

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

**The role of protein phosphatase-1 activation and inhibition
mechanisms in the regulation of cellular processes**

by Emese Tóth

Supervisor: Andrea Kiss, PhD



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By Emese Tóth, Biotechnology MSc

Supervisor:..Andrea Kiss, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Examination Committee:** László Csernoch, PhD, DSc
Members of the Examination Committee: Ágota Apáti, PhD
Éva Csósz, PhD, DSc

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Éva Csósz, PhD, DSc

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INTRODUCTION

Protein phosphorylation and dephosphorylation, catalyzed by kinases and phosphatases, are important mechanisms for the regulation of physiological processes. The number of phosphatases is about one third of the kinases, despite their role in regulating the levels of protein phosphorylation is equivalent to that of kinases. The limited number of phosphatases is counterbalanced by the diverse regulatory subunits associated with a small number of catalytic subunits. In eukaryotic cells, serine/threonine (Ser/Thr)-specific phosphatases are the most abundant, with protein phosphatase-1 (PP1) and -2A (PP2A) enzymes being of special importance.

Structure and regulation of PP1 and PP2A

PP1 is expressed in all eukaryotic cell types and is involved – among others - in cell division regulation, protein synthesis, cytoskeleton organization or in the regulation of apoptosis. PP1-type enzymes are composed of a catalytic subunit (PP1c) of 36-38 kDa and a regulatory subunit with variable structure. Catalytic subunits alone are capable of dephosphorylating substrate molecules, however, their substrate specificity is quite broad in this form. Although, PP1c occurs in cells in holoenzyme form bound to different regulatory subunits, which can affect both the substrate specificity and catalytic activity of the enzyme.

PP2A enzymes have prominent roles in the regulation of cell proliferation, apoptosis, cell motility and cell cycle. Like the PP1 enzyme family, the catalytic subunit of PP2A can interact with several regulatory subunits to form a holoenzyme. Structurally, the enzyme functions as a heterotrimer: it is composed of a 36 kDa catalytic C subunit (PP2Ac), a 65 kDa regulatory A subunit (PR65) and one of the diverse B subunits. The dissociable B subunits can be divided into four groups: B (B55 or PR55), B' (B56 or PR61), B'' (PR48/PR72/PR130) and B''' (PR93/PR110) proteins, each of which has 2-5 known isoforms.

Several membrane-permeable PP1 and PP2A inhibitory toxins produced by microorganisms occur naturally and their concentration-dependent selective inhibitory activity makes them essential for studying the role of specific phosphatases. Such toxin is for example calyculin A (CLA), which binds to the

catalytic subunit, thus prevents phospho-substrate binding. CLA is a more effective inhibitor of PP2A than PP1.

We have identified several plant-derived, tannin-type polyphenolic compounds as inhibitors of PP1 and PP2A, including the catechol-type epigallocatechin-3-gallate (EGCG), the most abundant component in green tea, which has been shown to induce apoptosis in several tumor cell lines. EGCG has been shown to bind to the PP1 catalytic subunit thus inhibiting PP1c activity.

Several phosphatase inhibitor proteins are also produced in cells, which are generally low molecular weight, thermostable proteins and exert their inhibitory effects mostly through phosphorylation on their threonine side chains. Such proteins include inhibitor-1 and inhibitor-2, the 32 kDa dopamine and cAMP-regulated phosphoprotein or the 17 kDa protein kinase C (PKC)-potentiated phosphatase inhibitor (CPI-17), which, in addition to the free catalytic subunit, may also inhibit the activity of the myosin phosphatase (MP) holoenzyme. Later, other CPI-17-like proteins (KEPI, PHI, GBPI) were described, all of which carry a central PP1 holoenzyme inhibitor domain (PHIN) and also possess a conserved activating phosphorylation site but differ from CPI-17 in their inhibitory effect on MP. An isoform of LIM-kinase 2 protein (LIMK2), the 75 kDa LIMK2v1, also contains high sequence similarity to the PHIN domain and a phosphorylation site (Thr596) corresponding to the Thr38 side chain of CPI-17. The occurrence and functions of LIMK2v1 are poorly understood, and its expression at protein level was first described only recently. LIMK2v1, although possessing kinase activity, does not directly phosphorylate cofilin, the traditional substrate of LIM kinases, but instead interacts with PP1c to inhibit its affinity for phosphorylated cofilin. However, the role of the Thr596 phosphorylation of LIMK2v1 in PP1 inhibition has not been investigated so far, and we have therefore sought to investigate these questions in detail.

