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Investigation of the role of TIMAP as a regulator of protein phosphatase 1 in pulmonary artery endothelial cells

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INTRODUCTION

Endothelial cells and their physiological functions

Endothelial cells line in a single layer of the lumen of the blood vessels and are essential for the normal function of the vessel wall. The closely packed and well-working cells form a barrier that regulates the movement of water, proteins, or blood cells between the bloodstream and tissues. Cells are able to respond in a coordinated, fast manner to physiological stress of the blood vessel. Gap-, adherent and tight junctions maintain the integrity of the endothelial cell layer. Various physical effects, inflammation, or bioactive stimuli can alter the barrier between endothelial cells, which can lead to the formation of paracellular gaps, thereby increasing vascular permeability, compromising normal organ function. Actin filaments, intermediate filaments, and microtubules make up the cytoskeleton playing an important role in the shape of cells and creating and maintaining a well-functioning barrier. In the regulation of barrier function of endothelial cells, protein phosphorylation and dephosphorylation is one of the key regulatory mechanism.

Regulation of reversible protein phosphorylation

Reversible phosphorylation and dephosphorylation of proteins is one of the most important and most common posttranslational modifications in which protein phosphatases and kinases play a key role. The presence or absence of a phosphate group on the Ser, Thr, or Tyr amino acid side chain of proteins can alter the conformation of the proteins, thereby regulating their activity or interaction with other proteins. Protein kinases are the largest group of kinases, catalysing the transfer of adenosine triphosphate (ATP) γ-phosphate to the hydroxyl group of a substrate protein. Phosphatases have the opposite function of kinases. They remove the phosphate group from phosphoproteins by hydrolysing phosphoric acid monoesters into phosphate group and a molecule with a free hydroxyl group. Protein phosphatases are classified into three major groups. Phospho-Ser/Thr specific phosphatases, phospho-Tyr specific phosphatases, and dual specificity phosphatases, that remove a phosphate group from both Ser/Thr and Tyr side chains. The family of Ser / Thr specific phosphatases includes phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases (FCP / SCPs). Protein phosphatase 1 (PP1), PP2A, PP2B (also known as calcineurin), PP4, PP5, PP6, and PP7 belongs to the PPP family.

Protein phosphatase 1

PP1 plays a role in normal cellular function maintenance in endothelial cells, protecting barrier function or regulating the cytoskeletal system. The PP1 holoenzyme consists of a highly conserved catalytic subunit (PP1c) and one or two regulatory subunits. There are several isoforms of the catalytic subunit. In mammals, we can distinguish PP1cα, PP1cβ / δ (in the following PP1cδ), PP1cγ catalytic subunits, which are encoded by three genes PPP1CA, PPP1CB and PPP1CC. PP1 isoforms are about 90% identical in amino acid sequence and the main differences are located on the N- and C-terminals. Mammalian PP1c isoforms show different tissue and intracellular distributions. More than 100 proteins have been identified forming specific dimeric or trimeric PP1-holoenzymes with the catalytic subunit of PP1 in mammals. The interacting proteins can be inhibitors of the catalytic activity (inhibitors), substrate specificity subunits (S-S subunits), targeting subunits or substrates.

Protein phosphatase 1 regulatory subunits

The catalytic subunit of PP1 associates with a wide variety of regulatory subunits that do not share significant sequence similarities (unless isoforms), thus cannot be identified by database search. More than 50 types of regulatory subunits have been identified creating unique holoenzyme forms with PP1c, involved in the regulation of many intracellular processes. Most of the regulatory subunits has a short, conserved PP1c-binding motif: K/RVxF, where X can be any amino acid residue except proline. There are interacting proteins that do not have this K/RVxF motif. Such protein is human factor C1 (HCF, also known as host cell factor) or retinoblastoma protein. Different regulatory subunits can directly affect the intracellular localization and substrate specificity of PP1 holoenzyme.

