Pulmonary Endothelial Cell Barrier Enhancement by Sphingosine 1-Phosphate

ROLES FOR CORTACTIN AND MYOSIN LIGHT CHAIN KINASE

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Steven M. Dudek‡§, Jeffrey R. Jacobson‡§, Eddie T. Chiang‡, Konstantin G. Birukov‡, Peiyi Wang‡, Xi Zhang‡, and Joe G. N. Garcia‡¶

From the ‡Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and the ¶Department of Experimental Pathology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855

We recently reported the critical importance of Rac GTPase-dependent cortical actin rearrangement in the augmentation of pulmonary endothelial cell (EC) barrier function by sphingosine 1-phosphate (S1P). We now describe functional roles for the actin-binding proteins cortactin and EC myosin light chain kinase (MLCK) in mediating this response. Antisense down-regulation of cortactin protein expression significantly inhibits S1P-induced barrier enhancement in cultured human pulmonary artery EC as measured by transendothelial electrical resistance (TER). Immunofluorescence studies reveal rapid, Rac-dependent translocation of cortactin to the expanded cortical actin band following S1P challenge, where colocalization with EC MLCK occurs within 5 min. Adenoviral overexpression of a Rac dominant negative mutant attenuates TER elevation by S1P. S1P also induces a rapid increase in cortactin tyrosine phosphorylation (within 30s) critical to subsequent barrier enhancement, since EC transfected with a tyrosine-deficient mutant cortactin exhibit a blunted TER response. Direct binding of EC MLCK to the cortactin Src homology 3 domain appears essential to S1P barrier regulation, since cortactin blocking peptide inhibits both S1P-induced MLC phosphorylation and peak S1P-induced TER values. These data support novel roles for the cytoskeletal proteins cortactin and EC MLCK in mediating lung vascular barrier augmentation evoked by S1P.

The pulmonary endothelium is a functionally dynamic tissue that serves as a semipermeable barrier between circulating vascular contents and the interstitium and airspaces of the lung. The regulatory mechanisms involved in maintenance of this barrier are poorly understood; however, we recently reported that sphingosine 1-phosphate (S1P), a potent phospholipid angiogenic factor released from activated platelets (1), produces significant endothelial cell (EC) barrier enhancement through Edg receptor ligation and Rac GTPase-dependent cortical actin rearrangement (2). Although the rapid, sustained, and dose-dependent increase in EC transmonolayer electrical resistance (TER) generated by S1P requires an intact actin cytoskeleton capable of undergoing dynamic rearrangement (2), the specific mediators and regulatory mechanisms that effect these actin cytoskeletal changes remain unclear.

The 80/85-kDa actin-binding protein, cortactin, has been implicated in cortical actin rearrangement (3). Ideally suited for integrating multiple signals at sites of dynamic actin rearrangement, the amino acid structure of cortactin contains an N-terminal acidic region that stimulates actin polymerization by the Arp2-Arp3 complex (murine AA 1–90), a unique tandem repeat sequence for actin binding (AA 91–326), a Pro- and Tyr-rich area containing sites for p60src phosphorylation (AA 401–495), and a C-terminal SH3 domain (AA 496–546) (3). Cortactin stimulates and stabilizes Arp2-ARP3-mediated polymerization of branched actin filaments at peripheral sites of cytoskeletal rearrangement (4, 5), but regulation of cortactin’s activity at these sites is poorly understood. Originally described as a target for p60src tyrosine kinase (6), increased cortactin tyrosine phosphorylation is observed after many stimuli associated with cytoskeletal rearrangement, including epidermal growth factor, fibroblast growth factor, thrombin, integrin activation, and shear stress (3, 7). The functional role of phosphorylated cortactin is unclear, but we recently reported an increased direct association of phosphocortactin with the barrier regulatory enzyme, EC myosin light chain kinase (MLCK), relative to nonphosphorylated cortactin (8). EC MLCK coprecipitates with both cortactin and p60src in cultured lung EC (9), and the interaction of cortactin with EC MLCK attenuates cortactin-stimulated, Arp2-ARP3-catalyzed actin polymerization in vitro (8). Thus, cortactin-MLCK interaction may modulate EC cytoskeletal rearrangement.

EC MLCK is an ATP- and Ca2+/calmodulin-dependent enzyme essential for generation of centripetal cellular tension via

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‡To whom correspondence should be addressed: Johns Hopkins Division of Pulmonary and Critical Care Medicine, 1830 Monument St., Rm. 527, Baltimore, MD 21205. Tel.: 443-287-3343; Fax: 443-287-3349; E-mail: drgarcia@jhmi.edu.