Structure, regulation and functions of myosin phosphatase

MP is composed of a 38 kDa PP1 catalytic subunit (PP1c δ), a 110-130 kDa myosin-binding regulatory subunit (MYPT1) and a 20 kDa subunit (M20) of unknown function. The function of MP is mainly regulated by the MYPT1 regulatory subunit, which interacts with the catalytic subunit and the substrate molecule. Its major structural elements are the PP1c-binding KVKF sequence motif located in the N-terminal region of MYPT1, the domain containing

ankyrin-like repeats, and two nuclear localization signals (NLS). An acidic region, two ionic regions, a Ser/Thr-rich section, and the middle insert and leucine zipper motifs can also be identified, although the latter does not occur in all isoforms.

MP can be regulated by phosphorylation of the regulatory subunit, dissociation of the holoenzyme, by change in its localization or inhibition of the enzyme by inhibitor proteins. Among these options the possibilities of the regulatory subunit's phosphorylation are discussed below. The two most extensively characterized inhibitory phosphorylation sites of MYPT1 are the Thr696 and Thr853 residues, which play prominent role in the regulation of MP-regulated cellular processes. Phosphorylation of the Thr696 site of MYPT1 can be catalyzed by several kinases (e.g. ROCK, ILK, ZIPK, PAK, MDPK), among which the RhoA-activated kinase (ROCK) is also able to phosphorylate the Thr853 residue of MYPT1. The process of MP activation is less well described but it is known to be associated with an increase in the concentration of cyclic nucleotides. MP activation can also be induced via the EGCG/67LR/PP2A pathway. 67LR is a non-integrin type laminin receptor that is overexpressed on the cell surface of many tumor cell types and also functions as a cell surface receptor for EGCG binding. 67LR activates PP2A via the adenylate cyclase/cAMP/PKA pathway, leading to inhibition of oncoproteins and activation of the tumor suppressor merlin. There are also data suggesting that EGCG can activate MP via 67LR, which may play a role in EGCG-induced cell proliferation reduction.

In addition to smooth muscle cells, MYPT1 can also be found in non-muscle cells and is expressed in almost all subcellular fractions. The firstly identified substrate of MP was myosin light chain (MLC20), a component of the myosin II protein, which is essential for the regulation of muscle contraction, cell division, cell motility and cell adhesion, and whose dephosphorylation by MP is followed by muscle relaxation. Several studies have shown that dephosphorylation of ERM proteins is also catalyzed by MP. Merlin, a product of the NF2 tumor suppressor gene, has also been shown to be dephosphorylated by MP at the Ser518 residue, thereby activating and implicating it in contact inhibition and growth inhibition. One of the most important interacting proteins of MP, which plays a key role in the regulation of cell cycle, is the

retinoblastoma protein (pRb), also a tumor suppressor, whose regulation is impaired in several tumor types. pRb regulates cell cycle and proliferation by inhibiting the transcription of E2F-mediated genes. Our group has identified MP as an enzyme involved in the dephosphorylation of the Thr826 side chain of pRb in THP-1 leukemia cells. MP is also involved in the regulation of gene expression, mitosis and vascular tone. In addition, its neuronal substrates have also been identified.

Role of PP1 and MP in leukemia and macrophage differentiation

In case of leukemias, as is common in other cancers, the delicate balance of phosphorylation-dephosphorylation processes is disrupted. For example, important kinase cascade pathways involved in survival and apoptosis may be activated or inhibited, and elevated activity of PP1 and PP2A enzymes, as well as low expression and hyperphosphorylation of the tumor suppressor pRb, have been described. Our group studied the effect of PP1-inhibitor tautomycin (TM) and calyculin A (CLA) at PP2A-inhibitory concentrations on the sensitivity of THP-1 leukemic cells to daunorubicin (DNR) and found that although the phosphatase inhibitors alone reduced cell viability, when used in combination with DNR they had a relative survival-promoting (protective) effect. DNR treatment resulted in decreased phosphorylation of the Thr826 side chain of pRb, whereas when used in combination with CLA, we detected increased phosphorylation of pRb, which promotes tumor cell survival. These results suggest that the elucidation of the role of phosphatases may be of particular importance, as these enzymes may become novel targets for chemotherapy.

