Microtubule (MT) destabilization promotes the formation of actin stress fibers and enhances the contractility of cells; however, the mechanism involved in the coordinated regulation of MTs and the actin cytoskeleton is poorly understood. LIM kinase 1 (LIMK1) regulates actin polymerization by phosphorylating the actin depolymerization factor, cofilin. Here we report that LIMK1 is also involved in the MT destabilization. In endothelial cells endogenous LIMK1 co-localizes with MTs and forms a complex with tubulin via the PDZ domain. MT destabilization induced by thrombin or nocodazole resulted in a decrease of LIMK1 colocalization with MTs. Overexpression of wild type LIMK1 resulted in MT destabilization, whereas the kinase-dead mutant of LIMK1 (KD) did not affect MT stability. Importantly, down-regulation of endogenous LIMK1 by small interference RNA resulted in abrogation of the thrombin-induced MTs destabilization and the inhibition of thrombin-induced actin polymerization. Expression of Rho kinase 2, which phosphorylates and activates LIMK1, dramatically decreases the interaction of LIMK1 with tubulin but increases its interaction with actin. Interestingly, expression of KD-LIMK1 or small interference RNA-LIMK1 prevents thrombin-induced microtubule destabilization and F-actin formation, suggesting that LIMK1 activity is required for thrombin-induced modulation of microtubule destabilization and actin polymerization. Our findings indicate that LIMK1 may coordinate microtubules and actin cytoskeleton.

Microtubules, polymers of α- and β-tubulins, are key components of the cytoskeleton and are involved in multiple cellular processes such as migration, mitosis, protein, and organelle transport (1, 2). Microtubule dynamics and their spatial arrangements are affected by a number of signaling molecules. Conversely, changes in microtubule dynamics modulate intracellular signal transduction (1). LIMK1, a serine/threonine kinase that regulates actin cytoskeleton stability and actin polymerization, expression of LIMK1, and cofilin, a key actin depolymerization factor, are intimately involved in regulating the contractile status of the cells (3). Actin dynamics is regulated by the Rho GTPase family members, Rho, Rac, and Cdc42 (8, 9). Cofilin regulates actin dynamics by severing actin filaments and sequestering the actin monomer from the pointed end of actin filaments (10). LIMK1 is regulated by the members of the Rho-GTPase family, including Rho, Rac, and Cdc42 (8, 10–15) through the activation of the effectors serine/threonine kinases, Rho kinase (ROCK) and the p21-activated kinases (PAK). These kinases phosphorylate LIMK1 at Thr-508 located in the activation loop of the kinase domain, resulting in its activation.

Here we demonstrate that LIMK1 coordinates both microtubule disassembly and actin polymerization. We provide experimental data to show that LIMK1 interacts with microtubules and the actin cytoskeleton in an agonist-dependent manner. Stimulation of the cells with thrombin decreases the interaction of LIMK1 with tubulin and increases its interaction with actin. Moreover, we show that LIMK1 induces microtubule destabilization and actin stress fiber formation that requires the kinase activity of LIMK1.

**MATERIALS AND METHODS**

**DNA and RNA Constructs**—Myc-LIMK1, kinase-dead (KD) Myc-LIMK1, and glutathione S-transferase-tagged cofilin deletion mutants of LIMK1, FLAG-LIMK1/2, FLAG-PDZ, FLAG-KD, were described previously (16). ROCK2 was from Dr. R. Ye (University of Illinois at Chicago, Chicago, IL). Double-stranded small interference RNA-LIMK1, 1

1 The abbreviations used are: LIMK1, LIM domain-containing kinase 1; ROCK2, Rho kinase 2; HUVEC, human umbilical vein endothelial cells; GFP, green fluorescent protein; siRNA, small interference RNA; PIPES, 1,4-piperazinediethanesulfonic acid; MT, microtubule.
LIMK1 Coordinates Microtubules and Actin

