Ph.D. THESIS

The control of protein phosphatase-1 by phosphorylation of the regulatory subunit and binding of inhibitors to the catalytic subunit

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INTRODUCTION

Protein phosphorylation and dephosphorylation is an important regulatory mechanism in the control of many cellular functions. Protein phosphorylation is catalyzed by the protein kinases, while protein phosphatases are responsible for the reversibility of this modification since they dephosphorylate the phospho-serin (P-Ser)/threonine (P-Thr) or phospho-tyrosine (P-Tyr) bonds. Protein phosphatase-1 (PP1) and -2A (PP2A) are key players among the P-Ser/Thr specific phosphatases, and they are estimated to be responsible for up to 90% of the P-Ser/Thr specific dephosphorylation in cells.

Structure and regulation of protein phosphatase-1 holoenzymes

In cells PP1 exists in holoenzyme forms, in which PP1 catalytic subunit (PP1c) is complexed to regulatory subunits. PP1c is expressed in all eukaryote cell types as three major isoforms, termed PP1α, γ or δ. The substrate molecule is bound in the active centre of PP1c to an Y-shaped surface consisting of C-terminal, hydrophobic and acidic grooves. It is thought that the hydrophobic and acidic grooves are involved in the interaction with the substrate. Two metal ions - probably Fe^{2+} and Zn^{2+} - are coordinated by the active centre, which facilitate the hydrolysis of the P-Ser/Thr side chain in the substrate by activation of a structural water molecule.

The catalytic activity of PP1c can be inhibited by naturally occurring membrane-permeable toxins, such as microcystin-LR (MC-LR), okadaic acid (OA), calyculin-A (CL-A), tautomycin (TM), and tautomycetin (TMC). Binding of these inhibitors to the hydrophobic groove of the enzyme results in occupation of part of the catalytic site and this blocks the binding and hydrolysis of the phosphosubstrate. These toxins are also able to suppress the activity of PP2A. We found that gallotannin also inhibits the activity of PP1 and PP2A, however, the mechanism of the inhibition and the molecular details of the interaction between tannin components and phosphatases are still not known.

Protein phosphatase catalytic subunit alone can catalyze the dephosphorylation of various phosphoproteins. However, in cells PP1c is bound to regulatory subunits, which target the catalytic subunit to the substrate or to the subcellular compartment containing the substrate molecule. Binding of the regulatory subunit to both the catalytic subunit and the substrate may result in a favourable position for the catalytic subunit to carry out dephosphorylation, thus the regulatory subunit may control substrate specificity too. The regulatory proteins have various structure but they share a common short sequence, called PP1c-binding motif (K/R-

x_1-V/I-x_2-F/W), which ensure specific binding to PP1c. Reversible phosphorylation of the regulatory subunit, generally mediated by second messenger molecules, can affect its association with the catalytic subunit and/or influence the enzyme activity. Nevertheless, many aspects of these diverse functions of the targeting subunits have remained to be elucidated.

Interaction of PP1 with proteins of various structure and function, play a key role in numerous cellular processes, such as the regulation of cell cycle, metabolism, transcription, translation, cell motility and reproduction. We investigated the myosin phosphatase holoenzyme, therefore the structure and possible regulatory mechanisms of this enzyme will be described.

Structure, function and regulation of myosin phosphatase

Myosin phosphatase (MP) consists of PP1cδ (38 kDa), a large regulatory subunit (110-130 kDa) termed myosin phosphatase target subunit-1 (MYPT1), and a 20 kDa subunit of yet unknowm function. MYPT1 plays an important role in regulation of the activity of the enzyme, since it targets PP1cδ to the substrate molecule. The N-terminal region of MYPT1 is involved in the interaction with PP1c, in which the ^{35}KVKF^{38} binding motif has an essential role. Binding of the PP1c-binding motif initiates the interaction of MYPT1$^{1-22}$, which then facilitate the binding of the ankyrin repeats (MYPT1$^{40-296}$) and the acidic cluster (MYPT1$^{304-511}$). During the formation of the PP1cδ–MYPT1 complex, the N-terminal part of MYPT1 will elongate the hydrophobic groove of the Y-shaped catalytic centre of PP1cδ, thereby it helps the binding of the hydrophobic substrate sequence such as found C-terminal to the phosphorylated residue in the 20 kDa light chain of myosin (MLC20).

