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Interaction of the cryptic fragment of myelin basic protein with mitochondrial voltage-dependent anion-selective channel-1 affects cell energy metabolism

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Abstract

In demyelinating nervous system disorders, myelin basic protein (MBP), a major component of the myelin sheath, is proteolyzed and its fragments are released in the neural environment. Here, we demonstrated that, in contrast with MBP, the cellular uptake of the cryptic 84-104 epitope (MBP84-104) did not involve the low-density lipoprotein receptor-related protein-1, a scavenger receptor. Our pull-down, mass-spectrometry and molecular modeling studies suggested that, similar with a number of other unfolded and aberrant proteins and peptides, the internalized MBP84-104 was capable of binding to the voltage-dependent anion-selective channel-1 (VDAC-1), a mitochondrial porin. Molecular modeling suggested that MBP84-104 directly binds to the N-terminal α -helix located midway inside the 19 β -blade barrel of VDAC-1. These interactions may have affected the mitochondrial functions and energy metabolism in multiple cell types. Notably, MBP84-104 caused neither cell apoptosis nor affected the total cellular ATP levels, but repressed the aerobic glycolysis (lactic acid fermentation) and decreased the L-lactate/D-glucose ratio (also termed as the Warburg effect) in normal and cancer cells. Overall, our findings implied that because of its interactions with VDAC-1, the cryptic MBP84-104 peptide invoked reprogramming of the cellular energy metabolism that favored enhanced cellular activity, rather than apoptotic cell death. We concluded that the released MBP84-104 peptide, internalized by the cells, contributes to the reprogramming of the energy-generating pathways in multiple cell types.

Keywords

myelin basic protein; voltage-dependent anion-selective channel-1; glycolysis; mitochondria; Warburg effect; neurotrauma; cancer

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INTRODUCTION

Specialized glial cells [Schwann cells and oligodendrocytes in the peripheral and central nervous systems (PNS/CNS), respectively] assemble the myelin sheath by enwrapping their cell plasma membranes around axons. Myelin basic protein (MBP) is a major component of the myelin sheath that protects neurons in the PNS/CNS. The major 18.5 kDa and the minor 17 kDa, 20.2 kDa and 21.5 kDa MBP isoforms are all transcribed from a single Golli-MBP gene in humans and rodents [1, 2]. MBP is an intrinsically unstructured protein that continually changes conformation as a result of its local disorder-to-order transitions and upon association with its multiple ligands [3]. Extensive autoimmune demyelination caused by neuraxial traumatic damage, diseases and exposure to drugs, toxins and viral pathogens affect the integrity of the myelin sheath [4], [5] and facilitate the proteolysis of MBP. This limited proteolysis releases the cryptic antigenic 84-104 epitope, which is normally hidden in the intact MBP globule [6-10]. The cryptic immunodominant MBP epitope ([exemplified in our study by a synthetic MBP84-104 peptide (ENPVVHFFKNIVTPRTPPPSQ); residues are numbered according to the GenBank #AAH08749 human MBP sequence] is homologous in all mammals and includes a conserved ⁸⁷VVHFF⁹¹ α -helix. The positively charged MBP exhibits multiple physiological functions through both its interactions with polyanionic cellular partners (e.g., tubulin and actin cytoskeletal proteins, and anionic lipids) and regulation of calcium flux, myelin compaction and the structural assembly of the axon-glia unit [11-16].

Converging lines of work emphasize the pathophysiological importance of the MBP cryptic sequences, including 84-104 epitope: (*i*) following proteolysis, the liberated cryptic epitopes contribute to autoimmune demyelination in multiple sclerosis [17-19], (*ii*) immunization of animals with these epitopes serves as a model of neurodegenerative conditions in humans [4, 20], (*iii*) these epitopes are released in mononeuropathy associated with focal trauma to peripheral nerve in rodents [7, 10], and (*iv*) injection of MBP84-104 into the intact sciatic nerve triggers auxiliary cellular and biochemical events, leading to mechanical hypersensitivity, an increase in the T cell response and the MHCII-reactive cell populations, and the elevated interleukin-6 levels at the injection site [8-10, 21, 22]. These effects of the injected MBP84-104 implied that the peptide interacts with certain cellular proteins and that these interactions contribute to the subsequent downstream cascade specifically in the injured, ipsilateral nerve.

The low-density lipoprotein (LDL) receptor-related protein-1 [LRP-1; synonyms - CD91/ α 2-macroglobulin receptor], a member of the LDL receptor family [23-26], consists of a 85 kDa membrane-spanning light β -chain and a non-covalently associated 515-kDa extracellular α -chain. Ligand interactions with LRP-1 are antagonized by RAP, a receptor-associated 39-40 kDa glycoprotein [27]. LRP-1 performs as a cell-surface endocytic scavenger receptor for numerous extracellular ligands, including MBP, myelin-associated glycoprotein, matrix metalloproteases, thrombospondins, α 2-macroglobulin, fibronectin, lipoprotein lipase, urokinase- and tissue-type plasminogen activators, and this list is continually growing. LRP-1 internalizes and then targets the bound ligands to the lysosomal compartment for degradation [28, 29]. LRP-1 also binds to and affects the activity of adaptor proteins, integrins and tyrosine kinase transmembrane receptors [28, 30-33]. Because LRP-1

internalizes the myelin debris released by damage to the nervous system, this scavenger receptor plays an important role in neurotrauma [31, 34-36].

Voltage-dependent anion-selective channel-1 (VDAC-1), one of the three VDAC isoforms, is an evolutionary conserved ion channel. VDAC-1 is the most abundant protein in the mitochondrial outer-membrane (MOM) [37, 38]. All VDAC isoforms exhibit a conserved three-dimensional structure with a 19 β -strands barrel and a 25-residue-long N-terminal α -helix normally located inside the pore [39], but able to exit the pore barrel [40, 41]. VDAC-1 plays a crucial role in the energy fluxes across the MOM, and in the metabolite exchange, including ADP and ATP, between the cytoplasm and the mitochondria [40, 42, 43]. There are some still controversial reports that VDAC-1 is also located in the plasma membrane [44, 45]. At low, 10-20 mV, transmembrane potential VDAC-1 is in its open conformation. In turn, the VDAC-1 pore is largely closed at the potential over 20-30 mV [43, 46]. Contrary to other channels, VDAC-1 is rarely present in its fully inert configuration, and the 'closed state' largely refers to a subconductance state that remains permeable for small size metabolites.

VDAC-1 also acts as a functional anchor for over 150 proteins [47], such as hexokinase (HK; 2 isoforms HK1 and HK2), adenine nucleotide translocator (ANT), apoptotic Bcl-2 family members [40, 45, 48, 49] and cytoskeletal proteins, including α - and β -tubulin and actin [50]. Through its direct interaction with the anti-apoptotic HK1, HK2 and Bcl-2, the N-terminal α -helix of VDAC-1 plays a crucial role in protecting cells from apoptosis [51]. Intriguingly, VDAC-1 also plays a noticeable role in the stress response and directly binds to the unfolded and aberrant proteins and peptides, including amyloid β ($A\beta$) and Tau in Alzheimer's disease, α -synuclein in Parkinson's disease and several superoxide dismutase (e.g. SOD1) mutants in amyotrophic lateral sclerosis [52-56].

To function normally, tissues require glucose, the central macronutrient. Glycolysis, the major glucose metabolism pathway, occurs in the cell cytosol. The first step of glycolysis is initiated by HK that catalyzes the phosphorylation of a glucose molecule (imported from the extracellular space *via* glucose transporters) to glucose-6-phosphate. The latter is subsequently converted into two pyruvate molecules with the concomitant production of 2 ATP molecules. Among the four mammalian HK isoforms, HK1 and HK2 are known to bind to the N-terminal α -helical region of VDAC-1 in order to gain a preferential access to the mitochondrially-generated ATP. Depending on the oxygen (O_2) level, the pyruvate metabolism pathway takes place either aerobically or anaerobically (Figure 1). In aerobic condition, the energy is generated from oxidative breakdown of pyruvate. Thus, pyruvate is transported to the mitochondria and then oxidized into acetyl-CoA (+2 ATP/glucose) and metabolized in the Krebs cycle (+2 ATP/glucose) followed by the electron transfer chain and oxidative phosphorylation (OxPhos; ~32 ATP/glucose). In anaerobic condition, also called lactic acid fermentation, pyruvate is reduced by L-lactate dehydrogenase (L-LDH) into L-lactate (+1 ATP/pyruvate) that is excreted into the extracellular space. As compared with normal cells, cancer cells are characterized by a high rate of glycolysis, which occurs even in the presence of a high O_2 level (aerobic glycolysis) and the properly functional mitochondria. The common feature of this rewired energy-generating pathway (frequently called the Warburg effect) [57, 58] is a difference in the ratio of aerobic glycolysis to

respiration characterized by an increased glucose uptake and enhanced lactate formation [57, 59, 60] (Figure 1). Although less ATP per unit of glucose is produced using the Warburg effect [61], cancer cells take advantages of this pathway. Thus, in aerobic glycolysis, glucose catabolism generates NADPH and molecular precursors *via* the pentose phosphate shunt for the reductive biosynthesis and anabolic metabolism, which is a response to the high demand of cancer cells for amino acids, nucleotides, and lipids that are necessary for the biosynthesis of proteins, nucleic acids and membranes, respectively [62]. Importantly, because of the rate of glucose metabolism *via* aerobic glycolysis is 10-100 times faster than the complete glucose oxidation through mitochondrial respiration [61, 63], the total cellular amount of ATP produced over any given period of time is comparable when either form of glucose catabolism is utilized [64, 65].

In this study, we shed light on VDAC-1, a mitochondrial target of the cryptic MBP 84-104 fragment released following nerve damages. In contrast with full-length MBP that is internalized *via* the binding to cellular LRP-1 scavenger receptor, the MBP84-104 cryptic epitope escapes LRP-1-mediated endocytosis and, thus, its uptake involves an additional mechanism yet to be identified. Our follow-on pull-down, mass spectrometry and molecular modeling studies implied that the internalized MB84-104 potentially interacts with the N-terminal α -helix inside the porin barrel of VDAC-1 and that these interactions affect cell energy metabolic pathways. In agreement, using highly proliferative cell lines known to exhibit high rates of glycolysis, we recorded that, without exhibiting any significant effect on apoptosis nor the total ATP levels, MBP84-104●VDAC-1 interactions reduced the L-lactate/D-glucose (L/G) ratio to favor the energy efficient OxPhos pathway in cells, rather than the lactic acid fermentation.

MATERIALS AND METHODS

Reagents.

All reagents were purchased from Millipore-Sigma unless indicated otherwise. The horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (#711-035-152) and mouse anti-LAMP-1 antibodies (#9835-01) were from Jackson ImmunoResearch and Southern Biotech, respectively. A 3,3',5,5'-tetramethylbenzidine HRP substrate (TMB/E) was from Surmodics. The rabbit monoclonal VDAC-1 (D73D12; #4661) and HK2 (C64G5; #2867), and polyclonal α -tubulin (#2144) antibodies were obtained from Cell Signaling Technology. A murine monoclonal antibody to LRP-1 (clone 8G1; #ab20384) was from Abcam. The secondary species-specific antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 594 were from Molecular Probes. The mitochondria isolation kit, Coomassie Protein Assay kits, SuperSignal West Dura Extended Duration Substrate, EZ-Link Sulfo-NHS-LC-biotin, and the 3-8% NuPAGE Tris-Acetate and 4-12% NuPAGE MOPS gels were from Thermo Scientific.

Cells.

Cells were obtained from American Type Culture Collection. Human fibrosarcoma HT1080 and glioma U251, and rat-mouse hybrid F11 [a somatic cell hybrid of a rat embryonic dorsal root ganglion (DRG) and mouse neuroblastoma cell line N18TG2] cells were routinely

maintained in DMEM supplemented with 10% FBS and gentamicin (10 µg/ml). Human mammary epithelial 184B5 cells [derived from the normal mammary cells mutagenized by benzo(a)pyrene] were grown in mammary epithelial cell growth medium (MEGM)-5% FBS supplemented with bovine pituitary extract (26 µg/ml) and gentamicin [66].

Preparation of cultured DRG cells.

