Regulation of vascular endothelial cell barrier function and cytoskeleton structure by protein phosphatases of the PPP family

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1Department of Medicine, Division of Biological Sciences, The University of Chicago, Chicago, Illinois; 2Department of Medical Chemistry, Research Center for Molecular Medicine, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary; 3Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, Case Western Reserve University, Cleveland, Ohio; and 4Medical College of Georgia, Vascular Biology Center, Augusta, Georgia

Csortos C, Kolosova I, Verin AD. Regulation of vascular endothelial cell barrier function and cytoskeleton structure by protein phosphatases of the PPP family. Am J Physiol Lung Cell Mol Physiol 293: L843–L854, 2007. First published August 10, 2007; doi:10.1152/ajplung.00120.2007.—Reversible phosphorylation of cytoskeletal and cytoskeleton-associated proteins is a significant element of endothelial barrier function regulation. Therefore, understanding the mechanisms of phosphorylation/dephosphorylation of endothelial cell cytoskeletal proteins is vital to the treatment of severe lung disorders such as high permeability pulmonary edema. In vivo, there is a controlled balance between the activities of protein kinases and phosphatases. Due to various external or internal signals, this balance may be shifted. The actual balances at a given time alter the phosphorylation level of certain proteins with appropriate physiological consequences. The latest information about the structure and regulation of different types of Ser/Thr protein phosphatases participating in the regulation of endothelial cytoskeletal organization and barrier function will be reviewed here.

endothelial barrier function; Ser/Thr protein phosphatases

THE VASCULAR ENDOTHELIAL CELL (EC) monolayer serves as a dynamic, semiselective barrier that regulates transport of fluid and macromolecules between blood and the interstitium. A variety of physical, inflammatory, and bioactive stimuli alter the endothelial barrier, leading to formation of paracellular gaps thereby increasing vessel permeability and compromising organ function (37, 106). Appropriate functioning of the EC barrier, therefore, necessitates proper shape/cytoskeletal structure of individual cells and integrity of the EC monolayer. Integrity, however, requires uncompromised intercellular junctions and cell-matrix contacts. Vascular EC have gap, adherens, and tight junctions; the latter two play a role in paracellular permeability. These are communicating structures and contain proteins for several signaling pathways (8, 31, 120). The integral, transmembrane proteins (VE-cadherin, occludin, claudins, PECAM, etc.) of junctions, at the cytoplasmic side, are connected to cytoskeletal filaments (microfilaments, and intermediate filaments) through linker intracellular proteins (catenins, α-actinin, zonula occludens proteins, etc). The dynamic structures of the three major components of the EC cytoskeleton, F-actin, microtubules (MT), and intermediate filaments, are of critical importance in the balance of the competing contractile and tethering forces determining the actual shape of the cell. Multiple signal transduction pathways, regulating EC contraction and barrier function, involve the activity of several protein kinases [myosin light chain kinase (MLCK), MAP kinases, protein kinase C, etc.] on junctional and cytoskeletal/cytoskeleton-associated proteins (37, 106, 173). The activity of protein phosphatases is required to keep balance with, or to reverse the effect of, protein kinases either in resting cells or after stimuli. The activity of both the kinases and the phosphatases is strictly controlled, many of them by phosphorylation/dephosphorylation. Therefore, to better understand the regulation of EC barrier function, the molecular and functional analyses of protein kinases and phosphatases in EC are equally important. Numerous recent findings pertaining to EC support their complexity both in structure and regulation. Here we provide an overview on the present information available about Ser/Thr-specific protein phosphatases involved in EC barrier regulation.

CLASSIFICATION AND GENERAL PROPERTIES OF SER/THR PROTEIN PHOSPHATASES

Protein phosphorylation/dephosphorylation is a well-recognized element in almost all signaling pathways of the cell. The presence or absence of a phosphate group on the Ser, Thr, or Tyr amino acid side chain of a protein affects its conformation and may serve as an on and off signal in the regulation of its physiological activity. As a primary effect, enzymes, for example, may become active or inactive; protein complexes may form or loosen, etc. The covalent attachment of phosphate groups is catalyzed by protein kinases, whereas protein phosphatases exhibit the opposite activity.

Protein phosphatases are sorted into three families according to their capability to dephosphorylate amino acid residues. Ser/Thr-specific protein phosphatases dephosphorylate phosphoserine/ phosphothreonine residues. Tyr-specific protein phosphatases dephosphorylate phosphotyrosine residues alone. Dual specificity
protein phosphatases dephosphorylate both phosphoserine/phosphothreonine and phosphotyrosine residues. The initial classification as type 1 (protein phosphatase 1, PP1) or type 2 (protein phosphatase 2, PP2) of Ser/Thr-specific protein phosphatases was based on their biochemical properties (72), such as their substrate specificity and their sensitivity toward heat-stable inhibitor proteins. Type 2 phosphatases were further assorted according to their metal ion dependency as PP2A, PP2B, and PP2C. Cloning of Ser/Thr-specific protein phosphatases revealed that they are encoded by two different gene families, termed PPP and PPM (6, 7, 27). PPP and PPM comprise both classic and more recently identified, but less characterized, members from different species (4, 7, 27). Tyrosine-specific and dual-specificity protein phosphatases belong to a distinct family gene, called PTP.

