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

Role of myosin phosphatase in wound healing and neurotransmitter release

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UNIVERSITY OF DEBRECEN

Doctoral School of Molecular Medicine

Debrecen, 2019

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The Examination takes place at Library of Department of Physiology, Faculty of Medicine, University of Debrecen

04th of June, 2019. 10:00 AM

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04th of June, 2019. 12:00 AM

INTRODUCTION

Protein phosphatases and PP1 enzymes

Many proteins in the living systems contain covalently-bound phosphate groups. Protein kinases catalyze protein phosphorylation, whereas protein phosphatases are responsible for the reversibility of this process. Based on their target amino acid residues we can classify them to serine/threonine (Ser/Thr)-, tyrosine (Tyr)- and dual-specificity protein phosphatases. In mammalian cells, PP1 and PP2A enzyme families are responsible for more than 90% of the Ser/Thr-specific phosphatase activity.

PP1 enzymes are heterodimers composed by a catalytic (PP1c) and a regulatory subunit, and the latter can be extremely variable. PP1c-interacting proteins can have many functions: they could either target the holoenzyme towards distinct subcellular regions or substrates, or influence the substrate specificity or inhibit the catalytic activity of PP1c or serve as substrates of the PP1c subunit. PP1 holoenzymes can play a role in various cellular processes such as cell cycle, apoptosis, protein synthesis, glycogen metabolism, muscle contraction or even cytoskeleton remodeling.

Structure, regulation and role of myosin phosphatase

Myosin phosphatase (MP) holoenzyme is a member of the PP1 enzyme family. Unlike PP1 holoenzymes, MP is not a heterodimer, but a heterotrimer. Besides β/δ isoform of PP1c (PP1c β/δ) it contains two subunits: a 110-130 kDa myosin phosphatase targeting subunit (MYPT1) and a 20 kDa protein with unknown function (M20). MP was first isolated from chicken gizzard and characterized as an enzyme which promotes smooth muscle relaxation by dephosphorylating the 20 kDa myosin light chain (MLC20) protein. MYPT1 regulatory subunit belongs to the MYPT family. All the members of this family contain a PP1c-binding RVxF motif followed by ankyrin repeats. MYPT1 peptide chain is more than 1000 amino acids long and is ubiquitously expressed - mainly in smooth muscle.

Activity of MP can be regulated by the phosphorylation of MYPT1 on Thr696 or Thr853 inhibitory residues by RhoA-activated kinase (ROK). Thr696 can also be phosphorylated by other kinases such as lucine zipper interacting protein kinase (ZIPK) or integrin-linked kinase (ILK). Enzyme activity of MP can be also altered by inhibitory proteins. Protein kinase C-potentiated inhibitory protein of PP1 (CPI-17) and its homologue, the kinase enhanced PP1 inhibitor (KEPI) are proteins capable to inhibit not only free PP1c but also MP holoenzyme effectively. Moreover, the activity of the holoenzyme can be modulated by MYPT1-interacting inhibitory proteins, such as smoothelin-like protein 1 (SMTNL1).

Besides MLC20, numerous new substrates of MP have been discovered. Ezrin, radixin, moesin (ERM proteins), adducin, Tau and MAP2 are playing a role in cytoskeleton remodelling and they are all substrates of both MP and RhoA-activated kinase (ROK). MP was also localized in the nucleus where it regulates gene expression and cell cycle. Histone deacetylase 7 (HDAC7) is one of its substrates, which translocates into the nucleus and regulates transcription after being dephosphorylated by MP. According to a novel finding ROK phosphorylates whereas MP dephosphorylates protein arginine methyltransferase 5 (PRMT5) on Thr80 residue, thus regulating tumorigenesis of hepatocellular carcinoma cells. PRMT5 phosphorylated at Thr80 is activated and catalyzes symmetrical dimethylation of histone proteins on arginine amino acid residues which accompanies tumorigenesis. Moreover MP dephosphorylates retinoblastoma protein (pRB), which protein regulates cell cycle progression. Merlin is also a substrate of MP, which modulates cell proliferation. Dephosphorylation of merlin by MP activates its anti-proliferative and tumor suppressor effect. MP also controls mitosis by dephosphorylating and inactivating polo-like kinase 1. Above all, MP has an effect on vascular tone through the dephosphorylation of endothelial nitric oxide synthase (eNOS). Through this eNOS is being activated which leads to NO production and vasodilation.

