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Molecular Characterization of Myosin Phosphatase in Endothelium

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Abstract

The phosphorylation status of myosin light chain (MLC) is regulated by both MLC kinases and type 1 Ser/Thr phosphatase (PPase 1), MLC phosphatase (MLCP) activities. The activity of the catalytic subunit of MLCP (CS1 β) towards myosin depends on its associated regulatory subunit, namely myosin PPase targeting subunit 1 (MYPT1). Our previously published data strongly suggested the involvement of MLCP in endothelial cell (EC) barrier regulation. In this paper, our new data demonstrates that inhibition of MLCP by either CS1 β or MYPT1 siRNA-based depletion results in significant attenuation of purine nucleotide (ATP and adenosine)-induced EC barrier enhancement. Consistent with the data, thrombin-induced EC F-actin stress fiber formation and permeability increase were attenuated by the ectopic expression of constitutively active (C/A) MYPT1. The data demonstrated for the first time direct involvement of MLCP in EC barrier enhancement/protection. Cloning of MYPT1 in human pulmonary artery EC (HPAEC) revealed the presence of two MYPT1 isoforms, long and variant 2 (V2) lacking 56 amino acids from 553 to 609 of human MYPT1 long, which were previously identified in HeLa and HEK 293 cells. Our data demonstrated that in Cos-7 cells ectopically-expressed EC MYPT1 isoforms co-immunoprecipitated with intact CS1 β suggesting the importance of PPase 1 activity for the formation of functional complex of MYPT1/CS1 β . Interestingly, MYPT1 V2 shows decreased binding affinity compared to MYPT1 long for radixin (novel MLCP substrate and a member of ERM family proteins). These results suggest functional difference between EC MYPT1 isoforms in the regulation of MLCP activity and cytoskeleton.

Keywords

MYPT1; MLCP; ERM; ezrin; radixin; moesin

INTRODUCTION

The pulmonary vascular endothelium acts as a semi-selective diffusion barrier between blood and interstitial space, which regulates fluid current, macromolecular transport, and leukocytes trafficking through the vessel wall. Inflammatory molecules, such as

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lipopolysaccharide (LPS), histamine, and thrombin cause disruption of the endothelial cell (EC) barrier and leads to lung edema, a hallmark of acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS) (Dudek and Garcia, 2001; Ishii et al., 1995; Kolosova et al., 2004; Verin et al., 1995). Therefore, the integrity of pulmonary EC barrier is required to preserve normal pulmonary function. One of the major factors that regulate intercellular gap formation and barrier dysfunction of EC is reversible phosphorylation of the myosin regulatory light chains (MLC). It is tightly regulated by the balanced activity between Ser/Thr protein kinases including MLC kinase (MLCK), Rho kinase, and MLC phosphatase (MLCP). MLCP is a type 1 protein phosphatase (PPase 1), one of the major protein Ser/Thr PPases in mammalian cells, and the only PPase that is able to dephosphorylate native MLC in smooth muscle (Chisholm and Cohen, 1988; Csontos et al., 2007). MLCP is composed of a catalytic subunit (CS1 β , initially described as CS1 δ) and two regulatory subunits: a 20kDa small subunit (M20), and a 110–130 kDa myosin PPase targeting subunit 1 (MYPT1), which determines substrate specificity towards myosin (reviewed in (Hartshorne et al., 1998; Ito et al., 2004)) and the subcellular localization of catalytic subunit, CS1 β . The role of M20 in MLCP activity regulation currently is not clear. Thus, the studies on MLCP are primarily conducted on the complex of CS1 β and MYPT1. MYPT1 is abundantly expressed in smooth muscle and most of the nonmuscle cells. MYPT1 increases the accessibility of CS1 β toward myosin because it has separate binding domains for CS1 β at its N-terminal region (amino acids 35–38, R/KVxF), which is followed by seven ankyrin repeats (amino acids 39–296), as well as its substrate, MLC. Therefore, the enzyme activity of the catalytic subunit is increased greater than 10 times in the form of CS1 β -MYPT1 holoenzyme compared to the catalytic subunit itself (Johnson et al., 1997). When it is phosphorylated at Thr696, MYPT1 acts as an inhibitory protein of CS1 β . Furthermore, several proteins related to the cytoskeleton organization or signal transduction are reported to interact with MYPT1 (Matsumura and Hartshorne, 2008). Thus, the role of MYPT1 would be extended to the integration of signal transduction pathways regulating cytoskeletal organization in various cell types. Multiple isoforms of MYPT1 are reported from several species. It was shown that they are products of alternative splicing from one gene, and that some of them are transiently expressed according to the developmental stages (Ito et al., 2004). The gene of human MYPT1 is located on human chromosome 12q15-q21.2, and its spliced variant (V2) was identified in HeLa and HEK293 cell lines (Takahashi et al., 1997; Xia et al., 2005). However, the structural features of MYPT1 in pulmonary EC have yet to be reported even though the published data suggested that MLCP contributes to the maintenance of endothelial barrier integrity (Kolosova et al., 2005; Verin et al., 1995). In this study, we directly demonstrated the involvement of MLCP in EC barrier regulation, identified MYPT1 isoforms in human endothelium, and examined the differences in the physiological role of these isoforms. We demonstrated that each isoform has similar properties in localization and binding affinity to CS1 β . However, they showed different binding affinity to radixin, one of the ERM proteins.

