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Adaptor proteins in the vascular endothelium

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Introduction

Endothelial barrier function and endothelial cytoskeleton

Vascular endothelium is the layer of closely connected cells lining the inner surface of blood vessels. The major function of vascular endothelium is to provide a semipermeable barrier that controls exchange of fluids, nutrients, and metabolic wastes between blood and tissues while preventing pathogens from entering the latter. It regulates a variety of processes including blood perfusion, angiogenesis or metabolic and synthetic functions. Integrity of the endothelial cell (EC) monolayer as well as proper cytoskeletal structure of individual cells are required for appropriate EC barrier function. The well-established vascular endothelial barrier function is indicated by the balance of contractile and tensile forces. The shifted balance towards the contractile forces results in gap formation between the cells. Endothelial barrier dysfunction occurs in a variety of diseases and injurious conditions, including inflammation, trauma, ischemia, diabetes mellitus, multiple sclerosis, thrombosis and metastatic tumor development. Loss of structural integrity of pulmonary endothelium plays a key role in the development of acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS). Lost ability of the alveoli to exchange oxygen and carbon dioxide, collapsion and fluid leakage (edema) into the alveoli are trademarks of these diseases.

Reversible protein phosphorylation

Reversible phosphorylation of proteins can be found almost in all biological activities of eukaryotic cells, such as controlling gene expression, protein complex formation, lipid raft assembly/disassembly, protein degradation, etc. It is one of the most important mechanisms influencing the activity of proteins. The enzymes that play a key role in reversible phosphorylation are protein kinases, responsible for the phosphorylation of proteins and protein phosphatases, which catalyze the cleavage of phosphate groups. There are more than 500 protein kinases in the human genome that catalyze the transfer of inorganic phosphate supplied by ATP onto serine (Ser; S), threonine (Thr; T) or tyrosine (Tyr; Y) amino acid residues of numerous proteins. The specificity of protein phosphorylation is determined by the local amino acid sequence, consensus sequence, surrounding the S/T/Y residue to be

phosphorylated. These consensus amino acid sequences are unique recognition sequences for phosphorylation by individual kinases. Molecular and functional analyses of protein kinases and protein phosphatases in EC are equally important for the better understanding of the regulation of EC barrier function.

Characterization of the Ser/Thr-specific protein phosphatases

Classification of phosphatases is based on molecular biological aspects, the amino acid sequence homology and the three-dimensional structure. Based on the structure of the catalytic subunit, three families of protein phosphatases were created: phosphoprotein phosphatases (PPP), metal ion-dependent protein phosphatases (PPM) and the phosphotyrosine and dual specificity protein phosphatases (PTP). Protein phosphatase 2A, 2B (PP2A and PP2B) and protein phosphatase 1 (PP1) catalytic subunits have similar amino acid sequences, while the protein phosphatase 2C (PP2C) is different. Accordingly, PP1, PP2A and PP2B belong to the phosphoprotein phosphatases group, while PP2C is a PPM group member. With exception of PP2C, Ser/Thr specific phosphatases exist in oligomeric form, consisting of the catalytic subunit and one or more additional regulatory/targeting subunits.

Protein phosphatase 1

PP1 is one of the most conserved eukaryotic proteins and one of the most significant Ser/Thr phosphatases. It was found in all cell types examined so far. Due to its broad substrate specificity it plays a major role in many physiological processes such as the cell cycle, apoptosis, and protein synthesis. The PP1 catalytic subunit has three isoforms: PP1α, PP1β (also known as PP1δ) and PP1γ.

The catalytic subunit is able to catalyze the dephosphorylation of many proteins itself, however, in cells it exists as a holoenzyme, mostly in dimer form with one or more of the regulatory subunit(s). These regulatory subunits target the catalytic subunit to the phosphoproteins, and control the substrate specificity of the holoenzyme. The interaction between the catalytic and regulatory subunits of PP1 plays an important role in the regulation of the enzyme. The primary structure of the more than 50 potential regulatory subunits shares no apparent similarities. Still, during mapping of the PP1 binding sites, a consensus (R/K)V*x*F sequence was found in the regulatory subunits, where *x* can be any residue.

Protein phosphatase 2A

PP2A is a heterotrimeric holoenzyme with a very diverse structure. As a consequence of this diversity, PP2A is involved in many biological processes, including cell cycle, growth, heat shock process, signal transfer, cell transformation, DNA replication or apoptosis. The PP2A trimer consists of a catalytic subunit and two regulatory proteins, PR65 (or A) and the B subunit.

