FINAL REPORT

Regulatory role of TIMAP-PP1 complex at the crossroad of PKC and endothelin signaling

Introduction and major aims of the study

Reversible protein phosphorylation of proteins at Ser/Thr residues is one of the most important posttranslational modifications of proteins to regulate cellular processes (1). The phosphorylation status of proteins depends on the balance of the activities of protein kinases and phosphatases. Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are the two major phospho-Ser/Thr specific enzymes and are responsible for the dephosphorylation of more than 90% of cellular proteins at phospho-Ser/Thr residues.

The major function of pulmonary endothelial cells is to form a continuous monolayer on the luminal surface of the lung vasculature. The endothelium is a metabolically active surface, producing various vasoactive mediators, such as NO, prostacyclin, endothelin-1 (ET-1), serotonin, and thromboxane. Structural changes in the pulmonary vasculature, caused by inadequate (de)phosphorylation of proteins or altered production of vasoactive agents, can lead to serious diseases. Our research focuses on pulmonary endothelial signaling pathways with specific emphasis on the role and substrate specificity of TIMAP (TGF-β inhibited membrane-associated protein) and PP2A B55α, regulatory subunits for PP1 and PP2A, respectively. TIMAP protein has been considered as a member of the myosin phosphatase targeting protein (MYPT) family (2). Our workgroup previously demonstrated specific protein-protein interaction between TIMAP and δ isoform of the catalytic subunit of PP1 (PP1cδ) (3,4) and identified ERM (ezrin-radixin-moesin) proteins and eEF1A1 (eukaryotic elongation factor 1A1) as TIMAP-PP1c substrates (3-5). Barrier protective role of TIMAP has been also shown in human pulmonary artery endothelial cells (HPAEC) (3) and the regulatory effect of PKA-primed GSK-3β-mediated phosphorylation of TIMAP (4,6) on PP1c activity was studied in details (4,7). To uncover novel cellular functions, we investigated the role of TIMAP in cell functions related to angiogenesis. Based on the obtained results we commenced to study ET-1 signaling in endothelial cells with specific emphasis on the regulation of endothelin production.

The major aims for this proposal, based on the literature and the preliminary results were:

(i) ***explore the physiological significance of the newly discovered TIMAP-ECE-1 interaction and the increased endothelin-1 level in TIMAP depleted cells***

(ii) ***identify the PKC site(s) on TIMAP and characterize the effect of phosphorylation on PP1c activity and substrate specificity***

The project has been executed according to the major lines of the original aims, therefore we state that it conforms to the original research plan. However, regarding the detailed research questions, during the course of study there have been some necessary modifications and we have also pursued a few novel developments related to the planned studies.

RESULTS AND DISCUSSION

I. *Study of the physiological significance of TIMAP-ECE-1 interaction and the increased endothelin-1 level in TIMAP depleted cells*

Endothelin-1 (ET-1) is a predominant isoform of endothelins expressed in the vasculature and the most potent vasoconstrictor (8), produced primarily by vascular endothelial cells. Endothelin induced signaling pathways can affect blood pressure and vascular tone, but the influence of endothelins on tumor cells is also significant. First, we tested the effect of TIMAP depletion in pulmonary artery endothelial cells. Using specific siRNA, the mRNA and protein level of TIMAP decreased greatly after 48-72 hrs of the treatment in endothelial cells. Depletion of TIMAP induces morphological change, as cells showed "angiogenic" phenotype (Fig.1). In contrast, viability and proliferation of endothelial cells were not affected. To investigate the potential role of TIMAP in angiogenesis, we examined the effect of TIMAP depletion on endothelial tube formation *in vitro*. Silencing of TIMAP accelerated *in vitro* tube formation measured by Matrigel 3D tube formation assay (Fig. 2). ECIS (electric cell-substrate impedance sensing) measurement is a non-invasive method to monitor the cell movement, shape changes or migration in real time. Migration speed of TIMAP depleted cells was also enhanced compared to control or nonsiRNA treated cells. By Proteome Profiler™ Human Angiogenesis Antibody Array analysis we showed that secreted **endothelin-1** level increased in pulmonary artery endothelial cells in the absence of TIMAP. Endothelial cells express ET_B type endothelin receptor and the produced ET-1 induces an autocrine signal. BQ788 is a specific ET_B receptor antagonist (9-11). To test whether the changes observed in TIMAP-depleted cells are actually evoked by the elevated ET-1 secretion, experiments were repeated with the usage of BQ788. The antagonist treated cells showed no significant difference in their morphology, tube formation or migration compared to non-siRNA treated cells suggesting that BQ788 diminished the effect of TIMAP depletion. The next question we had to answer, at which point of endothelin signaling is TIMAP a key player. Formation of mature ET-1 requires a proteolytic cleavage catalyzed by endothelin converting enzymes (ECEs) (12,13). The generation of ET-1 is crucially