The MYPT family

Myosin phosphatase (MP) is a key enzyme in maintaining the endothelial barrier. In terms of its structure, it is a heterotrimeric enzyme of PP1cδ, that is responsible for catalytic activity, myosin phosphatase regulatory subunit (MYPT1), the major regulatory subunit that responsible for substrate specificity in dephosphorylation processes and a smaller regulatory subunit (M20). MYPT1 is a member of the MYPT family, which includes the MYPT2, MBS85, MYPT3, and TIMAP proteins. They contain several conserved domains, such as the K/RVxF motif responsible for PP1c binding, ankyrin repeats, which play a role in protein-protein interactions. Ankyrin repeats are followed by a central region primarily involved in the regulation of MYPT

function. In addition to conserved domains, MYPT1, MYPT2, and MBS85 also contain several phosphorylation sites and leucine zipper domains that are involved in dimerization and protein-protein interactions. The other two members of the family, MYPT3 and TIMAP, do not have a leucine zipper domain, but at their C-terminal part contain a CAAX box (prenylation site; where "A" represents an aliphatic amino acid) which is involved in the membrane targeting of proteins. Members of the MYPT family are involved in several regulatory processes and have been associated with many diseases such as hypertension, Parkinson's disease, cancer, and chronic gastritis caused by Helicobacter pylori.

Role of TIMAP, a regulatory subunit of PP1, in the physiological processes of endothelial cells

The MYPT family member TGFβ-inhibited membrane-associated protein (TIMAP) was identified in glomerular endothelial cells by differential analysis, where TGF-β1 decreased TIMAP expression levels. In human pulmonary artery endothelial cells (HPAEC), endogenous TIMAP is localized in the nucleus and in the plasma membrane, where it plays a role in the regulation of the barrier as a regulatory subunit of PP1c. Examination of the interaction between TIMAP and PP1c revealed that TIMAP specifically binds the PP1cδ isoform, playing a role in dephosphorylation of certain proteins as a holoenzyme. Interaction studies of TIMAP have shown that it can bind to the 37/67 kDa laminin receptor (LAMR1). Recent results revealed that binding of the TIMAP-PP1c complex to LAMR1 activates PP1c phosphatase activity and regulates phosphorylation of the Thr125 side chain of LAMR1. RACK1 (activated protein kinase C receptor 1) has also been identified as an interacting partner of TIMAP. The structure of RACK1 protein allows to interact with several proteins simultaneously, like TIMAP and the farnesyl transferase enzyme, which is required for the prenylation of TIMAP. The interaction between TIMAP and eukaryotic elongation factor 1 A (eEF1A1) was confirmed by pull-down, LC-MS/MS and immunoprecipitation experiments. eEF1A1 phosphorylated by ROCK on a Thr side chain has been identified as a substrate of TIMAP-PP1c complex and it has been shown that the localization of eEF1A1 is regulated by TIMAP, as eEF1A1 together with TIMAP is localized in the endothelial cell membrane. ERM proteins play a role in the interaction between actin filament and plasma membrane proteins, thereby regulating barrier function. The TIMAP-PP1c complex plays a role in the dephosphorylation of Thr side chain of phosphorylated ERM proteins in endothelial cells, resulting in changes in cytoskeleton-membrane interactions and cell shape. Merlin is related to ERM proteins and it has also been identified as a substrate of

TIMAP-PP1c, as the TIMAP-PP1c complex has been shown to dephosphorylate the Ser518 side chain of merlin.

Phosphorylation of Ser331, Ser333, or Ser337 side chain of TIMAP plays a prominent role in the regulation of the TIMAP-PP1c complex. Protein kinase A (PKA) phosphorylates Ser337 side chain of TIMAP as a priming site for Ser333 phosphorylation by glycogen synthase kinase 3 beta (GSK3β). Phosphorylation of TIMAP at Ser333/Ser337 sites do not or moderately affects the association of TIMAP and PP1c but does affect PP1c activity. TIMAP phosphorylation by PKA and GSK3β is involved in the regulation of phosphorylation levels of ERM proteins and in the regulation of cellular barrier function. TIMAP is also a substrate of PKC enzyme which phosphorylates Ser331. Upon activation of PKC, TIMAP-PP1c is unable to dephosphorylate phospho-ERM proteins and their levels increase in the cell membrane. Phosphorylation of TIMAP at Ser331 by PKC is an inhibitory phosphorylation that reduces dephosphorylation of phospho-ERM and thus results in barrier dysfunction.