MATERIALS AND METHODS

Materials and reagents

Rabbit polyclonal anti-MYPT1 antibody was purchased from Upstate (Lake Placid, NY); mouse monoclonal anti-HA and anti-myc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-V5, lipofectamine 2000, TRIzol, and RT-PCR kit were from Invitrogen (Carlsbad, CA). FuGene HD transfection reagent was from Roche (Indianapolis, IN), and siPORT Amine was from Ambion (Austin, TX). Alexa 488-, Alexa 594-conjugated secondary antibodies, and ProLong Gold Antifade medium were purchased from Molecular Probes (Eugene, OR). Protease Inhibitor Cocktail Set III was purchased from EMD Biosciences (San Diego, CA). QuickChange Site-Directed mutagenesis kit was

from Stratagene (La Jolla, CA), and HRP-conjugated secondary antibody as well as lumiglo was from Cell Signaling (Danvers, MA). AlkPhos Direct Labeling reagent for labeling of the DNA probe with alkaline phosphatase, and protein G sepharose 4 fast flow were purchased from Amersham, GE Healthcare (Little Chalfont, UK). All other chemicals were from Sigma (St. Louis, MO).

Cell culture

Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza Group Ltd. (Walkersville, MD), propagated in culture medium EGM-2-MV (Lonza) supplemented with 5% (v/v) fetal bovine serum (FBS) (Hyclone), and used at passages 3–7. HUVEC was generously provided by Dr. J.D. Catravas (Georgia Health Sciences University, Augusta, GA). Cos-7 and HeLa cell lines (ATCC) were maintained in DMEM-F12 (Dulbecco's modified Eagle's medium) with 10% FBS. All cells were maintained at 37°C in humidified atmosphere of 5% CO₂-95% air.

Ectopic expression using plasmid or adenovirus

Cells were grown to 70% of confluency, incubated with appropriate constructs in the presence of FuGene HD (Roche, Indianapolis, IN) for HPAEC or Lipofectamin 2000 (Invitrogen, Carlsbad, CA) for Cos-7 cells according to manufacturer's protocol (Kolossova et al., 2004). On the other hand, HPAEC was infected with adenoviral construct containing constitutively active (C/A) MYPT1 (amino acid 1–300) at 20 multiplicities of infection (MOI). After incubation in the basal medium for 8 hrs, purified adenovirus was added to HPAEC. The medium was replaced with fresh complete medium containing serum and growth supplements after overnight incubation and then the infected cells were used for further experiments after 24 hrs. The infection efficiency of C/A MYPT1, which is determined by immunostaining with anti-myc antibody, was more than 90%. In addition, the overexpression of corresponding protein, induced by each plasmid or adenovirus, was confirmed by Western blotting.

Depletion of endogenous proteins using siRNA

HPAEC seeded on the gold microelectrodes were transfected with specific siRNA duplex or non-specific siRNA (final concentration 20 nM) designed according to Dharmacon Research (Lafayette, CO, www.Dharmacon.com) to decrease the protein expression of CS1 α , CS1 β , or MYPT1 using siPORT Amine (Ambion, Austin, TX). After incubating in the serum free medium for 2 hrs, the complex of siRNA and siPORT Amine was added to HPAEC. 6 hrs later, fetal bovine serum (final concentration 5%) was supplemented to the cell and the medium was replaced with complete medium containing serum and growth supplements after overnight incubation. After 24 hrs of additional incubation, the infected cells were used for further experiments.

