SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Novel interacting partners of the protein phosphatase 2A B55α holoenzyme and their role in angiogenesis

by Zsófia Thalwieser

Supervisor: Anita Boratkó (PhD)

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2025

Novel interacting partners of the protein phosphatase 2A B55α holoenzyme and their role in angiogenesis

By Zsófia Thalwieser, Biotechnology (MSc degree)

Supervisor: Anita Boratkó, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Defense Committee**:	László Csernoch, PhD, DSc
Reviewers:	Krisztina Köröskényi, PhD Szabolcs Sipeki, PhD
Members of the Defense Committee:	András Balla, PhD Endre Károly Kristóf, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 12 p.m. on June 24, 2025.

1. Introduction

1.1.Endothelial cells and their physiological role

Endothelial cells (ECs) are one of the most important regulatory elements of the vasculature, as they form a semi-permeable monolayer (endothelium) that separates circulating blood from the surrounding tissues. They regulate blood vessel elasticity, vascular permeability, coagulation, and inflammatory processes. The structure and functional integrity of the cells forming the endothelium are essential for maintaining the proper function of the vascular wall and circulation. The extracellular matrix (ECM) plays a crucial role in EC vascular development processes, cell migration, proliferation, morphogenesis, and maintaining cellular stability. The reversible phosphorylation of proteins has a pivotal role in intracellular regulatory mechanisms. ECs respond to physical and chemical stimuli within the circulation, thereby regulating processes such as blood coagulation, vasomotor tone, and immune and inflammatory responses. They are also key players in the processes of vasculogenesis and angiogenesis.

1.2. The process of angiogenesis

The initial blood vessels in the embryo are formed through a process known as vasculogenesis, during which precursor cells (angioblasts) differentiate into ECs and form a vascular system. In contrast, angiogenesis involves the formation of new blood vessels from the existing vasculature, expanding and developing the vascular system. Angiogenesis plays an important role in many physiological processes, such as development, reproduction and wound healing. However, it also has critical significance in pathological conditions, including cancer. Angiogenesis is a tightly regulated process involving a variety of soluble and membrane-bound molecules, as well as other factors. This process is governed by cytokines, growth factors and matrix proteins, such as vascular endothelial growth factor (VEGF), angiopoietins and Tie receptors, transforming growth factor-beta (TGF-β), and members of the fibroblast growth factor (FGF) family. The first step in angiogenesis is activation, primarily modulated by low oxygen and nutrient levels or inflammation. These physiological conditions enhance the expression of cytokines and other growth factors through various processes. Chronic inflammation can activate endothelial cells, further promoting vascular sprouting, which results in abnormal vascular structure and function.

1.3.Protein phosphatases

Reversible protein phosphorylation forms the basis of regulating numerous cellular processes. In eukaryotic cells, proteins can be phosphorylated on Ser, Thr, and Tyr side chains. The phosphorylation state of proteins varies according to physiological needs and is regulated by protein kinases and protein phosphatases. Protein phosphatases remove phosphate groups from phosphoproteins, hydrolyzing phosphoric acid monoesters into phosphate ions and molecules with free hydroxyl groups. Based on substrate specificity, protein phosphatases are classified into three groups: Ser/Thr-specific phosphatases, Tyr-specific phosphatases, and dual-specificity phosphatases. Ser/Thr-specific protein phosphatases can be further categorized by structure into phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and CTD-phosphatases (FCP/SCP). PPPs can be divided into seven subfamilies: PP1, PP2A, PP2B (calcineurin), PP4, PP5, PP6, and PP7.

1.4. Structure of protein phosphatase 2A (PP2A)

PP2A is a widely studied Ser/Thr-specific protein phosphatase expressed in many cell types and plays an important role in several biological processes. In cells, PP2A exists in both heterodimeric and heterotrimeric forms. The heterodimeric form (core dimer), consists of a structural A subunit (PP2A A/ PPP2AR1/PR65) and a catalytic C subunit (PP2A C/PPP2C). The regulatory B subunit can bind to this dimer to form the heterotrimeric structure. Both the structural A subunit and the catalytic C subunit have two nearly identical isoforms based on their nucleotide sequences. In its heterotrimeric form, the structural A subunit first binds to the catalytic C subunit, facilitating interactions with the regulatory subunit and other substrates. The regulator B subunit family has four structurally distinct subfamilies (B/B55/PR55, B'/B56/PR61, B"/B72/PR72 and B"'/PR93(SG2NA)/PR110(Striatin)). These subfamilies exhibit no structural similarity, and their diversity is further increased by multiple isoforms within each subfamily. Regardless of the subfamily or isoform, the regulatory subunits recognize and bind the same sequence on the structural A subunit. Genetic and biochemical studies have shown that the regulatory B subunits determine the subcellular localization and substrate specificity of the holoenzymes. The estimated number of holoenzymes that can be assembled from different A-B-C subunits is 96, but the exact number of existing complexes is unknown.