MP is expressed in neurons, too. Synapsin and syntaxin proteins are characterized as neuronal substrates of MP and ROK. Moreover, the inhibition of PP1 by tautomycetin (TMC) decreases while suppression of ROK by Y27632 increases neurotransmitter release proved by exocytosis assay. Pull-down assay and mass spectrometry analysis revealed that synaptosomal-associated protein of 25 kDa (SNAP-25) and MYPT1 are interacting partners. This observation made us study whether SNAP-25 is a substrate of MP.

The wound healing process and its regulation

Wound is a damage to the normal structure and function of the influenced tissues. Acute wound healing process involves four – didactically divided – phases: coagulation, inflammation, proliferation and remodelling. These phases take place successively, however, overlap one another in time and are regulated in many ways.

After injury coagulation immediately begins. Cytokines and growth factors are released from the platelets and surrounding tissues, which initiate the inflammatory phase. At first neutrophil granulocytes arrive at the site of damaged area and by their phagocytic activity they start to eliminate the microorganisms. 2-3 days after wounding, macrophages also appear. These cells continue to destroy pathogens and activate those cell populations which carry out more steps of proliferative phase. This proliferative phase begins approximately 3 days after wounding and lasts for 2 weeks. Fibroblasts begin to synthetize matrix proteins and the creation

of new connective tissue fibers is being initiated. Novel capillary vessels are also generated leading to more satisfactory blood supply of the tissues. During re-epithelialization epithelial cells are proliferating and migrating towards the surface of the wound in order to restore the barrier function of the epidermis. Remodelling is the latest phase of the wound healing process which could take up to few years. During this phase extracellular matrix is completely regenerated.

As we previously discussed, re-epithelialization is a key event of the wound healing process, which involves proliferation, migration and differentiation of the keratinocytes. Events of proliferation and migration are spatially separated: those cells close to the wound edge are migrating while keratinocytes distant from this place are proliferating. Many growth factors influence re-epithelialization, but protein kinases and phosphatases by dephosphorylating their target proteins can also regulate cell migration. However, their accurate role is not clearly understood. Okadaic acid or calyculin A-treated HaCaT cells showed slower migration rate in scratch assay which suggests that inhibition of both PP1 and PP2A together delays cell migration. In contrast, another research group states that not the suppressed but the active PP2A blocks keratinocyte migration through the dephosphorylation of ERK. Our goal was to clarify the role of MP and ROK in wound healing.

The neurotransmitter release

Chemical synapses enable communication between neurons. Neurotransmission is a multiple-stage process whose first step is the loading of the vesicles with neurotransmitters via ATP consuming active transport. Then vesicles migrate to the active zone where docking takes place. In the next step, called priming, molecular realignment happens which makes the vesicles ready for fusion with plasma membrane. Finally, instreaming calcium ions initiate vesicle exocytosis by opening a fusion pore.

Soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex plays a key role in vesicle docking and fusion. This complex is built up from three proteins: synaptobrevin is anchored to the vesicle membrane whereas SNAP-25 and syntaxin are bound to the plasmalemma. Every protein contains a 60-70 amino acid long and evolutionary conserved SNARE motif through they are anchored to each another. The three proteins are zippering up in an N- to C-terminal direction and the mechanical force generated by this process pushes the vesicle and presynaptic membrane towards each other so that their fusion takes place. A fusion pore is opened and neurotransmitters start to flow out through this pore. SNARE complex formation is spontaneous and exergonic process; energy is needed for the disgregation of the complex.

Regulation of SNAP-25 by phosphorylation and dephosphorylation

Neurotransmitter release is initiated by the increase in calcium ion concentration, but the completion of the process is also regulated by protein phosphorylation/dephosphorylation. According to the literature, the two most important phosphorylation sites of SNAP-25 are Thr138 and Ser187, both of them are phosphorylated by protein kinase A (PKA) and C (PKC). Thr138 amino acid residue regulates vesicle priming as its phosphorylation by PKA enhances the sizes of the readily releasable vesicle pools. According to *Risinger and Bennett* phosphorylation of Thr138 does not alter interaction between SNAP-25 and syntaxin, however *Gao et al* state that this phosphorylation inhibits SNAP-25-syntaxin interaction, SNARE complex assembly and noradrenalin secretion of PC12 cells. Ser187 phosphorylation regulates SNARE complex assembly, vesicle docking and the inhibition of voltage-gated calcium channels by SNAP-25. According to previous publications, Ser187 can be dephosphorylated by protein phosphatase 2A (PP2A) but cannot by PP1 or protein phosphatase 2B (PP2B). However, PP1 is the major enzyme which acts on Thr138 amino acid residue.