PP1, PP2A, as well as PP2B, belong to the PPP family (Table 1), whereas PP2C belongs to the PPM family. These four enzymes account for the majority of the Ser/Thr phosphatase activity in vivo. The catalytic subunits of mammalian PP1, PP2A, and PP2B share a highly conserved homologous catalytic domain (40–60% identity). Interestingly, although PP2C, a monomer enzyme with two domains, does not have resemblance in sequence, its three-dimensional structure is remarkably similar to that of the PPP family members (30).

The high versatility of PPP activities is due to multisubunit structure of its members (Table 1). One or two subunits from a diverse array of phosphatase regulatory or targeting (R) proteins associate with the catalytic subunit and create various holoenzyme forms. The R subunits, i.e., the holoenzyme composition, have high impact on the substrate specificity, localization, and regulation of Ser/Thr-specific phosphatases. Posttranslational modifications (phosphorylation, methylation) of the catalytic and regulatory subunits are also possible, although their exact role in the regulation is not yet completely understood (26, 35, 170).

PP1 holoenzymes are mostly dimers of one of the four isoforms, α-, β-, γ1-, or γ2-, of the catalytic subunit (PP1c, ~330 aa) (29, 118), and an R subunit (Table 1). The primary structure of the more than 50 potential R subunits share no apparent similarities, except multiple, short interaction sites with a conserved PP1c binding motif, (R/K)VxF (7, 28). It seems that the isoforms of PP1c may associate with discrete pools of R and possess diverse cellular functions. Different R subunits may direct the PP1 holoenzyme to distinct subcellular locations and enhance or suppress its activity toward different substrates (7, 28).

PP2A also has a multimeric structure with a common core of a dimer of the catalytic (PP2Ac, 36 kDa, 2 isoforms) (135) and the structural A (PP2Aa, 65 kDa, 2 isoforms) (62) subunit (Table 1). The latter one serves as a coordinator (57) to assemble PP2Ac with one of the variable third subunits, usually called the B subunit. The tertiary structure of PP2Aa and its regions binding the PP2Ac and B subunits are well characterized. As PP2Ac is thought to be constitutively associated with the PP2Aa, it implies that this association probably does not represent a direct regulatory mechanism. Rather, the activity of the heterotrimeric PP2A forms is controlled by means of the B subunit termed B', B", and B". At least three unrelated gene families encode the many isoforms and splice variants of B subunits (79) (Table 1). The core dimer may also associate with viral proteins (103, 132), and, in addition, with a phosphotyrosyl phosphatase activator (PTPA) protein that reversibly stimulates its variable phosphotyrosyl phosphatase activity (25). The specific role and regulation of the individual holoenzyme forms of PP2A are not yet clarified, although PP2A regulates many cellular processes, and many of its in vitro substrates were described as reviewed in Ref. 79.

PP2B, also called calcineurin, is a Ca2+-dependent enzyme and it is the least varied in structure among the PPP family of phosphatases. The enzyme consists of two tightly connected subunits. Calcineurin A (58–64 kDa) is the catalytic subunit, and calcineurin B (19 kDa) is a Ca2+-binding regulatory subunit (87, 88) (Table 1). The latter subunit is highly conserved and encoded by a single gene, whereas the three isoforms of calcineurin A are products of three different genes (157). The NH2-terminal region of the A subunit is the cata-

Table 1. Subunit composition, isoforms, and nomenclature of common serine/threonine protein phosphatase (PPP) enzymes

<table>
<thead>
<tr>
<th>Abbreviation Used in the Present Paper</th>
<th>Alternative Name/Abbreviation</th>
<th>Isoforms</th>
<th>Gene Symbol (HUGO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1</td>
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<td>α, β, γ, 2</td>
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</tr>
<tr>
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<td>PP1c</td>
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<tr>
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</tr>
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</tr>
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<td>PPP2R5A-E</td>
</tr>
<tr>
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<td>CaP4</td>
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Abbreviation Used in the Present Paper: PP1, PP1c, R, MYPT, MBS, MYPT1, MYPT2, PR65, PR52, PR56, PR72, PR130, PR53, CaP, CaP2, CaP3, CaP4.

