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TIMAP-protein phosphatase 1-complex controls endothelin-1 production via ECE-1 dephosphorylation

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Abbreviations:

BPAEC, bovine pulmonary artery endothelial cell; EC, endothelial cell; ECIS, electric cell-substrate impedance sensing; ET-1, endothelin-1; ECE-1, endotheiln converting enzyme-1; ERM, ezrin-radixin-moesin; HPAEC, human pulmonary artery endothelial cells; MYPT, myosin phosphatase targeting subunit; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP1, protein phosphatase 1; PP1c, catalytic subunit of protein phosphatase 1; TIMAP, TGF-β inhibited membrane associated protein;

Abstract

Endothelin induced signaling pathways can affect blood pressure and vascular tone, but the influence of endothelins on tumor cells is also significant. We have detected elevated endothelin-1 secretion from TIMAP (TGF-β inhibited membrane associated protein) depleted vascular endothelial cells. The autocrine signaling activated by the elevated endothelin-1 level through the ET_B receptors evoked an angiogenic-like phenotype, the cells assumed an elongated morphology, and enhanced tube formation and wound healing abilities. The depleted protein, TIMAP, is a highly specific and abundant protein in the endothelial cells, and it is a regulatory/targeting subunit for the catalytic subunit of protein phosphatase 1 (PP1c). Protein-protein interaction between the TIMAP-PP1c complex and the endothelin converting enzyme-1 (ECE-1) was detected, the latter of which is a transmembrane protein that produces the biologically active 21-amino acid form of endothelin-1 from proendothelin. The results indicate that silencing of TIMAP induces a reduction in TIMAP-PP1c activity connected to ECE-1. This leads to an increase in the amount of ECE-1 protein in the plasma membrane and a consequent increase in endothelin-1 secretion. Similarly, activation of PKC, the kinase responsible for ECE-1 phosphorylation increased ECE-1 protein level in the membrane fraction of the endothelial cells. The elevated ECE-1 level was mitigated in time in normal cells, but was clearly preserved in TIMAP-depleted cells. Overall, our results indicate that PKC-phosphorylated ECE-1 is a TIMAP-PP1c substrate and this phosphatase complex has an important role in endothelin-1 production of EC through the regulation of ECE-1 activity.

Keywords: protein phosphatase 1, TIMAP, endothelial cells, endothelin-1, endothelin converting enzyme-1

1. Introduction

Vascular endothelial cells (EC) respond to physical and chemical signals by production of a wide range of factors, which regulate vascular tone, cellular adhesion, smooth muscle cell proliferation, and vessel wall inflammation. Structural changes in the pulmonary vasculature, disordered EC proliferation, andan altered production of endothelial vasoactive mediators (NO, prostacyclin, endothelin-1, serotonin, and thromboxane) has been recognized in patients with hypertension, coronary artery disease, chronic heart failure or preeclampsia.

Endothelins are a family of peptides, which comprises of endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), each consisting of 21 amino acids. The predominant isoform in the vasculature is ET-1, one of the most potent vasoconstrictors (Masaki, 1998), produced primarily by vascular EC. Initially an inactive, large precursor form, prepro-ET-1 is produced (Kawanabe and Nauli, 2011), which is converted to pro-ET-1 after removal of a signal sequence. The formation of mature ET-1 requires a proteolytic cleavage catalyzed by one of the endothelin converting enzymes (ECEs) (Matsuoka et al., 1996, Meidan et al., 2005). Four isoforms of ECE-1 have been identified, all encoded by the same gene but under the control of different promoter regions (Meidan et al., 2005). ECE-1c isoform is the most dominant and therefore, it is the main physiological regulator of ET-1 production. PKC mediated phosphorylation of ECE-1c promotes its trafficking to the cell surface and increases its activity (Jafri and Ergul, 2006, Kuruppu et al., 2010).

Elevated level of ET-1 has been detected in several cardiovascular diseases, such as atherosclerosis, heart failure or primary pulmonary hypertension (Lerman et al., 1991, Pacher et al., 1996, Stewart et al., 1991, Agapitov and Haynes, 2002), and it has been suggested as a potential indicator of endothelial dysfunction (Stewart et al., 1991). ET-1 acts both on endothelial- and smooth muscle cells of the pulmonary arteries (Boscoe et al., 2000). ET-1 evolves its biological effects through activation of receptors belonging to the superfamily of G-protein coupled receptors, ET_A and ET_B. ET_A receptors are found in smooth muscle cells, whereas ET_B receptors found on both endothelial- and smooth muscle cells (White et al., 1993, Avedanian et al., 2010). ET-1 can also play an important role in tumor growth and survival through regulated sprouting of new vessels into the tumor and surrounding tissues (Dawas et al., 1999).

TGF-β inhibited membrane-associated protein (TIMAP) is a typical endothelial cell (EC) type member of the myosin phosphatase targeting (MYPT) family of the regulatory subunits of protein phosphatase 1 (PP1) (Cao et al., 2002, Czikora et al., 2011, Csortos et al., 2008). Although TIMAP is highly expressed in EC, its importance is poorly recognized. Our group has shown a barrier protective role of TIMAP in human pulmonary artery endothelial cells (HPAEC) (Csortos et al., 2008). We demonstrated specific protein-protein interaction between TIMAP and the β isoform of the catalytic subunit of PP1 (PP1cβ) (Csortos et al., 2008, Czikora et al., 2011), and identified a few substrates of the TIMAP-PP1c complex in pulmonary EC (Csortos et al., 2008, Czikora et al., 2011, Boratko et al., 2015). Regulation of PP1c activity in the TIMAP-PP1c complex is affected by PKA-primed GSK-3β-mediated phosphorylation of TIMAP (Li et al., 2007, Czikora et al., 2011, Boratko et al., 2013).

To uncover novel cellular functions of TIMAP as targeting subunit of PP1, we initiated a search for new interacting partners and substrates of TIMAP-PP1c. In the present study, morphological changes and angiogenesis related proteome profiling of TIMAP depleted EC turned our attention toward the regulation of ECE-1 mediated ET-1 secretion from EC. We show results suggesting ECE-1 being a novel substrate of the TIMAP-PP1c complex. In the absence of TIMAP, the targeting of PP1c is insufficient, and eventually, dephosphorylation of ECE-1 becomes disabled. This leads to sustained ECE-1 activity and increased secretion of ET-1, which evokes an angiogenic EC phenotype.

