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Insertion of Proteolipid Protein into Mitochondria but not DM20 Regulates Metabolism of Cells

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Abstract

Proteolipid protein (PLP), besides its adhesive role in myelin, has been postulated to have multiple cellular functions. One well-documented function of PLP is regulation of oligodendrocyte (Olg) apoptosis. In contrast, DM20, an alternatively spliced product of the PLP1/Plp1 gene, has been proposed to have functions that are unique from PLP but these functions have never been elucidated. Here, we compare metabolism of PLP and DM20, and show that oxidative phosphorylation (OxPhos) was significantly decreased in Plp1 but not DM20 or EGFP expressing cells. The reserve OxPhos capacity of Plp1 expressing cells was half of control cells, suggesting that they are very vulnerable to stress. ATP in media of Plp1 expressing cells is significantly increased more than two-fold compared to controls; markers of apoptosis are increased in cells over-expressing Plp1, indicating that abnormal metabolism of PLP is most likely the direct cause leading to Olg apoptosis. We hypothesize that abnormal metabolism, mediated by increased insertion of PLP into mitochondria, underlies demyelination in Pelizaeus-Merzbacher Disease (PMD) and in models of PMD. To understand why PLP and DM20 function differently, we mutated amino acids located in the PLP-specific region. All these mutations and deletions of the PLP-specific region prevented insertion of PLP into mitochondria. These findings demonstrate that the PLP-specific region is essential for PLP's import into mitochondria, and now offer an explanation for deciphering unique functions of PLP and DM20.

Keywords

Myelin; Proteolipid protein; Pelizaeus-Merzbacher Disease; Mitochondria; Oligodendrocytes; Metabolism

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INTRODUCTION

Mutations in the human PLP1 (X-linked proteolipid protein 1) gene in the Central Nervous System (CNS) causes Pelizaeus–Merzbacher Disease (PMD) (1–3). The hallmark of PMD mutations is dysmyelination, hypomyelination, and demyelination dependent upon the type of mutation (4). PLP1 duplications, which account for nearly 70% of human PLP1 mutations (5), lead to shortened lifespan and often death within the first decade. Duplications of the native Plp1 gene in rodents, which share the same amino acid sequence as human PLP1 (6), closely model the phenotype of PMD patients (7–8). All neural cell types: including oligodendrocytes (Olg) (9), neurons (10), astrocytes (11), and microglia (12) exhibit numerous abnormalities in these Plp1 models but deciphering the sequence of abnormalities between them has been challenging. Studies *in vivo* and *in vitro* with missense Plp1 mutations (13–15) have shown, not surprisingly, these mutations induce an unfolded protein response (UPR). In contrast to mice with Plp1 missense mutations, retention of PLP in mice with duplications in the endoplasmic reticulum (ER) is minimal, without significant activation of the UPR (16). This may be partially due to the fact that PLP itself is only modestly increased, although there may be many copies of the native Plp1 gene, (17–18). These studies raise again the question of what causes demyelination in mammals with duplications of the PLP1/Plp1 gene. More recently, our laboratory showed over-expression of wt-Plp1 cDNA's in COS-7 cells and in Plp1 transgenic mice led to insertion of PLP into mitochondria (19–20). Specific cysteine motifs in the N-terminus of PLP allow for its translocation to the mitochondria (21–23). The above data suggest that increased insertion of PLP into mitochondria alters oxidative phosphorylation (OxPhos) and disrupts mitochondrial function. Our results show that O₂ consumption decreased by half, extracellular levels of ATP and lactate were increased in Plp1 expressing cells compared to control cells. Markers of apoptosis were quickly increased in Plp1-expressing cells.

We were unable to demonstrate DM20 constructs co-localized DM20 to mitochondria; even though both PLP and DM20 have the same cysteine motifs required for insertion into mitochondria. Our finding that DM20 does not co-localize to mitochondria, and functions differently in metabolism than PLP is not that surprising because numerous studies show that the functions of DM20 are not fully comparable to PLP (24). A likely candidate to regulate these functions is the 35 amino acid intracellular loop of PLP that is lacking in DM20. We made different mutations in the PLP-specific region and all mutations prevented PLP's insertion into mitochondria, indicating that the PLP-specific region is required for import into mitochondria.