Alternative Name/Abbreviation: catalytic subunit (PP1), catalytic subunit (PP1c), R subunit (R), myosin phosphatase target subunit (MYPT), myosin phosphatase subunit 85 (MBS85), myosin phosphatase target subunit 3 (MYPT3), TGF-β-inhibited membrane-associated protein phosphatase 2A (TMAP), regulatory subunit (R), catalytic subunit (PP2Ac), regulatory, A subunit (PP2Aa), regulatory, B subunit (B subunit), regulatory, B’ subunit (B’ subunit), and regulatory, B” subunit (B” subunit).
lytic domain, whereas its COOH-terminal region contains binding sites for the B subunit, calmodulin, and an autoinhibitory domain as well. In the absence of Ca\(^{2+}\)/calmodulin, the autoinhibitory domain inhibits the enzyme by binding to the active site cleft. Ca\(^{2+}\) stimulation of PP2B requires association of Ca\(^{2+}\) to both the B subunit and to calmodulin. Calcineurin is involved in the regulation of several cellular events in mammals, such as apoptosis, gene regulation, T cell activation, etc. (116).

The study of individual PPP type activities is not easy, because the in vivo and in vitro substrate specificities of protein phosphatases are not the same; usually the latter one is broader. Therefore, several specific inhibitory toxins are utilized in in vitro assays to differentiate and identify the family members. For example, okadaic acid and calyculin A are both potent inhibitors of PP1 and PP2A at nanomolar concentrations. PP2B is much less sensitive, and PP2C is not affected. Okadaic acid inhibition of PP2A is stronger than that of PP1, K\(_i\) = 0.2 nM and K\(_i\) = 3 nM, respectively (128). Many of the naturally occurring toxins are cell permeable and are utilized in in vivo studies.

Western blot analysis and cloning work indicates that EC expresses all four major types of Ser/Thr phosphatases; moreover, a growing body of evidence points to their involvement in the regulation of endothelial barrier function (34, 100, 121, 139, 151, 152, 155).

**PP1: MYOSIN PHOSPHATASE AND MORE?**

Myosin phosphorylation is critical in the regulation of endothelial barrier function. Phosphorylation of the myosin regulatory light chain (MLC) at Ser19 and Thr18 by Ca\(^{2+}\)/calmodulin-dependent MLCK results in actin-myosin interaction, stress fiber formation, and cell contraction in nonmuscle and smooth muscle (SM) cells. ATP, Ca\(^{2+}\), calmodulin, and MLCK were shown as required elements for EC retraction (9, 167, 168), indicating that the phosphorylation level of MLC has a central, although not exclusive, role in the control of EC contraction-relaxation and intercellular gap formation. Indeed, it was shown that inflammatory agonists, thrombin and histamine, produce a rapid increase in MLC phosphorylation and actomyosin interaction and increased EC permeability (51, 53, 126, 147). Transforming growth factor (TGF)-\(\beta\) also induces MLC phosphorylation and increases EC permeability; however, the effects are deferred by 1–2 h, suggesting a less direct effect of the agonist, supposedly involving changes in gene expression (55). The critical roles of EC MLCK activity and phosphorylation level of MLC are recognized in the endothelial barrier function based on the above-mentioned and further experimental data. MLC phosphorylation and permeability increases were detected after direct introduction of activated MLCK (143). MLCK inhibitors significantly attenuate the effects of thrombin and histamine on EC permeability and actin stress-fiber formation (51, 69, 147). EC MLCK is expressed as a high-molecular-mass protein (214 kDa) from a single gene on chromosome 3 in humans; the same gene also encodes the smaller SM MLCK. Detailed characterization of EC MLCK revealed both its similarities and unique properties compared with its counterpart in SM (10, 52, 93, 153, 154).

**PP1 activity in EC.** Studies with the combined usage of semiselective PPP inhibitors, such as okadaic acid and calyculin A, and thrombin treatment followed by cell fractionation indicated that a substantial portion of PP1 activity is associated with the myosin filaments and suggested the predominant role of PP1 activity in MLC dephosphorylation. Calyculin A (0.1–10 nM), but not okadaic acid (1–100 nM), produced significant dose-dependent enhancement of both MLC phosphorylation and EC permeability in bovine pulmonary artery EC (BPAEC); moreover, the majority of the phosphatase activity in the myosin-enriched fraction of BPAEC was attributed to PP1 (155). Thrombin treatment evoked barrier disruption and partial translocation of the PP1 catalytic subunit from the myosin-enriched fraction and inhibited myosin phosphorylation activity (125, 151, 155). The mechanism responsible for thrombin-induced PP1 translocation is unclear; one possible explanation is the dissociation of the catalytic subunit from its targeting subunit. This mechanism was also proposed for the regulation of glycogen-associated PP1 (127). Further results also point out the significance of the interaction between PP1c and its regulatory subunits in the determination of subcellular localization (40, 166). Experiments with rat pulmonary microvascular EC led to a similar conclusion about the pivotal role of PP1 activity in barrier function. A substantial increase was detected in the MLC phosphorylation level after PPP inhibition; however, the authors imply that the inhibition of PPP activities results in loss of barrier function by a mechanism independent from MLC phosphorylation (34). These results suggest a critical, but not exclusive, role for MLC phosphorylation and indicate a complex task for PP1 activity in EC. Further studies with PPP inhibitors suggest a possible wider substrate spectrum of PP1 among cytoskeleton-associated/regulatory proteins, but the particular regulatory subunits, i.e., the actual holoenzyme forms, are not clear (83, 146).