LIMK1 Co-localizes with Microtubules in Endothelial Cells—To examine the relative intracellular distribution of LIMK1 in endothelial cells, HUVEC, were serum-starved, fixed with glutaraldehyde, and co-stained with rabbit anti-LIMK1 and mouse anti-α-tubulin antibodies. Optical sections (0.5-μm-thick confocal sections) of stained cells revealed a striking co-localization of LIMK1 and MTs (Fig. 1A, upper panel), where most of the LIMK1 protein was found along microtubules. Because high levels of actin monomer, tubulin subunits, and cytoskeletal-binding proteins are present in the cytoplasm, the resolution of cytoskeletal polymers could be reduced, making the detailed analysis of the localization of polymer binding proteins rather difficult. Therefore, to determine whether a certain pool of LIMK1 protein is associated with microtubule cytoskeleton, we have adapted a novel fixation approach that allows the extraction of cytosolic proteins, including monomeric tubulin, from living cells, preserving only polymeric cytoskeletal structures (17). The extraction of cytosolic LIMK1 and tubulin from living HUVEC was confirmed by Western blotting (Fig. 1B). Importantly, we showed that a certain fraction of endogenous LIMK1 could not be extracted from the living cells and was associated with MTs (Fig. 1A, lower panel). To quantify the relative amount of the co-localized LIMK1 and tubulin, we used the Zeiss enhanced co-localization tool software. Relative cell surface area was selected for each cell. Co-localization coefficient was calculated as $c_1(%) = 100\% \times \frac{\text{pixels}_{\text{Ch},1, \text{coloc}}}{\text{pixels}_{\text{Ch},1, \text{total}}} \times 100\%$, and found to be equal to 85 ± 4.5%, suggesting a high extent of co-localization.

Calculations of the Co-localization Coefficients and Total Intensities of Protein Staining—Images of Alexa Fluor 488- or 594-stained HUVEC monolayers stimulated with thrombin were captured as described above and analyzed using Zeiss enhanced co-localization tool software. Images were differentially segmented between cytosol (black) and microtubules or F-actin (highest gray value) based on image grayscale levels. The microtubule disassembly and actin stress fiber formation were expressed as a ratio of the cytoskeletal polymer area to the area of the whole image and normalized against controls. Co-localization coefficients and the number of pixels in each channel were calculated using Zeiss enhanced co-localization tool software. Data were collected from 20 cells for each experiment. Three independent experiments were performed. The values were statistically processed using Sigma Plot 7.1 (SPSS Science, Chicago, IL) software.

RESULTS

LIMK1 Co-localizes with Microtubules in Endothelial Cells—To quantify the relative intracellular distribution of LIMK1 in endothelial cells, HUVEC, were serum-starved, fixed with glutaraldehyde, and co-stained with rabbit anti-LIMK1 and mouse anti-α-tubulin antibodies. Optical sections (0.5-μm-thick confocal sections) of stained cells revealed a striking co-localization of LIMK1 and MTs (Fig. 1A, upper panel), where most of the LIMK1 protein was found along microtubules. Because high levels of actin monomer, tubulin subunits, and cytoskeletal-binding proteins are present in the cytoplasm, the resolution of cytoskeletal polymers could be reduced, making the detailed analysis of the localization of polymer binding proteins rather difficult. Therefore, to determine whether a certain pool of LIMK1 protein is associated with microtubule cytoskeleton, we have adapted a novel fixation approach that allows the extraction of cytosolic proteins, including monomeric tubulin, from living cells, preserving only polymeric cytoskeletal structures (17). The extraction of cytosolic LIMK1 and tubulin from living HUVEC was confirmed by Western blotting (Fig. 1B). Importantly, we showed that a certain fraction of endogenous LIMK1 could not be extracted from the living cells and was associated with MTs (Fig. 1A, lower panel). To quantify the relative amount of the co-localized LIMK1 and tubulin, we used the Zeiss enhanced co-localization tool software. Relative cell surface area was selected for each cell. Co-localization coefficient was calculated as $c_1(%) = 100\% \times \frac{\text{pixels}_{\text{Ch},1, \text{coloc}}}{\text{pixels}_{\text{Ch},1, \text{total}}} \times 100\%$, and found to be equal to 85 ± 4.5%, suggesting a high extent of co-localization.