Sprague-Dawley rats of both sexes (6-26 day-old; n=16) were obtained from Envigo Labs. The rats were sacrificed by deep anesthesia using CO₂ or 5 % isoflurane in O₂ (Aerrane; Baxter), followed by decapitation. For cell isolation, all thoracic and lumbar ganglia were collected, dissociated enzymatically in low-glucose DMEM containing 1% collagenase, 2% trypsin in HEPES buffer (Thermo Scientific). The cells were seeded into plates coated with Poly-D-lysine and laminin. Neurobasal medium (Thermo Scientific) supplemented with B27 Gem21 Neuroplex (Gemini Bioproducts), nerve growth factor (25 ng/ml) and glutamine (2 mM; Thermo Scientific) was used as culture media. For the pull-down assay and mass spectrometry analysis (see below), isolated cells were cultured for 24, 48 and 72 h. The animal procedures were performed according to the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the protocols approved by the Institutional Animal Care and Use Committee at the Veterans Affairs San Diego Healthcare System.

Intact MBP and MBP peptides.

Human full-length MBP (an 18.5 kDa isoform) was from Meridian Life Science. Where indicated, MBP was labeled for 30 min on ice at a 1:20 protein - biotin molar ratio using EZ-Link Sulfo-NHS-LC-biotin. Excess biotin was removed using a Micro Bio-spin column (Bio-Rad). The synthetic wild-type (WT; ENPVVHFFKNIVTPRTPPPSQ), mutant (H89G; ENPVVGFFKNIVTPRTPPPSQ, the mutation is underlined) and scrambled (SCR; SHVPFNEFQPEPVNVPKPRIE) 84-104 sequence region of MBP (MBP84-104) and the Alexa Fluor 488-labeled peptides (97-99% purity) were synthesized by GenScript. Where indicated, the peptides were protected from exoprotease degradation by C-terminal amidation and either N-terminal biotinylation or acetylation (Table 1). Peptides are numbered according to the human MBP sequence (GenBank #AAH08749).

Endocytosis assay using indirect immunofluorescence microscopy.

Cells (25-50% confluent) grown on a coverslip were washed with ice-cold DMEM containing 20 mM HEPES-0.2% BSA, pH 7.0, and then incubated on ice for 15 min in the same medium. Full-length MBP (10 µg/ml) was added to the cells, and incubation was continued for an additional 1 h at 4°C to allow MBP to bind to cell surfaces. To remove the unbound MBP, cells were extensively washed using the same buffer. Cells were then either kept at 4°C (no uptake) or transferred for 1 h to 37°C (uptake) to allow the internalization/endocytosis of the surface-bound MBP. Cells were next fixed and permeabilized with either methanol at -20°C for 10 min or 4% paraformaldehyde for 15 min at ambient temperature followed by 0.1% Triton X-100. To visualize cellular MBP and LRP-1, cells were blocked for 1 h at ambient temperature in PBS-10% BSA, and stained for 16-18 h at 4°C using the polyclonal MBP antibody (AB980; 1:200 dilution) and the monoclonal LRP-1 antibody (8G1; 1:250 dilution), followed by the secondary species-specific antibodies conjugated with

either Alexa Fluor 488 or Alexa Fluor 594 (1:200 dilution; 1 h). For the nuclear staining, the slides were mounted in the VectaShield anti-fading embedding medium (Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI). Images were acquired using an x100-400 original magnification on an Olympus BX51 fluorescence microscope equipped with an Olympus MagnaFire digital camera and MagnaFire 2.1C software. Where indicated, cells were co-incubated for 1 h at 4°C with Alexa Fluor 488-labeled WT MBP84-104 (25 µM) alone or jointly with a 4-fold molar excess of the unlabeled WT peptide. The samples were then washed to remove the unbound material and transferred for 1 h to 37°C. The immunofluorescence of the labeled peptide was observed in live cells using a fluorescent microscope. In addition, cells were fixed, stained with the monoclonal LAMP-1 antibody and then the relevant immunofluorescence was observed using a fluorescent microscope.

LRP-1 and α -tubulin pull-down assays.

Cells ($5-10 \times 10^6$; 90% confluent) were solubilized for 1 h at 4°C using 50 mM octyl- β -D-glucopyranoside (Octyl) in Tris (25 mM Tris-HCl, pH 7.4) or HEPES (25 mM HEPES, pH 6.0) buffered saline supplemented with 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 µM GM6001 (a broad-specificity inhibitor of matrix metalloproteinases), and a protease inhibitor mixture set III. Insoluble material was removed by centrifugation ($14,000 \times g$; 20 min). The supernatant fractions were diluted in the respective detergent-free buffered saline to reach a 20 mM concentration of the detergent. Supernatant aliquots (0.5 mg total protein, each) were pre-cleared for 2 h at 4°C using Streptavidin-agarose beads (100 µl; 50% slurry). Pre-cleared samples were incubated for 3 h at 4°C with the individual biotin-labeled WT, H89G and SCR MBP84-104 peptides or biotin-labeled full-length MBP (3 µg, each). Supernatant samples incubated with unlabeled full-length MBP were used as controls. The biotin-labeled material was then pulled-down for 16-18 h at 4°C using Streptavidin-agarose beads (70 µl; 50% slurry). Following washes with 20 mM Octyl in the respective buffered saline, the beads were incubated at 95 °C for 5 min in the non-reducing 2xSDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue, and 20% glycerol; 60 µl). Sample aliquots (35 µl) were separated by SDS gel-electrophoresis in a 3-8% NuPAGE Tris-Acetate gel and analyzed by Western blotting with the monoclonal LRP-1 antibody (1:1,000 dilution). The remaining aliquots (25 µl) were reduced using 1 M DTT (3 µl) and then separated in a 4-12% NuPAGE MOPS gel and analyzed by Western blotting with the polyclonal α -tubulin antibody (1:1,000 dilution) followed by the HRP-conjugated donkey anti-rabbit IgG antibody and a SuperSignal West Dura Extended Duration Substrate kit. Where indicated, cell-surface proteins were biotinylated using membrane-impermeable EZ-Link NHS-LC-biotin prior to cell solubilization [67].

MBP84-104 peptide-conjugated agarose beads.

Biotin-labeled WT, H89G and SCR MBP84-104 peptides (50 µg, each) were allowed to bind to Streptavidin-agarose beads (150 µl, 50% slurry) for 4 h at 4°C in 25 mM Octyl in 50 mM Tris-HCl buffer, pH 7.4, supplemented with 150 mM NaCl, 10 mM CaCl_2 , 1 mM MgCl_2 , 1 mM Na_3VO_4 , 1 mM PMSF and a protease cocktail inhibitor set III (pull-down buffer). The

beads were then extensively washed to remove the unbound peptides and next used in the pull-down experiments described below.

Pull-down assay using the DRG cells and mass spectrometry analysis.

Unless indicated otherwise, all procedures were performed at 4°C. Isolated DRG cells were propagated for 24, 48 and 72 h, washed with PBS and then solubilized for 1 h using 50 mM Octyl in the pull-down buffer. Insoluble material was removed by centrifugation (14,000×g; 20 min). The individual cell lysates were pooled and the protein concentration was determined using the Coomassie Protein Assay kit. Lysate aliquots (500 µg total protein; 2 ml, each) were 2-fold diluted using the detergent-free pull-down buffer to reach a 25 mM final concentration of the detergent. To remove the non-specific binders, the samples were precleared for 4 h using the biotin-labeled SCR MBP84-104 immobilized on Streptavidin-agarose beads (200 µl, 50% slurry). The fall-through fraction was co-incubated for 16-18 h with the biotin-labeled WT MBP84-104 immobilized on Streptavidin-agarose beads. After extensive washing with 25 mM Octyl in pulldown buffer, the bound material was eluted from the individual WT- and SCR peptides immobilized-beads using 2x reducing SDS-loading buffer. The eluted samples were separated in a 4-12% NuPAGE MOPS gel followed by silver staining. Four individual gel section discriminating the WT sample from the SRC sample were subjected to in-gel reduction (50 mM DTT, 60°C, 60 min), alkylation (50 mM iodoacetamide, 40°C, 45 min in the dark), and digestion using Sequencing Grade Modified Trypsin (Promega; 25 µg/ml, 37°C). Peptides were analyzed by LC-MS/MS using a Proxeon EASY nanoLC system (Thermo Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific). Peptides were separated using an analytical C₁₈ Acclaim PepMap column 0.075×500 mm, 2 µm particles (Thermo Scientific). The mass spectrometer was operated in positive data-dependent acquisition mode. MS1 spectra were measured with a resolution of 60,000, an AGC target of 1×10⁶ and a mass range from 350 to 1400 m/z. Up to 10 MS2 spectra per duty cycle were triggered, fragmented by collision-induced dissociation, and acquired in the ion trap with an AGC target of 1×10⁴, an isolation window of 2.0 m/z and a normalized collision energy of 35. Mass spectra were analyzed using MaxQuant software version 1.5.5.1. MS/MS spectra were searched against the *Rattus norvegicus* Uniprot protein sequence and the GPM cRAP sequence database (the latter is the database of commonly known protein contaminants). Carbamidomethylation of cysteines was searched as a fixed modification, while oxidation of methionines and acetylation of protein N-terminal were searched as variable modifications. Enzyme was set to trypsin in a specific mode and a maximum of two missed cleavages was allowed for searching. The target-decoy-based false discovery rate filter for spectrum and protein identification was set to 1%.

Pull-down assays using the mitochondria fractions.

The mitochondria isolation from adherent HT1080, F11 and U251 cells (~12×10⁷) was performed using the Dounce homogenization method and a mitochondria isolation kit. The kit buffers were supplemented with 1 mM PMSF, 1 mM Na₃VO₄ and a protease inhibitor cocktail set III (1:100 dilution). The mitochondria-enriched pellet was lysed with 50 mM Octyl in the pull-down buffer. The insoluble material was removed by centrifugation (14,000×g; 15 min). The protein concentration of the soluble material was determined using the Coomassie Protein Assay kit. To remove the non-specific binders, the isolated samples

(0.5 mg total protein) were precleared using Streptavidin-agarose beads (100 μ l, 50% slurry). Freshly prepared biotin-labeled WT and SRC MBP84-104 immobilized on Streptavidin-agarose beads were used to pull-down the specific binders from the precleared samples. After extensive washing, the bound material was eluted using 2x reducing SDS-loading buffer. The eluates were separated in a 4-12% NuPAGE MOPS gel and then analyzed by Western blotting with the polyclonal VDAC-1 antibody followed by HRP-conjugated donkey anti-rabbit IgG antibody and a SuperSignal West Dura Extended Duration Substrate kit or a TMB/M substrate. Where indicated, whole cell extracts were also analyzed by Western blotting with the rabbit polyclonal VDAC-1, HK2 and α -tubulin antibodies to assess the original level of the proteins in the cells.

Cell-surface biotinylation and pull-down of biotin-labeled proteins.

Cells (80-90% confluent) were washed twice with an ice-cold Soerensen Buffer (SBS), containing 14.7 mM KH_2PO_4 , 2 mM Na_2HPO_4 , and 120 mM sorbitol, pH 7.8, and then incubated for 20 min in ice-cold SBS. Cell surface-associated proteins were left intact or biotinylated for 30 min on ice with SBS supplemented with membrane-impermeable EZ-Link Sulfo-NHS-LC-biotin (0.3 mg/ml). Excess biotin was removed by washing the cells in ice-cold SBS. The residual amounts of biotin were quenched by incubating the cells for 10 min in SBS containing 100 mM glycine. Quenched cells were extensively washed with ice-cold SBS, and lysed in 50 mM Octyl in the pull-down buffer. Biotin-labeled proteins (0.5 mg total protein) were captured from cell lysates using Streptavidin-agarose beads. The precipitated samples were separated in a 4-12% NuPAGE MOPS gel, transferred onto a PVDF membrane and then analyzed by Western blotting with the VDAC-1 antibody followed by the HRP-conjugated donkey anti-rabbit IgG antibody and a SuperSignal West Dura Extended Duration Substrate kit. To confirm efficient biotinylation of the cell surfaces, as well as equal loading of the total protein, the whole cell extract aliquots (5 μ g each) were separated by electrophoresis and transferred onto a PVDF membrane. The membrane was next stained with 0.1% Ponceau S in 5% acetic acid to visualize the solubilized cellular proteins and then also analyzed using HRP-ExtrAvidin to visualize the biotin-labeled material.