AIMS

Our goal was to study the role of MP and ROK in (1) wound healing and (2) neurotransmitter release.

(1) Our aims in order to clarify the role of MP in wound healing were:

- to study the effects of PP1 and ROK inhibitors (tautomycin, H1152) on epidermal wound healing using mouse model.
- to examine how PP1 and ROK inhibitors influence the viability, protein phosphatase activity, proliferation, migration, and cell-cell adhesion of human keratinocytes (HaCaT cells).
- to study the role of MP itself in the above mentioned processes.
- to explore the role of MP in molecular machineries and signaling pathways influencing keratinocyte migration and wound healing by silencing MYPT1 in *ex vivo* epidermal tissues and applying tissue lysates on MAPK array.

(2) Our goals in order to study the role of MP in neurotransmitter release were:

- to verify the protein-protein interaction of SNAP-25 and MYPT1 by immunoprecipitation.
- to verify that SNAP-25 is a substrate of ROK and MP. Incubation of purified SNAP-25 with ROK *in vitro*, than detection of phosphorylation sites by mass spectrometry.

Dephosphorylation of phospho-SNAP-25 by the subunits of MP and verifying this by Western blot using specific antibody for phosphorylated SNAP-25.

- to examine the *in vivo* regulation of SNAP-25 by ROK/MP in B50 neuroblastoma cells and murine brain slices. Introduction of KEPI into mouse cortical synaptosomes in order to inhibit MP and study the effect of MP inhibition on SNAP-25 phosphorylation.
- to study the effect of MP on neurotransmitter release by exocytosis assay using control and KEPI-treated synaptosomes.

MATERIALS AND METHODS

Wound healing assay on mouse dorsal skin

The dorsal fur of male Balb/c mice (n=6), aged 6-7 weeks, was removed then three full thickness round biopsies with 4 mm diameter were made on each mouse. Wounds were treated with 1 μM tautomycin (TM) or 10 μM H1152 twice a day for 7 days. The wounds were photographed with Olympus camera every day, and sizes of the wound areas were determined by ImageJ program.

Trichrome staining

At the end of the previous experiment wounds were excised, fixed with 10% formalin and embedded into paraffin. 4 μm cross-sections were cut off, mounted on glass slides, deparaffinized by xylene, dehydrated by ascending concentration of ethanol series, then stained using Masson-Goldner trichrome staining.

Immunohistochemistry

Human epithelial tissue sections were incubated with anti-transglutaminase 1 (anti-TG1) or anti-MYPT1 primary antibodies overnight, and after washing with horseradish peroxidase conjugated secondary antibodies for 2 hours. Immunosignals were developed by 3,3'-diamino-benzidine chromogen substrate. The sections were cleared with xylene, embedded, mounted on glass slides then visualized with Leica S40 microscope.

Cell culture

Human keratinocyte (HaCaT), B50 neuroblastoma and tsA201 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, whereas normal human epidermal keratinocytes (NHEK) were cultured in EpiLife medium at 37 °C in a 5% CO_2-humidified atmosphere.

Gene silencing in HaCaT cells

Downregulation of MYPT1 expression in HaCaT cells was carried out by siRNA. 5 µl Lipofectamine 2000 transfection reagent was added to 100 pmol siRNA, both of them were previously diluted in 250-250 µl OptiMEM. Cells were trypsinized, serum-free DMEM was added to them, and then transfection mixture was pipetted to the cells. After 4 hours 10% FBS was also given to them and after another 44 hours cells were lysed.

Gene silencing in *ex vivo* human biopsies

Lipofectamine 2000 and siRNA were diluted in a special medium which was provided by Genoskin for the maintenance of *ex vivo* human biopsies. After two days, samples were frozen in liquid nitrogen, ground in a mortar and homogenized in lysis buffer. Finally tissue lysates were analyzed by Western blot or by human MAPK array. In the latter case we followed the manufacturer's instructions.

MTT assay

Cells were grown in 96-well plates and treated with 1 µM TM or 10 µM H1152 for 12 hours. After treatment medium was removed and 100 µl serum-free DMEM and 10 µl 5 mg/ml MTT solution were pipetted into the wells. After 30 minutes incubation at 37 °C, formazan crystals were dissolved in 100 µl dimethyl sulfoxide (DMSO) and the absorbance was detected by spectrophotometer at 540 nm wavelength.