1.5. Biological function of PP2A

Due to the diverse holoenzyme structure, PP2A participates in several biological processes, including cell cycle regulation, DNA replication, transcription and translation, signal transduction, cell proliferation, cytoskeletal dynamics, cell motility and apoptosis. Through interactions with viral proteins, PP2A influences cell transformation and has been shown to have tumor suppressor properties. Depending on its structure, the holoenzyme can promote both pro-apoptotic and anti-apoptotic signaling pathways. In cytoskeletal regulation, PP2A contributes to the dephosphorylation of various cytoskeletal and microtubule-associated proteins.

1.6. Flotilin-1 protein

The flotillin protein family consists of the flotillin-1 (reggie-2) and flotillin-2 (reggie-1) proteins. These proteins are present in many cell types, although the localization varies depending on the cell type. A characteristic feature of flotillin proteins is the SPFH (stomatin, prohibitin, flotillin, and HflK/C) or PHB (prohibitin) domain at the N-terminal region and the alanine- and glutamate-rich short repetitive motifs (flotillin repeats) in the C-terminal region. These membrane-associated proteins play roles in forming connections between surface receptors and the cytoskeleton and in signal transduction processes. In terms of post-translational modifications, flotillin-1 has a single palmitoylation site (Cys^{34}) and is phosphorylated on the Tyr^{160} residue by Fyn kinase.

1.7. Thrombospondin-1 (TSP1) protein

Thrombospondins (TSPs) are matricellular glycoproteins expressed in many cell types. Under physiological conditions, their levels in the ECM are low, but they significantly increase in response to tissue damage or pathological conditions. The TSP family comprises five proteins (TSP1, TSP2, TSP3, TSP4 and TSP5/COMP). TSP proteins play a key role in mediating cell-cell and cell-matrix communication. TSP1 was first identified as a "thrombin-sensitive" protein. It is secreted by many cell types, including endothelial cells. Structurally, TSP1 consists of an amino-terminal (N-terminal) and a carboxy-terminal (C-terminal) globular domain, a homologous procollagen (PC) region (also known as von Willebrand C repeat (vWC)) followed by three specific repeat sequences: type 1, type 2 and type 3 repeats (TSR). Its domains can bind to multiple molecules, giving the protein a broad range of functions. Through interactions with integrins, TSP1 is involved in cell adhesion, migration, proliferation and spreading, influencing the biological function of ECs. While TSP1 plays diverse regulatory roles in cellular processes, its most important function is inhibiting angiogenesis through various interactions.

Although the post-translational modifications of TSP1 are not well studied, its sequence contains several potential modification sites.

2. Aims

One of the main research interests of our group is the detailed investigation of the role of the PP2A enzyme in endothelial cells. The Ser/Thr specific PP2A regulates many crucial cellular functions. In our previous studies, we demonstrated that the B55α regulatory subunit containing PP2A holoenzyme plays a key role in regulating endothelial barrier function and β-catenin dephosphorylation. The aim of our present work was to gain further insight into the substrate proteins of PP2A holoenzyme and its role in endothelial cell physiology. To achieve this, we planned to identify a novel interacting partner of PP2A and investigate the physiological significance of this interaction. Our specific research objectives were:

- Identification of a new interacting partner in endothelial cells using a previously created recombinant GST-PP2A B55α protein.
- Confirmation of the interaction between the newly identified interacting partner and PP2A B55α proteins by pull down assays, Western blot, Far Western blot, immunoprecipitation and immunofluorescence techniques.
- Investigation of the enzyme-substrate relationship between PP2A and the interacting partner, with particular emphasis on the potential role of dephosphorylation in endothelial cell function.

PP2A is known to play an important role in numerous biological processes, such as cell division, cytoskeletal dynamics and cell motility. Furthermore, it acts as a tumor suppressor and through its phosphatase activity, can also influence angiogenesis in endothelial cells. Based on this, we aimed to explore the role of PP2A B55α in angiogenesis-related signaling pathways through the following objectives:

- Investigation of the effect of PP2A B55α depletion on angiogenesis-related proteins using Proteome profiler angiogenesis array.
- Mapping the interaction through molecular biology techniques.
- Studying the relationship between PP2A B55α and its target proteins, including the enzyme-substrate connection.
- Examining the role of PP2A B55α in angiogenesis by regulating its newly identified interacting partner.

3. Material and methods

Polymerase chain reaction (PCR). Specific primer pairs were used to amplify the coding sequences of PP2A B55α, flotillin-1, and TSP1 from cDNA or template sequences. The resulting DNA sequences were cloned into vectors suitable for bacterial or mammalian expression.