Macrophage differentiation is a process mediated by different signaling cascades and its inappropriate progression may lead to a variety of diseases, for example, excessive macrophage differentiation may lead to a chronic inflammatory condition with the formation of atherosclerotic plaques, called atherosclerosis, macrophages may therefore be attractive targets for the development of atherosclerosis-preventive therapy. Protein kinases and phosphatases, as important regulators of cell signaling, may also play a role in controlling this process. It is known, that during differentiation of HL-60 leukemic cells, PP1 activity decreases in the cytosolic fraction and increases in the membrane and nuclear fractions, and CLA treatment enhances cell differentiation, suggesting that PP1 and PP2A enzymes may play a role in the

regulation of macrophage differentiation. Increased expression of the $\text{Mg}^{2+}/\text{Mn}^{2+}$ -dependent protein phosphatase-1A (PPM1A) at the onset of differentiation inhibits cell arrest and the production of inflammatory cytokines. In a recent study, the role of the RhoA/ROCK signaling pathway in macrophage differentiation of U937 lymphoma-derived cells was investigated and a significant increase in the expression of ROCK1 and ROCK2 was observed upon PMA induction concomitant with an increase in the phosphorylation level of the MYPT1 Thr853 residue. In addition, silencing the components of the RhoA/ROCK pathway significantly inhibited macrophage differentiation, suggesting that this pathway may play an important role in the regulation of macrophage differentiation. However, the role of MP as a downstream effector of the RhoA/ROCK pathway in the differentiation process has not been investigated, and therefore, our work aims to investigate this pathway in more detail.

AIMS

PP1-type enzymes dephosphorylate many proteins, and a detailed understanding of their regulation may contribute to a better understanding of the signaling pathways they are involved in. One possible mechanism of PP1 regulation is inhibition by inhibitory proteins. The LIMK2v1 protein contains a PP1 inhibitory domain, but the role of its phosphorylation at the Thr596 residue in the inhibitory effect of the protein is not clear. The role and regulation of PP1-type MP has been extensively studied, but its involvement in the regulation of chemosensitivity and macrophage differentiation is not clearly understood. Our previous results have shown that phosphatase inhibitors reduce the sensitivity of THP-1 leukemic cells to chemotherapeutic agents, and MP has been identified as an enzyme mainly responsible for the phosphorylation status of pRb. Based on these findings, we aimed to investigate the possible chemosensitizing effect of MP activation against chemotherapeutic agents. Stimuli that induce macrophage differentiation have also been known for long, but their precise regulatory mechanisms are not clear yet. A recent study has suggested a role for the RhoA/ROCK signaling pathway - which catalyses the inhibitory phosphorylation of MYPT1 – in the regulation of differentiation, but the role of MP in macrophage differentiation has not been investigated yet.

Based on this, the main objectives of our work were to study the following questions:

1. Identification of the kinases that catalyze the phosphorylation of LIMK2v1 at Thr596 and investigation the role of this phosphorylation in PP1 inhibition.
2. Studying the effect of EGCG-induced MP activation on the phosphorylation status of pRb and merlin proteins, and the investigation of the effect of EGCG treatment on the sensitivity of THP-1 leukemic cells to DNR.
3. Investigating the expression and phosphorylation of MYPT1 during macrophage differentiation of THP-1 cells. Studying the effect of MP activation on the proliferation and the ability of attachment of differentiating macrophages.

MATERIALS AND METHODS

Cell culture, treatment and lysis

Human acute monocytic leukemia THP-1 cells were maintained in RPMI 1640 and tsA201 human kidney-derived cells in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin/streptomycin. Cells were cultured at 37 °C in a mixture of 5% CO₂ and 95% air saturated with water vapor.

