



Dephosphorylation of annexin A2 by protein phosphatase 1 regulates endothelial cell barrier

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Abstract

Annexin A2 (ANXA2) is a multifunctional protein expressed in nearly all human tissues and cell types, playing a role in various signaling pathways. It is subjected to phosphorylation, but no specific protein phosphatase has been identified in its posttranslational regulation yet. Using pull-down assay followed by liquid chromatography–mass spectrometry analysis we found that ANXA2 interacts with TIMAP (TGF-beta-inhibited membrane-associated protein) in pulmonary artery endothelial cells. TIMAP is highly expressed in endothelial cells, where it acts as a regulatory and targeting subunit of protein phosphatase 1 (PP1). TIMAP plays an important role in the regulation of the endothelial barrier maintenance through the dephosphorylation of its several substrate proteins. In the present work, phosphorylation of Ser25 side chain in ANXA2 by protein kinase C (PKC) was shown both in vivo and in vitro. Phosphorylation level of ANXA2 at Ser25 increased greatly by inhibition of PP1 and by depletion of its regulatory subunit, TIMAP, implying a role of this PP1 holoenzyme in the dephosphorylation of ANXA2. Immunofluorescence staining and subcellular fractionations revealed a diffuse subcellular localization for the endogenous ANXA2, but phospho-Ser25 ANXA2 was mainly detected in the membrane. ANXA2 depletion lowered the basal endothelial barrier and inhibited cell migration, but had no significant effect on cell proliferation or viability. ANXA2 depleted cells failed to respond to PMA treatment, indicating an intimately involvement of phospho-ANXA2 in PKC signaling. Moreover, phosphorylation of ANXA2 disrupted its interaction with S100A10 suggesting a phosphorylation dependent multiple regulatory role of ANXA2 in endothelial cells. Our results demonstrate the pivotal role of PKC-ANXA2-PP1

Abbreviations: ANXA2, Annexin A2; BPAEC, bovine pulmonary artery endothelial cells; CAMK, calmodulin dependent protein kinase; ECIS, electric cell-substrate impedance sensing; EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; HPAEC, human pulmonary artery endothelial cells; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PKA, protein kinase A or cAMP dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP1, protein phosphatase 1; PP1c, catalytic subunit of PP1; PP2A, protein phosphatase 2A; TIMAP, TGF-beta-inhibited membrane-associated protein.

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pathway in endothelial cell signaling, especially in barrier function and cell migration.

KEYWORDS

annexin A2, PKC, protein dephosphorylation; protein phosphatase 1

1 | INTRODUCTION

Annexin A2 (ANXA2) belongs to the annexin protein family that includes 12 members present in mammals.^{1,2} ANXA2 is a 36-kDa protein expressed in the majority of cells and tissues; it binds to several ligands and is involved in multiple and diverse cellular processes. ANXA2 has a strong impact in cancer progression as well^{3,4}; an increased expression of ANXA2 correlates with invasion and metastasis in a variety of human cancers.⁵ ANXA2 has a core structure consisting of four highly conserved homologous domains. The N-terminal domain contains a nuclear export sequence and a binding site for its main ligand, S100A10, while the C-terminal region bears binding sites for calcium ion, phospholipids, heparin, and F-actin.^{6,7}

ANXA2 has diverse cell type-specific subcellular localization, it can be present in the cell membrane, cytoplasm, or even in the nucleus.⁸ ANXA2 is transported to the cytoplasm from the nucleus via the interaction with exportin-1, but no nuclear localization signal has been identified in its sequence and the mechanism of cytoplasmic-nuclear translocation has not been explored yet.⁹ In the nucleus, it participates in DNA replication and protects DNA from harmful effects and plays a role in the regulation of transcription and translation.^{10–13} In cell membranes, ANXA2 participates in cell–cell interactions and cell adhesion.^{14,15} ANXA2 is able to bind F-actin and it was found to regulate cellular processes involving cytoskeletal rearrangement.^{16,17}

Beside the monomeric form, ANXA2 exists as a heterotetramer with S100A10 (a.k.a. p11),^{18,19} but interactions with other members of the S100 protein family like S100A6, S100A4, and S100A11 were also reported.^{20,21} S100A10 is unique among the S100 proteins as it does not contain an EF-hand motif.²² ANXA2 has a strong impact on S100A10 as it protects S100A10 from ubiquitination and proteosomal degradation,²³ furthermore, knockdown of ANXA2 leads to a decrease in the protein expression level of S100A10.²⁴ The ratio of monomeric and heterotetrameric forms is cell type specific, and diverse cellular functions are suggested for the different forms. Monomeric ANXA2 was detected to associate with cell membranes, and association with S100A10 increased its membrane appearance in HepG2 cells.²⁵

Furthermore, it was also shown that monomeric ANXA2 can localize in the nucleus of LNCaP cells.²⁶

The N-terminal region of ANXA2 is subjected to post-translational modifications, like acetylation or phosphorylation. Ser11, Ser25, and Tyr23 (counting Ser1 as the first amino acid) are thought to be important phosphorylation sites.^{3,27–29} S100A10 binds to the first 12 amino acids of ANXA2; therefore, Ser11 phosphorylation is considered as a regulator for the interaction, although it has been studied *in vitro* only.^{7,24} Protein kinase A (or cAMP-dependent protein kinase) and CAMK (calmodulin dependent protein kinase) were shown as possible regulators for Ser11 phosphorylation³⁰ and calcineurin-dependent dephosphorylation of the side chain was also found.²⁴

Protein kinase C (PKC) was reported phosphorylating ANXA2 *in vitro* and Ser25 was suggested to be a potential PKC site.³¹ Using a Ser25Glu ANXA2 mutant, it was shown that Ser25 phosphorylation may mediate nuclear entry of ANXA2; conversely, immunostaining with a pSer25ANXA2-specific antibody proved that ANXA2 phosphorylated on Ser25 is absent from the nucleoplasm of PC12 cells.^{32,33} Further results suggest that Ser25 may induce the phosphorylation of Ser11 by a sequential mechanism.³¹ The protein phosphatase which is involved in the dephosphorylation of phospho-Ser25 in ANXA2 has not yet been identified.

Several works report that Tyr23 of ANXA2 can be phosphorylated by pp60(src),³⁴ or by activation of the insulin receptor or the epidermal growth factor receptor.^{24,35} This phosphorylation results in an alteration of the cytoskeletal system, changes the cell shape, and increases the plasma membrane.^{36,37} Tyr23 and Ser25 sites are close to each other. No double-phosphorylated ANXA2, however, has been detected so far,³⁸ and these sites are considered as mutually exclusive sites.³⁹

Protein phosphatase 1 (PP1) is one of the main Ser-/Thr phosphatases. The catalytic subunit (PP1c) of the holoenzyme forms complexes with different regulatory/targeting subunits. The uniquely high expression level of TIMAP (HGNC: 15850, PPP1R16B) an endothelial cell-specific regulatory subunit of PP1c β/δ (HGNC: 9282), implies its significance in fundamental activities of this cell type.⁴⁰ Recent results proved its indispensable role in endothelial barrier integrity, angiogenesis, and cell migration.⁴¹ To better understand and reveal cross-talks

of TIMAP in related signaling pathways, pull-down assays were utilized to identify possible TIMAP-PP1c target proteins. ANXA2 was identified as an interacting protein partner of TIMAP by liquid chromatography–mass spectrometry (LC–MS/MS) analysis. Here, we provide evidence that the TIMAP-PP1c complex regulates ANXA2 dephosphorylation.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

Bovine pulmonary artery endothelial cells (BPAEC, culture line CCL 209, American Type Tissue Collection, Rockville, MD) and human pulmonary artery endothelial cells (HPAEC, Lonza Group Ltd, Switzerland catalogue Nr: CC-2530) were maintained as described earlier.^{42,43} Phorbol 12-myristate 13-acetate (PMA), tautomycin (TM), Gö6983, and okadaic acid (OA) purchased from Sigma-Aldrich were dissolved in DMSO, and they were utilized in serum-free medium.