The role of MP in contractile activity of smooth muscle was characterized first, where it is involved in dephosphorylation of MLC20. Phosphorylation of MLC20 induces contraction of the actomyosin complex, while dephosphorylation by MP results in relaxation.

MP activity can be regulated by phosphorylation of the MYPT1 subunit. Phosphorylation of Thr695 side chain by RhoA activated Rho-kinase (ROK) cause the inhibition of MP. Simultaneously, ROK can phosphorylate myosin and therefore it is involved in the induction of a Ca^{2+}-independent contraction of smooth muscle. ROK can also catalyze the phosphorylation of the Thr850 side chain in MYPT1. Initial studies suggested that phosphorylation of MYPT1 on Thr850 weakens the interaction between MYPT1 and myosin. However, the effect of Thr850 phosphorylation on MP activity was not studied in details yet.

Dephosphorylation of the phosphorylated side chains of MYPT1 is supposed to be catalyzed by PP2A, which seems to be more effective in dephosphorylation of phospho-Thr850.

Despite the initial assumptions, MP (and MYPT1) is expressed not only in smooth muscle cells, but in non-muscle cells too, and it is present in almost all subcellular compartments. The diverse localization pattern of MP, and its numerous interaction partners suggest that it can also be involved in the dephosphorylation of phosphosubstrates other than phosphorylated myosin.

Possible role of PP1 and MP in controlling the cell cycle

The retinoblastoma protein (pRb) is the product of the retinoblastoma susceptibility gene and it functions as a key regulator of cell cycle progression. In hypophosphorylated state, pRb interacts with and inactivates E2F and other transcription factors, thereby suppressing the synthesis of proteins necessary to progression of the cell cycle form G1 to S phase. Hyperphosphorylation of pRb upon extracellular signals relieves this inhibition by dissociating E2F from pRb. Phosphorylation of pRb is catalyzed by cyclin-dependent kinases, while studies with cell-permeable inhibitors of protein phosphatase suggest the role of PP1 and PP2A in the dephosphorylation process. Inhibition of PP2A affects pRb phosphorylation via decreasing the activity of cyclin-dependent kinases. Interaction of pRb with various PP1c isoforms and dephosphorylation of several sites in pRb by PP1c has been demonstrated. However, the regulatory protein targeting PP1c to pRb has remained to be identified. pRb is largely confined to the nucleus, therefore the nuclear PP1c-binding proteins such as nuclear inhibitor of PP1 (NIPP1) and phosphatase nuclear targeting subunit (PNUTS) were assumed as possible regulatory proteins. Transfection of MYPT1 as a GFP fusion protein into NIH3T3 cells resulted in a predominant nuclear localization of the protein and induced G1/S arrest accompanied by apoptotic cell death, supposedly by increased dephosphorylation of pRb. These data imply that MP (and MYPT1) may be involved in the dephosphorylation of pRb and in the regulation of cell cycle progression.

AIMS

While the regulation of our model enzyme, MP, has been extensively investigated, the function of the phosphorylation sites in MYPT1 is still not elucidated in all regards. Similarity of amino acid sequences surrounding the Thr850 phosphorylation site to that of the inhibitory Thr695 site raises the possibility that phosphorylation of Thr850 may also be inhibitory. ROK phosphorylates Thr695 and Thr850 *in vitro*, however, it is also important to address whether other kinases are involved in the phosphorylation of these sites in smooth muscle and non-muscle cells. The role of protein phosphatases in cell death is not clear in details concerning the molecular mechanisms, especially with respect to protein-protein interactions and regulatory phosphorylation of targeting subunits such as MYPT1. Our previous findings suggest that tannin components inhibit the activity of protein phosphatase-1 and -2A, but the selectivity toward PP1 and PP2A as well as the detailed inhibitory mechanisms have not been examined yet.

