SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of novel functions of myosin phosphatase and smoothelin-like 1 protein

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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The Examination took place at 2.306 office at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen.
24th of June, 2015. 11:00 AM

Head of the **Defense Committee**:	László Csernoch, PhD, DSc
Reviewers:	Ágota Apáti, PhD András Mádi, PhD
Members of the Defense Committee:	Norbert Szentandrássy, MD, PhD Virág Vas, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen.
16th of June, 2017. 2:00 PM

INTRODUCTION

Protein phosphorylation is one of the major post-translational modifications. Myosin phosphatase (MP) is one of the most prominent PP1 holoenzymes consisting of a PP1 catalytic subunit and a MYPT protein family regulatory subunit. Myosin phosphatase targeting (MYPT) protein family members are the MYPT1, MYPT2, MYPT3, MBS85 and TIMAP. Mammalian MYPT family members are the products of five genes and they target protein phosphatase type 1δ catalytic subunit (PP1cδ) to its substrates and localize it within the cell as a regulatory subunit.

Structure of MYPT proteins

MYPT proteins represent high sequence similarity and share several conserved domains. MYPTs include the PP1c binding RVxF motif on their N-terminus and the conserved central region of PP1c is associated to MYPTs via this region. Binding of MYPTs to PP1cδ is supported by additional conserved regions such as residues 10-17 called MyPhoNE (myosin phosphatase N-terminal element), which is a consensus RxxQV/I/LK/RxY/W sequence. RVxF motif is followed by eight or less ankyrin repeat domain of which ankyrin repeats 1, 5, 6 and 7 attach to C-terminus of PP1cδ during interaction with PP1c. Ankyrin repeats serve the major platform for substrate coupling is followed by the central region containing conserved phosphorylation sites from which Thr^{696} and Thr^{853} (by human sequence) are the best characterized ones, found in MYPT1 and MYPT2. MBS85 comprises the Thr^{696} site but neither MYPT3 nor TIMAP contains any of the side chains. C-terminal halves of MYPT proteins are more diverse. Leucin zipper sequence takes part of protein-protein interactions and dimerization and it is found in MYPT1, MYPT2 and MBS85 but not in MYPT3 or TIMAP. MYPT1 contains additional two bipartite nuclear localization signals (NLS).

Basic role of myosin phosphatase in cytoskeletal processes

Regulation of myosin II by phosphorylation is essential in muscle contraction or non-muscle cell functions *e.g.* shape changes, cell division and cytokinesis, cell adhesion, cell migration or regulation of ion channels. After phosphorylation of the 20 kDa myosin light chain (MLC20), myosin II reversibly binds to actin filaments via cross-bridges initiating contractile or motile events. MLC20 phosphorylation depends on the balance between Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and myosin phosphatase. Phosphorylation of MLC20 at Ser^{19} mainly by MLCK triggers the contraction of the acto-myosin complex and its dephosphorylation by MP is followed by relaxation. An agonist-dependent signalling can also provoke muscle contraction at a constant Ca^{2+} level. During Ca^{2+} sensitization ROK phosphorylates MYPT1 on its inhibitory sites in response to external stimuli and decreases the myosin phosphatase activity that induces the contraction of cytoskeletal elements. Myosin phosphatase can also be activated by cAMP-PKA or

cGMP-PKG signaling pathway during Ca^{2+} desensitization. Activation of PKA/PKG induces the phosphorylation of MYPT1 on Ser^{695} thus prevents the subsequent phosphorylation of inhibitory Thr^{696} site and activates myosin phosphatase.

Novel role of myosin phosphatase in neurotransmitter release

In the nervous system phosphorylation level of neuronal proteins depends on the balance between the catalytic activities of protein kinases and phosphatases. Many of protein kinases were identified as key regulators of neuronal protein phosphorylation but less information is available regarding protein phosphatases responsible for the reversibility of the process. MYPT1 was detected in rat brain and primary cell cultures of neurons and the activity of MP holoenzyme was also proved. Synaptosomes derived from the cerebral cortex presented significant myosin phosphatase activity. The complex of MYPT1 and PP1cδ was colocalized and coprecipitated with synaptophysin, a presynaptic marker protein, which is phosphorylated by Ca^{2+}/calmodulin-dependent kinase II. The ROK and myosin phosphatase were found to act on both pre- and post-synaptic target proteins influencing neurotransmission. ROK- and MP-dependent phophorylation of syntaxin-1 Ser^{14} and synapsin-I Ser^{9} was observed suggesting the regulatory role of ROK and MP in neurotransmitter release.

Role of myosin phosphatase in cell proliferation

Nuclear retinoblastoma protein (pRb) is a tumor suppressor protein plays a central role in cell proliferation and cell death. Loss of pRb function is a recognized initiator in cancer development. Colocalization and association of MYPT1 with pRb was detected in THP-1 leukemic cells suggesting that MYPT1 targets catalytic subunit PP1c to pRb. The absence of MYPT1 increased the pRb phosphorylation and resulted in a decrease in the cell death of THP-1 cells concluding that myosin phosphatase mediates chemoresistance of leukemic cells during cell cycle through the dephosphorylation of pRb.

