Project closing scientific report (2014.09.01. – 2019.08.31.)

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The project closing final report of the grant NKFI PD 111715 entitled **"Mechanism of nucleocytoplasmic trafficking of myosin phosphatase targeting subunit and its significance in the regulation of distinct cellular processes"** is as follows.

Background

Myosin phosphatase (MP) holoenzyme is a Ser/Thr-specific protein phosphatase composed of a protein phosphatase 1 catalytic subunit (PP1cδ) and a regulatory subunit called myosin phosphatase target subunit 1 (MYPT1). MYPT1 plays a targeting role, determines the substrate specificity, and its phosphorylation is known to modulate MP activity. MP is a multi-faceted enzyme: above its well-characterized function as the regulator of smooth muscle contractility mediating the dephosphorylation of myosin light chain, several newly identified substrates (e.g. retinoblastoma protein, merlin, HDAC7, polo-like kinase, PRMT5) support its role in other, non-contractile functions, including cell cycle, mitosis, gene expression regulation and neurotransmitter release. MYPT1 is generally distributed between the cytoplasm and the nucleus of cells, and it was suggested that trafficking of MYPT1 between different cellular locations requires phosphorylation, but the mechanism of nuclear translocation, the important phosphorylation site(s) on MYPT1, as well as the kinases and other interacting partners involved have not been identified yet.

Results

I. Revealing the mechanism of the nuclear-cytoplasmic trafficking of MYPT1

(a) Identifying the phosphorylation site(s) that may mediate the localization of MYPT1

Our previous experiments showed that in THP-1 leukemic cells - where MYPT1 appears to be mainly nuclear – the phosphatase inhibitor calyculin A (CLA) treatment causes nucleocytoplasmic translocation of MYPT1 with a parallel dissociation from PP1cδ. These results suggest that the phosphorylation site mediating the nuclear trafficking of MYPT1 may be located close to both the N-terminal NLS and the PP1c-binding motif. Hence, immunoprecipitation of Flag-MYPT1 from untreated and CLA-treated cells and subsequent phosphoproteomic analysis was performed in collaboration with the Laboratory of Proteomics Research, Biological Research Centre, Szeged. This analysis identified Ser20 as the only phosphorylation site in the N-terminal region of MYPT1.

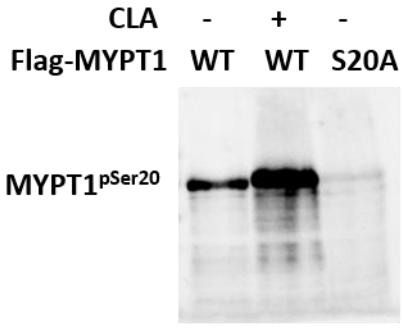

Figure 1. The specificity of the anti-MYPT1^{pSer20} antibody

To be able to detect MYPT1-Ser20 phosphorylation, we have initiated phosphopeptide synthesis and generation of phospho-specific antibody. The specificity of the anti-MYPT1pSer20 antibody was tested in Western blot experiments using lysates from cells overexpressing either wild-type or S20A mutant Flag-MYPT1. In agreement with our previous phosphoproteomic data, Flag-MYPT1 was significantly phosphorylated at Ser20 in untreated cells and the phosphorylation increased markedly upon CLA-treatment. In contrast, no phosphorylation was detected in case of Flag-MYPT1S20A mutant, proving the specificity of the antibody (Fig. 1). The antibody can also detect endogenous levels of MYPT1-Ser20 phosphorylation in different cell types, although with much weaker signal, suggesting that the basal Ser20 phosphorylation is relatively low.

(b)Determination of the subcellular targeting of phosphorylated and unphosphorylated forms of MYPT1

In order to determine whether the newly identified Ser20 phosphorylation site is critical for the subcellular targeting of MYPT1, we have carried out site-directed mutagenesis to produce phosphorylation site (Ser20Ala) and phosphomimic mutants (Ser20Asp). Transfection of the mutant plasmids into Cos-7 cells showed that the wild-type Flag-MYPT1 localizes almost exclusively in the nucleus of Cos-7 cells with discrete areas of higher fluorescence as revealed by confocal microscopy. Both phosphosite mutants exhibit similar localization pattern, suggesting that the nuclear export of MYPT1 is independent of Ser20 phosphorylation.