Treatments with the chemotherapeutic agent were performed in RPMI media containing 1% FBS at a cell density of 5×10^5 cells/ml for 3, 24 or 48 h alone or in combination with 5 μ M EGCG pretreatment for 1 h.

Macrophage differentiation experiments were carried out in RPMI medium containing 10 % FBS for 24, 48 or 72 hours in the presence of 100 nM PMA or 100 nM vitamin D3 (VD₃) alone or in combination with 20 μ M EGCG (1 hour) or 10 μ M H1152 pretreatment (30 minutes).

After 36 h post-transfection, tsA201 cells were serum-starved overnight in serum-free DMEM solution, then treated with 5 μ M GF109203X, 200 nM Gö6983 or 10 μ M H1152 for 30 min, and incubated with 50 nM CLA for 30 min.

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl, 1% Triton X-100, 0.25% Na-deoxycholate, 2 mM EDTA freshly supplemented with 1% protease inhibitor cocktail, 2% phosphatase inhibitor cocktail, or 1 μ M microcystin (MC-LR)), then sonicated in an ultrasonic water bath after scraping on ice and centrifuged (16 000 g, 10 min, 4°C) to remove cell debris. After addition of 5 x SDS sample buffer, the supernatant was boiled at 100 °C for 5 min.

SDS polyacrylamide gel electrophoresis and Western blot

The cell lysates were separated by denaturing polyacrylamide gel electrophoresis using a Bio-Rad MiniProtean (200 V) and blotted onto nitrocellulose membrane using a Bio-Rad Criterion Blotter. A 3% BSA and 0.05% Tween-20 containing PBS solution was used to block the membrane and dilute the antibodies. Following the washing, blocking and antibody labeling steps, immunoreactions were detected using a high-sensitivity chemiluminescence-based (ECL) reagent.

Directed mutagenesis and transfection of Flag-LIMK2v1

Point mutations in the Flag-LIMK2v1 T596 side chain were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions. Following the PCR reaction, template DNA was digested with DpnI enzyme and transformed into XL-10 Gold ultracompetent cells. For Flag-LIMK2v1 protein overexpression, pReceiver-M11/Flag-LIMK2v1 plasmid was transfected into tsA201 cells using polyethylenimine (PEI) transfection reagent with a ratio of 1 μ g DNA:2 μ l PEI. The transfection mixture was added to cells in serum- and antibiotic-free culture medium, and after 6 hours the medium was supplemented with serum.

Immunoprecipitation

Following lysis of Flag-LIMK2v1-producing tsA201 cells, the lysate was incubated with Protein A Sepharose resin coupled with normal mouse serum, then the pre-purified lysate was added to anti-Flag M2 affinity resin and incubated for 2 hours at 4°C with continuous rotation of the tubes. After centrifugation, the unbound lysate was aspirated by pipette, then the resin-bound protein was treated according to the purpose of further use.

***In vitro* phosphorylation and purification of Flag-LIMK2v1**

In vitro phosphorylation of recombinant Flag-LIMK2v1 by PKC and ROCK enzymes were carried out with anti-Flag M2 affinity resin immobilized protein. Phosphorylation by PKC was performed using 0.1 μ g/ml of enzyme in Hepes containing buffer [20 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM DTT in the presence of 0.65 mM CaCl₂, 1 μ M MC-LR, 0.2 mM ATP] and phosphatidylserine-diolene micelles at 30°C. For phosphorylation by ROCK, 2 U/ml of ROCK enzyme was applied in Tris buffer (50 mM Tris-HCl, 0.1 mM EGTA; pH 7.5) in the presence of 1 μ M MC-LR, 5 mM MgCl₂ and 0.2 mM ATP. Samples were incubated in a water bath at 30°C for 0, 10, 30, 60, 90 and 120 min and kinase reactions were stopped by boiling with 1 $\times$ SDS sample buffer. The success of immobilization and phosphorylation was detected by Western blot.

