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The role of TIMAP in the regulation of protein phosphatase 1 and endothelial barrier function

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1. INTRODUCTION

1.1. Reversible phosphorylation of proteins

Protein phosphorylation/dephosphorylation is a principal regulatory mechanism is the in the control of almost all cellular processes. The covalent attachment of phosphate groups is catalyzed by protein kinases, whereas protein phosphatases exhibit the opposite activity. The presence or absence of a phosphate group on the Ser, Thr, or Tyr amino acid side chain of a protein affects its conformation and may serve as an on and off signal in the regulation of its physiological activity. As a primary effect, enzymes, for example, may become active or inactive; protein complexes may form or loosen, etc. There are approximately 150 phosphatases encoded by the human genome and based on the substrate specificity, reaction mechanisms, and amino acid sequences they can be sorted into several families.

1.2. Classification of protein phosphatases

Protein phosphatases are sorted into three families according to their capability to dephosphorylate amino acid residues. Ser/Thr-specific protein phosphatases dephosphorylate phosphoserine/phosphothreonine residues. Tyr-specific protein phosphatases dephosphorylate phosphotyrosine residues alone. Dual specificity protein phosphatases dephosphorylate both phosphoserine/phosphothreonine and phosphotyrosine residues. The initial classification as type 1 (protein phosphatase 1, PP1) or type 2 (protein phosphatase 2, PP2) of Ser/Thr-specific protein phosphatases was based on their biochemical properties, such as their substrate specificity and their sensitivity toward heat-stable inhibitor proteins.

Ser/Thr-specific protein phosphatases are encoded by two different gene families, termed PPP (phosphoprotein phosphatases) and PPM (Mg^{2+}/Mn^{2+} dependent protein phosphatases). PPP and PPM comprise both classic and more recently identified, but less characterized members from different species. Tyrosine-specific and dual-specificity protein phosphatases belong to a distinct gene family, called PTP (protein tyrosin phosphatases).

The holoenzyme forms of Ser/Thr-specific phosphatases consist of the catalytic subunit and one or more regulatory subunit(s), which may direct the holoenzyme to diverse subcellular locations, to substrate proteins, and may regulate the activity. PP1, PP2A and PP2B phosphatases are the most studied Ser/Thr specific phosphatases.

1.3. Protein phosphatase 1

PP1 is highly expressed in almost every compartment of eukaryotic cells. PP1 dephosphorylates the β subunit of phosphorylase kinase, whereas PP2 phosphatases rather

dephosphorylate the α subunit. The I-1 and I-2 heat-stable inhibitors inhibit the activity of PP1. It is involved in the regulation of several cellular events, such as cell cycle, apoptosis, protein synthesis, cytoskeleton rearrangement and regulation of membrane receptors or channels. In connection with its diverse cellular functions the holoenzyme forms of PP1 possess wide substrate specificity. PP1 holoenzymes mostly consist of the catalytic subunit (PP1cα, β (also called δ), γ1 or γ2 isoform), and one or two of its regulatory subunits (R). Different R subunits may direct the PP1 holoenzyme to distinct subcellular locations and enhance or suppress its activity toward different substrates. The interaction between the catalytic and the regulatory subunits plays crucial role in the function of PP1. In spite of the differences in sequences of the regulatory subunits, a common structural element of R proteins is a short, conserved PP1c binding motif, (R/K)VXF. This conserved consensus motif has an essential role in the interaction of these proteins to PP1. However, sequences neighboring this motif are also important in defining the affinity and specificity of these regulators for PP1 isoforms. There are additional PP1-binding domains that allow a single PP1 catalytic subunit to recruit more than one regulator, and several heterotrimeric PP1 complexes have been observed.