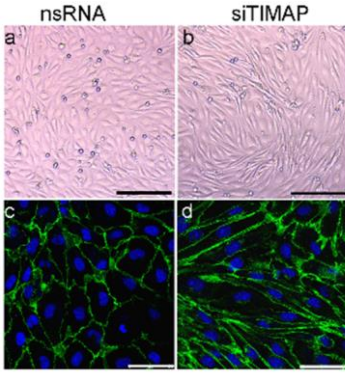

Figure 1. ***Morphology of control and TIMAP depleted endothelial cells***. Light microscopy images of nsRNA (a) or siTIMAP RNA (b) treated cells or immunofluorescent staining using CD31 primary antibody of nsRNA (c) or siTIMAP RNA (d) treated cells 48 h post-transfection. Nuclei of the cells were stained using TO-PRO-3 Iodide (blue). Scale bars: 500 µm (a and b), 50 µm (c and d).

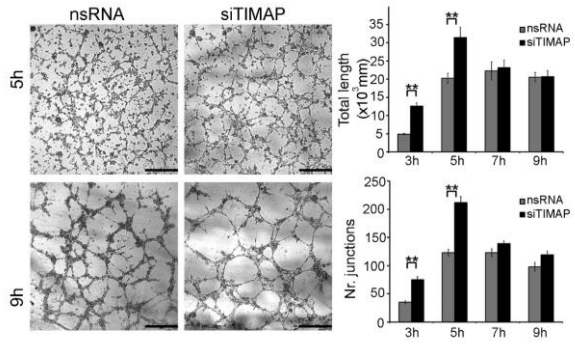

Figure 2. T*ube formation ability of control and TIMAP depleted endothelial cells*. Endothelial cells were treated with non-silencing or TIMAP-specific siRNA. Cells were seeded onto Matrigel 48h post-transfection and observed in time. Quantification of capillary formation was determined using the ImageJ program. Data are reported as mean ± SE. Statistical analysis was done with Student's t-test. Significant changes are indicated by asterisks;** ($P < 0.01$). Scale bar: 200 µm.

dependent on the presence and activity of ECE-1 in the membrane of the cells (14). PKC phosphorylates ECE-1c and the phosphorylated form translocates to the plasma membrane (15). The phosphatase catalyzing the dephosphorylation of ECE-1 was not known. As a potential phospho-target of TIMAP-PP1, first we tested and confirmed a protein-protein interaction between TIMAP, PP1c and ECE-1 by immunoprecipitation. Depletion of TIMAP or activation of PKC increased the amount of ECE-1 in the membrane region and a consequent increase in endothelin-1 secretion was also detectable. The elevated ECE-1 level was mitigated in time in normal cells, but was clearly preserved in TIMAP-depleted cells. Our results indicated that silencing of TIMAP induces a reduction in TIMAP-PP1c activity connected to ECE-1. Shifting the kinase to phosphatase activity ratio either by activation of PKC by PMA treatment, or by decreasing the TIMAP-PP1c activity via depletion of the targeting subunit, TIMAP, of the phosphatase complex, resulted in an elevated ECE-1 protein level in the plasma membrane fraction of cells. 30 min after the removal of the PKC activator, the ECE-1 level in the membrane was significantly decreased. In TIMAP-depleted cells, ECE-1 protein level was already elevated and activation of PKC could not evoke any further increase. Using specific phosphatase inhibitors against PP1, PP2A and PP2B, we showed, that only PP1 inhibition caused an increase in ET-1 level. These results strongly suggest that the protein phosphatase, which controls ECE-1 trafficking and activity at the cell membrane through its dephosphorylation, is the TIMAP-PP1 complex.