Quantitative Real-Time PCR (qPCR). qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) according to the manufacturer protocol. A LightCycler 480 Thermocycler (Roche, Basel, Switzerland) was used for measurements.

Agarose gel electrophoresis and DNA gel extraction. Depending on the DNA size, gel containing 0.9%-1.2% agarose and GelRed nucleic acid stain (20000x dilution, Biotium Inc) were used. DNA fragment sizes were checked using a 1kb DNA ladder (Thermo Fischer Scientific). Electrophoresis was performed at 60-80 V in 1xTAE buffer. DNA bands were excised under UV light and DNA was extracted using Thermo GeneJET Gel Extraction (Thermo Fischer Scientific) kit following the manufacturer's protocol. DNA elution was performed in nuclease-free water at 50-55°C. The purity and concentration of the isolated DNA were measured using a NanoDrop 2000 spectrophotometer.

Restriction digestion. Inserts and vectors for cloning, along with the back-to-back PCR amplified products, were digested in reaction mixtures containing appropriate FastDigest restriction endonucleases (Thermo Scientific^{TM}) for 1 hour in a 37°C.

Ligation. Prepared vectors and inserts, as well as linearized back-to-back phosphomutant plasmids, were ligated using T4 DNA ligase (Thermo Fischer Scientific) under sterile conditions for 1 hour at room temperature or overnight at 4°C. Ligated products were transformed into JM109 *E. coli* competent cells.

Transformation. Ligation mixtures were added to 100µl of JM109 *E.coli* competent cells and incubated on ice for 20 min. Heat shock was performed at 42°C for 45 seconds, followed by 2 minutes on ice. SOC medium containing 20mM glucose was added to the cells and incubated at 37°C for 45 minutes at 180rpm to develop antibiotic resistance.

Plasmid purification and DNA sequencing. Colonies were inoculated into antibiotic-containing LB medium and cultured at 37°C for 16-18 hours at 180rpm. Depending on the volume, plasmids were purified using the Thermo GeneJET Plasmid Miniprep or Maxiprep Kits (Thermo Fischer Scientific). Isolated DNA was analyzed by NanoDrop 2000 and restriction digestion. Mutations and recombinant plasmids were validated by sequencing (BIOMI Kft, Gödöllő).

Bacterially protein expression and GST-tagged protein production. GST-tagged protein encoding plasmids were transformed into BL21(DE3) *E.coli* competent cells. After culturing for 16-18 hours, cell suspensions were diluted 1:1000 in 2xYT medium and cells were grown at 37°C with shaking at 180rpm until OD_{600} reached 0.5. Protein expression was optimized by varying isopropylthio-β-galactoside (IPTG) concentrations and incubation temperatures (13°C, 25°C and 37°C).

Recombinant protein purification. Bacterial cells were lysed in protease inhibitor cocktail (1:1000) containing lysis buffer and homogenized using a Branson Sonifer (AMS Materials). After centrifugation at 12000 rpm for 20 minutes at 4°C, lysates were added to pre-washed glutathione Sepharose 4B resin and rotated at 4°C for binding.

Bovine pulmonary artery endothelial cell culture. Bovine pulmonary artery endothelial cells (BPAEC) were cultured in Minimum Essential Medium (Lonza) supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO_2. Cells between passages 16 and 21 were used.

Transfection, gene silencing. BPAECs at 80% confluence were transfected with recombinant plasmids using Lipofectamine 3000 and P-reagent (Invitrogen™) according to the manufacturer's protocol. Gene silencing was performed using specific siRNA with Lipofectamine RNAiMAX transfection reagent (Invitrogen™). Post-transfection intervals were optimized.

Pull down. Recombinant proteins immobilized on glutathione Sepharose 4B were incubated with endothelial cell lysates at 4°C for16-18 hours.

SDS-PAGE, Western blot and Far Western blot. Proteins were separated by molecular weight using polyacrylamide gels containing 8–12% acrylamide. The separated proteins were transferred onto a 0.45 μm pore-size nitrocellulose membrane (Advantec MFS Inc.). The membrane was blocked in 5% skim milk dissolved in TBST for 1 hour, followed by incubation in TBST containing 0.1% BSA and primary protein-specific antibodies for 16–18 hours at 4°C. The next day, the membrane was incubated for 1 hour at room temperature with HRP-conjugated anti-mouse/anti-rabbit IgG secondary antibody. Proteins were detected using the Chemidoc Touch (Bio-Rad) instrument.
For Far Western blot, GST-tagged proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked in 5% BSA for 1 hour and then incubated with endothelial cell lysates for 16–18 hours at 4°C. The next day, the membranes were incubated for 4 hours at 4°C with specific primary antibodies (0.1% BSA, TBST), followed by incubation with HRP-conjugated anti-rabbit/anti-mouse IgG secondary antibody. Chemiluminescent signals were detected using the Chemidoc Touch system.

