THESIS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (Ph.D.)

Localization and regulation of myosin phosphatase in non-muscle cells

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1, INTRODUCTION

The reversible phosphorylation of proteins is a basic regulatory mechanism of cellular processes in eukaryotic organisms. Protein kinases catalyze the phosphorylation of proteins by transferring the terminal phosphate of nucleoside-triphosphates (in most cases ATP serves as a phosphate donor) to the serine (Ser), threonine (Thr) and tyrosine (Tyr) residues. Protein phosphatases hydrolyse the phosphoester bond in proteins. Protein phosphatase holoenzymes consist of catalytic subunit and regulatory subunit(s). Regulatory subunits influence the substrate-specificity and catalytic activity of the holoenzyme, and they target the enzymes to different subcellular compartments by binding to both the catalytic subunit and the substrate. Myosin phosphatase (MP) is a member of the Ser/Thr specific protein phosphatase 1 (PP1) enzyme family, and consists of a PP1 catalytic subunit (PP1c), a myosin phosphatase target subunit (MYPT) and a 20 kDa small subunit with a currently unknown function.

Myosin phosphatase was first described in smooth muscle and it functions in the regulation of contractility by the dephosphorylation of the 20 kDa light chain of myosin II (MLC20). The myosin II isoform can be found in smooth muscle and in non-muscle cells, as well. Phosphorylation of MLC20 is an important event in the regulation of many cellular processes such as contractility and motility of smooth muscle and non-muscle cells, cell adhesion, cytokinesis, cytoskeleton reorganization, platelet activation and ion channel functions. Phosphorylation of MLC20 induces the contraction of the actomyosin complex while dephosphorylation by myosin phosphatase results in relaxation. The reorganization of actin-cytoskeleton as well as its dynamics are regulated by reversible protein phosphorylation and one of the most important factor in this process is the phosphorylation of MLC20. The increase in the phosphorylation of MLC20 enhances stress fiber formation and development of focal adhesions, while its dephosphorylation causes disassembling of these structures.

The level of myosin phosphorylation, therefore, an important regulatory factor in a number of cellular processes and is determined by the activity ratio of the phosphorylating kinases and the dephosphorylating phosphatases. The activity of the kinases and the activity of myosin phosphatase can be regulated via several signal transduction pathways. For a long time, the study of smooth muscle contractility served as the first model to investigate these signaling events. According to the classic theory of smooth muscle contraction a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ by agonist and/or membrane depolarization results in the formation of the Ca^{2+}-calmodulin (CaM) complex, which increases the activity of CaM-dependent myosin light chain kinase (MLCK). These lead to an increase in the MLCK/MP activity ratio resulting in the elevation of the level of myosin phosphorylation and the development of smooth muscle contraction. The kinase/phosphatase activity ratio decreases by the decrease in $[Ca^{2+}]_i$ and the dephosphorylation of myosin becomes preferred resulting in the relaxation of muscle. It was presumed that the role of myosin phosphatase in this mechanism was only to assure the reversibility of the process.

The importance of the regulation of myosin phosphatase (MP) in muscle contractility became obvious by the use of membrane permeable phosphatase inhibitory toxins on intact or permeabilized smooth muscles. These toxins alone induced myosin phosphorylation and smooth muscle contraction at low $[Ca^{2+}]_i$ and MLCK activity. Besides the importance of inhibition of MP, these experiments also drew attention to the fact that Ca^{2+}-independent kinases may also be involved in the phosphorylation of myosin. Similar effects on the action of phosphatase inhibitors were observed, namely, the inhibition of phosphatase and phosphorylation of myosin at low $[Ca^{2+}]_{i,}$, accompanied with slow and maintained contraction in GTPγS-treated permeabilized smooth muscles. It was revealed by study of the molecular background of this process that GTPγS activates the GTP-binding RhoA protein and it increases the activity of RhoA

associated protein kinase (ROK). It was shown that ROK is able to phosphorylate both the myosin and the MYPT subunit of myosin phosphatase. The latter phosphorylation results in the inhibition of myosin phosphatase and also helps to increase the level of myosin phosphorylation by ROK. The phenomenon of myosin phosphorylation and smooth muscle contraction induced at constant (and relatively low) $[Ca^{2+}]_I$ was termed Ca^{2+}-senzitization. In contrast, the activation of myosin phosphatase causes Ca^{2+}-desenzitization. This effect is often related to the elevated intracellular concentration of cyclic nucleotides (cAMP, cGMP) and to that of the protein kinases (PKA, PKG) activated by these messengers, but the precise mechanism is not yet known.