1.4. The holoenzyme form of myosin phosphatase and its regulatory subunits

Myosin phosphatase (MP), is composed of PP1cβ and two regulatory subunits, namely, a larger targeting/regulatory subunit (MP target subunit 1, MYPT1; ~110 kDa) and a smaller regulatory subunit (M20; 20 kDa). MYPT1 binds the PP1c catalytic subunit at its N-terminal end, the M20 subunit is associated with the C-terminal end of MYPT. The most characteristic features of MYPT1 structure are the short PP1c binding motif (KVKF), which is close to the NH_2 terminus and is followed by seven ankyrin repeats, and a regulatory/inhibitory phosphorylation sites, which may increase or inhibit the activity of MP. The expression level of MYPT1 is the highest in smooth musle, but it is also present in other tissues. The first cloning of MYPT1 was from chicken gizzard and rat aorta. Human MYPT1 and its spliced variants are encoded by one single gene.

1.4.1. The MYPT family

Several MYPT1-related proteins, MYPT2, MBS85 (myosin binding subunit 85), MYPT3, and TIMAP (TGF-β-inhibited membrane associated protein) were identified and partially characterized recently. The similarity of MYPT2 and MBS85 structure is high, the identities to MYPT1 are of 61 and 39%, respectively. Each MYPT family member contains

the PP1c binding motif. In the structure of MYPT-related proteins there are several conserved regions: the N-terminal ankyrin repeats; a central sequence spanning the inhibitory phosphorylation site; and C-terminal leucine zipper motif. Contrary to MYPT1/2 the MYPT3 and TIMAP contain C-terminal prenylation motif (CAAX box), which suggest possible membrane association.

1.4.2. The TIMAP protein

Representational difference analysis of glomerular endothelial cell response to transforming growth factor- β1 (TGF- β1) revealed a novel gene, TIMAP (TGFβ-inhibited membrane associated protein). TIMAP mRNA synthesis is strongly downregulated by TGF-β1; it is possible to assume that TIMAP may be an important component of endothelial response to TGF-β1, including apoptosis, capillary morphogenesis, and barrier dysfunction. TIMAP, a 64-kDa protein expressed at high levels in endothelial cells. It shares structural features of MYPT-related proteins, i.e., PP1c binding motif, ankyrin repeats, and prenylation motif. The high level of homology with MYPT1 implies that TIMAP is a potential regulatory subunit of PP1. Yeast and bacterial two-hybrid screening revealed several potential protein partners for TIMAP. For instance, TIMAP interacts with the 37/67-kDa laminin receptor (LAMR1), and it was suggested that TIMAP targets PP1c to LAMR1, and LAMR1 is a TIMAP-dependent PP1c substrate.

1.5. The role of phosphatases in endothelial barrier function

Endothelial cells (EC) form confluent monolayer on the surface of inner wall of blood vessels. One of their major functions is the separation of blood from underlying tissues, allowing only tightly controlled passage of macromolecules, and cells. The adherence of endothelial cells is formed by transmembrane adhesion proteins, which mediate homophilic adhesion and junctional structures. The transmembrane proteins are linked to specific intracellular partners, which mediate anchorage to the actin cytoskeleton and, as a consequence, stabilize junctions. The changes in the components of the EC cytoskeleton are of critical importance in the determination of the actual shape of the cell. The actin filaments and the phosphorylation/dephosphorylation controlled actomyosin interaction are dramatically involved in the increase of the vascular permeability. Phosphorylation/dephosphorylation of cytoskeletal/cytoskeleton-associated proteins also have regulatory role in EC barrier regulation. ATP, Ca^{2+}, calmodulin, and myosin light chain kinase (MLCK) were shown as required elements for EC retraction. The increase in EC

MLCK activity evokes rapid increase in MLC phosphorylation, thus EC contraction and intercellular gap formation. There are two signaling pathways regulating the barrier function via the inhibition of MP. One of them is the vasoactive agent-induced Rho pathway, which increases the endothelial permeability. ROCK may increase MLC phosphorylation indirectly via phosphorylation of MYPT1 at Thr696 and Thr853, which leads to MP inactivation, accumulation of diphospho-MLC, and cell contraction. PKC potentiated inhibitory protein of 17 kDa, called CPI-17 may also affect isolated PP1c or the holoenzyme form of MP without dissociating its subunits. Increased release of ATP from EC during acute inflammation and ATP-induced EC barrier enhancement were reported. In agreement with the assumed barrier-protective role of MP, it was shown, that the mechanism of ATP-induced barrier enhancement involves MP activity. The inflammatory agonists, thrombin and histamine, produce a rapid increase in MLC phosphorylation, actomyosin interaction, and increased EC permeability, in connection with intercellular gap formation and cytoskeletal rearrangement induced barrier dysfunction. Transforming growth factor (TGF)-β1 also induces MLC phosphorylation and increases EC permeability.

