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Proteolipid protein dimerization at cysteine 108: Implications for protein structure

Gurdip Daffu[a,*], Jasloveleen Sohi[b], John Kamholz[a,b]

[a] Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201, United States
[b] Department of Neurology, Wayne State University School of Medicine, Detroit, MI 48201, United States

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ABSTRACT

Proteolipid protein (PLP) and its alternatively spliced isoform DM20 comprise ~50% of central nervous system (CNS) myelin protein. The two proteins are identical in sequence except for the presence of a 35 amino sequence within the intracellular loop of PLP that is absent in DM20. In this work, we compared the expression of PLP/DM20 in transfected cells, oligodendrocytes and brain. In all 3 tissues, PLP exists as both a monomer and a disulfide-linked dimer; in contrast, DM20 is found mainly as a monomer. PLP dimers were increased by both chemical crosslinking and incubation with hydrogen peroxide, and were mediated by a cysteine at amino acid 108, located within the proximal intracellular loop of both PLP and DM20. The PLP-specific sequence thus influences the accessibility of this cysteine to chemical modification, perhaps as a result of altering protein structure. Consistent with these findings, several mutant PLPs known to cause Pelizaeus–Merzbacher disease form predominantly disulfide-linked, high molecular weight aggregates in transfected COS7 cells that are arrested in the ER and are associated with increased expression of CHOP, a part of the cellular response to unfolded proteins. In contrast, the same mutations in DM20 accumulate fewer high molecular weight disulfide-linked species that are expressed at the cell surface, and are not associated with increased CHOP. Taken together, these data suggest that mutant PLP multimerization, mediated in part by way of cysteine 108, may be part of the pathogenesis of Pelizaeus–Merzbacher disease.

1. Introduction

Myelin is a multi-lamellar membrane structure that ensheaths vertebrate axons and acts to facilitate nerve conduction. Myelin is composed mainly of lipids, but also contains a number of myelin-specific structural proteins. In the central nervous system (CNS), proteolipid protein (PLP) and its alternatively spliced isoform DM20, both highly hydrophobic tetramembrane spanning proteins synthesized by oligodendrocytes, are the major myelin structural proteins (Norton and Poduslo, 1973). Interestingly, PLP and DM20 are not necessary for myelin synthesis, although in their absence myelin periodicity is increased and becomes permeable to ferric ions (Rosenbluth et al., 2006). In addition, axons ensheathed by myelin without PLP and DM20 undergo length-dependent Wallerian degeneration, suggesting that PLP/DM20 also have a trophic effect on axons (Griffiths et al., 1998; Stecca et al., 2000; Garbern et al., 2002). The molecular basis for these functions of PLP and DM20, however, are not known.

Mutations in the *PLP1* gene, encoding PLP, cause Pelizaeus–Merzbacher disease (PMD), an X-linked dysmyelinating disease with onset during childhood causing nystagmus, ataxia and a spastic paraparesis (Garbern et al., 1999). The most common form of PMD is the result of a duplication of a region of the X-chromosome encoding *PLP1*, and animal studies have demonstrated that overexpression of *PLP1* is the likely cause of the disease phenotype. Over 100 point mutations, however, have also been identified in the *PLP1* gene, and produce a variety of clinical phenotypes, from severe weakness and hypotonia at birth, to a mild, adult-onset spastic paraparesis syndrome. Gow and co-workers have hypothesized that mutations that effect the folding of both PLP and its alternatively spliced isoform, DM20, cause the most severe disease, while those that preserve DM20 function cause a milder form of PMD (Gow and Lazzarini, 1996).

Several studies have shown that wild-type PLP can form homo-oligomers (dimers, trimers, or hexamers) in transfected cells and in solution (Brophy et al., 1984; Smith et al., 1984; Timsit et al., 1992; Sinoway et al., 1994; Swanton et al., 2005) suggesting that these interactions are important for PLP function. In addition,

* Corresponding author. Current address: Diabetes Research Program, Department of Medicine, New York University School of Medicine, 550 First Avenue, Smilow 906, New York, NY 10016, United States. Tel.: +1 212 263 9471.
E-mail address: Gurdip.Daffu@nyumc.org (G. Daffu).