The catalytic C subunit (PP2Ac) has two isoforms (α and β) in mammalian tissues. In the early embryonic stage of development, the two isoforms have different intracellular localizations and expression levels, but have the same catalytic activity. Expression of the catalytic subunit is regulated by an autoregulatory process, which keeps the amount of protein at a constant level.

The PR65 or A subunit has a structural function, it is strongly bound to the catalytic subunit of the enzyme and thereby forms a core dimer of PP2A which is connected to the third variable component, the B subunit. In mammals, two isoforms of the A subunit have been identified, which are 86% identical in their primary sequence. Northern analysis, using RNA isolated from several human cell lines, indicated that the Aβ isoform has a lower expression level than the Aα. The A subunit can associate with other proteins as well, engaging PP2A in specific signaling pathways. It should also be mentioned, that in the absence of a regulatory B subunit, it modifies the substrate specificity of the catalytic subunit. Data obtained from the crystal structure of the protein confirmed the previous hypothesis, that the α-helical structures in the 2-2 repeating units are arranged so that the tertiary structure of the protein is elongated and asymmetric, similar to the letter C.

The third subunit of the holoenzyme occurres in many molecular weight forms and it is generally called B subunit. Individual B subunits are classified as members of one of four subfamilies B, B', B" or B"'. Protein structure or function of the subfamilies do not show any similarities. The diversity is further increased by the multiple isoforms that can be found within the subfamilies. The various B subunits determine the substrate specificity and intracellular localization of the PP2A holoenzyme. Within each B subfamily, the members contain a highly conserved region (80% identity), which is located at the central part of the proteins, whereas the C- and N-terminals are different. This implies that the conservative regions play a role in the binding of the A and C subunit, while the various ends are responsible of the divers functions.

Role of reversible phosphorylation in EC cytoskeleton regulation

The fast and flexible change of the cytoskeleton is crucial in many cellular events such as cell motility and cell division. The organization and the plasticity of the cytoskeleton is determined primarily by the forces or tension generated within the cytoskeleton. Reversible phosphorylation of cytoskeletal and cytoskeleton-associated proteins is a significant element of cytoskeletal changes and endothelial barrier function regulation.

Phosphorylation of myosin light chain (MLC20) at Ser19 side chain results in cell contraction, stress fiber formation and intercellular gap formation. The phosphorylation is catalysed by the high molecular weight (210 kDa) Ca^{2+}-calmodulin dependent myosin light chain kinase (MLCK), which is highly homologous with the smooth muscle MLCK. In endothelial cells dephosphorylation of MLC20 is catalysed by myosin phosphatase, a type 1 phosphatase. Binding of thrombin to the protease activated receptor 1 increases the intracellular Ca^{2+} level, the calcium calmodulin complex binds to and activates MLCK that catalyzes the phosphorylation of MLC20. In addition, the level of MLC20 phosphorylation may increase by the activation of the Rho/Rho-kinase pathway, which inhibits the myosin phosphatase and consequently disrupts the equilibrium between kinase-phosphatase activities.

Myosin phosphatase (MP)

Myosin phosphatase is a key regulator of endothelial barrier function. It was originally purified from chicken gizzard myofibrils and was shown to be composed of three subunits - the delta isoform of the PP1 catalytic subunit (PP1cδ), the 130 kDa myosin binding subunit (MBS) and a 20 kDa small subunit (M20) which form a heterotrimer. MBS is also called MYPT1, myosin phosphatase targeting subunit 1. It has two isoforms (110 kDa and 130 kDa) encoded by the same gene. The MYPT gene seems to be a housekeeping gene since it is widely expressed in many tissues, although it is present at higher concentrations in smooth muscle. MYPT1 interacts with PP1cδ via its N-terminal PP1c binding motif, and the C-terminus binds M20. Two nuclear localization signals (NLS) and seven further ankyrin repeats were identified within the sequence.

TIMAP, a MYPT family regulator subunit

TIMAP (TGF-β inhibited membrane-associated protein) was originally identified in glomerular endothelial cells by representational difference analysis. It is highly expressed in all cultured endothelial and hematopoietic cells compared to non-endothelial cells. Immunofluorescence studies of rat tissues revealed that the protein mainly localizes in the vascular endothelium. Based on its structural features TIMAP has been considered as a member of the MYPT family of the regulatory subunits of PP1. TIMAP and MYPT3 are the most closely related members within the family. Both proteins contain ankyrin repeats, a PP1c binding motif and a C-terminal prenylation motif; the latter one mediates their association with the plasma membrane. A bipartite nuclear localization signal is also present in TIMAP; accordingly, it was detected in the nucleus of vascular endothelial cells, but its significance is not known yet.