Much less is known about the involvement of PP2A activity in the regulation of cytoskeletal structure compared with PP1. *In vitro* experiments suggest that PP2A is responsible for the dephosphorylation of CPI-17 in SM. Moreover, okadaic acid-induced phosphorylation and translocation of MYPT1 is dependent on PP2A, and to varying extents, on ROCK in HepG2 cells. These results suggest that PP2A might be involved in the regulation of MP. Human endothelium expresses all three isoforms of PP2B, also known as calcineurin. Thrombin treatment considerably increases the activity of PP2B; the activation correlates with the phosphorylation of the PP2B catalytic subunit. Furthermore, inhibition of PP2B attenuates thrombin-induced cytoskeletal protein dephosphorylation. These results suggest that PP2B may also be involved in the regulation of EC barrier function.

1.6. The ERM family

It is highly possible that MLC is not the only *in vivo* substrate for MP; for example, it may bind to F-actin associated proteins, i.e. ERM proteins. The so-called ERM family consists of three closely related proteins: ezrin, radixin, and moesin. Their NH_2-terminal region, the F-ERM domain (Fourpoint-one ERM) contain binding sites for membrane adhesion molecules, whereas their COOH-terminal part may bind actin; thus they mediate binding of actin filaments with membrane proteins. They are involved both in the morphogenesis of the membrane structures and in cell adhesion, involving molecular

mechanisms that are not yet understood fully. Close to their C-terminal end in the actin binding domain there is a conserved Thr residue present in all three ERM proteins.Their conformations may change upon phosphorylation (by ROCK or PKC) of this conserved Thr side chain. Non-phosphorylated forms of the ERM form head to tail association between the NH_2- and COOH-terminal domains. They exist as monomers, dimers and oligomers. The conformation of ERM may change via phosphorylation, which allows translocation of proteins; the soluble form becomes membrane-cytoskeleton-associated form *in vivo*. In various experimental cell and tissue systems, ERM association with the membrane-cytoskeleton always correlates with an enhanced state of ERM phosphorylation.

2. AIMS

TIMAP is a member of the MYPT family. It is likely to assume by its structural features that TIMAP may be a regulatory subunit of PP1c. However, its regulatory role is not yet completely understood.

TIMAP has high level of its expression in endothelial cells compared to other cell lines and associates to the plasmamembrane. Furthermore, it may have a regulatory function toward PP1c. Therefore, we hypothesized that TIMAP may have a regulatory role in endothelial barrier regulation by the regulation of the level of phosphorylation of membrane associated proteins.

Accordingly we formulated the following aims:

- To study the interaction between TIMAP and PP1c using several methods.
- To verify the regulatory effect of TIMAP on the activity of PP1c, and identification of possible substrates.
- To study the phosphorylatable side chains of TIMAP and their role in the regulation of TIMAP-PP1c interaction and in the regulation of PP1c activity
- To investigate the role of TIMAP in regulation of the endothelial barrier function.

3. MATERIALS AND METHODS

Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA was isolated from endothelial cells and after reverse transcription the cDNA was used as a template in PCR reaction.

The sequences of genes were amplified by primers containing specific cleavage sites for subcloning. The PCR products were verified by gel electrophoresis in 1-1.2% agarose gel.

Restriction digestion

Restriction digestions were performed according to the manufacturer's protocol using the appropriate buffers. The amounts of the enzymes never exceeded 10% of the reaction mixture.

Ligase reaction

The DNA was ligated with the aid of T4 DNA ligase. The manufacturer's ligase buffer was used and the reaction was carried out under the standard manufacturer's conditions.