To measure the phosphatase inhibitory effect of Flag-LIMK2v1, the immobilized protein was thiophosphorylated in the presence of 0.1 μ g/ml PKC using 0.2 mM ATP γ S as described above. Both the unphosphorylated and thiophosphorylated proteins bound to the resin were eluted with TBS containing

300 µg/ml Flag peptide. Protein concentration was measured at 595 nm by the Bradford method and the purity of the protein was checked by Coomassie Brilliant Blue staining.

Protein phosphatase activity measurement

The effect of Flag-LIMK2v1 on PP1 activity was determined using native PP1c with ^{32}P -labelled myosin light chain (^{32}P -MLC20) as substrate. For the determination of protein phosphatase activity, a ^{32}P -labelled myosin light chain (^{32}P -MLC20) was used as substrate. PP1c was diluted with 20 mM Tris-HCl (pH 7.4) buffer containing 0.1% BSA and 0.1% 2-mercaptoethanol, and incubated with 0-200 nM unphosphorylated or thiophosphorylated Flag-LIMK2v1 for 10 min at 30°C. The reaction was initiated by the addition of substrate, incubated for 10 min and stopped by the addition of 200 µl 10% TCA and 200 µl 6 mg/ml BSA.

To determine the phosphatase activity of PMA-treated THP-1 cells, the cells were lysed in TBS-EDTA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM EDTA) and after freezing in liquid nitrogen and sonication, the lysate was centrifuged (16 000 g, 10 min, 4°C). From the supernatant, 10 µl was used, supplemented with 10 µl of TM buffer [20 mM Tris-HCl (pH 7.4), 0.1% 2-mercaptoethanol]. Dephosphorylation of ^{32}P -MLC20 substrate was performed for 15-30 sec and the radioactivity of the released $^{32}\text{P}_i$ was determined in a scintillation counter after separation of the precipitated proteins by centrifugation.

Immunofluorescence

THP-1 cells were plated on a collagen-coated (5 µg/cm² collagen, 1 hour) 96-well Cell Carrier Ultra glass bottom plate and after 24 hours cells were fixed with 3.7% paraformaldehyde solution for 15 min, and then permeabilized with 0.1% Triton X-100 containing PBS solution for 10 min. Blocking was performed with PBS containing 1 % BSA for one hour at room temperature. The primary antibody was diluted 1:100 in 0.1 % BSA/PBS solution and the cells were incubated overnight at 4 °C. After incubation with Alexa 488-conjugated anti-rabbit IgG diluted 1:200 for 90 min, nuclei were stained with 2.5 µg/ml DAPI for 2 min. Confocal images were obtained using an Opera Phenix High Content Screening device with a 40x magnification aqueous objective lens.

Cell viability measurement with Alamar Blue assay

THP-1 cells were treated in 96-well plates at a cell concentration of 5×10^6 /ml. DNR was applied at several sublethal concentrations (0-200 ng/ml) alone or in combination with 5 μ M EGCG pretreatment for 24 h. Alamar Blue reagent was then added to the medium at a final concentration of 20 μ M, incubated for 2 hours and fluorescence was measured at 560/590 nm using a Tecan Spark Multimode Plate Reader (Thermo Fischer Scientific).

Measuring cell adherence by Sulforhodamine B assay and Electric Cell Substrate Impedance Measurement (ECIS)

The extent of adherence of differentiating macrophages was assessed by sulforhodamine B (SRB) assay. THP-1 monocytes were differentiated with 100 nM PMA alone, in combination with 10 μ M H1152 or 20 μ M EGCG pretreatment, and after 24, 48 and 72 hours, the medium was removed together with non-adherent cells and 10% (v/v) ice-cold trichloroacetic acid (TCA) was added to the cells. The precipitated proteins were stained with a 1 % acetic acid solution containing 0.4 % (m/v) SRB for 10 min and finally solubilized with 10 mM Tris base. Absorbance values were measured at 515 nm wavelength using a Tecan Spark Multimode Plate Reader.

The intensity of the attachment of differentiating cells to the surface was measured using an ECIS instrument (Applied Biophysics). Cells were pre-treated in 24-well plates as described in the previous section, then following PMA-induction cells were immediately pipetted into 8W10E ECIS 8 electrode culture dishes. Adherence was monitored by real-time, minute-by-minute measurement of resistance for 72 h using an electrical pulse at a frequency of 4000 Hz.