In summary the regulation of myosin phosphatase is accomplished both by inhibition and activation. Inhibition could occur by either the phosphorylation of MYPT1 at certain residues, the dissociation of the myosin phosphatase holoenzyme (losing the targeting effect of MYPT), the changes in the localization of the holoenzymes or its inhibition by inhibitory proteins. The activation can be related to the increase in the concentration of cyclic nucleotides and it may involve phosphorylation of MYPT by PKG/PKA. The Thr695 residue, the inhibitory phosphorylation site, appears to be primarily important concerning the regulation of enzymic activity. This site is also phosphorylated by other kinases, beside ROK, including integrin-linked kinases (ILK), leucine zipper motif interacting protein kinase (ZIPK) and p21-activated protein kinase (PAK). ROK, ZIPK and ILK, besides regulating MP, can also phosphorylate MLC20 at Ser19 and Thr18, therefore, these enzymes may also be involved in inducing Ca^{2+}-independent smooth muscle contraction.

The above results indicate the importance of MP in cellular processes and due to this fact widespread studies have been carried out in the last decade to uncover the structure and regulation of myosin phosphatase.

2. AIMS

Myosin phosphorylation and the role of myosin phosphatase was studied extensively in smooth muscle cells, where its importance was demonstrated in the regulation of muscle contraction and in other myosin-related cytoskeletal changes. Myosin phosphatase is able to dephosphorylate other protein beside myosin, such as adducin, moesin, Tau, MAP-2, that bound to actin and other filaments. Consequently it could be involved in the regulation of cellular processes other then contractility. It is also apparent that the MYPT1 isoform is present in every eukaryotic cell studied so far, and its intracellular localization is not restricted to subcellular compartments containing myosin.

Our hypothesis was that uncovering the localization of MP in different non-muscle cells as well as the identification of novel interacting partners for MYPT1 could contribute to the understanding of further physiological functions and ways of regulation of this enzyme. This dissertation is based on experiments with cells (neuronal cells and hepatocytes) derived from brain and liver tissues. The composition of the brain is the most complex of any organ in the human body and its function is specialized. Active proliferation or cell migration that requires extensive cytoskeletal changes is not characteristic of this tissue, but cytoskeletal elements could be important in the nervous system in synaptic plasticity or in the transport of vesicles carrying neurotransmitters and in the release of neurotransmitter, as well. Liver is characterized by a more homogenous structure compared to that of the brain. The presence of myosin phosphatase, the MYPT1 regulatory subunit and myosin II was detected in brain and liver tissues. It was demonstrated in our preliminary experiments that the myosin phosphatase can associate with several subcellular compartments in both neuronal cells and in hepatocytes. It indicates that the enzyme could associate with substrate(s) important in cell functions other than contractility.

The MYPT subunit of the myosin phosphatase can be phosphorylated at a number of residues and several protein kinases can be involved in these

phosphorylation processes *in vitro*. In contrast, less is known about the kinases and phosphatases that act on the MYPT1 *in vivo*. *In vitro* studies suggested that protein phosphatase 2A (PP2A) is a candidate as MYPT1 phophatase, but the physiological role of this enzyme in the dephosphosphorylation of the phosphorylated Thr695 and Thr850 of MYPT1 has not been established.

Based on the questions raised above the aims of our studies were as follows.

1. Study of the localization of myosin phophatase and MYPT1 in rat brain and primary cultures of neuronal cells:

- Region specific and subcellular distribution of myosin phosphatase and MYPT1 as revealed by immunoblots, phosphatase avtivity assays and immunofluorescence,
- Identification of MYPT1 interacting proteins by immunoprecipitation and pull-down assays.