**The catalytic subunit.** All four isoforms of the PP1 catalytic subunit are present in human and bovine endothelium, as shown by Northern blot analysis; however, only the \(\beta\)-isofrom is stably associated with the actomyosin complex (152). This suggests that, like in smooth muscle cells, PP1c\(\beta\) is responsible for the dephosphorylation of MLC.

The holoenzyme form of myosin phosphatase. SM PPP/holoenzyme, termed myosin phosphatase (MP), is composed of PP1c\(\beta\) and two regulatory subunits, namely, a larger targeting/regulatory subunit (MP target subunit 1, MYPT1; ~110 kDa) and a smaller regulatory subunit (M20; 20 kDa) (2, 75). The activity of the holoenzyme is increased by a factor of 10 toward phosphorylated myosin compared with the PP1c monomer (80). Human MYPT1 and its spliced variants are encoded by one single gene on human chromosome 12q15–q21.2 (136). The domain structure, phosphorylation sites, and regions involved in various interactions of SM MYPT1 are well explored and understood. The most characteristic features of MYPT1 structure are the short PP1c binding motif (KVKF), which is close to the NH\(_2\) terminus and is followed by the seven ankyrin repeats and a regulatory/inhibitory phosphorylation site, Thr966 (75) (Fig. 1). While MYPT1 is expressed in SM and in most of the nonmuscle cells, its isoform, MYPT2, is expressed predominantly in skeletal and cardiac muscles and was also found in the brain (45). The specific role(s) of the small MP subunit (M20), however, has not yet been established.

Western immunoblot and immunoprecipitation experiments with cell extracts and fractions from porcine and bovine EC verified that the endothelial counterpart of SM MYPT1 has a similar size, ~115–130 kDa (65, 156, 158). However, an
additional 70-kDa immunoreactive protein band was also detected in BPAEC with antibodies specific for the entire SM MYPT1 protein or its NH2- or COOH-terminal fragments, suggesting the presence of further MYPT1-related proteins in EC (156). Screening the porcine aortic EC cDNA library yielded two partial NH2-terminal fragments closely related to SM MYPT1 (65). Our cloning work also revealed two MYPT1 variants in BPAEC (Kolosova, Verin, unpublished observations) and the same two, and two more potential, variants in human pulmonary artery EC (HPAEC) (Csortos, Verin, unpublished observations). The two variants differ by the presence or absence of a central 168-bp/56-aa insert and correspond to MYPT1 splice variants 1 and 4, which were identified in rat aorta earlier (33), and to human MYPT1 and variant 2, described by Xia et al. (169) (Fig. 1). Although this region is not yet recognized as having any special feature, still, it is questionable whether the two alternate forms of MYPT1 have different localizations and physiological functions in SM and EC.

Other MP substrates. It is highly possible that MLC is not the only in vivo substrate for MP; for example, association of MYPT1 and moesin was shown in Madin-Darby canine kidney cells, and the same work suggests that MP and Rho-activated kinase (ROCK) regulate the phosphorylation state of moesin (47). Moesin is a member of the so-called ERM family of three closely related proteins: ezrin, radixin, and moesin. Their NH2-terminal regions contain binding sites for membrane adhesion molecules, whereas their COOH-terminal part may bind actin; thus they mediate binding of actin filaments with membrane proteins (144). Their conformations may change upon phosphorylation (by ROCK or PKC) of a conserved Thr side chain close to their COOH terminus (101, 104). The conformation of nonphosphorylated ERM is folded; the binding sites are masked; they become unmasked after phosphorylation. Another MYPT-associated protein was identified as adducin (84). Adducin is a ubiquitously expressed membrane-skeletal protein, which in unphosphorylated (active) form directly binds actin and bundles actin filaments. As a consequence, adducin can modulate the lattice structure of the cytoskeleton and the exposure of transmembrane proteins. Similar to ezrin, the phosphorylation state of adducin is regulated by activity balance between ROCK and MP (84). The discovery that ERM and adducin can bind MYPT1 expanded our understanding of the MP function from strictly myosin-targeting enzyme to a broader involvement of MP in control of actomyosin cytoskeleton.