Measurement of transendothelial electrical resistance

Transendothelial electrical resistance (TER) was measured in response to barrier protective agents (ATP or adenosine) or a barrier disruptive agent (thrombin) using an electrical cell-substrate impedance sensing system (ECIS) (Applied BioPhysics, Troy, NY) as previously described (Bogatcheva et al., 2007; Kolossova et al., 2005). All of the results obtained were presented as means $\pm$ SE from more than three independent experiments.

Cloning of human endothelial MYPT1

The coding sequences of human MYPT1 in HPAEC were generated by reverse transcription-coupled polymerase chain reaction (RT-PCR). Total RNA was extracted with Trizol, and the first strand of cDNA was synthesized by reverse transcription with both

random hexamer and oligo (dT) primers. To subclone the entire coding sequence of MYPT1, the following PCR primers based on GeneBank sequence of human MYPT1, D87930, were utilized: forward primer 5' - **GAGATAGGTACCATGAAGATGGCGGACGCG**-3' and reverse primer 5' - **GCGGCAGAAATTCATTATTTGGAAAGTTTGCTTATAAC**-3'. The PCR primers consisted not only of the 5' - or 3' -end coding sequences (bold letters) of MYPT1 but also the restriction site sequences (italicized letters) *KpnI* or *EcoRI*, respectively, to insert the amplified cDNA into pcDNA3.1/c-myc-HisB (Invitrogen) mammalian expression vector. The PCR product was purified using Qiagen PCR purification kit and was digested with *KpnI* and *EcoRI* restriction enzymes, and then was ligated into *KpnI* and *EcoRI* digested pcDNA3.1/c-myc-HisB. TOP10 One shot competent cells (Invitrogen) were transformed with the ligation mixture. Plasmid DNA from ten transformed colonies was isolated and sequenced. The sequencing analysis proved that the clones contain two different forms of MYPT1, namely long, the full length form (GenBank accession number D87930), and a splice variant (V2), GenBank accession number AY380574 (Xia et al., 2005). MYPT1 variant 1 (GenBank accession number AF458589) was not detected in HPAEC, HUVEC, and HeLa cells.

Library screening

Screening of 10⁶ clones from random hexamer and oligo dT primed HUVEC λ gt11 cDNA library, generously provided by Dr. D. Ginsburg (Univ. Michigan, Ann Arbor, Michigan), was performed according to the manufacturer's (GE Healthcare, Little Chalfont, UK) protocol, as previously described (Verin et al., 2000). Briefly, human lung MYPT1 DNA probe that is amplified by RT-PCR was randomly labeled with alkaline phosphatase, and hybridization was carried out at 55°C with the immobilized cDNA clones on the nitrocellulose membrane. Non-specifically bound DNA probes were removed from the membrane by washing with 2×SSC at 60°C. Positive chemiluminescent signals were detected by autoradiography using CDP-Star as a substrate. After three cycles of subsequent screening, positive clones were isolated and were subcloned into pcDNA 3.1/c-myc-HisB for further sequencing analysis.

Preparation of constructs

A full length sequence of a catalytic subunit of PPase 1 (CS1 β) was generated by RT-PCR from HPAEC and was subcloned into pCMV-HA or pcDNA3.1/c-myc-HisB. This plasmid was used as a template of the site-directed mutagenesis to make an enzymatically inactive mutant construct (N124A). For the construction of plasmid containing each human ERM proteins (ezrin, radixin, moesin), cDNA of each ERM proteins was amplified by RT-PCR from HPAEC and inserted into pcDNA3.1-V5 vector. Plasmid containing constitutively active (C/A) MYPT1 (amino acids 1–300) with myc tag, which was generously provided by Dr. M. Eto (Thomas Jefferson University, Philadelphia, PA), was used as a template for subcloning of C/A MYPT1 (1–300) into an adenoviral vector. Construction of adenovirus containing C/A MYPT1 was performed in the laboratory of Dr. D. Fulton (Georgia Health Sciences University, Augusta, GA) according to his published protocols (Qian et al., 2009). The truncated mutant of MYPT1 missing the first ankyrin repeat (amino acid 39–64), MYPT1 Δ Ank1, was generated from MYPT1 long in pcDNA3.1/c-myc.