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mutant forms of PLP identified in patients, can also form homo-oligomers, and may thus participate in the pathophysiology of the disease process (Dhaunchak and Nave, 2007). Swanton and co-workers (Swanton et al., 2005) have shown, however, that PLP-dimers analyzed from transfected cells and rodent brain were both SDS- and DTT-resistant, implying they were formed by irreversible hydrophobic interactions, possibly induced during protein isolation. To resolve this issue, and to elucidate the role of homo-oligomers in PMD, in the present work we have analyzed and compared the interactions of PLP and DM20 in transfected COS7 cells, oligodendrocytes and brain using membrane extracts isolated with the non-ionic detergent n-dodecyl-β-D-maltopyranoside, a method known to preserve mitochondrial respiratory complexes in an enzymatically active state (Schagger and von Jagow, 1991). In all 3 tissues, we have found that PLP exists as both a monomer and a disulfide-linked dimer; in contrast, DM20 is found mainly as a monomer. PLP dimers were increased by both chemical crosslinking and incubation with hydrogen peroxide, and were mediated by a cysteine at amino acid 108, located within the proximal intracellular loop of both PLP and DM20. Consistent with these findings, several mutant PLPs known to cause Pelizaeus–Merzbacher disease form predominantly disulfide-linked, high molecular weight aggregates in transfected COS7 cells that are arrested within the ER and are associated with increased expression of CHOP, a part of the cellular response to unfolded proteins. In contrast, the same mutations in DM20 produce fewer high molecular weight disulfide-linked species that are expressed at the cell surface, and are not associated with increased CHOP. Taken together, these data suggest that mutant PLP multimerization, mediated in part by way of cysteine 108, may be part of the pathogenesis of Pelizaeus–Merzbacher disease.

2. Material and methods

ICC: Immunocytochemistry; WB: Western blot

2.1. Reagents and antibodies

All reagents were from Sigma unless otherwise stated. The following antibodies were used: α-rat AA3 monoclonal serum (WB: 1:100) raised to a carboxy terminal peptide of PLP was kindly provided by Dr. Marjorie Lees (Yamamura et al., 1991); α-rabbit TRIF polyclonal (ICC: 1:250) that recognizes the PLP-specific domain was a gift from Dr. Bob Skoff; α-chicken HA (ICC: 1:50, WB: 1:1000) was from Chemicon (AB3254); α-mouse FLAG monoclonal (ICC: 1:50, WB: 1:1000) was from Sigma (F1804); α-rabbit calnexin polyclonal (WB: 1:20,000) was from Stressgen (SPA-860); α-Golgi 58 K monoclonal (WB: 1:1000) was from Sigma (G-2404); α-mouse monoclonal IgM O10 antibody was kindly provided by Dr. Klaus-Armin Nave; this sera recognizes an extracellular conformational epitope encoded by PLP/DM20 (Jung et al., 1996). HRP antibodies were from Santa Cruz: α-rat (sc-2006), α-mouse (sc-2005), α-rabbit (sc-2004); α-chicken AlexaFluor 488 (ICC: 1:500) was from Molecular Probes (A-11039), α-mouse AlexaFluor 594 (ICC: 1:500) was from Molecular Probes (A-21225); IgM red Cy3 (ICC: 1:500) was from Jackson ImmunoResearch Laboratories (715-165-020).

2.2. Molecular cloning and DNA manipulations

The cDNAs encoding human PLP and human DM20 coding sequences (Puckett et al., 1987), were inserted into the pCGN vector (Tanaka and Herr, 1990) containing a human CMV promoter and had either an HA or FLAG tag fused to the N-terminus of the protein. Site-directed mutagenesis was carried out using QuikChange II Kit (Stratagene #200524-5). All constructs were confirmed by DNA sequencing.