Structure and physiological role of Annexin A2

The most studied member of the annexin family is annexin A2 (ANXA2), which is expressed in most cells and tissues and it binds to several ligands. ANXA2 can be present in the cell membrane, cytoplasm, in the nucleus and extracellular matrix. It is made up of two main domains, a very diverse amino-terminal part, also called a "head region," and a carboxy-terminal "core" domain. The N-terminal region of the protein contains numerous post-translational modifications, such as phosphorylation sites, a Ser1 acetylation site, in addition a nuclear export sequence (NES) and a binding site for main ligands and proteins, such as S100A10. The C-terminal region bears binding sites for calcium ion, phospholipids, heparin, and F-actin. ANXA2 exists as two main forms: a monomeric and a heterotetrameric complex, the ratio of them depends on the cell type. The heterotetrameric form consist of two molecules of ANXA2 bounded to S100A10 homodimer. Interaction with the S100A10 protein protects S100A10 from ubiquitination and proteosomal degradation, increases Ca^{2+} sensitivity of ANXA2 and its ability to bind membrane and F-actin. Three main phosphorylation sites of ANXA2 are Ser11, Ser25 and Tyr23. Studies of Ser11 side chain phosphorylation focused on the interaction between ANXA2 and S100A10. The reason is that the S100A10 binding site and the Ser11 phosphorylation site overlap, as the first 12 amino acids of ANXA2 play a role in the interaction. Some literature data suggest that PKC is responsible for phosphorylation of

this side chain, while other research groups suggest that cAMP kinase and calmodulin kinase show higher affinity than PKC.

The potential PKC phosphorylation of the Ser25 side chain has been suggested by *in vivo* and *in vitro* experiments, however, the phosphatase responsible for the dephosphorylation of this side chain has not been identified yet. The major phosphorylation site of ANXA2 is the Ser25 side chain, which can induce phosphorylation of the Ser11 side chain by a so-called hierarchical phosphorylation mechanism. ANXA2 proteins have also been identified as substrates for pp60src (Rous sarcoma virus transforming protein), which phosphorylates the Tyr23 side chain. In addition, this side chain is also phosphorylated in response to the activation of various receptors, such as the insulin receptor, epidermal growth factor receptor (EGFR), or platelet-derived growth factor (PDGF). Phosphorylation of the Tyr23 side chain resulted in a change in the cytoskeletal system, which caused a change in cell shape and motility, and showed increased localization of the protein in the cell membrane. The phosphatase involved in the dephosphorylation of the Tyr23 side chain has also not been identified.

AIMS

The high expression level of the TIMAP protein, a regulatory subunit of protein phosphatase 1 (PP1) in endothelial cells, draws our attention to the prominent role of the protein. Over the past decade, our group has identified several substrates and interacting partners of the TIMAP-PP1c complex through which it has described the regulation of cellular processes such as barrier maintenance, angiogenesis, and cell proliferation.

Therefore, in our study we aimed:
I. Identification of a new interacting partner of the TIMAP protein and investigate the physiological significance of the interaction.

- Using recombinant GST-N-terminal and C-terminal TIMAP proteins to search for a new interacting partner in BPAEC cells by pull-down experiment.
- Investigation of the interaction between the new interacting partner and TIMAP-PP1c proteins by pull-down, Western blot, immunoprecipitation experiments, and immunofluorescent staining.
- Investigation of the role of the TIMAP-PP1c complex in the dephosphorylation of the new interacting partner.
- Investigation of the role of the new interacting partner in endothelial cells.

Based on the results of phosphoprotein analysis, the Ser69 side chain of TIMAP is a potential phosphorylation site located near the PP1c binding motif and the nuclear localization signal sequence. This suggests that phosphorylation may affect the activity and affinity of PP1c and may affect its localization.

Therefore, our goal was to:
II. Study of phosphorylation of TIMAP Ser69 side chain.

- Generation of phosphomimic Ser69Asp and phosphonull Ser69Ala mutant TIMAP proteins in bacterial and mammalian expression systems.
- Investigation of the interaction of TIMAP mutants with PP1c, ERM and phospho-ERM proteins by pull-down and Western blot.
- Investigation of the intracellular localization of TIMAP mutants in endothelial cell
- Identification of the kinase responsible for Ser69 side chain phosphorylation.
- Investigation of the effect of TIMAP mutants on endothelial cell barrier function and cell migration.

RESULTS AND DISCUSSION

I. Identification of a new interacting partner of TIMAP and investigation of the physiological significance of the interaction

Identification of ANXA2 protein as a new interacting partner of TIMAP in endothelial cells