(c)Identifying the phosphorylating kinase(s) that determine the subcellular localization of MYPT1

Ser20 side chain is predicted to be phosphorylated by casein kinase 2 (CK2), but the ability of CK2 to phosphorylate MYPT1 was not investigated before. Our i*n vitro* phosphorylation studies using recombinant kinase proved that CK2 is able to phosphorylate full-length MYPT1. Subsequently, we have used the specific CK2 inhibitor, TBBt in cell culture treatments. In THP-1 cells TBBt has decreased the mobility shift of MYPT1 on SDS-PAGE caused by CLA, but did not inhibit the translocation of MYPT1 from the nucleus to the cytoplasm during these treatments. TBBt also failed to decrease the CLA-induced Ser20 phosphorylation, as it was proved later with the help of the phospho-specific antibody (see Figure 2.) These suggest that CK2 might phosphorylate residues other than Ser20 in MYPT1.

In the meanwhile, independently of our research project, Hu and his co-workers have also identified Ser20 as a novel phosphorylation site of MYPT1, and check point kinase 1 (Chk1) as the phosphorylating kinase (Hu et al. Cell Cycle, 2018). Using rabusertib, a specific inhibitor of Chk1, we also found that it can substantially decrease the CLA-induced phosphorylation of endogenous as well as overexpressed MYPT1 at Ser20, proving that Chk1 can control the phosphorylation of this site (Figure 2.).

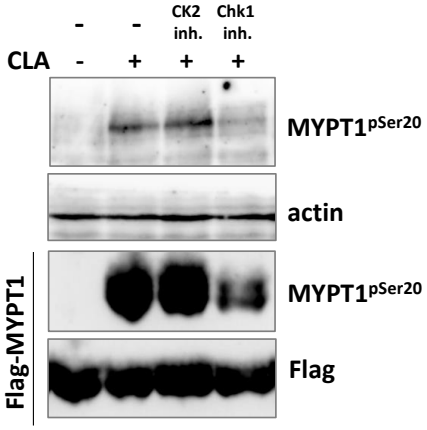

Figure 2. Effect of CK2 and Chk1 inhibition on MYPT1-Ser20 phosphorylation

We have also tested the effect of rabusertib on MYPT1 localization, and it was found that inhibition of Chk1 can attenuate, but not fully prevent the CLA-induced nucleocytoplasmic translocation of MYPT1 in THP-1 cells, although these results require further verification, and the involvement of other kinases and/or other phosphorylation site(s) in mediating the subcellular targeting of MYPT1 cannot be excluded.

(d) Studying the effect of MYPT1 phosphorylation on the subunit interaction of MP

We have hypothesized that phosphorylation of Ser20 sidechain will initiate the dissociation of MYPT1 from PP1cδ. Hu et al., however, suggested that Ser20 phosphorylation of MYPT1 is essential for its interaction with PP1cδ. To elucidate the effect of MYPT1 phosphorylation on the subunit interaction