Adaptor proteins

The specific and well-constructed response of cells to external stimuli requires the integration of multiple signaling pathways. Stimulation of cell surface receptors initiates cellular signals, which may require recruitment of protein binding partners to specific subcellular domains, such as the membrane. Adaptor proteins mostly function as flexible molecular scaffolds. They tend to lack enzymatic activity, but instead, mediate specific protein-protein interactions and facilitate the formation of larger signaling complexes. Adaptor proteins usually contain several protein binding modules (domains) within their structure. The specificity of signaling is achieved by the type of these domains, which predicts the identity of binding partners, as well as the subcellular localization.

NHERF1/EBP50 adaptor protein

ERM binding phosphoprotein of 50 kDa (EBP50) is a member of the Na^+/H^+ exchanger regulatory factor (NHERF) family which consists of four related PDZ domain containing scaffolding proteins termed as NHERF1/EBP50, NHERF2/E3KARP,

NHERF3/PDZK1, and NHERF4/IKEPP. NHERF1 was originally recognized as Na^+/H^+ exchanger-3 binding partner, and it has later been identified as an ERM binding phosphoprotein. NHERFs are highly abundant in the epithelium and their role in Na^+/H^+ exchanger-3 regulation is well established, therefore EBP50 was characterized mainly in polarized epithelial cells up to the present.

EBP50 has two PDZ domains and a C-terminal ERM-binding domain. It is believed that EBP50 forms bridges among plasma-membrane and cytoskeleton proteins through these domains. Most of the interacting proteins bind to the first PDZ domain, only a few partners described so far relate with the second PDZ, like beta-catenin. Self association of EBP50 through the PDZ domains, and the intramolecular interactions between the PDZ2 and C-terminal domains of EBP50 result in an autoinhibition of complex formation with other protein partners. Protein-protein interactions with the other members of the NHERF family have been described, as well.

EBP50 is a subject to phosphorylation by several kinases and these modifications have been suggested to alter its binding activity. Oligomerization of EBP50 was shown to be regulated via site-specific phosphorylation. During mitosis EBP50 is phosphorylated on Ser^{279} and Ser^{301} by cyclin dependent kinase 1 (Cdk1) and that phosphorylation inhibits its oligomerization, but allows association with Pin1, a peptidylprolyl isomerase. Dephosphorylation of the above mentioned sites is an equally important element of the reversible phosphorylation, however, phosphatases specific for EBP50 have not been identified yet.

RACK1 adaptor protein

RACK1 is a highly conserved scaffolding/anchoring protein that contains seven WD repeats predicted to form a seven-bladed propeller structure. The protein was named Receptor for Activated C Kinase 1, given the association of RACK1 with the active conformation of PKCβII. Due to its structural features it has the ability to interact simultaneously with several signaling molecules which suggests the possibility for multiple protein interactions. The role of RACK1 was identified in many signaling pathways. Activation of the cAMP/PKA pathway in hippocampal neurons leads to the dissociation of RACK1 from Fyn kinase, results in the phosphorylation of the NR2B subunit of N-methyl D-aspartate receptor and the enhancement of the channel's activity. RACK1 interacts with several transmembrane receptors including

the insulin-like growth factor receptor I, β-integrin receptor, androgen receptor as well as several ion channels.

Recently, it was also demonstrated that RACK1 is a core component of the eukaryotic 40S ribosomal subunit. It is localized on the head region close to the mRNA exit channel, suggesting a physical link between the eukaryotic ribosome and cell signaling pathways *in vivo*. In accord with its structural features, all these recent findings imply the involvement of RACK1 in numerous signaling pathways.