Further MP targeting/regulatory proteins. Several MYPT1/2-related proteins, MYPT3, TIMAP, and MBS85, were identified and partially characterized recently (Fig. 1). MYPT3 is a novel PP1c-interacting protein cloned from 3T3-L1 adipocyte cDNA library (130). It shares some structural features with MYPT1/2, namely NH2-terminal ankyrin repeats and a preceding PP1c binding motif; on the other hand, its size is considerably smaller, 58 kDa, and it has a COOH-terminal prenylation motif suggesting possible membrane association. It was shown that MYPT3 binds PP1c and inhibits PP1c activity toward phospho-MLC in vitro (130). The regulatory phosphorylation site identified in MYPT1 is not present in MYPT3 (Fig. 1). However, a more recent paper reports that MYPT3 can be a substrate for protein kinase A (PKA) and that the phosphorylation of MYPT3 resulted in PP1c activation toward phospho-MLC (172). This is in agreement with previous data showing that PKA attenuates agonist-induced EC barrier dysfunction (15, 99, 111). Furthermore, it was also found that activation of PKA may improve vascular endothelial dysfunction (124). Still, the exact physiological role and regulation of MYPT3 is not clear.

Another potential regulator of PP1c is TIMAP (for TGF-β1-inhibited membrane-associated protein), a 64-kDa protein expressed at high levels in endothelial cells. It is highly homologous to MYPT3 and shares its structural features, i.e., PP1c binding motif, ankyrin repeats, and prenylation motif (Fig. 1).
(21). Yeast and bacterial two-hybrid screening revealed several potential protein partners for TIMAP (1, 83). For instance, TIMAP interacts with the 37/67-kDa laminin receptor (LAMR1). It was suggested that TIMAP targets PP1c to LAMR1, and LAMR1 is a TIMAP-dependent PP1c substrate (83). Although protein-protein interaction between TIMAP and PP1c was shown by immunoprecipitation, its role in regulating PP1c activity is not yet clarified. TIMAP mRNA synthesis is strongly downregulated by TGF-β1 (21); it is possible to assume that TIMAP may be an important component of endothelial response to TGF-β1, including apoptosis, capillary morphogenesis, and barrier dysfunction.

A third putative regulatory subunit, called myosin binding subunit 85, MBS85 (or p85), was cloned from human genomic library (138). Similar to other members of MYPT family, it contains PP1c binding motif and ankyrin repeats, and it also contains an inhibitory phosphorylation site equivalent to Thr696 in human MYPT1 (Fig. 1). Using Northern blot and RT-PCR methods, we found that mRNA homologous to MBS85 is present both in human and bovine pulmonary arterial EC, although it apparently differs from MBS85 at the 5'-region. We employed different RACE-PCR approaches using HPAEC and BPAEC mRNA, and human heart mRNA where higher expression level was detected (138), but failed to identify the very 5'-piece of the coding region (Tar K, Kolosova I, Verin AD, unpublished observations). Our previous Western analysis using anti-MYPT1 antibodies, however, showed the presence of a ~70-kDa protein in EC, which might be the endothelial homolog of MBS85 (156).

Regulation of MP via Rho pathway. Studies with SM provided evidence that the level of PLC phosphorylation can be modulated by changes in Ca^{2+} concentration, as MLCK requires Ca^{2+}/calmodulin for its activity, or without a change in the Ca^{2+} concentration, a process called Ca^{2+} sensitization. The latter process includes the inhibition of MP activity, i.e., the balance between MLCK and MP is shifted toward phosphorylation (131). Although the exact mechanism for MP inhibition has not yet been completely clarified, several mechanisms were suggested, including phosphorylation of MYPT1, dissociation of the MP holoenzyme, change in localization, and the effect of inhibitory proteins (61, 75). Phosphorylation of Thr696, the inhibitory site of human MYPT1, can be catalyzed by several kinases including ROCK (43, 81, 85). ROCK, therefore, may increase MLC phosphorylation by two mechanisms: directly via phosphorylation of MLC at Ser19 and Thr18 and indirectly via phosphorylation of MYPT1 at Thr696 and Thr853, which leads to MP inactivation and dissociation from myosin (46).