Proteome Profiler Angiogenesis Array. Proteome Profiler Human Angiogenesis Array Kit (Bio-Techne R&D System Ltd) was used to analyze non-targeting and siRNA-transfected cell lysates according to the manufacturer's protocol. Results were evaluated using ImageJ software.

Immunoprecipitation (IP). Endothelial cells were lysed by sonication and centrifuged. During a pre-clearance step, non-specifically bound proteins were removed. Antibodies specific to the protein of interest and protein G-Sepharose (GE Healthcare) were added to the supernatant, and samples were incubated at 4°C for 16–18 hours. The following day, samples were washed, and bound proteins were eluted by boiling in 2× SDS sample buffer.

Immunofluorescence (IF) and confocal microscopy. Cells grown on coverslips were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in TBS, and blocked with 2% BSA. Cells were then incubated with protein-specific primary antibodies for 1 hour, followed by light-protected incubation with light-sensitive secondary antibodies for 1 hour. Coverslips were mounted onto slides using SlowFade Gold antifade reagent (Molecular Probes). Images were captured with a Leica TCS SP8 confocal microscope using an HC PL APO CS2 63× 1.40NA oil immersion objective. Immunofluorescence images were analyzed using the Las AF software.

Proximity Ligation Assay (PLA). After fixing, permeabilizing, and blocking endothelial cells, they were incubated with protein-specific primary antibodies for 1 hour. The PLA was performed using the Duolink In Situ Kit (Sigma-Aldrich) following the manufacturer's instructions. Images of cells were taken using a Leica TCS SP8 confocal microscope.

In vitro PKC phosphorylation and in vitro dephosphorylation. For in vitro phosphorylation, purified recombinant proteins were incubated with active PKC enzyme at 30°C for 2 hours in the buffer recommended by the manufacturer. Phosphorylation efficiency was verified using Western blot with phospho-specific antibodies. For in vitro dephosphorylation, phosphorylated recombinant proteins immobilized on glutathione Sepharose beads were incubated with lysates from differently treated endothelial cells. Dephosphorylation was assessed via Western blot using phospho-specific antibodies.

Cell fractionation. Membrane and cytoplasmic fractions of cells were isolated using the ProteoJET Membrane Protein Extraction Kit (Thermo Scientific) according to the manufacturer's protocol. Fraction purity was verified using membrane, cytoplasmic, and nuclear markers.

NanoBiT system. As a positive control, we used SmBiT-PRKACA and LgBiT-PRKAR2A plasmids encoding the catalytic and regulatory subunits of protein kinase A (PKA) provided by the manufacturer. For negative controls, HaloTag-SmBiT was co-transfected with our LgBiT-PP2A B55α plasmid. Luminescence signals arising from interactions were detected using a Tecan Spark multimode microplate reader (Tecan Group).

ECIS measurements (electric cell-substrate impedance sensing). Cell attachment, migration and wound healing were studied using a high-sensitivity, non-invasive biophysical method. For wound healing experiments, a single high voltage pulses were used to introduce wounds, and healing was monitored in real time.

Scratch Assay. A uniform scratch was made on the endothelial cell monolayer. Wound closure was photographed hourly using a Leica MC 120 HD microscope with a 5× objective lens. Wound healing was quantified using ImageJ software.

In vitro angiogenesis. Endothelial cells were plated in µ-Slide plates (Ibidi) containing Matrigel and conditioned medium. Tube formation was monitored hourly using a Leica MC 120 HD microscope. Results were analyzed using ImageJ software.

3D magnetic cell culture. Endothelial cells were magnetized using NanoShuttle-PL magnetic beads according to the manufacturer's instructions. The magnetized cells were placed into a cell-repellent plate, and a specialized magnetic module was placed beneath the plate, forming 3D spheroids within 16-18 hours of incubation. After removing the module changes in the spheroids were monitored at defined time points. Images were analyzed using ImageJ software.

Anti V5-agarose affinity gel. BPAEC cells were transfected with recombinant constructs containing V5-His tags. The following day, transfected cells were lysed via sonication, and lysates were incubated with anti-V5 agarose resin for 5 hours at 4°C. After washing, samples were eluted with 1× SDS sample buffer and analyzed by Western blot.

Statistical analysis. Results are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism (version 8.0.1) software according to the indicated tests. Significant differences were marked with asterisks (*) ($p<0.05$ (*); $p<0.01$ (**); $p<0.001$(***)). Densitometry of immunoblots was performed using ImageJ (version 1.54h) software.