In Vitro Kinase Assays—LIMK1 activity was determined as described previously (19). Briefly, Myc-tagged LIMK1 (Myc-LIMK1) was transfected with various cDNA constructs as described under “Results.” Forty-eight hours later the cells were lysed in lysis buffer containing 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate with 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin for 30 min at 4 °C. Debris was removed by centrifugation at 12,000 × g for 15 min at 4 °C. Forty-eight hours later the cells were lysed in lysis buffer containing 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate with 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin for 30 min at 4 °C. Debris was removed by centrifugation at 12,000 × g for 15 min at 4 °C. Forty-eight hours later the cells were lysed in lysis buffer containing 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate with 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin for 30 min at 4 °C. Debris was removed by centrifugation at 12,000 × g for 15 min at 4 °C. Forty-eight hours later the cells were lysed in lysis buffer containing 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate with 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin for 30 min at 4 °C. Debris was removed by centrifugation at 12,000 × g for 15 min at 4 °C.
FIG. 1. LIMK1 co-localizes with microtubules in HUVEC. A, immunofluorescence analysis of LIMK1 and tubulin colocalization in HUVEC (top panels). To remove soluble cytosolic proteins, including monomeric tubulin, living cells were extracted with 0.5% Triton X-100 supplemented with 2 μM taxol before fixation with 0.1% glutaraldehyde, 0.1 M sodium cacodylate and staining with anti-LIMK1 and anti-α-tubulin antibodies (bottom panels). Images were captured using dual-wavelength laser scanning confocal microscope Zeiss LSM 510. Bars, 10 μm. Shown are representative images from three independent experiments. B, LIMK1 co-sediments with tubulin after extraction of cytosolic proteins from living cells. Cytosolic proteins were extracted from living cells with extraction solution (see “Materials and Methods”). Cells lysates were loaded on buffered cushion and centrifuged for 40 min at 100,000 × g. The pellet (P) and supernatant (S) were assayed by Western blotting, and the membrane was probed with anti-LIMK1 and anti-α-tubulin antibodies. C, endogenous LIMK1 co-immunoprecipitates with tubulin. Cell lysates of confluent HUVEC (grown in 100-mm dishes) were immunoprecipitated with anti-Myc or anti-FLAG antibodies. Tubulin was immunoprecipitated together with endogenous LIMK1, whereas protein A/G-agarose (Fig. 1C) and non-immune sera did not precipitate tubulin or LIMK1 (data not shown), suggesting that the interaction between tubulin and LIMK1 was specific.

The PDZ Domain Mediates the Interaction between LIMK1 and Tubulin—LIMK1 interacts with F-actin via its kinase domain, determined using an in vitro co-sedimentation assay (9). To identify the LIMK1 domain(s) that mediates the interaction with tubulin, we transfected COS-7 cells with Myc-tagged full-length LIMK1 or FLAG-tagged deletion mutants consisting of the two LIM domains, the PDZ domain, or the kinase domain of LIMK1 (Fig. 2A). Tubulin was immunoprecipitated from cell lysates, and the presence of the LIMK1 proteins was determined using either anti-Myc or anti-FLAG antibodies. Tubulin was immunoprecipitated together with Myc-tagged LIMK1 but not with Myc-tagged zyxin used as a negative control (Fig. 2B, left panel). In addition, Myc-tagged LIMK1 was immunoprecipitated together with endogenous tubulin, whereas non-immune serum did not precipitate Myc-tagged LIMK1 or tubulin (Fig. 2B, right panel). Immunoprecipitation of cell lysates with anti-tubulin antibody followed by Western blotting with anti-FLAG antibody revealed that the PDZ and not the LIM or the kinase domain interacted with tubulin (Fig. 2C).

Modulation of the Microtubule Cytoskeleton Induces Changes in LIMK1 Localization—We analyzed the intracellular distribution of LIMK1 in HUVEC stimulated with thrombin, a multifunctional enzyme that plays a central role in the regulation of biochemical, transcriptional, and functional responses of endothelial cells (for review, see Ref. 20). To test the effect of thrombin on microtubule organization in endothelial cells, we treated HUVEC with 25 nM thrombin for 10 min and found pronounced MT destabilization, resulting in disassembly of the peripheral microtubule network (Fig. 3A). Changes in the relative amount of MTs were measured using Zeiss Enhanced Colocalization Tool software. The degree of MT disassembly was expressed as a ratio of the MT area to the area of the whole cell. The data showed that ∼44% of the MTs underwent disassembly upon thrombin stimulation (Fig. 3A), consistent with previously published results (21). In addition, the level of acetylated tubulin, representing the stable microtubule pool, was decreased, further confirming the destabilization of MT upon thrombin stimulation (Fig. 3A).