Several interacting partners and substrates of TIMAP protein have been identified to better understand its physiological role in endothelial cell, as it is highly expressed in these cells. Previously, our group successfully used the recombinant GST-TIMAP protein to search for interacting partners in a pull-down experiment in which RACK1 and eEF1A1 proteins were identified. Unfortunately, the full-length recombinant TIMAP protein is easily fragmented, therefore N- and C-terminal TIMAP fragments cloned into the pGEX-4T-3 vector were used to search for a new interacting partner. The N-terminal fragment consists of 290 amino acids and contains the NLS sequence, the PP1c binding motif, and the 5 ankyrin repeats. The C-terminal part of TIMAP (291-567aa) contains a disordered regions and a CAAX prenylation signal sequence. After bacterial expression and protein purification by affinity chromatography, the recombinant proteins were used in an *in vitro* pull-down experiment. Purified recombinant proteins were incubated with bovine pulmonary artery endothelial (BPAEC) cell lysate and with lysis buffer as a negative control. After the necessary washing steps, the eluted proteins were separated by SDS-PAGE. Extra bands were identified in the sample of GST-N-terminal TIMAP incubated with BPAEC lysate compared to control samples (sample incubated with GST and lysis buffer). The extra band at 36 kDa were cut from the gel and were further analysed by LC-MS/MS and ANXA2, RACK1, eEF1A1 and PP1cδ proteins were identified. Since RACK1, eEF1A1, and PP1cδ proteins are already known TIMAP interacting partners, we focused on the ANXA2 protein as a possible new interacting partner.

Investigation of the interaction of ANXA2 and TIMAP proteins in endothelial cells

The interaction of recombinant N-terminal as well as wild-type full-length TIMAP protein with ANXA2 was confirmed by Western blot analysis of pull-down samples. The C-terminal fragment of TIMAP had no interaction with ANXA2, despite the higher amount of loaded protein as shown by Coomassie stained SDS-PAGE gels. As ANXA2 protein interacts with the N-terminal part of TIMAP, therefore shorter GST fusion fragments of the N-terminal TIMAP region were used in further pull-down experiments to investigate which TIMAP domain plays

a role in the interaction. In the interaction study, we demonstrated that ANXA2 binds to ANK1–ANK3 repeats in the N-terminal part of TIMAP. All identified substrates and interacting protein partners of TIMAP interacted with the N-terminal region (1-290aa), which is not surprising because ankyrin repeats involved in protein-protein interactions are found in this region. Interaction of endogenous proteins was proved by immunoprecipitation utilizing TIMAP and ANXA2-specific antibodies. ANXA2 was detected in the sample immunoprecipitated by anti-TIMAP antibody from endothelial cell lysate, and vice versa, TIMAP was detected in the ANXA2 immunoprecipitated samples. TIMAP is a regulatory subunit of the PP1c and the TIMAP-PP1c complex is involved in the dephosphorylation of several substrates, so we also examined the presence of PP1cδ in IP complexes. PP1cδ was present in TIMAP and ANXA2 immunoprecipitation samples. To test whether ANXA2 binds PP1c directly or via TIMAP, immunoprecipitation was made from control and TIMAP siRNA-treated cells using ANXA2 specific antibody. No interaction was detected between ANXA2 and PP1cδ in TIMAP-depleted cells, therefore we concluded that ANXA2 interacts with PP1cδ via TIMAP.

Generation of recombinant GST-ANXA2 protein and investigation of its interaction with IMAP-PP1c

To further investigate the interaction of TIMAP with ANXA2 protein, a recombinant plasmid pGEX-4T-2-ANXA2 were created. Using a recombinant GST-ANXA2 protein, we examined the interaction of TIMAP and recombinant GST-ANXA2 in a pull-down experiment. Western blot following the pull-down experiment demonstrated the interaction of the recombinant GST-ANXA2 protein with TIMAP and PP1cδ.

Phosphorylation of Ser25 in ANXA2 is regulated by PKC in endothelial cells

To investigate ANXA2 phosphorylation by PKC, in vitro PKC phosphorylation assays were performed. The amount and purity of loaded GST, GST-ANXA2, and GST-TIMAP proteins were confirmed by SDS-PAGE. After incubation with PKC, the antibody specific for phospho-Ser PKC substrate confirmed the phosphorylation of both GST-TIMAP and GST-ANXA2 proteins. The phospho-Ser25 ANXA2-specific antibody verified that indeed PKC phosphorylated the Ser25 side chain in ANXA2. TIMAP is also a substrate for the PKC enzyme, so we examined whether TIMAP phosphorylated by PKC on the Ser331 side chain has an effect on the interaction with the ANXA2 protein. There was no difference in the amount of bounded ANXA2 in the phosphomimic S331D or phosphonull S331A TIMAP pull-down

samples, compared to the wild-type TIMAP, suggesting that PKC phosphorylation of TIMAP does not affect its interaction with ANXA2.