Caspase-3 activity assay

HaCaT cells were cultured in 6-well plates and after 24-hour serum starvation cells were treated with inhibitors (1 µM TM, 10 µM H1152, 12 hours). Cells were lysed, centrifuged then Ac-DEVD-AMC substrate was added to the supernatant. Eppendorf tubes were incubated at 37 °C for 60 minutes and the fluorescence intensity AMC was measured by Fluoroskan FL instrument (excitation: 3600 nm, emission: 460 nm).

Western blot

Proteins were separated by polyacrylamide gel electrophoresis due to their molecular sizes then transferred to nitrocellulose membrane. Membrane was blocked in 5% bovine serum albumin solution and then incubated with the primary antibodies at 4 °C overnight. Secondary antibodies were also applied onto the membrane for 2 hours at room temperature and immunosignals were detected by SuperSignal West Pico Chemiluminescent Substrate Kit.

Protein phosphatase activity assay

Cells were treated with inhibitors (1 µM TM, 10 µM H1152, 1 hour) or with siRNA and then they were lysed. The lysates were centrifuged, then supernatants were diluted in 20 mM Tris-HCl (pH 7.4) containing 0.1% β-mercaptoethanol at 3-fold final dilution and analyzed at

30 °C in the presence of ^{32}P-labelled turkey gizzard MLC20. The reaction was terminated by addition of 200 µl 10% trichloroacetic acid and 200 µl 6 mg/ml BSA. The released radioactively labelled phosphate was detected by a scintillation counter.

Scratch assay

HaCaT cells were grown in 96-well plate and then scratched by Tecan Freedom EVO 150 instrument. Wideness of the initial scratches were the same. After scratching cells were treated with inhibitors (1 µM TM, 10 µM H1152), then we let them grow for 24 hours. In case of MYPT1-silencing, cells were transfected by siRNA 24 hours before scratching. At the end of the experiment images were taken from the scratches and their sizes were determined using ImageJ program.

Sulforhodamine B (SRB) cell proliferation assay

At the end of scratch assay ice-cold trichloroacetic acid was added to the cells at 10% final concentration. After 1 hour-long incubation medium was discarded and 100 µl 1% acetic acid containing 0.4% (m/V) SRB was pipetted into the wells. Following 10 minutes' incubation unbound dye was removed by washing and remaining dye bound to cellular proteins were solubilized with 10 mM Tris base. Absorbance was recorded at 540 nm by spectrophotometer.

JuLI^{TM} real-time cell history recording

HaCaT cells were cultured in Petri dishes and the monolayer was scratched by a pipette tip. HaCaT cells then were kept in 10% FBS/DMEM supplemented with the inhibitors (1 µM TM or 10 µM H1152). The siMYPT1- or non-target siRNA-transfected cells were scratched after 24 hours from the beginning of the transfection. Scratch closure was recorded for 24 hours by JuLI^{TM} Br instrument which made images about the scratches in every hour.

Electric cell-substrate impedance sensing (ECIS)

HaCaT cells were grown on 8W10 ECIS electrode arrays. 5 mA current at 60 kHz frequency was applied two times for 30 seconds and by this electric shock cells were burnt out on the surface of the electrodes. Then electric impedance was measured. Reaching maximum plateau value was considered as the restoration of the monolayer's integrity. We also studied the barrier function of HaCaT cells in this case without wounding. Impedance was recorded in real-time for at least 6 hours.

Immunofluorescence staining

Cells grown on glass coverslips were washed with PBS, fixed by 4% paraformaldehyde (HaCaT) or by ascending ethanol concentrations (B50), and permeabilized with 0.02-0.2% Triton X-100. Then cells were blocked in 1% BSA or horse serum diluted in PBS for 1 hour at 4 °C. Primary antibodies were diluted at 1:250, whereas secondary antibodies and Texas Red-

X phalloidint at 1:2000 in this blocking solution and cells were incubated in them overnight and 2 hours respectively. Finally nuclei were stained with DAPI and coverslips were mounted on glass plates by ProLong Gold Antifade medium. Immunosignals were visualized by confocal microscopy.

Immunoprecipitation

Antibodies were bound to Protein A-Sepharose (PAS) beads, then precleared B50 cell lysate was incubated with PAS-antibody complexes at 4 °C for 90 minutes. Supernatant was discarded and beads were washed with immunoprecipitation buffer and finally with PBS. Beads were boiled with 1x SDS-PAGE sample buffer at 100 °C for 5 minutes then Western blot analysis was carried out.