2. Material and Methods

2.1 Reagents

Materials were obtained from the following vendors: paraformaldehyde, dimethylsulfoxide, bovine serum albumin, BQ788, Thiazolyl Blue Tetrazolium Bromide (MTT), Phorbol 12-myristate 13-acetate, Gö6983, anti-actin antibody: Sigma (St Louis, MO); anti-rabbit IgG HRP-linked and anti-mouse IgG HRP-linked secondary antibodies, CD31(PECAM-1) antibody: Cell Signaling Technology, Inc. (Beverly, MA); anti-PP1 delta antibody, mouse anti-human endothelin-1 antibody: Upstate Biotechnology (Lake Placid, NY); anti-PPP1R16B antibody: Abgent Inc, (San Diego, CA); anti-lamin A/C (H-110) antibody: Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); ECE-1 monoclonal antibody, anti-rat IgG HRP affinity purified antibody: R&D Systems, Inc., (Minneapolis, MN); TO-PRO-3 Iodide, Lipofectamine RNAiMax: Life Technologies (Carlsbad, CA); Alexa 488-conjugated secondary antibody and ProLong Gold Antifade medium: Molecular Probes (Eugene, OR);Protease Inhibitor Cocktail

Set III: EMD Biosciences (San Diego, CA); Substances for cell culturing were from PAA (Austria). All other chemicals were obtained from Sigma (St Louis, MO).

2.2 Cell cultures

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 4–6. 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 15-20. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in MEM supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1% sodium pyruvate, 0.1mM MEM non-essential amino acids solution.

2.3 siRNA silencing

TIMAP (PPP1R16B) was silenced using 50 nM ON-TARGETplus SMARTpool siRNA (L-004065-00-0005 HumanPPP1R16B, Dharmacon) in complex with Lipofectamine RNAiMAX reagent (Life Technologies) in serum-free medium. ON-TARGETplus siCONTROL non-targeting pool (D-001810-10-01-05; Dharmacon) was used as an irrelevant control. After 6 h the medium was changed to complete medium. Cells were further incubated for 48 h.

2.4 Immunofluorescence and microscopy

Cells were grown on glass coverslips, washed once with 1X TBS and fixed with 3.7% paraformaldehyde in 1X TBS for 10 min at room temperature. Between each step, the cells were rinsed three times with 1X TBS. The cells were permeabilized with 0.5 % Triton X-100 in TBS at RT for 15 min, blocked with 2% BSA in TBS for 30 min at RT, and incubated with primary, then with secondary antibodies diluted in blocking solution for 1 h at RT. Cover slips were rinsed and mounted in ProLong Gold Antifade medium. Confocal images were acquired with a Leica TCS SP8 confocal microscope using HC PL APO CS2 63x 1.40 NA oil immersion objective on an DMI6000 CS microscope at 25°C. Images were processed using LAS AF v3.1.3 software. Non-specific binding of the secondary antibodies was checked in control experiments (not shown). Light microscopy images were taken by Leica DM IL LED microscope using N Plan 5x/0.12 objective.

2.5 Immunoprecipitation

Cells were grown in 100-mm tissue culture dishes, rinsed three times with 1x TBS and then collected and lysed with 600 µl of immunoprecipitation buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM sodium vanadate, 1% Nonidet P-40) containing protease inhibitors. The lysates were centrifuged with 10,000 *g* for 15 min at 4°C. To avoid non-specific binding, the supernatants were precleared with 50 µl 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 50 µl of fresh protein G Sepharose at 4°C overnight with gentle rotation. The resin was washed three times with 500 µl of IP buffer and then resuspended in 150 µl of 1X SDS sample buffer, boiled, and microcentrifuged. The supernatant was further analyzed by Western blot.

2.6 Subcellular fractionation

Membrane fraction was isolated using Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific, Inc.,Vantaa, Finland) according to the manufacturer's protocol. The efficiency of fractionation was analyzed by immunoblotting using CD31 antibody as a membrane marker, and β-tubulin antibody as a cytoplasmic marker.

2.7 Western blotting

Protein samples were separated by 10-15% SDS-PAGE and transferred to 0.45 µm pore sized Hybond ECL Nitrocellulose Membrane (GE Healthcare, Piscataway, NJ). Membranes were blocked with TBST containing 5% low-fat milk for 1 h. Primary and secondary antibodies were diluted (according to the manufacturer's recommendation) in TBST containing 1% BSA for 1 h or overnight. Western blots were imaged using an Alpha Innotech FluorChem® FC2 Imager or Kodak Medical X-ray Developer.

2.8 ECIS measurements

ECIS (Electric cell-substrate impedance sensing) model Zθ, Applied BioPhysics Inc. (Troy, NY) equipment was used to monitor transendothelial electric resistance (Giaever and Keese, 1993) of control or transfected cells seeded on type 8W10E arrays. In wound healing experiments an alternate current of 5 mA at 60 kHz frequency was applied for 30 sec duration

to establish wounds in the cell layer, and then the impedance was measured. The impedance in each wounded well increased gradually, until it reached a maximum plateau value.

2.9 In vitro tube formation assay

BD Matrigel™ Basement Membrane Matrix (BD Biosciences) was used to study the effect of TIMAP silencing on endothelial capillary tube formation in accordance with the manufacturer's instructions. Non-silencing RNA or TIMAP-specific siRNA treated EC (~1 × 10^3cells) were plated in µ-Slide (Ibidi, Germany) previously coated with Matrigel and incubated in triplicates at 37°C. Images at different time points of the experiment were taken with Leica DM IL LED microscope using N Plan 5x/0.12 objective. Quantification of capillary-like structures was made with Angiogenesis Analyzer ImageJ toolkit.

2.10 Proteome Profiler™ Human Angiogenesis Antibody Array

The Proteome Profiler Human Angiogenesis Antibody Array from R&D Systems, Inc., (Minneapolis, MN) was used to estimate the level of angiogenesis-related proteins in non-silencing RNA or TIMAP-specific silencing RNA treated cell culture supernatants. Data analysis was done according to the recommendations in the manufacturer's manual. Pixel densities of duplicated spots were measured by Image J and negative control background value was subtracted from the averaged signal. The experiment was repeated three times, therefore all single measurement was normalized against the positive controls, and then statistical analysis was done.

2.11 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. At different time points (24, 48 ,72 h) 10µl of MTT (5 mg/ml) was added to each well, and incubated at 37 °C for 2h. Untransformed MTT was removed by aspiration, and the formazan product was dissolved in DMSO. After vigorous shake of plates the absorbance was measured at wavelength of 540 nm.

2.12 Statistical analysis

All experiments were repeated at least three times independently. Data are presented as mean ± SE. Statistical comparisons between groups were performed using Student's t-test or

ANOVA. Significant changes are indicated by asterisks; * ($P<0.05$), ** ($P<0.01$), or *** ($P<0.001$).