Aims of the study

Endothelial cytoskeleton structure and the vascular barrier are critical in the maintenance of proper lung function. A significant and sustained increase in vascular permeability is a hallmark of acute inflammatory diseases. Approaches designed to understand endothelial cell paracellular gap formation and barrier function have revealed the complexity of these processes as the involvement of various signaling pathways were identified, but our knowledge about their regulation is still limited. ERM proteins are actin-binding linkers connecting F-actin and the plasma membrane, either directly or indirectly via scaffolding proteins. EBP50, an adaptor phosphoprotein, was shown to interact with ERM in epithelial cells, but it has not been studied in the endothelium yet. TIMAP protein is a targeting partner of type 1 protein phosphatase and regulates the phosphorylation level of ERM proteins in pulmonary EC. TIMAP is highly expressed in endothelial cells compared to other cell types suggesting its importance in the endothelium. Indeed, previous results of our research group revealed that it has a crucial role in the regulation of endothelial barrier. Thus, exploring protein-protein interactions of TIMAP is essential to understand its unique role in this cell type.

Our specific aims listed below are divided into two major parts of investigation of EBP50 (part A) and identification of a new protein partner of TIMAP (part B).

Part A: - detection of EBP50 protein in endothelial cells and verification of its phosphorylation during mitosis
- investigation of the effect of phosphorylation on the localization of EBP50
- identification of the phosphatase that plays a role in the dephosphorylation of EBP50

Part B: - identification of a new TIMAP interacting protein in endothelial cells
- structural domain mapping of the interaction
- investigation of the physiological role of the interaction.

Materials and methods

Cell cultures

Bovine pulmonary artery endothelial cells (BPAEC) (culture line-CCL 209) were obtained frozen at passage 8 (American Type Tissue Culture Collection, Rockville, MD), and were utilized at passages 17-22. MCF7 (catalogue No: 86012803) cells were obtained frozen at passage 11 (European Collection of Cell Cultures, Salisbury, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in MEM supplemented with 10% (v/v) fetal bovine serum (heat inactivated), 1% sodium pyruvate, 0.1 mM MEM non-essential amino acids solution. HeLa cells (catalogue No: 93021013) were obtained frozen at passage 4 (European Collection of Cell Cultures, Salisbury, UK) and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and 0.1 mM non-essential amino acids solution. Human Pulmonary Artery Endothelial Cells (HPAEC) (catalogue No: CC-2530) were obtained frozen at passage 3 (Lonza Group Ltd, Switzerland) and were cultured in EGM-2 Endothelial Cell Growth Medium-2 supplemented with 10% FBS and EGM-2 SingleQuots of Growth Factors. Cells were utilized at passages 5–7.

Cell synchronization

BPAEC were synchronized at G1/S phase using double thymidine block as follows. Cells were treated with 2mM thymidine for 16 h. After this first thymidine block cells were released for 8 h and then treated with 2mM thymidine for 16 h again. G2/M phase cells were obtained by 14-16 h treatment with 80ng/ml nocodazole.

Transfection

To express recombinant wild type or mutant pCMV-myc EBP50, 1μg total DNA/3μl FuGENE® HD reagent was mixed and added to the culture plates containing cells at ~80% confluency according to the manufacturer's instruction. Cells were analyzed 24 h later.
HeLa cells were transfected with pEGFP-C1, pEGFP-C1 TIMAP WT or pEGFP-C1 TIMAP Δpp1c plasmids using Lipofectamine 2000 transfection reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions.

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

Total RNA was extracted from HPAEC using ZR RNA MicroPrep™ (Zymo Research Corporation, CA). cDNA was synthesized from 2μg of total RNA using 0.111μM oligo-dT

primer and 200 U M-MLV reverse transcriptase (Promega Corporation, USA) with 5mM dNTP in 1x RT buffer. The reaction assay was incubated at 37°C for 1 hour.

Phire® or Phusion® High-Fidelity DNA Polymerases (Thermo Scientific, Inc.,Vantaa, Finland) were used for PCR. Coding regions of EBP50 (SLC9A3R1, NM_001077852), TIMAP (PPP1R16B, NM_015568), RACK1 (GNB2L1, NM_006098.4) were amplified using specific primer pairs. The inserts were subcloned into the appropriate vectors than transformed into *E.coli* cells.

Transformation

Plasmids were added to100µl competent cells thawed on ice. After 30 min incubation on ice, 45 sec heat shock was applied (42°C), then cells were replaced on ice. 900µl SOC medium was added to the samples and they were cultured at 180 rpm and 37°C for 45 min. After that bacteria were spread onto LB agar with appropriate antibiotic. Controls were spread onto LB agar with and without the antibiotic. Plates were incubated for 16 h, at 37°C.