Our studies aimed the investigation of the following questions:

1. Investigation of the role of Thr850 phosphorylation in MYPT1 in the regulation of MP activity. Study of phosphorylation of Thr695 and Thr850 in MYPT1 in cells in the presence of phosphatase inhibitors and activators of signalling pathways.
2. Study of the role of PP1 and PP2A inhibition in the regulation of the viability of leukemic cells treated with chemotherapeutic agents.
3. To uncover the interaction of MP and pRb and the role of MP in the control of the phosphorylation level of pRb.
4. The study of the inhibitory effect of the tannin constituents (epigallocatechin-3-gallate (EGCG) and penta-O-galloyl-D-glucose (PGG)) on PP1c and to investigate the PP1c-inhibitor interactions to characterize the structure-activity relationship.

MATERIALS AND METHODS

Protein preparation

Myosin and MLC20 was isolated from turkey gizzard, and phosphorylated by myosin light chain kinase. Myosin phosphatase holoenzyme was prepared from chicken gizzard. PP1c was isolated from rabbit skeletal muscle and was separated from PP2Ac on heparin-Sepharose and further purified by FPLC-MonoQ chromatography.

Full length MYPT1 (chicken sequence) (GST-MYPT1), the Thr695 to Ala mutant (MYPT1^{T695A}), the Thr850 to Ala mutatnt (MYPT1^{T850A}), the triple mutant (Ser694 to Ala, Thr695 to Ala, Thr850 to Ala) (MYPT1^{AAA}), and the C-terminal framgment (GST-MYPT1$^{667-1004}$) were expressed in bacteria, and purified by ion-exchange and/or glutathione-Sepharose 4B affinity chromatography. Recombinant PP1cδ (rPP1cδ) and deletion mutants of MYPT1 (His-MYPT1$^{1-296}$, His-MYPT1$^{1-633}$) were expressed in *E. coli* with hexahistidine tag (His), and purified by chromatography on Ni-agarose. Recombinnat PP1cδ was renatured and activated in the presence of Mn^{2+}-ions before purification.

Cell culture and treatment

Human rat aorta (A7r5) cells were grown in D-MEM, while human monocytic leukemic (THP-1) cell suspension in IMDM culture medium completed with 10 % FBS. Before treatments cells were serum-starved for 16 h. A7r5 cells were treated with 100 nM CL-A (20 min), or 1 μM LPA (20 min) or 10 μM Y27632 (30 min). In some experiments, serum-starved cells were pretreated for 30 min with 10 μM Y27632 before treatment with CL-A or LPA. THP-1 cells (~10^6/ml) were treated with 2 μg/ml DNR, or with 50 nM CL-A for 60 min, and then with or wihout 2 μg/ml DNR for 6, or 12 hours.

Preparation of subcellular fractions of THP-1 cells

THP-1 cells were lysed in 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM NaF, 1 mM Na-vanadate, 1 mM PMSF and 0.5% protease inhibitor coctail. The suspension was briefly vortexed and kept on ice for 15 min. Triton X-100 was added to a final concentration of 0.5% followed by gentle vortexing for 10 s and centrifugation (15 000 *g*, 45 s). The supernatant obtained was considered the cytosolic fraction. The pellet containing the nuclei and cell debris, was resuspended in 20 mM Hepes (pH 7.9), 420 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM NaF, 1 mM Na-

vanadate, 1 mM PMSF and 0.5% protease inhibitor coctail. The suspension was kept on ice for 25 min and then centrifuged (15 000 x *g*, 10 min), and the supernatant obtained was considered the nuclear extract.

Western blot

Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins from the gel were blotted onto nitrocellulose membranes. After blocking with inert proteins, membranes were incubated with primary antibodies, followed by HRP-conjugated secondary antibodies, and the immunoreactions were detected by enhanced chemiluminescence (ECL).