3. Results

3.1 Depletion of TIMAP induces morphological change, but does not affect viability and proliferation of endothelial cells

TIMAP depletion was established in HPAEC and BPAEC by siRNA mediated silencing. The efficiency of silencing was confirmed by Western blot. About 70-80% decrease in the protein level of TIMAP was detected in the depleted cells compared to the control or non-silencing RNA transfected cells (Fig.1A). To test whether loss of the protein affects cell viability, MTT assay was performed at 24, 48 and 72 h with non-specific or TIMAP-specific siRNA treated cells seeded onto 96–well plates as described in Materials and Methods. The depletion of the protein had no significant effect on EC viability (Fig.1B). Cell proliferation was studied by ECIS measurements starting from a sub-confluent cell number seeded onto 8W10E array 48 h post-treatment, and the impedance being followed in time. During the 24 h of the experiment, no significant difference was observed in their proliferation (Fig.1C).

On the other hand, silencing of TIMAP induced morphological changes in EC shown both by light microscopy and by immunofluorescent staining using TO-PRO Iodide as nuclear marker and CD31 as membrane marker (Fig.1D). In the absence of TIMAP, the morphology of the cells appeared as in shear stress-induced aligned state, resembling an angiogenic phenotype.

3.2 Silencing of TIMAP accelerates tube formation and migration of EC

To investigate the potential role of TIMAP in angiogenesis, first we examined the effect of TIMAP depletion on endothelial tube formation *in vitro*. The assay measures the ability of EC to form capillary-like structures, “tubes” in Matrigel extracellular matrix. Non-siRNA or TIMAP-specific siRNA treated cells were used 48 h post-transfection. The behavior of the cells during the tube formation process was analyzed (Fig.2). The depleted cells showed faster dynamics of tube formation as they already aligned end-to-end 2 h after plating on Matrigel and started to form capillary like structures earlier than the non-siRNA treated cells. Images taken during the experiment were evaluated by measuring the number of junctions and total length of the tubes as indexes of angiogenesis. As shown in Fig.2, TIMAP depletion greatly accelerated the formation of tube-like structures. Both total length of tubes and number of

junctions were significantly increased in TIMAP-specific siRNA treated cells up to 5 h after seeding, but the differences have disappeared at the longer time-points of the experiment.

The migration of cells was assessed by *in vitro* wound healing assay carried out by ECIS measurements. Cells were grown until forming a confluent monolayer on ECIS arrays, and then an alternate current was applied to establish wounds in the cell layer, which led to the death of cells in a well-defined area. The impedance was monitored to chart the migration of EC from nearby and the ultimate healing of the wound. We did not find any difference in the proliferations of non-siRNA treated or TIMAP depleted EC (Fig.1c), therefore variations detected during the wound healing assay reflect variation in cell migration. The *in vitro* wound healing assay showed that knockdown of TIMAP expression increases EC migration, as evidenced by the increased speed of wound closure (Fig.3).

Together, these data suggest that TIMAP depletion promotes angiogenesis without promoting cell proliferation. Since TIMAP was identified earlier as a regulatory/targeting subunit of PP1cβ, one can assume that phosphorylation level of an angiogenesis-related protein can not be modulated by the TIMAP-PP1c complex in the absence of TIMAP.

3.3 Secretion of ET-1 is regulated by TIMAP

A human cytokine array including 55 different angiogenesis-related proteins was used to further screen the effect of TIMAP depletion. In our experiments we used cell culture supernates of nonsiRNA and siTIMAP RNA treated cells, and shown are the results for the 13 strongest positive hits, and we found that the level of ET-1 was elevated due to the TIMAP depletion (Fig.4). EC express ET_B type endothelin receptor, therefore, a specific ET_B receptor antagonist, BQ788 (Ishikawa et al., 1994, Hersch et al., 2004, Okada and Nishikibe, 2002), was employed to test whether the changes observed in TIMAP-depleted EC are actually evoked by the elevated ET-1 secretion. Cells were treated with 1μM BQ788 in the last 24 h of silencing and their morphology was studied by immunofluorescent staining. BQ788 diminished the effect of TIMAP depletion, as the antagonist treated cells showed no significant difference in their morphology compared to non-siRNA treated cells (Fig.5A).

To verify the role of the elevated ET-1 level in the tube formation process, *in vitro* tube formation assays with non-siRNA and TIMAP-specific siRNA treated cells were made in the presence or absence of BQ788. The tube formation ability of the cells was compared at 5 h after seeding on Matrigel, since the biggest difference was found at this time point in the

previous experiment (see Fig.2). Again, treatment of the cells with the ET_B receptor antagonist diminished the effect of the TIMAP depletion (Fig.5B). Pretreatment with receptor antagonist for 30 min and then performing an *in vitro* wound healing assay revealed that BQ788 decreased the wound healing ability of the cells, furthermore, there was no difference in the wound healing of the non-siRNA treated or TIMAP-depleted cells in the presence of BQ788 (Fig.5C).

These results imply a connection between the elevated level of ET-1 and the change in morphology, the faster tube formation and wound healing/cell migration of TIMAP-silenced EC suggesting that TIMAP/TIMAP-PP1c may play an essential role in affecting the properties of EC through a regulation of ET-1 secretion.

3.4 TIMAP-PP1c is involved in the membrane localization of ECE-1

The generation of ET-1 is crucially dependent on the local presence and activity of ECE-1 on the surface of vascular EC (Kuruppu and Smith, 2012). PKC phosphorylates ECE-1c and the phosphorylated form translocates to the plasma membrane (Kuruppu et al., 2010). As a potential phospho-target of PP1, first we tested and confirmed a protein-protein interaction between TIMAP, PP1c and ECE-1 in EC by immunoprecipitation (Fig.6A). Next, the effect of TIMAP depletion on the localization of ECE-1 was studied in membrane fractions of the non-siRNA or TIMAP-specific siRNA treated cells. Although there was no significant change in the ECE-1 level in total cell lysates, about 2-3fold increase of the ECE-1 protein was detected in the membrane fraction of TIMAP-depleted cells (Fig.6B). This result suggests an alteration in the kinase to phosphatase activity ratio in the silenced EC.

To change the phosphorylation level of ECE-1 by shifting the kinase-phosphatase equilibrium in non-depleted EC, PMA treatment was utilized to activate PKC and initiate phosphorylation and translocation of ECE-1 to the cell membrane (Fig.7), or Gö6983 pre-treatment , specific inhibitor of PKC, was used before the PMA-challenge in control experiments. PMA induced translocation of ECE-1, which was inhibited by the Gö6983 pre-treatment. Protein-protein interactions between ECE-1 and TIMAP-PP1c were proven by immunoprecipitation of either TIMAP or ECE-1 from the membrane fractions of PMA treated EC (Fig.7A). It is important to note the fixed ratio of the detected proteins although the efficiency of the two antibodies utilized for immunoprecipitation was different.