2.3. Cell culture and transfection

COS7 Cells: Cells obtained from ATCC (Manassas, Virginia) were grown in DMEM (Invitrogen) containing 10% Fetal Bovine Serum (Invitrogen) and 1% Pen/Strep (Invitrogen). Cells were transfected following the Lipofectamine 2000 protocol (Invitrogen cat #11668-019) and analyzed after 18–24 h by immunocytochemistry or isolated native membrane extracts (NME) for SDS-PAGE.

Primary Culture Rat Oligodendrocytes: Oligodendrocytes (OLs) were cultured as previously described by Benjamins et al. (2003) and modified from McCarthy and de Vellis (1980). Briefly, 2-day-old rats were decapitated and heads were placed on an alcohol wipe. The brains from three animals were dissected out and placed in a Petri dish with 5 ml of media without serum. The brainstems were cut off, thalami dissected away and the cerebra were cut in half. The meninges were removed by rolling the brains on sterilized filter paper. The brains were then transferred to a new Petri dish containing 5 ml of media without serum [197 ml DMEM (Invitrogen #11995, 3 ml 7.5% Sodium Bicarbonate (Gibco #25080-094)], and chopped into small pieces with a single-edged razor blade. The chopped up cerebra were transferred with a glass pipette to a 15 ml conical tube and mixed with 3.5 ml of media without serum, 1 ml of 2.5% trypsin (Invitrogen #15090-046) and 0.5 ml of 200 μg/ml DNase (#D5025). This was incubated for 10 min in a 37 °C water bath, and then centrifuged for 5 min at 1000 rpm at 22 °C (RT). Supernatant was discarded and pellet was resuspended in 5 ml of media + 10% Newborn Calf Serum (NCS) [172 ml DMEM, 3 ml 7.5% Sodium Bicarbonate, 2 ml Antibiotic/Antimycotic (Gibco #15240-096), 20 ml NCS (Invitrogen)]. Pieces of cerebra were triturated 8–10 times through a sterile 9″ Pasteur pipette. The large cell clumps were allowed to settle and supernatant containing single and small clumps of cells were drawn off and placed in another 15 ml tube. To the large settled clumps of cells, another 5 ml of media +10% NCS was added and triturated another 8–10 times with a 9″ Pasteur pipette that had its bore size reduced with a flame. The large cell clumps were again allowed to settle, the supernatant was drawn off and combined with previous supernatant of single and small clumps of cells. This combined supernatant was centrifuged for 5 min at 1000 rpm at RT. The supernatant was discarded, the pellet was resuspended in 8 ml of media +10% NCS and placed in a poly-lysine (#7280) coated T-75 flask (Corning #430720). The cells were grown at 37 °C for 7–10 days in media +10% NCS and the media was changed every 3–4 days.

Shake-off cultures: After 7–10 days of culture, primary culture OLs were shaken off from the primary flask. The flat bottom of flasks were slammed about 50 times onto the top of a lab bench covered with a blue pad with paper towels as cushioning support between the flask and the hand. The removal of small, dark cells (OLs) from the astrocytic bed layer was checked under the microscope after the first 30–40 times and shake-offs were continued if needed. The supernatant was removed from the flask and centrifuged for 5 min at 1000 rpm at RT. To the primary flask, 8 ml of media + 10% NCS was added and incubated as before. The supernatant from shake-off's was discarded, leaving about 10 ml remaining on top of pellet. To remove microglial, the cells were resuspended and transferred to a sterile, untreated 100 mm Petri dish and incubated at 37 °C for 1 h. The supernatant was then transferred to a new untreated Petri dish for another 2–4 h. Following this, unattached cells enriched in OLs were removed and placed in a 15 ml tube and centrifuged for 5 min for 1000 rpm at RT. The supernatant was discarded and the pellet was resuspended in 1–2 ml of Chemically Defined Media (CDM) (Bottenstein, 1986) [180.75 ml DMEM, 3 ml 7.5% Sodium Bicarbonate, 2 ml Antibiotic/Antimycotic,