2.2 | Preparation of TIMAP and ANXA2 constructs

TIMAP (NM_015568) wild-type full-length (1-567aa), N-terminal (1-290aa), C-terminal (291-567aa), TIMAP 1-165aa, 52-165aa, 67-165aa, and 165-290aa, TIMAP S331A, and S331D recombinant constructs were made as described earlier.^{42,44,45} Human cDNA prepared from HeLa cells was used to amplify ANXA2 coding sequence with the following primer pair containing *Bam*HI and *Xho*I restriction sites for cloning: 5'-TAT AGG ATC CAT GTC TAC TGT TCA CGA AAT CC-3'; 5'-TAT CTC GAG CTA GTC ATC TCC ACC ACA CA-3'. The amplified DNA was inserted into a pGEX-4T-2 vector suitable for bacterial protein expression. All primers were synthesized by Integrated DNA Technologies (Leuven, Belgium). DNA sequences of the constructs were confirmed by sequencing (BIOMI Kft., Gödöllő, Hungary).

2.3 | Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and western blotting

Proteins in extracts (20–50 µg) were subjected of separation on 10–12% SDS polyacrylamide gels and transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked in 5% w/v non-fat dry milk or 5% w/v BSA (for phospho-specific antibodies) containing TBST (Tris-Buffered Saline, 0.1% Tween[®] 20 Detergent)

TABLE 1 List of antibodies used for western blot

Antibody	Dilution	Vendor
Actin	1:1,000	Sigma Aldrich (A5060)
ANXA2 (N-term)	1:500	Abgent Inc. (AP7420a)
β-tubulin	1:2,000	Millipore (#05-661)
CD31 monoclonal (HEC7)	1:1,000	Thermo Fisher Scientific (MA3100)
lamin A/C (H-110)	1:2,000	Santa Cruz (sc-20681)
PP1cδ	1:1,000	Millipore (#07-270)
S100A10	1:500	Abgent Inc. (AP909c)
PKCα	1:1,000	Proteintech Group Inc. (21991-1-AD)
Phospho-Annexin A2 (Ser26)	1:1,000	Invitrogen (#PA5-37474)
pSer PKC substrate antibody	1:1,000	Cell Signaling (#2261)
PPP1R16B (TIMAP)	1:1,000	Abgent Inc. (AP17590c)
Anti-rabbit IgG, HRP-linked antibody	1:5,000	Cell Signaling (#7074)
Anti-mouse IgG, HRP-linked antibody	1:5,000	Cell Signaling (#7076)

solution for 1 hr. Membranes were incubated with primary antibodies overnight at 4°C. After washing steps membranes were probed with Horseradish Peroxidase (HRP)-conjugated immunoglobulin G (IgG) secondary antibodies. Bands were visualized by enhanced chemiluminescence reaction (Western Bright™ ECL, Advansta) and blots were imaged using Alpha Innotech FluorChem[®] FC2 Imager. Blots were quantified by densitometry using the ImageJ software. The primary and secondary antibodies are listed in Table 1.

2.4 | LC–MS/MS analysis

Protein samples were separated by 10–12% SDS-PAGE and stained with Coomassie Brilliant Blue solution. Liquid chromatography with tandem mass spectrometry detection was performed by Dr. Tamás Janáky and Zoltán Szabó at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged, as described in Reference 42.

2.5 | Bacterial protein expression and GST pull-down assay

Escherichia coli BL21 (DE3) cells were transformed with pGEX-4T-2 coding glutathione S-transferase (GST), pGEX-4T-3 and -2 recombinant vectors containing TIMAP or ANXA2 coding sequences, respectively.

TIMAP protein expression was induced as described earlier in References 42 and 45. ANXA2 transformed *E. coli* cells were grown (37°C, 180 rpm) till $OD_{600} = 0.5$ and recombinant protein expression was induced by 0.1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Cells were further cultured at room temperature with shaking for 3 hr. Cells were harvested by centrifugation and protein purification followed by pull-down assay was carried out as described in Reference 42.

2.6 | Immunoprecipitation, immunofluorescence, and microscopy

Immunoprecipitation was made as described in Reference 42. Cells grown on glass coverslips were utilized for immunofluorescent staining.⁴² Primary (1:100) and secondary (1:300) antibodies were diluted in blocking solution. Nonspecific binding of the secondary antibodies was checked in control experiments. Actin filaments were stained using Texas Red Phalloidin and nuclei were visualized by DAPI. Confocal images were acquired with a Leica TCS SP8 confocal microscope using an HC PL APO CS2 63 \times 1.40 numeric aperture oil immersion objective on a DMI6000 CS microscope at 25°C. Images were processed using LAS AF software.

2.7 | In vitro PKC assay

Purified recombinant GST, GST-TIMAP, and GST-ANXA2 proteins were incubated with or without active PKC α (Signal Chem) in kinase assay buffer offered by the manufacturer. After 60 min incubation at 30°C, the reaction was terminated by the addition of 5X SDS sample buffer and heating the samples to 100°C for 5 min.

2.8 | siRNA silencing

TIMAP (PPP1R16B) was silenced using 50 nM siRNA (ON-TARGET plus SMARTpool siRNA (L-004065-00-0, Dharmacon) and ANXA2 was silenced using 10 nM annexin A2 siRNA (Santa Cruz Biotechnology, sc-270,151) in complex with Lipofectamine RNAiMAX transfection reagent (Invitrogen) in serum-free medium for 48 hr. ON-TARGETplus siCONTROL nontargeting pool was used as a negative control.

2.9 | Subcellular fractionation

Membrane, cytoplasmic, and nuclear fractions were isolated as described earlier.^{42,46}

The efficiency of fractionation was analyzed by immunoblotting using CD31 antibody as a membrane marker, lamin A/C antibody as a nuclear marker, and β -tubulin antibody as a cytoplasmic marker.

2.10 | MTT assay

Cell viability was determined with MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay. Cells in triplicates were plated on flat-bottomed 96-well microtiter plates and treated with non-siRNA or siANXA2 RNA. At indicated time points 10 μ l of MTT (5 mg/ml) was added to each well, and incubated at 37°C for 2 hr. Untransformed MTT was removed by aspiration, and the formazan product was dissolved in DMSO. After vigorous shake of the plates, the absorbance was measured at wavelength of 540 nm.

2.11 | ECIS measurements

Electric cell-substrate impedance sensing (ECIS) model Z0 (Applied BioPhysics Inc.) was used to monitor endothelial barrier resistance and cell migration using non-siRNA or ANXA2-specific siRNA-treated cells as described in Reference 46.