To further test whether TIMAP-PP1c is involved in the dephosphorylation of ECE-1 in EC, membrane fractions were isolated from cells without addition of PMA, after 10 min PMA treatment, and 30 minutes after removing PMA. In non-siRNA treated cells, PMA greatly increased the membrane presence of ECE-1, but it was reduced after 30 min suggesting dephosphorylation of ECE-1. However, the already elevated ECE-1 protein level in the membrane fraction of TIMAP-depleted cells showed no significant alteration after PMA challenge (Fig.7B). Finally, we showed the involvement of PP1 activity in ET-1 production utilizing type-specific phosphatase inhibitors. ET-1 secretion from EC treated with 1 μM tautomycetin, a PP1-specific inhibitor was comparable with the ET-1 production of TIMAP depleted cells (Fig.7C). However, specific inhibition of PP2A and PP2B phosphatases, by okadaic acid or cyclosporine A, respectively, did not increase ET-1 secretion at all.

4. Discussion

Several recent studies demonstrate the contribution of the TIMAP-PP1c complex in major physiological activities of EC through the regulation of phosphorylation levels of various proteins (Boratko et al., 2013, Czikora et al., 2011, Csortos et al., 2008, Kim et al., 2005, Shopik et al., 2013). PP1c can be targeted by many regulatory subunits (Cohen, 2002) and dephosphorylates a great number of phosphoproteins in all subcellular fractions of the cell. Therefore, in order to learn more about the specific role of the TIMAP-PP1c complex in EC without perturbing too many signaling pathways, experiments of this work aimed at TIMAP primarily.

The apparent morphological change evoked by silencing of TIMAP was manifested in an elongated shape and alignment and in accelerated tube formation and cell migration of EC. Another recent study, on the contrary, has demonstrated reduced HUVEC migration due to PP1cβ knockdown, and the Authors claim that silencing/inhibition of PP1c activity had no effect on EC the tube formation (Iacobazzi et al., 2015). Knockdown of PP1cβ could affect several signaling pathways through different targeting of the phosphatase catalytic subunit.

Investigation of the possible effect of TIMAP depletion on the secretion of angiogenesis related proteins demonstrated that in case of TIMAP-silenced cells there is about 30% increase in the secreted ET-1 level compared to the non-siRNA transfected EC 48 h post-transfection. Treatment with specific ET_B receptor antagonist, BQ788, demonstrated that the raised level of secreted ET-1 activated an autocrine signaling and was indeed responsible for the changes in TIMAP-depleted EC. Another study, using a different endothelial cell line,

also reported ET-1 inducing angiogenic phenotype of cultured HUVEC through the ET_B receptor (Salani et al., 2000), however, detailed mechanism was not provided.

ET-1 is a potent vasoconstrictor and it is intimately employed in several normal and pathological activities of EC (Kedzierski and Yanagisawa, 2001, Grant et al., 2003, Kelly and Whitworth, 1999, Salani et al., 2000, Bagnato and Rosano, 2008, Tanfin et al., 2011). Role of ET-1 in lung diseases is recognized, for example increased circulating ET-1 is associated with pulmonary arterial hypertension (Fagan et al., 2001, Galie et al., 2004, Chester and Yacoub, 2014). Myosin phosphatase (MP) plays a central role in myosin light chain dephosphorylation and smooth muscle cell contraction/relaxation. MP consists of a PP1 catalytic subunit and its targeting subunit, MYPT1. ET-1 was shown to inhibit MP activity in smooth muscle through phosphorylation of MYPT1 via G protein signaling mediated by ET_A (Hersch et al., 2004). However, connection between TIMAP-PP1c and ET-1 has not been recognized earlier.

Production of active ET-1 depends on the metalloprotease, ECE-1. Therefore, one may assume that ECE-1 is the link between TIMAP depletion and elevated ET-1 secretion. The ECE-1 protein level studied 48 h post-transfection in total lysates of TIMAP-depleted cells showed no significant difference compared to the controls. Thus, it is not likely that altered ECE-1 protein expression would be responsible for the elevated ET-1 level in our system, although one earlier work done on rat EC (Naomi et al., 1998) suggested that ECE-1 mRNA variations may affect ET-1 production. On the other hand, we detected an increased ECE-1 level in the membrane fraction of TIMAP-depleted EC, which rather suggests post-translational modification and trafficking of ECE-1.

Several isoforms (a-d) of ECE-1 are known; their catalytic domains at the C-terminal part of the protein are identical, but they differ in length and composition of the cytosolic N-terminal regions. Isoforms b-d are constitutively phosphorylated (at Ser18 and Ser20 in ECE-1c), and despite the differences in the primary structure of the N-terminus, they share further potential phosphorylation sites (Kuruppu and Smith, 2012, MacLeod et al., 2002). Importantly, it is well known that PKC regulates the cell surface activity of the ECE-1c isoform through its phosphorylation (likely additional to the constitutively phosphorylated sites) and consequent trafficking to the cell surface (Smith et al., 2006, Kuruppu et al., 2010). For that reason, although isoform specific antibody against ECE-1 is not available, our results strongly suggest that the isoform concerned in the presented work is ECE-1c.

The protein phosphatase counterpart for the reversible phosphorylation of ECE-1 which establishes an appropriate equilibrium with PKC, however, has not been known earlier. Therefore, we tested the hypothesis of TIMAP-PP1c being involved in ET signaling by the regulation of ECE-1 dephosphorylation. First of all, our results showed protein-protein interaction between ECE-1 and TIMAP-PP1c. Then, shifting the kinase to phosphatase activity ratio either by activation of PKC by PMA treatment, or by decreasing the TIMAP-PP1c activity via depletion of the targeting subunit, TIMAP, of the phosphatase complex, resulted in an elevated ECE-1 protein level in the plasma membrane fraction of EC. In non-siRNA transfected cells not only ECE-1, but TIMAP-PP1c protein levels were also increased in the plasma membrane after the PMA challenge. Notably, 30 min after the removal of the PMA, the ECE-1 level in the membrane was significantly decreased. Conversely, when TIMAP-depleted EC was challenged with PMA, activation of PKC could not evoke any further increase in the already elevated ECE-1 protein level. These results strongly suggest that the protein phosphatase, which controls ECE-1 trafficking and activity at the cell membrane through its dephosphorylation, is the TIMAP-PP1 complex.