Cytoskeletal merlin protein is a scaffolding protein linking actin filaments to the cell membrane or membrane-associated glycoproteins and it inhibits cell proliferation. Merlin, which is a tumor suppressor, can be phosporylated at C-terminal Ser^{518} by PAK 1 and 2 which initiates the translocation of merlin from the cell membrane to the cytoplasm. Merlin can also be phosphorylated by PKA at Ser^{518} and at additional N-terminal phosphorylation sites. Myosin phosphatase can activate merlin by dephosphorylating Ser^{518}.

Regulation of myosin phosphatase by phosphorylation

Phosphorylation of MYPT1 on Ser/Thr residues by a variery of protein Ser/Thr kinases regulates myosin phosphatase causing enzyme activation or inhibition. Several phosphorylation sites were found in the sequence of MYPT1 and there are two major inhibitory sites, namely Thr^{696} and Thr^{853}. RhoA-associated protein kinase (ROK) was first found to catalyze phosphorylation on both of them. Phosphorylation of MYPT1 on Thr^{696} or on Thr^{853} attenuated the activity of myosin phosphatase and the phosphorylation of Thr^{853} by ROK cause the dissociation of MYPT1 from its substrate myosin. There are two serine residues, Ser^{695} and Ser^{852}, immediately adjacent to the two threonine inhibitory phosphorylation sites. Phosphorylation of Ser^{695} by PKA/PKG was found to hamper subsequent phosphorylation of Thr^{696} by ROK, releasing the inhibitory effect of myosin phosphatase. Many of other kinases are known to phosphorylate MYPT1 on Thr^{696}. Phosphorylation of MYPT1 on Ser^{445}, Ser^{472} and Ser^{910} has an inhibitory effect on cell adhesion. During mitosis, MYPT1 phosphorylation on Thr^{435} and/or Ser^{432} by cdc2 kinase enhances the affinity of the holoenzyme towards phosphorylated myosin increasing MP activity.

Regulation of myosin phosphatase by interacting proteins

Two small heat stable proteins, inhibitor-1 (I-1) and -2 (I-2) were first described as inhibitor proteins of protein phosphatase 1. Both proteins bind to free PP1c subunit hampering its activity while if PP1c is in complex with MYPT1, their inhibitory effect decreases or even disappears. CPI-17 inhibitor affects on MP holoenzyme as well as on isolated PP1c. Another member of CPI-17 family, kinase-enhanced protein phosphatase type 1 inhibitor (KEPI) also attenuates PP1c activity. Additional small regulatory protein is DARPP-32 that is primarily expressed in brain and shows sequence homology with I-1. 14-3-3β was found to contribute cytoskeletal reorganization with binding to MYPT1, which diminishes the association of MYPT1 to myosin II and cause dissociation of MP complex from its substrate increasing phosphorylation of MLC20. Telokin was found to activate MP without altering phosphorylation of Thr^{696} or Thr^{853} of MYPT1. Telokin does not have any kinase activity but its phosphorylation on Ser^{13} by PKG or PKA is required for MP activation. However, it is still undefined how telokin activates myosin phosphatase. Par-4 protein was also found to support activation of MP. In vascular smooth muscle cells Par-4 co-localizes with the actin filaments and directly binds to and interacts with MYPT1. A novel inhibitory protein of myosin phosphatase was recently identified in smooth muscle contraction namely the smoothelin-like 1 (SMTNL1) protein which is a member of smoothelin family of muscle proteins.

Structure and functions of smoothelins and SMTNL1

Smoothelin-like 1 protein (SMTNL1) was identified as a new member of smoothelin family of muscle proteins, which contains smoothelin A (SMTN-A) and smoothelin B (SMTN-B). SMTN-A, a 59 kDa short isoform is expressed in visceral smooth muscle and the 100 kDa long isoform, SMTN-B is expressed in vascular smooth muscle. SMTNL1 was also found to be expressed in vascular smooth muscle and in skeletal muscle as well. The three members of SMTN family shares sequence similarity mainly at their carboxyl-termini containing a single type-2 calponin homology (CH) domain. Although SMTNL1 contains the CH-domain, it was not able to bind filamentous actin. SMTNs have other novel actin-binding domains (double within SMTN-B, single within SMTN-A) and both isoforms contain additional tropomyosin-binding domain. SMTNL1 was also found to bind tropomyosin and this interaction promoted its localization to the thin filament through the CH-domain. SMTNL1 is also able to interact with calmodulin (CaM) by an IQ motif at the N-termini of the CH-domain (apo-CaM-binding site or CBD2) and by another CaM-binding region namely Ca^{2+}-CaM-binding domain (CBD1). SMTNL1 has a single serine phosphorylation site, Ser^{301}, which is responsible for PKA and PKG and plays a central role in SMTNL1 function. Additional family member is the functionally uncharacterized SMTNL2.