DNA gel extraction

The DNA fragments were verified in UV light after gel electrophoresis. The appropriate bands were cut out by 96% alcohol sterilized scalpel. The purification was performed using the QIAquick gel extraction kit according to the manufacturer's protocol.

E. coli transformation

Freshly grown (OD= 0,4) DH5α and BL21 *E.* coli cells were treated with calcium-chloride and the competent cells were divided into equal aliquots. After 10 minutes incubation on ice, we added the DNA and incubated the mixture for further 45 minutes on ice. After the heat shock step (50 seconds, 42^{o} C) the cells were next incubated on ice for 2 minutes followed by the addition of SOC medium. The transformation mixture was cultured for one hour at 37^{o} C. The cells were spread onto antibiotic containing LB agar plates and incubated for 16 hours at 37^{o} C.

Plasmid isolation

Plasmid DNA was isolated with the following plasmid isolation kits: QIAprep Spin Miniprep (Novagen) and Qiagene midi or maxi Plasmid Kits according to the manufacturer's

protocol. The size of the inserted DNA was determined by agarose gel electrophoresis after digestion with appropriate enzymes.

DNA sequencing

DNA was sequenced in the Sequencing Laboratory of Biological Research Center of the Hungarian Academy of Sciences in Szeged and at Sequencing Facility of the Cancer Research Center of University of Chicago using vector-specific pGEX primers.

Recombinant protein expression

Escherichia coli BL-21 (DE3) transformed with pGEX-4T containing glutathione S-transferase (GST) alone, truncated mutant cDNA of TIMAP, wild type cDNA of TIMAP, or moesin fused with N-terminal GST were induced with 0.1 mM isopropyl β-D-thiogalactoside and grown at RT with shaking for 3 h. Cells were harvested by centrifugation and the fusion proteins were isolated from the sonicated lysates by affinity chromatography on glutathione Sepharose 4B according to the manufacturer's protocol. Eluted GST fusion proteins were tested by SDS-PAGE and the identity of proteins were confirmed by Western blot.

Glutathione S-transferase tag was removed by proteolytic cleavage with thrombin according to the manufacturer's protocol. After that thrombin was removed from the eluted mixture by its binding to p-aminobenzamidine-agarose by rotation at RT for 30 min. The eluted thrombin cleaved protein was analyzed by SDS-PAGE.

Cell culture

Bovine pulmonary artery endothelial cells (BPAEC) and human pulmonary artery endothelial cells (HPAEC) were cultured in MEM supplemented with 20% (v/v) fetal bovine serum or in EBM-2 medium supplemented with 10% FBS.

Depletion of TIMAP in HPAEC

HPAEC were treated with four pooled SMARTselection-designed TIMAP-specific siRNA duplexes (SMARTpool reagent) to decrease the amount of endogenous TIMAP. HPAEC were transfected at 60–70% confluence with 50 nM final concentration of both TIMAP-specific and nonspecific, nontargeting siRNA duplexes using Dharma-FECTI transfection reagent and used for further experiments at 48 h posttransfection.

In vitro GST Pull-Down Assay

Confluent HPAEC monolayers were washed, scraped, and lysed. The lysates were incubated with 1 μmol each of GST, GST-TIMAP and mutant GST-TIMAP fusion proteins coupled to glutathione-Sepharose beads for 1 h at 4°C. Afterward, the beads were washed three times with 1xPBS, resuspended in 150 μl of 5xSDS sample buffer, boiled for 5 min, and cleared by centrifugation, and each of the supernatants were analyzed by Western blot.

Immunoprecipitation

HPAEC or BPAEC monolayers were rinsed three times and then lysed with immunoprecipitation (IP) buffer. The cells were scraped, centrifuged and the supernatants were precleared with protein G Sepharose at 4°C for 3 h with gentle rotation. Protein G Sepharose was removed by centrifugation and the supernatant was incubated with the appropriate volume of antibody at 4°C for 1 h and then with 60 μl of fresh protein G Sepharose at 4°C overnight with gentle rotation. The resin was washed and then resuspended in 1xSDS sample buffer, boiled, and microcentrifuged. The supernatant was further analyzed by Western blot.