5. Conclusions

The results presented in this study show the contribution of TIMAP-PP1c holoenzyme form of PP1 in the regulation of ET-1 secretion/ET-1 signaling of EC via affecting the phosphorylation level of ECE-1. This may imply that TIMAP is involved in the regulation of important processes of EC such as cardiovascular homeostasis, tumorigenesis, vascular permeability, and angiogenesis.

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Figure 1.

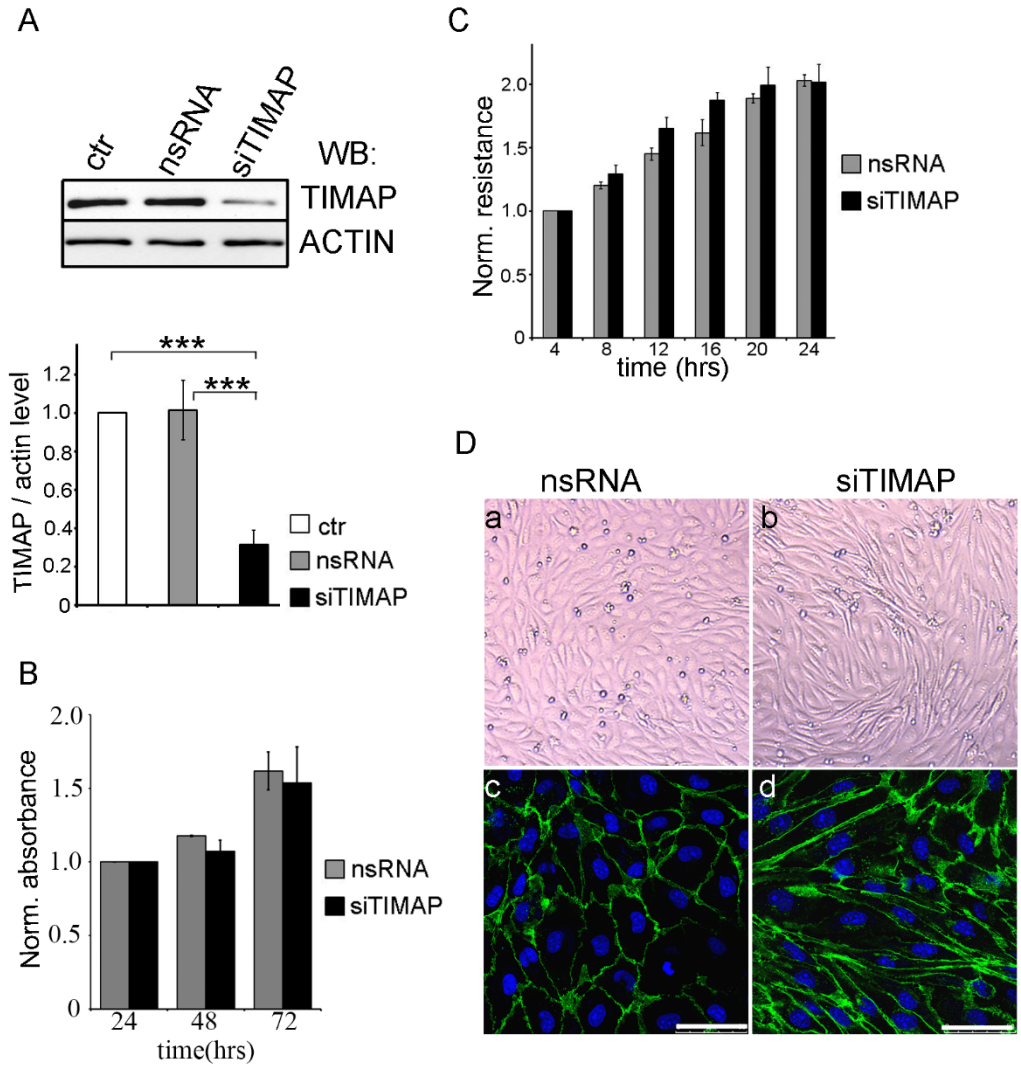


Figure 1. ***Viability, proliferation and morphology of TIMAP depleted EC.*** (A) HPAEC cells were transfected with non-silencing RNA (nsRNA) or with 50 nM siPPP1R16B RNA (siTIMAP) as described in *Materials and Methods*. Efficiency of depletion was analyzed by Western blot of non-transfected (ctr), non-silencing RNA (nsRNA), or siPPP1R16B transfected cells using TIMAP-specific antibody. Actin was tested as loading control. The amount of TIMAP signal was expressed as ratio of TIMAP:β-actin signal density. The error bars correspond to SE from 3 independent transfections. Statistical analysis was done with Student's t-test. Significant changes are indicated by asterisks;*** ($P < 0.001$). (B) Cell viability was measured by MTT assay as described in *Materials and Methods*. Cells transfected with non-siRNA or siTIMAP RNA were seeded onto 96–well plates and measurements were carried out at 24, 48 and 72 h later. Values are means ± SE. Results are

representative of 3 separate experiments. (C) Non-silencing or TIMAP specific silencing RNA transfected cells were plated (5 x 10^5 cells/well) onto two 8W10E arrays 48 h post-transfection. The initial resistance values at the beginning of the measurement were about 300 Ω and the impedance was measured for 24 h after the seeding. (D) Light microscopy images of nsRNA (a) or siTIMAP RNA (b) treated cells or immunofluorescent staining using CD31 primary antibody of nsRNA (c) or siTIMAP RNA (d) treated cells 48 h post-transfection. Nuclei of the cells were stained using TO-PRO-3 Iodide (blue). Non-specific binding of the secondary antibodies was checked in control experiments (not shown). Representative data of at least three independent experiments are shown. Scale bars: 500 μm (a and b), 50 μm (c and d).

Figure 2.