Statistical analysis

Statistical analysis and charts were created using GraphPad Prism 8 software. Data were analyzed using Student's t-test or two-way ANOVA with post hoc Šidak-, Tukey- or Dunnett-tests. Columns represent the mean of at least three independent measurements, error bars represent the standard deviation of the data.

RESULTS AND DISCUSSION

Investigating the phosphatase inhibitory role of LIMK2v1

LIM kinases may be involved in the regulation of many cellular functions, such as cell motility, cell division, differentiation, apoptosis or tumorigenesis. Quite few is known about the functional differences between the three LIMK2 isoforms, but some data suggest, that these isoforms are likely to have non-overlapping functions, as they differ in both tissue and subcellular expression and are expressed at different developmental stages. In our work we have investigated the least described LIMK2v1 isoform, which differs structurally from the other isoforms by the presence of a C-terminal PP1 inhibitory sequence.

In the *in vitro* phosphorylation assay of Flag-LIMKv1, we found that both protein kinase C (PKC) and ROCK enzymes phosphorylate the protein at its CPI-17-like phosphorylation sequence, the Thr596 residue. It is known that ROCK is able to phosphorylate all the three isoforms of LIMK2 on the Thr505 site for LIMK2a and on the Thr484 residue for LIMK2b and LIMK2v1, but no study has reported phosphorylation by ROCK on the Thr596 site. Phosphorylation of LIMK2a by PKC has also been reported, but PKC-induced phosphorylation of LIMK2v1 has not been investigated yet. Phosphorylation of LIMK2v1 also occurs in living cells, as phosphorylation of the overexpressed Flag-LIMK2v1 at the Thr596 site in tsA201 cells was increased upon the phosphatase inhibitor CLA treatment. PKC inhibitors (GF10923x and Gö6983) in a negligible extent, whereas the ROCK inhibitor H1152 significantly reduced the elevated phosphorylation of the Thr596 side chain observed in the presence of CLA. To investigate the role of the Thr596 site of LIMK2v1, non-phosphorylatable (Thr596A) and phosphorylation mimicking (Thr596E) mutants were generated. Our immunoprecipitation experiments with the phosphosite-mutant protein showed that the phosphorylation of Thr596, which is detectable in the presence of CLA in case of the wild-type Flag-LIMK2v1, is absent in the Thr596A mutant protein, confirming that phosphorylation occurs on this residue. The Thr596E mutant protein showed a slight cross-reactivity with the anti-CPI-17^{pT38} antibody. In the study of the phosphatase inhibitory effect of LIMK2v1, we found that even the unphosphorylated protein slightly reduced the phosphatase activity of PP1c in the 100-200 nM concentration

range, whereas thiophosphorylated Flag-LIMK2v1 already exerted a similar phosphatase inhibitory effect at 10 nM concentration, and when it was applied at 200 nM concentration, it reduced the activity of PP1c to 10%. These data suggest that the phosphatase inhibitory effect of LIMK2v1 is significantly enhanced by phosphorylation, like other members of the CPI-17 protein family.

Nowadays there is increasing evidence suggesting that LIMK2 may play a role in tumor development and metastasis, suggesting that LIMK2v1 may serve as a novel therapeutic target for tumor treatment as an inhibitory regulator of protein phosphatases.

Role of MP in the regulation of chemosensitivity of THP-1 cells

One of the most challenging problems in cancer therapy is the development of chemoresistance of cancer cells. Our group has previously shown in THP-1 cells that DNR-induced cell death is associated with activation of phosphatases, moreover inhibition of phosphatases reduced chemosensitivity against DNR, which was associated with increased phosphorylation of the Thr696 residue of MYPT1 and Thr826 residue of pRb. All these data suggest that activation of MP may have the potential to enhance the sensitivity of THP-1 leukemic cells to chemotherapeutic agents. No direct agonist of MP is available, but it is known that binding of EGCG to the 67 kDa laminin receptor (67LR) results in dephosphorylation of the MYPT1 Thr696 inhibitory phosphorylation site, thereby activating the MP holoenzyme. However, little is known about how the EGCG-induced phosphatase activation contributes to its cell growth inhibitory and antiproliferative effect. The activation of MP by EGCG via the 67LR/PKA/PP2A pathway may provide an opportunity to understand these functions, and we therefore investigated the presence of this signaling pathway's components and the localization of the receptor in THP-1 cells. Our results show that the components of the EGCG/67LR/PKA/PP2A pathway are all present in THP-1 cells, and the receptor is localized mainly in the cytoplasm and plasma membrane region, suggesting that treatment of leukemia-derived THP-1 cells with EGCG may induce MP activation via the EGCG/67LR/PKA/PP2A signaling pathway.

