in lymphocytes and skin fibroblasts (Cambi et al., 1996; Campagnoni et al., 1992; Lagenaur et al., 1992; Pribyl et al., 1996).

The rigorous analyses used to determine the topologies of DM-20 and PLP have not yet been applied to other lipophilin family members, nonetheless a common topology among the lipophilins is anticipated (Kitagawa and Colman, 1994). Western blot analyses of CNS proteins from many vertebrates (Waehnelde, 1990; Yoshida and Colman, 1996) have shown that the lipophilins are N-glycosylated in several species (neither DM-20 nor PLP are themselves glycosylated). Cloning of the cDNAs encoding M6a and M6b (Yan et al., 1993) has revealed that the functional N-glycosylation consensus sites (N-X-S/T) in the primary structures of these glycoproteins (Baumrind et al., 1992; Lagenaur et al., 1992) reside in

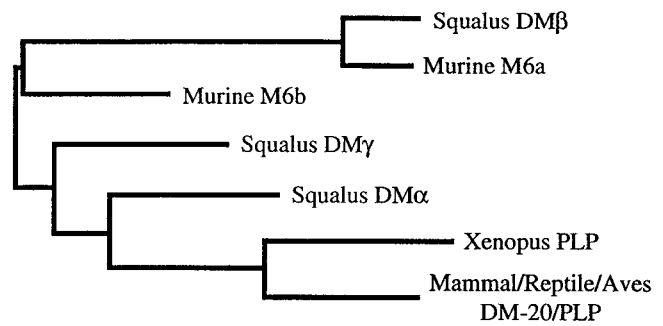


Fig. 2. A dendrogram of the lipophilin family derived from published amino acid sequences and constructed using the CLUSTAL W software (Thompson et al., 1994). The similarity of the primary structures of any two proteins is proportional to their distance apart and their relative positions within the tree.

hydrophilic domains corresponding to the extracellular *a-b* and *c-d* loops of DM-20/PLP and are thereby consistent with the topological model shown in Figure 1.

HOW MANY FAMILY MEMBERS?

In evolutionary terms, the lipophilins are related to one another as indicated by the dendrogram in Figure 2 which illustrates the statistically most likely links between ancestral genes in the marine vertebrates and those found in terrestrial vertebrates (Thompson et al., 1994). Given the high degree of overall similarity of lipophilins in marine vertebrates compared to those in amphibians and mammals, it is more likely that genes from different species are homologous rather than distinct family members. DM-20/PLP in reptiles, birds, mammals, and amphibians appear to be homologs of *Squalus* DM α while murine M6b is homologous to *Squalus* DM γ . Finally, murine M6a appears to be homologous to *Squalus* DM β and has been more highly conserved from marine to terrestrial vertebrates than either DM-20/PLP or M6b to their ancestral forms. Thus, at present there appears to be three distinct lipophilins that diverged in their primary sequences, if not functions, at least 425 million years before present (Kitagawa and Colman, 1994).

CAN THE LIPOPHILINS BE DIVIDED INTO SUB-FAMILIES?

Although the lipophilin family consists of a small number of genes there may be some merit in placing members into subgroups to highlight particular features in the hope of gleaning information about function or, at the very least, of allowing us to formulate ideas with which to investigate potential functions. For example, we can use the major patterns of expression of the lipophilins in mice as a basis for subdivision. Clearly, M6a is

GROUP	DM γ	CQT ISSPPVNLTTVIEE IC VDVRQYGI IPWNAS PGK AC --G STL TT IC
A	M6b	CEVIK SPQSN GTSG VEQ IC VDVRQYGI IPWNA FP PGK IC--G SALE NI IC
	DM α	CQM VKYVTEN-GTGFDD IC VDARQYGI L PW NAS PGK IC --G LSLA AV IC
	DM-20/PLP	CQ SI A FP S KT-SASIG SLC ADAR MY GV L PW NA FP PGK V C --G SNLL S IC
	Xen. PLP	CQ SI A FP G KT-TTSV STL CSDRR MY GV L PW NA FP PGK V C --G TSL LA IC
GROUP	DM β	CQ NVT IM ES----- TDL CFDLRQFGIV PI HE Q - KT V CT LNEN FS KL C
B	M6a	CR NTTL VE G----- ANL CLDLRQFGIV T IG EE - KK I CT ASE N FL RM C

Fig. 3. The lipophilins can be divided into two groups according to the presence (group A) or absence (group B) of a short peptide motif in the second extracellular domain (bolded). The four cysteine residues in this domain (bolded) are conserved in all lipophilins and have been shown for DM-20/PLP to participate in disulfide bonds (see Fig. 1). These covalent interactions may conceivably confer a compact stable structure