Protein production and purification

Flag-SNAP-25 or Flag-KEPI plasmids were transfected into tsA201 mammalian cells using polyethyleneimine (PEI). Cells were lysed 24 hours after transfection and produced proteins were purified on Anti-Flag M2 affinity gel. For eluation 300 µg/ml Flag-peptide/TBS was used.

***In vitro* kinase assay**

5 µM Flag-SNAP-25 protein was incubated with 20 ng/µl ROK enzyme, 1 mM γ-[^{32}P]ATP and 1 µM microcystin-LR protein phosphatase inhibitor for 120 minutes at 30 °C. Kinase reaction was terminated by addition of hot 5x SDS-PAGE sample buffer. After polyacrylamide gel electrophoresis incorporated phosphate was detected by autoradiography.

Protein phosphorylation/dephosphorylation assay

Flag-SNAP25 protein was produced in tsA201 cells then cell lysate was incubated with Anti-Flag M2 affinity gel at 4 °C-on for 2 hours. After washing of the beads ROK, ATP and microcystin-LR were pipetted into the Eppendorf-tubes, into control sample no ROK was added. Following 30 min incubation at 30 °C supernatant was discarded, resin was washed by TBS and ROK-phosphorylated sample was divided into two Eppendorf tubes. Into one tube only TBS was measured while into the second tube subunits of MP were added: 25 nM Flag-MYPT1 and 5 nM PP1cδ-t. Tubes were replaced into the water bath (30 °C) for 15 min, then beads were washed with TBS and boiled with 1x SDS-PAGE sample buffer.

Mass spectrometry

Bands of interest were subjected to in-gel digestion using trypsin. 80% of the peptide mixture was subjected to phosphopeptide-enrichment by titanium dioxide, while remaining 20% was analyzed by liquid chromatography-mass spectrometry and by PAVA software peak lists were generated from the MS/MS data. Peak lists were searched against the human data of

Swissprot database using ProteinProspector. With the confidently identified peptides another search was conducted, but this time Ser/Thr phosphorylation was also added to the variable modifications. For phosphopeptide site assignments SLIP score threshold was 6 which indicates 95% confidence of site localization.

Synaptosome preparation

C3H mice were euthanized using CO_2 then cortexes were removed, homogenized and homogenate was centrifuged. Supernatant was measured onto the top of Percoll gradient (23-15-10-3%) in polycarbonate tubes, then centrifuged at 32500 *g* for 5 min at 4 °C. Fractions between 10/15% and 15/23% phases were collected and diluted with four-times volume of Krebs buffer, then centrifuged. The pellets were washed with Krebs buffer and after another centrifuge step synaptosomes were resuspended in Krebs buffer.

Introduction of Flag-KEPI into synaptosomes

Synaptosomes were resuspended in HBS buffer containing 25% DMSO. The suspension was divided into three tubes, and 0.3 μg/μl Flag-peptide or 0.3 μg/μl Flag-KEPI was added to the synaptosomes; the third tube was the control. The tubes were placed into dry ice-isopropanol bath for 3 min (freezing step) then into 4 °C water bath for 10 min (thawing step). Finally, tubes were centrifuged, the pellets were washed with Krebs buffer twice, then pellets were resuspended in Krebs buffer.

Exocytosis assay

The Eppendorf tubes containing synaptosomes were incubated at 37 °C for 3 min, then FM 2-10 fluorescent dye was pipetted into them. The tubes were kept at 37 °C for another 1 min and FM 2-10 uptake into synaptosomes was stimulated by adding 30 mM KCl. Samples were centrifuged followed by the incubation at 37 °C for 2 min (dye internalization) and the pellets were resuspended in Krebs buffer, then 100-100 μl volumes were pipetted into the wells of a black 96-well plate. Exocytosis was initiated by 30 mM KCl and the changes of the fluorescence intensity was recorded by Fluoroskan FL instrument (excitation: 488 nm, emission: 540 nm).

Preparing brain slices and treatment

Cortex was removed from a C3H mouse then 200 μm thick brain slices were prepared using Microm HM 650 V vibratome. Slices were immersed into artificial cerebrospinal fluid in culture plate inserts, then inhibitors (5 μM TMC or 10 μM H1152) and 8 mM KCl were added and the inserts were incubated under moderately pressurized carbogen gas at 1 hour.

Statistical analysis

Statistical analysis and graph making were carried out using GraphPad Prism 6 program. Normalized data were analyzed by either Student t-test or by ANOVA and *post hoc* Šidak- or Dunnett-test. All data show in the thesis represent mean ± standard error of the mean (SEM).