4. Results

4.1. Identification of a novel interaction partner of PP2A and investigation of its physiological significance

4.1.1. Identification of flotillin-1 protein as a novel interaction partner of PP2A B55α in endothelial cells

To identify new interaction partner of the PP2A B55α subunit, we created a recombinant pGEX-4T-2-PP2A B55α plasmid. After purification of GST and GST-PP2A B55α proteins using glutathione Sepharose 4B resin, the immobilized proteins were incubated with BPAEC lysates. Compared to control GST samples, a faint extra band at 45 kDa was observed, excised from the gel, and identified by LC-MS/MS mass spectrometry as flotillin-1. The interaction between PP2A B55α and flotillin-1 proteins was further confirmed by Western blot analysis of the pull down samples.

4.1.2. Production of recombinant GST-flotillin-1 protein

The flotillin-1 coding sequence was amplified by PCR from human pulmonary artery endothelial cell cDNA. The PCR product was separated by agarose gel electrophoresis, isolated, ligated, transformed, and purified. Recombinant plasmids were analyzed by restriction digestion and sequencing (confirmed by BIOMI Kft.).

The recombinant plasmids were transformed into BL21 (DE) *E. coli* competent cells and induced at various temperatures and IPTG concentrations. After optimalization, the GST-tagged flotillin-1 protein was purified on glutathion Sepharose 4B resin.

Pull-down assays and immunoprecipitation confirmed that flotillin-1 interacts directly or indirectly with the PP2A holoenzyme B55α regulatory subunit. Using anti-V5 agarose affinity gel, we demonstrated that flotillin-1 isoform interacts specifically with the PP2A B55α subunit.

4.1.3. PP2A B55α and flotillin-1 proteins co-localize in endothelial cells

Immunofluorescence staining of endogenous proteins in endothelial cells revealed that PP2A B55α protein was evenly distributed in the cytoplasm, while flotillin-1 displayed a filamentous pattern also in the cytoplasm. The two proteins colocalized in the cytoplasm, predominantly around the nucleus.

4.1.4. Impact of PP2A activity on the intracellular localization of flotillin-1 protein

ECs were treated with different drugs to affect PP2A and PKC activity. PKC enzyme was activated by PMA and inhibited by Gö6976 treatment. PP2A enzyme activity was inhibited by okadic acid treatment. Immunofluorescence staining after various treatments affecting PP2A and PKC activities revealed that inhibiting PP2A with okadaic acid or silencing the B55α subunit resulted in the perinuclear accumulation of flotillin-1. PKC activation with PMA produced similar changes, while PKC inhibition caused flotillin-1 to relocate to the cell membrane. Western blot analysis after cell fractionation confirmed these results.

4.1.5. Creation of phosphor-mutant flotillin-1 proteins via site-directed mutagenesis

Based on literature, flotillin-1 is phosphorylated at Ser^{315} side chain. Using site-directed mutagenesis, we generated phosphonull (S315A) and phosphomimetic (S315D) mutants. Sequencing confirmed the mutations, and GST-tagged S315A and S315D proteins were expressed and purified. Localization studies showed that wild-type and S315A flotillin-1 displayed a homogeneous cytoplasmic distribution, while S315D accumulated around the nucleus, consistent with endogenous protein behavior after PKC activation or PP2A inhibition.

4.1.6. Phosphorylation of Ser315 side chain of flotillin-1 affects its interaction with PP2A

Proximity Ligation Assay (PLA) was carried out on control and PMA treated cells. PLA showed that while flotillin-1 interacted with PP2A in untreated cells, PMA-induced PKC activation significantly enhanced this interaction.

In vitro PKC phosphorylation assay was performed using immobilized wild type, S315A, S315D GST-flotillin-1 proteins. Phosphorylation of the recombinants was examined by Western blot technique using phospho-Ser PKC substrate specific antibody. In vitro phosphorylation assays revealed that PKC specifically phosphorylates flotillin-1 at Ser315, as only the wild-type GST-flotillin-1 exhibited signs of PKC-mediated phosphorylation, whereas the phosphonull and phosphomimetic mutants did not. This was further confirmed in PMA-treated endothelial cells transfected with pcDNA3.1-myc/His-flotillin-1 WT and S315A constructs, using immunoprecipitation techniques.

4.1.7. PP2A dephosphorylates flotillin-1 in the endothelial cells

The dynamics of the interaction between PP2A B55α and flotillin-1 proteins were investigated using the NanoBiT system. This system consists of two subunits, LgBiT (17.6 kDa) and SmBiT (11 amino acids), which were recombinantly fused to the target proteins. Upon interaction between the proteins, the LgBiT and SmBiT subunits associate, generating a luminescent signal. The required constructs were generated through cloning, and endothelial cells were co-transfected with LgBiT-PP2A B55α and SmBiT-flotillin-1 wild-type or phosphomutant constructs. The luminescent signal confirmed that PP2A B55α interacts with all three forms of flotillin-1. The interaction between PP2A B55α and the phosphomimetic flotillin-1 S315D produced the strongest luminescence signal. The interaction dynamics between wild-type flotillin-1 and PP2A B55α were linked to the reversible phosphorylation of flotillin-1.