Immunoprecipitation

Transfected Cos-7 cells were washed with ice-cold PBS and lysed with lysis buffer (20mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3mM EGTA, 1 mM DTT and protease inhibitor cocktail). Immunoprecipitation was performed as previously described (Csontos et al., 2008). The cells were scraped, and the lysate was centrifuged with 15,000 *g*

for 15 min at 4°C. The supernatant was incubated with appropriate volume of anti-myc antibody with fresh protein G Sepharose (GE Healthcare, Little Chalfont, UK) at 4°C overnight with gentle rotation. The beads were washed three times with IP buffer, resuspended with 1× SDS sample buffer, and then boiled for 5 min. The supernatant was used for Western blotting.

Western Blotting

Cells were washed with ice-cold PBS, lysed with lysis buffer that was used for immunoprecipitation; and then, lysate was mixed with SDS sample buffer and boiled for 5 minutes. Extracts were separated on SDS-PAGE, transferred to nitrocellulose membranes, reacted with primary antibody of interest, and then with HRP-conjugated secondary antibody. Immunoreactive proteins were visualized with Lumiglo reagents (Cell Signaling, Danvers, MA). The relative intensity of each protein band was quantified using ImageJ software (NIH, Bethesda, MD).

Immunofluorescence

Immunofluorescence microscopy was performed as previously described (Bogatcheva et al., 2007). Transfected cells grown on the coverslips were washed with ice-cold PBS, fixed in 3.7% paraformaldehyde in PBS for 10 minutes, and then washed three times with PBS. The cells were permeabilized with 0.25% Triton X-100 in TBST (0.1% Tween 20 in Tris buffered saline pH 7.4) for 10 minutes, and blocked with 5% normal goat serum in TBST for 30 min at room temperature. After overnight incubation with primary antibody, the coverslips were washed with PBS and exposed to corresponding secondary antibody conjugated with fluorescent dye. Then, the cover slips were rinsed with PBS, mounted with ProLong Gold Antifade (Molecular Probes, Eugene, OR), and observed with an x60 objective on a Nikon Eclipse TE300 microscope. Images were processed using PhotoShop Imaging software.

RESULTS

MLCP directly involves in EC barrier regulation

Our previously published data demonstrated increased PPase 1 activity, which paralleled with increased association of CS1 β (PP1 δ) with the myosin-enriched fraction of pulmonary endothelium in response to ATP treatment (Kolosova et al., 2005), suggesting the involvement of CS1 β in EC barrier enhancement induced by extracellular purines. To directly examine the involvement of CS1 β in EC barrier regulation, we specifically depleted CS1 β or CS1 α from HPAEC using siRNA approach and measured the changes in TER in response to ATP and its degradation product, adenosine, as described in “Materials and Methods”. Any change in basal resistance (inlets in Fig.1 *C*, *D*, and *F*) or cytoskeletal arrangement (data not shown) was not detected in each siRNA transfected cells. Fig. 1 demonstrated that both purines increased TER in a time-dependent manner reflecting EC barrier enhancement. Depletion of CS1 β , but not CS1 α significantly attenuated the effect of adenosine (panels *A* and *B*) and ATP (panels *C* and *D*) on the increase of TER. Consistent with these results, depletion of MYPT1 leads to significant attenuation of increase in TER induced by either ATP or its non-hydrolysable analog, ATP γ S (Fig. 1E and F). Collectively, the data strongly implicated the involvement of MLCP, which is composed of CS1 β and MYPT1, in EC barrier enhancement induced by extracellular purines. To investigate the role of MLCP in EC barrier regulation further, we infected HPAEC by adenoviral construct containing C/A MYPT1 (amino acids 1–300) missing the inhibitory phosphorylation site, Thr696 (Eto et al., 2005). We found that thrombin-induced decrease in TER was significantly attenuated by ectopic expression of C/A MYPT1 (Fig. 2A). To link the effects of C/A MYPT1 on EC TER with cytoskeletal changes, HPAEC were transiently transfected

with either empty eukaryotic expression vector (pcDNA3.1/c-myc-HisB) or plasmid, which contained C/A MYPT1. As shown in Fig. 2B, thrombin-induced stress fiber formation was significantly decreased (almost completely abolished) in C/A MYPT1 overexpressing cells compared to surrounding untransfected cells. Taken together, these results clearly demonstrate that MLCP is critically involved in EC barrier enhancement and protection.