¶The abbreviations used are: S1P, sphingosine 1-phosphate; CBP, cortactin SH3 blocking peptide; DN, dominant negative; DPV, diperoxovanadate; EC, endothelial cells; GFP, green fluorescent protein; HGF, hepatocyte growth factor; IF, immunofluorescence; MLCK, myosin light chain kinase; TER, transendothelial electrical resistance; AA, amino acids; SH3, Src homology 3; Ab, antibody; HPAEC, human pulmonary artery endothelial cells; HUVEC, human umbilical vein endothelial cell(s); GST, glutathione S-transferase; HA, hemagglutinin; IP, immunoprecipitation; MLCK, myosin light chain.
its ability to enhance actomyosin motor activity (10). Nonskeletal muscle MLCK exists as high molecular mass (≈210 kDa) and low molecular mass (130–160 kDa) isoforms that are derived from a single gene (11). Human EC express only the high molecular weight form (EC MLCK) cloned by our group (12), with multiple splice variants also detected by reverse transcription-PCR (13). The two most predominantly expressed variants (EC MLCK 1 and 2) differ only by a single exon deletion (encoding AA 458–505), which results in EC MLCK 2 containing 69 fewer amino acids than MLCK 1. The deleted region contains two sites for p60src-catalyzed tyrosine phosphorylation (Tyr464 and Tyr471) that serve to produce differential regulation of EC MLCK splice variant activity (14). In multiple in vitro and in vitro models of EC permeability, increased MLCK activity produces increased MLC phosphorylation within newly formed stress fibers, intracellular tension, cell rounding, paracellular gap formation, and subsequent EC barrier disruption (15–17). However, we recently described increased MLC phosphorylation in a cortical distribution during S1P EC barrier enhancement (2), suggesting possible spatially defined MLCK activation that can differentially regulate permeability.

These observations led us to speculate integral roles for cortactin and EC MLCK in regulating the dynamic actin changes seen at the EC periphery during S1P-induced vascular barrier enhancement (2). In this study, we demonstrate that S1P induces rapid cortactin colocalization with EC MLCK at the EC periphery with functional roles for these proteins and their interaction in EC barrier enhancement. These results support important regulatory functions for cortactin and EC MLCK in dynamic actin rearrangements involved in enhancement of vascular integrity.

MATERIALS AND METHODS

Reagents and Antibodies—Unless otherwise specified, reagents (including S) were obtained from Sigma. Hepatocyte growth factor (HGF) was obtained from Research Diagnostics (Pfandlers, NJ). PP2 (5,10 used for experimentation). Dominant negative HA-tagged RacN17 and dominant negative Myc-tagged PAK1 transient transfection constructs were kindly provided by M. Nicolic (Harvard University). Adenoviral dominant negative Rac (triple HA-tagged) and control vectors were kindly provided by C. Waters (University of Tennessee, Memphis, TN).

GFP Tag/Fusion Activity Assay—Determination of Rac activation (quantitation of Rac-GTP) was performed using a commercially available kit (UBI) as previously described (2).

Transendothelial Monolayer Electrical Resistance—EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and TER measurements were then performed using an electrical cell-substrate impedance sensing system obtained from Applied Biophysics (Troy, NY) as previously described in detail (2). TER values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± S.E. (19).

Transfection and Infection of EC—EC plated on coverslips or dishes to 50–70% confluence were exposed for 4–6 h to a transfection mixture consisting of 1 µg of plasmid DNA per ml of FuGene6 per ml of Opti-MEM medium (Invitrogen). EC were then incubated in complete culture medium for an additional 48–72 h to allow protein expression. For antisense oligonucleotide studies, HPACE were incubated with 1–2 µg of oligonucleotides/ml of medium plus FuGene6 for 4–10 h before being replaced with complete medium. Cortactin protein expression was assayed by Western blotting 48–72 h after oligonucleotide treatment. For experimenting using myristoylated peptides, EC were incubated with 10 µM CBP or control peptide for 45 min prior to beginning other manipulations. HUVEC lines were infected with retroviral GFP-tagged wild-type cortactin or tyrosine-deficient mutant cortactin as previously described (20). Adenoviral infection with dominant negative Rac or control vector (multiplicity of infection = 20–40) was performed 48 h prior to the TER assay.

Endothelial Imaging—EC were grown on gelatinized coverslips before being exposed to various conditions as described for individual experiments. EC were then fixed in 3.7% formaldehyde and permeabilized with 0.25% Triton X-100 for 5 min. Cells were washed in phosphate-buffered saline, blocked with 2% bovine serum albumin in phosphate-buffered saline for 30 min, and then incubated for 60 min at room temperature with the primary Ab of interest. After washing, EC were then incubated with appropriate secondary Ab conjugated to FITC (or Texas Red-conjugated phalloidin for actin staining) for 60 min at room temperature. After further washing with phosphate-buffered saline, coverslips were mounted using Slow Fade (Molecular Probes, Inc., Eugene, OR) and analyzed using a Nikon Eclipse TE 300 microscope and Sony Digital Photo camera DRC 5000. Confocal microscopy was performed using the Radiance Laser scanning 2100 system (Bio-Rad). Images were recorded and saved in Adobe Photoshop.