MATERIALS AND METHODS

Cell Culture

COS-7 cells were maintained on 10 cm tissue culture dishes in DMEM supplemented with 10% fetal bovine serum (Invitrogen Thermo Fisher, Waltham, MA) and 1% Penicillin/Streptomycin. Cells were grown at 37°C under 5% CO₂ atmosphere and the medium was replaced every third day. For passaging cells, confluent plates were treated with non-enzymatic cell dissociation solution (Sigma Aldrich, St. Louis, MO USA) for 5 to 10 mins,

cells were collected, centrifuged, and re-suspended in fresh medium for counting, then added to new plates containing fresh medium.

Transfections

For immunocytochemistry, COS-7 cells were seeded on 12mm coverslips in 6 well plates when 40–50% confluent one day before transfections. Cells were transfected with Lipofectamine^{TM}2000 (Thermo Fisher Scientific) containing 3 µg of plasmid cDNAs: (wt-PAcGFPPlp1, wt-PAcGFP, PAcGFPDM20, PAcGFPc>S139Plp1, PAcGFPc141>SPlp1, PCMVwt-hPLP1, PcGNwt-hPLP1, del142Plp1, del143Plp1, del126_130Plp1, del146_150Plp1, del141_145Plp1, wt-hPlp1HA, PcGNC7>APlp1HA, wt-Plp1-EGFP according to the manufacturer's protocols (Life Technologies). Media was changed 4 hours after addition of constructs, and cells grown at 37°C in 5% CO₂ for 18, 24, or 48 hrs. Transfection efficiency ranged between 65% and 70%. For Seahorse and Western blotting experiments, COS-7 cells were seeded on 10cm tissue culture plates at 30–40% confluency one day prior to transfections. 30µg of plasmid cDNA was used.

Immunocytochemistry of cultured cells

Immunocytochemistry was performed using a slight modification of a previously used protocol (20). COS-7 cells were grown on 12 mm coverslips in 6-well plates until they were 40–50% confluent. At 48h post-transfection, cells were fixed in 4% paraformaldehyde for 10 min at 4°C, rinsed in PBS, permeabilized with 0.1% Triton X-100 for 2min and rinsed in PBS. For studying Plp1 localization in mitochondria with the GFP constructs, cells were incubated with a monoclonal mouse electron transport chain complex IV, cytochrome *c oxidase*, subunit 1 (COX1) antibody (Invitrogen, Carlsbad, CA USA) diluted 1:100 or a rabbit polyclonal anti-complex III antibody diluted 1:200 in PBS for 1h at RT. Coverslips were rinsed 3X in PBS and incubated with a goat anti-mouse IgG antibody conjugated to Alexa 488 (Jackson ImmunoResearch, West Grove, PA) or with a goat anti-rabbit IgG antibody conjugated to Texas Red (Jackson ImmunoResearch) diluted 1:300 in PBS for 1h at RT and rinsed 3X with PBS. The coverslips were then stained with DAPI diluted 1:10,000 in methanol for 2 mins at RT, rinsed 3X with PBS and mounted on slides with Aqua-mount (Polysciences, Warrington, PA). In some experiments, cells transfected with PcGN plasmids were fixed in 4% paraformaldehyde for 10–15min, rinsed in PBS first, processed with 10mM citric acid, 0.1% Tween-20 pH 6.0 for 10mins at 95°C and then 20min at RT for antigen retrieval. Coverslips were then rinsed twice with PBS and incubated with mouse anti-COX1 diluted 1:200, mouse anti-KDEL (ENZO, Farmingdale NY) 1:200 and PLP C-term (25) 1:100 for 1h at RT, rinsed 3X with PBS, and then with an anti-mouse IgG antibody conjugated to Alexa 488 (Jackson ImmunoResearch) then incubated with goat anti-rabbit IgG antibody conjugated to Texas Red (Jackson ImmunoResearch). Finally, the cells were stained with DAPI 1:10,000 in methanol and coverslips mounted with Aquamount. Cells were imaged on a Leica TCS S5P microscope and images were processed in Photoshop.