Plasmid preparation

One single colony of plasmid containing bacteria was inoculated into LB medium containing antibiotic and was grown at 37°C, 180 rpm for 16 h. GeneJET plasmid miniprep kit (Thermo Fisher Scientific, Inc., Waltham) was used to purify plasmid DNA. For larger scale plasmid preparation, the overnight culture was diluted 1:100 and further grown for 16 h and GeneJET plasmid maxiprep kit (Thermo Fisher Scientific, Inc., Waltham) was employed.

Immunofluorescence and microscopy

Cells were plated onto 0.2% gelatin coated glass coverslips and grown, washed once with 1x PBS and fixed with 3.7% paraformaldehyde in 1x PBS for 15 min at room temperature. Between each following step, the cells were rinsed three times with 1x PBS. The cells were permeabilized with 0.5% Triton X-100 in PBS at room temperature for 15 min, blocked with 2% BSA in PBS for 30 min at room temperature, and incubated with primary then with secondary antibodies diluted in blocking solution for 1 h at room temperature. Cover slips were rinsed and then mounted in ProLong Gold Antifade medium.

Images were acquired with a Carl Zeiss Axioskope-20 microscope using Zeiss Plan-NEC FLUAR 63x 1.25 NA oil immersion objective and Axiocam color camera (Zeiss, model 412-312). Confocal images were acquired with an Olympus Fluoview FV1000 confocal microscope using UPLSAPO 60x 1.35 NA oil immersion objective on an inverted microscope (Olympus IX81) at 25°C. Images were processed using FV10-ASW v1.5 software.

Nonspecific binding of the secondary antibodies was checked in control experiments.

Immunoprecipitation

Cells with or without thymidine or nocodazole treatment grown in 100 mm tissue culture dishes were rinsed three times with 1x PBS and then collected and lysed with 600µl of immunoprecipitation (IP) buffer (20mM Tris-HCl (pH 7.4), 150mM NaCl, 2mM EDTA, 2mM sodium vanadate, 1% NP-40) containing protease inhibitors. The lysate was centrifuged with 10,000 *g* for 15 min at 4°C. To avoid nonspecific binding, the supernatants were precleared with 50µl of protein G Sepharose (GE Healthcare, Piscataway, NJ) at 4°C for 3 h with end-over-end rotation. Protein G Sepharose was removed by centrifugation at 4°C for 10 min, and the supernatant was incubated with the appropriate volume of antibody at 4°C for 1 h and then with 50µl of fresh protein G Sepharose at 4°C overnight with gentle rotation. The resin was washed three times with 300µl of IP buffer and then resuspended in 150µl of 1x SDS sample buffer, boiled, and microcentrifuged for 5 minutes. The supernatant was analyzed by Western blot.

Subcellular fractionation

ProteoJET^{TM} Cytoplasmic and Nuclear Protein Extraction Kit (Thermo Fisher Scientific, Inc., Waltham) was used for subcellular fractionation. Cells were collected in cell lysis buffer, containing 0.01M DTT and protease inhibitors, vortexed and kept on ice for 10 min. Cytoplasmic fraction (CP1) was obtained by centrifugation at 500*g* for 7 min and further cleaned by centrifugation at 13,000*g* for 15 min at 4°C (CP2). Nuclear protein fraction (N) was obtained after washing the pellet from the first centrifugation two times with Nuclei washing buffer. The efficiency of fractionation was analyzed by immunoblotting using β-tubulin antibody as a cytoplasmic and lamin A/C antibody as a nuclear marker.

Membrane fraction was isolated using ProteoJET™ Membrane Protein Extraction Kit (Thermo Fisher Scientific, Inc., Waltham) according to the manufacturer's protocol. The efficiency of fractionation was analyzed by immunoblotting using CD31 antibody as a membrane marker.

siRNA transfection

RACK1 (GNB2L1) and TIMAP (PPP1R16B) were silenced using 50nM ON-TARGET plus SMARTpool siRNA, L-006876-00-0 HumanGNB2L1 and L-004065-00-0 HumanPP1R16B, respectively, (Thermo Fisher Scientific, Inc., Waltham) with DharmaFECT-4 transfection reagent (Thermo Fisher Scientific, Inc., Waltham) in serum-free

medium. ON-TARGETplus siCONTROL nontargeting pool (D-001810-10-01-05; Thermo Fisher Scientific, Inc., Waltham) was used as an irrelevant control. After 6 h the medium was changed to complete medium. Cells were further incubated for 48-72 hours.