2. Investigation of the effect of okadaic acid on the phosphorylation of MYPT1 and myosin, and on the motility in HepG2 cells

-Distribution of myosin phosphatase and MYPT1 in HepG2 cells and in its subcellular fractions,

- Study of OA-induced myosin and MYPT1 phosphorylation, their localization and their relation to the changes in the activity of PP2A,
- Establish the effect of OA on stress fiber formation and the migration of HepG2 cells.

MATERIAL AND METHODS

3.1 Preparation of lysates from dissected regions of rat brain for Western blotting

The following brain regions were dissected: olfactory bulb; cortex; striatum; hippocampus; diencephalon; midbrain; hindbrain and cerebellum. Dissected tissues were washed in ice-cold phosphate buffered saline (PBS: 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 137.9 mM NaCl, pH 7.4) Ice-cold homogenization buffer (20 mM HEPES (pH 7.5), 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail (1:10 dilution)), 1 ml, was added to each dissected brain sample. The tissues were homogenized and incubated on ice for 10 minutes with 0.1 % Triton X-100 followed by sonication.

3.2 Preparation of primary hippocampus and cochlear nucleus cell culture

The hippocampus and cochlear nuclei were removed from 3 day-old Wistar rats and immersed in ice-cold dissection solution containing 10 mM HEPES, (pH 7.4), 120 mm NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 20 mM sucrose, 15 mM glucose, 12 U/ml penicillin and 10 μg/ml streptomycin. After dispersion of the cells, proteolysis was carried out in dissection solution buffer containing 0.25 % trypsin. Digested pieces of rat brain were triturated in minimum essential medium (MEM) with 10 % FCS and plated in MEM containing 10 % FCS, 0.1 μg/ml NGF, 12 U/ml penicillin and 10 μg/ml streptomycin.

3.3 Isolation of synaptosome and postsynaptic density fractions from rat cerebral cortex

Synaptosome and postsynaptic densities were isolated from rat cerebral cortices as described in the scientific literature with some modifications. A homogenate containing synaptosome and mitochondria was applied to sucrose density gradient (0,8 M, 1,0 M és 1,2 M sucrose) centrifugation (2 hours at 83,000*g*). The synaptosome fraction was used to isolate postsynaptic densities

by sucrose gradient (0,8 M, 1,5 és 2,0 M sucrose) centrifugation (2 hours, 202,300*g*).

3.4 HepG2 cell culture and treatment

Human hepatocarcinoma cells (HepG2) were used for our experiments. HepG2 cells were grown in a humidified 5% CO_2 atmosphere at 37 ^{o}C in D-MEM culture medium completed with 10 % FBS, 2 mM L-glutamine, Penicillin/Streptomycin (100 U/ml) and Antibiotic-Antimycotic solution (500 U/ml). Cells were then treated with 5-50 nM okadaic acid (OA) or 10 μM Y27632, or Y27632 plus OA for 60 minutes. After the incubation with these effectors cells were lysed in 100 μl RIPA buffer (1% Nonidet P-40, 1% sodium deoxycolate, 0.1% SDS, 0.15 M NaCl, 0,01 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride) and sonicated.

3.5 Preparation of HepG2 subcellular fractions

Nuclear, cytoplasmatic, microsomal/mitochondrial, cytoskeletal and plasma membrane fractions were isolated from HepG2 lysate. Cells were homogenized in four volumes of homogenization buffer (0.5 M sucrose, 20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA and protease inhibitors), then Nonidet P-40 was added to a final concentration of 0.5%. Lysates were microcentrifuged (8000*g*, 15 sec). The pellet was resuspended in buffer A (0.5 M sucrose, 10 mM HEPES (pH 7.9), 3.3 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and protease inhibitors) and centrifuged. This pellet was resuspended in buffer B (the same as buffer A except that it contains 0.35 M sucrose) and centrifugated. The pellet was resuspended in buffer B and sonicated, the sonicated suspension was used as the *nuclear fraction.* The 8000*g* supernatant was centrifuged (1250*g*, 20 min) and the component of the supernatant was ultracentrifuged: The pellet yielded the *microsomal fraction*, the supernatant was considered as the *cytosolic fraction.* The pellet of the 1250*g* centrifugation was resuspended in homogenization buffer containing 1% Nonidet P-40 and ulracentrifuged. The

resultant supernatant was the *plasmamembrane fraction,* the pellet was considered as the *cytoskeletal fraction.*