Importantly, upon thrombin treatment the cell morphology changed together with the pattern of LIMK1 staining; the apparent filamentous staining of LIMK1 became more homogeneous as it translocated to the periphery of the cell (Fig. 3B). Co-localization coefficient calculated as c*, (%) = 100% × pixelsLIMK1, color/pixelsCh1, total of LIMK1 and tubulin significantly decreased from 85 ± 4.5 to 47 ± 6.2%. To determine whether thrombin-induced changes to the LIMK1-staining pattern and localization was due to MTs destabilization, we treated endothelial cells with 1 μM nocodazole for 5 min. Treatment with nocodazole resulted in MT destabilization similar to that seen after incubation with thrombin (Fig. 3B). Similar to cells treated with thrombin, the addition of nocodazole also resulted in more homogenous LIMK1 staining and its re-distribution to the periphery of the cell (Fig. 3B). The co-localization coefficient of LIMK1 and tubulin was significant, from 85 ± 4.5 to 25 ± 7.9%.
LIMK1 Coordinates Microtubules and Actin

To determine whether MT destabilization was required for thrombin-dependent changes of LIMK1 intracellular distribution, microtubule cytoskeleton was stabilized by taxol, an agent that binds microtubules and counteracts the effects of GTP hydrolysis (22). In cells treated with taxol, LIMK1 was co-localized with MTs (Fig. 3C). Stimulation of these cells with thrombin did not affect microtubule structure and did not change the pattern of LIMK1 localization (Fig. 3C). Together, these results indicate that modulation of the microtubule cytoskeleton induces changes in LIMK1 localization.

**Thrombin Enhances the Interaction of LIMK1 with F-Actin**—We have determined that MTs and F-actin are located at different intracellular compartments, with the greater proportion of MTs found in the apical part of the cell, whereas F-actin is found in the basal part of the cell (Fig. 4A). Thrombin that binds microtubules and counteracts the effects of GTP hydrolysis (22). In cells treated with taxol, LIMK1 was co-localized with MTs (Fig. 3C). Stimulation of these cells with thrombin did not affect microtubule structure and did not change the pattern of LIMK1 localization (Fig. 3C). Together, these results indicate that modulation of the microtubule cytoskeleton induces changes in LIMK1 localization.

**The Kinase Activity of LIMK1 Is Required for the Interaction with Tubulin and Actin**—In endothelial cells, thrombin was shown to activate Rho and its target Rho kinase (25). The Rho-associated kinase ROCK activates LIMK1 by phosphorylation at threonine 508 within the kinase activation loop (23). Inhibition of Rho kinase activity by the pharmacological inhibitor Y27632 prevented thrombin-induced actin stress fiber formation in endothelial cells (21). Similarly, inhibition of Rho kinase activity prevented thrombin-induced depolymerization of microtubules (21). Initially, we determined whether thrombin could induce activation of LIMK1 by ROCK. Endogenous LIMK1 was immunoprecipitated from HUVEC treated with or without 25 nM thrombin and was used in *in vitro* kinase assays using cofilin as a substrate. Data showed that stimulation of HUVEC with thrombin for 10 min significantly increased LIMK1 kinase activity, as was detected using specific anti-phospho-cofilin antibodies (Fig. 5A). Pretreatment of HUVEC with ROCK inhibitor Y27632 for 10 min completely abolished thrombin-induced cofilin phosphorylation (Fig. 5A), which suggests that LIMK1 is activated upon thrombin stimulation by ROCK. Therefore, we have tested the possibility that inhibition of ROCK would also prevent the thrombin-dependent change of LIMK1 localization in endothelial cells. Pretreatment...
of HUVEC with Y27632 for 10 min inhibited thrombin-induced actin polymerization and microtubule depolymerization (Fig. 5B). Interestingly, Y27632 also abolished LIMK1 translocation in cells challenged with thrombin (Fig. 5B), suggesting that ROCK was involved in the thrombin-dependent LIMK1 translocation.