GST Binding Assay—In vitro binding of recombinant MLCK proteins with GST-tagged cortactin constructs was performed exactly as previously described (6).

Immunoprecipitation and Western Blotting—Cortactin IP from confluent 60-mm HPACE dishes was performed as follows. After treatment with vehicle or S1P, EC were rinsed with phosphate-buffered saline and then lysed with 50 µl of lysis buffer (1% SDS, 1 mM NaVO4, 10 mM Tris, pH 7.4). Lysates were boiled for 5 min, passed several times through a 26-gauge needle, and centrifuged for 5 min (16,000 × g). Supernatant was collected and added to 200 µl of H2O plus 250 µl of 2× IP buffer (20 mM Tris, pH 7.4, 2% Triton X-100, 1% Nonidet P-40, 300 mM NaCl, 0.4 mM Na2, 2 mM EDTA, 2 mM EGTA, 0.4 mM NaVO4, 1,250 dilution of Calbiochem protease inhibitor mixture 3). The mixture was incubated at 4 °C for 2 h with 10 µl of monoclonal cortactin Ab 30 µl of protein G-Sepharose (Amersham Biosciences) was added and then rotated at 4 °C for 60 min. The complex was washed in 1× IP buffer prior to the addition of Laemmli sample buffer, boiling, and subsequent analysis by SDS-PAGE. After transfer to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), Western blotting was performed using α-phosphotyrosine primary Ab and horseradish peroxidase-conjugated secondary Ab prior to visualization using enhanced chemiluminescence (Amersham Biosciences).

Statistical Analysis—Student’s t test was used to compare the means of data from two or more different experimental groups. Results are expressed as means ± S.E.
Cortactin depletion significantly attenuates S1P-induced endothelial barrier enhancement. The above results suggest that phosphorylation of cortactin by p60src activation and focal contact alteration (19, 23). DPV induces substantial early translocation of cortactin to the periphery (Fig. 2E) in association with peak DPV barrier augmentation (15–30 min) (23). This localization was followed by loss of cortactin staining intensity as the DPV barrier disruptive response evolved. Conversely, the potent edemagenic agent thrombin causes immediate and profound EC barrier impairment (24), actin stress fiber formation, and paracellular gap formation but fails to alter the base-line cytoplasmic distribution of cortactin (Fig. 2D). Thus, cortactin is rapidly concentrated in a cortical distribution by barrier-enhancing agents but not by thrombin, a barrier-disrupting agonist. Together, these observations strongly suggest an important role for cortactin at the EC periphery during stimuli-induced barrier enhancement.

**Essential Role for Cortactin Tyrosine Phosphorylation in S1P-Induced EC Barrier Enhancement—Tyrosine phosphorylation of cortactin is associated with multiple stimuli that evoke cell shape and cytoskeletal changes (3). Whereas the functional role of tyrosine-phosphorylated cortactin is unclear, models of cell migration (18, 25) and H2O2-mediated morphological changes (20) demonstrate the importance of cortactin phosphorylation in regulating specific types of cytoskeletal changes. In our EC model, S1P rapidly increases tyrosine phosphorylation of cortactin (within 30 s) (Fig. 3A), with levels declining by 5 min but remaining above base line for over 30 min. S1P barrier enhancement is therefore temporally associated with both rapid tyrosine phosphorylation of cortactin and translocation of cortactin to the cell periphery. Consistent with our prior experiments (7), the specific Src inhibitor PP2 (5 μM) failed to block S1P-induced cortactin translocation (Fig. 3B), suggesting that p60src activity may not be integral to this effect. To avoid difficulties inherent in chemical inhibitor specificity, HPAEC were transiently transfected with a mutant cortactin deficient in three critical tyrosine residues (Tyr421, Tyr466, and Tyr482), which account for >90% of p60src-catalyzed cortactin phosphorylation (18). As illustrated in Fig. 3B, the tyrosine-deficient mutant cortactin strongly localizes to the periphery following S1P in a manner similar to endogenous protein.

We confirmed that cortactin phosphorylation by p60src is not required for peripheral translocation of cortactin after S1P by using HUVEC infected with a retroviral form of the tyrosine-deficient mutant cortactin. Prior characterization of these retrovirally infected HUVEC demonstrated that similar amounts of GFP-tagged cortactin wild-type and cortactin tyrosine-deficient mutant protein are expressed by the respective HUVEC, whereas endogenous cortactin protein expression is relatively unaffected (20). In the present study, S1P induced cortical translocation of endogenous cortactin as well as GFP-tagged overexpressed cortactin protein in retroviral vector controls, cortactin wild-type, and tyrosine-deficient mutant cortactin HUVEC (data not shown). However, phosphorylation at these three critical tyrosine residues (Tyr421, Tyr466, and Tyr482) appears to be necessary for peak S1P barrier enhancement (Fig. 3C). HUVEC overexpressing the tyrosine-deficient mutant cortactin exhibited a sustained decrease in the TER response compared with vector controls or wild-type cortactin-overexpressing EC (Fig. 3D). Moreover, HUVEC overexpressing wild-type cortactin showed enhanced resistance after S1P, providing further novel evidence for the essential role of cortactin in EC barrier regulation.