RESULTS

Tautomycin inhibits murine excisional wound healing

We studied the effect of PP1 and ROK on murine excisional wound healing using tautomycin (TM) and H1152. TM, a selective PP1 inhibitor completely hindered wound closure, the size of the wounds did not alter during 6 days. The difference between TM-treated and control wounds became significant after 2 days. Inhibition of ROK by H1152 caused a slightly but not significant acceleration of wound closure.

On the sixth day wounds were excised and we evaluated the re-epithelialization of control, H1152- and TM-treated wounds with Masson-Goldner trichrome staining, which showed that the re-epithelialization was almost completely inhibited in TM-treated mice. These results suggest that PP1 enzymes have an important role in wound healing as PP1 inhibition impedes re-epithelialization.

Effect of TM and H1152 on the physiological functions of keratinocytes

Neither MTT assay nor caspase-3 activity assay showed significant difference between the viability of control and TM/H1152-treated keratinocytes. Inhibition of ROK had no effect on protein phosphatase activity of HaCaT cells, however, TM decreased it to the 57.1% of control level. By scratch assay carried out on HaCaT cells grown in 96-well plate we observed that H1152 accelerated scratch closure by 19% compared to control cells. However, migration of TM-treated cells completely hampered, in this case scratch areas were similar to the negative (serum-free) control. Based on SRB assay, TM arrested cell proliferation and the proliferation rate of TM-treated cells was lower by 24% compared to control. In contrast, H1152 had no influence on proliferation. Scratch assay was also conducted in the presence of mitomycin, a cell cycle inhibitor. SRB assay verified the inhibitory effect of mitomycin on cell proliferation and we found applying scratch assay that this compound delays scratch closure, which suggests that cell proliferation is crucial in the effective completion of wound healing.

In another experiments HaCaT cells were grown in Petri dishes and scratched by a pipette tip, then wound closure was analyzed by JuLI^{TM} time-lapse microscopy. By two-way ANOVA and *post hoc* Dunnett-test we proved that TM inhibited whereas H1152 accelerated

scratch closure also in this case. H1152-treated cells overran significantly greater area than control cells even 8 hours after scratching, in case of TM treatment difference became significant after 16 hours. ECIS experiments strengthened our previous results showing that control, H1152- and TM-treated cells needed 2.1, 1.6 and 2.7 hours, respectively to the full closure of the wound areas burnt out on the electrodes. Our results clearly show that PP1 and ROK oppositely regulate the wound healing of keratinocytes: PP1 enhances while ROK suppresses it.

MYPT1 silencing inhibit the movement of keratinocytes

Scratch assay showed that the free area of the scratch after MYPT1 silencing was three-times larger than that cells transfected with non-target siRNA. Th proliferation rate decreased by 7% in response to MYPT1 silencing. Using JuLI^{TM} real-time cell history recorder we investigated the movement of HaCaT cells previously scratched by a pipette tip and our results showed that the difference in the size of occupied areas between control and siMYPT1 transfected cells became significant 8 hours after scratching. Silencing of MYPT1 resulted in increased time to wound closure (by 1 hour) compared to control cells in ECIS plates. All these data suggest that MP accelerates scratch closure.

MP disrupts the barrier function of HaCaT cells

The role of ROK in the formation and maintenance of cell-cell attachments and barrier function has already been described previously in corneal epithelial cells. However, no data were proven the role of MP in these processes. By immunofluorescent staining of zonula occludens 1 (ZO-1) protein we observed an increased amount of ZO-1 at the sites of cell-cell connections in response to MYPT1 silencing, which refers to enhanced tight junctions. We also observed an alteration of actin fiber construction as they accumulated rather in cortical region of MYPT1 silenced cells.

Effect of PP1/ROK inhibitors and MYPT1 silencing on barrier function was also investigated by ECIS. Gene silencing was conducted either in the presence or in the absence of calcium; increased calcium ion concentration triggered the differentiation of HaCaT cells. According to our results calcium itself increased impedance suggesting that keratinocyte differentiation causes the enhancement of cell-cell contacts. MYPT1-silencing also resulted in a significant increase in impedance compared to control. TM treatment did not alter the electric impedance significantly, however, H1152 decreased it. Based on our results, ROK activity strengthens while MP weakens the cell-cell connections.