To investigate the dephosphorylation of the flotillin-1 protein, immobilized GST-flotillin-1 was phosphorylated using PKC enzyme, and the phosphorylated protein was incubated with lysis buffer, cell lysate, okadaic acid, tautomycetin (TM, a PP1 inhibitor), and cell lysates pre-treated with either non-targeting siRNA or PP2A B55α-specific siRNA. Incubation with untreated, TM-treated, or non-targeting siRNA-treated lysates resulted in dephosphorylation of the recombinant protein. However, when incubated with lysates treated with okadaic acid (OA) or PP2A B55α-specific siRNA, dephosphorylation was inhibited, leaving the protein in its phosphorylated state. These findings indicate that flotillin-1 is a substrate of PP2A B55α.

4.1.8. Non-phosphorylatable flotillin-1 shows increased membrane localization and plays a role in cell migration and angiogenesis.

Endothelial cells were transfected with plasmids encoding wild-type, S315A (phosphonull), and S315D (phosphomimetic) flotillin-1 proteins and analyzed by immunofluorescence staining. Wild-type flotillin-1 was primarily localized in the cytoplasm. Phosphomimetic flotillin-1 (S315D) was enriched around the nucleus, mimicking the localization of endogenous flotillin-1 following PKC activation or PP2A inhibition. In contrast, phosphonull flotillin-1 (S315A) was distributed throughout the cell, excluding the nucleus. Cell fractionation experiments were also performed on transfected cells. Among the recombinant proteins, only phosphonull flotillin-1 (S315A) was consistently detected in the membrane

fraction. Wild-type flotillin-1 was also present in the membrane fraction, but only after PKC activity was inhibited.

The effects of flotillin-1 phosphomutants on cell adhesion and migration were assessed using Electric Cell-Substrate Impedance Sensing (ECIS) measurements. Cells expressing wild-type or phosphonull flotillin-1 adhered and spread more rapidly than control cells or those expressing phosphomimetic flotillin-1. Migration rates were evaluated using in vitro wound healing and scratch assays. Cells expressing wild-type or phosphonull S315A flotillin-1 demonstrated significantly higher migration rates compared to control cells or cells expressing the phosphomimetic S315D mutant.

To assess the angiogenic properties of transfected cells, a Matrigel extracellular matrix assay was performed. The vascular characteristics of the cells were evaluated by measuring total vessel length and the number of branching points. Both metrics were significantly higher in cells expressing wild-type or phosphonull flotillin-1 compared to control cells or those expressing phosphomimetic flotillin-1.

4.2. Investigation of the role of PP2A B55α in signaling pathways related to angiogenesis

4.2.1. Effect of PP2A B55α depletion on TSP1 expression in endothelial cells

To investigate the role of PP2A B55α in angiogenesis, BPAEC cells were transfected with PP2A B55α specific (siPPP2R2A) siRNA. Cell lysates were analyzed using Proteome Profiler Angiogenesis Array Kit, which simultaneously detects 55 angiogenesis-related proteins. Silencing PP2A B55α resulted in a significant decrease in TSP1 protein levels compared to lysates from cells treated with non-targeting siRNA. This reduction in TSP1 protein was further confirmed by Western blot analysis. Additionally, qPCR analysis demonstrated that the absence of PP2A B55α significantly reduced TSP1 expression at both the protein and mRNA levels.

4.2.2. Detection of the interaction between PP2A B55α and TSP1 proteins

To investigate the interaction between the PP2A holoenzyme containing the B55α regulatory subunit and TSP1 protein, immunoprecipitation experiments were performed in BPAEC cells. The results revealed that TSP1 interacts, either directly or indirectly, with the subunits of the PP2A holoenzyme. Co-localization of PP2A B55α and TSP1 was confirmed by immunofluorescence staining and the proximity ligation assay (PLA). Furthermore, using an

anti-V5 agarose affinity gel, it was determined that TSP1 specifically interacts with the PP2A holoenzyme containing the B55α regulatory subunit.

4.2.3. TSP1 protein is a substrate of the PKC enzyme

The specific interaction between TSP1 and PP2A suggested reversible phosphorylation of TSP1. To investigate this, the effects of protein kinase A (PKA) and protein kinase C (PKC) activity and inhibition on TSP1 protein levels were examined. Endothelial cells were subjected to various treatments, and Western blot and qPCR analyses indicated that PKC phosphorylation plays a role in the quantitative regulation of TSP1 protein levels.