Initial studies focused on the interactions between SMTNL1 and the contractile apparatus although SMTNL1 is also expressed in steroid hormone sensitive tissues such as endometrium and myometrium. SMTNL1 was found to bind to progesterone receptor (PR) *in vivo* and *in vitro*. Smtnl1 knock out mice were characterized by a reproductive phenotype by decreased fertility with higher embryonal lethality, longer intervals between pregnancies and difficulties to get pregnant. Direct role in the regulation of PR was confirmed since RNA interference of SMTNL1 caused significant increase in PR expression and gene expression analysis suggested a co-regulator effect of SMTNL1 on PR transcriptional activity. In addition, SMTNL1 translocated from the cytosol to the nucleus upon phosphorylation at Ser^{301} and the translocation attenuated its binding to the B isoform of PR and inhibited transcriptional activity of PR-B.

AIMS

Cytoskeletal regulator and muscle contractility mediator myosin phosphatase impacts through the dephosphorylation of phosphorylated 20 kDa light chain of motor protein myosin II and governs contractility, cell motility and migration processes of muscle or non-muscle cells. Extensive regulatory role of MP is indicated by the additional substrates besides myosin II and supported by the diverse subcellular localization of the regulatory subunit of MP (MYPT1) in different cell types. Possible translocation of MYPT1 from the cytosol to the plasma membrane and to the nucleus upon phosphorylation was previously reported and active nuclear conduction of MP was proved *in vitro* suggesting a possible regulatory role of MP in different nuclear processes. Based on the above mentioned preliminary data our goal was:

- To gain more information about nuclear roles of myosin phosphatase by investigating exact subnuclear localization of MYPT1.
- To screen novel substrates of MP through the determination of the nuclear interactome of MYPT1.
- To determine the regulatory effect of myosin phosphatase towards its nuclear substrates.

On the other hand, smoothelin-like 1 protein (SMTNL1) was demonstrated as a regulator of MP activity during exercise adaptation and pregnancy but the molecular background of it is still undetermined. In the second part of the present study our aims were the followings:

- To investigate the possible direct interaction between SMTNL1 and MYPT1 by binding assays and to determine the regions of MYPT1 are responsible for the interaction.
- To describe the molecular mechanism regulated by SMTNL1 and the physiological relations connected to SMTNL1 in skeletal muscle adaptation during pregnancy.

MATERIALS AND METHODS

Surface plasmon resonance (SPR)

SPR-based binding studies were carried out using a Biacore 3000 instrument to monitor the interaction of MYPT1 with PRMT5 or SMTNL1. GST-MYPT1$^{1-1004}$ and GST-MYPT1$^{667-1004}$ were immobilized on CM5 sensor chips coupled with anti-GST. Recombinant GST as control surface, GST-MYPT1$^{1-1004}$ and GST-MYPT1$^{667-1004}$ were bound in running buffer. His-MYPT1$^{1-633}$ was directly immobilized via amine-groups of protein by amine coupling. A surface lacking any captured ligand and blocked by 1M ethanolamine was used as control surface. FT-His-PRMT5 was injected over the cell surfaces in a concentration range of 0.3125-6.7 μM or FT-SMTNL1 was applied from 0.5 to 7.14 μM. Unspecific binding was screened by the control surface. Resonance signal was expressed as response units (RU). Interaction of PRMT5 or SMTNL1 with MYPT1 fragments was characterized by kinetic parameters of sensorgrams and association constant (K_a) values determined by BIAevaluation 3.1 software.

Cell cultures

Human hepatocyte carcinoma (HepG2) and human embryonal kidney (tsA201) cells were grown using DMEM with high glucose content supplemented with 2 mM L-glutamine and 10 % heat-inactivated FBS. Human breast adenocarcinoma (MCF-7) cells were maintained in MEM completed with 2 mM L-glutamine, 1% NEAA and 10 % heat-inactivated FBS. All three human cell lines were subcultured in 37 °C incubator with humidified atmosphere of 5 % CO_2 between 60-90% confluency, and were passed after 2-3 days by trypsin-EDTA solution.

Transient transfections

The endogenous MYPT1 protein level was knocked down using mixture of double-stranded siRNAs and a non-target sequence with the G–C content of siMYPT1 was used as a control. Transfection mixture containing 50 nM siRNAs and Dharmafect 2 reagent was added to serum-free medium comprising HepG2 or MCF-7 cells. After 30 min, the medium was completed with 10% FBS and cells were incubated for 6 h and transfection medium was replaced to complete medium. After 42 h of incubation cells were processed.

TsA201 cells were transfected with pReceiver-M11 plasmids containing FT-PRMT5^{wt}, FT-PRMT5^{T80A} and FT-MYPT1 inserts or pcDNA 3.1 plasmid with FT-SMTNL1 insert using 1 mg/ml PEI transfection reagent in serum-free medium, based on the transient transfection protocol of adherent cells by PolyPlus-transfection. After 6 h incubation medium was replaced to complete DMEM and after an additional 24 h cells were lysed.