Rac GTPase Mediates S1P-Induced Cortactin Translocation and Barrier Enhancement—The above results suggest that phosphorylation of cortactin by p60src is not essential for EC cortactin translocation by S1P. We next investigated the possible involvement of the Rac GTPase pathway in this translo-
expression of dominant negative Rac (Fig. 4C). Furthermore, this Rac GTPase-dependent inhibition of S1P-induced cortactin translocation is Rac GTPase-dependent in HPAEC transiently transfected with dominant negative PAK1 (Fig. 4A). Although we have shown that PAK1 is a key Rac downstream effector activated after S1P (2), PAK1 activity does not appear to mediate cortactin translocation. Transient transfection with dominant negative PAK1 fails to inhibit S1P-induced cortactin translocation (Fig. 4B). Thus, S1P-induced cortactin translocation is Rac GTPase-dependent but does not require PAK1 kinase activities. Moreover, this Rac activity is necessary for peak S1P-induced EC barrier enhancement, since inhibition of this activation by adenosine overexpression of dominant negative Rac (Fig. 4D) significantly inhibits this barrier response (Fig. 4C).

S1P Stimulation Translocates EC MLCK and Cortactin to the EC Periphery—We next utilized IF techniques to characterize EC MLCK cellular localization in living cells under various barrier-regulatory conditions. HPAEC were transiently transfected with a GFP-EC MLCK 2 construct, which we previously reported increased stress fiber formation compared with control cells (28). Using live cell imaging, under basal conditions (Fig. 5A), EC MLCK 2 is predominantly distributed along the actin fiber network within the cytoplasm. However, after exposure to physiologic levels of S1P, MLCK is clearly translocated within 5–10 min to peripheral areas of membrane ruffling (Fig. 5A, arrows). Thus, overexpressed EC MLCK appears to dynamically localize to the periphery during barrier augmentation in a manner similar to cortactin (see Fig. 2).

We previously reported that cortactin, an actin-binding protein involved in dynamic cortical actin rearrangement, coprecipitates with EC MLCK in stimulated EC (9) with direct interaction subsequently demonstrated in vitro (8). To explore the interaction of cortactin and EC MLCK in situ, we transiently transfected HPAEC with GFP-EC MLCK 2 and examined protein localization with confocal microscopy. Under basal conditions, overexpressed EC MLCK 2 is again observed along cytoplasmic actin cables, whereas cortactin exhibits a more diffuse cytoplasmic distribution (Fig. 5B). Within 5 min of exposure to S1P, EC MLCK 2 is rapidly redistributed to areas of active membrane ruffling where colocalization with translocated cortactin occurs (Fig. 5B, merged image). Although these observations must be interpreted cautiously because of the artificial elevation in MLCK levels, they suggest rapid interaction of cortactin and EC MLCK in areas of dynamic cortical rearrangement during S1P barrier enhancement.

Cortactin SH3 Domain Interaction Mediates S1P Barrier Enhancement—Cortactin contains a highly conserved C-terminal SH3 region (Fig. 6A) that interacts with cytoskeleton-associated proteins such as CortBP-1, dynamin, ZO-1, and WIP (29–32). We recently reported that EC MLCK binds directly with cortactin in vitro (8) and identified via competitive peptide studies proline-rich sites within the EC MLCK sequence as sites of cortactin binding. Since these results suggest interaction with cortactin through its SH3 domain, we performed in vitro binding experiments using cortactin deletion mutants to confirm the site of binding to recombinant EC MLCK 1 and 2 splice variants. Incubation of EC MLCK 1, MLCK 2, or rabbit uterine SM MLCK with GST-tagged cortactin deletion constructs revealed that all three MLCK variants bind to the full-length cortactin protein (amino acid residues 1–546) as well as to its isolated SH3 domain (AA 496–546) (Fig. 6B), but interaction is not observed with GST-cortactin deficient in the SH3 domain (AA 1–495). Therefore, the cortactin SH3 domain is both necessary and sufficient for EC MLCK (and SM MLCK) interaction with cortactin. To address the possibility of additional cortactin binding sites within the unique N-terminal region (AA 1–922) of EC MLCK, we incubated recombinant truncated EC MLCK 1 N-terminal protein (AA 1–922) with GST-tagged cortactin (Fig. 6C). No binding with cortactin is observed, providing further evidence that cortactin-MLCK interaction occurs solely within the portion of the enzyme common to both SM and EC MLCK isoforms (AA 923–1914) that contains the proline-rich motifs we have identified as sites of interaction (8).