MitoBloCK-6 treatment and Western blotting

For MitoBloCK-6 treatment, COS-7 cells were grown and transfected with PCMVwt-hPLP. Four hours after transfection, media was changed and supplemented with 100µM MitoBloCK-6, (EMD Millipore-Chemicon, Temecula, CA). Cells were incubated an

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additional 24 and 48hr, mitochondria were isolated and fractions were Western blotted. Western blotting was performed using a modification of a previously used protocol (19). Mitochondrial/cytosolic fractionation kits (Biovision Mountain View, CA) were used according to the manufacturer's protocol. To confirm the purity of the mitochondrial fractions, Western blots were probed with markers for mitochondria (complex III) as well as an estrogen receptor β for cytosol. Anti-Flag, anti-HA or monoclonal anti-PLP (AA3) antibodies were used to probe for PLP in the different fractions. Blots for MitoBloCK-6, cytochrome *c*, Parp, Cleaved Parp, AIF (Cell Signalling Technologies, Danvers Massachusetts) were lightly stripped and re-probed. Fifty-100 μ g of protein was loaded into each well. Proteins were transferred onto a methanol activated PVDF membrane and blocked with 5%(w/v) non fat milk for 1h and then incubated with primary antibody (anti-actin 1:1000, anti-FLAG 1:1000, anti-HA 1:1000, anti-AA3 1:50, anti-cyc I 1:1000, anti-estrogen receptor beta 1:1000) overnight at 4°C. The blots were washed 3X and then probed with secondary antibody (goat anti-mouse HRP conjugated (GE Healthcare, Pittsburgh PA) or goat anti-rabbit HRP conjugated 1:5000) for 1h, washed and detected using the ChemiLucent^{TM} ECL Detection system (EMD Millipore-Chemicon). Blots were digitally imaged on a ProteinSimple (San Jose, CA) imager; image densitometry of images was done in Adobe Photoshop.

Breeding pairs of wt-, heterozygous and homozygous Plp1tg line 66 (provided by K-A Nave, Max Planck Institute, Gottingen, Germany) mice are maintained in Wayne State University DLAR facilities, a federally approved AAALAC facility. All animal procedures (harvesting of animals for Westerns) for this study were approved by IACUC.

ATP and lactate measurements

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For the measurement of lactate production, COS-7 cells were plated on 6 well plates. Lactate released from cells, incubated for 18, 24 and 48hrs post PLP and DM20 transfections were measured in media, using a colorimetric lactate assay (Sigma Biovision, Mountain View, CA) following the manufacturer's protocol. A standard curve was generated and used to calculate the amount of lactate in each sample. All the experiments were performed in triplicates. For ATP assay, media and cell pellets at 18, 24 and 48hrs post PLP transfection were immediately frozen in liquid nitrogen, stored at -80°C, and ATP concentrations determined in triplicate per sample using the ATP bioluminescence assay kit HSII (Roche Applied Science) according to the manufacturer's protocol in conjunction with boiling. Data were standardized to protein concentration determined by the DC protein assay (Bio Rad).

Respiration assay

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The Seahorse Extracellular Flux Analyzer XF®24 (Agilent, Santa Clara, CA) was used to measure O₂ consumption rate in a 24-well format. The machine detects changes in O₂ content in a very small volume of 7 μ l above plated cells with a fluorescence biosensor. Measurements are taken from intact cells in short, repeated intervals making it non-invasive and physiologically relevant. XF assay medium (Seahorse), pH 7.2, was supplemented with pyruvate and glucose and used in the XF analysis. COS-7 cells transfected with EGFP, DM20, and PLP were seeded in an XF® 24-well cell culture micro-plate previously coated with 0.1% gelatin at 40,000 cells/well in 500 μ l of DMEM medium supplemented with 10%

serum and 1% penicillin/streptomycin and incubated overnight at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Prior to assay, medium was removed and replaced by 750µl of assay medium and the cells were placed in a CO₂ free incubator for 1hr at 37°C in air.

Statistical Analysis

Data was analyzed using Microsoft Excel and GraphPad Prism. All experiments were done using an n of 3 or more. P values were calculated using two-tailed t tests.