Protein phosphatase assay

Phosphatase activity was determined at 30°C with 3 μM ^{32}P-myosin, 2 μM ^{32}P-MLC20 or 0.5 μM ^{32}P-MBP-pRb-C as substrate. The incubation time was 0.5 to 30 min depending on the phosphatase sample. The released $^{32}P_i$ was determined (after precipitation of proteins and centrifugation) from the supernatant in scintillation counter.

Immunoprecipitation and pull-down assay

Polyclonal anti-MYPT1$^{1-296}$ or monoclonal anti-pRb antibody was incubated with Protein-A Sepharose in immunoprecipitation buffer (IP buffer: 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 1 mM NaF, 1 mM Na-vanadate, 1 mM PMSF and 0.5% protease inhibitor coctail), and then the suspension was centrifuged. THP-1 cell lysate was precleared with Protein-A Sepharose, and then incubated with the antibody-coupled Protein-A Sepharose or non-immune serum-coupled resin as control. Beads were washed with IP buffer and boiled in SDS sample buffer. Pull-down assay was carried out with C-terminal fragment of pRb fused to maltose binding protein (MBP-pRb-C). THP-1 lysate was precleared with uncoupled amylose resin, and then incubated with MBP-pRb-C fusion protein-coupled amylose resin. Bound proteins (pRb, MYPT1) were resolved by SDS-PAGE and detected by immunoblotting using specific antibodies.

Immunofluorescence and confocal microscopy

After treatment with different effectors THP-1 cells were plated on coverlips by cytospin. Cells were fixed with paraformaldehyde (4 %) and permeabilized with 0.02% (v/v) Triton X-100 dissolved in PBS. After blocking in 1% BSA/PBS, coverlips were incubated

with primary antibodies diluted in 0.1% BSA/PBS, and then with Alexa 488- or Alexa 543-conjugated secondary antibodies. For nuclear staining, 2.5 μg/ml DAPI was applied. Finally, coverlips were covered with mounting medium using Antifade Light Kit. Cell cultures prepared for immunofluorescence were imaged on Zeiss LSM 510 confocal laser scanning microscope equipped with Helium/Neon, Krypton/Argon and UV laser detectors.

Cell viability assays

After the treatments, methyl-thiazol tetrazolium (MTT) was added to the culture medium of THP-1 cells ($1x10^6$/ml), which can be converted by viable cells into colored formazan product that can be measured spectrophotometrically at 562 nm. The optical density is linearly related to the number of viable cells. To investigate the influence of tannin constituents on cell viability, THP-1 cells were treated with 0-100 μM PGG or EGCG for 24 h, and then 20 μM Alamar Blue was added, and fluorescence was measured at 530/590 nm.

Caspase- activity assay

The change in caspase-3 activity was determined by the increase in the cleavage of DEVD-AMC substrate. After the treatments, THP-1 cells were lysed in lysis buffer (10 mM Hepes (pH 7.4) 2 mM EDTA, 0.1% CHAPS, 1 mM PMSF, 5 mM DTT and 0.5% protease inhibitor coctail) and kept on ice for 10 min. Lysate was centrifuged, and the supernatant was assayed with 2 μg/ml DEVD-AMC substrate in 2x reaction buffer (100 mM Hepes (pH 7.25), 20% sucrose, 0.1% CHAPS, 5 mM DTT). Samples were incubated for 60 min at 37°C and fluorescence was measured at 355/460 nm.

Surface plasmon resonance

Interactions of pRb with PP1c or MYPT1, and PP1c with EGCG or PGG were analyzed by surface plasmon resonance (SPR)-based binding experiments using the Biacore 3000 instrument. Proteins were immobilized on sensor chip CM5 through anti-GST antibody or via primary amine groups of the proteins. The putative interacting molecules were injected over the surface at different concentrations. Binding to the immobilized proteins was monitored as a sensogram where response unit (RU) values were plotted against time. The sensograms were analyzed using the BIAevalution 3.1 software.