To explore the functional importance of this cortactin SH3 site in S1P barrier regulation, we generated a cell-permeable myristoylated peptide derived from CortBP-1 that mimics the optimal binding sequence for the cortactin SH3 domain (33) in order to competitively block interactions at this site. We recently demonstrated that this cortactin SH3 blocking peptide (CBP) abolishes cortactin interaction with EC MLCK in vitro (8). Preincubation of cultured EC for 45 min with 10 μM CBP does not appreciably alter cortactin translocation or the increased cortical actin produced by S1P (Fig. 7A); however, CBP significantly reduced peak EC barrier enhancement relative to control peptide (Fig. 7B), suggesting that interactions via
significantly decreased S1P response relative to wild-type cortactin controls at 90 min. EC expressing Tyr-deficient cortactin exhibit a wild-type and Tyr-deficient mutant cortactin compared with vector depicts the relative S1P-stimulated TER elevation in EC expressing pooled data deficient mutant cortactin attenuates this response compared with wild-type cortactin augments the S1P TER response, whereas the Tyr- treated with vehicle or 0.5 μM S1P for 0–30 min before cortactin IP as described under “Materials and Methods.” Cortactin phosphotyrosine content (top row) dramatically increased within 30 s and decreased over time but remained above base line for 30 min. Pancortactin staining is also shown (bottom row). B, EC were stained for cortactin immunoreactivity after vehicle (first panel), S1P (second panel, 0.5 μM, 5 min), S1P-challenged EC previously preincubated with the p60src inhibitor, PP2 (5 μM, 60 min) (third panel), or S1P-challenged EC previously transfected with a α-c-Myc-tagged Tyr-deficient cortactin resistant to p60src Tyr phosphorylation (fourth panel, α-c-Myc immunoreactivity, representative of at least 10 transfected cells evaluated per experiment). PP2 fails to inhibit S1P-stimulated cortactin translocation (panel 3), and c-Myc Tyr-deficient cortactin translocates after S1P challenge. These observations are representative of the entire cell monolayer and were reproduced in multiple independent experiments (n = 3 for each condition). C, established HUVEC lines retrovirally infected with either GFP control vector, GFP-tagged wild-type cortactin, or GFP-Tyr-deficient mutant cortactin were plated on gold microelec- trodes and then challenged with S1P (0.5 μM, 5 min). Overexpression of wild-type cortactin augments the S1P TER response, whereas the Tyr-deficient mutant cortactin attenuates this response compared with vector controls in these HUVEC lines. The TER tracing represents pooled data ± S.E. from five independent experiments. D, bar graph depicts the relative S1P-stimulated TER elevation in EC expressing wild-type and Tyr-deficient mutant cortactin compared with vector controls at 90 min. EC expressing Tyr-deficient cortactin exhibit a significantly decreased S1P response relative to wild-type cortactin (*, p = 0.03).

the cortactin SH3 domain participate in S1P EC barrier enhancement.

We next determined whether interaction with the SH3 domain of cortactin is involved in stimulation of EC MLCK activity by S1P. The role of EC MLCK in vascular barrier regulation has previously been confined to its involvement as a central participant in several models of EC permeability (10). However, we previously noted the critical importance of the cortical actin ring in S1P-mediated barrier enhancement, which was accompanied at this site by strong increases in Ser19 and Thr18 phosphorylation of MLC that are highly suggestive of EC MLCK activation (2). CBP-mediated inhibition of MLCK interaction with cortactin appears to have important functional consequences on this MLCK phosphorylation. Fig. 8A depicts significant CBP-mediated attenuation of the S1P-induced (but not thrombin-induced) increased MLC phosphorylation on Ser19 and Thr18 as detected by Western blotting (quantified by densitometry and normalized to total MLC content in Fig. 8B).
In this study, we explored the cortical actin rearrangements necessary for S1P to modulate EC integrity. The central role of the actomyosin cytoskeleton in multiple models of barrier disruption is well supported (10), although cytoskeletal contributions to barrier-promoting events are less well studied. We now demonstrate novel and essential EC barrier-enhancing roles for two cytoskeleton-associated proteins, EC MLCK and cortactin. EC MLCK is a well-established and essential participant in multiple in vitro and in vivo models of EC barrier disruption (15–17) where increased MLCK activity results in stress fiber formation and subsequent increases in EC permeability. Although EC MLCK has not previously been reported to mediate enhanced EC barrier function, our data suggest an important functional role for MLCK-catalyzed MLC phosphorylation, spatially localized in the cell periphery, in mediating EC barrier disruption and subsequent alterations in the cortical actin cytoskeleton (2). The impressive barrier enhancing potency of this system is demonstrated by the S1P analog, FTY720, currently the focus of ongoing clinical trials as a potent immuno-suppressive agent. FTY720 via engagement of Edg receptors seals the EC barrier so tightly as to severely limit lymphocyte migration through lymphoid tissues and the blood-EC barrier (35). Understanding the regulatory mechanisms involved in S1P-induced EC barrier enhancement, therefore, may direct further development of clinically useful approaches for modulating or restoring vascular integrity.