The DNR-induced cytotoxicity combined with EGCG pretreatment was significantly increased at DNR concentrations of 25, 50 and 100 ng/ml, suggesting that EGCG enhanced the sensitivity of THP-1 cells against DNR

treatment. Analysis of the expression of the protein proliferating cell nuclear antigen (PCNA), used as a marker of cell proliferation, also showed that PCNA expression was most significantly reduced in the presence of both EGCG and DNR, suggesting that the combined treatment has a more potent proliferation inhibitory effect than the two agents separately. It is known that DNR treatment induces apoptosis in THP-1 cells, therefore we examined the extent of PARP protein cleavage as a marker of apoptosis in THP-1 cells subjected to the above treatments. Significantly increased levels of PARP cleavage were observed following combined treatment compared to the treatments when agents were used alone, suggesting that EGCG significantly increases the extent of DNR-induced apoptosis in THP-1 cells.

EGCG treatment alone did not have a significant effect on cell viability during long-term incubations (24-48 h), but significantly reduced phosphorylation of MYPT1 Thr696 residue after 4 h, as well as DNR treatment alone. We also examined the phosphorylation state of MP tumor suppressor substrates and showed that EGCG alone slightly, whereas DNR significantly reduced phosphorylation of the Thr826 residue of pRb. However, a much more pronounced dephosphorylation of both MYPT1 Thr696 and pRb Thr826 residues was observed upon combined treatment. The MP activation observed in the presence of EGCG may explain the decrease in phosphorylation of the pRb Thr826 residue, which may partly explain the increased cell death in response to the combined treatment. The phosphorylation state of the Ser518 residue of the other tumor suppressor substrate of MP, merlin was not significantly affected by either EGCG or DNR alone, but a notable (although not statistically significant) decrease in phosphorylation was detected in the presence of both agents, which is required for the cell growth inhibitory function of merlin via the Hippo signaling pathway. These data suggest that EGCG induces dephosphorylation of pRb and merlin proteins by enhancing MP activity and, through this, may potentiate their tumor suppressor functions. These processes may explain how EGCG is able to sensitize THP-1 cells against DNR.

The regulatory role of myosin phosphatase in macrophage differentiation

PP1 and PP2A enzymes play important role in regulating cell signaling pathways through the dephosphorylation of several proteins, but little is known about their functions in macrophage differentiation. To investigate the changes in the activity of PP1 and PP2A during macrophage differentiation, *in vitro* phosphatase activity of PMA-stimulated THP-1 cells was measured using phosphorylated MLC20 substrate. We found that the phosphatase activity of untreated THP-1 cells decreased successively, but the decrease in phosphatase activity of PMA-treated cells was even more pronounced, resulting in only 42% residual activity after 48 hours of differentiation. These data suggest that PMA-induced differentiation of THP-1 cells may be associated with inhibition of PP1 and/or PP2A enzymes.