Overall, our results indicate that PKC-phosphorylated ECE-1 is a TIMAP-PP1c substrate and this phosphatase complex has an important role in endothelin-1 production of endothelial cells through the regulation of ECE-1 activity. Results of the research were published in ***The International Journal of Biochemistry & Cell Biology*** *(16).*

II. *Identify the PKC site(s) on TIMAP and characterize the effect of phosphorylation on PP1c activity and substrate specificity*

The regulatory effect of TIMAP on PP1c activity depends on its phosphorylation state. So far two phosphorylation sites were identified, namely TIMAP Ser333 and Ser337, sites of a PKA-primed GSK-3β-mediated phosphorylation (3,4). Phosphorylation of these sites has no significant effect on the binding of PP1c, but considerably attenuates the inhibitory effect of TIMAP on PP1c activity (4,6,7). The primary structure of TIMAP also raises the possibility of its PKC phosphorylation. Amino acid sequence analysis based on consensus PKC sites predicts phosphorylation of TIMAP by PKC at one or more side chains, out of which most are present in the C terminal domain.

Protein-protein interaction was detected in bovine pulmonary endothelial cells between endogenous TIMAP and activated PKCα by immunoprecipitation (Fig. 3A). In vehicle treated control cells PKC was present in the cytoplasm, while TIMAP was mostly visible in the plasma membrane region of the cells

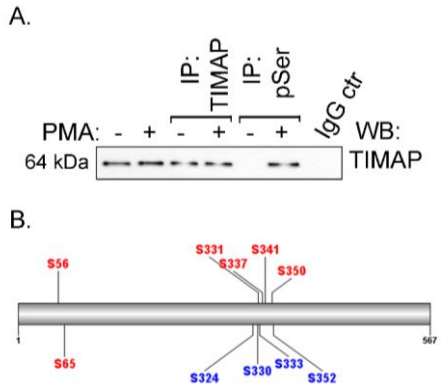

Figure 3. ***Interaction of TIMAP with PKC.*** A) BPAEC were treated with vehicle or with PMA for 30 min after which TIMAP or phospho-Ser PKC substrate antibodies were used for immunoprecipitation. Total cell lysates (lane 1 and 2) and IP complexes were probed for TIMAP in Western blot experiments. B) Schematic representation of the results of the GPS 3.0 Kinase-specific phosphorylation site prediction program for potential PKCα phosphorylation sites in TIMAP protein (NM_015568.2). Sites marked with red color have high score values, blue ones have medium scores.

shown by immunofluorescent staining. After activation of PKC by PMA treatment, PKC translocated into the membrane region and co-localized with TIMAP. Also, the amount of TIMAP increased in the membrane region due to PKC activation, shown by cell fractionation experiments from control and treated cells. To test whether TIMAP is a PKCα substrate, *in vitro* kinase assay was carried out using GST-tagged purified TIMAP protein. PKCα phosphorylated the full length recombinant TIMAP. The possible phosphorylation sites were mapped using GPS3.0 – a group-based prediction system (Fig. 3B) (17). To identify the PKC target side chain, several truncated or Ser-Ala mutant forms of TIMAP were created at the potential residues. We identified **Ser331 as the only PKC phosphorylation site** in TIMAP. In endothelial cells PKC depletion was carried out using specific siRNA. While phosphorylation of TIMAP upon PKC activation in control endothelial cells results in enrichment of TIMAP in the membrane that fails in PKC depleted cells. However, no crosstalk was detected with the previously identified PKA/GSK-3β induced enrichment of TIMAP at the plasma membrane (Fig. 4). Next, we tested the effect of PKC phosphorylation on the interaction with the previously identified TIMAP-PP1c substrates, namely phospho-ERM (ezrin, radixin, moesin), eEF1A1, LAMR1 and ECE-1. ERM proteins serve as cross-linkers between cortical actin filaments and integral membrane proteins through direct interaction. Their function depends on their phosphorylation state on a conserved threonine residue (18). Althrough PKA/GSK-3 phosphorylated TIMAP dephosphorylates ERM protein, we showed that binding of PKC phosphorylated TIMAP to ERM is severely reduced. This suggests an inhibitory effect of phospho-Ser331 on TIMAP-PP1 activity toward phospho-ERM. Constructs of phospho-mimicking, S331D and phospho-null, S331A mutants of TIMAP were created and endothelial cells were transfected. Phospho-ERM level in the membrane fraction of the phospho-mimic S331D TIMAP mutant transfected cells was increased, but the S331A mutant overexpressing endothelial cells had a lower phospho-ERM level. Activation of PKC leads to barrier disruption of endothelial cells, on the other hand, it was also shown that TIMAP is a positive regulator of endothelial barrier function. ECIS measurements were carried out on wild type, S331A and S331D TIMAP overexpressing endothelial cells. Consistent with the phospho-ERM level, electric cell substrate impedance sensing measurements showed that the S331A mutation of TIMAP resulted in faster recovery from the PMA treatment.