In Fig. 6, we report MLCK immunoprecipitation analysis that demonstrates novel and essential roles for MLCK- and cortactin-EC MLCK interaction. The data shown are representative of two independent experiments. C, recombinant N-terminal EC MLCK 1 protein (AA 1–922) fails to bind GST-cortactin bound to glutathione-Sepharose beads (lane 2) in the same incubation assay described in B. Since this N-terminal EC MLCK construct does not contain a His tag, an N-terminal EC MLCK 1-specific antibody was generated (described under “Materials and Methods”) for detection of this protein. Lane 3, a positive control of purified N-terminal protein loaded directly onto the gel to demonstrate specificity of this antibody. The blot shown (IB) is representative of two independent experiments.

DISCUSSION

The disruption of vascular integrity with the resultant profound increase in permeability is an essential feature of inflammatory processes such as acute lung injury and contributes significantly to the high morbidity and mortality of these conditions. Despite its obvious clinical importance, functional understanding of the molecular basis for EC barrier regulation remains imprecisely defined. The contributions of platelets and platelet-derived products to EC barrier maintenance are well recognized, since thrombocytopenia-induced EC permeability can be reversed with infusions of whole platelets or platelet-released products (34). Recently, we reported the critical importance of the platelet-derived phospholipid, S1P, in augmentation of pulmonary EC barrier function through Edg receptor ligation and subsequent alterations in the cortical actin cytoskeleton (2). The impressive barrier enhancing potency of this system is demonstrated by the S1P analog, FTY720, currently the focus of ongoing clinical trials as a potent immuno-suppressive agent. FTY720 via engagement of Edg receptors seals the EC barrier so tightly as to severely limit lymphocyte migration through lymphoid tissues and the blood-EC barrier (35). Understanding the regulatory mechanisms involved in S1P-induced EC barrier enhancement, therefore, may direct further development of clinically useful approaches for modulating or restoring vascular integrity.

In this study, we explored the cortical actin rearrangements necessary for S1P to modulate EC integrity. The central role of the actomyosin cytoskeleton in multiple models of barrier disruption is well supported (10), although cytoskeletal contributions to barrier-promoting events are less well studied. We now demonstrate novel and essential EC barrier-enhancing roles for two cytoskeleton-associated proteins, EC MLCK and cortactin. EC MLCK is a well-established and essential participant in multiple in vitro and in vivo models of EC barrier disruption (15–17) where increased MLCK activity results in stress fiber formation and subsequent increases in EC permeability. Although EC MLCK has not previously been reported to mediate enhanced EC barrier function, our data suggest an important functional role for MLCK-catalyzed MLC phosphorylation, spatially localized in the cell periphery, in mediating EC barrier augmentation by S1P. These results are consistent with a precedent in non-EC tissues for MLCK participation in restoration of barrier integrity, since the specific MLCK inhibitor, ML-7, reportedly blocks closure and sealing of single cell epithelial defects (36) as well peripheral actin formation and spread morphology in foreskin fibroblasts (37). Moreover, over-
expression of mutant MLC that cannot be phosphorylated by MLCK leads to decreased resistance across epithelial monolayers (38), providing further support for the importance of MLCK activity in the maintenance of barrier function.

The novel observation that overexpressed EC MLCK colocalizes with cortactin in the cortical actin ring and in lamellipodia (Fig. 5) offers the opportunity to shed new light on important barrier-regulatory mechanisms. Cortactin is a multidomain actin-binding protein (Fig. 6A) found in peripheral areas of dynamic actin rearrangement and participates in cell migration as well as tumor invasiveness (18, 39). Cortactin acts to stimulate and stabilize Arp2-Arp3-mediated actin polymerization of branched networks at the leading edge of cell protrusions and other sites of weblike actin polymerization (4, 5); however, the spatial and temporal regulation of cortactin interaction with Arp2-Arp3 is poorly defined. Our results now demonstrate a novel role for cortactin in pulmonary EC barrier regulation. In human lung EC, cortactin rapidly translocates to the cell periphery within 5 min of exposure to S1P, a response also seen with other barrier-promoting stimuli such as HGF, early DPV (Fig. 2), shear stress (7), and simvastatin (40), but not with the barrier disrupting agent, thrombin. As evidence for tight linkage of cortactin to EC barrier regulation, antisense reduction of cortactin expression significantly inhibits S1P TER elevation (Fig. 1).