Immunofluorescence

HPAEC or BPAEC were plated onto glass coverslips and grown to confluence. The cells were washed with 1xPBS and fixed in 3.7% paraformaldehyde in 1xPBS for 10 min at room temperature. The cells were permeabilized with 0.25% Triton X-100 in TBST and blocked with 2% BSA in TBST and incubated with primary, then with secondary antibodies diluted in blocking solution for 1 h at room temperature. Cover slips were rinsed and mounted in ProLong Gold Antifade and observed with a x60 objective on a Nikon Eclipse TE300 or Olympus Fluoview FV1000 microscope.

SDS-PAGE, Western-blot

The separation of proteins by their size was performed using SDS-polyacrylamid gelelectrophoresis (SDS-PAGE). After that the proteins were transferred onto a nitrocellulose membrane. Next we blocked the membrane and then incubated with specific primary and peroxidase-coupled secondary antibodies. To detect the antibodies we used chemiluminescence method (ECL reagent) and the result was developed on X-ray films.

In vitro phosphatase activity assay

Phospho-moesin was prepared by phosphorylation of recombinant moesin by Rho-kinase. Recombinant PP1cβ was assayed for 30 min at 30^{o}C in the presence or absence of wt, mutant or thiophosphorylated forms of GST-TIMAP. The reaction was started by the addition of the substrate (P-moesin), and stopped by the addition of hot 5X SDS loading buffer. Phosphorylation level of P-moesin before and after the phosphatase assay was determined by Western blot using anti-phospho-ERM antibody.

Phosphatase assays with [γ-^{32}P]-ATP phosphorylated MLC20 substrates were performed as well. One third of the total reaction mixture volume contained the enzyme extract, one third contained the appropriate effector or TM buffer. The reaction was started with one third volume of the substrate. The mixture was incubated for 10 min at 30 ^{o}C and it was stopped by the addition of TCA solution. After the centrifugation we measured the amount of liberated $^{32}P_i$ in a scintillation counter.

Measurement of transendothelial electrical resistance

To study the endothelial barrier function transendothelial electrical resistance (TER) was measured dynamically across confluent monolayers using an electrical cell-substrate impedance sensing system (ECIS). Cells were grown to 60% confluency on golden electrodes in ECIS chambers and basal TER was measured.

Surface plasmon resonance

Protein-protein interaction between different forms of GST-TIMAP and PP1cβ was studied by surface plasmon resonance (SPR) based binding technique on Biacore 3000 equipment. Proteins were coupled onto the surfaces of CM5 sensor chips by anti-GST antibody. To determine the kinetic parameters of protein-protein interactions recombinant PP1c was injected over the surfaces. The coupled amounts of PP1c were plotted against the time and these sensograms were evaluated using BIAevaluation 3.1 software.

In vitro phosphorylation of TIMAP

Mono-phosphorylated TIMAP was prepared by thiophosphorylation of recombinant GST-TIMAP with PKA catalytic subunit at 30^{o}C for 60 min using ATP-γ-S. Double-phosphorylated TIMAP was prepared by further thiophosphorylation of the mono-phosphorylated TIMAP with GSK3β at 30^{o}C for 60 min. These thiophosphorylated forms of TIMAP were further used in SPR measurements and *in vitro* phosphatase activity assays.

Analysis and evaluation of data

Primers used for DNA amplification were designed by the DNASTAR software [URL: http://www.dnastar.com/]. DNA sequences were compared with the align function on the NCBI website [URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi].

Adobe Photoshop CS5 imaging software was used to edit the immunofluorescent pictures.

The statistical evaluation of the results was done with the Excel software of the Microsoft Corporation.

The density/intensity of the protein bands of the Western blot results was evaluated with the ImageJ 1.42q software (http://rsbweb.nih.gov/ij/download.html).