2.12 | Statistical analysis

All results are presented as means \pm SD ($n = 3$). Statistical analysis was done with GraphPad Prism program using ANOVA. Differences were considered significant for $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)). Densitometry of immunoblots was done by the ImageJ software.

3 | RESULTS

3.1 | TIMAP interacts with ANXA2 in endothelial cells

Protein phosphatase 1 has over 50 potential regulatory subunits to fulfill its function in different cell types. TIMAP is highly expressed in endothelial cells, therefore, to reveal new substrate/interacting partner for this PP1 holoenzyme, GST-tagged TIMAP fragments were used as baits in pull-down assays. Bovine pulmonary endothelial cell lysate was added to N-terminal (1-290aa) and C-terminal (291-567aa) TIMAP fragments immobilized to glutathione Sepharose 4B beads. Pull-down samples were resolved by SDS-PAGE and stained with Coomassie Blue dye solution (Supplementary Figure 1). The extra band

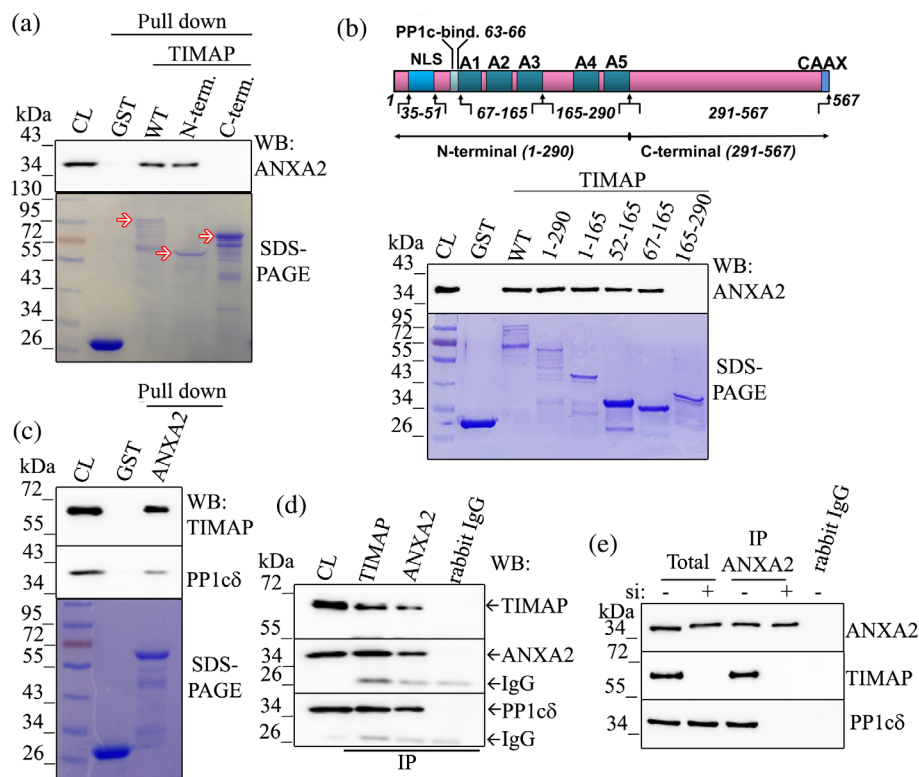


FIGURE 1 ANXA2 interacts with TIMAP-PP1c complex. (a, b) Bacterially expressed glutathione S-transferase (GST) and GST-tagged full length wild-type TIMAP, N-terminal (1-290aa) or C-terminal (291-567aa) TIMAP fragments (a) or GST, GST-TIMAP wild type, N-terminal (1-290aa) and shorter (1-165aa, 52-165aa, 67-165aa, 165-290aa) TIMAP fragments of the N-terminal region (b) were loaded onto glutathione Sepharose and the washed resin samples were incubated with BPAEC lysate (pull down). Western blot of the endothelial cell lysate (CL) and the eluted fractions after the pull-down probed with ANXA2-specific antibody are shown. The pull-down complexes from were visualized by Coomassie blue staining as loading controls (SDS-PAGE). Red arrows indicate the undegraded TIMAP proteins. (c) Pull down of endothelial cell lysate was made using GST and GST-tagged ANXA2 as baits. Samples were tested using TIMAP and PP1c δ -specific antibodies by western blot. The pull-down complexes were visualized by Coomassie blue staining as loading controls. (d) TIMAP or ANXA2 was immunoprecipitated from lysates of BPAEC. IP complexes were probed for TIMAP, ANXA2, and PP1c δ . Rabbit IgG was used as control. The extra bands seen at about 25 kDa correspond to IgG. (e) ANXA2 was immunoprecipitated from nonsiRNA or siTIMAP (+) RNA-treated BPAEC. IP complexes were probed for TIMAP, ANXA2, and PP1c δ

present in the GST-TIMAP N-terminal pull-down sample approximately at 36 kDa was identified by LC-MS/MS as annexin A2 (ANXA2; UniProt: P04272). The interaction between TIMAP and ANXA2 was confirmed by western blot analysis of the pull-down samples (Figure 1a). ANXA2 bound to wild type full-length TIMAP and the N-terminal fragment that contains the PP1c binding motif, nuclear localization signal, and five ankyrin (ANK) repeats. The C-terminal fragment of TIMAP containing a disordered region and the prenylation motif had no interaction with ANXA2, despite the higher amount of loaded protein as shown by Coomassie stained SDS-PAGE gels (Figure 1a). To further specify the interacting area, additional shorter N-terminal TIMAP recombinants were tested (Figure 1b). Using TIMAP 1-165 and 165-290 fragments, we found that ANK4 and ANK5 domains are not involved in the interaction. Deletion of either the NLS (TIMAP 52-165) or the PP1c binding site

(TIMAP 67-165) had no effect on ANXA2 binding; therefore, it was concluded that ANK1-ANK3 repeats are the critical regions in TIMAP-ANXA2 interaction.

ANXA2 was cloned from HeLa cDNA and pGEX-4T-2 constructs were created. GST-ANXA2 protein expression was optimized to produce GST-ANXA2 recombinant. The recombinant was used in pull-down assays. The TIMAP-PP1c δ complex interacted with GST-ANXA2, but no interaction was detected with control GST, as expected (Figure 1c). Interaction of endogenous proteins was proved by immunoprecipitation utilizing TIMAP and ANXA2-specific antibodies (Figure 1d). ANXA2 was detected in the immunoprecipitated yielded by a TIMAP-specific antibody from endothelial cell lysate, and vice versa. PP1c δ was present in both IP complexes. To test whether ANXA2 binds PP1c directly or via TIMAP immunoprecipitation was made with ANXA2 antibody from control and TIMAP siRNA-treated cells

(Figure 1e). No interaction was detected between ANXA2 and PP1c δ in TIMAP-depleted cells, therefore we concluded that ANXA2 interacts with PP1c δ via TIMAP.