Cell viability assays.

Assays were conducted in wells of a 96-well flat bottom plates. Cells (7.5×10^4) were grown for 16-18 h in DMEM-10% FBS or MEGM-5% FBS. After washing in PBS, cells were replenished with serum-free DMEM containing 10 mM glucose (100 μ l) and incubated for an additional 16 h in the presence of the individual WT and SCR MBP84-104 (50 μ M, each). Cells incubated with methyl jasmonate (MJ; 3 mM), an inducer of apoptosis in cancer cells, served as a control [68]. Cell viability was assessed by monitoring the total cellular ATP levels using a luminescent ATP-Lite assay (PerkinElmer). Each datum point represents the results of three independent experiments performed in triplicate.

Measurement of the L/G ratio in conditioned cell media.

Assays were conducted in wells of a 96-well cell culture flat bottom plates. Cells (7.5×10^4) were grown for 16 h in DMEM-10% FBS or MEGM-5% FBS. After three washes with PBS, cells were replenished with DMEM containing 10 mM glucose (150 μ l) and 1% dialyzed

FBS, and then incubated for an additional 16 h in the presence of the individual WT or SCR MBP84-104 (50 μ M, each). Cells incubated with MJ (3 mM) served as a control [68]. The levels of D-glucose and L-lactate in conditioned culture medium aliquots (25 μ l) were measured using an YSI 2950D Biochemistry Analyzer (YSI Life Sciences) equipped with the sample chamber configured with D-glucose oxidase and L-lactate oxidase conjugated enzymes membranes, respectively. Measurements obtained using DMEM-1% dialyzed FBS alone allowed the calculation of the glucose uptake and lactate production. The each datum point represents the results of at least two independent experiments performed in several replicates.

Molecular modeling of VDAC-1 complexed with the MBP peptide.

Molecular modeling of the MBP84-104●VDAC-1 complex was initiated using the NMR structure of human VDAC-1 (PDB 2JK4) [69]. Molecular modeling of the Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-Pro-Ser-Gln peptide was performed according to the configuration of the S⁷²-S¹⁰⁷ peptide of 18.5kDa murine MBP (PDB 2LUG; S⁷⁶-S¹¹¹ peptide in human MBP) [70]. In these structures, both VDAC-1 and MBP peptide were kept rigid during the docking. For docking of the peptide to VDAC-1 we used ZDOCK program [71]. For the further optimization we performed limited energy minimization involving the position of amino acids side chains using Amber16 program and its ff14SB force field [72]. The final models were displayed using PYMOL (www.pymol.org).

Data analyses.

Statistical analysis was performed using one-way analysis of variance (ANOVA) with multiple comparisons followed by Tukey's post-hoc test. $p < 0.05$ values were considered statistically significant.

RESULTS

Cellular endocytosis of MBP and MBP84-104.

Earlier reports demonstrated that full-length MBP directly binds to LRP-1, a ubiquitous scavenger cell receptor involved in the internalization of multiple ligands, including degraded myelin [30-32, 35]. Accordingly, we speculated that LRP-1 also represented a receptor for the cryptic MBP84-104 fragment that is hidden in the intact MBP globule and that is released only in a result of the MBP proteolysis. Because neural cells express low level of LRP-1 [34], in our experiments we used a surrogate cell line - human fibrosarcoma HT1080 cells, which naturally express high level of LRP-1 [73]. Using pull-down of the biotin-labeled plasma membrane-associated proteins followed by Western blotting with the LRP-1 antibody, we confirmed the high expression level of the cell-surface 515-kDa LRP-1 α -chain in HT1080 cells (Figure 2A). To determine if the plasma membrane-associated LRP-1 acted as a cellular receptor for MBP84-104, we performed endocytosis assays. First, we confirmed that LRP-1 was a receptor for the full-length MBP in HT1080 cells. Thus, we co-incubated HT1080 cells with the full-length MBP, initially at 4°C to allow MBP to bind to its cellular receptors. Next, incubation was continued at 37°C to allow the internalization of the bound MBP, followed by cell staining using the LRP-1 and MBP antibodies.

Immunofluorescence microscopy revealed cellular uptake of the MBP-LRP-1 complex, which was originally present at the cell surface, but then internalized and trafficked to the cellular perinuclear region (Figure 2B), most likely to the lysosomal compartment in which the LRP-1-associated ligands are typically delivered for their subsequent degradation [28]. In agreement with the observations by others [31, 32, 35], we concluded that LRP-1 was the primary cell receptor involved in the binding to and the follow-on cellular endocytosis of the full-length MBP protein.

The Alexa Fluor 488-labeled WT MBP-84-104 peptide was internalized and then accumulated in the vesicles in the perinuclear region of HT1080 cells (Figure 2C). No internalization of the labeled peptide by the cells was observed in the presence of a 4-fold molar excess of the unlabeled peptide or if the cells were incubated only at 4°C. According to cell staining for LAMP-1 (a lysosomal marker) the internalized MBP84-104 accumulated in the cell compartment that is distinct from the lysosomes. Unfortunately, cell fixation or embedding caused a gross reduction of the peptide fluorescence, making the more in-depth immunostaining studies nearly impossible (*data not shown*).

MBP, but not MBP84-104, binds to LRP-1 and α -tubulin.

We next determined if LRP-1 was capable of binding to MBP84-104 and, accordingly, of functioning as its cellular receptor. For these purposes, we performed the pull-down assays of LRP-1 from the HT1080 cell lysates. Because the MBP84-104 peptide contains the His-89 (normally, pKa His = 6.0-6.1) and because protonation could affect the peptide properties [74], we performed our experiments at both pH 6.0 and pH 7.4. Cells were lysed, and lysate aliquots were incubated with the biotin-labeled WT or control SCR peptides. The unlabeled and biotin-labeled full-length MBP served as additional controls. The samples were next pulled-down using Streptavidin-agarose beads. The isolated material was analyzed by Western blotting with the LRP-1 antibody. The data demonstrated that the biotin-labeled full-length MBP efficiently pulled-down LRP-1 from cell lysates at both pH 6.0 and pH 7.4. In contrast, the ability of the WT MBP84-104 to pull-down LRP-1 was exceedingly low (Figure 3A). These data suggested that the protonation of His-89 in the MBP peptide does not significantly affect the peptide-LRP-1 interactions.

To corroborate these observations, we employed the H89G mutant MBP84-104 peptide in which Gly-89 substituted for His-89. Thus, HT1080 lysates were incubated with the biotin-labeled WT and H89G mutant peptides at pH 6.0. The unlabeled MBP and both the biotin-labeled MBP and SCR MBP84-104 served as controls. Our results clearly indicated that both the WT and H89G peptides were similarly inefficient in the LRP-1 pull-down (Figure 3B). In sum, we concluded that LRP-1, a scavenger cell receptor for MBP and degraded myelin, did not efficiently bind to MBP84-104 and, because of that, could not function as a MBP84-104 cellular receptor.

To further highlight the difference between MBP and MBP84-104, we tested their ability to bind to the tubulin microtubules, a known target of MBP [75-77]. As expected, biotin-labeled MBP was able to pull-down α -tubulin from the HT1080 cell lysate, while the three biotin-labeled WT, H89G and SCR peptides were inefficient (Figure 3B). Overall, our

results indicate that, relative to full-length MBP, the interactions of the MBP84-104 peptide are distinct and additional to cellular LRP-1 and α -tubulin.

MBP84-104 pulls-down mitochondrial proteins from DRG cells.

To determine the identity of the MBP84-104 cellular targets, we employed cultured DRG-neurons (a mixture of neuronal and glial cells) and both the biotin-labeled WT and SCR MBP84-104 peptides immobilized on Streptavidin-agarose beads as baits. Thus, first, the rat DRG samples were enzymatically dissociated and the mixed DRG cells were propagated for up to 72 h. The cells were then lysed. To remove the non-specific binders, the lysate was pre-cleared on the biotin-labeled SCR MBP-84-104 immobilized on Streptavidin-agarose beads. The fall-through material was allowed to bind to biotin-labeled WT MBP84-104 immobilized on Streptavidin-agarose beads. The proteins bound to the SCR and WT peptide columns were eluted and the purified samples were separated by SDS-gel electrophoresis followed by silver staining of the gel. The four protein bands which appeared distinct in the WT sample relative to the SCR sample were excised, digested with trypsin and the tryptic peptides were analyzed by LC-MS/MS (Figure 4A). Interestingly, the major proteins identified by LC-MS/MS were mitochondrial-associated VDAC-1 (GI: 13786200; 9 peptides, 34.6% sequence coverage), ATP- α [ATP synthase subunit α (GI: 40538742); 7 peptides, 20.8% sequence coverage] and ATP- β [ATP synthase subunit β (GI: 54792127); 17 peptides, 52.4% sequence coverage], cytoplasmic L-LDH α (GI: 8393706; 8 peptides, 30.4% sequence coverage), and endoplasmic reticulum (ER) GRP78 [78kDa glucose-regulated protein (GI: 25742763); 11 peptides, 26.6% sequence coverage] (Table 2). The identified peptides of VDAC-1 are shown in Figure 4B. All of these proteins, including chaperone ER-associated GRP78 [78], are directly related to cellular energy metabolism (Figure 1).

MBP84-104 interacts with rodent and human VDAC-1.

The interactions of MBP84-104 with VDAC-1, the most abundant protein in the MOM [37] and a key in the cellular energy production and metabolic cross-talk [50], were studied in a more detail. Because of the ubiquitous expression of mitochondrial VDAC-1, we performed our follow-on experiments in well-established, robust cell lines instead of using the rat primary DRG cells, which are not available at the required amounts. Thus, to corroborate our data, we isolated the mitochondrial fraction from human HT1080, U251 and rat-mouse hybrid F11 cells (a DRG neuron-derived immortal cell line extensively used in the in vitro model systems that mimic native peripheral sensory neurons). VDAC-1 was then pulled-down from the mitochondria lysate using biotin-labeled WT MBP84-104 as bait and the biotin-labeled SCR MBP84-104 as a control. Western blotting with the VDAC-1 antibody clearly demonstrated that the WT bait, but not the SCR peptide, was capable of binding to both human and rodent mitochondrial VDAC-1 in HT1080 and F11 cells, respectively (Figure 5A). This result was not surprising since VDAC-1 shares ~99% (279/283) sequence homology between humans and rodents. The H89G mutant MBP84-104 was also capable of interacting efficiently with VDAC-1, suggesting that the His-89 was not critical for these associations (Figure 5B). Unexpectedly, we were unable, under the same experimental conditions, to pull-down VDAC-1 from human glioma U251 cells, regardless of the significant level of VDAC-1 in the isolated mitochondrial fraction (Figure 5B).

Modeling of the VDAC-1●MBP84-104 complex.

To model the VDAC-1●MBP84-104 complex (Figure 6), we employed the structure of human VDAC-1 (PDB 2JK4) as a template. The fold of MBP84-104 was modeled according to the structure of the S⁷²-S¹⁰⁷ sequence region of 18.5 kDa murine MBP (PDB 2LUG). ZDOCK software was used to predict the potential VDAC-1●MBP84-104 complex. The ten top scoring structures suggested that MBP84-104 directly interacted with the 25-long residue N-terminal α -helix of VDAC-1. This α -helix is located midway inside the 19 β -strands barrel of the porin (Figures 6A, S1 and S2). Based on the top scoring position configuration, the models suggests that there are three hydrophobic interacting clusters. These clusters involve six MBP84-104 residues and four residues of the VDAC-1 α -helix. These predicted clusters consist of (i) Pro-86 and Phe-90 of MBP and Leu-10 of VDAC-1, (ii) Val-87 of MBP and Val-17 of VDAC-1, and (iii) Phe-91, Ile-94 and Val-95 of MBP and Phe-18 and Phe-24 of VDAC-1 (Figure 6B, Table 3). Three (underline, bold) of the predicted six MBP residues are in the evolutionary conserved ⁸⁷VVHFF⁹¹ α -helix of MBP (Table 3). According to our modeling, there is a significant reduction in the open space within the barrel in the complex relative to free VDAC-1 (Figure S2). By itself, our modeling data do not allow us to suggest that VDAC-1, if complexed with MBP84-104, is now biased to its “closed” or “opened” gate configuration [40, 41, 79-81]. Importantly, because of the evolutionary sequence conservation (100%) of both MBP84-104 and VDAC-1 α -helix in mammals, it is tempting to hypothesize that the predicted VDAC-1●MBP84-104 complex may play a similar functional role in all mammals, rather than in humans alone.