3.6 Western blot

The protein concentration was determined by the BCA method. Proteins were resolved by SDS-PAGE. The gels were blotted onto nitrocellulose membranes and for blocking 5% non-fat dried milk in PBS/0.05 % Tween 20 was used. After blocking, the membranes were incubated with primary antibodies followed by application of the HRP-conjugated secondary antibodies. Immunoreactions were detected by enhanced chemiluminescence (ECL). Semi-quantitative Western blot analysis was performed to determine the relative MYPT1 content of the dissected brain regions. The intensity of the blots was compared to standards of His-MYPT1$^{1-296}$. Densitometry of the blots was performed and the scans were analyzed by the Volume Analyse feature of the Molecular Analyst Software (BioRad).

3.7 Protein phosphatase assay

Phosphatase activity in the lysates of dissected rat brain regions, synaptosome, postsynaptic density fractions and HepG2 cells, and in the subcellular fractions of HepG2 cells, was determined with 2 μM ^{32}P-myosin or 2 μM ^{32}P-MLC20 as substrate. The phosphatase activity in 2-fold diluted HepG2 lysate was measured in the presence of OA (5-1000 nM) with 0.5 ^{32}P-myosin or 0.5 μM ^{32}P-MLC20 as substrate (0,5-1 min incubation time, 30 ^{o}C).

3.8 Immunohistochemistry

Wistar rats were perfused transcardially. The whole brain was removed and dissected into the following parts: olfactory bulb, forebrain, midbrain, cerebellum and lower brainstem, and horizontal or sagittal sections were cut. Sections were fixed with 4 % paraformaldehyde (PFA) or with ice-cold acetone. Tissue sections were blocked (10 % normal horse serum, 0.1 % BSA, 0.01 % NaN_3, 0.3 % Triton-X 100 /PBS) then incubated with anti-MYPT1$^{1-38}$ polyclonal antibody followed by incubation with biotin-conjugated anti-rabbit IgG, or

alternatively with AP-conjugated anti-rabbit IgG. Development was carried out with avidin-conjugated FITC or with alkaline phosphatase reaction.

3.9 Immunofluorescence and confocal laser scanning microscopy (CLSM)

Cells were fixed by ethanol or 4% PFA then washed by PBS. Cells were permeabilized in 0.05 % Triton X-100/PBS. After blocking in 2 mg/ml BSA/PBS, coverslips were incubated with primary antibodies diluted in 1 mg/ml BSA/PBS. It was followed by incubation with Alexa 488-conjugated anti-rabbit IgG, or Alexa 633-conjugated anti-mouse IgG as secondary antibodies, or for staining F-actin Alexa 633-conjugated phalloidin was used. Coverslips were covered with mounting medium using the Antifade Light Kit. For nuclear staining 1 mg/ml propidium iodide was applied. Coverslips were imaged on a Zeiss LSM 410 confocal scanning laser microscope equipped with Helium/Neon and Krypton/Argon laser detectors.

3.10 Immunoprecipitation

Affinity purified anti-MYPT$^{1\text{-}296}$ antibody was incubated with Protein-A Sepharose in immunoprecipitation buffer (IP buffer: 20 mM Tris-HCl (pH 7.0), 20 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0,01 % SDS plus protease inhibitors) and centrifuged. Aliquots from rat hippocampus extract were precleared by Protein A Sepharose then were incubated with anti-MYPT$^{1\text{-}296}$ antibody-coupled to Protein A Sepharose. Beads were washed with increasing salt concentration in the presence of detergents, then with PBS. Proteins were applied for SDS-PAGE and the proteins were identified by Western blots with the respective antibodies.

3.11 GST-MYPT pull-down assay

Glutathione-Sepharose equilibrated with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 protease inhibitors (binding buffer) was coupled with 0.1 μM GST or 0.1 μM GST-MYPT1 in binding buffer. A sample from the synaptosome fraction was precleared with uncoupled Glutathione-Sepharose and then incubated with GST (as a control) or GST-

MYPT1 coupled glutathione-Sepharose. The resin was washed with binding buffer, bound proteins were solubilized by boiling in SDS sample buffer.