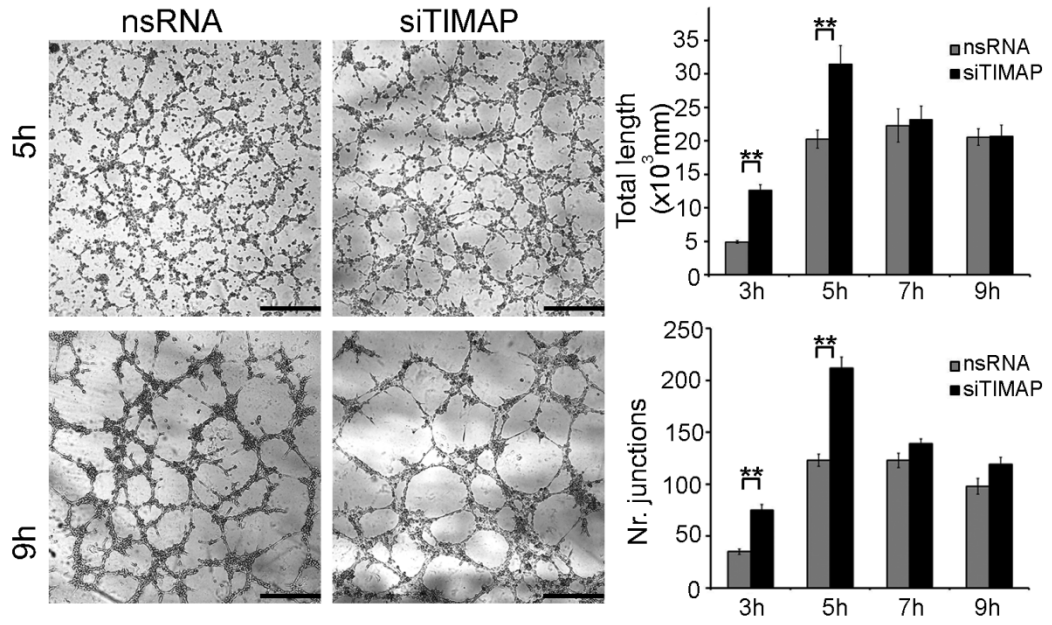


Figure 2. ***The effect of TIMAP depletion on tube formation.*** BPAEC cell were treated with non-silencing or TIMAP-specific siRNA. Cells were seeded onto Matrigel 48h post-transfection and observed in time. Quantification of capillary formation was determined using the ImageJ program. Data are reported as mean ± SE. Statistical analysis was done with Student's t-test. Significant changes are indicated by asterisks;** ($P < 0.01$). Scale bar: 200 µm.

Figure 3.

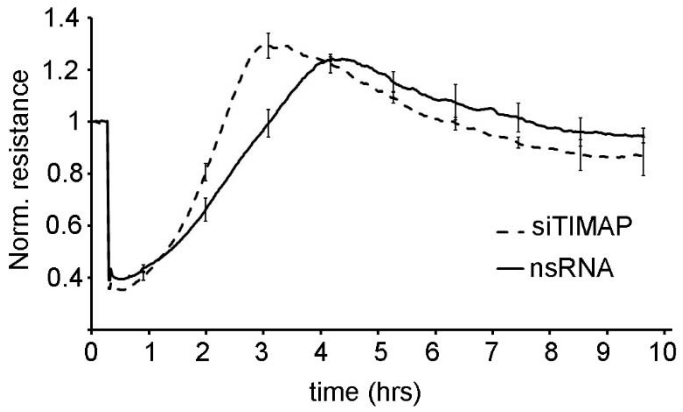


Figure 3. ***Faster in vitro wound healing of TIMAP depleted HPAEC.*** Cells plated onto two 8W10E arrays were treated with non-siRNA or siTIMAP RNA. 48 h post-transfection (about 1000 Ω impedance) an alternate current of 5 mA at 60 kHz frequency was applied for a 30 sec duration to establish wounds in the cell layer, after which the impedance was measured. The average results from three independent experiments each made with four parallel measurements are shown. Error bars represent SE.

Figure 4.

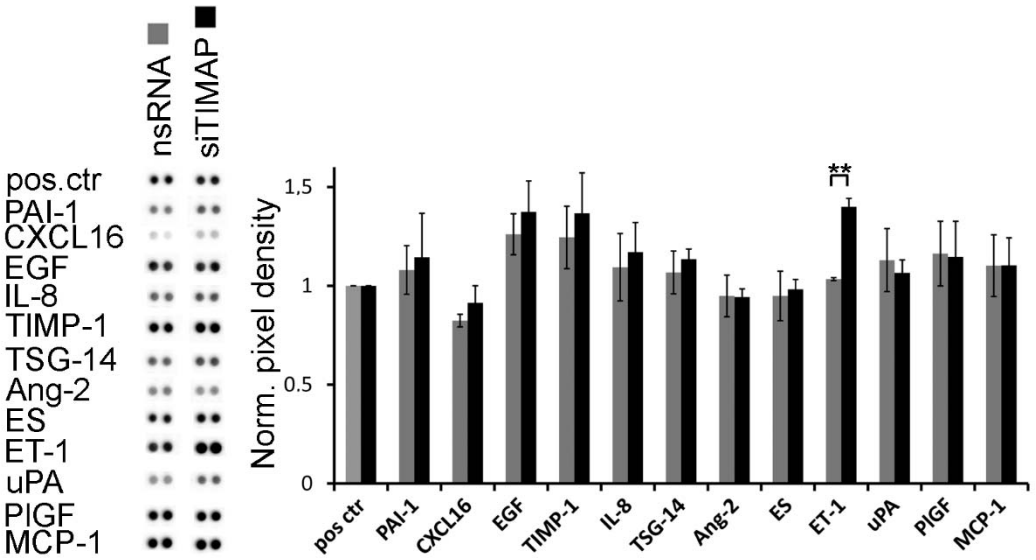


Figure 4. ***Angiogenesis protein array.*** Relative levels of human angiogenesis-related proteins were evaluated from HPAEC and BPAEC cell culture supernatants of non-siRNA or siTIMAP RNA- treated cells using a human angiogenesis array kit (Proteome Profiler, R&D Systems) according to the manufacturer's recommendations. Shown are pixel densities of positive hits quantified by ImageJ. Data are reported as mean ± SE from three independent experiments. Statistical analysis was performed with ANOVA. Significant changes are indicated by asterisks;** ($P < 0.01$).