Substantial work demonstrates the involvement of the vasoactive agent-induced Rho pathway in increased endothelial permeability (163). For instance, inactivation of RhoA in cultured endothelial monolayers with C3 toxin caused a decline in PLC phosphorylation and attenuated the hyperpermeability evoked by either thrombin, TNF-α, or dipheroxovanadate (23, 39, 54, 162). The permeability enhancing activity of RhoA appears to be mainly mediated through ROCK, which phosphorylates and inactivates MP in EC (22, 39, 148, 149). Our recent works have provided further evidence that thrombin-induced EC barrier dysfunction in BPAEC and HPAEC involves membrane translocation and direct activation of small GTPase Rho and ROCK. Translocation of the upstream Rho activator, guanosine nucleotide exchange factor, p115-Rho-GEF, to the membrane was also detected, demonstrating its involvement in Rho regulation in EC. Furthermore, ROCK induced phosphorylation of MYPT1 at the inhibitory Thr696 site and at Thr853, which resulted in MP inactivation, accumulation of diphospho-MLC, and cell contraction (11, 16) (Fig. 2). An interesting and novel observation of our laboratory demonstrates that MT disruption correlates with increased lung permeability as well as with decreased MP activity and increased PLC phosphorylation, suggesting the possibility for cross talk between specific signaling pathways of the MT and microfilament network (17, 150) (Fig. 2). The critical role of MT dynamics in thrombin- or TGF-β1-induced lung vascular EC barrier dysfunction and the G protein-dependent mechanisms of MT alterations were also shown (12, 14).
MP activity can also be regulated through a PKC potentiated inhibitory protein of 17 kDa, called CPI-17 (41). It may affect isolated PP1c or the holoenzyme form of MP without dissociating its subunits (122). Recent phospho-pivot modeling predicts specific interactions of PP1c with CPI-17 (105). The MP inhibitory effect of CPI-17 is dramatically increased by its phosphorylation at Thr38. Even though in vitro, numerous kinases are capable of phosphorylating CPI-17, including ROCK, MYPT1-kinase, etc. (75). PKC is thought to be primarily responsible for the in vivo phosphorylation of CPI-17 (41). PKC activation in vascular smooth muscle by phorbol ester was shown to induce MP inhibition, which led to an increase both in MLC phosphorylation and in contraction (102). In addition, contraction of permeabilized SM was induced by phosho-CPI-17 (86, 94). We studied endogenous and overexpressed CPI-17 in microvascular and macrovascular EC, respectively. Both the inflammatory agonist histamine, and, to a much smaller extent, thrombin, were able to evoke CPI-17 phosphorylation (Fig. 2). Utilizing various approaches, we found that the contribution of PKC is more significant than that of ROCK in agonist-induced CPI-17 phosphorylation (90). These data suggest that MP inhibition/MLC phosphorylation can be regulated via Rho-ROCK or/and PKC-CPI-17 pathways depending on the agonist.

The observation that MP activity is inhibited during agonist-induced EC barrier dysfunction suggests a barrier-maintaining/protective role for MP. Increased release of ATP from EC during acute inflammation (19) and ATP-induced EC barrier enhancement (59, 78) were reported. We found, in agreement with the assumed barrier-protective role of MP, that the mechanism of ATP-induced barrier enhancement involves MP activity (91).

**PP2A AND CYTOSKELETON**

Much less is known about the involvement of PP2A activity in the regulation of cytoskeletal structure compared with PP1, although many data suggest its critical role. Inhibition of PP2A in the nonmetastatic Lewis lung carcinoma cells, for example, caused a rapid change in morphology and an increase in their in vitro invasiveness (77). Translocation and activation of PP2A during mast cell secretion were reported; furthermore, both secretion and cytoskeletal reorganization were affected by PP2A inhibition (66, 98). Gene disruption studies in fission yeast indicated that PP2A plays a critical role in cell morphogenesis, probably through the regulation of the cytoskeletal network and cell wall synthesis (107). There are also data demonstrating a possible role of PP2A in the regulation of cytoskeletal protein complex formation (107, 113). Cytoskeletal targets of PP2A, and its participation in cell contraction/relaxation, are not well characterized, although recent data support its involvement in the regulation of several cytoskeletal and cytoskeleton-associated protein targets. For instance, in vitro experiments suggest that PP2A is responsible for the dephosphorylation of CPI-17 in SM (137). A recent paper reports that okadaic acid-induced phosphorylation and translocation of MYPT1 is dependent on PP2A, and to varying extents, on ROCK in HepG2 cells (97). These results suggest that PP2A might be involved in the regulation of MP.

Some actin-binding proteins, like caldesmon, coflin, the small heat shock protein HSP27, and the MT-associated protein tau, are further cytoskeletal targets of PP2A (3, 20, 110, 171). A growing body of evidence indicates the association between PP2A and microtubules and shows the substantial role of PP2A in MT stability (64, 133, 134). In vitro experiments demonstrate that only PP2A was able to dephosphorylate βIII-tubulin and inhibit MT assembly (82), and PP2A was found to be the major phosphatase dephosphorylating tau in human brain extract (96). Several data suggest that only the B, not the B’ subunit containing PP2A heterotrimer form, is able to bind to MT (64, 113). Indeed, B subunit binding is critical for cell viability under conditions of MT damage in yeast (42).