3.12 Cell migration assays

Cell migration was measured by using fibronectin-coated (FN) Quantitative Cell Migration Assay. HepG2 cells were starved 18 hours prior passage in serum-free D-MEM medium. 2.5 x 10^5 cells were plated on FN-coated membrane in a Boyden Chamber. HGF (50 ng/ml) was applied as chemoattractant in the lower chamber. Cells were incubated in the absence or in the presence of 5-50 nM OA, or 10 μM Y27632, or in the presence of Y27632 plus OA, for 18 hours. Migration of the cells was assessed by measuring the optical density of dye infiltered cells at 540 nm.

3. RESULTS AND DISCUSSION

4.1 Localization of myosin phophatase and MYPT1 in rat brain and primary cultures of neuronal cells

MYPT1 and MYPT2 isoforms are both expressed in rat brain, but the MYPT1 content is much higher then that of MYPT2 as judged by Western blot analysis using the antibody specific for both isoforms. These data suggest that in brain MYPT1 represents the major isoform and we investigated the localization of MYPT1 and its interactions with other proteins.

4.1.1. Region specific and subcellular distribution of myosin phosphatase and MYPT1 as assayed by immunoblots, phosphatase avtivity assays and immunofluorescence

The expression of MYPT1 is observed in each brain region and its concentration was determined by semi-quantitative Western blot analysis. MYPT1 is enriched in cortex, olfactory bulb, striatum, hippocampus and diencephalon, whereas lower levels were detected in midbrain and hindbrain. The range of MYPT1 concentrations (30-78 nmol/g total protein) in different brain regions is similar to concentrations estimated in smooth muscle cells and in non-muscle cells. The specific activity of MP in the lysates of the distinct brain regions (1.1-2.7 nmol/min/mg) also is comparable to that found in smooth muscle and non-muscle cells. In the midbrain and the hindbrain the MYPT1 level and MP activity is 1.5-2 fold lower than in other regions. The functional consequence(s) of these differences is not known yet.

MYPT1-immunoreactive material was found in all areas of the brain but it displayed different staining in distinct brain nuclei and nerve bandles. MYPT1 is present in the brain stem, which and this is involved in transmitting information between higher and lower compartments of the central nervous system. MYPT1 is present in parts of the limbic system and connecting axon branches, which region is involved in controlling emotion and instinct behavior. MYPT1 and MP

may also be implicated in memory since MYPT1 was detected in the perikarya of the entorhinal cortex and afferents of the hippocampus. MYPT1 is present in the nuclei and nucleoli in many, but not all neuronal cells. The presence of MYPT1 in the nucleolus of neuronal cells is a novel observation regarding the subcellular localization of this protein. It should be noted, however, that neither in primary hippocampus cells nor in cochlear nucleus cells was MYPT1 identified in the nucleoli. Partial differences were found in the nuclear staining of primary neuronal cells: in hippocampus cell cultures MYPT1 was found in the cytoplasm and in the nucleus, whereas in cochlear nucleus cells, MYPT1 displayed mainly a cytoplasmic localization. The above results suggest that the localization of MYPT1 may be influenced by a number of factors, including the physiological state as well as the age and the developmental stage(s) of the cells. It also has to be noted, that subcellular staining for MYPT1 may be dependent on the conditions of fixation and the type of cell studied. Our results suggest that the distribution of MYPT1 in brain/neuronal cells is non-uniform but is related to specialized brain regions and subcellular compartments. This localization pattern of MYPT1 is quite distinct from that of myosin II and suggests that the MP and MYPT1 are involved in more general cellular functions and MP may dephosphorylate alternative neuronal substrates distinct from myosin.