Gene silencing of MYPT1 increases transglutaminase-1 (TG1) expression

We proved by Western blot analysis that MYPT1-silencing results in an increase in transglutaminase-1 (TG1) protein expression both in human keratinocytes and in *ex vivo* native human skin. We also studied the epidermal distribution of MYPT1 and TG1 by immunohistochemistry. MYPT1 localized rather in the deeper layers of the epidermis in normal skin while TG1 could be found primarily in the *stratum granulosum* and *stratum spinosum*. In response to injury MYPT1 staining was intensified in the basal layers, whereas TG1 was expressed in all layers except *stratum basale*. Based on these results our hypothesis is that MP facilitates keratinocyte proliferation and migration and inhibits their differentiation by downregulating TG1 expression in basal layers. However, in the *stratum granulosum* MYPT1 expression drops off and TG1 expression is upregulated. Here TG1 promotes keratinocyte differentiation and cornification.

MP accelerates wound healing through Akt signaling pathway

Non-target siRNA- and siMYPT1-transfected *ex vivo* human native skin lysates were applied on human phospho-MAPK protein profiler array. Based on our results the elements of Akt signaling pathway – Akt1, GSK-3β, p70 S6 kinase – became dephosphorylated in response to MYPT1-silencing. *Charette and McCance* have published previously that active Akt downregulates RhoA activity and facilitates keratinocyte migration through inhibition of ROK. Furthermore, *Esposito et al* successfully stimulated PI3K – Akt pathway and accelerated wound healing in mice using homobrassinolide, a plant hormone. As silencing of MYPT1 resulted in Akt1 dephosphorylation in our experiments, we conclude that MP acts not directly on Akt but on one of its upstream effector. Our hypothesis is that MP activates Akt indirectly and this upregulation initiates the proliferation and migration of keratinocytes.

In *ex vivo* human skin MYPT1-silencing also resulted in an upregulated phosphorylation level of heat shock protein 27 (Hsp27). Previous results suggest that Hsp27 expression and its phosphorylation are indispensable to the differentiation of keratinocytes and the formation of a properly layered epidermis. We presume that MP downregulation activates Hsp27 in the uppermost layers of the epidermis which facilitates differentiation.

ROK phosphorylates, while MP dephosphorylates SNAP-25 *in vitro* and *in vivo*

Immunoprecipitation (IP) experiment carried out from B50 neuroblastoma cell lysates showed a co-precipitation of SNAP-25 and MYPT1. By immunofluorescent staining we proved the co-localization of these two proteins in the cytoplasm and in the neuronal projections of B50. Purified Flag-SNAP-25 was phosphorylated by ROK *in vitro* and Thr138 amino acid side chain was determined as the phosphorylation site for ROK by mass spectrometry (LC-MS/MS)

analysis. Flag-SNAP-25 protein was also bound to anti-Flag agarose and incubated with ROK at first, then – following a washing step – with the subunits of MP. Using phospho-specific antibody we verified the phosphorylation and dephosphorylation of SNAP-25 on Thr138 by Western blot analysis.

B50 neuroblastoma cells were treated with TMC or with H1152 and we analyzed the effect of the treatments on SNAP-25 Thr-138 phosphorylation by semi-quantitative Western blot analysis. Inhibition of ROK by H1152 resulted in a decrease in phosphorylation by 57.0% compared to control, while suppression of PP1 increased it by 46.9%. These treatments were carried out on mouse brain slices too, and we received similar results. It was also revealed that depolarization by KCl itself is coupled with a decrease in SNAP-25 Thr138 phosphorylation.

MP facilitates neurotransmitter release

Flag-KEPI was introduced into synaptosomes in order to inhibit specifically MP among the PP1 holoenzymes. We verified the successful transduction of KEPI and its phosphorylation by an endogenous kinase by Western blot analysis. Based on the exocytosis assay results, Flag-KEPI significantly reduced the level of neurotransmitter release compared to control even after 30 sec of KCl-induced depolarization. Flag-KEPI transduction also elevated SNAP-25 phosphorylation on Thr138 amino acid residue as proven by Western blot. Our results suggest that MP facilitates neurotransmitter release via dephosphorylating SNAP-25 on Thr138.

SUMMARY

Myosin phosphatase (MP) is a Ser/Thr-specific protein phosphatase 1 (PP1) holoenzyme. It consists of a protein phosphatase 1 catalytic subunit (PP1cδ), a regulating subunit which can bind to myosin (MYPT1) and a 20 kDa subunit with unknown function (M20). MP was named after its first identified substrate, the 20 kDa myosin light chain (MLC20). In the past few decades it has become clear that MP is expressed not only is muscle cells but perform ubiquitous expression and has a role in many physiological processes, like cell cycle, gene expression or neurotransmitter release. Its activity can be regulated by RhoA-activated kinase (ROK) among others, which inhibits MP via phosphorylating MYPT1. Moreover, MP and ROK share a number of substrates in the living systems.