RESULTS AND DISCUSSION

1. Phosphorylation of Thr695 and Thr850 in MYPT1 and its effect on myosin phosphatase activity

Wild-type (GST-MYPT1) and Thr to Ala point mutants (MYPT1^{T695A}, MYPT1^{T850A} and MYPT1^{AAA}) of MYPT1 were phosphorylated by ROK, and we found that the extent and rate of phosphorylation of Thr695 and Thr850 were similar. To confirm that the expected site in each mutant was phosphorylated phosphospecific antibodies, that recognized phopsho-Thr695 or phospho-Thr850, were used.

We investigated the effect of thiophopsphorylation at Thr695 and/or Thr850 on the phosphatase activity of native PP1c with P-myosin and P-MLC20 as substrate. Our data indicate that phosphorylation or thiophosphorylation of either Thr695 or Thr850 results in inhibition of native PP1c activity, and the efficacy of inhibition with P-myosin, the physiological substrate, is similar for each phosphorylated site.

The phosphorylation of MYPT1 in A7r5 cells was investigated in the presence of lysophosphatidic acid (LPA), an activator of the RhoA/ROK pathway, the ROK inhibitor Y27632, or the protein phosphatase inhibitor calyculin-A (CL-A). For control cells, both Thr695 and Thr850 were partially phosphorylated. Y27632-treatment eliminated the phosphorylation of Thr850, but it only slightly diminished the phosphorylation of Thr695. The phosphatase inhibitor CL-A markedly increased phosphorylation of Thr850, but had little effect on phosphorylation of Thr695. In the presence of CL-A, only the phosphorylation of Thr850 was sensitive to inhibition of ROK by Y27632. LPA induced a marked increase in phosphorylation of both Thr695 and Thr850, however pretreatment with Y27632 inhibited only the phosphorylation of Thr850 significantly. These data suggest that both sites in MYPT1 are phosphorylated in A7r5 cells. Thr850 is the primary phosphorylation site for ROK under physiological and pathological conditions, while phosphorylation of Thr695 may be catalyzed by other kinases.

Under *in vitro* conditions phosphorylation of Thr695 and Thr850 by ROK is indistinguishable, while distinct kinase(s) are involved *in vivo* in phosphorylation of Thr695. The mechanism involved in discrimination of these sites is unknown. There is a possibility that in different cells the accessibility of these two sites to distinct kinases may vary. An attractive hypothesis is that the distinction in phosphorylation sites offers a mechanism for integration of different signalling pathways converging on MYPT1, and resulting in inhibition of MP.

2. Role of inhibition of PP1 and PP2A enzyme activity in survival of leukemic cells

The influence of cell-permeable inhibitors of PP1 and PP2A on viability of cells is contradictory, since they can induce apoptosis or survival depending on cell type or experimental conditions. Here we show, that CL-A alone at 10-100 nM concentration cause moderate decrease in cell survival. The cytotoxic daunorubicin (DNR) decreased the number of viable cells dramatically, but pre-treatment of the cells with CL-A significantly attenuated DNR-induced cell death.

Protein phopshatase activity in THP-1 cells was slightly increased by DNR, while 50 nM CL-A suppressed phosphatase activity by more than 85%, both in the absence or in the presence of DNR. DNR-treatment of THP-1 cells caused degradation of pRb and this was attenuated in the presence of CL-A. CL-A increased the level of phosphorylation of pRb and decreased the DNR-induced caspase-3 activation. Our data imply that the inhibition of pRb dephosphorylation and the phosphorylation-dependent inactivation of caspase-3 can account for the effect of CL-A. Phosphorylation of pRb results in protection against proteolytic degradation, therefore CL-A can also contribute to antiapoptotic effects via increasing the level of phosphorylation of pRb. Inactivation of caspase-3 can be a consequence of the inhibition of PP2A by CL-A, which cause the inhibition of caspase-3 dephosphorylation and thereby it maintains the inactive, phosphorylated form of caspase-3. In addition, CL-A can increase the phosphorylation and activation of p38-MAP kinase, which in turn phosphorylates and inactivates caspase-3.