The participation of PP2A activity in maintaining EC cytoskeletal organization has been suggested rarely until recently (50, 89). Colocalization of PP2A with MT was shown in unstimulated EC, but exposure to carcinoma-derived angiogenic products resulted in a more diffuse distribution of PP2A and a loss of filamentous tubulin (161). Similarly, we found a substantial amount of PP2A, along with HSP27 and tau, two phosphorylatable regulators of the cytoskeleton, in the MT-enriched fraction of pulmonary artery EC (139). Small HSPs are proposed to control actin filament dynamics and to stabilize microfilaments after dissociating from large to small aggregates and after phosphorylation. Following exposure to stress, HSP27, as a terminal substrate of p38 MAPK-pathway, becomes phosphorylated and promotes F-actin formation (58, 68, 92) (Fig. 2). However, we detected the majority of HSP27 in a MT-enriched fraction of pulmonary artery EC (139), and interaction between tubulin/MT and HSP27 in other cell types has also been reported (63), raising the question of whether HSP27 is a link between MT and microfilaments in agonist-evoked responses. On the other hand, tau is directly related to MT; its unphosphorylated form supports MT assembly and inhibits the rate of depolymerization (36). Phosphorylation of tau by several kinases decreases its ability to bind MT and to promote MT assembly (60, 95). Tau is predominantly found in neuronal cells but has been reported in several nonneuronal cells as well (73, 140, 142). Both HSP27 and tau become phosphorylated after thrombin treatment or MT disruption (13, 14). We studied both endogenous and overexpressed PP2A in HPAEC using pharmacological effectors, thrombin and nocodazole, and specific phosphatase inhibitors, calyculin A and okadaic acid (139, 140). Our data suggest that HSP27 and tau are potential substrates of PP2A activity with an impact on EC barrier function. PP2A overexpression significantly attenuated thrombin- or nocodazole-induced phosphorylation of HSP27 and tau, suggesting a barrier-protective role for this phosphatase (Fig. 2). PP2A-mediated dephosphorylation of these two cytoskeletal proteins correlates with the PP2A-induced preservation of EC cytoskeleton and barrier maintenance (140).

Most likely, tau and HSP27 are not the sole substrates of PP2A; rather, the high versatility of PP2A holoenzyme forms raises the possibility of a more complex role of PP2A in EC cytoskeleton regulation at different levels. For example, PAK1, one of the p21-activated kinases (PAKs), which are a group of recognized p38 activators (174), was found in a protein complex with PP2A isolated from rat brain (160), suggesting that PP2A may regulate the p38 MAP kinase pathway at different levels. Another interesting, but unexplored, issue pertains to intermediate filaments (IF). The organization of the IF network depends on its phosphorylation state. Selective inhibition of PP2A, but not PP1, led to hyperphosphorylation and concom-
itiant disassembly of vimentin, characterized by a collapse into bundles around the nucleus in mammalian fibroblasts (145). Impaired dephosphorylation of vimentin and increased phosphorylation of tau were described in mutant PP2A transgenic mice (119). Vimentin contains many sites that can be phosphorylated by a variety of kinases with variable impact on IF structure at different physiological conditions (38, 71, 76). It will be a difficult task to identify the physiological phosphatase enzyme(s) of these sites and to learn about their regulation.

Endothelial nitric oxide synthase (eNOS) is considered to be a major vasoprotective molecule; its function is fundamentally modulated by phosphorylation at multiple sites (49, 165). Several agonists, like vascular endothelial growth factor (VEGF), sphingosine-1-phosphate, or hydrogen peroxide, may produce Akt activation and phosphorylation of Ser1179 (bovine)/1177 (human) of eNOS, a site with a central role in eNOS regulation (32, 48, 70, 141). Dephosphorylation of serine 1179 and one more site (Thr497) by PP2A was reported, suggesting a key role for PP2A in the control of nitric oxide-dependent signaling pathways in vascular EC (56). Moreover, recent work revealed a new pathway for eNOS regulation including PP2A, namely, proteasome inhibition did not modulate eNOS phosphorylation and activity by affecting its turnover, but it caused ubiquitination and translocation of PP2A from cytosol to membrane (159).

**PP2B AND EC BARRIER FUNCTION**

PP2B is thought to be involved in the maintenance of a prolonged state of contraction of molluscan smooth muscle (24), and PP2B isolated from scallop smooth muscle dephosphorylated regulatory MLC prepared from the same source (74). In nonmuscle cells, tight association was found between the cytoskeleton and PP2B/calcineurin (44, 109). Human endothelium expresses all three isoforms of calcineurin A, the catalytic subunit of PP2B (Bako E, Verin AD, unpublished observations). We also showed PP2B associating with a detergent-insoluble actin-enriched cellular fraction of pulmonary artery EC (151). Thrombin treatment considerably increased the activity of PP2B; the activation correlated with the phosphorylation of the PP2B catalytic subunit. Furthermore, pharmacological inhibition of PP2B attenuated thrombin-induced cytoskeletal protein dephosphorylation and increased thrombin-induced albumin clearance (151). These results suggest that PP2B may also be involved in the regulation of EC barrier function. Recent results of Lum et al. (100) confirmed our observations, as their data provided further evidence that PP2B activity is a significant participant in EC barrier regulation in response to thrombin. PP2B inhibition potentiated thrombin-induced increase in PKC activity and PKCα but not PKCβ phosphorylation in pulmonary microvascular EC. PP2B inhibition also extended the thrombin-induced barrier dysfunction; however, downregulation of PKC by phorbol ester treatment rescued the inhibition of recovery, indicating that PP2B may play a physiologically important role in returning EC barrier function to normal through the regulation of PKC (100). In addition, our data demonstrated that PKA inhibition increased costaining of actin with both PP2B and MLCK, which correlated with reorganization of focal adhesions, and increased F-actin content and organization into stress fibers, suggesting a link between PP2B, MLCK, and PKA activities (111). A further question is whether there is a direct or indirect connection between PP2B and other enzymes involved in the regulation of EC cytoskeleton. This requires future studies.