PKCα-silenced endothelial cells were used to further investigate the intracellular phosphorylation of the Ser25 side chain of ANXA2. Endothelial cells were silenced with non-specific siRNA (nonsiRNA) and PKCα-specific siRNA. There was no change in the amount of ANXA2 in the PKCα silenced samples, however, phospho-Ser25 ANXA2 specific antibody did not give a signal in silenced (siPKCα) samples, confirming the role of PKC in the phosphorylation of this ANXA2 side chain in endothelial cells. No phosphorylation of ANXA2 was detected on the Ser25 side chain in PKCα-silenced endothelial cells after PMA treatment, similar to PKCα silenced sample without PMA treatment. However, we obtained an intense signal for phosphorylation in nonsiRNA-transfected and control cells by Western blot. After pretreatment with PKC inhibitor (Gö6983) in control, nonsiRNA, and siPKCα silenced cells, PMA did not induce phosphorylation on the Ser25 side chain of ANXA2. These results further demonstrate that the PKCα enzyme is responsible for the phosphorylation of the Ser25 side chain of ANXA2 in endothelial cells.

The interaction of the TIMAP-PP1c complex with ANXA2 raises the question of whether the TIMAP-PP1c complex is involved in the dephosphorylation of the Ser25 side chain of ANXA2. To identify phosphatases involved in dephosphorylation, PP1 activity in endothelial cells was inhibited by tautomycetin, while PP2A was inhibited by okadanoic acid treatment. Treatment with tautomycetin significantly increased, while treatment with okadanoic acid did not affect the amount of phospho-Ser25 ANXA2. From this, we concluded that PP1 may indeed play a role in the dephosphorylation of the Ser25 side chain. Silencing of TIMAP as a PP1c regulatory subunit significantly increased the amount of phospho-Ser25 ANXA2, similar to the inhibition of PP1 by tautomycetin, confirming our hypothesis that TIMAP-PP1c is involved in the dephosphorylation of the Ser25 side chain of ANXA2.

The ANXA2 protein is transported to the cell membrane by phosphorylation

Localization of ANXA2 in endothelial cells was first tested by immunofluorescent staining using ANXA2 and TIMAP specific antibodies. We detected colocalization of ANXA2 and TIMAP predominantly in the cell membrane. Similar to TIMAP protein, ANXA2 was enriched in the cell membrane upon PKC activating PMA treatment. We also examined the localization of ANXA2 in TIMAP-silenced endothelial cells. Following transfection with TIMAP-specific siRNA, the same staining was observed for ANXA2 as for PKC activation after PMA treatment.

The fact that both PKC activation and TIMAP silencing caused enrichment of ANXA2 in the membrane suggests that the localization of ANXA2 changes depending on its phosphorylation. Immunofluorescent staining with phospho-Ser25 ANXA2-specific antibody showed weak staining in nonsiRNA-transfected and control cells, which was significantly enhanced in the cell membrane by PMA treatment and by TIMAP silencing, further indirectly demonstrating the role of TIMAP in ANXA2 Ser- dephosphorylation of this side chain.

To further investigate the intracellular localization of ANXA2, subcellular fractions were isolated from nonsiRNA and siTIMAP RNA-treated endothelial cells. Phospho-Ser25 ANXA2 was detectable in the membrane in nonsiRNA transfected cells, parallel with the results of immunofluorescent staining. TIMAP gave a more intense signal mainly in the nuclear and membrane fractions and ANXA2 was mainly in the nuclear fraction, however, both proteins were present in the other fractions as well as it was found with the immunostaining. Upon TIMAP silencing, ANXA2 did not show any signal in the nuclear fraction, whereas the amount of phospho-Ser25 ANXA2 in both total and cell membrane fractions was significantly increased upon silencing compared to the control. Thus, our results show that TIMAP silencing increases the phosphorylation level of the Ser25 side chain of ANXA2, and changes the localization of ANXA2, as the level of ANXA2 in the nucleus of TIMAP depleted cells decreased, but its localization in the membrane increased. All these results suggest that the TIMAP-PP1c complex plays an important role in the dephosphorylation of ANXA2, and that phosphorylation of ANXA2 regulates the intracellular localization of the protein.