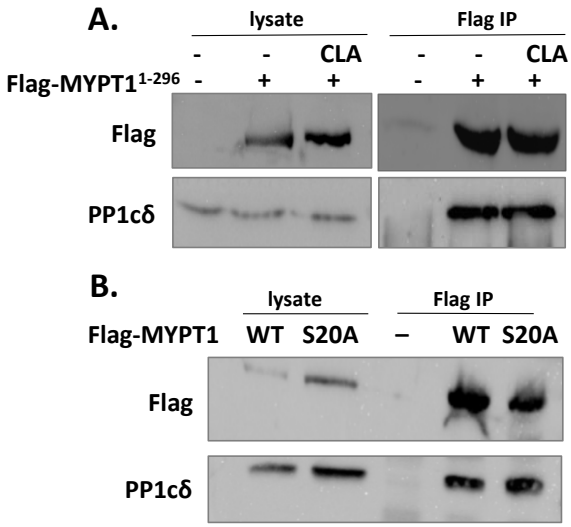

Figure 3. Effect of MYPT1-Ser20 phosphorylation on subunit intercation of MP

of MP we performed immunoprecipitation experiments using Flag tagged MYPT1 proteins as bait. Treatment of the cells with CLA was used to trigger Ser20 phosphorylation. PP1cδ co-precipitated with wild-type Flag-MYPT1 as well as the truncated MYPT1(1-296)-Flag either in the absence or in the presence of CLA suggesting that Ser20 phosphorylation does not exclude the binding of MYPT1 to PP1cδ (Figure 3A.). Furthermore, the phosphorylation site mutants Flag-MYPT1S20A and the truncated MYPT1(1-296)S20A-Flag were both able to precipitate PP1cδ, making the role of Ser20 phosphorylation in subunit interaction doubtful (Figure 3B.). We initiated surface plasmon resonance binding experiments with unphosphorylated and thiophosphorylated forms of MYPT1 to investigate whether Ser20 phosphorylation changes its affinity to PP1cδ, but these experiments have not been completed yet.

II. Studying the role of nucleocytoplasmic trafficking of MYPT1 in the regulation of distinct cellular processes

We have found that Thr696 – an inhibitory site of MYPT1 – becomes phosphorylated during PMA-induced macrophage differentiation of THP-1 cells. Blocking MYPT1-Thr696 phosphorylation by pharmacological inhibition of Rho kinase, thereby activation of MP reduces THP-1 adhesion and attenuates macrophage differentiation (Figure 4A.). In addition, we detected a decrease in the amount of nuclear MYPT1 upon PMA stimulation (Figure 4B.). These suggest that the nucleocytoplasmic shuttling of MYPT1 and/or inhibition of MP may be involved in the regulation of macrophage differentiation. However, we don't have a clear experimental evidence on the involvement of MYPT1-Ser20 phosphorylation yet. We plan to complete this subproject in the next few months.

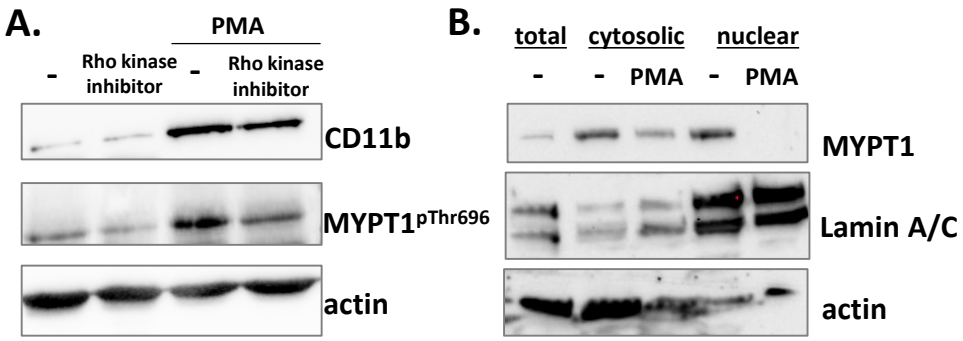

Figure 4. Involvement of MP inhibition and MYPT1 nucleocytoplasmic translocation in macrophage differentiation

We have also tested the involvement of MYPT1-Ser20 phosphorylation in an adipogenesis model studied in our group. It was found that phosphorylation of Ser20 shows a transient increase during the adipocyte differentiation of mesenchymal stem cells, and it is suspected to mediate the proteasomal degradation of MYPT1. Experiments testing the effect of the Chk1 inhibitor on adipocyte differentiation are in progress.

During the period of the grant, three posters were presented at international conferences with the support of NKFI. Three BSc and three MSc dissertations have been completed. One PhD student was involved in the subprojects of the grant.

Two papers were supported by the project and have been published in peer-reviewed papers:

Dedinszki D, **Kiss A**, Márkász L, Márton A, Tóth E, Székely L, Erdődi F: Inhibition of protein phosphatase-1 and -2A decreases the chemosensitivity of leukemic cells to chemotherapeutic drugs. Cell Signalling 27: 363-372, 2015

Kiss A, Erdődi F, Lontay B. Myosin phosphatase: Unexpected functions of a long-known enzyme. Biochim Biophys Acta Mol Cell Res. 1866(1): 2-15, 2019

There is one manuscripts in preparation:

Tóth E, Erdődi F, **Kiss A.**: Activation of myosin phosphatase by epigallocatechin-gallate sensitizes cancer cells to daunorubicin through dephosphorylation of tumor suppressor proteins