3.2 | Phosphorylation state of Ser25 in ANXA2 is regulated by PKC and TIMAP-PP1c

Ser25 side chain of ANXA2 is a potential PKC phosphorylation site.^{31,47} To investigate ANXA2 phosphorylation by PKC, *in vitro* PKC phosphorylation assays were performed (Figure 2a). The amount and purity of

loaded GST, GST-ANXA2, and GST-TIMAP proteins were confirmed by SDS-PAGE. Samples were tested in western blot experiments using an antibody specific for phospho-Ser side chains phosphorylated by PKC (phospho-Ser PKC substrate antibody) and an anti-phospho-Ser25 ANXA2 antibody. GST was used as negative control, and GST-TIMAP served as a positive control for *in vitro* phosphorylation, as it was shown earlier to be phosphorylated by PKC on the Ser331 sidechain.⁴⁵ PKC phosphorylated both TIMAP and ANXA2, moreover, the phospho-Ser25 ANXA2-specific antibody verified that indeed PKC phosphorylated the Ser25 side chain in ANXA2 (Figure 2a). To test whether PKC

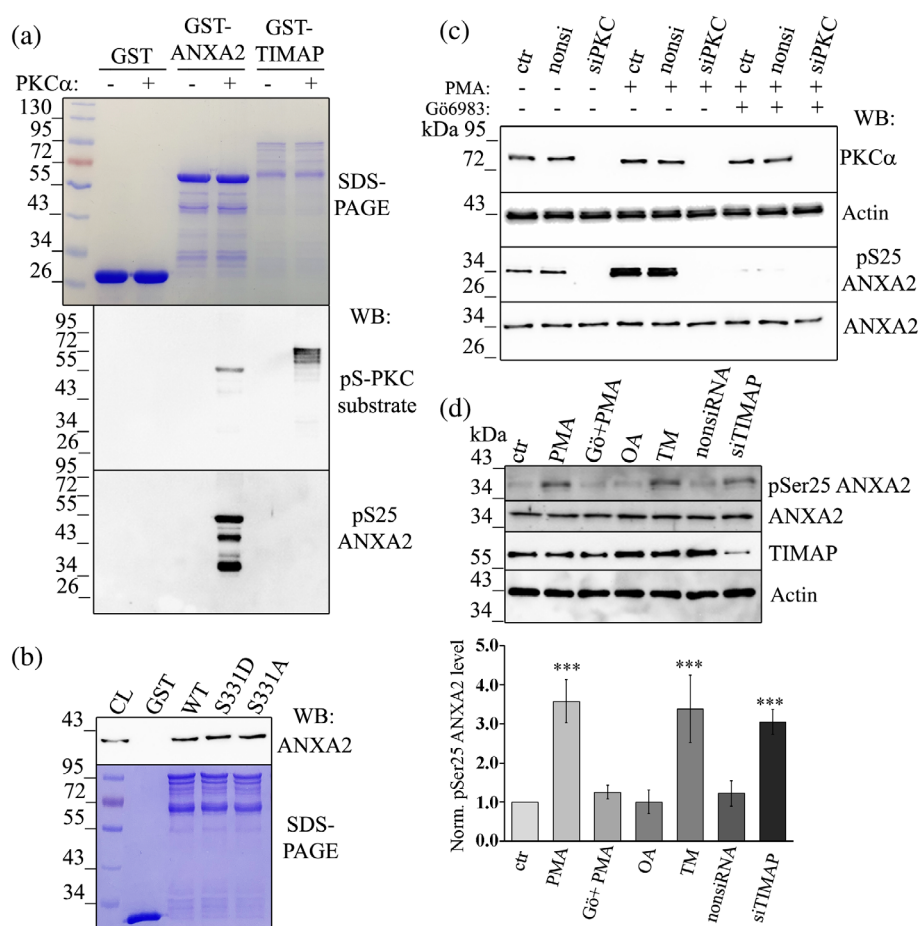


FIGURE 2 PKC phosphorylates ANXA2 on Ser25 side chain. (a) Purified recombinant GST, GST-ANXA2, and GST-TIMAP proteins were incubated with or without active PKC α for 30 min. Amount of loaded proteins were verified by SDS-PAGE followed by Coomassie staining. Phosphorylation of proteins was analyzed by western blot using anti-phosphoSer PKC substrate and phospho-Ser25 annexin A2-specific antibodies. (b) Pull down of endothelial cell lysate was made using GST, GST-tagged TIMAP wild type, S331D or S331D recombinant full-length TIMAP proteins as baits. Samples were tested for ANXA2 in western blot experiment. (c) Total cell lysates of control, nonsilencing RNA and PKC-specific siRNA transfected HPAEC treated with PMA (1 μ M for 30 min) or PMA (1 μ M for 30 min) after a pretreatment with G66983 (1 μ M for 30 min) were analyzed by western blot using PKC α , actin, ANXA2, and phospho-Ser25 ANXA2-specific antibodies. (d) Western blot analysis of control, PMA (1 μ M, 30 min), PMA (1 μ M, 30 min) with G66983 pretreatment (1 μ M, 30 min), okadaic acid (5 nM, 30 min), tautomycetin (1 μ M, 30 min), nonsiRNA, or TIMAP-specific siRNA-treated BPAEC cells are shown. Phospho-Ser25 ANXA2 level was normalized to ANXA2 protein level. Densitometric analysis of western blots are shown as mean \pm SD ($n = 3$). Statistical analysis was done with ANOVA and marked by asterisks; *** $p < .001$

phosphorylation of TIMAP has an effect on ANXA2 binding, recombinant Ser331 phosphomutant TIMAP proteins were used in pull-down assay (Figure 2b). There was no difference in the amount of bounded ANXA2 in the phosphomimic S331D or phosphonull S331A TIMAP pull-down samples, compared to the wild-type TIMAP. Next, to study more detailedly ANXA2 phosphorylation at Ser25, PKC α was depleted by specific siRNA transfection. HPAEC cells were treated with nonspecific RNA and PKC α -specific siRNA for 48 hr. PMA was used to activate PKC, and Gö6983 pretreatment was applied to block PKC activity. Western blot analyses of the samples using specific antibodies (Figure 2c) demonstrated *in vivo* phosphorylation of Ser25 in ANXA2 after activation of PKC by PMA.