Annexin A2 is involved in endothelial barrier maintenance and cell migration

To reveal the physiological role of ANXA2 in endothelial cells, siRNA mediated depletion of ANXA2 was carried out. We first checked the effect of ANXA2 silencing on cell viability. Neither the nonsiRNA treated nor the depleted cells showed any significant changes in their viability compared to control cells. In the next step, the endothelial barrier of the transfected cells was also monitored using ECIS. As a result of silencing, lower resistance values were measured compared to the control, which indicated a weakening of the barrier function. The effect of PMA treatment on the barrier function of ANXA2 silenced cells was also examined by ECIS measurement. NonsiRNA-treated cells responded first with an increase of resistance that fell below the initial level 2–3 hr after the addition of PMA and then, in a second phase, gradually returned to a normal value (about 1,000 Ω). In contrast, ANXA2-depleted cells had a significantly smaller elevation of the resistance upon addition of PMA and after a significant

decrease detected 2–3 hr later, the resistance of the silenced cells remained much lower compared to the nonsilenced cells. These results demonstrate that ANXA2 plays a role in maintaining endothelial cell barrier function. Finally, we examined the cell migration rate of control and ANXA2 silenced cells in an *in vitro* wound healing experiment, which was also performed by ECIS measurement. ANXA2 silenced cells showed significantly lower cell migration rates compared to control cells, suggesting that ANXA2 plays an important role not only in maintaining barrier function of endothelial cells but also in cell migration.

Investigation of the interaction of phosphorylated ANXA2 and S100A10 protein

The most well-known interacting partner of ANXA2 are the members of the S100A protein family with which it forms so-called heterotetrameric complexes. The classic ANXA2 binding S100 protein is S100A10, and their complex plays a role in cell-cell interactions and cell adhesion processes. The presence of S100A10 protein in the sample immunoprecipitated with the ANXA2 specific antibody was detected as expected and vice versa, the presence of ANXA2 in the sample immunoprecipitated with the S100A10 specific antibody. We examined the effect of phosphorylation of the Ser25 side chain of ANXA2 on its interaction with the S100A10 protein. There was no detectable interaction between endogenous ANXA2 and S100A10 in immunoprecipitated samples from PMA-treated endothelial cells. In untreated cells the presence of phospho-Ser25 ANXA2 was not detected in the S100A10 immunoprecipitated sample. In control cells, ANXA2–S100A10 interaction was detected from both sides, but after PMA treatment the interaction was no longer detectable, therefore, we concluded that phosphorylation on the Ser25 side chain of ANXA2 abolishes its interaction with S100A10 protein.

Our results revealed a new interaction of ANXA2 with TIMAP–PP1c complex and a phosphorylation dependent regulation that affects endothelial cell migration, barrier, and signaling pathways.

II. Study of phosphorylation of TIMAP Ser69 side chain

Generation of recombinant Ser69Asp and Ser69Ala TIMAP mutant proteins

A search of the Phosphosite Plus database revealed several potential Ser / Thr and Tyr phosphorylation sites on the TIMAP protein. The Ser69 phosphorylation site in the primary

sequence of TIMAP, due to its proximity to the PP1-binding motif and NLS, raises the question of whether its phosphorylation state may affect PP1 binding ability or the subcellular localization of TIMAP. To examine this potential phosphorylation of the protein, we generated a Ser69 phosphomimic and a phosphonull mutant of TIMAP. For the phosphomimic mutant, serine at position 69 was replaced with aspartic acid, a negatively charged side chain. For the phosphonull mutant, Ser69 was modified to alanine, which belongs to the group of apolar amino acids. For amino acid substitution, specific primers were designed that contained a codon corresponding to the amino acid substitution at the appropriate site, as well as a phosphate group at their 5 'end. Following back-to-back PCR, the linear plasmids were ligated and transformed into JM109 *E. coli* cells. The success of the mutation was verified by sequencing the DNA samples. To produce GST fusion proteins, pGEX-4T-3 wild-type and mutant (S69A, S69D) TIMAP plasmids were transformed into *E. coli* BL21 (DE3) bacterial cells, and expression of the recombinant proteins was induced by IPTG. Successfully produced recombinant proteins were purified on Glutathione Sepharose 4B resin. Total, soluble, and immobilized samples of the purification process of GST-TIMAP WT, -S69A, and -S69D proteins were checked by SDS-PAGE.