Two isoforms of MYPT1 exist in HPAEC

The existence of two isoforms of human MYPT1 were reported in HeLa cells; the larger isoform being dominantly expressed compared to the smaller isoform, namely variant 2. In contrast to the HeLa cells, Western blot and RT-PCR results show that the expression level of the two isoforms detected are equivalent in both HPAEC and HUVEC (Fig. 3). We designed two sets of primers to detect the presence of previously reported human MYPT1 variant 1 (V1, GenBank number AF458589) or variant 2 (V2, GenBank number AY380574), because the molecular mass of these two variants are quite similar and it is difficult to separate them on SDS-PAGE, even though Western blot with whole cell extract clearly showed two bands. As shown in Fig. 3B, primer 1 (P1) and primer 2 (P2) were designed to verify the existence of MYPT1 V1. The longer band (480 bp) corresponding to MYPT1 long was detected in the PCR reaction, but the expected smaller band (around 370 bp) corresponding to MYPT1 V1 was not detected in HPAEC and HeLa. However, two bands corresponding to the MYPT1 long (450 bp) and V2 (around 280 bp) were detected in the PCR product with the set of primer 3 (P3) and primer 4 (P4). Sequence analysis of this smaller PCR product proved that this smaller band corresponds to the previously reported sequence of human MYPT1 V2. The data demonstrate that MYPT1 long and V2 are expressed in HPAEC, HUVEC and HeLa while MYPT1 V1 does not exist in these cell lines. In MYPT1 V2, 168 bp (56 amino acids) from 1657 to 1824 are deleted with no frame shift. We also used an alternative method, library screening, to confirm the existence of MYPT1 variants and test the existence of further variants that are not reported yet. 163 positive clones were selected in the screening of a random hexamer/oligo dT primed HUVEC λ gt11 cDNA library with probe encoding a human MYPT1 cDNA fragment. In the further experiments, all of these positive clones were determined as either MYPT1 long or V2.

MYPT1 is distributed in the whole cells

Each MYPT1 variant with a C-terminal c-myc tag were transfected into Cos-7 cells, and their localization were examined (Fig. 4). In agreement with earlier publications (Bannert et al., 2003; Koga and Ikebe, 2005; Koga and Ikebe, 2008), most of the overexpressed MYPT1 long-myc was located in the cytosol, and MYPT1 V2-myc transfected cells showed a similar distribution pattern in the immunofluorescent staining. In addition, several intensive fluorescent areas were detected and these areas are supposed to be part of the nucleus, as it was identified by DAPI staining. However, the truncated mutant of MYPT1 missing a part of the first ankyrin repeat (amino acid 39–64) next to the CS1 β binding motif, namely MYPT1 Δ Ank1, is concentrated in the nucleus.

MYPT1 isoforms demonstrate different binding affinity to CS1 β

The interaction of MYPT1 variants and CS1 β was confirmed by co-immunoprecipitation (Fig. 5). Cos-7 cells were co-transfected with each MYPT1 variants with myc tag and CS1 β -HA. The cell lysates were immunoprecipitated with anti-myc antibody to pull down MYPT1 variants and immunoblotted with anti-HA to visualize CS1 β -HA which is bound to the ectopically expressed MYPT1 variants. Next, we tested whether the enzyme activity of CS1 β affects the binding affinity between these two proteins. Based on a previous paper (Zhang et al., 1996), we generated catalytically inactive CS1 β mutant (N124A) with possible loss of metal binding ability, which is crucial for enzyme activation; and the same

immunoprecipitation experiment was repeated with cells cotransfected with the catalytically inactive CS1 β mutant (N124A) and MYPT1 variants. Interestingly, none of the MYPT1 variants was able to bind the catalytically inactive CS1 β mutant (N124A).

Different binding of ERM proteins with MLCP subunits

We tested the difference in the binding ability of MYPT1 variants with ERM proteins (Fig. 6). We co-transfected plasmids containing CS1 β (wild type, Wt) and one of the ERM proteins into Cos-7 cells. Our new data demonstrated that CS1 β preferentially bound to moesin compared with ezrin (Fig. 6A). The band that corresponds to ezrin detected in CS1 β immunoprecipitate is faint. However, we did not consider this band as nonspecific binding because any detectable band in the radixin transfected cells was not observed. Next, we co-transfected Cos-7 cells with both a plasmid encoding one of the MYPT1 variants and ERM proteins. Using co-immunoprecipitation, we observed strong interaction between MYPT1 long and radixin; however, much weaker interaction was detected between MYPT1 V2 and radixin.