Initially, we determined the ability of overexpressed wild type LIMK1 or kinase-dead LIMK1 (D446A) to phosphorylate cofilin and to be phosphorylated by the Rho kinase, ROCK2. In vitro kinase assay showed that wild type LIMK1 phosphorylated cofilin (Fig. 5C) and that this phosphorylation was significantly increased by ROCK2. As previously demonstrated, LIMK1 (D446A) could not phosphorylate cofilin (Fig. 5C). Similarly, antibody specific to phosho-Thr-508 of LIMK1 detected basal phosphorylation of wild type LIMK1 and kinase-dead LIMK1 (D446A) that was further enhanced by ROCK2 (Fig. 5C).

To determine whether ROCK2 could affect the interaction between wild type LIMK1 and kinase-dead mutant with tubulin and actin, COS-7 were transfected with wild type LIMK1 or LIMK1 (D446A) in the absence or presence of ROCK2. Forty-eight hours later, the cells were lysed and immunoprecipitated with either anti-α-tubulin or anti-β-actin antibodies. Western blot analysis showed that the amount of wild type LIMK1 and LIMK1 (D446A) associated with tubulin was greatly decreased in the presence of ROCK2 (Fig. 5D). These data suggest that kinase activity is not required for complex formation with tubulin and for the modulation of LIMK1 association with tubulin upon activation.

In contrast, the amount of wild type LIMK1 associated with actin was greatly increased in the presence of ROCK2 (Fig. 5D), supporting the notion that activation of LIMK1 led to increased association with actin. Importantly, although the interaction between LIMK1 (D446A) and actin is similar to that of wild type LIMK1, no changes were observed in the presence of ROCK2 (Fig. 5D). These data suggest that LIMK1 activation increases its association with actin.

**LIMK1 Is Required for Thrombin-induced MT Destabilization and Actin Polymerization—**To determine the ability of LIMK1 to modulate the microtubule and actin cytoskeleton in HUVEC, we studied the effects of overexpressed wild type LIMK1, LIMK1 (D446A), and siRNA. Overexpression of wild type LIMK1 or kinase-dead LIMK1 (D446A) in HUVEC decreases the amount of acetylated microtubules. HUVEC grown on gelatin-coated coverslips and serum-starved for 3 h were stimulated with 25 nM thrombin for 10 min. Soluble cytosolic proteins, including monomeric tubulin, were extracted from the living cell. The cells were fixed and stained with either anti-α-tubulin or anti-acetylated tubulin antibodies. Shown are representative images from three independent experiments.

In contrast, LIMK1 (D446A) could not phosphorylate cofilin (Fig. 5C). Similarly, antibody specific to phosho-Thr-508 of LIMK1 detected basal phosphorylation of wild type LIMK1 and kinase-dead LIMK1 (D446A) that was further enhanced by ROCK2 (Fig. 5C).

To determine whether ROCK2 could affect the interaction between wild type LIMK1 and kinase-dead mutant with tubulin and actin, COS-7 were transfected with wild type LIMK1 or LIMK1 (D446A) in the absence or presence of ROCK2. Forty-eight hours later, the cells were lysed and immunoprecipitated with either anti-α-tubulin or anti-β-actin antibodies. Western blot analysis showed that the amount of wild type LIMK1 and LIMK1 (D446A) associated with tubulin was greatly decreased in the presence of ROCK2 (Fig. 5D). These data suggest that kinase activity is not required for complex formation with tubulin and for the modulation of LIMK1 association with tubulin upon activation.