RESULTS

PLP not DM20 lowers respiration in COS-7 cells

OCR was calculated in real-time using a Seahorse Bioscience XF^e24 extracellular flux analyzer in order to determine if basal rates of O₂ consumption differed between non-transfected COS-7 cells, COS-7 cells expressing EGFP, COS-7 cells expressing DM20 (DM20+ive), and COS-7 cells expressing PLP (Plp1+ive). Figure 1A reviews differences between ATP production from OxPhos versus glycolysis. Baseline OCR is 900pMols/min for EGFP+ive cells versus 550pMols/min for Plp1+ive cells, roughly a 35% reduction. The addition of oligomycin A, an inhibitor of ATP synthase, is used to measure ATP-linked respiration by subtracting the oligomycin rate from the addition of proton leak and non-mitochondrial oxygen consumption. For Plp1+cells, ATP linked respiration was approximately 75pMol/min versus 150pMol for EGFP+ive cells, a reduction of 50%. To determine maximal respiratory capacity, FCCP was added as an uncoupling agent that disrupts ATP synthesis and allows the electron transport chain to function at its maximal rate. Maximal respiratory capacity is measured by subtracting non-mitochondrial respiration after the addition of FCCP. For Plp1+cells, it was approximately 175pMol/min versus 350pMol/min for EGFP+ive cells, a 50% reduction. The reserve capacity is measured following the addition of Rotenone and Antimycin A; which blocks transfer of electrons from complexes I and III to ubiquinone. Reserved capacity is measured by subtracting basal respiration from maximal respiratory capacity. For Plp1+ive cells, reserve capacity was 75pMol/min versus 150pMols/min for EGFP+ive cells. In Fig. 1C, we compare OCR between non-transfected and transfected cells (48hr data is derived from Fig. 1B). OCR in EGFP+ive cells is the same as non-transfected cells, indicating cDNAs did not alter OCR. OCR in DM20+ive cells is somewhat reduced compared to non-transfected and EGFP+ive cells but is still significantly higher than Plp1+ive cells. OCR in Plp1+ive cells at 18, 24 and 48hrs is significantly reduced 30–35% post Plp1 transfection. An immortalized oligodendrocyte cell line (26) transfected with a Plp1 cDNA construct also exhibited a significantly decreased basal OCR compared to both EGFP and DM20 transfected cells after 48hrs (Figure 1D).

PLP increases extracellular ATP and lactate levels in COS-7 cells

To determine how over-expression of PLP affected cytosolic and extra-cellular ATP levels, COS-7 cells were transfected with wt-Plp1-EGFP, wt-DM20-EGFP and EGFP constructs for 18, 24 and 48hrs. Our data showed a slight but not significant drop in cytosolic ATP levels at 48hrs in PLP1+ive cells as compared to EGFP+ive or DM20+ive transfected cells (Fig. 2A)

but this trend was not found at 18hr or 24hr time points. In contrast, we observed significantly higher levels of ATP in the media in Plp1+ive cells 48hrs post transfection as compared to EGFP+ive and DM20+ive COS-7 cells (Fig. 2B).

Reduced OCR cultured cells and pH in media and ECF but higher ATP in media in Plp1+ive cells and tissues from Plp1tg mice compared to control cells/tissues suggests these cells also use glycolysis to produce ATP likely by increased lactate production. Not surprisingly, the amount of lactate in the media was significantly higher in wt-Plp1+ive cells at 24hrs and 48hrs compared to EGFP+ive cells (Fig. 2C).

PLP-specific region is required for translocation of PLP to mitochondria

The only difference between PLP and DM20 at the protein level is a 35 amino acid in the intra-cellular loop in PLP. Surprisingly DM20 constructs, in contrast to PLP constructs, do not co-localize to mitochondria (Fig. 3E–H). Numerous DM20 cDNA constructs (DM20-EGFP, DSRED-DM20, untagged DM20) rarely co-localized to mitochondria. Different topological features of PLP and DM20 in the trans-membrane domains may explain these differences (Discussion). Deleted single amino acids, single amino acid substitutions, and deleted 5 amino acids of PLP failed to show co-localization of mutated PLP with mitochondria stained for COX1 (Figs. 3I–O). wt-PLP is distributed differently than mutated PLP: wt-PLP is peri-nuclear in ER, Golgi, and mitochondria and transported to the plasma membrane but mutated PLP, while also peri-nuclear, does not enter mitochondria. Using site-directed mutagenesis, we mutated the 2 cysteines in the intra-cellular loop to a serine (139C>SPlp1-EGFP cDNA and 141C>SPlp1 EGFP cDNA) and these mutations do not permit transport of PLP into mitochondria (Fig. 3N–O). However, mutations of other cysteines (7C>APLP) (Fig. 3P) show considerable amount of co-localization of wt-PLP in the mitochondria (Fig. 3P). Cysteine mutations in the PLP extracellular loop also do not prevent co-localization of PLP into mitochondria (20).