4. RESULTS

4.1. Protein-protein interaction between TIMAP and PP1c

The protein-protein interaction was studied using several methods. Immunofluorescence staining of human or bovine endothelial cells revealed colocalization between TIMAP and PP1c, especially after thrombin treatment. To confirm the interaction of endogenous PP1c with TIMAP in endothelial cells, we employed immunoprecipitation. We detected the 64 kDa TIMAP protein in PP1c(α,β)-immunoprecipitate and also PP1c protein in TIMAP immunoprecipitate. We detected about the same amount of proteins in immunoprecipitates after various treatments of endothelial cells affecting the barrier function. These results prove the interaction of native TIMAP and PP1c proteins in endothelial cell lysates. To study isoform specificity of the interaction we employed recombinant GST-TIMAP in pull-down experiments. Analysis of the bound proteins revealed preferential binding of the β-isoform of PP1c to wild-type TIMAP compared with the weak/no binding of the α-isoform.

The results of immunofluorescence, immunoprecipitation, and pull-down experiments confirmed the interaction of these proteins. To further characterize the interaction we employed surface plasmon resonance (SPR) binding studies and all characteristic values (association constant (K_A=1,8 x 10^6 1/M), the rate constant of complex formation and the rate constant of dissociation) showed specific interaction between TIMAP and PP1c.

4.2. TIMAP regulates EC barrier function

To study whether TIMAP is involved in EC barrier regulation we used TIMAP-specific siRNA duplexes to deplete TIMAP in HPAEC; and measured TER of the monolayers in the presence of various effectors. Changes in TER reflect changes in EC barrier integrity: increased or decreased resistance of a monolayer reflects increased barrier stability, or barrier compromise, respectively. The effects of both S1P and ATP, two barrier-protective agents, were attenuated, whereas the effects of thrombin and nocodazole, both evoking barrier dysfunction, were enhanced by TIMAP depletion. These results clearly indicate that TIMAP is involved in the regulation of EC barrier function as a barrier protective protein.

4.3. Moesin/ERM, possible targets of TIMAP

It was shown that ERM proteins mediate binding of actin filaments to membrane proteins, therefore they stabilize the cytoskeleton. Their conformation and binding ability may change upon phosphorylation of a threonine side chain close to their COOH terminus. We

observed moesin in both TIMAP and PP1c immunoprecipitates and also detected moesin and phospho-ERM in pull-down experiments as associated proteins to recombinant TIMAP. Immunofluorescent staining of untreated cells did not show colocalization of moesin and TIMAP, however, thrombin treatment markedly enhanced moesin and phospho-moesin staining along the cell membrane and we observed colocalization between these proteins suggesting possible interaction between moesin and TIMAP. Our results suggest that TIMAP might serve as a targeting subunit of PP1c, and may regulate ERM dephosphorylation.

4.4. The effect of TIMAP phosphorylation on the phosphorylation level of ERM proteins and on the endothelial barrier function

In contrast with the other members of the MYPT family TIMAP does not contain inhibitory phosphorylation sites. However, MYPT3, the closest homologous protein of TIMAP can be phosphorylated by PKA which results in PP1c activation. We have shown that TIMAP can be phosphorylated *in vitro* by both PKA and GSK3β. Next we treated HPAEC with adenylate cyclase activator, forskolin, to induce phosphorylation of TIMAP. Compared to control cells we did not observe significant change in the pattern of phospho-ERM proteins in consequence of the phosphorylation of TIMAP. However, the level of phospho-ERM proteins increased and concentrated at the plasmamembrane after thrombin treatment. Interestingly forskolin suppressed thrombin induced stress fiber and gap formation, and diminished the phospho-ERM signal along the cell edges. In TIMAP depleted cells, forskolin failed to affect the level of moesin phosphorylation at the cell edges. This result was supported by TER measurements, as well. The above data suggest that TIMAP and its phosphorylation level have essential role in the endothelial barrier regulation via the regulation of ERM phosphorylation.