Cell fractionation method

HepG2 and MCF-7 cells were homogenized in 400 µl buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 % (v/v) Nonidet P-40, 0.5 mM PMSF, 1 x concentrated protease inhibitor cocktail) by suspending with a 26 gauge needle 10 times for HepG2, 5 times for MCF-7 cells using a 1 ml syringe then vortexed for 15 sec. The efficiency of lysis was judged by trypan blue staining monitoring intact nuclei. The lysate was centrifuged at 4°C at 16,000 x g for 1 min and the supernatant was used as cytosolic fraction. The pellet was resuspended in 200 µl buffer A and passed through a 26G needle 5 times as a washing step. After centrifugation 100 µl buffer B (20 mM Hepes, pH 7.9, 420 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 x concentrated protease inhibitor cocktail) was added to the pellet, sonicated and used as nuclear fraction.

Mouse colony maintenance and pregnancy studies

Congenic 129 SvEv *smtnl1-/-* mouse was created and pregnancy and pseudo-pregnancy studies were conducted by the Haystead group at Duke University, NC. For pregnancy studies, 8 week old mice were sacrificed at day 14-17 of the pregnancy. Animal studies were approved by the Duke University Institutional Animal Care & Use Committee. Procedures of human skeletal muscle biopsies were approved by the University and Medical Center Institutional Review Board at East Carolina University.

Processing of tissue samples

For Western blotting murine plantaris muscles were frozen in liquid N_2 and were homogenized on ice in glass dounce homogenizer with 40-50 strokes in a buffer containing 50 mM Tris-HCl, pH 6.8, 1% (m/v) SDS, 10% (v/v) glycerol, 20 mM DTT, 127 mM 2-mercaptoethanol supplemented with protease and phosphatase inhibitors. After 20 min incubation on ice samples were centrifuged at 13,000 x g for 5 min on 4°C and supernatants were heated for 5 min on 100°C with 5x sample buffer. Protein concentration of the samples was determined by the BCA method.

Immunofluorescence microscopy

HepG2 cells were grown on rat tail collagen-coated cover slips and were fixed by ethanol and permeabilized by 0.5% Triton-X-100 for 3 min at room temperature. Cells were washed three times for 5 min by TBS then blocked by 1% BSA for 60 min at 4°C. Primary and secondary antibodies were applied in 1:100 and 1:200 dilutions, respectively, at least for 60 min at 4°C. To-Pro-3 iodide (1:1000) or DAPI (1:2000) were added as nuclear markers. After washing, cover slips were mounted with ProLong Gold Antifade Reagent. Images were taken by Leica X8 confocal microscope and were processed using Leica X8 software program and PhotoShop Imaging software.

Pull-down assays

FT-MYPT1 and Flag-peptide as a control were bound to anti-Flag M2 affinity gel for LC-MS/MS analysis of FT-MYPT1 interacting proteins. The samples were precleared on the anti-Flag M2 affinity gel. After washing, beads were incubated with HepG2 nuclear extract for 2 hr on 4°C. The FT-MYPT1 with the anchored proteins was eluted from the beads by 300 μg/ml Flag-peptide and was subjected to SDS-PAGE. Bands of interest were visualized by MS compatible silver staining. FT-PRMT5^{wt}, -PRMT5^{T80A} and FT-MYPT1 was purified from transfected tsA201 lysates using anti-flag M2 affinity gel. FT-PRMT5^{wt} and -PRMT5^{T80A} proteins were bound to anti-flag M2 affinity gel during kinase and phosphatase assays and *in vitro* protein arginine methyltransferase assays. FT-MYPT1 was eluted from the beads with 300 μg/ml Flag peptide for execution of phosphatase assays and *in vitro* protein arginine methyltransferase assays.

Protein kinase and phosphatase assays for recombinant PRMT5

For Western blots, non-radioactive phosphorylation reaction by ROK (0.4 U/ml) was carried out in the absence or in the presence of 10 μM H1152 with 0.5 mM ATP, 5mM $MgCl_2$ at 30°C for 30 min using FT-PRMT5^{wt} or FT-PRMT5^{T80A} bound to anti-Flag M2 affinity gel. The kinase assay medium was removed and beads were washed by TBS. Dephosphorylation of ROK-phosphorylated FT-PRMT5^{wt} bound to the beads was initiated by adding 25 nM FT-MYPT1, 5 nM rPP1cδ or the combination of these two proteins at 30°C for 15 min, then the beads were washed by TBS and were incubated at 100°C for 5 min in 1 x SDS sample buffer followed SDS-PAGE. Phosphorylation level of Thr80 of PRMT5 was detected by phospho-PRMT5^{T80} specific antibody by Western blot and analysed by densitometry normalized to the PRMT5 input.