In contrast, the amount of wild type LIMK1 associated with actin was greatly increased in the presence of ROCK2 (Fig. 5D), supporting the notion that activation of LIMK1 led to increased association with actin. Importantly, although the interaction between LIMK1 (D446A) and actin is similar to that of wild type LIMK1, no changes were observed in the presence of ROCK2 (Fig. 5D). These data suggest that LIMK1 activation increases its association with actin.
type LIMK1 induced MT destabilization, with the relative amount of microtubules decreased by ~52% (Fig. 6A). Similarly, acetylated tubulin was dramatically decreased in cells expressing wild type LIMK1 (Fig. 6A). Thrombin that induced MT destabilization in non-transfected cells did not cause any further changes in microtubule organization in the cells transfected with wild type LIMK1 (Fig. 6A). In contrast, expression of kinase-dead LIMK1 did not induce MT destabilization in endothelial cells (Fig. 6B). Importantly, it attenuated thrombin-induced MT destabilization and preserved ~80% of acetylated microtubules (Fig. 6B).

We used small interfering RNA targeted against LIMK1 to determine the LIMK1 role in the thrombin-induced modulation of microtubule cytoskeleton. HUVEC were transfected with or without siRNA against LIMK1 or Gα12 (negative control). Twenty-four hours later, cells were lysed and probed with antibodies against LIMK1, LIMK2, and Hsp90 (Fig. 6C). Data showed that siRNA-LIMK1 but not siRNA-Gα12 induced significant down-regulation of the LIMK1 protein. Importantly, LIMK2 expression was not affected under any experimental conditions, suggesting that LIMK1 siRNA was specific (Fig. 6C).

To demonstrate that LIMK1 is required for MT destabilization induced by thrombin, HUVEC were transfected with green fluorescent protein (GFP) in the absence or presence of siRNA-LIMK1, and MT stability was analyzed in the cells expressing GFP. In the control experiment using oligonucleotide conjugated to a fluorescent probe, we determined that the cotransfection efficiency with GFP was ~95%. Data showed that siRNA-LIMK1 did not affect MT stability in non-stimulated HUVEC. Importantly, down-regulation of LIMK1 inhibited MT destabilization induced by thrombin (Fig. 6D).

Similar to its effect on fibroblasts and epithelial cells, wild type LIMK1 induced formation of actin stress fibers in endothelial cell (Fig. 7A), whereas stimulation of the HUVEC with thrombin did not further increase F-actin staining. LIMK1 (D446A) did not promote stress fiber formation, but it attenuated actin stress fiber formation upon thrombin stimulation (Fig. 7B). Importantly, down-regulation of endogenous LIMK1 using siRNA also attenuated stress fiber formation upon thrombin stimulation (Fig. 7C). Together, these data indicate that LIMK1 is required for thrombin-induced MTs destabilization and actin polymerization.

**DISCUSSION**

**LIMK1 Interacts with Tubulin**—We have demonstrated here that in endothelial cells LIMK1 is colocalized with MTs and can form a complex with tubulin via its PDZ domain. Depolymerization of MTs induced by thrombin or nocodazole resulted in decreased LIMK1 interaction with tubulin and changes LIMK1 localization to sites with increased actin dynamics. This thrombin-dependent translocation of LIMK1 was prevented by stabilization of MTs with taxol.

In contrast to previous findings, we did not detect any significant co-localization of LIMK1 with F-actin in non-stimulated endothelial cells; however, co-localization was dramatically increased upon stimulation with thrombin. Finally, LIMK1 was required for the thrombin-induced modulation of MTs destabilization and actin polymerization. These results indicate that LIMK1 may coordinate microtubules and actin cytoskeleton in endothelial cells.

**Microtubules and LIMK1**—In endothelial cells, MT destabilization is associated with stress fiber formation, contraction, and endothelial cell barrier dysfunction (27). Recent studies suggest direct involvement of MTs in the regulation of endothelial integrity and wound repair, as depolymerization by the microtubule inhibitors nocodazole and vinblastine results in rearrangement of the actin cytoskeleton, increased stress fiber formation, cell contraction, and permeability (6, 28). Here we showed that LIMK1 could induce MTs destabilization (Fig. 6A). Because the kinase-dead mutant of LIMK1 and LIMK1 down-regulation using siRNA prevented thrombin-induced MTs destabilization, this suggests that LIMK1 is required for the thrombin-induced MT destabilization.