We further explored the signaling pathways that direct cortactin cellular localization as well as post-translational modifications that may contribute to peripheral cytoskeletal changes producing barrier enhancement. Increased cortactin tyrosine phosphorylation occurs after many stimuli associated with cytoskeletal rearrangement. Although long appreciated as a target for p60Src (6), we were unable to link p60Src to cortactin peripheral translocation after S1P (Fig. 3B), despite rapid increases in the phosphotyrosine content of cortactin in HPAEC (Fig. 3A). While consistent with our previously published data that the Src inhibitor, PP2, does not block shear-induced cortactin translocation (7), these results differ from those of Vouret-Craviari et al. (41), who found PP2 to reduce cortactin peripheral localization after S1P in HUVEC. Differences in the cell lines studied may account for this discrepancy; however, it is interesting to note that whereas this study (41) did not

![Fig. 7](image-url) Inhibition of cortactin SH3 domain binding blunts S1P-induced barrier enhancement. A, HPAEC were fixed and stained for cortactin (a and b) and actin (c and d) after a 45-min preincubation with 10 μM control peptide (a and c) or CBP (b and d) and then treatment with 0.1 μM S1P for 5 min. The cortactin and actin images (representative of the entire cell monolayer in two independent experiments) show matched cell fields for each condition. CBP did not inhibit cortactin translocation or increased cortical actin after S1P. B, CBP significantly attenuates the S1P TER response compared with control peptide incubation. The TER tracing represents pooled data ± S.E. from three independent experiments. The bar graph (inset) depicts pooled TER data (n = 3) as maximal percentage of S1P-induced TER elevation above base line achieved within 30 min ± S.E. (*, p = 0.04 relative to control peptide plus S1P).

![Fig. 8](image-url) Inhibition of cortactin SH3 domain binding decreases S1P-induced MLC phosphorylation. HPAEC were preincubated with 10 μM CBP or scrambled control peptide for 45 min prior to stimulation with vehicle, 1 μM S1P (5 min), or 0.1 μM thrombin (2 min). A, after lysis, MLC phosphorylation was assayed by Western blotting with α-diphosphorylated MLC Ab (pp-MLC). CBP (which abolishes cortactin-MLCK interaction in vitro (8)) attenuated S1P-, but not thrombin-induced MLC phosphorylation. B, blots from multiple independent experiments were quantified by densitometry and normalized to total MLC content. Data represent mean ± S.E. from four blots expressed as percentage of α-diphosphorylated MLC Ab compared with unstimulated controls. *, p = 0.05 for CBP-treated versus control peptide-treated cells after S1P. C, HPAEC were fixed and stained for diphosphorylated MLC. Incubation with CBP decreased S1P-induced peripheral MLC phosphorylation (d) compared with incubation with control peptide (b, arrows). No differences in MLC phosphorylation are observed after vehicle (a and c) or thrombin stimulation of cells (data not shown). These images are representative of three independent experiments (bar, 10 μm).
evaluate the role of cortactin in EC barrier regulation, consistent with our observations, we found that PD2 did not inhibit S1P EC barrier enhancement (2). We extended these pharmacologic approaches and utilized human EC transiently overexpressing mutant cortactin (Fig. 3B) deficient in three critical tyrosine residues (Tyr421, Tyr466, and Tyr482), which account for >90% of p60src cortactin phosphorylation (18), to definitively show that p60src signaling is not required for S1P-induced cortactin translocation. Phosphorylation of one or more of these three critical residues appears to be necessary, however, for peak S1P EC barrier enhancement, since HUVECs overexpressing the tyrosine-deficient mutant cortactin exhibit a blunted TER response (Fig. 3, C and D).

Chemical inhibitor data suggest that p60src may not be the only tyrosine kinase involved in this response. We previously reported that the specific Src inhibitor PD2 failed to alter S1P EC barrier enhancement, whereas significant inhibition was observed after incubation with the nonspecific tyrosine kinase inhibitor genistein, herbimycin A, or erbstatin (2). Thus, it is likely that tyrosine kinases other than p60src are able to phosphorylate cortactin at these three critical residues (Tyr421, Tyr466, and Tyr482) during S1P EC barrier enhancement. For example, both Fyn and FER are known to catalyze tyrosine phosphorylation of cortactin during osmotic stress, with FER phosphorylation targeting these same three residues (42). In addition, the EC barrier-enhancing agent HGF appears to induce Src-independent cortactin phosphorylation through activation of the intrinsic tyrosine kinase activity of the HGF receptor (c-MET) (43). Although multiple tyrosine kinases may be involved in cortactin phosphorylation, a recent report indicates that Rac-mediated translocation to the cell periphery is required for this phosphorylation to occur (44).