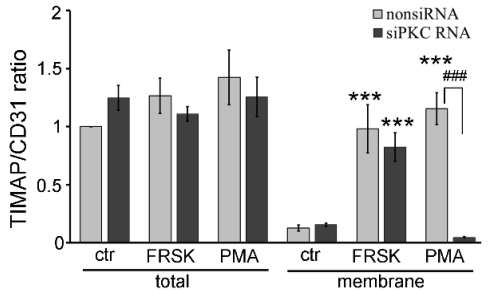

Figure 4. ***PKC and PKA/GSK3β pathways act independently on the localization of TIMAP.*** Densitometry analysis of Western blots of total cell lysates and membrane fractions of non-silencing RNA and PKC specific siRNA transfected BPAEC treated with vehicle, PMA or forskolin. CD31, membrane marker was used for protein level normalization. The results are presented as means ± SE (n=3). Statistical analysis was done with ANOVA. Significant changes are indicated (P<0.001). Star (*) marks statistical significance of the indicated bar graphs versus membrane fraction vehicle control; # marks statistical significance between the indicated bar graph pair.

In conclusion, compared to other MYPT family members of PP1 regulatory proteins, Ser331 is a unique phosphorylation site in TIMAP. Phosphorylation of TIMAP at Ser331 by PKC inhibits dephosphorylation of phospho-ERM upon PKC activation, therefore, it is a significant factor in PKC mediated barrier disruption and endothelial barrier regulation. Results of the research were published in ***Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*** *(19). The PI is the first author and also a corresponding author for the research.*

TIMAP protein, as a PP1c regulator contains a PP1c-binding motif and a bipartite nuclear localization signal (NLS) on its N-terminus (2). Accordingly, it was detected in the nucleus of vascular endothelial cells (3), but the significance of this localization is not known yet. Several phospho-site studies reported that TIMAP can be phosphorylated on **Ser69 side**

chain as well, near to the PP1c binding motif and the NLS. We hypothesized, that phosphorylation of Ser69 may affect the localization of TIMAP or its PP1c binding affinity. Phosphorylation mimicking S69D and phosphonull S69A TIMAP mutant encoding plasmids were created. Interactions between PP1c and wild type, S69A and S69D TIMAP proteins were investigated. Our results showed that although the Ser69 side chain is located near to the PP1 binding motif, this phosphorylation has no significant effect on the interaction. The recombinant plasmids were transfected into endothelial cells and the expression and localization of the proteins were checked by Western blot and immunofluorescent experiments. The overexpression of the proteins was successful but showed no difference compared to wild type. *In the future we would like to identify the protein kinase which is responsible for the phosphorylation of Ser69 and explore the relevance of the phosphorylation in endothelial cells. The research was presented on several conference and also a master's thesis was written by an undergraduate student under the supervision of the PI.*