Figure 5

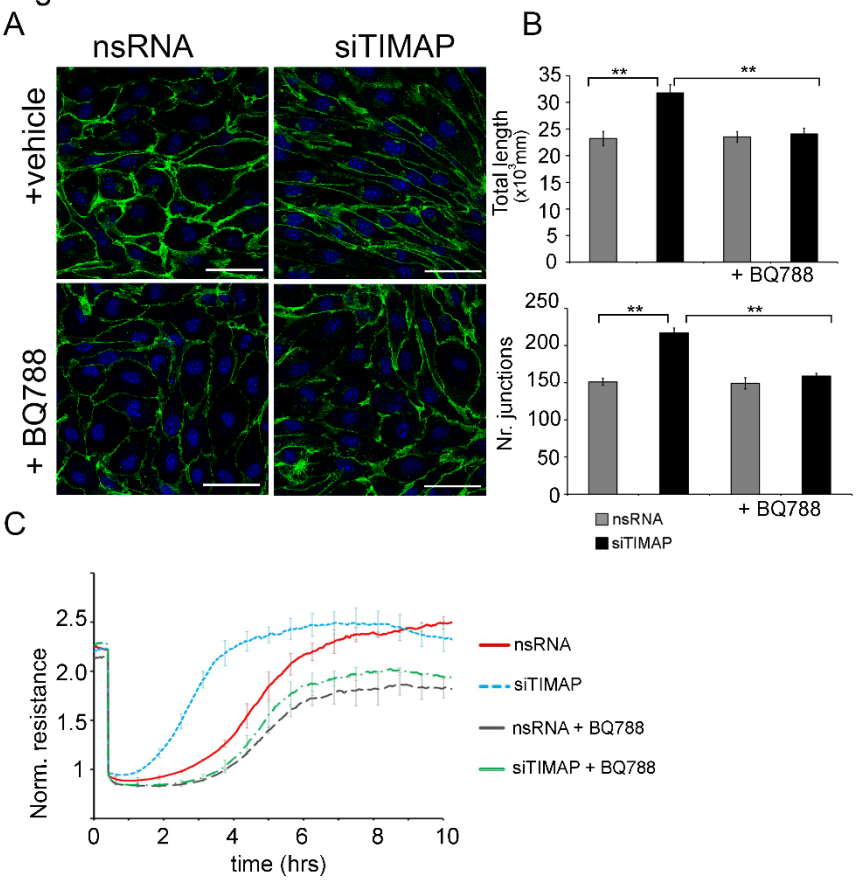


Figure 5. ***ET_B antagonist diminishes the effects of TIMAP silencing.*** (A) Immunofluorescent staining was made using CD31 primary antibody to show the morphology of non-siRNA or siTIMAP RNA treated BPAEC 48 h post-transfection with or without BQ788 in the last 24 h of silencing. Nuclei of the cells were stained using TO-PRO-3 Iodide (blue). Representative pictures of at least three independent experiments are shown. Scale bar: 50 µm. (B) Non-silencing or TIMAP-specific siRNA treated cells were seeded on Matrigel 48 h post-transfection with or without BQ788. Quantification of capillary formation was determined using ImageJ at 5 h after seeding. Data are reported as mean ± SE. Statistical analysis was done with ANOVA. Significant changes are indicated by asterisks;** ($P < 0.01$). (C) Cells plated onto two 8W10E arrays were treated with non siRNA or siTIMAP RNA. 48 h post-transfection vehicle or BQ788 was added and an alternate current of 5 mA at 60 kHz frequency was applied for 30 sec duration to establish wounds in the cell layer; after that the impedance was measured. The average results from three independent experiments each made

with four parallel measurements are shown. Error bars represent SE.

Figure 6

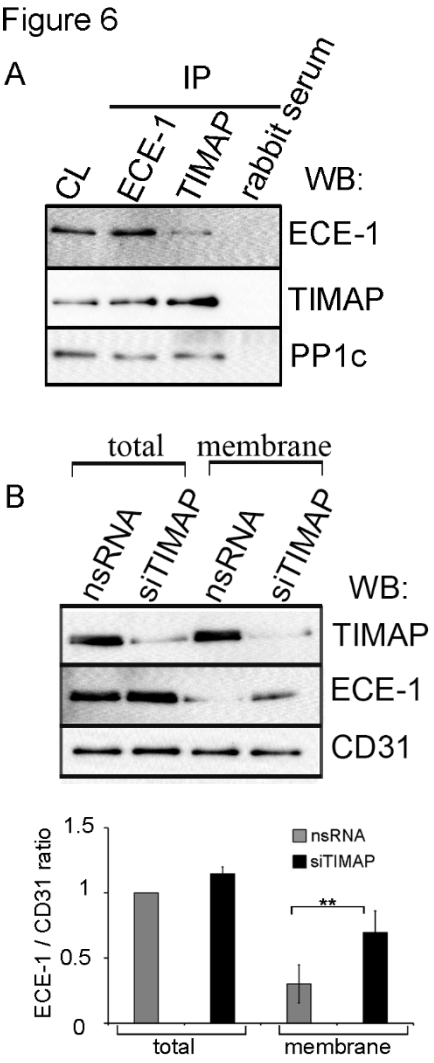


Figure 6. ***Protein-protein interaction between ECE-1 and TIMAP-PP1c.*** (A) TIMAP or ECE-1 was immunoprecipitated from lysates of BPAEC as described in *Materials and Methods*. IP complexes were probed for TIMAP, ECE-1 and PP1c. (B) Membrane fractionation of non-silencing RNA and TIMAP-specific siRNA treated BPAEC was performed as described in *Materials and Methods*. The fractions were analyzed with anti-TIMAP, anti-ECE-1 and anti-CD31 (membrane marker) antibodies. The error bars correspond to SE from 3 independent experiments. Statistical analysis was done with Student's t-test. Significant changes are indicated by asterisks;** ($P < 0.01$). Shown are representative data of at least 3 independent experiments.