To confirm PKC-mediated phosphorylation of TSP1, immunoprecipitation was performed on control and PMA-treated endothelial cells using a phospho-specific antibody. Western blot analysis of total lysates and immunocomplexes demonstrated that the PKC enzyme phosphorylates TSP1 on Ser residue(s).

4.2.4. Generation of bacterially expressed recombinant TSP1 constructs

The coding sequence of TSP1 was amplified from HeLa cDNA using nested primers, resulting in a product longer than the coding sequence. The coding sequence was then amplified from this extended product. The PCR product was ligated into the pGEM T-Easy vector. Following transformation and selection, DNA was isolated. Restriction digestion was used to excise the TSP1 coding sequence from the vector, which was subsequently purified and prepared for ligation into the pGEX-4T-2 vector. After ligation and transformation, recombinant pGEX-4T-2-TSP1 plasmids were verified by restriction digestion. The sequence of the recombinant plasmids was confirmed by BIOMI Kft via sequencing.

4.2.5. Generation of recombinant TSP1 protein fragments

To identify potential serine residues that could be phosphorylated by PKC, GPS 5.0 phosphorylation prediction software was utilized. The analysis identified five potential phosphorylation sites within the full-length TSP1 sequence: Ser^{44}, Ser^{93}, Ser^{297}, Ser^{1113} and Ser^{1163}. Based on these predictions, plasmids encoding recombinant GST-tagged TSP1 protein fragments were generated from the full-length pGEX-4T-2-TSP1 plasmid. Production conditions for the recombinant protein fragments were optimized by testing various IPTG concentrations, incubation temperatures, and production time intervals. Once the optimal

production conditions were determined, protein purification on affinity chromatography was also optimized.

4.2.6. TSP1 is regulated on the Ser^{93} side chain by PKC/PP2A B55α

To identify the PKC-phosphorylated serine residues, an in vitro PKC phosphorylation assay was performed with recombinant TSP1 protein fragments immobilized on glutathione Sepharose 4B resin. Western blot analysis using a phospho-Ser-PKC substrate-specific antibody indicated that Ser^{44} and/or Ser^{93} were likely phosphorylation sites. To confirm these findings, point mutations were introduced to generate single (S44A or S93A) and double (S44A/S93A) phosphonull mutants. Repeating the in vitro PKC phosphorylation experiment with these mutants confirmed that PKC specifically phosphorylates TSP1 at Ser^{93}.

The interaction between PP2A B55α and recombinant GST-TSP1 fragments was examined using a pull-down assay. The results demonstrated that B55α interacts with the N-terminal fragment containing the PKC phosphorylation site. Furthermore, a GST-TSP1 S93D phosphomimic mutant was generated by point mutation and tested in pull-down assays. The findings showed that PP2A B55α interacts less strongly with the phosphomimic mutant compared to the wild-type and phosphonull mutant.

In vitro dephosphorylation experiments confirmed that the PP2A B55α holoenzyme is responsible for dephosphorylating TSP1 phosphorylated at Ser^{93} by PKC.

4.2.7. PP2A B55α is essential for the regulation of TSP1 during wound healing

The physiological role of the PP2A B55α regulatory subunit was studied using a scratch assay to assess wound-healing capacity in endothelial cells. The results showed that B55α-depleted cells exhibited slower wound closure compared to control cells treated with non-targeting siRNA. During the wound-healing process, TSP1 and PP2A B55α protein levels were monitored by Western blot analysis. The findings suggest that PP2A B55α plays a key role in regulating TSP1 during wound healing.

4.2.8. PP2A B55α is involved in 3D spheroid stability and endothelial cell vascularization.

The impact of PP2A B55α depletion on the angiogenic properties of endothelial cells, including spheroid formation and vascularization, was also investigated. Time-lapse imaging of spheroids showed that B55α depletion affected their structural integrity. Spheroids formed by B55α-depleted cells were initially larger but became fragmented after 48 hours.

The angiogenic capacity of endothelial cells was assessed using a Matrigel 3D matrix assay. In the absence of the B55α regulatory subunit, cells exhibited significantly more branching points and longer vessel segments than control cells.

.

4.2.9. The phophomimic S93D form of TSP1 inhibits angiogenesis in endothelial cells

To explore the physiological role of TSP1 phosphorylation at Ser93, pcDNA3.1 myc-His-TSP1 recombinant plasmids were generated for expression in mammalian cells. BPAEC cells transfected with these plasmids were tested for their ability to form spheroids. Cells overexpressing c-myc-tagged TSP1 formed larger spheroids than control cells at the initial time point (0 minutes). In contrast, cells expressing the phosphomimic TSP1 S93D formed smaller but more compact spheroids.