We stained cells transfected with wt-Plp1 and Plp1 deletions in the Plp1 specific domain, with KDEL, target peptide sequence that retains protein in the ER, and must be cleaved off in order for the protein to leave the ER. In wt-Plp1+ive cells, co-localization is present as expected because PLP message is translated in the ER (Fig. 3Q) but transport to the plasma membrane is easily visualized. In contrast, cells transfected with deletion constructs had intense co-localization of PLP and KDEL in the ER in the form of a ring structure around the nucleus (Figs. 3R–T). The mutations do not completely prevent transport of PLP out of ER as wispy strands are present in the cytoplasm and some trafficks to the plasma membrane.

The Mia40/Erv1 pathway is required for import of PLP into mitochondria

PLP contains CX₃C and CX₉C motifs in the N-terminal region that are required for the Mia40/Erv1 redox-coupled mitochondrial import pathway (27). Confirmation that PLP localizes to Mia40/Erv1 complex is demonstrated with MitoBloCK-6, an Erv1 inhibitor. In cells without MitoBloCK-6, co-localization between Mia40, COX1 and PLP is clearly observed (Fig. 4A–C) but absent in MitoBloCK-6 treated cells (Fig. 4D–F). Cells treated with MitoBloCK-6 does not prevent PLP from trafficking to the plasma membrane (Fig.

4D–F) indicating there are two separate pathways for trafficking of PLP, one for insertion into the membrane and another for insertion into mitochondria. Our results showed PLP was present in mitochondrial fractions of COS-7 cells that were not treated with MitoBloCK-6, whereas no or faint PLP bands were in the mitochondrial fractions treated with MitoBloCK-6 (Fig. 4G–H). MitoBloCK-6 also reduces the amount of Mia40 entering mitochondrial fractions, as previously reported (27).

PLP insertion into mitochondria causes increased release of apoptotic markers

We examined cytochrome *c* levels in mitochondrial and cytosolic fractions *in vitro* and *in vivo*, cleaved PARP, and apoptosis inducing factor (AIF) in Plp1 over-expressing cells and in 1-month-old Plp1tg mice. In both cases, we observed an increase in the level of cytochrome *c* in the cytosol (Figs. 5A–B). Cleaved PARP (95 kD fragment due to caspase-3 mediated cleavage of full-length PARP) is increased in cells over-expressing PLP 48hrs post transfection (Fig. 5C), in agreement with our *in vivo* study showing cleaved PARP was increased 50% in Plp1tg mice (16). Lastly, the level of AIF in the nuclear fractions of Plp1tg mice was increased compared to nuclear fractions in normal/control mice (Fig. 5D). Normally AIF is found in mitochondria but when mitochondria are damaged, it translocates to the nucleus. (28). We found an average 2.2-fold increase in nuclear AIF brain fractions from Plp1tg mice compared to fractions from wt-mice in 3 experiments. Both nuclear and mitochondrial preparations contain actin as it is present in nuclei and mitochondrial matrices as well as being anchored to outer membranes of nuclei and mitochondria.