4.1.2. Identification of MYPT1 interacting proteins by immunoprecipitation and pull-down assay

In primary neuronal cells colocalization of MYPT1 and PP1cδ was observed with synaptophysin, a presynaptic marker and integrate membrane protein of synaptic vesicules. Immunoprecipitation from the lysates of brain regions with antibody specific for MYPT was performed to screen for those proteins that may be associated with MYPT1 in the brain and to prove the potential interaction between MYPT1 and synaptophysin. ROK, synaptophysin, PP1cδ and other PP1c isoforms were indentified as interacting partners of MYPT1. In part, the above findings imply that PP1cδ may be the major isoform

in the MP holoenzyme in neuronal cells similarly to smooth muscle cells, since the distribution of PP1cδ found previously in various brain regions is similar to that shown here for MYPT1. Consistent by we found that PP1cδ coprecipitated with MYPT1 from brain tissue extracts. However, the partially distinct localizations of MYPT1 and PP1cδ in primary neuronal cells suggest that the MYPT1 subunit may exist free i.e., dissociated from PP1c in cells, or that MYPT1 may interact with PP1c isoforms other than PP1cδ. Our results support the latter possibility, since PP1cα and/or PP1cγ1 coprecipitated with MYPT1 from brain extracts. Other neuronal targeting subunits, including neurofilament-L, neurabin I and neurabin II were found to be associated primarily with PP1cα and PP1cγ1. Our data thus suggest that MYPT1 is the first PP1c targeting subunit identified in neuronal cells that recruit PP1cδ predominantly.

The interaction of MYPT1 with synaptophysin may indicate that MP is enriched in synaptic vesicles and it might predict an important function for MP in the regulation of phosphorylation events in the presynaptic terminals. Synaptosomes and postsynaptic densities were isolated and the distribution of MP, MYPT1 and MYPT1-interacting proteins (ROK, synaptophysin and PP1cδ) were assayed in these fractions. Myosin phosphatase activity was high in the synaptosome, but was relatively low in the postsynaptic density fraction. MYPT1, ROK and PP1cδ were also higher in synaptosomes than in the postsynaptic densities, whereas synaptophysin was detected only in the synaptosomes. The interaction of MYPT1 with ROK, synaptophysin and PP1cδ was also supported by pull-down assays carried out with GST-MYPT1. Western blots with specific antibodies indicated the presence of ROK, synaptophysin and PP1cδ in the GST-MYPT1 pull-down fractions. Other neuronal targeting subunits, including neurofilament-L, neurabin I and neurabin II were identified in postsynaptic densities. Our data support the conclusion that MYPT1 is the first c PP1c regulatory subunit identified in the presynapsis.

Synaptophysin is a phosphoprotein and its phosphorylation is implicated in the neurotransmitter release from the synaptic vesicles. It is hypothesized further that since synaptophysin and MYPT1 interact with each other, MYPT1 may target this neuronal protein to PP1c and may regulate the level of its phosphorylation, thereby affecting neurotransmitter release. ROK could be another component of this mechanism, since MP is thought to be regulated via RhoA/ROK signaling and phosphorylation of MYPT1 by ROK at the inhibitory site (Thr695). Several common substrates (MAP2, Tau) of ROK/MP are known but further experiments are needed to prove the coordinated action of this pair of enzymes on the phosphorylation/dephosphorylation of synaptophysin.

4.2 Effect of okadaic acid on the phosphorylation of MYPT1, myosin and on the motility in HepG2 cells

4.2.1 Distribution of myosin phosphatase and MYPT1 in HepG2 cells and in its subcellular fractions

The subcellular localization of MYPT1 in HepG2 cells were investigated by Western blot analysis, protein phosphatase activity assay and immunofluorescence. MYPT1 concentration was higher in the nuclear, cytoskeletal and in the microsome-mitochondria fractions compared to the cytosolic and the plasmamembrane fractions. It is assumed that MP is targeted to these subcellular compartments by the MYPT1 subunit since the phosphatase activity determined by intact myosin was in proportion with the amount of MYPT1 detected by Western blots. These results indicate novel functions of MP in the dephosphorylation of microsomal/mitochondrial and nuclear phosphoproteins. MYPT1 was stained throughout the cytoplasm and it accumulated also in speckle-like structures in the nucleus. We intended to examine how treatment of HepG2 cells with okadaic acid (OA), an inhibitor of PP1 and PP2A types of phosphates, would influence phosphorylation and localization of MYPT1 in HepG2 cells. OA in 50 nM resulted in the

translocation of MYPT1 from the cytoplasm to the plasma membrane but the nuclear staining of MYPT did not change significantly.