VDAC-1 is not a cell-surface receptor for MBP84-104.

Several reports suggested that VDAC-1 was present both in the mitochondria and the plasma membrane [44, 45]. To determine if VDAC-1 functioned as a cell-surface receptor of MBP84-104 in our cell systems, plasma membrane of the cells were biotin-labeled using membrane-impermeable EZ-Link Sulfo-NHS-LC-biotin. Cells were next lysed and the biotin-labeled proteins were pulled-down from the lysate using Streptavidin-agarose beads. The isolated samples were then analyzed by Western blotting analysis with the VDAC-1 antibody. The respective unlabeled cells served as a control. Western blotting using HRP-conjugated ExtrAvidin confirmed the efficient biotinylation of cell surfaces under our experimental conditions (Supplemental Figure S3). Our tests did not reveal any significant presence of the 31 kDa VDAC-1 porin in the plasma membrane of biotin-labeled cells (Figure 7A). These results suggest that a non-VDAC-1 plasma membrane receptor was involved in the cellular uptake of MBP84-104.

MBP84-104 affects cellular energy metabolism, but not apoptosis, in multiple cell types.

To determine if MBP84-104 *via* its interactions with VDAC-1 affects cell energy metabolism or apoptosis or both, we employed multiple cell types, including highly proliferative human cancer fibrosarcoma HT1080 and glioma U251 cells, human immortalized mammary epithelial 184B5, and rodent DRG neuron-derived hybrid F11 cells. For these purposes, cells were co-incubated for 16-18 h with or without the WT MBP84-104 peptide, and cell viability was assessed by monitoring the total cellular ATP levels using a

luminescent ATP-Lite assay. The SCR peptide was used as a control. To induce mitochondria dysfunction leading to cell death, we also co-incubated the cells with the pro-apoptotic MJ compound. MJ is known to bind to HK2 and, as a result, to stimulate dissociation of HK2 from its complex with VDAC-1, repression of glycolysis and upregulation of apoptosis in the affected cells [68, 82-85]. According to our Western blotting tests with the respective antibodies, all of these cells express the substantial levels of HK2, α -tubulin and VDAC-1 (Figure 7B).

As depicted by the significantly reduced level of the total cellular ATP, apoptosis was induced in all MJ-treated cancer cells relative to the untreated cell control, but not in normal immortalized 184B5 cells [86, 87]. Thus, no significant cell survival (<10%) was detected in fibrosarcoma HT1080 cells and rat hybrid F11 cells that harbor a murine neuroblastoma genome, whereas glioma U251 cells appeared readily more resistant to apoptosis with a survival rate of 63% (62.6 ± 11.5). In turn, our measurements revealed that the total ATP levels were not affected by none of the MBP84-108 peptides, even at a high concentration (50 μ M) in all cells we tested (Figure 8A), suggesting that the peptides were non cytotoxic, and that they did not significantly stimulate nor repress cellular ATP production. In addition, using a bright-field microscope, we did not observe any significant effect on the general morphology of the MBP84-104-treated cells relative to the intact cells (*data not shown*), corroborating the lack of cytotoxicity of the peptides.

Next, to determine if MBP84-104 affected cell energy metabolism, HT1080 cells were incubated with or without the WT MBP84-104 peptide in DMEM containing 10 mM glucose and 1% dialyzed FBS, and the conditioned medium aliquots were then analyzed using an YSI 2950D Biochemistry Analyzer to determine the concentrations of D-glucose and L-lactate. The measurements of both the D-glucose uptake and L-lactate production by the cells allowed us to calculate the L/G ratio, an indicator of the difference in the ratio of aerobic glycolysis to mitochondrial respiration. Cells incubated with MJ and the SCR MBP84-104 serve as controls. As compared with both the untreated cells and the SCR peptide, we recorded a noticeable effect of the WT peptide on the glycolytic L/G ratio. Thus, in 16 h, untreated HT1080 cells consumed 6.07 mM of glucose and produced 11.55 mM of lactate resulting in a glycolytic L/G ratio of 1.90 (Figure 8B). This is not surprising since normally over 85% of incoming glucose is converted to lactate by cancer cells under the Warburg effect (Figure 1) [61].

Because of its combined effect on cell death and metabolism [88, 89], MJ-treated cells demonstrated a reduced L/G ratio, which was less by 12.4% as compared with the untreated HT1080 cells. In HT1080 cells, the WT peptide, but not the SCR peptide, also reduced the L/G ratio by 7.5% relative to the untreated cells. However, in contrast to MJ, this metabolic effect of the WT MBP84-104 peptide took place without involving cell apoptosis (Figure 8A).

To test the effect of MBP84-104 further, we evaluated if the peptide was able to reverse the Warburg effect, or at least to affect the glycolytic rate, in cells that were distinct and additional to HT1080 cells. For this purpose we co-incubated HT1080, U251 and hybrid F11 cells with the WT MBP84-104 peptide in DMEM supplemented with 10 mM glucose and

1% dialyzed FBS. Normal 184B5 cells were used as a control. Where indicated, cells were also treated with MJ. In 16 h, conditioned medium aliquots were analyzed using a YSI 2950D Biochemistry Analyzer. Our measurements indicated that the untreated F11 cells were similar to HT1080 cells and exhibited the Warburg effect ($L/G=1.88$ vs 1.90). In both cell lines, the glycolytic rate was significantly affected with similar reduced L/G ratios following the treatment with MJ (15.6% vs 12.4%) and WT peptide (5.0% vs 7.5%) (Figure 8C).

In contrast, U251 cells were distinct and their glycolytic parameters remained unaffected by the treatment with the peptide (the L/G ratio of 1.37 corresponding to 4.46 mM glucose uptake and 6.12 mM lactate production), while MJ increased the L/G ratio without inducing a strong apoptotic effect in these cells (Figure 8A). Our results suggest that there is no, or exceedingly limited, Warburg effect in glioma cells that favor the oxidative breakdown of pyruvate through respiration (OxPhos and electron transfer chain), rather than the lactic acid fermentation, to maintain their high proliferative rate and migration capabilities. Our unexpected results agree well with the recent data by others who reported the similar characteristics of glioma U251 and U87 cells, both of which exhibited an L/G ratio equaled to $1-1.10$ [90].

Furthermore, normal, albeit immortalized, 184B5 mutant cells [66], performed like cancer cells and displayed a high, 2.06 , glycolytic L/G ratio, suggesting that these cells are also subjected to the Warburg effect. The WT MBP84-104 peptide reduced the L/G ratio by 9.6% (although insignificantly relative to the untreated cell control), while MJ was ineffective. These results support the data by others that normal cells were resistant to apoptosis mediated by MJ [86, 87] (Figure 8A).

In general, we concluded here that the treatment of cells with MBP84-104 did not induce apoptosis, but in turn, most frequently, resulted in the partial reversal of the Warburg effect. These results offer the first evidence for the potential interactions of the MBP84-104 peptide with mitochondrial VDAC-1 to promote the reprogramming of the energy-generating pathway in multiple cell types.

DISCUSSION

Proteolysis of myelin sheath proteins accompanies demyelination caused by traumatic and disease conditions in the nervous system [6, 7, 9, 10, 91]. More specifically, cleavage of MBP by proteinases releases the cryptic epitope sequences that are hidden in the native MBP fold. We previously demonstrated that when injected into naive sciatic nerve, the synthetic peptide that harbors the 84-104 sequence of the cryptic immunodominant MBP epitope induces long-lasting and robust pathological hypersensitivity in rodents [7-10, 21, 22, 91, 92]. However, the cellular targets of MBP84-104 that may be involved in these mechanisms remained unidentified. Our study is focused on the identification of these targets. As a result of our study, we now provide the first evidence that MB84-104 is internalized by the cells and then the peptide is likely to interact directly with the mitochondrial VDAC-1 and affect cell energy metabolism.

Thus, our results indicate that, in contrast with full-length MBP [28, 31, 32, 34-36], LRP-1, a major scavenger receptor in phagocytic macrophages and Schwann cells in the damaged PNS, is not efficiently involved in the cellular uptake of the cryptic MBP84-104 peptide. The ligands internalized by the LRP-1-dependent mechanism, including MBP, are normally trafficked to the lysosomal compartment for degradation [29] and, as a result, full-length MBP is readily removed from circulation. Conversely, based on our results it is likely that MBP84-104 escapes the rapid degradation by the lysosomes and targets certain intracellular proteins.

In our attempt to determine the identity of the intracellular targets of MBP84-104 we initially used the primary cultured cells isolated from the rat DRG neurons, which predominantly included sensory neurons and glia (Schwann and satellite cells). We then asked a question: which specific protein(s) from the DRG lysate would bind to the MBP84-104 bait relative to the MBP84-104 scrambled sequence? As a result of our pull-down assays followed by mass-spectrometry analysis of the selected protein bands, we identified five potential MBP84-104 interacting proteins consisting of the three mitochondrial-associated VDAC-1, α - and β -subunits of ATP synthase, the cytoplasmic L-LDH, and the ER chaperone GRP78, a protein that targets mitochondria in the ER-stress response in apoptosis signaling pathway [93-96] (Figure 1; Table 2). Notably, all of these proteins are key players in cellular energetic and metabolic pathways.

VDAC-1, a mitochondrial porin, plays a central role in the metabolism and energy exchange between the mitochondria matrix and the cytoplasm [40, 42, 43, 45, 48-50, 81]. VDAC-1 acts as a functional anchor for multiple molecules that interact with the mitochondria, including glycerol kinase, HK1 and HK2, and creatine kinase [45]. Octameric creatine kinase interacts with VDAC-1 [97] to decrease its affinity for HK2 and pro-apoptotic Bax [98]. VDAC-1 also forms hetero-complexes with ANT [99], tubulin [79], actin [100], the dynein light chain, the ORDIC channel, glyceraldehyde 3-phosphate dehydrogenase [84, 101], as well as with apoptotic members of the Bcl-2 family. The association of N-terminal α -helix domain of VDAC-1 with HK1/2 sterically protects VDAC-1 from its binding to pro-apoptotic proteins and from the Bax-induced cytochrome c release [51, 88, 89, 102]. In addition, VDAC-1 is known to be the main mitochondrial docking site for multiple misfolded protein and peptides, including α -synuclein in Parkinson's disease, A β in Alzheimer's disease and several SOD1 mutants in amyotrophic lateral sclerosis [52-55]. These interactions with VDAC-1 have a strong impact on the alteration of both cellular bioenergetics and apoptosis pathways [53]. Thus, we centered our subsequent studies on VDAC-1, a potential target of MBP84-104 in the neuronal and glial cells of the DRG.

Because the spatial structures of human VDAC-1 and the murine 72-107 MBP fragment were known, we modeled the potential human VDAC-1●MBP84-104 complex [39, 69, 70]. The structure of VDAC-1 includes a β -barrel composed by 19 β -strands and a N-terminal 25-long residue α -helix horizontally positioned midway within the pore [69], but able to exit the barrel [50, 103]. An uneven number of β -strands confers an intrinsic instability of the β 1- β 4-strands, which are less stable than the other barrel regions, resulting in a barrel ability for a conformational switch [69]. The N-terminal α -helix domain plays a central role in channel gating, acting as the voltage sensor that regulates the conductance of ions and

metabolic molecules through the VDAC-1 pore [39, 69, 103]. Intriguingly, our modeling suggests that, similar to other pathogenic peptides, MBP84-104 directly associates with the N-terminal α -helix of VDAC-1 inside the pore barrel. Our cell surface labeling experiments using membrane impermeable biotin suggested that VDAC-1 was not the primary cell surface receptor of MBP84-104. Currently, we cannot exclude that the plasma membrane VDAC-1 may exist in pathological conditions. Thus, Smilansky et al. reported that VDAC-1 is involved in the A β cell entry into both rat PC12 and human SH-SY5Y neuroblastoma cells [56].