DISCUSSION

PMD, caused by mutations in the PLP1 gene, often results in death of affected individuals within the first decade of life. Duplications of the PLP1 gene in PMD account for more than half of the mutations. Demyelination was a common finding in these patients at the end of life (4) but what exactly causes demyelination in PMD patients with duplications remains unclear. Numerous studies have examined the processing and transport of PLP in animal models with duplications, and in all these Plp1 animal models, abnormalities were found but which of these defects directly causes altered myelin composition remains elusive. Understanding how PLP1/Plp1 mutations lead to myelin deficiencies is further complicated by the fact that all neural cells are abnormal. Deciphering the sequence of abnormalities has not been resolved (29; 5; 10). For example, a massive inflammatory response has been described in *jimpy* and in Plp1tg mice (12) but is the inflammation caused by demyelination, axonal degeneration, or still other unknown factors like abnormal metabolism? Given the interactions between inflammation and metabolism (eg., 30–31), few studies of metabolism in PMD and in animal models have been conducted. Trying to understand these observations, we investigated whether duplications of the Plp1 gene and its insertion into mitochondria led to aberrant metabolism and downstream abnormalities.

Using Seahorse respiration assays, we found OxPhos is abnormal in rodents with wt-Plp1 gene duplications. The OCR pattern we found is typical of OCR described in other Seahorse studies (32). Basal OCR, a measure of OxPhos, was significantly reduced about 35% in COS-7 Plp1+ive cells compared to controls at several time points after transfection (Fig. 1).

After 48hrs, PLP toxicity leads to increased death so the experiments were terminated. The mitochondrial reserve capacity for Plp1+ive cells was half compared to EGFP+ive cells, indicating their reserve capacity is severely impacted. The reduced reserve capacity of Plp1+ive cells is revealing because reserve capacity is the ability of a cell/tissue to generate additional ATP in energy demanding states (33). *In vivo*, we found sharply decreased MMP in Plp1tg mice at 1 month, suggesting that mitochondria are uncoupled, and this initially should lead to higher respiration rates. In later stages, as shown here, higher mitochondrial activities cannot be maintained due in part to release of cytochrome *c* and decreased OxPhos. The data shown here may represent a latter phase of mitochondrial dysfunction. To determine whether OlgS would exhibit similar metabolic abnormalities as COS-7 cells with increased expression of PLP, we used an immortalized Olg cell line (158N) that makes very low levels of PLP (26). These Plp1 transfected OlgS cells exhibit a significantly reduced OCR 48hrs after transfection, confirming that OxPhos deficiency in Plp1+ Olg-like cells is not a peculiarity of COS-7 cells but occurs in OlgS.

Since OCR is a measure of ATP production, reduced OCR in Plp1+ive cells suggests processing of ATP is likewise abnormal. Previously, we showed extra-cellular levels of ATP from both *in vitro* and *in vivo* samples of Plp1 over-expression were significantly increased (20). In the present study, we re-confirmed that ATP in tissue culture media was increased about 2.5-fold compared to EGFP and DM-20 expressing cells. Although cytosolic ATP was not significantly reduced in Plp1+ive cells in this study, our previous study (19) showed ATP levels were significantly decreased by ½ in whole brain homogenates of Plp1tg mice; however, these mice were starved for 5–6hrs, stressing their reserve capacity. The levels of ATP in ECF and in media have been calculated to be in the nanomolar-micromolar range (34–35) as we calculated; single digit increases are sufficient to activate numerous receptors, particularly purinergic receptors (eg., 36–37). More strikingly, media from cultured Plp1+ive cells and ECF from Plp1tg mice is highly acidic (25); this acidity is clearly due to PLP's insertion into mitochondria as Plp1 mutations that prevent PLP's insertion did not alter pH (20). Lactate is a contributor to this acidity as it is increased in media from Plp1+ive cells. Lactate is generally considered a marker of glycolysis and presumably Plp1+ive cells generate ATP via glycolysis. Interestingly, adult OlgS mainly use glycolysis to generate ATP (38). Given these studies, we speculate increased expression of the PLP1/Plp1 gene switches OPCs from OxPhos to glycolysis as they mature.

Insertion of PLP into mitochondria was predicted by algorithms, one essential component being CX₃C and CX₉C motifs in PLP. These motifs permit PLP's import as an unfolded protein through the Mia40/Erv1 pathway; genetic mutations of these cysteines prevented import into mitochondria (20). Application of MitoBlock-6 (27), which inhibits Erv1 activity, to Plp1+ive cells blocks PLP's insertion into mitochondria as demonstrated by Westerns and immunofluorescence. While this drug is toxic, presumably because essential proteins required for mitochondrial function are blocked from insertion, this data is further confirmation that PLP enters mitochondria via the Mia40/Erv1 import pathway.