Identification of MYPT1 interacting proteins and PRMT5 phosphorylation sites from LC-MS/MS data

Bands of interest were subjected to in-gel digestion (for protocol see https://msf.ucsf.edu/protocols.html) using side-chain protected porcine trypsin (37 ˚C, 4h). For the identification of MYPT1 interacting proteins, the resulting peptide mixtures were analyzed directly by data-dependent „triple play" LC-MS/MS using a 3D-ion trap mass spectrometer (LCQ-Fleet, Thermo Fisher Scientific). For phosphorylation studies, approximately 80% of the peptide mixtures were subjected to phosphopeptide-enrichment using TiO_2 and the phosphopeptide fractions as well as the remaining 20% of the original samples were analyzed by data-dependent LC-MS/MS using an Orbitrap Elite mass spectrometer (MS spectra acquired in the Orbitrap, MS/MS spectra in the linear ion trap). Peak lists generated from the MS/MS data by the PAVA software (v2010/september) were

searched against the Swissprot database (downloaded 06/27/2013; 540546 protein sequences) using the ProteinProspector search engine.

***In vitro* protein arginine methyltransferase assays**

FT-PRMT5^{wt} was bound to anti-Flag M2 affinity gel following phosphorylation by ROK and was applied for the methylation assay. Methyltransferase assays were carried out in a buffer containing 50 mM Tris, 150 mM NaCl supplemented with protease inhibitor cocktail, pH 7.4 and 2 μM S-adenosyl-L-methionine (SAM) and 0.02 mg/ml of histone mixture were added to the reaction in a total volume of 50 μl. Methylation reaction was allowed to proceed for 2 hours at 30°C in the presence or in the absence of 5 nM rPP1cδ, 25 nM FT-MYPT1 or the combination of rPP1cδ and FT-MYPT1 (assumed as MP holoenzyme). Products were analysed by Western blots using antibodies specific for symmetrical dimethylated H2AR3 and H4R3. Membranes were stripped in every case and were assayed with anti-H2A and -H4 antibodies. Changes of symmetrical dimethylation levels were calculated by densitometry normalized to H2A and H4 internal controls.

Tissue array analysis

SomaPlex protein microarray slides containing human cancered liver and normal tissue samples from 25 clinical cases and 15 common cancer cell line lysates in triplicates were blocked by 3% bovine serum albumin dissolved in TBST assayed with anti-phospho-PRMT5^{T80}, -phospho-MYPT1^{T850} and -histone H2A antibodies at 4°C. Slides were incubated with HRP-labelled secondary antibodies for 2 hours and the antibody binding was developed by enhanced chemiluminescence (ECL) using x-ray films. Each slide was stripped and incubated by antibodies raised against PRMT5, MYPT1$^{1-296}$, H2AR3me2s and α-tubulin. Dots of interest were analysed by densitometry and were all normalized to their adequate tubulin internal control. Values of the phosphorylation of PRMT5 at Thr^{80}, -MYPT1 at Thr^{850} and H2AR3me2s were normalized to PRMT5, MYPT1 and H2A, respectively. Values of cancer tissues were related to normal tissue data and plotted as relative numbers.

Statistical analysis

Normalized data were analyzed using either unpaired parametric Student's t-test (for two groups) or analysis of variance (one-way ANOVA, for >2 groups) or by general linear models (GLM, for >2 groups). Post hoc testing for one-way ANOVA was determined by Tukey's test. When any covariate or factor was significant in GLMs, we applied Tukey's procedure to test for pair-wise differences in group means. Groups sharing the same letter do not show significant deviations. Tests were conducted using GraphPad for Windows. All data presented in this work represent mean ± SE or SEM, *n* means the number of independently performed experiments.

RESULTS

Subnuclear localization of MP and MYPT1 nuclear interactome in HepG2 cells

We exhibited subcellular localization of MYPT1 and different isoforms of PP1c subunits in HepG2 cells by Western analysis using antibodies specific for MYPT1, for PP1cδ or for both PP1cα and PP1cγ1. MYPT1, PP1cα/γ1 and PP1cδ were distributed in cytoplasmic and nuclear subcellular fractions. MYPT1 and PP1cδ showed nuclear colocalization by fluorescent confocal microscopy in HepG2 cells indicating the nuclear presence of MP. MYPT1 and PP1cδ isoform exhibited staining in the cytoplasm as well. MYPT1 also accumulated in spliceosomes and colocalized with histone H1b protein but did not localize to the nuclear membrane or nucleoli. These data suggest that MYPT1 is a putative PP1-targeting subunit during pre-mRNA splicing and that it may play a role in the regulation of chromatin structure.

Nuclear interactome of Flag (FT)-MYPT1 was established from nuclear extract of HepG2 cells by pull-down followed by mass spectrometry. Potential nuclear MYPT1-binding proteins mostly play roles in RNA processing and splicing and in gene expression. All three members of the methylosome complex were identified, namely the protein arginine methyltransferase 5 (PRMT5/JBP1), the PRMT5/JBP1-interacting protein (pICln), and the methylosome protein 50 (MEP50/WDR77). PRMT5 plays a unique and specific role in the generation of the ω-N^G, N^G-symmetric dimethylarginine (SDMA) as a type II PRMT. It is implicated in the regulation of transcription, RNA transport and cellular signaling.