**INTERCELLULAR JUNCTION PROTEINS AND PPPS**

Tight junctions (TJ) and adherens junctions (AJ) are formed by several transmembrane adhesion proteins and their intracellular partners. For a detailed review of EC cell-cell junctions, see Refs. 8 and 31. The organization of AJ and TJ depends on protein-protein interactions, which in turn are determined in many cases by the phosphorylation state of proteins. It seems that both Ser/Thr and Tyr phosphorylation, in many cases of the same protein, play a regulatory role in TJ and AJ structure and eventually influence paracellular permeability. For example, serine phosphorylation of cadherin, the major transmembrane component of AJ, leads to specific interactions with one of its intracellular partners, β-catenin; on the other hand, tyrosine phosphorylation of β-catenin reduces its affinity to bind to cadherin (67, 115). There are further recent data showing that tyrosine phosphorylation of VE-cadherin prevents binding of β-catenin and p120, another member of the catenin family, and inhibits EC barrier function (112). Furthermore, serine phosphorylation and proteosomal degradation of β-catenin are modulated by platelet EC adhesion molecule-1 (PECAM-1), another transmembrane protein in AJ (18). Similarly, tyrosine and serine/threonine phosphorylation of TJ components is a critical factor in TJ assembly and endothelial permeability. Localization of the transmembrane protein occludin changes with its phosphorylation state; its highly phosphorylated form was detected to be selectively concentrated at TJ (117). VEGF increased the phosphorylation level of occludin and its intracellular partner, zona occludens-1 (ZO-1) (5). However, the amino acid side chains involved are not yet identified.

Much less is known about the dephosphorylation of AJ and TJ proteins. The inflammatory barrier-disruptive agents histamine and VEGF both induce a decrease in Ser/Thr phosphorylation of p120 (114, 164), and that must imply protein phosphatase activity. Okadaic acid and calyculin A, inhibitors of PP1 and PP2A, induce complete disruption of cell-cell contacts and increase the Ser/Thr phosphorylation level of β-catenin in epidermal cells (123). Another work showed cellular redistribution of ZO-1 after okadaic acid treatment in epithelial cells (129). However, the applied concentrations of PPP inhibitors in these experiments are not suitable to differentiate between PP1 and PP2A activities. A specific study of PP2A in epithelial cells indicated that it negatively regulates TJ assembly (108). Expression of PP2Ac prevented TJ assembly; on the other hand, okadaic acid inhibition of PP2A supported the phosphorylation and recruitment of ZO-1, occludin, and claudin-1 to the TJ during junctional biogenesis (108). Overall, the above data clearly indicate the crucial involvement of PPP activities in AJ/TJ-mediated cellular permeability.

**CONCLUSION**

During the last decade, it has become clear that EC barrier function is finely regulated via the coordinated complex actions of protein kinases and phosphatases on cytoskeletal/cytoskeleton-associated and intercellular junction proteins. The phosphorylation level of these proteins is crucial in determining the
actual physical state of cytoskeletal elements, their interaction with regulatory, membrane-associated, or intercellular junction proteins, and furthermore, in the assembly of intercellular junctions, each having impact on EC barrier function. The role of PP1 in MLC dephosphorylation is fairly well understood (Fig. 2). However, the exact regulation of MP through CPI-17 and MYPT1 is still unclear, as the physiological function of the alternate forms of MYPT1, whether they direct MP to different substrates or to different locations, is still unknown. Beyond structural similarities to MYPT1, information about MYPT3, TIMAP, and MBS85 is also very limited. Based on a growing body of evidence, it is also clear that both PP2A (Fig. 2) and PP2B are of critical importance, but their exact role in agonist-induced EC cytoskeletal remodeling has to be elucidated. The wide substrate specificity of PP2A, due to the extremely high number of B regulatory subunits and holoenzyme forms, implies multiple functioning, most probably at different levels of EC barrier function regulation, and does not exclude the possible opposite outcome of the activity of this enzyme. The role of PPPs in EC AJ and TJ regulation is still virtually unexplored; the experimental data suggest rather complex regulation involving likely cooperation between protein phosphatases of different classes.

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44. Ferreira A, Kincaid R, Kosik KS. Calcinurin is associated with the cytoskeleton of cultured neurons and has a role in the acquisition of polarity. Mol Biol Cell 4: 1225–1238, 1993.


71. Inagaki N, Goto H, Ogawa M, Nishi Y, Ando S, Inagaki M. Spatial patterns of Ca2+-signals define intracellular distribution of a signaling by 10.220.33.5 on September 7, 2017 http://ajplung.physiology.org/ Downloaded from
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Invited Review