3. The role of myosin phosphatase in controlling the phosphorylation level of retinoblastoma protein

To clarify the role of protein phosphatase types in the dephosphorylation of pRb we carried out *in vitro* phosphatase activity measurements with purified PP1c, PP2Ac and MPH with phosphorylated MBP-pRb-C as substrate. P-MLC20 was applied as a reference substrate and the amounts of phosphatases were choosen to dephosphorylate this substrate to a similar extent (25-35%). At these amounts PP1c resulted in only a slight dephosphorylation of P-pRb (2,6%), while PP2Ac appeared to be ineffective. In contrast, MPH dephosphorylated both substrates to similar extent, which suggest that MYPT1 can be involved in dephosphorylation of pRb by PP1c. In accordance with this finding, an inhibitory antibody (anti-MYPT1$^{1\text{-}296}$) which was shown to interfere with the targeting function of MYPT1, inhibited the pRb phosphatase activity of both purified MPH and THP-1 cell lysate.

To differentiate PP1 and PP2A activity we determined the pRb phosphatase activity in THP-1 cell lysate at different okadaic acid (OA) concentrations. OA inhibited pRb phosphatase activity significantly only in the 10-1000 nM concentration range, suggesting that PP1-type phosphatase is predominantly involved in pRb dephosphorylation. To dissect the phosphatase activity due to MP in cell lysate thiophosphorylated CPI-17 was applied in the assays. CPI-17 was shown to specifically inhibit MP without any effect on other PP1 holoenzymes. The results show that more than 50% of the total phosphatase activity in the cell lysate was inhibited by CPI-17 both with P-MLC20 and P-pRb substrates, indicating that MP may substantially contribute to the dephosphorylation of pRb in THP-1 cells. Phosphorylation of Thr695 and Thr850 in MYPT1, which results in inhibition of MP activity, can account for the increased phosphorylation of pRb in the presence of CL-A. In untreated and DNR-treated cells, a relatively low level of phosphorylation of Thr695 was detected, whereas no phosphorylation of Thr850 was observed. CL-A induced phosphorylation of MYPT1 at both inhibitory sites either in the presence or in the absence of DNR. These data imply, that phosphorylation-dependent inhibition of MP can be involved in the regulation of pRb phosphorylation level in THP-1 leukemic cells. The CL-A-induced, indirect inhibition of MP is consistent with the findings that during 50 nM CL-A treatment of the cells the toxin causes complete inhibition of PP2A. The suggested mechanism for MP inhibition is that CL-A may unmask the activity of MYPT1 kinase(s) and/or suppress the activity of protein phosphatases responsible for the dephosphorylation of these inhibitory sites in MYPT1. This mechanism is in accordance with previous finding that PP2A can catalyze the dephosphorylation of these sites *in vitro*.

Involvement of MP in dephosphorylation of pRb suggests the interaction of MYPT1 and pRb which was proved by reciprocal immunoprecipitation and pull-down assay with MBP-pRb-C fragment in THP-1 cell lysate. To characterize further the interaction between pRb-C with MYPT1 or PP1c we initiated surface plasmon resonance (SPR)-based binding studies. Injection of PP1c over the surface covered by MBP-pRb-C proved the interaction of PP1c with pRb, which was notably decreased in the presence of the PP1c-binding peptide of MYPT1, suggesting the role of a PP1c-binding motif-like sequence of pRb in this interaction. It was shown that pRb-C binds to the immobilized full-lengths and also to the N- and C-terminal fragments of MYPT1, among which the interaction with the N-terminal peptide can play important role in the targeting function. Using the BIAevaluation 3.1 software, association constants (K_a) were estimated from the sensograms for the interaction of pRb-C with PP1c (K_a = 1,42 ± 0,12 x 10^6), GST-MYPT1 (K_a = 1,34 ± 0,42 x 10^7), His-MYPT1$^{1-633}$,

(K_a = 4,46 ± 3,1 x 10^6) and GST-MYPT1$^{667-1004}$ (K_a = 3,28 ± 1,11 x 10^6). These data suggest the following mechanism: in PP1c-pRb complex PP1c has low catalytic activity toward pRb. MYPT1, which binds to both PP1c and pRb, can direct the catalytic centre of PP1c toward the pRb substrate forming a functional complex.