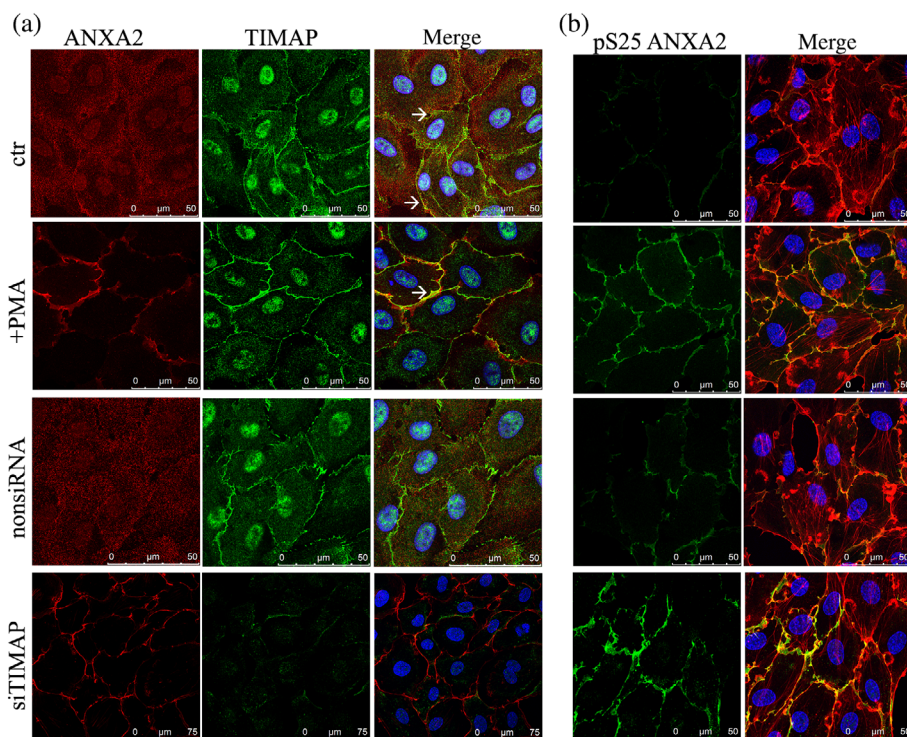
Next, we compared the effect of PKC activation/inhibition or inhibitions of phosphatases on the phosphorylation level of ANXA2 (Figure 2d). PKC was activated by PMA challenge, while inhibition of PKC activity was achieved by a pretreatment with Gö6983. Protein phosphatase 2A (PP2A) activity was inhibited by OA (in the applied concentration OA selectively inhibits PP2A), while PP1 was blocked by tautomycin. Effect of TIMAP depletion was also tested by employing TIMAP-specific siRNA. Normalized data show that phosphorylation level of ANXA2 increased after PMA treatment or after blocking PP1c either by using tautomycin or by depleting TIMAP, the PP1 regulatory subunit (Figure 2d).

3.3 | Subcellular localization of ANXA2 is affected by its phosphorylation state

Localization of ANXA2 in endothelial cells was first tested by immunofluorescent staining. HPAEC cells were costained with ANXA2 and TIMAP-specific antibodies (Figure 3a). Also, phospho-Ser25 ANXA2 and actin filaments were visualized by specific antibody and Texas-Red Phalloidin, respectively (Figure 3b). TIMAP was present mainly in the membrane and nuclear area of cells as expected. ANXA2 staining did not show any particular accumulation of the protein at any specific location of the cells. We detected colocalization of ANXA2 and TIMAP predominantly in the cell membrane (white arrows). Staining of phospho-Ser25 ANXA2 in untreated, control cells resulted in a faint signal that considerably increased in the cell membrane upon PMA treatment. TIMAP was also enriched in the cell membrane after PKC activation in agreement with our earlier findings.⁴⁵ The localization changes and the increase in the phosphorylation level of Ser25 of ANXA2 detected in TIMAP-depleted cells compared to nonsiRNA or control cells were similar to the changes observed after the above described changes evoked by PKC activation.

Next, subcellular fractions were isolated from non-siRNA and siTIMAP RNA-treated HPAEC. Lamin A/C, β -tubulin, and CD31-specific antibodies were used to confirm the purity of nuclear, cytoplasmic, and membrane fractions, respectively. Western blot analysis of the

FIGURE 3 Phospho-Ser25 ANXA2 localizes in cell membrane. Immunofluorescent staining of control, PMA (1 μ M for 30 min), non siRNA or TIMAP-specific siRNA-treated HPAEC was performed using (a) ANXA2 and TIMAP-specific primary antibodies or (b) phospho-Ser25 ANXA2-specific antibody and Texas Red Phalloidin (actin, red). Nuclei of the cells were stained using DAPI (blue). White arrows on merged images of control and PMA-treated cells point colocalization of TIMAP and ANXA2 in the membrane region. Nonspecific binding of the secondary antibodies was checked in control experiments (not shown). Representative data of at least three independent experiments are shown



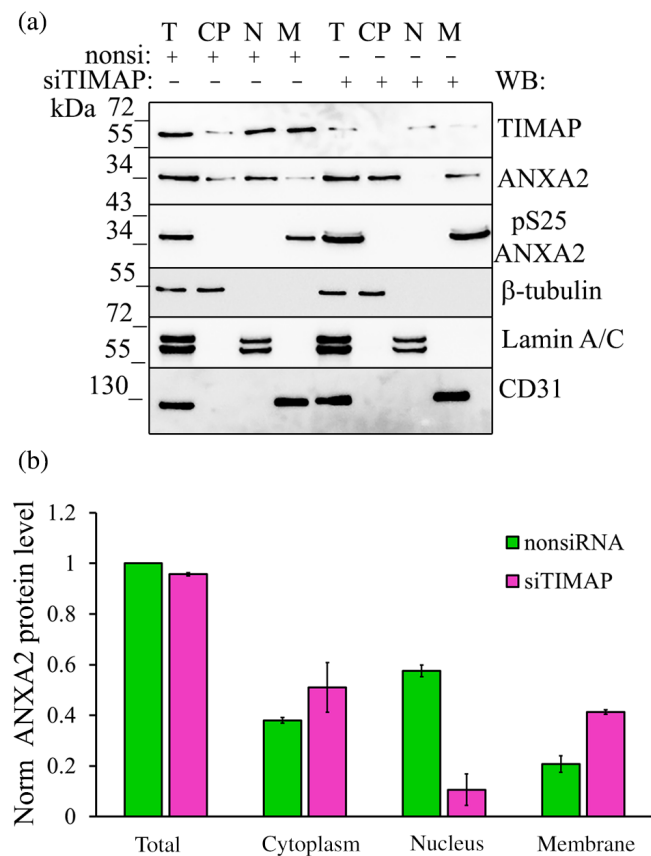


FIGURE 4 TIMAP regulates cellular localization of ANXA2.

(a) Subcellular fractionation of nonsilencing RNA and TIMAP-specific siRNA-treated HPAEC. Total cell lysates (T) and subcellular fractions were analyzed with TIMAP, ANXA2, phospho-Ser25 ANXA2, CD31 (membrane marker, M), β -tubulin (cytoplasmic marker, C), and anti-lamin A/C (nuclear marker, N) specific antibodies. (b) ANXA2 level in different cell fractions were analyzed by densitometric analysis of western blots. ANXA2 bands were normalized to the corresponding cell fraction marker bands

fractions proved that ANXA2 is present in the nuclear, cytoplasmic, and membrane fraction of HPAEC cells (Figure 4a). Phospho-Ser25 ANXA2, however, was only detectable in total cell lysates and the membrane fraction. In parallel with the results of immunofluorescent staining, TIMAP-depleted cell lysates showed increased phosphorylation level of ANXA2. Cellular distribution of ANXA2 was also affected by TIMAP depletion, as the nuclear ANXA2 level decreased, while its level in the membrane increased (Figure 4b).