I investigated the role of MP and ROK in wound healing and neurotransmitter release. TM, a selective PP1 inhibitor impeded wound healing in mice. TM and gene silencing of MYPT1 significantly slackened, while H1152, a ROK inhibitor significantly accelerated the scratch closure of human keratinocytes (HaCaT). MYPT1 silencing had an effect on cell-cell

adhesion and increased electric impedance. Silencing of MYPT1 also elevated the transglutaminase-1 (TG1) expression both in HaCaT cells and *ex vivo* native human skin. According to our results MP facilitates the proliferation and migration of keratinocytes in the basal layers of the skin, but inhibits their differentiation through the downregulation of TG1 expression. MP exerts its effect via Akt signaling pathway and regulation of hsp27.

By mass spectrometry analysis and *in vitro* phosphorylation/dephosphorylation assay we proved that SNAP-25, one of the members of vesicle exocytosis regulator SNARE complex, is a substrate of ROK/MP, and this enzyme pair acts on SNAP-25 at Thr138 amino acid residue. Introduction of KEPI, a MP inhibitor protein into mouse cortical synaptosomes resulted in an increase in SNAP-25 phosphorylation on Thr138 and a decrease in the level of exocytosis. Our results suggest that MP promotes neurotransmitter release via dephosphorylating SNAP-25.

This work was supported by grants from the National Research, Development and Innovation Office (FK 125043, K108308, EFOP-3.6.2-16-2017-00006, OTKA PD104878, K109249), by TÁMOP 4.2.2.A-11/1/KONV-2012-0025, TÁMOP-4.2.2/B-10/1-2010-0024, TÁMOP-4.2.4.A/2-11-1-2012-0001 and by University of Debrecen (RH/751/2015).

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Registry number: DEENK/24/2019.PL
Subject: PhD Publikációs Lista

Candidate: Dániel Horváth
Neptun ID: VLX0HJ
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Horváth, D.**, Sipos, A., Major, E., Kónya, Z., Bátori, R. K., Dedinszki, D., Szöllősi, A. G., Tamás, I., Iván, J., Kiss, A., Erdődi, F., Lontay, B.: Myosin phosphatase accelerates cutaneous wound healing by regulating migration and differentiation of epidermal keratinocytes via Akt signaling pathway in human and murine skin.
   Biochim. Biophys. Acta. Mol. Basis Dis. 1864 (10), 3268-3280, 2018.
   DOI: http://dx.doi.org/10.1016/j.bbadis.2018.07.013
   IF: 5.108 (2017)
2. **Horváth, D.**, Tamás, I., Sipos, A., Darula, Z., Bécsi, B., Nagy, D., Iván, J., Erdődi, F., Lontay, B.: Myosin phosphatase and RhoA-activated kinase modulate neurotransmitter release by regulating SNAP-25 of SNARE complex.
   PLoS One. 12 (5), 1-23, 2017.
   IF: 2.766

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List of other publications

3. Kónya, Z., Bécsi, B., Kiss, A., **Horváth, D.**, Hadháziné Raics, M., Kövér, K. E., Lontay, B., Erdődi, F.: Inhibition of protein phosphatase-1 and -2A by ellagitannins: structure-inhibitory potency relationships and influences on cellular systems.
J. Enzym. Inhib. Med. Chem. "Accepted by Publisher", 3-56, 2019.
IF: 3.638 (2017)

4. Sipos, A., Iván, J., Bécsi, B., Darula, Z., Tamás, I., **Horváth, D.**, Medzihradszky-Fölkl, K., Erdődi, 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: 4.122

5. Iván, J., Major, E., Sipos, A., Kovács, K., **Horváth, D.**, Tamás, I., Bai, P., Dombrádi, V., Lontay, B.: The Short-Chain Fatty Acid Propionate Inhibits Adipogenic Differentiation of Human Chorion-Derived Mesenchymal Stem Cells Through the Free Fatty Acid Receptor 2.
Stem Cells Dev. 26 (23), 1724-1733, 2017.
DOI: http://dx.doi.org/10.1089/scd.2017.0035
IF: 3.315

Total IF of journals (all publications): 18,949
Total IF of journals (publications related to the dissertation): 7,874

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

04 February, 2019