Colocalization of MYPT1 and pRb and the effect of CL-A on their localization was shown in THP-1 cells using confocal microscopy. In untreated and DNR-treated cells both MYPT1 and pRb were predominantly localized in the nucleus. Merged images indicated partial colocalization of these two proteins. Upon CL-A treatment, both MYPT1 and pRb translocated to the cytoplasm. After combined treatment with CL-A and DNR only MYPT1 was translocated into the cytoplasm. pRb^{pT826} displayed similar staining pattern to pRb, which suggest that the nucleo-cytoplasmic shuttling of pRb induced by CL-A was not due to the phosphorylation of Thr826, rather than the involvement of another phosphrylation site in pRb could be considered. The translocation pattern of MYPT1 was further confirmed by Western blotting of the subcellular fraction of the differentially treated THP-1 cells. In untreated and DNR-treated cells, MYPT1 were localized to the nuclear fraction, and its phosphorylation level was low. After CL-A-treatment MYPT1 translocated to the cytosolic fraction, and a parallel upshift of the MYPT1 bands on the blots was observed after phosphorylation. Phosphorylation of Thr695 and Thr850 does not result in slower migration of MYPT1 during SDS-PAGE, thus this upshift in the MYPT1 bands may be the result of phosphorylation at additional site(s). We hypothesize that phosphorylation of these additional, yet unidentified site(s), may influence nuclear export of MYPT1 during CL-A treatment. Distribution pattern of PP1c isoforms in the subcellular fractions did not change by the different treatments. These data implies that translocation of MYPT1 from the nucleus to the cytoplasm takes place without the involvement of PP1c.

4. Effect of tannin constituents on phosphatase activity

Penta-O-galloyl-D-glucose (PGG), one of the main constituents of tannins, has an inhibitory effect on the phosphatase activity of native and recombinant PP1c (for native PP1c: IC_{50} = 6.5 μM and for rPP1cδ: IC_{50} = 0.7 μM), and PP2A (IC_{50} > 100 μM) enzymes. In addition to PGG, epigallocatechin-3-gallate (EGCG) (IC_{50} = 0.93 μM) and aleppo tannin, a naturally occurring gallotannin (0.52 μM) also inhibit the activity of PP1c, while gallic acid, a building block of tannin components is without any effect. These data suggest that PGG is a

partially selective inhibitor of PP1, and at lower concentrations it can differentiate between PP1 and PP2A activity *in vitro*.

SPR-based binding experiments proved the binding of PGG and EGCG to immobilized rPP1cδ. Kinetic studies of the interaction suggest slow association and slow dissociation, with an association constant of $K_a = 2{,}25 \pm 1{,}16 \times 10^5$ for PGG, and $K_a = 1{,}48 \pm 0{,}67 \times 10^6$ for EGCG. Binding of PGG and EGCG to the surface was decreased significantly when rPP1cδ was saturated with MC-LR or OA, and the dissociation of the formed complexes was also faster. Since MC-LR and OA are bound to the Y-shaped hydrophobic groove of the catalytic center of PP1, these competition assays suggest that PGG and EGCG also interact with this hydrophobic region of the enzyme.

Saturation transfer difference (STD) NMR-studies on the interaction of rPP1cδ with PGG and EGCG revealed the involvement of the hydrophobic rings and the hydroxyl groups of the gallic acid in binding to the enzyme. The results of the initial docking experiments imply also the essential role of the aromatic rings of EGCG in binding to the hydrophobic cleft of the Y-shaped catalytic center of PP1c, that is further stabilized by the H-bonding formed between the hydroxyl groups of EGCG.