PRTM5 interacts with MP

Nuclear and cytoplasmic colocalization of MYPT1 to the methylosome proteins MEP50 and PRMT5 was established by confocal microscopy in HepG2 cells. Both of the methylosome proteins exhibited a significant nuclear colocalization with MYPT1 quantified by the Pearson's correlation coefficient. Since PRMT5 is a key component in the methylosome complex giving the catalytic activity of the complex and it was defined by the most peptide sequence by mass spectrometry (8 for PRMT5, and 2-2- for MEP50 and pICln) from FT-MYPT1 nuclear interactome, we focused on PRMT5 in our further experiments. PRMT5 and MYPT1 were co-precipitated by each other during immunoprecipitation assays. To investigate the molecular background of MYPT1-PRMT5 interaction, binding of PRMT5 to N- and C-terminal regions of MYPT1 and to full length MYPT1 was investigated by SPR-based binding assays. PRMT5 interacted with full-length GST-$MYPT1^{1-1004}$ and N-terminal His-$MYPT1^{1-633}$ while no signal for binding was obtained with GST-$MYPT1^{667-1004}$. Binding of PRMT5 to the N-terminus of MYPT1 but not the C-terminal fragment suggest that PRMT5 forms a stable complex with MYPT1 through its N-terminal region.

PRMT5 is a substrate of ROK and MP

PRMT5 was phosphorylated by ROK but not by PKA or PKC in *in vitro* kinase assays when radioactive ATP (γ-^{32}P-ATP) was used. Thr^{80} residue was identified as a ROK phosphorylation site in PRMT5 by mass spectometry analysis of ROK-phosphorylated FT-PRMT5 samples compared to non-phosphorylated ones. ROK-specific phosphorylation of PRMT5^{T80} was confirmed by ROK-assay, in which the relative Thr^{80} phosphorylation level of wild type PRMT5 determined by anti- phospho-PRMT5^{T80} antibody was significantly reduced (nearly 50%) in response to H1152, a selective Rho-kinase inhibitor. To prove the regulatory role of MP on PRMT5, FT-PRMT5 was phosphorylated by ROK and *in vitro* phosphatase assays were carried out using recombinant PP1cδ and purified FT-MYPT1 proteins or their combination representing MP holoenzyme. FT-MYPT1 by itself had no effect on the phosphorylation level of PRMT5 at Thr80, whereas recombinant PP1cδ or the mixture of PP1cδ and FT-MYPT1 caused ~36% and ~63% decrease in phospho-PRMT5^{T80}, respectively, comparing to the ROK-phosphorylated samples. The increased dephosphorylation of PRMT5 at Thr^{80} by PP1cδ in the presence of FT-MYPT1 indicates that the phosphorylated PRMT5 is a substrate of MP holoenzyme and MYPT1 has a targeting role in this dephosphorylation process.

Methyltransferase activity of PRMT5 is regulated via phosphorylation and dephosphorylation of its Thr80 residue

To explore the effect of Thr^{80} phosphorylation of PRMT5 we assayed PRMT5 activity by determining symmetric dimethylation of H2A and H4 on their common arginine 3, the so called "R3 motif". Symmetrical dimethylation of H4R3 and H2AR3 was decreased by 46% and 64%, respectively, when the mixture of FT-MYPT1 and PP1cδ was applied during methyltransferase assay. MEP50 is essential for the activity of PRMT5. The relative amount of MEP50 binding to wt PRMT5 showed no significant differences either upon ROK phosphorylation or dephosphorylation by MP implying that phosphorylation of PRMT5 at Thr^{80} has no effect on MEP50 binding. Upon MYPT1-silencing of HepG2 cells, the phosphorylation of PRMT5 at Thr^{80} was increased by 46%. The dimethylation level of H2AR3 and H4R3 was elevated by 40% and 45%, respectively, whereas the expression of both histone proteins remained constant upon MYPT1 silencing. To clarify our data on PRMT5 regulation by MP, we applied the nuclear extract of non-target and MYPT1-silenced HepG2 on quantitative methyltransferase assay. We found that knocking down MYPT1 enhanced the specific activity of PRMT5 by ~65% comparing to the control samples and we also verified these data on human breast cancer cell line (MCF7 cells). These data indicate that Thr^{80} residue is a regulatory phosphorylation site of PRMT5 and the phosphorylation increases, while the dephosphorylation decreases its methytransferase activity.