Phosphorylation of TIMAP Ser69 side chain does not affect its interaction with PP1c, RACK1 and ERM proteins

The Ser69 side chain is located on the 1st ankyrin repeat near the PP1c-binding motif, so the question is whether the phosphorylation of Ser69 may affect the binding of the PP1c regulatory subunit. Therefore, we investigated the interaction of wild-type and phosphomutant recombinant TIMAP proteins with PP1cδ in a pull-down experiment. For both mutants and the wild-type TIMAP, an interaction with PP1cδ was detected, and no significant difference in the interactions was observed. In the next step, we examined whether phosphorylation of the TIMAP Ser69 side chain affects the dephosphorylation of phospho-ERM proteins by regulating PP1c activity. In our pull-down experiment, we showed the interaction of wild-type TIMAP and the Ser69 mutant TIMAP proteins with ERM and phospho-ERM proteins, however, no significant differences were observed in the interactions. RACK1 has also been identified as an interacting partner of TIMAP, which provides an interaction surface for TIMAP and farnesyl transferase and thus plays a role in the appearance of TIMAP in the membrane. Interactions between TIMAP mutants and RACK1 also showed the same binding, suggesting that the TIMAP Ser69 mutants may also be prenylated. Thus, in our pull-down experiments, we showed that phosphorylation of the TIMAP Ser69 side chain has no effect on the interaction with PP1cδ

and RACK1 protein and probably does not affect phosphatase activity toward previously identified substrates.

S69D TIMAP protein has enhanced membrane localization

To investigate the intracellular localization of mutants, the mutant TIMAP sequences were subcloned into a mammalian expression plasmid, pCMV-myc. To study the localization of mutant TIMAP proteins in endothelial cells, immunofluorescent staining was performed after transfection. The phosphonull TIMAP S69A protein, like the wild-type recombinant TIMAP protein, appeared in the cytoplasm of endothelial cells and in the cell membrane. The TIMAP S69D mutant overexpressing cells showed more intense membrane staining and they also induced formation of plasma membrane protrusions compared to the control. The immunofluorescent staining results were confirmed by cell fractionation. Cytoplasmic and membrane fractions were isolated from wild-type and mutant TIMAP transfected endothelial cells by cell fractionation. TIMAP S69A, like wild-type TIMAP protein, was equally localized in the cytoplasm and cell membrane, whereas TIMAP S69D was more detectable in the membrane. Our results from immunofluorescent staining and cell fractionation show that phosphorylation of the Ser69 side chain enhances the presence of TIMAP protein in the cell membrane.

Identification of a kinase involved in the phosphorylation of the TIMAP Ser69 side chain

There are no literature data on which kinase is responsible for the phosphorylation of the TIMAP Ser69 side chain, so we used GPS 5.0 software to search for a potential kinase that may play a role in the phosphorylation of this side chain. The software search gave the highest score for Polo-like kinase 4 (PLK4). NimA-linked kinase (NEK) gave the second highest score, however, many forms of NEK occur and the search results did not give specific results, so we examined the third hit, interleukin-1 receptor-associated kinase (IRAK1) in addition to PLK4. To investigate the interaction with possible kinases, an immunoprecipitation experiment was performed from BPAEC cell lysate using a TIMAP-specific antibody. Our results confirmed the interaction of TIMAP with PLK4 kinase. To further investigate the interaction, in addition to untreated endothelial cells, endothelial cells treated with a specific PLK4 inhibitor (YLT-11) were used in the immunoprecipitation experiment. The interaction of PLK4 and TIMAP in inhibitor-treated cells was no longer detectable compared to untreated cells. These results suggest that PLK4 kinase may be involved in the phosphorylation of TIMAP Ser69. No

interaction was detected between TIMAP and IRAK1 proteins in the immunoprecipitation experiment.

The effect of TIMAP Ser69 side chain phosphorylation on cell migration

TIMAP S69D mutant was detected in large amounts in the cell membrane by cell fractionation and immunofluorescence experiments. Thus, the question is whether phosphorylation of this side chain may affect the barrier function of endothelial cells. This was examined by ECIS measurement. No significant differences were found in the resistance of mutant recombinant TIMAP overexpressing cells, suggesting that phosphorylation of the Ser69 side chain has no effect on endothelial cell barrier function. Previous data in the literature suggest that PLK4 plays a role in the motility of osteocarcinoma and embryonic kidney cells. We demonstrated the interaction between TIMAP and PLK4, so we examined the effect of PLK4 on endothelial cell migration. Migration rate comparison shows that the TIMAP S69D overexpressing cells started to migrate earlier and faster to the wounded area compared to TIMAP wild type or TIMAP S69A overexpressing cells. This result suggests that phosphorylation of the Ser69 side chain of TIMAP enhances the migration capacity of cells. Next, we examined the rate of wound healing following S-1-P treatment of cells expressing wild-type and mutant TIMAP proteins. Sphingosine-1-phosphate (S-1-P), a lipid mediator formed during sphingosine metabolism, helps endothelial cell proliferation, migration, and increases the stability of barrier function. Addition of S-1-P significantly elevated the rate of cell migration as expected. When PLK4 inhibitor (YLT-11) was also applied to transfected endothelial cells after S-1-P treatment in the wound healing study, the rate of wound healing was reduced in wild-type and TIMAP S69A proteins overexpressing cells. However, TIMAP S69D overexpressing cells that mimicked the phosphorylation were not affected by the kinase inhibitor. Our data show that mimicking phosphorylation on Ser69 side chain enhances endothelial cell migration and PLK4 is a potential kinase for TIMAP S69 phosphorylation.