DISCUSSION

The transport of fluid, macromolecules, and leukocytes from blood into lung interstitium is regulated on the vascular endothelium level. The shape of endothelial cells is determined by the cytoskeletal structure which is composed of F-actin, microtubules, and intermediate filaments. The change in the arrangement of these cytoskeletal components regulates the formation of paracellular gaps that increase the endothelial permeability. The phosphorylation status of MLC, one of the key determinants of cell contraction, is precisely regulated by MLCK and phosphatase, MLCP. MLCP belongs to the PPase 1 subfamily and composed of a catalytic subunit (CS1) and two regulatory subunits. Three different genes encoding CS1 were cloned and five isoforms ($\alpha 1$, $\alpha 2$, β , $\gamma 1$, $\gamma 2$) are generated from these genes by alternative splicing (Cohen, 1988; Sasaki et al., 1990); however, only CS1 β was associated with MYPT1 to form MLCP in functional complex in smooth muscle (Alessi et al., 1992; Ishihara et al., 1989; Shimizu et al., 1994). In this study, we confirmed the role of PPase 1 in pulmonary barrier regulation using *in vitro* models. In addition, our data demonstrated the crucial role of CS1 β and MYPT1 subunits of MLCP in the barrier regulation when barrier protective (ATP and its hydrolysis product, adenosine) or barrier disruptive (thrombin) agents were applied. The result showing the involvement of MYPT1 in the barrier protection confirms the previously reported result that the inhibition of adenosine induced barrier enhancement by depletion of MYPT1 (Umapathy et al., 2010).

MYPT1 is considered as a housekeeping gene because it is ubiquitously expressed in many cell types (Yamawaki et al., 2001), particularly in smooth muscle cells (Okubo et al., 1994). Several isoforms are generated by alternative splicing in chicken as well as in rat (Dirksen et al., 2000); and the existence of human MYPT1 variants have also been tested in HeLa and HEK293 cell lines (Xia et al., 2005). Although the MYPT1 variants showed tissue specific expression pattern in chicken and rat, this expression pattern has not been reported in normal human tissues. We confirmed that two MYPT1 variants, long and V2, are present in HPAEC as well as in HeLa. The central region of MYPT1 corresponding to the exon 15 (168 bp from 1657 to 1824) is absent in MYPT1 V2. The same splice variant was already identified as isoform 4 in rat aorta (Dirksen et al., 2000). In rat lung tissue, isoforms that are generated by skipping an alternative exon (isoform 4 and 5) are predominantly expressed (Dirksen et al., 2000). The quantity of MYPT1 long and V2 expressed in HPAEC is similar.

Overexpressed MYPT1 variants in Cos-7 cells have shown similar localization in the cytosol. Furthermore, several intense punctuate MYPT1-myc signals were detected in the nucleus. Published data has shown similar intensive signals localized in the nucleus in

MYPT1 overexpressing NIH3T3 cells (Wu et al., 2005). They suggested the possibility that these punctuate signals are located in the nucleoli, Cajal bodies, interchromatin granule clusters, or promyelocytic leukemia bodies (Pederson, 2002; Wu et al., 2005). In spite of these highly concentrated areas, overexpressed MYPT1 variants are shown to be dispersed to the whole nucleus. Generally, nuclear localization signal (NLS) sequences are required for the nuclear transport of large proteins (bigger than 45kD) (Yoneda, 2000). MYPT1 possesses two NLSs, one in N-terminal region (PVVQRQK, residues 27–33) and the other in C-terminal region (RRRPREKRRS, residues 843–852 in MYPT1 long). These NLSs may enable nuclear localization of MYPT1. In the holoenzyme form, CS1 β binds to the CS1 binding motif (RVxF, residues 35–38) of MYPT1 and prevents the karyopherin-mediated import of MYPT1 to the nucleus because the adjacent NLS is masked by CS1 β . Most of the endogenous MLCP exists as a stable holoenzyme, composed of two regulatory and a catalytic subunit, and its dissociation is limited (Eto et al., 2005). Thus, the overexpressed MYPT1 has difficulty binding to endogenous CS1 β and possibly being recruited to the nucleus by the exposed N-terminal NLS. This hypothesis was already tested in rat embryo fibroblasts; overexpressed myc-MYPT1 localized in the cytoplasm as well as in the nucleus, although overexpressed MYPT1 that was co-overexpressed with CS1 β was present in the cytoplasm only (Eto et al., 2005). However, the effect of overexpression of CS1 β on the relocalization of the overexpressed MYPT1 was not significant in Cos-7 cells, and each of the MYPT1 variants was still detected in the nucleus as well as in the cytosol although the highlighted clusters disappeared (data not shown). Based on this immunostaining data, we can speculate that the central deleted region does not contribute to the MYPT1 localization and the interaction between the CS1 β and MYPT1 does not prevent the nuclear localization of MYPT1 in Cos-7 cells.