Why DM20 is not inserted into mitochondria is puzzling. Numerous studies have alluded to or described unique properties of PLP and DM20 in both CNS and PNS, especially from the developmental period (eg., 24; 39–41). In our own studies, numerous DM20 constructs

failed to show co-localization in mitochondria. Peculiarly, in our different studies comparing PLP to DM20 and controls, measurements of DM20 often laid somewhere between measurements of controls and PLP but their values were significantly different than PLP. (eg., 42). In the present study, OCR was decreased in DM20+ive COS-7 cells, compared to control cells but still significantly increased compared to Plp1+ive cells. DM20 remains an enigma compared to PLP.

The second intracellular loop of PLP, encompassing an additional 35 amino acids, is a likely candidate to explain functional differences between PLP and DM20, and one factor why it does not insert into mitochondria. Indeed, studies have examined the topology of PLP in the alpha helix and found that mutations in the trans-membrane domains alter helix-helix interactions, and can create surface exposed interactions that lead to functional changes of the protein at its exposed surface (43–44). Both deletions and point mutations in the PLP-specific region prevented PLP from entering mitochondria. However, not all point mutations, including certain cysteine mutations, prevented PLP's insertion into mitochondria (Fig. 3; 20). These findings clearly indicate that the PLP-specific region is required for PLP's insertion into mitochondria. Importantly, our findings for the first time demonstrate a molecular basis for functional differences between PLP and DM20. Not surprisingly, these DM20 deletions and mutations lead to exaggerated PLP retention in the ER similar to that found in previous studies (44).

With the exception of Plp1 null mice, any mutation in the Plp1 gene leads to sharply increased numbers of dying Olg (45–46; 16). In contrast, the number of Olg is increased roughly two-fold in Plp1 null mice and in Olg cultures of Plp1 null mice (47). Incubation of PLP anti-sense oligonucleotides into Olg cultures or jimpy mice reduced dying Olg several-fold and increased their survival (48). We previously showed in jimpy mice, Plp1tg mice or COS-7 Plp1+ cells that markers of apoptosis were increased in these mutants. Cytochrome *c* is one such protein which when translocated to the cytosol initiates apoptosis by formation of the apoptosome that activates effector caspase-3. Transfection of COS-7 cells with wt-Plp1 constructs shows cytochrome *c* leaks into the cytosol from mitochondria. Likewise, cleaved PARP, a downstream target for caspase 3 is increased in COS-7 Plp1+ive cells but not in EGFP or DM20+ive cells. AIF, normally located behind the outer mitochondrial membrane when mitochondria are damaged (28), is trans-located to the nucleus in Plp1+ive cells. Our data shows that increased production of PLP has wide ranging effects on metabolic parameters ranging from increases in ATP and lactate to increased cytochrome *c* in cytosol. A straightforward explanation for these multiple effects lies in the classical hypothesis that PLP functions as a channel protein, thus allowing increased leakage of metabolic molecules into ECF and cytosol. Data from the 1970's and '80's strongly suggests that PLP functions as a proton channel (49–52).

The present study shows PLP's insertion into mitochondria detrimentally alters OCR and, subsequently, numerous downstream molecules involved in apoptosis. Consequently, abnormal metabolism may be the basis for abnormal processing of myelin in PMD.

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HIGHLIGHTS

- Proteolipid protein but not DM20 regulates metabolism of glia.
- Proteolipid protein insertion into mitochondria leads to a decrease in OxPhos.
- Deletion of amino acids in PLP intracellular loop stop insertion into mitochondria.

Fig. 1.