3.4 | Annexin A2 is involved in endothelial barrier maintenance and cell migration

To reveal the physiological role of ANXA2 in endothelial cells, siRNA mediated depletion of ANXA2 was carried

out. ANXA2 protein level decreased close to undetectable level in specific siRNA-treated cells after 48 hr (Figure 5a). Effect of siRNA transfection on cell proliferation and viability was analyzed by MTT assay. Neither the nonsiRNA treated nor the depleted cells showed any significant changes in their viability compared to control cells (Figure 5b). The endothelial barrier of the transfected cells was also monitored using ECIS. Monolayers of endothelial cells were treated with nonsi- and ANXA2-specific siRNA 24 hr after seeding. Our results show that the depletion of ANXA2 decreases the resistance of endothelial cells (Figure 5c). An even more pronounced difference was found in the cell migration rates measured by in vitro wound healing assays of nonsiRNA and ANXA2-specific siRNA-treated cells (Figure 5d) suggesting that ANXA2 might be important not just in barrier maintenance but also in cell movement. This concept was further proved by challenging the confluent monolayer of nonsiRNA and ANXA2-specific siRNA-treated cells with PMA to activate PKC (Figure 6a). NonsiRNA-treated cells responded first with an increase of resistance that fell below the initial level 2–3 hr after the addition of PMA and then, in a second phase, gradually returned to a normal value (about 1,000 Ω).^{48,49} In contrast, ANXA2-depleted cells had a significantly smaller elevation of the resistance upon addition of PMA and after a significant decrease detected 2–3 hr later, the resistance of the silenced cells remained much lower compared to the nonsilenced cells.

Interaction of ANXA2 and S100A10 plays an important role in cell–cell interactions, actin binding, and cellular motility. We tested their interaction by immunoprecipitation. ANXA2 or S100A10 was immunoprecipitated from control or PMA-treated endothelial cell lysates (Figure 6b). In control cells, ANXA2–S100A10 interaction was detected from both sides, but the interaction was abolished after the activation of PKC suggesting a weaker or no interaction of the phosphorylated form of ANXA2 with S100A10. Indeed, S100A10 only interacted with the nonphosphorylated ANXA2, but the phospho-Ser25 ANXA2 was not detectable in the IP complexes isolated with the S100A10-specific antibody.

4 | DISCUSSION

Annexin A2 is one of the most studied members of the annexin family. A growing number of evidence reports that ANXA2 is involved in numerous signaling pathways. Its importance in normal biological functions and its clinical relevance as a cancer biomarker sets ANXA2 in the spotlight. Posttranslational modifications of ANXA2 regulate its function, subcellular localization, and

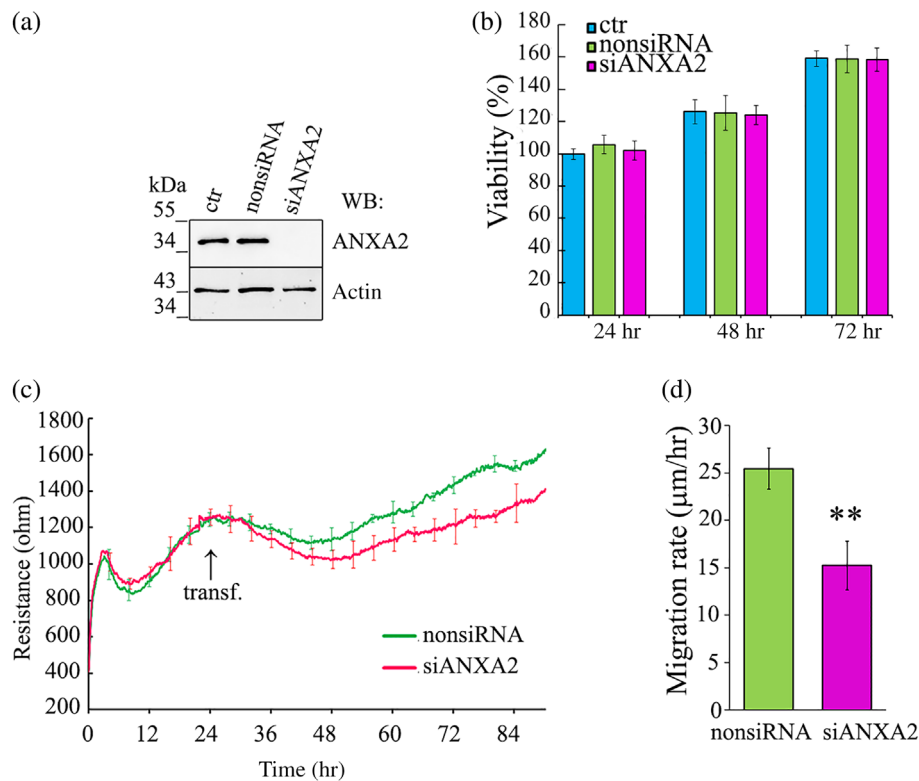


FIGURE 5 ANXA2 affects endothelial barrier regulation and cell migration. (a) Lysates of control, nonsiRNA and annexin A2-specific siRNA-treated HPAEC cells were analyzed by western blot. (b) Cell viability of control and nonsi- or siRNA-transfected HPAEC was measured by MTT assay 24, 48, and 72 hr after transfection. Cell viability was expressed as percent of viability observed for 0 hr samples. (c) Resistance of nonsi- and siRNA-transfected HPAEC was monitored using ECIS. Cells plated on 8W10E arrays were transfected with nonsiRNA and ANXA2-specific siRNA at 24 hr. Resistance was followed in time. Representative curves show the means \pm SD of three independent measurements. (d) In vitro wound healing assays were performed with ECIS to measure the rate of cell migration. Results are presented as means \pm SD of four independent measurements for each sample

interactions with other proteins. Although several important phosphorylation sites were identified so far, protein phosphatases responsible for the dephosphorylation of these side chains have not been described yet. In the present work, a specific interaction was detected between ANXA2 and a type1 phospho-Ser/phospho-Thr-specific protein phosphatase, namely, the TIMAP-PP1c complex in pulmonary endothelial cells. TIMAP, as a regulatory subunit of PP1c, interacts with other proteins and determines the substrate specificity of the holoenzyme. ANXA2 binds to the ANK1-ANK3 domains in the N-terminal part of TIMAP. Up to now, all identified substrates and interacting protein partners of TIMAP interacted with the N-terminal region (1-290aa) and no protein was described to bind to the disordered C-terminal region.⁴¹ The newly described interaction of ANXA2 with a phosphatase raised the question, whether phosphorylated ANXA2 can be a substrate of PP1. Several known TIMAP-PP1c substrates, for example, endothelin converting enzyme-1 or ERM proteins are subjected to phosphorylation by PKC.^{44,50} In vivo and

in vitro experiments proved that ANXA2 is phosphorylated by PKC enzyme on the Ser25 side chain. Drug-induced inhibition of the PP1 activity or depletion of TIMAP, the regulatory subunit of PP1c, both maintained the phosphorylated state of ANXA2. One can hypothesize that depletion of TIMAP reduces the PP1 activity toward TIMAP-PP1c substrates and that results in an increase in the phosphorylation level of the substrates. Due to the structural domain properties of TIMAP, it localizes in cell membrane and nuclear area of endothelial cells.⁴¹ ANXA2 colocalizes with TIMAP in the cell membrane region. Importantly, phospho-Ser25 ANXA2 was found only in the membrane region of endothelial cells and depletion of TIMAP caused an increased phosphorylation level of ANXA2. Also, translocation of ANXA2 was detected from the nucleus to the membrane in TIMAP depleted cells. It was reported that ANXA2 interacts with VE-cadherin in endothelial cells, regulating cell motility and actin dynamics.⁵¹ Others reported the involvement of ANXA2-S100A10 complex in E-cadherin-based adherent junction formation in MDCK cells.⁵² In line with