Whereas the functional importance of tyrosine-phosphorylated cortactin in promoting cell migration (18, 25), H2O2-mediated morphological changes (20), and now S1P EC barrier enhancement is well described, the mechanistic effects of this phosphorylation are poorly defined. It has been reported that p60src phosphorylation decreases cortactin F-actin cross-linking activity in vitro (45), and tyrosine phosphorylation probably modulates other protein interactions through which cortactin regulates the actin cytoskeleton. Cortactin tyrosine phosphorylation increases its affinity for EC MLCK, an interaction that down-regulates cortactin-stimulated Arp2-Arp3 actin polymerization in vitro (8), thereby providing a potential mechanism through which cortactin tyrosine phosphorylation may modulate the cytoskeleton.

An emerging concept from our work is the commonality of a marked increased cortical actin ring following barrier protective stimuli such as shear stress, HGF, simvastatin, and S1P (2, 7, 22, 40). This rearrangement appears to require Rac GTPase activation that is essential for peak S1P-induced barrier enhancement (Fig. 4). However, the downstream effector(s) that links Rac to cortactin movement remains unclear. Rac activation of the PAK family is well described and is essential for S1P-mediated cortical actin rearrangement (2). Overexpression of dominant negative PAK1 did not affect S1P-induced cortactin translocation (Fig. 4B); however, since this mutant PAK1 is kinase-dead but otherwise intact, PAK may mediate cortactin cellular movement through protein interactions apart from PAK kinase activity. PAK may play a role in mediating this process, since overexpression of a PAK1 construct mutated in its GTPase binding domain is sufficient to stimulate peripheral cortactin localization in fibroblasts (26). Interestingly, cortactin co-precipitates with PAK in resting platelets but dissociates during stimulated lamellipodia formation (46), suggesting a dynamic interaction between these two proteins.

A key finding of our study is that protein interactions involving the cortactin SH3 domain may participate in S1P EC barrier enhancement. Incubation of HPAEC with a myristoylated peptide that competitively blocks the SH3 binding site (CBP) results in significant inhibition of peak S1P-stimulated TER (Fig. 7B). Cortactin interacts with several known cytoskeleton-associated proteins via its C-terminal SH3 domain including the neuronal protein CortBP-1, the vesicle-associated GTPase dynamin, the junctional protein ZO-1, and the WASP-binding protein WIP (29–32). Despite these candidate cortactin-binding proteins, we present multiple lines of evidence indicating that peripherally distributed EC MLCK represents an essential cortactin SH3 binding partner for optimal S1P EC barrier enhancement. We definitively show that MLCK interacts with cortactin through the latter protein’s SH3 domain (Fig. 6B), with rapid colocalization in S1P-stimulated HPAEC at peripheral sites of cytoskeletal rearrangement (Fig. 5). The functional importance of regional, subcellular MLCK activation is being increasingly recognized, since recent studies have reported functional effects of localized MLCK activity in the periphery of intestinal epithelia (47) as well as the lamellipodia of COS-7 cells (48). CBP blocking experiments strongly suggest that EC MLCK-cortactin interaction is necessary for maximal S1P-induced MLCK phosphorylation at the cell periphery (Fig. 8).

Given that cortactin fails to directly stimulate EC MLCK activity in vitro (8), we speculate that cortactin increases EC MLCK-catalyzed MLC phosphorylation by either linking the kinase to enzymatic cofactors such as p60src, which binds directly with cortactin and increases splice variant EC MLCK 1 activity in vitro (14), or by facilitating substrate targeting (MLC) in this locale. For example, the Arp2-Arp3 complex is essential in yeast for cortical localization of calmodulin during endocytosis (49), and thus one function of Arp2-Arp3-cortactin interaction may be to target EC MLCK and its key regulator, calmodulin, to sites of active cortical rearrangement. The functional role of peripheral MLC phosphorylation in promoting S1P barrier enhancement remains unclear, but phosphorylating MLC at the periphery may stimulate actomyosin interactions that produce cell spreading, flattening, or other changes that strengthen the cortical actin ring. Alternatively, EC MLCK may serve as a scaffolding protein that stabilizes the cortical actin ring through multiple actin-binding sites of MLCK or though other potential, but as yet undefined, cytoskeletal interactions.

In summary, we have demonstrated essential and novel roles for the cytoskeletal proteins EC MLCK and cortactin in mediating S1P-induced EC barrier augmentation. Both proteins are rapidly concentrated at the cell periphery by S1P, where they participate in cytoskeletal rearrangements necessary for increased EC barrier function. These observations improve our understanding of the mechanisms regulating EC barrier function and provide potential molecular targets for the future development of clinical applications to modulate pulmonary vascular permeability.

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Steven M. Dudek, Jeffrey R. Jacobson, Eddie T. Chiang, Konstantin G. Birukov, Peiyi Wang, Xi Zhan and Joe G. N. Garcia

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