4.2.2. OA-induced myosin and MYPT1 phosphorylation and localization and their relation to the changes in the activity of PP2A

OA (at 50 nM) increased phosphorylation of MLC20 at the center of HepG2 cells. Inhibition of ROK by Y27632 partially decreased the OA-induced increase in the MLC20 phosphorylation. OA also increased the extent of phosphorylation of MLC20 and phosphorylation of MYPT1 on both Thr695 ($MYPT^{pThr695}$) and Thr850 ($MYPT^{pThr850}$). In addition, differential localization of the distinct phosphorylated MYPT forms was observed: $MYPT^{pThr695}$ was localized at the plasma membrane, whereas $MYPT^{pThr850}$ accumulated at the perinuclear regions and in the nucleus. The OA induced increase in the phosphorylation of Thr695 was affected slightly, while the increase in the phosphorylation of Thr850 was almost completely eliminated in the presence of ROK kinase inhibitor (Y27632). It implies that kinase(s) distinct from ROK could be involved in the phosphorylation of Thr695 while ROK regulates directly or indirectly the phosphorylation at Thr850.

The elevated phosphorylation of MLC20 resulted by the OA treatment suggests inhibition of MP. OA, at concentration of 5-50 nM, was without influence on the activity of myosin phosphatase in HepG2 cell lysate (where the concentration of phosphatases was similar to that in the cells), but inhibited the activity of PP2A enzymes. PP2A was shown to dephosphorylate $MYPT1^{pThr695}$ and $MYPT1^{pThr850}$ *in vitro*. This suggests that in HepG2 cells OA may not inhibit MP directly, therefore indirect mechanism via inhibition of PP2A activity could be assumed. The latter may increase the phosphorylation level of MYPT1, thereby inhibiting myosin phosphatase activity. Phosphorylation of Thr695, the inhibitory phosphorylation site, decreases the activity of MP, which was detected by both Western blot analysis and immunofluorescence. On the other hand, phosphorylation of MYPT1 on Thr850 is known to dissociate MYPT1

from myosin affecting the targeting of MP to myosin, thereby this could also contribute to the inhibition of myosin dephosphorylation in HepG2 cells. In addition, the translocation of MYPT1 to the plasma membrane could also result in the separation of MP from myosin II and lead to decreased myosin dephosphorylation.

Since Y27632 inhibited OA induced the phosphorylation of Thr850 in MYPT1, it is reasonable to assume that phosphorylation of this site occurs in a PP2A-and ROK-dependent manner. In OA-treated HepG2 cells similar localization of PP2Ac and ROK to $MYPT^{pThr850}$ (i.e. each is present in the nucleus and at the perinuclear region) also supports this assumption. In contrast, Y27632 had only a slight influence on the phosphorylation of Thr695, implying that cytoplasmic and/or plasma membrane kinase(s) distinct from ROK may be involved in the phosphorylation of Thr695 in MYPT1. ZIPK was also identified in HepG2 cells exhibiting a predominantly cytoplasmic localization. Besides ROK, ZIPK could play an important role in the OA induced phosphorylation of MLC20.

4.2.3 Effect of OA on the stress fiber formation and the migration of HepG2 cell

OA treatment caused development of stress fibers in HepG2 cells but this process was completely blocked by Y27632. OA decreased the chemoattractant-induced cell migration of HepG2 cells and this inhibition was not detected in the presence of Y27632. It appears that both ROK activity and inhibition of MP are required for increased myosin II phosphorylation, stress fiber assembly and inhibition of cell migration. During cell migration ROK-dependent phosphorylation of myosin II and inhibition of MP are thought to be involved in the cell body contraction and tail detachment. The continuous migration, requires dynamic reorganization and disassembly of stress fibers and focal adhesions. One suggested mechanism is that inhibition of MP could lead to a slower turnover rate of myosin II phosphorylation in the cell center that could

result in more stable adhesive structures and hence a slower migration rate. A novel finding of our present study is that inhibition of cell migration can be observed in the presence of relatively low concentration of OA, which causes the inhibition of PP2A. This observation implies, that besides the already described ROK- and MP-dependent mechanism, PP2A could also be involved in the regulation of cell motility under certain conditions. These data may also contribute to studies aiming to discover novel pharmacological molecules that influence cell migration.