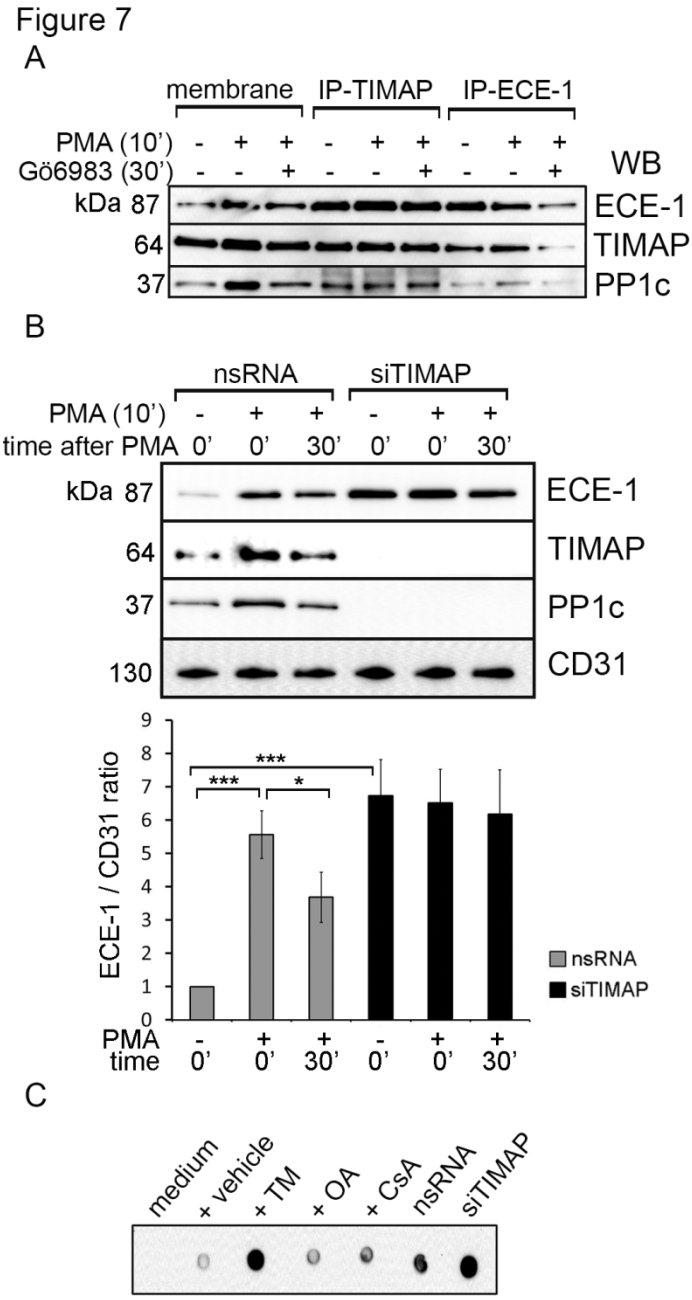
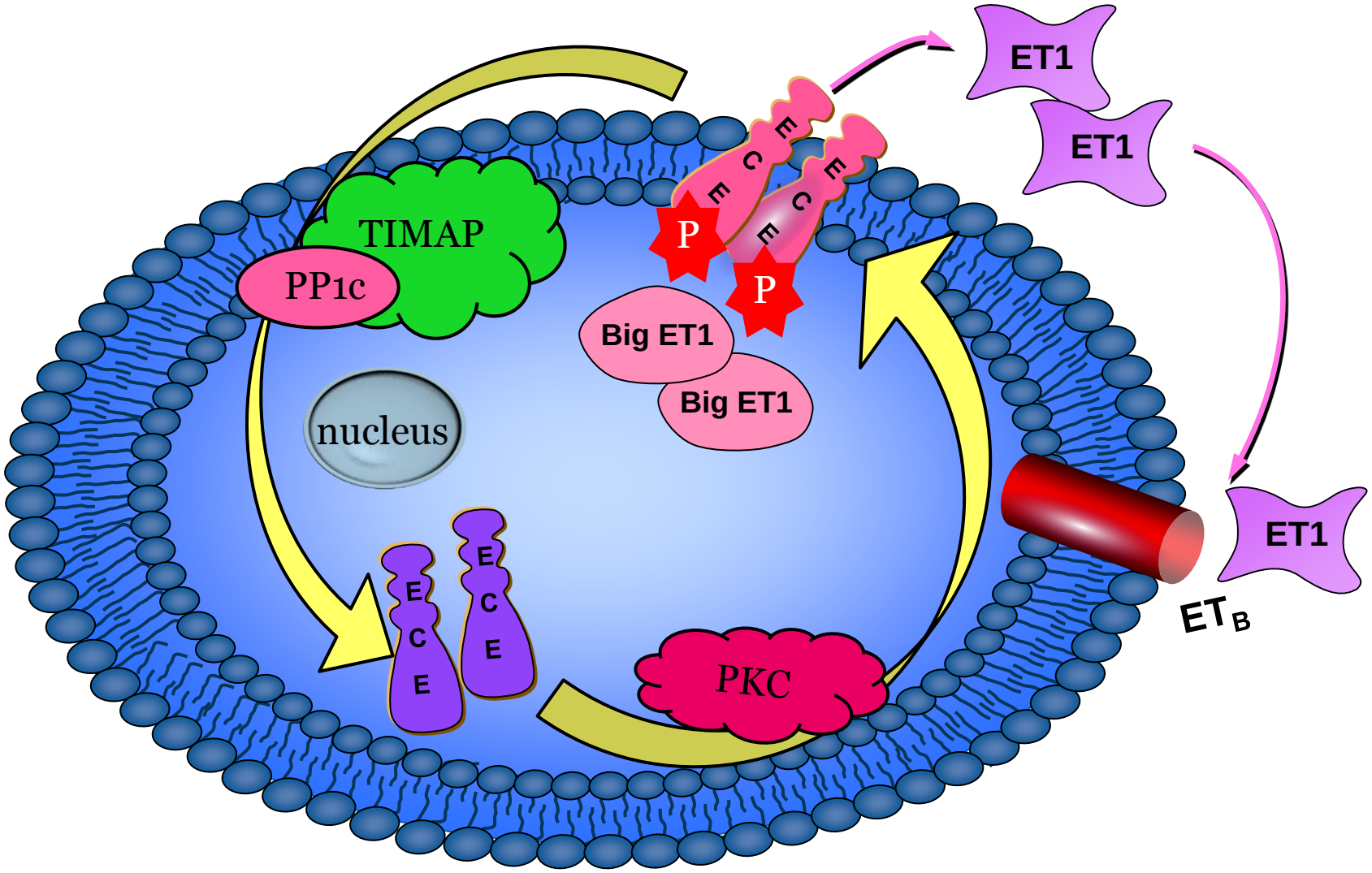


Figure 7. ***TIMAP-PP1c regulates membrane localization of ECE-1.*** (A) TIMAP or ECE-1 was immunoprecipitated from membrane fractions of untreated, PMA (1μM, 10') or Gö6983 pretreated (5μM, 30') and PMA challenged (1μM, 10 min) BPAEC cells as described in *Materials and Methods*. IP complexes were probed for TIMAP, ECE-1 and PP1c. (B) Membrane fractionation of nsRNA or TIMAP-specific siRNA treated BPAEC were made from untreated, PMA (1μM, 10 min) treated (right after the treatment or 30 min later) as described in *Materials and Methods*. The fractions were analyzed with anti-TIMAP, anti-

ECE-1, anti-PP1c and anti-CD31 (membrane marker) antibodies. The error bars correspond to SE from 3 independent experiments. Statistical analysis was done with ANOVA. Significant changes are indicated by asterisks; *($P < 0.05$),***($P < 0.001$). (C) ET-1 secretion was compared by dot blot from non-treated BPAEC (control) or BPAEC treated with vehicle (DMSO), 1 μM tautomycetin (TM), 5 nM okadaic acid (OA), or 2 μM cyclosporine A (CsA) for 24 h and BPAEC transfected with non-specific (nonsiRNA) or TIMAP-specific (siTIMAP) siRNA. Supernates' proteins were immobilized on nitrocellulose membrane, and the membranes were blocked with 5% low-fat milk containing TBST, and then incubated for 1h with ET-1 specific and another 1h with the secondary antibody.

Shown are representative data of at least 3 independent experiments.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