We also asked a question such as if MBP84-104 affects cell metabolism and, more specifically, glycolysis. Our data suggest that MBP84-104 does not promote cell death nor impact the total cellular levels of ATP, but, notably, affects cell energy metabolism as evidenced by partially reducing the level of Warburg effect and reprogramming the cells to respiration (the more energy efficient oxidative breakdown of pyruvate *via* OxPhos and electron transfer chain), rather than to aerobic glycolysis (lactic acid fermentation and acidification of the microenvironment). The reasons why most of the cancer cells exploit the Warburg effect can be explained because (i) exported lactate diffuses and acidifies the tumor environment, which, in turn, alters the tumor-stroma interface to enhance tumor invasiveness [104, 105]; (ii) lactate provokes an inflammatory response that attracts immune cells, which secrete cytokines and growth factors to drive tumor cell growth, invasion and metastasis [106, 107]; (iii) lactate impairs the immune response, disabling immune surveillance [108, 109]; (iv) in aerobic glycolysis, glucose catabolism generates NADPH and molecular precursors for the reductive biosynthesis and anabolic metabolism as a response to the high demand of cancer cells for amino acids, nucleotides, and lipids that are necessary for the biosynthesis of proteins, nucleic acids and membranes, respectively [62]. In turn, lactate utilization in cells of the nervous system is a debated issue. It is widely believed that glial cells produce lactate, which is then transferred to neurons as a substrate for mitochondrial oxidation [110].

In conclusion, our findings allow us to speculate that neurotrauma facilitates the proteolytic release of the cryptic MBP84-104 peptide in the damaged nerve. These events provide an opportunity for the MBP84-104●VDAC-1 interactions, which may affect cell metabolism in the damaged nerve microenvironment [111]. Future functional studies will have to be performed to better understand the role of the degraded MBP peptide in the bioenergetics of neuronal and non-neuronal cells of the nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations.

Aβ	Amyloid β
DRG	dorsal root ganglia
HK2	hexokinase 2
HRP	horseradish peroxidase
L/G	L-lactate/D-glucose
LC-MS/MS	liquid chromatography-tandem mass spectrometry
L-LDHα	L-lactate dehydrogenase A chain
LRP-1	low-density lipoprotein receptor-related protein-1
MBP	myelin basic protein
MBP84-104	the 84-104 residue sequence of MBP
MJ	methyl jasmonate
MOM	mitochondria outer-membrane
OxPhos	oxidative phosphorylation
pull-down buffer	50 mM Tris-HCl pH 7.4 supplemented with 150 mM NaCl, 10 mM CaCl ₂ , 1 mM MgCl ₂ , 1 mM Na ₃ VO ₄ , 1 mM PMSF and a protease cocktail inhibitor set III
VDAC-1	voltage-dependent anion-selective channel-1
WT, H89G and SCR	wild-type, H89G mutant and scrambled peptides

References

1. Campagnoni AT, Pribyl TM, Campagnoni CW, Kampf K, Amur-Umarjee S, Landry CF, Handley VW, Newman SL, Garbay B and Kitamura K (1993) Structure and developmental regulation of Golli-mbp, a 105-kilobase gene that encompasses the myelin basic protein gene and is expressed in cells in the oligodendrocyte lineage in the brain. *J Biol Chem.* 268, 4930–4938 [PubMed: 7680345]
2. Feng JM (2007) Minireview: expression and function of golli protein in immune system. *Neurochem Res.* 32, 273–278 [PubMed: 17024569]
3. Harauz G, Ishiyama N, Hill CM, Bates IR, Libich DS and Fares C (2004) Myelin basic protein-diverse conformational states of an intrinsically unstructured protein and its roles in myelin assembly and multiple sclerosis. *Micron.* 35, 503–542 [PubMed: 15219899]
4. Li J, Zhao X, Hao HW, Shaw MK and Tse HY (2011) T cells that trigger acute experimental autoimmune encephalomyelitis also mediate subsequent disease relapses and predominantly produce IL-17. *J Neuroimmunol.* 230, 26–32 [PubMed: 20826011]
5. Sommer C (2003) Painful neuropathies. *Curr Opin Neurol.* 16, 623–628 [PubMed: 14501847]
6. Chattopadhyay S, Myers RR, Janes J and Shubayev V (2007) Cytokine regulation of MMP-9 in peripheral glia: implications for pathological processes and pain in injured nerve. *Brain Behav Immun.* 21, 561–568 [PubMed: 17189680]

7. Hong S, Remacle AG, Shiryaev SA, Choi W, Hullugundi SK, Dolkas J, Angert M, Nishihara T, Yaksh TL, Strongin AY and Shubayev VI (2017) Reciprocal relationship between membrane type 1 matrix metalloproteinase and the algescic peptides of myelin basic protein contributes to chronic neuropathic pain. *Brain Behav Immun.* 60, 282–292 [PubMed: 27833045]
8. Kim Y, Remacle AG, Chernov AV, Liu H, Shubayev I, Lai C, Dolkas J, Shiryaev SA, Golubkov VS, Mizisin AP, Strongin AY and Shubayev VI (2012) The MMP-9/TIMP-1 axis controls the status of differentiation and function of myelin-forming Schwann cells in nerve regeneration. *PloS one.* 7, e33664 [PubMed: 22438979]
9. Kobayashi H, Chattopadhyay S, Kato K, Dolkas J, Kikuchi S, Myers RR and Shubayev VI (2008) MMPs initiate Schwann cell-mediated MBP degradation and mechanical nociception after nerve damage. *Mol Cell Neurosci.* 39, 619–627 [PubMed: 18817874]
10. Liu H, Shiryaev SA, Chernov AV, Kim Y, Shubayev I, Remacle AG, Baranovskaya S, Golubkov VS, Strongin AY and Shubayev VI (2012) Immunodominant fragments of myelin basic protein initiate T cell-dependent pain. *J Neuroinflammation.* 9, 119 [PubMed: 22676642]
11. Boggs JM (2006) Myelin basic protein: a multifunctional protein. *Cell Mol Life Sci.* 63, 1945–1961 [PubMed: 16794783]
12. Garbay B, Heape AM, Sargueil F and Cassagne C (2000) Myelin synthesis in the peripheral nervous system. *Prog Neurobiol.* 61, 267–304 [PubMed: 10727776]
13. Harauz G and Boggs JM (2013) Myelin management by the 18.5-kDa and 21.5-kDa classic myelin basic protein isoforms. *J Neurochem.* 125, 334–361 [PubMed: 23398367]
14. Harauz G, Ladizhansky V and Boggs JM (2009) Structural polymorphism and multifunctionality of myelin basic protein. *Biochemistry.* 48, 8094–8104 [PubMed: 19642704]
15. Paez PM, Spreuer V, Handley V, Feng JM, Campagnoni C and Campagnoni AT (2007) Increased expression of golli myelin basic proteins enhances calcium influx into oligodendroglial cells. *J Neurosci.* 27, 12690–12699 [PubMed: 18003849]
16. Zhang C, Walker AK, Zand R, Moscarello MA, Yan JM and Andrews PC (2012) Myelin basic protein undergoes a broader range of modifications in mammals than in lower vertebrates. *J Proteome Res.* 11, 4791–4802 [PubMed: 22420465]
17. Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL and Hafler DA (1990) T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature.* 346, 183–187 [PubMed: 1694970]
18. Pette M, Fujita K, Kitze B, Whitaker JN, Albert E, Kappos L and Wekerle H (1990) Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. *Neurology.* 40, 1770–1776 [PubMed: 1700336]
19. Ziganshin RH, Ivanova OM, Lomakin YA, Belogurov AA Jr., Kovalchuk SI, Azarkin IV, Arapidi GP, Anikanov NA, Shender VO, Piradov MA, Suponeva NA, Vorobyeva AA, Gabibov AG, Ivanov VT and Govorun VM (2016) The Pathogenesis of the Demyelinating Form of Guillain-Barre Syndrome (GBS): Proteo-peptidomic and Immunological Profiling of Physiological Fluids. *Mol Cell Proteomics.* 15, 2366–2378 [PubMed: 27143409]
20. Jansson L, Olsson T, Hojeberg B and Holmdahl R (1991) Chronic experimental autoimmune encephalomyelitis induced by the 89-101 myelin basic protein peptide in B10RIII (H-2r) mice. *Eur J Immunol.* 21, 693–699 [PubMed: 1707002]
21. Chernov AV, Dolkas J, Hoang K, Angert M, Srikrishna G, Vogl T, Baranovskaya S, Strongin AY and Shubayev VI (2015) The calcium-binding proteins S100A8 and S100A9 initiate the early inflammatory program in injured peripheral nerves. *J Biol Chem.* 290, 11771–11784 [PubMed: 25792748]
22. Ko JS, Eddinger KA, Angert M, Chernov AV, Dolkas J, Strongin AY, Yaksh TL and Shubayev VI (2016) Spinal activity of interleukin 6 mediates myelin basic protein-induced allodynia. *Brain Behav Immun.* 56, 378–389 [PubMed: 26970355]
23. Makarova A, Mikhailenko I, Bugge TH, List K, Lawrence DA and Strickland DK (2003) The low density lipoprotein receptor-related protein modulates protease activity in the brain by mediating the cellular internalization of both neuroserpin and neuroserpin-tissue-type plasminogen activator complexes. *J Biol Chem.* 278, 50250–50258 [PubMed: 14522960]

24. Migliorini MM, Behre EH, Brew S, Ingham KC and Strickland DK (2003) Allosteric modulation of ligand binding to low density lipoprotein receptor-related protein by the receptor-associated protein requires critical lysine residues within its carboxyl-terminal domain. *J Biol Chem.* 278, 17986–17992 [PubMed: 12637503]
25. Patel M, Morrow J, Maxfield FR, Strickland DK, Greenberg S and Tabas I (2003) The cytoplasmic domain of the low density lipoprotein (LDL) receptor-related protein, but not that of the LDL receptor, triggers phagocytosis. *J Biol Chem.* 278, 44799–44807 [PubMed: 12941948]
26. Strickland DK and Ranganathan S (2003) Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. *J Thromb Haemost.* 1, 1663–1670 [PubMed: 12871303]
27. Medved LV, Migliorini M, Mikhailenko I, Barrientos LG, Llinas M and Strickland DK (1999) Domain organization of the 39-kDa receptor-associated protein. *J Biol Chem.* 274, 717–727 [PubMed: 9873007]
28. Lillis AP, Van Duyn LB, Murphy-Ullrich JE and Strickland DK (2008) LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiol Rev.* 88, 887–918 [PubMed: 18626063]
29. Fuentealba RA, Liu Q, Zhang J, Kanekiyo T, Hu X, Lee JM, LaDu MJ and Bu G (2010) Low-density lipoprotein receptor-related protein 1 (LRP1) mediates neuronal Abeta42 uptake and lysosomal trafficking. *PLoS One.* 5, e11884 [PubMed: 20686698]
30. Emonard H, Theret L, Bennisroune AH and Dedieu S (2014) Regulation of LRP-1 expression: make the point. *Pathol Biol (Paris).* 62, 84–90 [PubMed: 24661974]
31. Gaultier A, Wu X, Le Moan N, Takimoto S, Mukandala G, Akassoglou K, Campana WM and Gonias SL (2009) Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. *J Cell Sci.* 122, 1155–1162 [PubMed: 19299462]
32. Gonias SL and Campana WM (2014) LDL receptor-related protein-1: a regulator of inflammation in atherosclerosis, cancer, and injury to the nervous system. *Am J Pathol.* 184, 18–27 [PubMed: 24128688]
33. Yamamoto K, Murphy G and Troeberg L (2015) Extracellular regulation of metalloproteinases. *Matrix Biol.* 44–46, 255–263 [PubMed: 25701651]
34. Campana WM, Li X, Dragojlovic N, Janes J, Gaultier A and Gonias SL (2006) The low-density lipoprotein receptor-related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury. *J Neurosci.* 26, 11197–11207 [PubMed: 17065459]
35. Fernandez-Castaneda A, Arandjelovic S, Stiles TL, Schlobach RK, Mowen KA, Gonias SL and Gaultier A (2013) Identification of the low density lipoprotein (LDL) receptor-related protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris. *J Biol Chem.* 288, 4538–4548 [PubMed: 23264627]
36. Gaultier A, Arandjelovic S, Li X, Janes J, Dragojlovic N, Zhou GP, Dolkas J, Myers RR, Gonias SL and Campana WM (2008) A shed form of LDL receptor-related protein-1 regulates peripheral nerve injury and neuropathic pain in rodents. *J Clin Invest.* 118, 161–172 [PubMed: 18060043]
37. Mannella CA and Bonner WD Jr. (1975) X-ray diffraction from oriented outer mitochondrial membranes. Detection of in-plane subunit structure. *Biochim Biophys Acta.* 413, 226–233 [PubMed: 1191691]
38. Menzel VA, Cassara MC, Benz R, de Pinto V, Messina A, Cunsolo V, Saletti R, Hinsch KD and Hinsch E (2009) Molecular and functional characterization of VDAC2 purified from mammal spermatozoa. *Biosci Rep.* 29, 351–362 [PubMed: 18976238]
39. Ujwal R, Cascio D, Colletier JP, Faham S, Zhang J, Toro L, Ping P and Abramson J (2008) The crystal structure of mouse VDAC1 at 2.3 Å resolution reveals mechanistic insights into metabolite gating. *Proc Natl Acad Sci U S A.* 105, 17742–17747 [PubMed: 18988731]
40. Shoshan-Barmatz V and Ben-Hail D (2012) VDAC, a multi-functional mitochondrial protein as a pharmacological target. *Mitochondrion.* 12, 24–34 [PubMed: 21530686]
41. Geula S, Ben-Hail D and Shoshan-Barmatz V (2012) Structure-based analysis of VDAC1: N-terminus location, translocation, channel gating and association with anti-apoptotic proteins. *Biochem J.* 444, 475–485 [PubMed: 22397371]
42. Colombini M (2012) VDAC structure, selectivity, and dynamics. *Biochim Biophys Acta.* 1818, 1457–1465 [PubMed: 22240010]