Silencing of MYPT1 results in altered gene expression pattern in HepG2 cells

To define more precisely the role of MP in functions related to the regulation of dimethylation of histone proteins, we performed microarray analysis using the Affymetrix Human Gene 1.0 ST Array. The comparison of non-target control and siMYPT1 samples of HepG2 cells identified 2429 genes differentially regulated between the two groups. Bioinformatic analysis was assigned to different canonical pathways such as LPS/IL-1 mediated inhibition of RXR function (PPAR α, γ, IL1R, JUN), antioxidant action of vitamin C (STAT5, MAPK3 and 9), cell cycle regulation (Not7, PPP2C, CDK2, E2F3, Rb protein) as well as IL-4 and IL-8 signaling. 39.5% of all related genes (960) were linked to cancer disease such as lymphohematopoietic, liver and breast cancer and renal-cell carcinoma formation. Genes related to infectious diseases and developmental disorders were also identified in 10.9% and 6.99%, respectively. Microarray analysis identified a number of signaling pathways strongly point to a role of MP in the regulation of gene expression. The evaluation of genes related to MYPT1-silencing revealed the significant downregulation of several tumor suppressors and transcription factors. The protein expressions of tumor suppressor retinoblastoma protein and c-Myc were significantly decreased upon MYPT1 silencing.

MP modulates indirectly the suppression mark of gene expression on H2A and H4 through the regulation of PRMT5 activity in human cancer

The microarray analysis indicated that in case of decreased level of MP cancer-related processes dominate. Tumor tissue lysates were obtained from a large group of human patients with grade 2 and 3 and state II and III hepatocellular carcinoma (HCC, n=20), with four different types of metastatic liver cancers as well as with 15 other types of cancer cell lines with their adequate normal cell type controls. Protein expression of PRMT5 showed an increase in ~75% of the investigated cancer tissues comparing to the healthy controls. THP-1 monocytic leukemia, Raji Burkitt lymphoma, epidermoid and uterina carcinoma cells presented the largest increase. The relative phosphorylation of PRMT5 at Thr^{80} in cancer tissues were significantly elevated in all cases especially in the leukemia, lymphoma, lung, liver and breast carcinoma as well as in HCC tissues. The relative MYPT1 expression exhibited a diverse expression pattern since it was downregulated in few cases, in others it showed a twofold increase, however, it did not change in the majority of cancer types (60%). Increased phosphorylation of $MYPT1^{Thr850}$ was found in almost all cases. Our results imply that the decreased activity of MP bears an obvious relation to the increased activity of PRMT5 and the increased gene repression mark on histones in almost every investigated cancer types. These data suggest the involvement of the ROK/MP/PRMT5/histone dimethylation pathway in tumorigenesis.

Protein-protein interaction between MYPT1 and SMTNL1

Since MP plays a crucial role not only in the regulation of cytoskeletal elements but in other cellular processes such as gene expression, our aim was also to investigate its other regulatory possibilities. A novel inhibitory protein of MP is SMTNL1. We attempted SPR binding studies between the two proteins using recombinant MYPT1 fragments (unpublished data). Full-length GST-MYPT1$^{1\text{-}1004}$, His-MYPT1$^{1\text{-}633}$ and GST-MYPT1$^{667\text{-}1004}$ were immobilized on sensor chips and FT-SMTNL1 was applied in variant concentrations ranging from 0.5 to 7.14 μM. Distinct interaction of SMTNL1 was observed with full-length GST-MYPT1$^{1\text{-}1004}$ and N-terminal mutant (His-MYPT1$^{1\text{-}633}$) or with C-terminal fragment represented by residues 667-1004. Association reached saturation when GST-MYPT1$^{1\text{-}1004}$ or His-MYPT1$^{1\text{-}633}$ was bound to the sensor chip, but a lower response was detected with His-MYPT1$^{1\text{-}633}$ than with the full-length protein. Slighter but distinct binding of SMTNL1 to GST-MYPT1$^{667\text{-}1004}$ indicates that the C-terminus of MYPT1 amplifies the interaction and it happens through multiple surfaces.

Pregnancy and SMTNL1 regulate glycolytic fiber switching in mice and humans

To clarify the role of SMTNL1 as the regulator of MP in pregnancy, changes of SMTNL1 were examined in SKM during pregnancy by immunoblot analysis. SMTNL1 expression was remarkably enhanced in pregnant and pseudopregnant mice compared to plantaris muscle of non-pregnant females (t_{max} day 16±2). Phosphorylation of SMTNL1 on Ser301 was 3.5-fold increased in pregnant females (t_{max} day 12±2) in proportion to non-pregnant ones.