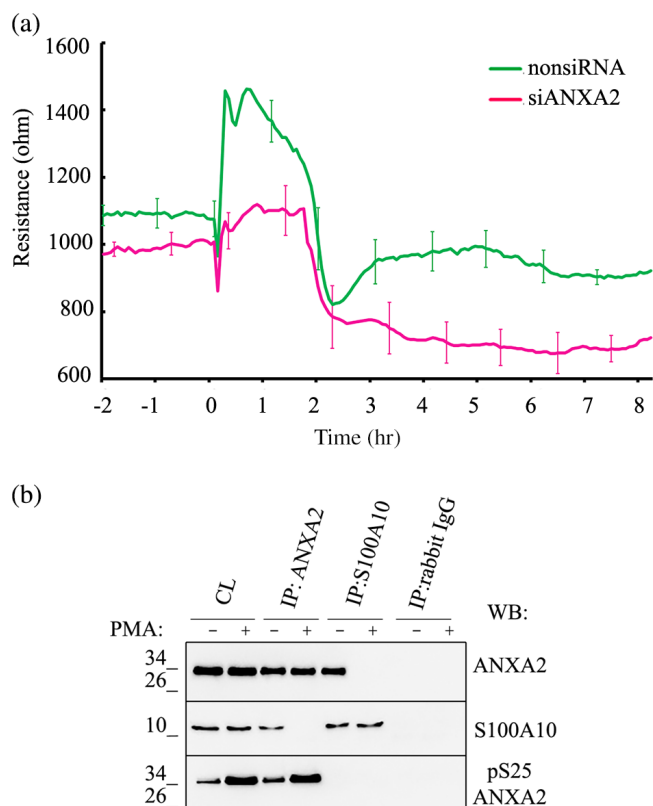


FIGURE 6 Annexin A2–S100A10 interaction is regulated by PKC. (a) Confluent monolayer of nonsiRNA and ANXA2-specific siRNA-treated HPAEC cells were challenged by PMA (0 hr) and resistance was measured using ECIS. (b) Annexin A2 or S100A10 proteins were immunoprecipitated from control and PMA (1 μ M, 30 min)-treated HPAEC cells. Rabbit IgG was used as IP control. IP complexes were analyzed with specific antibodies by western blot

these results of others, the membrane localization of phospho-Ser25 ANXA2 emphasizes its involvement in cell–cell interactions and endothelial barrier regulation. Depletion of ANXA2 had no significant effect on endothelial cell proliferation or viability, but lowered the basal barrier resistance of cells and decreased their migration rate. Importantly, ANXA2 depleted cells failed to respond to PMA challenge and they were unable to restore normal endothelial barrier. Moreover, activating PKC by PMA disrupted the ANXA2–S100A10 complex by a phosphorylation dependent manner, as no interaction was found between phospho-Ser25 ANXA2 and S100A10. S100A10 binds to the N-terminal region of ANXA2, therefore, phosphorylation of Ser25 can induce a conformational change in that part of the protein resulting in a dissociation of the complex. ANXA2 and S100A10 interaction is well studied, but there is no consistent view about the regulation of the complex.²⁰ It seems that ANXA2 regulation is cell type specific that further

reflects the importance to explore its many signaling pathways.

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

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

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REFERENCES

- Gerke V, Moss SE. Annexins: From structure to function. *Physiol Rev*. 2002;82:331–371.
- Moss SE, Morgan RO. The annexins. *Genome Biol*. 2004;5:219.
- Grindheim AK, Saraste J, Vedeler A. Protein phosphorylation and its role in the regulation of Annexin A2 function. *Biochim Biophys Acta Gen Subj*. 2017;1861:2515–2529.
- Christensen MV, Hogdall CK, Jochumsen KM, Hogdall EVS. Annexin A2 and cancer: A systematic review. *Int J Oncol*. 2018;52:5–18.
- Sharma MC. Annexin A2 (ANX A2): An emerging biomarker and potential therapeutic target for aggressive cancers. *Int J Cancer*. 2019;144:2074–2081.
- Filipenko NR, Waisman DM. The C terminus of annexin II mediates binding to F-actin. *J Biol Chem*. 2001;276:5310–5315.
- Johnsson N, Marriott G, Weber K. p36, the major cytoplasmic substrate of src tyrosine protein kinase, binds to its p11 regulatory subunit via a short amino-terminal amphiphatic helix. *EMBO J*. 1988;7:2435–2442.
- Waisman DM. Annexin II tetramer: Structure and function. *Mol Cell Biochem*. 1995;149-150:301–322.
- Eberhard DA, Karns LR, VandenBerg SR, Creutz CE. Control of the nuclear-cytoplasmic partitioning of annexin II by a nuclear export signal and by p11 binding. *J Cell Sci*. 2001;114:3155–3166.
- Kumble KD, Iversen PL, Vishwanatha JK. The role of primer recognition proteins in DNA replication: Inhibition of cellular proliferation by antisense oligodeoxyribonucleotides. *J Cell Sci*. 1992;101(Pt 1):35–41.
- Vishwanatha JK, Kumble S. Involvement of annexin II in DNA replication: Evidence from cell-free extracts of *Xenopus* eggs. *J Cell Sci*. 1993;105(Pt 2):533–540.
- Madureira PA, Hill R, Lee PW, Waisman DM. Genotoxic agents promote the nuclear accumulation of annexin A2: Role of annexin A2 in mitigating DNA damage. *PLoS One*. 2012;7:e50591.