expressed in most regions of the CNS but only in neurons; in CNS-derived cell cultures this protein is localized to neurite growth cones (Baumrind et al., 1992; Lagenaur et al., 1992; Yan et al., 1996). On the other hand, DM-20/PLP is expressed exclusively in oligodendrocytes in the CNS (Braun, 1984). The identities of the cell types that express DM-20 at low levels in embryos shortly after neural tube closure are at present somewhat controversial but may include oligodendrocyte and neural crest progenitor cells (Timsit et al., 1995; Wight et al., 1993). The expression pattern of M6b is more complex than other lipophilins that have been examined and to some extent appears in both neuronal and glial populations (Yan et al., 1996). However, the highest levels of expression predominate in white matter tracts and the protein is present in compact myelin (Klugmann et al., 1997). In the embryo, and for 1 to 2 weeks after birth, M6b is expressed throughout the ventricular zone and external granular layer of the cerebellum and appears to switch to the internal granular layer after birth. Coincident with the deposition of myelin in the spinal cord, M6b is strongly expressed in white matter tracts but also appears transiently in some large motor neurons. Intriguingly, the expression of DM γ in marine vertebrates (Kitagawa et al., 1993) and amphibians (M. Yoshida and D.R. Colman, personal communication) is coincident with DM α and P₀ in spinal cord white matter tracts and it is worth bearing in mind that the complex expression pattern of M6b during development in mice may be due to relatively recent modifications in the promoter/enhancer of the gene.

on the domain. Single letter codes are used to denote amino acids. Bolded amino acids for M6a and M6b are conserved in mouse and humans (Olinsky et al., 1996) and those for DM-20/PLP are conserved among all terrestrial vertebrates. DM α , DM β , and DM γ sequences are from *Squalus*. Xen, *Xenopus*.

The lipophilins can also be separated into two groups on the basis of short motifs within their primary structures. Portions of the second extracellular domains of each protein from shark, *Xenopus* and mouse are aligned to highlight several features (Fig. 3). All four cysteines in this region of the proteins are conserved between family members and biochemical data suggests that, at least in DM-20/PLP, these residues are likely to participate in two intramolecular disulfide bonds (Shaw et al., 1989; Weimbs and Stoffel, 1992) that might be expected to confer a stable three-dimensional structure to this domain (Fig. 1). In addition to the cysteines, proteins in group A exhibit a conserved "PWNA[F/S]PGK" motif which is absent in group B proteins. Interestingly, group B proteins are expressed in neurons while group A proteins are expressed strongly in glial cells (with exceptions for M6b as noted above). Currently there is no functional data regarding the function of the group A motif but it is tempting to speculate about possible roles by drawing parallels from recent crystallographic data obtained for the extracellular domain of P₀ (Shapiro et al., 1996). As previously mentioned, PLP can be viewed as having usurped P₀ as the major adhesive protein in the CNS of terrestrial vertebrates. Although there is no detectable homology between these two proteins, both may stabilize juxtaposed extracellular membrane surfaces of compact myelin in similar fashion by, for example, inserting a single tryptophan residue per monomer into the adjacent bilayer as suggested for P₀. For PLP, a tryptophan residue within the group A motif is situated toward the center of the extracellular domain between

cysteines two and three (stippled region in Fig. 1) and is flanked by proline residues which may contort the α -carbon backbone sufficiently to expose the aromatic sidechain of the tryptophan and allow it to intercalate into a closely apposed lipid bilayer. To return briefly to the function of PLP in compact myelin, perhaps ancestral group A members performed various adhesive roles in the CNS of marine vertebrates and that this property was so effectively enhanced by insertion of the PLP-specific segment into the DM α gene that selective pressure effected expunction of P₀ from the CNS. With regard to potential functions of group B lipophilins, there is currently very little functional data available. Previous studies have localized M6a to the growth cones of cultured neurons but for additional information we must await the results of standard transgenic mouse approaches such as overexpression and knockouts.

In the last few years we have witnessed a rapid surge in our understanding of the functions of DM-20 and PLP in the CNS. The topologies of these proteins have finally been determined and we can now devise experiments that probe the functions of individual domains of the proteins. The use of homologous recombination in embryonic stem cells to ablate the expression of the *Plp* gene in mice has also yielded invaluable information about the function of DM-20/PLP in vivo during embryonic development, myelinogenesis, and myelin stability. In addition, progress in other areas not discussed here have highlighted the detrimental effects of overexpression of the wild type *Plp* gene (Kagawa et al., 1994; Readhead et al., 1994) and have described a molecular mechanism for the pathogenesis of diseases such as Pelizaeus-Merzbacher disease and spastic paraplegia (Gow et al., 1994; Gow and Lazzarini, 1996) that are associated with mutations in the coding region of *PLP* (Hodes et al., 1993; Nave and Boespflug-Tanguy, 1996). The immediate goal of future research will be to develop therapeutic strategies to ameliorate the severity of these diseases (A. Gow, C.M. Southwood, R.P. Skoff, and R.A. Lazzarini, manuscript in preparation). Finally, the molecular cloning of lipophilin family members in marine vertebrates have provided invaluable insights into the evolution of the myelin membrane over 400 million years. The emergence of the PLP-specific segment has now been placed into the context of a gene family and has suggested a mechanism whereby PLP has replaced P₀ as the major adhesive molecule in the CNS.

ACKNOWLEDGMENTS

I would like to acknowledge Mika Yoshida for sharing unpublished data, and Cherie Southwood and Dan Pereira for constructive criticism of the manuscript. This work was supported by a research grant from the

International Human Frontier Science Program (RG318/97). This is manuscript number 236 from the Brookdale Center for Molecular Biology, Mount Sinai School of Medicine.

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