SDS-PAGE and Western blot

Protein samples were separated by 10-15% SDS-PAGE and transferred to 0.45μm pore sized Hybond ECL Nitrocellulose Membrane (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% low-fat dry milk powder in TBST and then they were incubated with primary antibody diluted in TBST containing 1% BSA for 1 hour or O/N at 4°C. After the washing steps (twice in TBST, once in TBS, 5 min each), the membranes were incubated with the HRP-conjugated secondary antibody diluted in 1% BSA-TBST for 1 hour. After washing, the membrane was incubated with Immobilon Western HRP Substrate (Millipore, Billerica, MA). Images were acquired using darkroom development techniques for chemiluminesence (Kodak Medical X-ray Developer) or using an Alpha Innotech FluorChem® FC2 Imager. Representative data of at least 3 independent experiments are shown.

GST pull-down assay

Escherichia coli BL21 (DE3) transformed with pGEX-4T-2/pGEX-4T-3 containing glutathione S-transferase (GST), pGEX-4T-3 containing TIMAP mutants or pGEX-4T-2 containing RACK1 or EBP50 constructs were induced with 1mM IPTG and grown at room temperature with shaking for 3h. Cells were harvested by centrifugation, sonicated in lysis buffer (50mM Tris-HCl (pH 7.5), 0.1% Tween 20, 0.2% 2-mercaptoethanol, protease inhibitors) and proteins were isolated by affinity chromatography on glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol. BPAEC grown in 100 mm culture flasks were washed twice with 1x ice-cold PBS, scraped, and lysed in 600μl lysis buffer. The lysates were incubated for 4 h at 4°C with GST or different GST-fused proteins coupled to glutathione Sepharose beads. The beads were washed three times with 1x PBS then the GST fusion proteins were eluted with 10mM glutathione and were tested for interacting proteins by SDS-PAGE and Western blot.

Anti-V5 Agarose Affinity Gel Chromatography

BPAEC grown in 6 well plates were transfected with pcDNA3.1 V5-His, pcDNA3.1 V5-His PP2A Bα, or pcDNA3.1 V5-His PP2A B'γ construct prepared in our laboratory. 24 h after transfection the cells were washed twice with 1x ice-cold PBS, scraped, and lysed in 600µl lysis buffer (50mM Tris-HCl (pH 7.5), 0.2% 2-mercaptoethanol and protease inhibitors). The cell lysates were sonicated then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was added to 50µl Anti-V5 Agarose conjugate and rotated for 5 h at 4°C. Beads were washed 3 times with PBS then boiled with 1x SDS loading buffer and analyzed by Western blot.

ECIS measurements and in vitro wound healing assay

To study endothelial barrier function, wound healing, and cell migration ECIS (Electric cell-substrate impedance sensing) model Zθ, (Applied BioPhysics Inc. Troy, NY) was used. To monitor transendothelial electric resistance, control or transfected cells were seeded onto type 8W10E arrays. Wild type, mock, or mutant EBP50 transfected cells were seeded on type 8W10E arrays. After the cells achieved monolayer density (about 1000Ω impedance), an alternate current of 5 mA at 60 kHz frequency was applied for 30 sec duration to establish wounds in the cell layer, which led to the death and detachment of cells present on the small active electrode, then the impedance was measured for 5 h. The impedance in each wounded well increased gradually, until it reached a maximum plateau value.

Statistical analysis

Analysis of Variance on Ranks was performed on Pearson coefficients using SigmaStat. Statistical significance was determined at $P<0.05$ by Dunn's Method, multiple comparisons versus control group (group of interphase cells).

Statistical analysis was done with Student's t-test. Densitometric analysis of immunoblots was done by Image J software.

Results and discussion

Nuclear localization of EBP50 in endothelial cells

EBP50 is a well characterized protein in epithelium, yet little is known about its role in other cell types. It is expressed at high levels in the kidney, liver, small intestine, and placenta, but also expressed at lower levels in the brain and lung. Studies made on epithelial cells revealed that EBP50 is localized at the plasma membrane and the cytoplasm, and it is accepted that its primary function is to act as a scaffold protein linking transmembrane proteins to various cytoskeletal proteins. In interphase endothelial cells (BPAEC and HUVEC) we detected EBP50 in the nucleus and in the perinuclear region in contrast with the above mentioned cytoplasmic location found by others in epithelial cells. Our results were verified by immunofluorescent staining, subcellular fractionation, and recombinant protein expression.