PGG and EGCG have different effect on the viability of THP-1 cells, since PGG causes higher extent of cell death than does EGCG at similar concentrations. To clarify the mechanism and the role of phosphatase inhibitory effect of tannin molecules in this process will be the subject of future studies.

CONCLUSIONS

1. The phosphorylation of myosin phosphatase (MP) target subunit (MYPT1) on Thr850 by ROK inhibits the activity of the type 1 phosphatase (PP1) catalytic subunit (PP1c). This phosphorylation can be detected in smooth muscle cells under physiological or pathological conditions implicating its involvement in the regulation of MP activity by distinct signalling pathways.

2. Treatments of cells with CL-A, a membrane-permeable phosphatase inhibitory toxin, suggest that PP1 and PP2A enzymes play important role in the regulation of the viability of leukemic cells. CL-A attenuates the extent of daunorubicin (DNR) induced cell death and it is thought to be due to increased phosphorylation and decreased proteolytic degradation of the retinoblastoma protein (pRb).

3. MP is involved in the dephosphorylation of pRb and in this process MYPT1 targets PP1c to pRb substrate and it increases pRb phosphatase activity. The phosphorylation of MYPT1 on Thr695 and Thr850 may play also an important role in decreasing MP activity in leukemic cells, thereby MP inactivation is involved in the increase of the pRb phosphorylation level. The phosphorylation level of pRb controls transitions between the different phases of cell cycle, therefore MP is implicated in the regulation of cell cycle and chemoresistance of leukemic cells.

4. Treatment of cells with CL-A induced changes of the localization pattern of MYPT1 and pRb resulting in translocation of both protein from the nucleus to the cytoplasm. The translocation of these proteins may be due to their increased phosphorylation and it could also influence the viability of the cells.

5. The tannins and their constituent molecules (PGG és EGCG) are identified as a novel family of the protein phosphatase inhibitory compounds. They inhibit PP1c preferentially and bind to the hydrophobic groove of the catalytic center in PP1c. Structural modifications of these compounds may be promising in producing novel, effective and pharmacologically important inhibitors of protein phosphatases.

PUBLICATION LIST

Publications the Ph.D. thesis is based on

Kiss A, Lontay B, Bécsi B, Márkász L, Oláh E, Gergely P, Erdődi F.: Myosin phosphatase interacts with and dephosphorylates the retinoblastoma protein in THP-1 leukemic cells: Its inhibition is involved in the attenuation of daunorubicin-induced cell death by calyculin-A. *Cell Signal*. **20**: 2059-2070. (2008) (IF: 4,887)

Murányi A, Derkach D, Erdődi F, **Kiss A**, Ito M, Hartshorne DJ.: Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. *FEBS Lett*. **579**: 6611-6115. (2005) (IF: 3,415)

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Márkász L, Hajas G, **Kiss A**, Lontay B, Rajnavölgyi E, Erdődi F, Oláh E.: Granulocyte Colony Stimulating Factor Increases Drug Resistance of Leukaemic Blast Cells to Daunorubicin. *Pathol Oncol Res*. (2008) (közlés alatt) (IF: 1,241)

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Lectures and posters related to the Ph.D. thesis

Conference lectures

Kiss A.: Role of myosin phosphatase in regulation of retinobalstoma. *Ph.D. Student Scientific Conference of University of Debrecen*, Hungary 2007

Kiss A, Lontay B, Márkász L, Oláh É, Gergely P, Erdődi F.: Role of myosin phosphatase in phosphorylation of retinoblastoma protein. *Conference of the Hungarian Biochemistry Society*, Pécs, Hungary, 2006

Kiss A.: Phosphorylation of Thr695 and Thr850 on the myosin phosphatase targeting subunit by Rho-kinase. *Ph.D. Student Scientific Conference of University of Debrecen*, Hungary, 2006

Kiss A.: Transduction of TAT-conjugated N-terminal fragment of myosin phosphatase target subunit in cells. *Ph.D. Student Scientific Conference of University of Debrecen*, Hungary, 2005

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