To identify the specific role of the central region deleted in MYPT1 V2, we examined the binding affinity with other proteins that are already reported to bind MYPT1 long. In spite of the fact that the binding site for CS1 β is located at N-terminal region, MYPT1 V2 showed decreased binding affinity toward CS1 β Wt compared with MYPT1 long. This data suggests the possibility that a conformational change in MYPT1 V2 reduces the binding affinity to CS1 β . To test the effect of CS1 β activity on the binding affinity between CS1 β and MYPT1, we generated a catalytically inactive mutant by point mutation of the metal binding site (N124A) to interrupt the association of metal ions crucial for CS1 β activation (Chu et al., 1996; Zhang et al., 1996). It has also been shown that the mutated site (Asn124) exists separately from the residues required for the association with MYPT1; Ile169, Leu243, Phe257, Leu289, Cys294, and Phe293 (Hartshorne et al., 2004; Terrak et al., 2004). However, the binding between CS1 β mutant (N124A) and each MYPT1 variant was completely diminished. This result suggests the importance of PPase 1 activity for MYPT1/CS1 β binding.

The other important role of MYPT1, as a multiple interactive platform for proteins, is accomplished by association of other proteins, such as RhoA-GTP, PRKG1, and moesin, etc. (Amano et al., 2000; Matsumura and Hartshorne, 2008; Nakai et al., 1997; Surks et al., 1999). Recently, we reported that another member of the MYPT family, TIMAP (TGF- β -inhibited membrane-associated protein) and CS1 β bind to moesin, one of the ERM proteins (ezrin-radixin-moesin family) in HPAEC (Csontos et al., 2008), but Fukata et. al reported that rat MBS (myosin binding subunit) also associates with mouse moesin in MDCK cells (Fukata et al., 1998). Moesin belongs to the ERM (ezrin, radixin, and moesin) family and serves as a bridge between actin filaments and integral proteins on the plasma membrane (Amano et al., 2000). Therefore, we generated several plasmids containing the human MYPT1 variants and human ERM proteins to test the interaction between these two proteins. As expected, each ERM protein has shown a distinct binding affinity toward the subunits of MLCP, CS1 β and MYPT1. Ezrin as well as moesin were detected in the CS1 β

immunoprecipitates; in contrast, radixin was detected in the MYPT1 immunoprecipitates. Furthermore, radixin demonstrated a stronger binding affinity to MYPT1 long compared with MYPT1 V2. This data suggest the possibility of a distinct role for each ERM protein and MYPT1 in cytoskeleton rearrangement despite their similar amino acid sequence.

In summary, our results demonstrate the role of CS1 β and MYPT1 in maintaining the integrity of endothelial barrier regulation and the existence of two MYPT1 variants in HPAEC. The similar characteristics in their distribution and binding pattern to CS1 β suggest that the deleted region in MYPT1 V2 is not required for localization of MYPT1 and binding to CS1 β . Nonetheless, different binding affinities detected by the co-immunoprecipitation with radixin suggest a distinct role for MYPT1 variants in controlling cytoskeleton structure.

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Fig. 1.

Role of the each subunit of protein phosphatase 1 (PPase 1) in the endothelial permeability. HPAEC plated on gold microelectrodes were transfected with small interfering RNA (siRNA) specific to the catalytic subunit of PPase 1 (CS1β in *A, C* or CS1α in *B, D*) or to the regulatory subunit, MYPT1 (*E, F*) as described in Experimental procedure. 48 hours later, changes of the transendothelial resistance (TER) induced by ATP, adenosine, or ATPγS (50 μM each) were measured. Arrow in the graph indicates the point that agonist is added to the medium. Nonspecific siRNA (nsRNA) was used as a control, and the data are presented as means ± SE from three independent experiments. The insets in *C, D*, and *F* demonstrate the effect of siRNA transfection on basal resistance of HPAEC. The resistance of monolayers from eight wells transfected with each specific siRNA was normalized to the resistance of nsRNA transfected well and presented as means ± SE. Western blots on the right top corner of *A* and *E* show the expression of CS1α, CS1β, or MYPT1; GAPDH was used as a loading control.