Phosphorylation dependent localization of EBP50

When BPAEC were co-stained with anti-EBP50 and anti-β-tubulin antibodies, we observed that EBP50 left the nuclear region in dividing cells. EBP50 was in the nucleus in interphase cells, however, during prophase its redistribution to the cytoplasm was apparent and we could detect its disappearance from the cytosol only in the phase of cytokinesis. It is known that EBP50 can be phoshorylated by protein kinases on multiple sites. During mitosis it is phosphorylated by Cdk1 at Ser^{279} and Ser^{301} and this phosphorylation inhibits its oligomerization. The SPX(K/R) preferred phosphorylation motifs of Cdk1 are conserved in EBP50 of different species, thus Ser^{279} and Ser^{301} residues in the rabbit protein correspond to Ser^{288} and Ser^{310} in the bovine EBP50. Therefore, we concluded that the cell cycle dependent localization change and mobility shift on SDS-PAGE of the endothelial EBP50 are in parallel with the phosphorylation and possibly the oligomerization state of the protein. Furthermore, the phospho-mimic mutant (S288D:S310D) form of EBP50 showed mobility shift on SDS-PAGE and cytoplasmic localization as did the endogenous EBP50 in dividing cells.

Identification of the protein phosphatase interacting with EBP50

Protein phosphorylation/dephosphorylation is a reversible process. We intended to identify the Ser/Thr specific protein phosphatase catalyzing the dephosphorylation of EBP50. BPAEC were arrested in mitotic phase and at the point of the release the media were implemented with specific protein phosphatase inhibitors. A specific PP2A inhibitor was able

to maintain the phosphorylation state of EBP50, suggesting that PP2A is involved in the dephosphorylation. Immunoprecipitation experiments indicated that PP2A interacts and co-localizes with EBP50 in a cell cycle dependent manner. Furthermore, we identified Bα (also named as PR55), besides the AC dimer, with pull-down assays as the third subunit in the PP2A holoenzyme interacting with EBP50.

BPAEC were co-stained with anti-EBP50 and anti-PP2Ac antibodies. The location change and the co-localization of EBP50 with PP2Ac during the course of mitosis were parallel, with the highest levels of co-localization during the phases from metaphase to early cytokinesis. Our results show that a protein thought to be a linker between the plasma membrane and cytoplasmic/cytoskeletal proteins may be involved in nuclear events as well. We aquired evidences for cell cycle dependent localization and phosphorylation of EBP50, and its interaction with PP2A during mitosis. Our results indicate that EBP50 may have a significant role in the course of the cell cycle and that PP2A can be the phosphatase dephosphorylating P-Ser^{288} and P-Ser^{310} of EBP50 in BPAEC.

RACK1 binds TIMAP-PP1c complex in endothelial cells

In a search for partners of TIMAP we recognized and proved by different methods that TIMAP binds the adaptor protein, RACK1. We found that PP1cδ is present in the RACK1-TIMAP complex as TIMAP is its regulatory/targeting subunit, but does not bind directly to RACK1. Our results indicate that RACK1 binds to the NLS region at the N-terminal of TIMAP, but there is/are further association site(s) within the C-terminal half region of TIMAP suggesting a more complex surface for the interaction. It should be noted that the PKA/GSK-3β phosphorylation sites, Ser337/333 of TIMAP are present in this region. Site directed mutations revelaed that the phosphorylation state of TIMAP is an important factor of the interaction. Moreover, we showed that the site/region in RACK1 responsible for TIMAP binding is within the N-terminal half (WD 1-4) of the protein.

Activation of the cAMP/PKA pathway affects localization of TIMAP and attenuates the RACK1-TIMAP interaction

Although RACK1 and PKC are intimately related to each other, that seems irrelevant in the TIMAP-RACK1 relation, as PKC activation of EC did not change their binding. On the other hand, phosphorylation of TIMAP by activation of the cAMP/PKA pathway had significant effect not only on the interaction, but also on the localization of TIMAP. Upon

cAMP/PKA activation of EC, we detected enrichment of TIMAP in the plasma membrane and its translocation from the nucleus. The phosphorylation may directly impair the connection by inducing conformation change of TIMAP, or may initiate interactions with other binding partners leading to the loss of RACK1-TIMAP complex. Our results clearly demonstrate that significant loss in TIMAP-RACK1 complex follows PKA primed GSK-3β phosphorylation of TIMAP.