SUMMARY

In endothelial cells, PP1 enzyme is one of the main Ser / Thr-specific phosphatases, consisting of a catalytic and a targeting regulatory subunit. One type of the regulatory subunit families is the myosin phosphatase regulatory subunit (MYPT) protein family and its member protein, TIMAP has high expression level in endothelial cells.

ANXA2 protein was identified in the study of TIMAP function in endothelial cells and in a search of its new interacting partners. We have shown that phosphorylation of Ser25 side chain of ANXA2 in endothelial cells is catalysed by the PKC enzyme. Phosphorylation level of ANXA2 at Ser25 increased greatly by inhibition of PP1 or by depletion of its regulatory subunit, TIMAP, implying the role of this PP1 holoenzyme form in the dephosphorylation of ANXA2. By immunofluorescence experiments and subcellular fractionation endogenous ANXA2 was revealed mainly in the cytoplasm and nucleus, whereas Ser25 phosphorylated ANXA2 was detected primarily in the membrane. Localization of ANXA2 with TIMAP protein was also detected in the membrane of endothelial cells. In parallel with the phosphorylation of ANXA2, the interaction with the S100A10 protein was abolished. ANXA2 depletion lowered the basal endothelial barrier and inhibited cell migration, but the lack of protein had no significant effect on cell proliferation or viability. Our results demonstrate that regulation of ANXA2 by PKC and PP1 may play a role in endothelial cell signaling, especially in barrier function and cell migration.

To study reversible phosphorylation of the Ser69 side chain of TIMAP protein, wild-type, phosphomimic S69D and phosphonull S69A recombinant TIMAP proteins were made. We have shown that phosphorylation of the Ser69 of TIMAP had no effect on the interaction with PP1c, ERM or RACK1 proteins. However, S69D TIMAP showed enhanced membrane localization in cells overexpressing this phosphomimic mutant form. In addition, an increased number of membrane protrusions were observed on the membrane of cells overexpressing TIMAP S69D protein. Significantly faster wound healing and migration rate of the S69D mutant overexpressing cells were detected by endothelial barrier resistance measurements (ECIS). By immunoprecipitation experiment, specific interaction was shown between TIMAP and polo-like kinase 4 (PLK4), which may be a potential kinase to phosphorylate Ser69.

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Registry number: DEENK/415/2021.PL
Subject: PhD Publication List

Candidate: Nikolett Király
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Király, N.**, Thalwieser, Z., Fonódi, M., Csortos, C., Boratkó, A.: Dephosphorylation of annexin A2 by protein phosphatase 1 regulates endothelial cell barrier.
   IUBMB Life. [Epub ahead of print], 2021.
   DOI: http://dx.doi.org/10.1002/iub.2538
   IF: 3.885 (2020)

2. **Király, N.**, Csortos, C., Boratkó, A.: Ser69 phosphorylation of TIMAP affects endothelial cell migration.
   Exp. Lung Res. [Epub ahead of print], 2021.
   DOI: http://dx.doi.org/10.1080/01902148.2021.1960651
   IF: 2.459 (2020)

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List of other publications

3. Aladdin, A., Yao, Y., Yang, C., Kahlert, G., Ghani, M., **Király, N.**, Boratkó, A., Uray, K., Dittmar, G., Tar, K.: The Proteasome Activators Blm10/PA200 Enhance the Proteasomal Degradation of N-Terminal Huntingtin.
Biomolecules. 10 (11), 1-33, 2020.
DOI: http://dx.doi.org/10.3390/biom10111581
IF: 4.879

4. Thalwieser, Z., **Király, N.**, Fonódi, M., Csortos, C., Boratkó, A.: Protein phosphatase 2A-mediated flotillin-1 dephosphorylation up-regulates endothelial cell migration and angiogenesis regulation.
J. Biol. Chem. 294 (52), 20196-20206, 2019.
DOI: http://dx.doi.org/10.1074/jbc.RA119.007980
IF: 4.238

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