5. SUMMARY

The myosin phosphatase holoenzyme (MP) consists of a protein phosphatase 1 (PP1) catalytic subunit (PP1c) and a 130/133 kDa myosin binding (targeting) subunit termed myosin phosphatase target subunit 1 (MYPT1). The MP primarily dephosphorylates the 20 kDa subunit of myosin-II (MLC20), thereby regulates the contractility and motility of smooth muscle and non-muscle cells. The activity of MP is mediated via phosphorylation of MYPT1 on Thr695 and Thr850 by RhoA-associated protein kinase (ROK). The MP also dephosphorylates substrates distinct from myosin; however, its localization and function in cellular processes (besides contractility) of non-muscle cells and tissues, is less understood.

Our results show that MP and MYPT1 are present in brain and in human hepatoma (HepG2) cells. The activity of MP and the amount of MYPT1 exhibited similar region- and subcellular distribution suggesting a targeting function for MYPT1 in both neuronal and HepG2 cells. The MP and MYPT1 are identified in all regions of brain, but within the regions MYPT1 accumulated in certain groups of neurons and axon bundles suggesting its involvement in specialized neuronal functions. MP and MYPT1 were identified in the presynaptic fraction of synaptosomes isolated from cerebral cortex, and the interaction of MYPT1 with synaptophysin, ROK and PP1cδ was confirmed. These data suggest that MP may be involved in the regulation of the transport of synaptic vesicles and in the release of neurotransmitters into the synaptic clefts

We have shown that the treatment of HepG2 cells with okadaic acid (OA) resulted in the inhibition of protein phosphatase 2A (PP2A) that was accompanied with the regulation of the phosphorylation level of MLC20 and MYPT1. OA induced the translocation of MYPT1 from the cytoplasm to the plasma membrane and increased the phosphorylation of MYPT1 on Thr695, which causes inhibition of MP activity. These effects were slightly influenced by Y27632, a specific ROK inhibitor. OA, increased the phosphorylation of

MYPT1 on Thr850 (MYPT1^{pThr850}), which was profoundly inhibited by Y27632. MYPT1^{pThr850} was localized in the nucleus and at the prinuclear regions in HepG2 cells. On treatment with OA the increased phosphorylation of MLC20 on Ser19 was observed and it was associated with the appearance of myosin II at the cell center. OA induced stress fiber formation and a decrease in cell migration, but both of these OA-induced effects were blocked by Y27632. It is concluded that OA induces differential phosphorylation and translocation of MYPT1 in a PP2A- and to varying extent in a ROK-dependent manner resulting in the inhibition of myosin dephosphorylation. These changes are associated with an increased and sustained level of myosin II phosphorylation and attenuation of motile features of the HepG2 cells.

LIST OF PUBLICATIONS

This thesis based on the following publications:

Lontay, B., Serfőző**,** Z., Gergely**,** P., Ito**,** M, Hartshorne**,** D. J. and Erdődi**,** F. Localization of myosin phosphatase target subunit 1 in rat brain and in primary cultures of neuronal cells *J. Comp. Neur.;* 478: 72-87 (2004) (IF=3,5)

Lontay, B., Kiss, A., Gergely P., Hartshorne D. J., Erdődi F. Okadaic acid induces phosphorylation and translocation of myosin phosphatase target subunit 1 influencing myosin phosphorylation, stress fiber assembly and cell migration in HepG2 cells. *Cell. Signal.* (in press) (2005) (IF=5,19)

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Palatka K, Serfőző Z, Veréb Z, Hargitay Z, **Lontay B**, Erdődi F, Bánfalvi G, Nemes Z, Udvardy M, Altorjay I. Changes in the expression and distribution of the inducible and endothelial nitric oxide synthase in mucosal biopsy specimens of inflammatory bowel disease. *Scan. J. Gastroenterol*. (2005) (in press) (IF=2,3)

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Szirák, K., Zeke, T., **Lontay, B**., Grosz, G., Dombrádi, V., Fehér, Zs., Identification of proteins interacting with Ser/Thr protein phosphatases of Neurospora crassa using the yeast two-hybrid system. *Yeast* **18**: S168 (2001)

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