Recently it has been shown that PMA-induced macrophage differentiation requires the activation of ROCK, which is involved in the regulation of cytoskeletal processes through phosphorylation of specific substrates (MLC20, ezrin/radixin/moesin, adducin). Phosphorylation levels of these proteins are cooperatively regulated mainly by ROCK and MP, but the possible role of MP in the differentiation process is currently unclear. We investigated the expression of the catalytic and regulatory subunits of MP and the phosphorylation state of MYPT1 during PMA and vitamin D3 (VD₃)-induced differentiation of THP-1 cells and we found, that neither the expression of the PP1c δ catalytic nor the MYPT1 regulatory subunit changed during differentiation, but a significant increase was observed in the phosphorylation state of the inhibitory phosphorylation sites of MYPT1, suggesting that PMA-induced macrophage differentiation requires not only activation of ROCK but also simultaneous inhibition of MP. MLC20-mediated actomyosin rearrangement appears to be essential for the differentiation of monocytes into macrophages. In agreement with this, we observed a significant increase in phosphorylation of MLC20 upon PMA stimulation of THP-1 cells. The effects of pharmacological activation of MP on macrophage differentiation were investigated by pretreatments with the ROCK inhibitor H1152 or with the MP-activating agent EGCG. The application of H1152, as well as EGCG prior to PMA stimulation, significantly reduced the phosphorylation level of both MYPT1 inhibitory phosphorylation sites and MLC20 Ser19 residue, reduced

proliferation inhibition and decreased cell scavenging ability, as well as differentiation capacity of monocytes as indicated by decreased expression of CD11b, as a marker of macrophage differentiation.

In PMA-differentiated macrophages EGCG has been shown to reduce the expression of matrix metalloproteinases via 67LR, which may lead to the stabilization of atherosclerotic plaques. As shown in our studies, EGCG or H1152 treatment of THP-1 cells prior to PMA stimulation significantly reduced the phosphorylation of MYPT1 inhibitory sites, thus activating the holoenzyme, and reduced phosphorylation of the Ser19 residue of MLC20. Considering the important role of MLC20 phosphorylation status in macrophage differentiation, our results may provide novel insights into the anti-atherosclerotic effects of EGCG.

SUMMARY

1. During the study of the phosphatase inhibitory role of LIMK2v1 protein, we identified the Thr596 residue, which was phosphorylated by both protein kinase C (PKC) and Rho-A-associated kinase (ROCK) in *in vitro*, while in cellular systems only the role of ROCK was apparent in the phosphorylation. Phosphorylated LIMK2v1 exhibited significantly higher PP1c inhibitory potency compared to the unphosphorylated protein, supporting our hypothesis that, like other members of the CPI-17 protein family, phosphorylation of LIMK2v1 is required to exert its efficient phosphatase inhibitory function.

2 We have shown that the sensitivity of leukemic THP-1 cells to daunorubicin may be enhanced by activation of myosin phosphatase (MP) upon EGCG treatment, which is associated with decreased proliferation and increased apoptosis of tumor cells. In addition to decreased phosphorylation of the Thr696 residue of MYPT1 – indicating the active state of the holoenzyme - we observed a decrease in phosphorylation of the MP tumor suppressor substrates, retinoblastoma (pRb) and merlin proteins, which may result in cell cycle inhibition and may explain suppressed cell viability.

3. During PMA-induced macrophage differentiation of THP-1 monocytes, we observed decreased activity of PP1 and PP2A enzymes, and increased phosphorylation of the inhibitory sites of the MP regulatory subunit, indicating an inhibited state of holoenzyme. Inhibition of MP was also observed during VD₃-induced macrophage differentiation. Activation of MP by inhibition of ROCK or stimulation of the the 67LR/PKA/PP2A pathway by EGCG was manifested in decreased expression of CD11b protein, a marker of differentiation, and was associated with a decrease in cell attachment ability, an essential feature of PMA-induced macrophage differentiation. In addition, PMA-induced phosphorylation of the MP substrate MLC20 at the Ser19 residue also showed a significant decrease in the presence of the ROCK inhibitor or EGCG, which may play a role in the differentiation process through regulation of the actomyosin complex. We concluded that inhibition of MP may be crucial for the progression of macrophage differentiation.



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PhD Publication List

Candidate: Emese Tóth

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Tóth, E.**, Erdődi, F., Kiss, A.: Activation of Myosin Phosphatase by Epigallocatechin-Gallate

Sensitizes THP-1 Leukemic Cells to Daunorubicin.

ACAMC. 21 (9), 1092-1098, 2021.

DOI: <http://dx.doi.org/10.2174/1871520620666200717142315>

IF: 2.507 (2020)

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Total IF of journals (all publications): 16,776

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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.

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