43. Hodge T and Colombini M (1997) Regulation of metabolite flux through voltage-gating of VDAC channels. *J Membr Biol.* 157, 271–279 [PubMed: 9178614]
44. De Pinto V, Messina A, Lane DJ and Lawen A (2010) Voltage-dependent anion-selective channel (VDAC) in the plasma membrane. *FEBS Lett.* 584, 1793–1799 [PubMed: 20184885]
45. Shoshan-Barmatz V, De Pinto V, Zweckstetter M, Raviv Z, Keinan N and Arbel N (2010) VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Mol Aspects Med.* 31, 227–285 [PubMed: 20346371]
46. Gincel D, Silberberg SD and Shoshan-Barmatz V (2000) Modulation of the voltage-dependent anion channel (VDAC) by glutamate. *J Bioenerg Biomembr.* 32, 571–583 [PubMed: 15254371]
47. Shoshan-Barmatz V, Krelin Y, Shteinfer-Kuzmine A and Arif T (2017) Voltage-Dependent Anion Channel 1 As an Emerging Drug Target for Novel Anti-Cancer Therapeutics. *Front Oncol.* 7, 154 [PubMed: 28824871]
48. Green DR and Reed JC (1998) Mitochondria and apoptosis. *Science.* 281, 1309–1312 [PubMed: 9721092]
49. Ma S, Hockings C, Anwari K, Kratina T, Fennell S, Lazarou M, Ryan MT, Kluck RM and Dewson G (2013) Assembly of the Bak apoptotic pore: a critical role for the Bak protein alpha6 helix in the multimerization of homodimers during apoptosis. *J Biol Chem.* 288, 26027–26038 [PubMed: 23893415]
50. Shoshan-Barmatz V, Ben-Hail D, Admoni L, Krelin Y and Tripathi SS (2015) The mitochondrial voltage-dependent anion channel 1 in tumor cells. *Biochim Biophys Acta.* 1848, 2547–2575 [PubMed: 25448878]
51. Abu-Hamad S, Arbel N, Calo D, Arzoin L, Israelson A, Keinan N, Ben-Romano R, Friedman O and Shoshan-Barmatz V (2009) The VDAC1 N-terminus is essential both for apoptosis and the protective effect of anti-apoptotic proteins. *J Cell Sci.* 122, 1906–1916 [PubMed: 19461077]
52. Israelson A, Arbel N, Da Cruz S, Ilieva H, Yamanaka K, Shoshan-Barmatz V and Cleveland DW (2010) Misfolded mutant SOD1 directly inhibits VDAC1 conductance in a mouse model of inherited ALS. *Neuron.* 67, 575–587 [PubMed: 20797535]
53. Magri A and Messina A (2017) Interactions of VDAC with proteins involved in neurodegenerative aggregation: an opportunity for advancement on therapeutic molecules. *Curr Med Chem*
54. Rostovtseva TK, Gurnev PA, Protchenko O, Hoogerheide DP, Yap TL, Philpott CC, Lee JC and Bezrukov SM (2015) alpha-Synuclein Shows High Affinity Interaction with Voltage-dependent Anion Channel, Suggesting Mechanisms of Mitochondrial Regulation and Toxicity in Parkinson Disease. *J Biol Chem.* 290, 18467–18477 [PubMed: 26055708]
55. Vina J, Lloret A, Valles SL, Borrás C, Badia MC, Pallardo FV, Sastre J and Alonso MD (2007) Effect of gender on mitochondrial toxicity of Alzheimer's Abeta peptide. *Antioxid Redox Signal.* 9, 1677–1690 [PubMed: 17822363]
56. Smilansky A, Dangoor L, Nakdimon I, Ben-Hail D, Mizrahi D and Shoshan-Barmatz V (2015) The Voltage-dependent Anion Channel 1 Mediates Amyloid beta Toxicity and Represents a Potential Target for Alzheimer Disease Therapy. *J Biol Chem.* 290, 30670–30683 [PubMed: 26542804]
57. Pedersen PL (2008) Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the "Warburg effect" in cancer. *J Bioenerg Biomembr.* 40, 123–126 [PubMed: 18780167]
58. Warburg O, Wind F and Negelein E (1927) The Metabolism of Tumors in the Body. *J Gen Physiol.* 8, 519–530 [PubMed: 19872213]
59. Mathupala SP, Ko YH and Pedersen PL (2006) Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene.* 25, 4777–4786 [PubMed: 16892090]
60. Pedersen PL, Mathupala S, Rempel A, Geschwind JF and Ko YH (2002) Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochim Biophys Acta.* 1555, 14–20 [PubMed: 12206885]
61. Vander Heiden MG, Cantley LC and Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science.* 324, 1029–1033 [PubMed: 19460998]

62. Ward PS and Thompson CB (2012) Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell*. 21, 297–308 [PubMed: 22439925]
63. Locasale JW and Cantley LC (2011) Metabolic flux and the regulation of mammalian cell growth. *Cell Metab*. 14, 443–451 [PubMed: 21982705]
64. Dai Z, Shestov AA, Lai L and Locasale JW (2016) A Flux Balance of Glucose Metabolism Clarifies the Requirements of the Warburg Effect. *Biophys J*. 111, 1088–1100 [PubMed: 27602736]
65. Shestov AA, Liu X, Ser Z, Cluntun AA, Hung YP, Huang L, Kim D, Le A, Yellen G, Albeck JG and Locasale JW (2014) Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. *Elife*. 3
66. Stampfer MR and Bartley JC (1985) Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. *Proc Natl Acad Sci U S A*. 82, 2394–2398 [PubMed: 3857588]
67. Remacle A, Murphy G and Roghi C (2003) Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. *J Cell Sci*. 116, 3905–3916 [PubMed: 12915589]
68. Goldin N, Arzoino L, Heyfets A, Israelson A, Zaslavsky Z, Bravman T, Bronner V, Notcovich A, Shoshan-Barmatz V and Flescher E (2008) Methyl jasmonate binds to and detaches mitochondria-bound hexokinase. *Oncogene*. 27, 4636–4643 [PubMed: 18408762]
69. Bayrhuber M, Meins T, Habeck M, Becker S, Giller K, Villinger S, Vornrhein C, Griesinger C, Zweckstetter M and Zeth K (2008) Structure of the human voltage-dependent anion channel. *Proc Natl Acad Sci U S A*. 105, 15370–15375 [PubMed: 18832158]
70. Ahmed MA, De Avila M, Polverini E, Bessonov K, Bamm VV and Haraux G (2012) Solution nuclear magnetic resonance structure and molecular dynamics simulations of a murine 18.5 kDa myelin basic protein segment (S72-S107) in association with dodecylphosphocholine micelles. *Biochemistry*. 51, 7475–7487 [PubMed: 22947219]
71. Pierce BG, Wiehe K, Hwang H, Kim BH, Vreven T and Weng Z (2014) ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers. *Bioinformatics*. 30, 1771–1773 [PubMed: 24532726]
72. Maier JA, Martinez C, Kasavajhala K, Wickstrom L, Hauser KE and Simmerling C (2015) ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J Chem Theory Comput*. 11, 3696–3713 [PubMed: 26574453]
73. Rozanov DV, Hahn-Dantona E, Strickland DK and Strongin AY (2004) The low density lipoprotein receptor-related protein LRP is regulated by membrane type-1 matrix metalloproteinase (MT1-MMP) proteolysis in malignant cells. *J Biol Chem*. 279, 4260–4268 [PubMed: 14645246]
74. Lu M and Park JS (2015) protonation behavior of histidine during HSF1 activation by physiological acidification. *J Cell Biochem*. 116, 977–984 [PubMed: 25560907]
75. Galiano MR, Lopez Sambrooks CH and Lak ME (2008) Insights into the Interaction of Myelin Basic Protein With Microtubules. Nova, New York
76. Modesti NM and Barra HS (1986) The interaction of myelin basic protein with tubulin and the inhibition of tubulin carboxypeptidase activity. *Biochem Biophys Res Commun*. 136, 482–489 [PubMed: 2423083]
77. Taketomi M, Kinoshita N, Kimura K, Kitada M, Noda T, Asou H, Nakamura T and Ide C (2002) Nogo-A expression in mature oligodendrocytes of rat spinal cord in association with specific molecules. *Neurosci Lett*. 332, 37–40 [PubMed: 12377379]
78. Prasad M, Pawlak KJ, Burak WE, Perry EE, Marshall B, Whittall RM and Bose HS (2017) Mitochondrial metabolic regulation by GRP78. *Sci Adv*. 3, e1602038 [PubMed: 28275724]
79. Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM and Sackett DL (2008) Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. *Proc Natl Acad Sci U S A*. 105, 18746–18751 [PubMed: 19033201]
80. Robey RB and Hay N (2006) Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. *Oncogene*. 25, 4683–4696 [PubMed: 16892082]