Merlin (moesin-ezrin-radixin like protein), the product of neurofibromatosis type 2 gene belongs to the same family as ERM proteins (18). It was primarily recognized as a tumor suppressor, but it also functions as a membrane-cytoskeletal linker and regulator of multiple signaling pathways (20-22). The activity and localization of merlin is regulated by head to tail folding that is controlled by phosphorylation of the Ser518 side chain. Merlin localizes in the nucleus when the Ser518 side chain is not phosphorylated, while the phosphorylated form is present in the cytoplasm and the plasma membrane (21,23). Ser518 side chain of merlin was reported to be phosphorylated by cAMP-dependent protein kinase (PKA) or p21-activated kinase (PAK) (24,25). The phosphatase dephosphorylating the Ser518 side chain in endothelial cells has not been identified yet. Previously, we found that EBP50, ERM-binding phosphoprotein 50 (also known as NHERF1), an adaptor protein for ERM and merlin (26,27), localizes in the nucleus of the endothelial cells (27). Recently, we showed that merlin (dephospho-Ser518 form) interacts in the nucleus of endothelial cells with EBP50. Upon EBP50 depletion, merlin translocated from the nucleus, suggesting that binding of merlin to EBP50 is critical in the nuclear localization of merlin. Along with the translocation, the phosphorylation level of phospho-Ser518-merlin was increased in EBP50 depleted cells. To test whether a PP1 type phosphatase does dephosphorylate phospho-Ser518 merlin, specific protein phosphatase inhibitors were applied. These results confirmed that the phosphorylation level of the Ser518 site is regulated via the activity of a type 1 protein phosphatase. TIMAP was recognized as an interacting partner for merlin in endothelial cells, suggesting that TIMAP directs PP1 to merlin. Domain mapping using truncated mutant forms in GST pull down revealed that the N-terminal half of TIMAP (aa 1-290) and the FERM domain of merlin are the regions responsible for the interaction. The catalytic subunit of PP1 (PP1c) was present in all merlin-TIMAP pull down or immunoprecipitation samples demonstrating that merlin actually interacts with the PP1c-TIMAP holoenzyme. On the other hand, from TIMAP depleted cells, without its targeting protein, PP1c could not bind to merlin. Also, when the phosphatase activity of PP1c-TIMAP was inhibited either with depletion of TIMAP or by treatment of the cells with specific PP1 inhibitor, there was an increase in the amount of phospho-Ser518 form of merlin in the membrane of the cells (Fig. 5). These data strongly suggest that the PP1c-TIMAP-

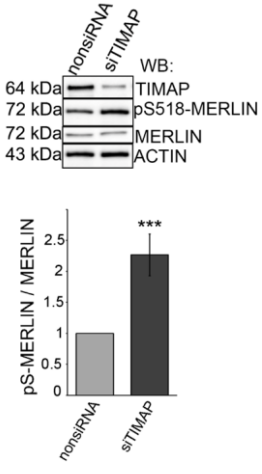

Figure 5. ***TIMAP regulates Ser518 dephosphorylation of merlin.*** Western blot analysis of nonsiRNA and siTIMAP RNA treated cells with the indicated antibodies are shown. Phospho-merlin to merlin ratios determined by densitometry of the Western blots are shown (lower panel). The error bars correspond to SE from 3 independent experiments. Statistical analysis was done with ANOVA. ($p<0.001$)

complex dephosphorylates phospho-Ser518-merlin. ECIS measurements indicated that phospho-merlin accelerates *in vitro* wound healing of the endothelial monolayer.

In conclusion, in endothelial cells, EBP50 is required for the nuclear localization of merlin and the PP1c-TIMAP holoenzyme plays an important role in the dephosphorylation of merlin on its Ser518 side chain, which influence cell migration and proliferation. The results of the research were published in ***The International Journal of Biochemistry & Cell Biology (28).***

TIMAP protein has long been in the focus of our attention and several studies were reported by our workgroup about its function, interacting partners and role in endothelial cells. Along with the information from the literature we wrote a review entitled *TIMAP, the versatile protein phosphatase 1 regulator in endothelial cells* and published in ***International Union of Biochemistry and Molecular Biology Life (29)***.

Results of additional research not planned in the proposal

In collaboration with Péter Bai and his workgroup, the mechanistic relationship between the gut microbiome and breast cancer was studied. We found that secondary bile acid, lithocholic acid (LCA) reduced cancer cell proliferation and VEGF production, aggressiveness and metastatic potential of primary tumors through inducing mesenchymal-to-epithelial transition, increased antitumor immune response, OXPHOS and the TCA cycle. The research was published in ***Biochimica et Biophysica Acta - Bioenergetics*** (30).

Students scientific activities within this research project

On this research project two undergraduate students worked under the supervision of the Principal Investigator. Nikolett Király, a molecular biology MSc student and Zsófia Thalwieser, a biotechnology MSc student completed students' scientific research within the project and have presentations on the local conference. They were recognized with a 2nd and 1st place, respectively. They wrote their student scientific paper which was recognized as their diploma thesis defense and fulfillment to their final examination. Nikolett Király became a PhD student of the PI in 2017, and Zsófia Thalwieser in 2018.

Additional projects related to the research

Anita Boratkó won a Bolyai Fellowship from the The Hungarian Academy of Sciences in 2017 for 3 years. The research project is to explore the role of *TIMAP-PP1 complex in neuronal cell signaling*. In 2018, another fellowship has been won, namely ÚNKP-18-4 New National Excellence Program of the Ministry of Human Capacities. The research plan is based on the Bolyai Fellowship. These projects have not been completed yet.

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