During fiber typing experiments of murine and human SKM samples MHC2b showed increased expression in pregnant *smtnl1-/-* mice and concomitant reduction of MHC2a expression was detected. IHC of plantaris showed that SMTNL1 expression localized only in type2a muscle fibers in non-pregnant WT females. Above all, SMTNL1 expression was not confined only to MHC2a fibers in plantaris of pregnant animals but SMTNL1 staining was detected in fibers different from type2a *e.g.*type 2x fibers that are in the transition from type 2a to 2b fibers. Pregnancy and pseudo-pregnancy induced a 15-20% increase in expression of type2b fibers. In spite of the increasing expression of MHC2b during pregnancy both in mouse and human SKM, SMTNL1 can also be found in fibers undergoing type2a/type2b transition. Pregnancy or pseudo-pregnancy did not affect the expression of MHCI, the marker of oxidative slow type 1 fibers. The decrease of protein expression of type2a marker MHC2a was only tendentious significant, while MHC2b increased by 20% indicating that fibers switched from oxidative to the more glycolytic phenotype in human pregnant SKM. The increased type2b content in pregnancy is accompanied by a 24% increase in glycogen content. These data suggest that pregnancy promotes the transformation of SKM fiber type to a more glycolytic phenotype and that SMTNL1 may play a regulatory role in this process.

SUMMARY

Myosin phosphatase (MP) holoenzyme is a protein phosphatase-1 type Ser/Thr specific enzyme consisting of a PP1cδ catalytic subunit, a myosin phosphatase target subunit-1 (MYPT1) and a 20 kDa subunit with unknown function. We identified the protein arginine methyltransferase 5 (PRMT5) enzyme of the methylosome complex as a novel interacting partner of MYPT1 in hepatocellular carcinoma cells. PRMT5 was found to be regulated by phosphorylation at Thr80 by RhoA-associated protein kinase and MP. Loss of MP function increased the level of the PRMT5-specific symmetric dimethylation on arginine residues of histone 2A/4, a repressing gene expression mark. It resulted in an overall change in the expression of genes affecting cellular processes like growth, proliferation and cell death, also affecting the expression of the retinoblastoma protein and *c*-Myc. Increased phosphorylation of PRMT5 at Thr80 and elevated symmetric dimethylation of H2A was described in human hepatocellular carcinoma and in other types of cancers in accordance with the increased phosphorylation of MYPT1 at inhibitory site Thr850. Our results suggest a novel mechanism of tumorigenesis governed by ROK and MP via regulation of PRMT5 thereby modulating gene expression through histone arginine dimethylation.

MP activity can be regulated by the smoothelin-like 1 protein (SMTNL1) that is a regulator of vascular smooth muscle contractility and modulates cardiovascular and skeletal muscle adaptations. Under physiological conditions cytosolic SMTNL1 controls MP activity towards phosphorylated MLC20 causing changes in muscle contractility. Based on SPR binding assays, we verified binding of SMTNL1 to the regulatory subunit MYPT1 of MP suggesting direct protein-protein interaction between them.

During pregnancy, the metabolic properties of skeletal muscle are adapted to physiological challenges and increased expression of SMTNL1 was observed in uterine and vascular smooth muscle and sex-hormone related tissues. We found that pregnancy promotes fiber type changes from an oxidative to a glycolytic isoform in skeletal muscle regulated by SMTNL1, which alters the expression of transcriptional and enzyme regulators and structural molecules. We suggest that these events are natural adaptations of normal pregnancy and potentially infer evolutionary advantages to the mother by increasing her ability to store fat and her physical strength to carry the developing foetus.

Registry number: DEENK/53/2017.PL
Subject: PhD Publikációs Lista

Candidate: Adrienn Sipos
Neptun ID: I0DSCU
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MTMT ID: 10037471

List of publications related to the dissertation

1. **Sipos, A.**, Iván, J., Bécsi, B., Darula, Z., Tamás, I., Horváth, D., Medzihradszky-Fölkl, K., Erdödi, F., Lontay, B.: Myosin phosphatase and RhoA-activated kinase modulate arginine methylation by the regulation of protein arginine methyltransferase 5 in hepatocellular carcinoma cells.
Sci. Rep. 7 (40590), 1-15, 2017.
DOI: http://dx.doi.org/10.1038/srep40590
IF: 5.228 (2015)

2. Lontay, B., Bodoor, K., **Sipos, A.**, Weitzel, D. H., Loiselle, D., Safi, R., Zheng, D., Devente, J., Hickner, R. C., McDonnell, D. P., Ribar, T., Haystead, T. A. J.: Pregnancy and Smoothelin-like Protein 1 (SMTNL1) Deletion Promote the Switching of Skeletal Muscle to a Glycolytic Phenotype in Human and Mice.
J. Biol. Chem. 290 (29), 17985-17998, 2015.
DOI: http://dx.doi.org/10.1074/jbc.M115.658120
IF: 4.258

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List of other publications

3. Dedinszki, D., **Sipos, A.**, Kiss, A., Bátori, R., Kónya, Z., Virág, L., Erdődi, F., Lontay, B.: Protein phosphatase-1 is involved in the maintenance of normal homeostasis and in UVA irradiation-induced pathological alterations in HaCaT cells and in mouse skin.
Biochim. Biophys. Acta-Mol. Basis Dis. 1852 (1), 22-33, 2015.
DOI: http://dx.doi.org/10.1016/j.bbadis.2014.11.005
IF: 5.158

Total IF of journals (all publications): 14,644
Total IF of journals (publications related to the dissertation): 9,486

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

10 March, 2017

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