13. Hitchcock JK, Katz AA, Schafer G. Dynamic reciprocity: The role of annexin A2 in tissue integrity. *J Cell Commun Signal.* 2014;8:125–133.
14. Lee DB, Jamgotchian N, Allen SG, Kan FW, Hale IL. Annexin A2 heterotetramer: Role in tight junction assembly. *Am J Physiol Renal Physiol.* 2004;287:F481–F491.
15. Myrvang HK, Guo X, Li C, Dekker LV. Protein interactions between surface annexin A2 and S100A10 mediate adhesion of breast cancer cells to microvascular endothelial cells. *FEBS Lett.* 2013;587:3210–3215.
16. Hayes MJ, Rescher U, Gerke V, Moss SE. Annexin-actin interactions. *Traffic.* 2004;5:571–576.
17. Hayes MJ, Shao D, Bailly M, Moss SE. Regulation of actin dynamics by annexin 2. *EMBO J.* 2006;25:1816–1826.
18. Gerke V, Creutz CE, Moss SE. Annexins: Linking Ca²⁺ signaling to membrane dynamics. *Nat Rev Mol Cell Biol.* 2005;6:449–461.
19. Kwon M, MacLeod TJ, Zhang Y, Waisman DM. S100A10, annexin A2, and annexin a2 heterotetramer as candidate plasminogen receptors. *Front Biosci.* 2005;10:300–325.
20. Liu Y, Myrvang HK, Dekker LV. Annexin A2 complexes with S100 proteins: Structure, function and pharmacological manipulation. *Br J Pharmacol.* 2015;172:1664–1676.
21. Gerke V, Weber K. The regulatory chain in the p36-kd substrate complex of viral tyrosine-specific protein kinases is related in sequence to the S-100 protein of glial cells. *EMBO J.* 1985;4:2917–2920.
22. Donato R. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta.* 1999;1450:191–231.
23. He KL, Deora AB, Xiong H, et al. Endothelial cell annexin A2 regulates polyubiquitination and degradation of its binding partner S100A10/p11. *J Biol Chem.* 2008;283:19192–19200.
24. Brandherm I, Disse J, Zeuschner D, Gerke V. cAMP-induced secretion of endothelial von Willebrand factor is regulated by a phosphorylation/dephosphorylation switch in annexin A2. *Blood.* 2013;122:1042–1051.
25. Zobiack N, Gerke V, Rescher U. Complex formation and submembranous localization of annexin 2 and S100A10 in live HepG2 cells. *FEBS Lett.* 2001;500:137–140.
26. Liu J, Rothermund CA, Ayala-Sanmartin J, Vishwanatha JK. Nuclear annexin II negatively regulates growth of LNCaP cells and substitution of ser 11 and 25 to glu prevents nucleo-cytoplasmic shuttling of annexin II. *BMC Biochem.* 2003;4:10.
27. Jost M, Gerke V. Mapping of a regulatory important site for protein kinase C phosphorylation in the N-terminal domain of annexin II. *Biochim Biophys Acta.* 1996;1313:283–289.
28. Gould KL, Woodgett JR, Isacke CM, Hunter T. The protein-tyrosine kinase substrate p36 is also a substrate for protein kinase C in vitro and in vivo. *Mol Cell Biol.* 1986;6:2738–2744.
29. Glenney JR Jr, Tack BF. Amino-terminal sequence of p36 and associated p10: Identification of the site of tyrosine phosphorylation and homology with S-100. *Proc Natl Acad Sci USA.* 1985;82:7884–7888.
30. Johnsson N, Nguyen Van P, Soling HD, Weber K. Functionally distinct serine phosphorylation sites of p36, the cellular substrate of retroviral protein kinase; differential inhibition of reassociation with p11. *EMBO J.* 1986;5:3455–3460.
31. Regnouf F, Sagot I, Delouche B, et al. "In vitro" phosphorylation of annexin 2 heterotetramer by protein kinase C. Comparative properties of the unphosphorylated and phosphorylated annexin 2 on the aggregation and fusion of chromaffin granule membranes. *J Biol Chem.* 1995;270:27143–27150.
32. Luo W, Yan G, Li L, et al. Epstein-Barr virus latent membrane protein 1 mediates serine 25 phosphorylation and nuclear entry of annexin A2 via PI-PLC-PKCalpha/PKCbeta pathway. *Mol Carcinog.* 2008;47:934–946.
33. Aukrust I, Rosenberg LA, Ankerud MM, et al. Post-translational modifications of Annexin A2 are linked to its association with perinuclear nonpolysomal mRNP complexes. *FEBS Open Bio.* 2017;7:160–173.
34. Glenney JR Jr. Phosphorylation of p36 in vitro with pp60src. Regulation by Ca²⁺ and phospholipid. *FEBS Lett.* 1985;192:79–82.
35. Ayala-Sanmartin J, Gouache P, Henry JP. N-Terminal domain of annexin 2 regulates Ca(2+)-dependent membrane aggregation by the core domain: a site directed mutagenesis study. *Biochemistry.* 2000;39:15190–15198.
36. de Graauw M, Tijdens I, Smeets MB, Hensbergen PJ, Deelder AM, van de Water B. Annexin A2 phosphorylation mediates cell scattering and branching morphogenesis via cofilin activation. *Mol Cell Biol.* 2008;28:1029–1040.
37. Grindheim AK, Hollas H, Raddum AM, Saraste J, Vedeler A. Reactive oxygen species exert opposite effects on Tyr23 phosphorylation of the nuclear and cortical pools of annexin A2. *J Cell Sci.* 2016;129:314–328.
38. Yan G, Luo W, Lu Z, et al. Epstein-Barr virus latent membrane protein 1 mediates phosphorylation and nuclear translocation of annexin A2 by activating PKC pathway. *Cell Signal.* 2007;19:341–348.
39. Ecsedi P, Kiss B, Gogl G, et al. Regulation of the equilibrium between closed and open conformations of annexin A2 by N-terminal phosphorylation and S100A4-binding. *Structure.* 2017;25(1195–1207):e1195.
40. Cao W, Mattagajasingh SN, Xu H, et al. TIMAP, a novel CAAX box protein regulated by TGF-beta1 and expressed in endothelial cells. *Am J Physiol Cell Physiol.* 2002;283:C327–C337.
41. Boratko A, Csontos C. TIMAP, the versatile protein phosphatase 1 regulator in endothelial cells. *IUBMB Life.* 2017;69:918–928.
42. Boratko A, Gergely P, Csontos C. RACK1 is involved in endothelial barrier regulation via its two novel interacting partners. *Cell Commun Signal.* 2013;11:2.
43. Boratko A, Gergely P, Csontos C. Cell cycle dependent association of EBP50 with protein phosphatase 2A in endothelial cells. *PLoS One.* 2012;7:e35595.
44. Csontos C, Czikora I, Bogatcheva NV, et al. TIMAP is a positive regulator of pulmonary endothelial barrier function. *Am J Physiol Lung Cell Mol Physiol.* 2008;295:L440–L450.
45. Boratko A, Csontos C. PKC mediated phosphorylation of TIMAP regulates PP1c activity and endothelial barrier function. *Biochim Biophys Acta Mol Cell Res.* 2017;1864:431–439.
46. Thalwieser Z, Kiraly N, Fonodi M, Csontos C, Boratko A. Protein phosphatase 2A-mediated flotillin-1 dephosphorylation up-regulates endothelial cell migration and angiogenesis regulation. *J Biol Chem.* 2019;294:20196–20206.

47. Johnstone SA, Hubaishy I, Waisman DM. Phosphorylation of annexin II tetramer by protein kinase C inhibits aggregation of lipid vesicles by the protein. *J Biol Chem.* 1992;267:25976–25981.
48. Favaron M, Manev H, Siman R, et al. Down-regulation of protein kinase C protects cerebellar granule neurons in primary culture from glutamate-induced neuronal death. *Proc Natl Acad Sci USA.* 1990;87:1983–1987.
49. Hepler JR, Earp HS, Harden TK. Long-term phorbol ester treatment down-regulates protein kinase C and sensitizes the phosphoinositide signaling pathway to hormone and growth factor stimulation. Evidence for a role of protein kinase C in agonist-induced desensitization. *J Biol Chem.* 1988;263:7610–7619.
50. Boratko A, Vereb Z, Petrovski G, Csontos C. TIMAP-protein phosphatase 1-complex controls endothelin-1 production via ECE-1 dephosphorylation. *Int J Biochem Cell Biol.* 2016;73:11–18.
51. Heyraud S, Jaquinod M, Durmort C, et al. Contribution of annexin 2 to the architecture of mature endothelial adherens junctions. *Mol Cell Biol.* 2008;28:1657–1668.
52. Yamada A, Irie K, Hirota T, Ooshio T, Fukuhara A, Takai Y. Involvement of the annexin II-S100A10 complex in the formation of E-cadherin-based adherens junctions in Madin-Darby canine kidney cells. *J Biol Chem.* 2005;280:6016–6027.

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