81. Zhang D, Yip YM and Li L (2016) In silico construction of HK2-VDAC1 complex and investigating the HK2 binding-induced molecular gating mechanism of VDAC1. *Mitochondrion*. 30, 222–228 [PubMed: 27544294]
82. Chacko AD, Liberante F, Paul I, Longley DB and Fennell DA (2010) Voltage dependent anion channel-1 regulates death receptor mediated apoptosis by enabling cleavage of caspase-8. *BMC Cancer*. 10, 380 [PubMed: 20646307]
83. Schindler A and Foley E (2013) Hexokinase 1 blocks apoptotic signals at the mitochondria. *Cell Signal*. 25, 2685–2692 [PubMed: 24018046]
84. Shoshan-Barmatz V, Keinan N and Zaid H (2008) Uncovering the role of VDAC in the regulation of cell life and death. *J Bioenerg Biomembr*. 40, 183–191 [PubMed: 18651212]
85. Machida K, Ohta Y and Osada H (2006) Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells. *J Biol Chem*. 281, 14314–14320 [PubMed: 16551620]
86. Fingrut O and Flescher E (2002) Plant stress hormones suppress the proliferation and induce apoptosis in human cancer cells. *Leukemia*. 16, 608–616 [PubMed: 11960340]
87. Rotem R, Heyfets A, Fingrut O, Blickstein D, Shaklai M and Flescher E (2005) Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Res*. 65, 1984–1993 [PubMed: 15753398]
88. Azoulay-Zohar H, Israelson A, Abu-Hamad S and Shoshan-Barmatz V (2004) In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem J*. 377, 347–355 [PubMed: 14561215]
89. Zaid H, Abu-Hamad S, Israelson A, Nathan I and Shoshan-Barmatz V (2005) The voltage-dependent anion channel-1 modulates apoptotic cell death. *Cell Death Differ*. 12, 751–760 [PubMed: 15818409]
90. Zhang Y, Han D, Wei W, Cao W, Zhang R, Dong Q, Zhang J, Wang Y and Liu N (2015) MiR-218 Inhibited Growth and Metabolism of Human Glioblastoma Cells by Directly Targeting E2F2. *Cell Mol Neurobiol*. 35, 1165–1173 [PubMed: 26012781]
91. Shubayev VI, Strongin AY and Yaksh TL (2016) Role of myelin auto-antigens in pain: a female connection. *Neural Regen Res*. 11, 890–891 [PubMed: 27482203]
92. Nishihara T, Remacle AG, Angert M, Shubayev I, Shiryayev SA, Liu H, Dolkas J, Chernov AV, Strongin AY and Shubayev VI (2015) Matrix metalloproteinase-14 both sheds cell surface neuronal glial antigen 2 (NG2) proteoglycan on macrophages and governs the response to peripheral nerve injury. *J Biol Chem*. 290, 3693–3707 [PubMed: 25488667]
93. Hayashi T and Su TP (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell*. 131, 596–610 [PubMed: 17981125]
94. Lee AS (2014) Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. *Nat Rev Cancer*. 14, 263–276 [PubMed: 24658275]
95. Sun FC, Wei S, Li CW, Chang YS, Chao CC and Lai YK (2006) Localization of GRP78 to mitochondria under the unfolded protein response. *Biochem J*. 396, 31–39 [PubMed: 16433633]
96. Tsai YL, Zhang Y, Tseng CC, Stancianskas R, Pinaud F and Lee AS (2015) Characterization and mechanism of stress-induced translocation of 78-kilodalton glucose-regulated protein (GRP78) to the cell surface. *J Biol Chem*. 290, 8049–8064 [PubMed: 25673690]
97. Schlattner U, Dolder M, Wallimann T and Tokarska-Schlattner M (2001) Mitochondrial creatine kinase and mitochondrial outer membrane porin show a direct interaction that is modulated by calcium. *J Biol Chem*. 276, 48027–48030 [PubMed: 11602586]
98. Vyssokikh M and Brdiczka D (2004) VDAC and peripheral channelling complexes in health and disease. *Mol Cell Biochem*. 256–257, 117–126 [PubMed: 14977175]
99. Vyssokikh MY and Brdiczka D (2003) The function of complexes between the outer mitochondrial membrane pore (VDAC) and the adenine nucleotide translocase in regulation of energy metabolism and apoptosis. *Acta Biochim Pol*. 50, 389–404 [PubMed: 12833165]
100. Xu X, Forbes JG and Colombini M (2001) Actin modulates the gating of *Neurospora crassa* VDAC. *J Membr Biol*. 180, 73–81 [PubMed: 11284205]

101. Shoshan-Barmatz V and Israelson A (2005) The voltage-dependent anion channel in endoplasmic/sarcoplasmic reticulum: characterization, modulation and possible function. *J Membr Biol.* 204, 57–66 [PubMed: 16151701]
102. Pastorino JG, Shulga N and Hoek JB (2002) Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. *J Biol Chem.* 277, 7610–7618 [PubMed: 11751859]
103. Hiller S and Wagner G (2009) The role of solution NMR in the structure determinations of VDAC-1 and other membrane proteins. *Curr Opin Struct Biol.* 19, 396–401 [PubMed: 19665886]
104. Estrella V, Chen T, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, Bailey K, Balagurunathan Y, Rothberg JM, Sloane BF, Johnson J, Gatenby RA and Gillies RJ (2013) Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res.* 73, 1524–1535 [PubMed: 23288510]
105. Gatenby RA and Gawlinski ET (1996) A reaction-diffusion model of cancer invasion. *Cancer Res.* 56, 5745–5753 [PubMed: 8971186]
106. Shime H, Yabu M, Akazawa T, Kodama K, Matsumoto M, Seya T and Inoue N (2008) Tumor-secreted lactic acid promotes IL-23/IL-17 proinflammatory pathway. *J Immunol.* 180, 7175–7183 [PubMed: 18490716]
107. Yabu M, Shime H, Hara H, Saito T, Matsumoto M, Seya T, Akazawa T and Inoue N (2011) IL-23-dependent and -independent enhancement pathways of IL-17A production by lactic acid. *Int Immunol.* 23, 29–41 [PubMed: 21131367]
108. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, Gottfried E, Schwarz S, Rothe G, Hoves S, Renner K, Timischl B, Mackensen A, Kunz-Schughart L, Andreesen R, Krause SW and Kreutz M (2007) Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood.* 109, 3812–3819 [PubMed: 17255361]
109. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, Mackensen A and Kreutz M (2006) Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood.* 107, 2013–2021 [PubMed: 16278308]
110. Itoh Y, Esaki T, Shimoji K, Cook M, Law MJ, Kaufman E and Sokoloff L (2003) Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia in vitro and on glucose utilization by brain in vivo. *Proc Natl Acad Sci U S A.* 100, 4879–4884 [PubMed: 12668764]
111. Zhang J, Sun X, Zheng S, Liu X, Jin J, Ren Y and Luo J. (2014) Myelin basic protein induces neuron-specific toxicity by directly damaging the neuronal plasma membrane. *PLoS One.* 9, e108646 [PubMed: 25255088]

Figure 1. Schematics of cellular energy-generating pathways.

Glycolysis and energy metabolism pathways - Glycolysis is the major metabolic pathway in the cell cytoplasm. HK2 binds to the mitochondrial outer-membrane (MOM)-associated voltage-dependent anion-selective channel-1 (VDAC-1) to gain a preferential access to the mitochondrially-generated ATP. The first step of glycolysis is initiated by hexokinase 2 (HK2) that catalyzes the phosphorylation of one glucose molecule [imported from the extracellular space *via* glucose transporters (GT)] to glucose-6-phosphate (glucose-6P). Following 9 successive reactions, one glucose-6P molecule is converted into two pyruvate molecules (+2 ATP molecules). Depending on the oxygen (O₂) level, the pyruvate

metabolism pathway takes place either aerobically or anaerobically. (a) In aerobic conditions (+O₂), pyruvate is transported to the mitochondrial matrix *via* VDAC-1 and the mitochondria inner membrane (MIM)-associated pyruvate transporter (PT), where it is oxidized into acetyl CoA (+2 ATP/glucose) and then metabolized in the Krebs cycle (+2 ATP/glucose) followed by oxidative phosphorylation (OxPhos; ~32 ATP/glucose). (b) In anaerobic conditions (-O₂), pyruvate is reduced by L-lactate dehydrogenase (L-LDHa) into L-lactate (+2 ATP/glucose) that is secreted into the extracellular space *via* lactate transporter (LT). (c) In many cancer cells, the pyruvate anaerobic pathway occurs even in the presence of O₂ and fully functioning mitochondria – this is called the Warburg effect (aerobic glycolysis). In the latter, cancer cells show a high rate of glycolysis concomitant with an increased glucose uptake and lactate production. - *Other proteins depicted in the schematic* - ATP- α and ATP- β , ATP synthase α and β subunits form a complex that undergoes a sequence of conformational changes leading to the formation of ATP from ADP and inorganic phosphate (P_i); ANT, adenine nucleotide translocase serves as a transporter for ADP and ATP. GRP78, 78kDa glucose-regulated protein is a chaperone protein that targets mitochondrial compartment in the endoplasmic reticulum-stress response in apoptosis signaling pathway. *VDAC-1 “opened” and “closed” configurations* - In both VDAC-1●HK2 and VDAC-1●ANT complexes the VDAC-1 pore is in its ‘opened’ configuration allowing metabolites (e.g. pyruvate, ADP and ATP) to freely circulate between the cytoplasm and the mitochondrial matrix. Both methyl jasmonate (MJ) and free tubulin lead to VDAC-1 “closed” configuration and apoptosis. MJ binding to HK2 dissociates the latter from VDAC-1 that becomes free to interact with pro-apoptotic molecules, whereas free tubulin penetrates into the VDAC-1 channel pore to block channel conductance, leading to cell death.

Figure 2. Endocytosis of MBP and LRP-1 by HT1080 cells.

A, HT1080 cells naturally express cell-surface LRP-1. HT1080 cells were surface-biotinylated (BIOTIN) and then lysed. Biotin-labeled proteins were precipitated from the lysates and analyzed by Western blotting with the LRP-1 antibody. *B*, Full-length MBP co-localizes with LRP-1. HT1080 cells were incubated for 1 h at 4°C with full-length MBP, washed, and then left intact (– uptake) or transferred to 37°C (+ uptake) for 1 h. Cells were then fixed and stained with the MBP and LRP-1 antibodies. MBP (*green*), LRP-1 (*red*), DAPI (nuclei; *blue*). Scale bars, 10 μm. *C*, Internalization of the MBP84-104 peptide. *Top and middle panels*, HT1080 cells were incubated for 1 h at 4°C with Alexa Fluor 488-labeled WT peptide (488-WT; 25 μM) alone or jointly with the unlabeled WT peptide (WT; 100 μM), washed, and then transferred to 37°C for 1 h. Live cells were then directly

observed using a fluorescent microscope. *Bottom panel*, cells were fixed and stained with the LAMP-1 antibody. MBP (green), LAMP-1 (red), DAPI (nuclei; *blue*). Scale bars, 10 μ m.

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Figure 3. Pull-down assays of cellular LRP-1 and α -tubulin with full-length MBP and MBP84-104 peptides.

LRP-1 and α -tubulin both efficiently co-precipitates with intact MBP, but not with the cryptic MBP84-104 fragment. *A*, HT1080 cell lysates were incubated at pH 6.0 and pH 7.4 with biotin-labeled MBP (b-MBP) or the WT (b-WT) and SCR (b-SCR) peptides. Unlabeled MBP (MBP) served as a control. Biotin-labeled proteins were pulled-down (IP) and analyzed by Western blotting (WB) under non-reducing conditions with the LRP-1 antibody. *B*, HT1080 cell lysates were incubated at pH 6.0 with MBP, b-MBP, b-WT or b-SCR. Biotin-labeled H89G mutant (b-H89G) served as a control to assess the potential effect of His89 protonation on the binding efficiency. Biotin-labeled proteins were IP and analyzed by WB with the LRP-1 (*top panel*) and α -tubulin (*bottom panel*) antibodies under non-reducing and reducing conditions, respectively. *Beads*, whole cell extract was incubated with the Streptavidin-agarose beads alone. *CTR*, whole cell extract (5 μ g total protein) of human

colorectal adenocarcinoma HT-29 cells served as a control for α -tubulin. In *A-B*, the gels were overexposed to depict the insignificant pull-down of the peptides by LRP-1.

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Figure 4. Mass spectrometry analysis of the pulled-down proteins by the MBP84-104 peptide. **A**, Biotin-labeled wild-type (b-WT) and control scrambled (b-SCR) MBP84-104 peptides were individually conjugated on Streptavidin-agarose beads. The solubilized cultured DRG cell sample was precleared using the SCR peptide immobilized-beads, and then chromatographed using the WT peptide immobilized-beads. The captured materials from both WT- and SCR immobilized-beads were eluted, separated by SDS-gel electrophoresis, and silver stained. *Black rectangles*, four regions of the gel were cut and then analyzed by LC-MS/MS followed by the data Analysis with MaxQuant software version 1.5.5.1. MS/MS spectra were searched against the *Rattus norvegicus* Uniprot protein sequence database (version July 2017). The identified proteins with the combined highest score for peptide intensity, MS/MS count and sequence coverage are placed along the respective gel sections. GRP78, 78 kDa glucose-related protein; ATP- α and - β , ATP synthase subunit α and β ; VDAC-1, voltage-dependent anion-selective channel-1; L-LDH α , L-lactate dehydrogenase A chain. **B**, Representative sequence coverage of the 283-residue long rat VDAC-1 protein by the peptides identified by LC-MS/MS. *Yellow*, the 9 identified peptides that cover 98 residues (34.6%) of the VDAC-1 sequence.