4.5. The effect of TIMAP phosphorylation level on protein phosphatase 1 activity

The wild type GST-TIMAP was thiophosphorylated by PKA (GST-TIMAP-P, Ser337). Part of the sample was further thiophosphorylated by GSK3β (GST-TIMAP-PP, Ser333). The effect of these phosphorylated forms of TIMAP were studied in *in vitro* phosphatase activity assays. Bacterially expressed recombinant moesin was phosphorylated by Rho kinase and the phospho-moesin was used in the PP1c phosphatase activity assays as substrate. Wt non-phosphorylated and mono-thiophosphorylated GST-TIMAP reduced considerably (~60% and ~50%, respectively) the PP1c activity toward the substrate, while the double-thiophosphorylated GST-TIMAP had negligible (<10%) effect. We repeated the experiment

with P-MLC substrate and detected similar pattern of changes in PP1 activity indicating that the inhibitory effect of TIMAP is not substrate specific.

4.6. The effect of TIMAP phosphorylation on its interaction with PP1c

To study the interaction of non-phosphorylated and phosphorylated forms of TIMAP with PP1c we used SPR binding studies. We found that PKA thiophosphorylation of TIMAP and sequential thiophosphorylation by PKA and GSK3β slightly decreases the rate constants of complex formation (k_a), and dissociation (k_d) compared to the corresponding values for non-phosphorylated TIMAP-PP1c. The association constants for TIMAP-PP1c (K_A= 1.28 x 10^6 M^{-1}) and double-thiophosphorylated TIMAP-PP1c (K_A= 1.93 x 10^6 M^{-1}) interactions are very similar, but for the mono-thiophosphorylated TIMAP-PP1c (K_A= 7.39 x 10^6 M^{-1}) is about four times larger value, suggesting a moderately stronger interaction. Our results clearly indicate that thiophosphorylation of TIMAP affects the kinetics, but does not or only moderately modifies the affinity of TIMAP-PP1c binding.

4.7. Inhibition of GSK3β attenuates the effect of forskolin in HPAEC

Our *in vitro* phosphatase measurements indicated that the PKA and PKA/GSK3β phosphorylated mono- and double-thiophosphorylated forms of TIMAP affected the dephosphorylation of a phospho-moesin substrate differently. Therefore, next we tested whether PKA activity alone, or PKA primed GSK3β phosphorylation of TIMAP as well is important in the EC barrier regulation via the ERM phosphorylation level. Immunofluorescence stainings of BPAEC were performed without and with GSK3β inhibitor (AR-A014418) pretreatment of the cells followed by various additions. GSK3β inhibition in control or forskolin treated cells caused slight increase in ERM phosphorylation at the cell boundaries compared to cells without the addition of the inhibitor. After forskolin treatment the spikes at the cell boundaries disappeared on the majority of control cells, however the morphology of the GSK3β pre-treated cells was more similar to that of untreated cells suggesting that PKA activation by forskolin may be followed by GSK3β activation too. We detected ERM phosphorylation and barrier dysfunction (gap formation) after thrombin; and even more pronounced barrier dysfunction when the GSK3β activity was inhibited by the specific inhibitor in thrombin treated BPAEC. More importantly, forskolin was not able to rescue the cells from the effect of thrombin when GSK3β activity was inhibited as gaps between the cells and spikes on the cell boundaries were still present. Our data suggest that both PKA and GSK3β phosphorylation of TIMAP are important regulatory events in the

modulation of the phosphorylation level of the ERM proteins. We checked the P-ERM level in the cell lysates after the same treatments using Western blot. The variance of signals was less apparent, but the tendency of changes was the same. On the other hand our TER measurements showed that inhibition of GSK3β activity significantly attenuated the rescuing effect of forskolin in thrombin challenged cells supporting both PKA and GSK3β activities play critical role in the determination of ERM phosphorylation level and in thrombin-induced barrier disruption via phosphorylation of TIMAP.

Taken together, using immunoprecipitation, pull-down assays and surface plasmon resonance binding studies we showed specific protein-protein interaction between TIMAP and PP1cβ. Our results clearly indicate that TIMAP may control the ERM phosphorylation level via regulation of the activity of the PP1cβ; and TIMAP has a positive role in the regulation of endothelial barrier function.