RACK1 aids farnesylation/membrane transport of TIMAP

When the TIMAP-RACK1 interaction was diminished by depletion of RACK1, TIMAP was not found in the plasma membrane of the silenced cells suggesting a pivotal role of RACK1 in prenylation/membrane localization of TIMAP. Prenylation of TIMAP at the C-terminal CAAX box by farnesyl transferase is required for its membrane localization. Eventually, deficiency of membrane anchored TIMAP may be the result of the lack of its prenylation. Our results indicated that both TIMAP and farnesyl transferase bind to the N-terminal half of RACK1 and the interaction between TIMAP and farnesyl transferase was diminished in RACK1 depleted cells. These confirm the assumption of RACK1 being the anchoring surface for prenylation of TIMAP. By ECIS measurements we found that RACK1 should also be regarded as a participant in maintaining barrier integrity, through the regulation of TIMAP prenylation.

Summary

Vascular endothelial cell monolayer acts as a semiselective barrier between blood and the interstitium. Phosphorylation level of many cytoskeleton and cytoskeleton-associated proteins playing crucial role in the EC barrier function is critical to tissue and organ function. ERM-binding phosphoprotein 50 (EBP50) is a phosphorylatable PDZ domain-containing adaptor protein that is abundantly expressed in epithelium but was not yet studied in the endothelium. We found unusual nuclear localization of EBP50 in bovine pulmonary artery endothelial cells (BPAEC). Immunofluorescent staining and cellular fractionation demonstrated that EBP50 is present in the nuclear and perinuclear region in interphase cells. In the prophase of mitosis EBP50 redistributes to the cytoplasmic region in a phosphorylation dependent manner and during mitosis EBP50 co-localizes with protein phosphatase 2A. Furthermore, *in vitro* wound healing of BPAEC expressing phospho-mimic mutant of EBP50 was accelerated indicating that EBP50 is involved in the regulation of the cell division. Cell cycle dependent specific interactions were detected between EBP50 and the subunits of PP2A (A, C, and Bα) with immunoprecipitation and pull-down experiments. The interaction of EBP50 with the Bα containing form of PP2A suggests that this holoenzyme of PP2A can be responsible for the dephosphorylation of EBP50 in cytokinesis. Moreover, our results underline the significance of EBP50 in cell division via reversible phosphorylation of the protein with cyclin dependent kinase and PP2A in normal cells.

TIMAP, TGF-β inhibited membrane-associated protein, is most abundant in endothelial cells with a regulatory effect on the endothelial barrier function, yet little is known about its interacting partners. RACK1, receptor for activated protein kinase C, serves as an anchor in multiple signaling pathways. We found that RACK1 binds to the TIMAP-PP1c complex in endothelial cells. WD1-4 repeats of RACK1 were identified as critical regions of the interaction both with TIMAP and farnesyl transferase. Phosphorylation of TIMAP by activation of the cAMP/PKA pathway reduced the amount of TIMAP-RACK1 complex and enhanced translocation of TIMAP to the cell membrane in vascular endothelial cells. However, both membrane localization of TIMAP and transendothelial resistance were attenuated after RACK1 depletion. Farnesyl transferase, the enzyme responsible for prenylation and consequent membrane localization of TIMAP, is present in the RACK1-TIMAP complex in control cells, but it does not co-immunoprecipitate with TIMAP after

RACK1 depletion. Our results suggest that transient parallel linkage of TIMAP and farnesyl transferase to RACK1 could ensure prenylation and transport of TIMAP to the plasma membrane where it may attend in maintaining the endothelial barrier as a phosphatase regulator.

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List of publications related to the dissertation

1. **Boratkó, A.**, Gergely, P., Csortos, C.: RACK1 is involved in endothelial barrier regulation via its two novel interacting partners.
   Cell Commun Signal. 11 (2), 1-14, 2013.
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2. **Boratkó, A.**, Gergely, P., Csortos, C.: Cell cycle dependent association of EBP50 with protein phosphatise 2A in endothelial cells.
   PLoS One. 7 (4), e35595, 2012.
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List of other publications

3. Szilágyi, O., **Boratkó, A.**, Panyi, G., Hajdú, P.: The role of PSD-95 in the rearrangement of Kv1.3 channels to the immunological synapse.
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Presentations:

Anita Boratkó, Pál Gergely, Csilla Csortos**:** PP2A dephosphorylates ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 at the mitotic phase of the cell cycle, 2011, Signal transduction and skin biology: a training course, Galyatető

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