The effect of TSP1 phosphomutants on endothelial cell vascularization was also examined. The results suggest that modification of the PKC phosphorylation site at Ser93, and by extension PKC-mediated phosphorylation of TSP1, regulates the angiogenic properties of endothelial cells.

5. Summary

Endothelial cells perform numerous functions by forming a semipermeable monolayer (endothelium) that separates blood circulation from surrounding tissues. The phosphorylation and dephosphorylation of proteins are essential mechanisms in the organization of endothelial cells into blood vessels and the regulation of barrier function. Understanding the functions of endothelial cells and their molecular mechanisms in greater detail is critical for studying vascular diseases and other related conditions.

PP2A is one of the most significant Ser/Thr-specific protein phosphatases, with a wide range of functions due to its structure. Through its B55α regulatory subunit, PP2A participates in the establishment and maintenance of barrier function, as well as in the regulation of angiogenesis. In our research, we identified flotillin-1 as a novel interacting partner of PP2A B55α. We found that the interaction between these two proteins is phosphorylation-dependent and significantly influenced by the activity of the PKC enzyme. Furthermore, we demonstrated that the phosphorylation and dephosphorylation of flotillin-1 affect its intracellular localization, which plays a key role in the formation and maintenance of the endothelial barrier.

Our work also revealed that PP2A B55α is critically important in the process of angiogenesis. Its absence led to the instability of vascular-like structures formed by endothelial cells, as supported by our 3D extracellular matrix experiments. We observed that the depletion of B55α resulted in a significant reduction in the levels of TSP1 protein, which further contributed to the loss of vascular stability. Additionally, we showed that the absence of B55α adversely affected wound healing and the maintenance of 3D cellular structures.

Our findings highlight the complex regulatory network of endothelial cells and emphasize the role of PP2A B55α in maintaining vascular stability and regeneration. These insights may contribute to the development of new therapeutic strategies for the treatment of vascular diseases. By improving our understanding of the regulatory mechanisms affecting endothelial cell function, we can explore innovative drug development targeting vascular stability and regeneration. Moreover, uncovering new pathways can enhance the diagnostics of vascular diseases, enabling earlier interventions and improving patient outcomes.

6. Publications

DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR
H-4002 Debrecen, Egyetem tér 1, Pf.: 400
Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

Nyilvántartási szám: DEENK//2024.PL
Tárgy: PhD Publikációs Lista

Jelölt: Thalwieser Zsófia
Doktori Iskola: Molekuláris Orvostudomány Doktori Iskola

A PhD értekezés alapjául szolgáló közlemények

1. **Thalwieser, Z.**, Fonódi, M., Király, N., Csortos, C., Boratkó, A.: PP2A Affects Angiogenesis via Its Interaction with a Novel Phosphorylation Site of TSP1.
   Int. J. Mol. Sci. 25 (3), 1-23, 2024.
   DOI: http://dx.doi.org/10.3390/ijms25031844
   IF: 4.9 (2023)
2. **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

További közlemények

3. Fonódi, M., **Thalwieser, Z.**, Csortos, C., Boratkó, A.: TIMAP, a Regulatory Subunit of Protein Phosphatase 1, Inhibits In Vitro Neuronal Differentiation.
   Int. J. Mol. Sci. 24 (24), 1-17, 2023.
   DOI: http://dx.doi.org/10.3390/ijms242417360
   IF: 4.9
4. Király, N., **Thalwieser, Z.**, Fonódi, M., Csortos, C., Boratkó, A.: Dephosphorylation of annexin A2 by protein phosphatase 1 regulates endothelial cell barrier.
   IUBMB Life. 2021, 1-12, 2021.
   DOI: http://dx.doi.org/10.1002/iub.2538
   IF: 4.709

DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR
H-4002 Debrecen, Egyetem tér 1, Pf.: 400
Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

5. Boratkó, A., Péter, M., **Thalwieser, Z.**, Kovács, E., Csortos, C.: Elongation factor-1A1 is a novel substrate of the protein phosphatase 1-TIMAP complex.
Int. J. Biochem. Cell Biol. 69, 105-113, 2015.
DOI: http://dx.doi.org/10.1016/j.biocel.2015.10.021
IF: 3.905

A közlő folyóiratok összesített impakt faktora: 22,652
A közlő folyóiratok összesített impakt faktora (az értekezés alapjául szolgáló közleményekre): 9,138

A DEENK a Jelölt által az iDEa Tudóstérbe feltöltött adatok bibliográfiai és tudománymetriai ellenőrzését a tudományos adatbázisok és a Journal Citation Reports Impact Factor lista alapján elvégezte.

Debrecen, 2024.12.14.