5. SUMMARY

Protein phosphatase 1 (PP1) regulates numerous cellular processes by dephosphorylating phospho-Ser/Thr residues of proteins. TIMAP protein (64 kDa), as a member of the MYPT family, is a putative regulatory subunit of the catalytic subunit of PP1 (PP1c). Our aim was to study the interaction between TIMAP and PP1c; and to study the effect of the phosphorylation of Ser333 and Ser337 side chains in TIMAP on this interaction. TIMAP is highly expressed in endothelial cells (EC) compared to other cell lines, and it localizes to the plasma membrane. Therefore we studied the role of TIMAP in the regulation of barrier function of human and bovine pulmonary artery endothelial cells.

Using several methods we provided evidence for specific interaction between TIMAP and PP1c; TIMAP binds preferentially the beta isoform of PP1c (K_a=1.8x10^6 M^{-1}). Thiophosphorylation of TIMAP by PKA or sequential thiophosphorylation by PKA and GSK3β only slightly modifies the association constant for the interaction of TIMAP with PP1c. However, non- and mono-thiophosphorylated forms of TIMAP inhibit PP1cβ activity, while the double-thiophosphorylated form does not affect the phosphatase activity with the utilized substrates.

To investigate the role of TIMAP in EC barrier regulation, we depleted TIMAP in HPAEC. We found that depletion of TIMAP attenuates the increases in transendothelial electrical resistance induced by the barrier protective agents (S1P and ATP) and enhances the effect of barrier-compromising agents (thrombin, nocodazole) demonstrating a barrier-protective role of TIMAP in EC. PKA activation by forskolin treatment of EC prevents thrombin evoked barrier dysfunction and ERM phosphorylation at the cell membrane. On the contrary in TIMAP depleted cells forskolin failed to affect the thrombin effect, and the ERM proteins remained phosphorylated. These data demonstrate that TIMAP is involved in the EC barrier protection as part of PKA-mediated ERM (ezrin-radixin-moesin) dephosphorylation. Using a specific GSK3β inhibitor we have shown that PKA activation is followed by GSK3β activation in bovine pulmonary EC and activation of both kinases is required for the rescuing effect of forskolin and protects the EC barrier function.

6. PUBLICATIONS

Register Number: DEENKÉTK /143/2011.
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Subject: Ph.D. List of Publications

Candidate: István Czikora

Neptun ID: JDABTQ

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Czikora, I.**, Kim, K.m., Kása, A., Bécsi, B., Verin, A., Gergely, P., Erdődi, F., Csortos, C.: Characterization of the effect of TIMAP phosphorylation on its interaction with protein phosphatase 1.
   Biochimie. Epub ahead of print (2011)
   DOI: http://dx.doi.org/10.1016/j.biochi.2011.03.011
   IF:3.897 (2009)

2. Csortos, C., **Czikora, I.**, Bogatheva, N.V., Adyshev, D.M., Poirier, C., Oláh, G., Verin, A.: TIMAP is a positive regulator of pulmonary endothelial barrier function.
   Am. J. Physiol. Lung Cell. Mol. Physiol. 295 (3), L440-450, 2008.
   DOI: http://dx.doi.org/10.1152/ajplung.00325.2007
   IF:3.924

List of other publications

3. Tar, K., Csortos, C., **Czikora, I.**, Oláh, G., Ma, S.F., Wadgaonkar, R., Gergely, P., Garcia, J.G.N., Verin, A.: Role of protein phosphatase 2A in the regulation of endothelial cell cytoskeleton structure.
J. Cell. Biochem. 98 (4), 931-953, 2006.
DOI: http://dx.doi.org/10.1002/jcb.20829
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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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Presentations related to the thesis:

1. I. Czikora: The role of TIMAP in the regulation of endothelial barrier function
   A Molekuláris Orvostudomány Doktori Iskola PhD szimpóziuma, Debrecen, 2010. május 31 - június 1

Posters related to the thesis:

1. Czikora I., Oláh G., Csortos Cs., Verin A.: Characterization of TIMAP, the potential regulatory subunit of protein phosphatase 1 in human endothelial cells
   The American Society for Cell Biology 46th Annual Meeting 2006, San Diego, CA, December 9-13, 2006

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