Fig. 2. Effect of MYPT1 on thrombin-induced change of endothelial cell permeability. (A) Adenovirus containing constitutively active (C/A) MYPT1 fragment was infected to HPAEC for 24 hours, and then challenged with thrombin (20 nM) at indicated time (arrow in the graph). The change of TER was measured as described in Fig. 1. Adenovirus containing LacZ was used as a control. Data is presented as means $\pm$ SE from 3 independent experiments. Western blots show the expression amount of MYPT1 or C/A MYPT1; GAPDH was used as a loading control. (B) HPAEC monolayers were transfected with plasmid containing C/A MYPT1 with myc tag. After 36 hours, cells were incubated in serum free medium for 1 hr, followed by challenge with 50 nM thrombin for 5 min. After the indicated time of treatment, the cells were fixed and stained for F-actin (A and C) and myc (B and D). Arrows indicate the transfected cells.

Fig. 3.

Expression of MYPT1 and its variant in HPAEC. (A) Expression pattern of MYPT1 and its variant in HPAEC, HUVEC, and HeLa cells are shown at the protein level. (B) *Upper panel*, Schematic diagram of reported human MYPT1 variants. Specific primers were designed to test the existence of MYPT1 variants in mRNA extracted from HPAEC, HUVEC and HeLa. PCR with the primer mixture of P1 and P2 identify the MYPT1 V1 (GenBank number AF458589) and P3 and P4 identify the MYPT1 V2 (GenBank number AY380574). *Lower Panel* shows the PCR result obtained with the PCR primers described at the upper panel. P1 and P2 generated only one band that corresponds to the MYPT1 long. On the other hand, two bands corresponding to the MYPT1 long (450 bp) and MYPT1 V2 (280 bp) were detected in the PCR reaction with P3 and P4.

Fig. 4.

Localization of overexpressed MYPT1 variants in Cos-7 cells. Cos-7 cells were transiently transfected for 27 hr with plasmid encoding MYPT1 long, MYPT1 V2, or the truncated mutant (Δ Ank1) with myc tag at the C-terminus. Cells were fixed and stained with anti-myc antibody as described in Experimental procedures. Anti-myc was stained with Alexa 488-conjugated secondary antibody (A, C, and E), and the nucleus was stained with DAPI (B, D, and F).

Fig. 5.

Protein-protein interactions between MYPT1 variants and CS1β in Cos-7 cells. Cos-7 cells were transiently transfected with two plasmids containing each MYPT1 variant with myc tag and either CS1β (Wt) or catalytically inactive CS1β mutant (N124A). Each CS1β has HA tag. After 27 hr, cell lysates were immunoprecipitated with anti-myc antibody (IP), separated on SDS-PAGE gel, and the membrane was probed with anti-HA antibody to detect the interaction with CS1β (IB). The quantification shows the normalized binding affinity of each CS1β compared to the immunoprecipitated MYPT1 long-myc. Values are presented as an average ratio $\pm$ SE from three independent experiments (* $p < 0.05$ vs. Wt). An aliquot of the cell lysate was analyzed in the same experimental condition to compare the expression level of each protein. Same experiments were repeated more than three times.

Fig. 6.

Protein-protein interaction between MLCP and ERM proteins. (A) Specific interaction of CS1 β with ezrin and moesin. Cos-7 cells were transiently transfected with CS1 β -myc (Wt) and one of the ERM proteins (ezrin, radixin, moesin) with V5 tag. After 27 hr, cell lysates were immunoprecipitated with anti-myc antibody (IP), separated by SDS-PAGE electroblotted, and the membrane was probed with anti-V5 antibody to detect the interaction with ERM proteins (IB). An aliquot of the cell lysate was analyzed with the same experimental conditions to compare the expression level of each protein. (B) Specific interaction of MYPT1 variants with radixin. Cos-7 cells were transiently transfected with each MYPT1 variant with myc tag and one of the ERM proteins (ezrin, radixin, moesin) with V5 tag. After 27 hr, cell lysates were immunoprecipitated with anti-myc antibody (IP), separated by SDS-PAGE electroblotted, and the membrane was probed with anti-V5 antibody to detect the interaction with ERM proteins (IB). An aliquot of the cell lysate was analyzed with the same experimental condition to compare the expression level of each protein.