A. Cell metabolism was measured using an XFe24 Extracellular Flux Analyzer. **A.** Diagram illustrating generation of ATP via OxPhos or glycolysis. **B.** After transfection (Tx) with EGFP, DM20 or *Plp1* cDNAs, COS-7 cells were seeded onto an XFe24 Cell Microplate (60,000 cells/well, incubated at 37°C overnight in DMEM assay medium (pH 7.4), supplemented with 8mM pyruvate and 11mM glucose after 45–60 min equilibration). OCR changes in response to F1F0 ATP synthase inhibitor (1μM oligomycin), uncoupler (0.75μM FCCP), and ETC inhibitors (1μM rotenone plus 1μM antimycin A). The number of experiments for this 48hr (n=4) experiment and other time points is listed under **1C**. For each experiment, multiple coverslips (4–5) per plate for each condition are averaged. **C.** OCR quantification (2nd measurement) between non-transfected cells, EGFP, DM20 and Plp1 transfected cells shows only Plp1 transfected cells exhibit a significant OCR decline. **D.** An immortalized Olg cell line (158N) transfected (Tx) with EGFP, DM20 or Plp1 constructs exhibits a significant decline in OCR.

Fig. 2.

Measurement of ATP and lactate in cytosol and in media of COS-7 cells transfected with different cDNA constructs. **A.** Eighteen, 24 and 48hrs after transfection with a Plp1-EGFP cDNA, ATP is reduced in cytosol but not significantly compared to EGFP and DM20 constructs. **B.** In media, ATP is significantly increased 2.5x compared to EGFP and DM20 cDNA constructs. **C.** Lactate is slightly but significantly increased at 24 and 48hrs after transfection with a Plp1-EGFP construct compared to EGFP.

Fig. 3.

Confocal images of COS-7 cells fixed 48hrs after transfection with different PLP1/Plp1 and DM20 cDNAs. **A–D.** wt-PLP (green) shows extensive co-localization (yellow) with COX1 mitochondria (red). Many red mitochondria do not contain PLP in this cell and non-transfected cells, indicating specificity of staining. Note that PLP reaches the plasma membrane in some regions of the plasma membrane (arrows). **E–H.** wt-DM20 (green) shows little co-localization with mitochondria (red). DM20 is aggregated into the plasma membrane (arrow) as punctate spots. **I–L.** Deletions of individual amino acids or short peptides within the PLP-specific region show no co-localization with mitochondria. Mutated PLP (green) is aggregated into large clumps adjacent to the nucleus but some is spread into the cytoplasm. A well-defined plasma membrane is lacking in most of the mutations except for the 126_130 deletion where PLP appears fairly normally distributed. **N–O.** Site directed mutagenesis of cysteines to serines in the PLP-specific region shows lack of co-localization with mitochondria. **P.** Not all substitutions of cysteine residues in PLP prevent co-localization as shown with the cysteine to alanine mutant at residue 7 (7C>APLP). **Q.** Co-localization of wt-PLP and KDEL shows co-localization around nucleus but is not as intense as with single amino acid or short peptide deletions. **R–T.** Deletions of single or a short PLP amino acid peptide (green) lead to intense accumulation of mutated PLP product in endoplasmic reticulum (KDEL-red) around nucleus. Note that small amounts are transported towards plasma membrane.

Fig. 4.

The insertion of mitochondrial proteins into mitochondria using the Mia40/Erv1 pathway is blocked with MitoBloCK-6, an inhibitor of Erv1. **A–C.** COS-7 cells transfected with wt-hPlp1HA show co-localization with Mia40 and COX1. Plasma membranes (arrows) show PLP reaches the cell surface. **D–F.** Cells stained for PLP and COX1 or Mia40 show no co-localization with mitochondria. Even with MitoBloCK-6, plasma membranes are stained for PLP. **G.** When MitoBloCK-6 is added to COS-7 cells for 24hrs, it mostly prevents insertion of PLP into mitochondria. Western blot of PLP in cells transfected with a wt-Plp1 cDNA shows little PLP in a mitochondria preparation incubated with MitoBloCK-6 compared to cells not incubated with drug. **H.** Quantification of data of Western shown in **G**.

Fig. 5.

A–B. Cytochrome *c* is increased in cytosol of COS-7 cells 48hrs after transfection with a Plp1-EGFP construct compared to non-transfected cells and in cytosol of 1-month old Plp1tg mice. **C.** Cleaved PARP is increased 48hrs after transfection with a Plp1-EGFP construct compared to EGFP or DM20-EGFP transfected COS-7 cells. **D.** AIF is increased in nuclei of Plp1tg mice at 30 days.