Figure 5. The MBP84-104 peptide pull-downs mitochondrial VDAC-1.

A, Biotin-labeled wild-type (b-WT) and control scrambled (b-SCR) peptides were immobilized onto Streptavidin-agarose beads and then used to pull-down VDAC-1 from enriched mitochondrial fractions (Mito) obtained from human fibrosarcoma HT1080 (*left and middle panels*) and rat embryonic dorsal root ganglion F11 cells (*right panel*). The captured samples were eluted and then analyzed by Western blotting with the VDAC-1 antibody followed by the secondary HRP-conjugated antibody and either a TMB/M substrate (*left panel*) or a SuperSignal West Dura Extended Duration Substrate kit (*middle and right panels*). *Total* whole cell extract. *Beads*, Streptavidin-agarose beads alone. **B**, Enriched mitochondrial fraction aliquots of human fibrosarcoma HT1080 (*left panel*) and glioma U251 cells (*right panel*) were used to pull-down VDAC-1 using biotin-labeled wild-type (b-WT), mutant (b-H89G) and scrambled (b-SCR) MBP84-104 immobilized-beads. The captured materials were analyzed by Western blotting (WB) with the VDAC-1 antibody. *Initial*, the enriched mitochondrial fraction prior the pull-down assay.

Figure 6. Modeling of the VDAC-1-MBP84-104 peptide complex.

A, The predicted structure of the VDAC-1-MBP84-104 peptide complex. To initiate the modeling, we used the available NMR structure of the human VDAC-1 (PDB 2JK4) and the MBP84-104 peptide (configuration according to PDB 2LUG). Cartoon representation of the top score position for the binding of MBP84-104 (ice blue) to VDAC-1 N-terminal α -helix (red) located midway inside the 19 β -strands barrel (grey). The β 1 and β 19 blades are shown in lavender. N- and C-, the N- and C-terminus of the VDAC-1 protein. CYTO, cytoplasm; MOM, mitochondria outer-membrane; INTER, mitochondria intermembrane space. *Black rectangle*, two views of the VDAC-1-MBP complex from the CYTO (*left*) and INTER (*right*) spaces in which VDAC-1 barrel is shown as surface and both VDAC-1 α -helix and MBP84-104 are shown as cartoon. **B**, Clusters of interacting hydrophobic residues. Close-up of the three proposed clusters of interacting residues (yellow, green and brown) between L10, V17, F18 and F24 of VDAC-1 α -helix and P86, V87, F90, F91, I94 and V95 of MBP84-104. For more details, see Table 3.

Figure 7. VDAC-1 is not likely a cell-surface receptor of the MBP peptide.

A, Biotinylation of the cells. Plasma membrane-associated proteins of HT1080, F11, U251 and 184B5 cells were left intact (– BIOTIN) or biotinylated (+ BIOTIN). Cells were then lysed, and the biotin-labeled proteins were pulled-down (IP) with Streptavidin-agarose beads followed by WB analysis with the VDAC-1 antibody. *Arrow*, where VDAC-1 band should be detected. The figure shows an overexposed gel to depict the level of VDAC-1 below detection level. Asterisk, an unidentified contaminant that is present in F11 cells. **B**, Protein expression levels. HT1080, F11, U251 and 184B5 cells were lysed and whole cell extract aliquots (5 µg, each) were analyzed by Western blotting with the Hexokinase 2 (HK2; *top panel*), α -tubulin (α -TUB; *middle panel*) and VDAC-1 (*bottom panel*) antibodies.

Figure 8. Effect of the MBP84-104 peptides on the cellular energy metabolism.

A, The MBP peptides do not affect cell viability nor the total cellular ATP levels. HT1080, F11, U251 and 184B5 cells were incubated alone (CTR) for 16 h or jointly with wild-type (WT) or control scrambled (SCR) MBP84-104 (50 μ M, each) in DMEM containing 10 mM D-glucose and 1% dialyzed FBS. Methyl jasmonate (MJ; 3 mM), an inducer of apoptosis, serves as a control. Cell viability was assessed by monitoring the level of total cellular ATP using a luminescent ATP-Lite assay. Data are means $\pm$ SD from three individual experiments performed in triplicate. **, $p < 0.05$ values for the individual treated cells relative to the respective untreated cell controls (CTR). The figure depicts the changes in the total cellular ATP level in cells treated with MJ or the MBP84-104 peptides relative to the untreated cells. In the latter, the total level of cellular ATP was normalized to 100% for the individual cell

lines. *B*, The WT, but not SCR, peptide affects glucose uptake and lactate production ratio in cells. HT1080 cells were incubated alone (CTR) for 16 h or jointly with MJ (3 mM), or WT or SCR peptides (50 μ M, each) in DMEM containing 10 mM D-glucose and 1% dialyzed FBS. The levels of D-glucose and L-lactate in conditioned culture medium aliquots were then measured using an YSI 2950D Biochemistry Analyzer. *Left*, the glucose consumption (*black rectangles*) and lactate production (*grey rectangles*) in samples are calculated relative to the measurements in DMEM-1% dialyzed FBS alone. *Right*, the L-lactate/D-glucose (L/G) ratio. The numbers expressed in percent denote the reduction of the L/G ratio relative to the untreated cells. *C*, Effect of the MBP peptides on the L/G ratio in various proliferating cells. Cancer (HT1080, F11 and U251) and normal (184B5) cells were incubated alone (CTR) for 16 h or jointly with MJ (3 mM), WT or SCR peptides (50 μ M, each) in DMEM containing 10 mM D-glucose and 1% dialyzed FBS. Condition medium aliquots were then analyzed using an YSI 2950D Biochemistry Analyzer to measure both the D-glucose and L-lactate concentrations. Measurement using DMEM-1% dialyzed FBS alone was used to calculate the glucose uptake and lactate production, and determine the L/G ratios. *B-C*, each datum point represents the results of at least two independent experiments performed in two-three replicates. Statistical analysis was performed using one-way analysis of variance (ANOVA) with multiple comparisons followed by Tukey's post hoc test. **, $p < 0.05$ values were considered statistically significant.

Table 1.

The sequences and modifications of the MBP peptides used in our study. The H89G mutation is in bold and underlined.

Peptides	MBP84-104 sequences	N-terminal modification	C-terminal modification
Biotin-labeled WT	ENPVVHFFKNIVTPRTPPPSQ	Biotin	Amidated
Biotin-labeled H89G	ENPVV <u>G</u> FFKNIVTPRTPPPSQ	Biotin	Amidated
Biotin-labeled SCR	SHVPFNTSQPPVNVKPRIT	Biotin	Amidated
WT	ENPVVHFFKNIVTPRTPPPSQ	Acetylated	Amidated
SCR	SHVPFNTSQPPVNVKPRIT	Acetylated	Amidated
Alexa Fluor 488-WT	ENPVVHFFKNIVTPRTPPPSQ	Alexa Fluor 488	Amidated

Table 2.

Representative examples of the protein identification in the LC-MS/MS methodology. Pulled-down proteins identified by LC-MS/MS with the combined highest score for peptide intensity, MS/MS count and sequence coverage.

Proteins	Identified peptides	Coverage %
Voltage-dependent anion-selective channel-1 (VDAC-1) GI:13786200	² AVPPTYADLGK ¹² , ³⁵ SENGLEFTSSGSANTETTK ⁵³ , ⁶⁴ WTEYGLTFTEK ⁷⁴ , ⁹⁷ LTFDSSFPNTGK ¹⁰⁹ , ¹⁶² SRVTQSNFAVGK ¹⁷⁴ , ¹⁶⁴ VTQSNFAVGK ¹⁷⁴ , ²²⁵ YQVDPDACFSK ²³⁶ , ²⁵⁷ LTLALLDGK ²⁶⁶ , ²⁷⁵ GLGLEFQA ²⁸³	34.6
L-lactate dehydrogenase A chain (L-LDH) GI:8393706	⁴³ DLADELALVDVIEDK ⁵⁷ , ⁶⁰ GEMMDLQHGSFLK ⁷³ , ⁸² DYSVTANSK ⁹⁰ , ¹⁵⁸ VIGSGCNLDSAR ¹⁶⁹ , ²¹³ SLNPQLGTDADKEQWKDVHK ²³² , ²³³ QVVD SAYEVIK ²⁴³ , ³⁰⁶ VTLTPDEEAR ³¹⁵ , ³¹⁹ SADTLWGIQK ²³⁸	30.4
ATP synthase subunit β (ATP- β) GI:54792127	⁹⁵ LVLEVAQHLGESTVR ¹⁰⁹ , ¹¹⁰ TIAMDGTEGLVR ¹²¹ , ¹²⁵ VLD SGAPIKIPVGPETLGR ¹⁴³ , ¹⁴⁴ IMNVIGEPIDER ¹⁵⁵ , ¹⁴⁴ MNVIGEPIDERGPIK ¹⁵⁹ , ²¹³ TVLIMELINNAK ²²⁵ , ²²⁶ AHGGYSVFAGVGER ²³⁹ , ²⁴⁰ TREGNDLYHEMIESGVINLK ²⁵⁹ , ²⁴² EGNDLYHEMIESGVINLK ²⁵⁹ , ²⁶⁵ VALVYQGMNEPPGAR ²⁷⁹ , ²⁸² VALTGLTVAEYFR ²⁹⁴ , ³²⁵ IPSAVGYPQLATDMGTMQER ³⁴⁵ , ³⁵² GSITSVQAIYVPADDLTPAPATTFALHDATTVLSR ³⁸⁷ , ³⁸⁸ AIAELGIYPAVDPLDSTR ⁴⁰⁶ , ⁴⁰⁷ IMDPNIVGSEHYDVAR ⁴²² , ⁴⁶³ FLSQPFQVAEVFTGHMGK ⁴⁸⁰ , ⁴⁹⁰ GFQQILAGDYDHLPEQAFYMGVPIEEAVAK ⁵¹⁹	52.4
ATP synthase subunit α (ATP- α) GI:40538742	⁴⁶ TGTAEMSSILEER ⁵⁸ , ¹⁰⁴ GMSLNLEPDNVGVVFGNDK ¹²³ , ¹³⁴ TGAIVDVPVGDELLGR ¹⁴⁹ , ¹⁵⁰ VVDALGNAIDGK ¹⁶¹ , ²¹⁹ TSIAIDTIINQK ²³⁰ , ⁴⁷³ QQQYSPMAIEEQVAVIYAGVR ⁴⁹³ , ⁵⁰⁷ FESAFLSHVVSQHQSLGNIR ⁵²⁷	20.8
78kDa glucose-regulated protein (GRP78) GI:25742763	⁶¹ ITPSYVAFTPEGER ⁷⁴ , ⁸² NQLTSNPENTVFDK ⁹⁶ , ¹⁰² TWNDPSVQQDIK ¹¹³ , ¹³⁹ TFAPEEISAMVLTK ¹⁵² , ¹⁸⁶ DAGTIAGLNVMR ¹⁹⁷ , ³²⁷ FEELNMDLFR ³³⁶ , ³⁴⁵ VLEDSDLKK ³⁵³ , ⁴⁴⁸ SQIFSTASDNQPTVTIK ⁴⁶⁴ , ⁵⁶³ NELESYAYSLK ⁵⁷³ , ⁶²² ELEEIVQPIISK ⁶³³ , ⁶³⁴ LYGSGPPPTGEEDTSEKDEL ⁶⁵⁴	22.5

Table 3.

Clusters of hydrophobic interactions in the VDAC-1●MBP peptide complex. The three clusters of interacting residues between the 25-long residue α -helix of VDAC-1 and the MBP84-104 peptide. The interacting hydrophobic residues of the conserved ⁸⁷VVHFF⁹¹ α -helix of MBP84-104 are in bold and underlined.

VDAC-1 (PDB 2JK4)	MBP84-104 (according to PDB 2LUG)	Color code *
Leu-10	Pro-86 <u>Phe-90</u>	Yellow
Val-17	<u>Val-87</u>	Green
Phe-18 Phe-21	<u>Phe-91</u> Ile-94 Val-95	Brown

* the color code refers to the three clusters of interacting hydrophobic residues depicted in Figure 6B.