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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 or deleted 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.

1. 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 (Olgs) [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 O_2 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

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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.

2. Materials and methods

2.1. 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% CO_2 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 5–10 mins, cells were collected, centrifuged, and re-suspended in fresh medium for counting, then added to new plates containing fresh medium.

2.2. Transfections

For immunocytochemistry, COS-7 cells were seeded on 12 mm coverslips in 6 well plates when 40–50% confluent one day before transfections. Cells were transfected with Lipofectamine ™2000 (Thermo Fisher Scientific) containing 3 μg of plasmid cDNAs: (wt-PAcGFPPlp1, wt-PAcGFP, PAcGFPDM20, PAcGFPC > S139Plp1, PAcGFPC > S141Plp1, 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 h after addition of constructs, and cells grown at 37 °C in 5% CO_2 for 18, 24, or 48 h. Transfection efficiency ranged between 65% and 70%. For Seahorse and Western blotting experiments, COS-7 cells were seeded on 10 cm tissue culture plates at 30–40% confluency one day prior to transfections. 30 μg of plasmid cDNA was used.

2.3. 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 48 h 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 2 min 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 1 h 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 1 h 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 10–15 min, rinsed in PBS first, processed with 10 mM citric acid, 0.1% Tween–20 pH 6.0 for 10mins at 95 °C and then 20 min 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 1 h 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.

2.4. MitoBloCK-6 treatment and Western blotting

For MitoBloCK-6 treatment, COS-7 cells were grown and transfected with PCMVwt-hPLP1. Four hours after transfection, media was changed and supplemented with 100 μM MitoBloCK-6, (EMD Millipore-Chemicon, Temecula, CA). Cells were incubated an additiional 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 MA) were lightly stripped and re-probed. Fifty–100 μg 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 1 h 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 1 h, washed and detected using the ChemiLucent™ 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.

2.5. ATP and lactate measurements

For the measurement of lactate production, COS-7 cells were plated on 6 well plates. Lactate released from cells, incubated for 18, 24 and 48 h 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 48 h 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).

2.6. Respiration assay

The Seahorse Extracellular Flux Analyzer XF^e24 (Agilent, Santa Clara, CA) was used to measure O_2 consumption rate in a 24-well format. The machine detects changes in O_2 content in a very small volume of 7 μl above plated cells with a fluorescence biosensor.