The LIM and PDZ domains are known to mediate protein-protein interactions (for reviews, see Refs. 29 and 30). Deletion mutants lacking these domains show increased LIMK1 activity, suggesting that they regulate LIMK1 activity (8). We have shown that LIMK1 interacts with tubulin via its PDZ domain (Fig. 2C). Previous yeast two-hybrid and mammalian cell interaction analyses have revealed that LIMK1 interacts via its LIM domains with a number of proteins including protein kinase C (31), the cytoplasmic domain of the transmembrane ligand neuregulin (32), the cytoplasmic tail of bone morphogenic protein receptor type II (16), and LATS1 (33). Although no function was assigned to the interactions between LIMK1 and protein kinase C or neuregulin, the interaction with protein kinase C and LATS1 resulted in
down-regulation of LIMK1 activity. However, the PDZ domain was not shown previously to mediate LIMK1 interactions.

Microtubule dynamics is regulated by two groups of proteins; one stabilizes microtubules and the other destabilizes them. The proteins belonging to the first group are known as structural microtubule-associated proteins (MAPs), such as MAP1B and tau (34). Proteins that are potent destabilizers of microtubules have been identified more recently (35, 36) and include stathmin and SCG10, which belong to the same gene family. The mechanism by which LIMK1 induces MTs destabilization is not yet known. One possibility is that LIMK1 may phosphorylate proteins that either stabilize or destabilize microtubules thereby affecting their function. As cofilin is the only well studied LIMK1 substrate, it is of great importance to identify novel proteins that can be phosphorylated by LIMK1. Another possibility could be that LIMK1 directly affects microtubule dynamics. Such a scenario was described for the Gα subunits of heterotrimeric G proteins where the Gα subunits activate tubulin
GTPase and modulate microtubule polymerization dynamics (37).

LIMK1 and Cross-talk between Microtubules and Actin Cytoskeleton—The role of LIMK1 in regulation of actin dynamics is well established. LIMK1 regulates actin dynamics via phosphorylation and inactivation of cofilin (8, 9, 15). The functional significance of LIMK1 in actin organization was revealed in studies using LIMK1 (−/−) mice, demonstrating that LIMK1 is essential for normal spine morphology, synaptic regulation, and memory (38). In agreement with previous findings, our data also demonstrate that LIMK1 induces actin stress fiber formation. In addition, we have shown that the kinase-dead mutant of LIMK1 and down-regulation of LIMK1 with siRNA attenuated actin stress fiber formation induced by thrombin (Fig. 7B) and that stabilization of microtubules with taxol prevented both translocation of LIMK1 to F-actin (Fig. 3C).

Cross-talk between microtubules and the actin cytoskeleton is essential for the regulation of many cellular functions such as migration, locomotion, cytokinesis, and cell polarity (for review, see Ref. 39). The Rho family of small GTPases was shown to participate in the regulation of both microtubules and actin (7). Importantly, microtubule disassembly was shown to induce Rho activation (26). Microtubule disassembly releases the microtubule-bound Rho guanine nucleotide exchange factor (GEF), GEF-H1, to activate RhoA (13). However, the mechanism of agonist-dependent microtubule disassembly is not yet understood. We have shown here that LIMK1 induces MTs destabilization and actin polymerization and propose that LIMK1 may serve as a molecular switch.
that regulates both MTs destabilization and formation of actin stress fibers, thereby providing the molecular mechanism that explains how microtubule disassembly promotes the formation of actin stress fibers.

In conclusion, the results presented here suggest that the regulation of microtubules and actin polymerization by LIMK1 provides a mechanism for the coordination of microtubule and the actin cytoskeletons. We propose that in resting endothelial cells LIMK1 is associated with microtubules (Fig. 8). Ligand-induced activation of the Rho-ROCK pathway activates LIMK1, which in turn causes MTs destabilization and release of LIMK1. Consequently, activated LIMK1 associates with actin, thereby inducing its polymerization via cofilin phosphorylation.

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FIG. 7. LIMK1 activity is required for actin polymerization. HUVEC were grown on gelatin-coated coverslips and transfected with Myc-tagged wild type (wt) LIMK1 (A), LIMK1 (D446A) DNA (B), or GFP with or without siRNA-LIMK1 (C). Twenty-four hours later cells were serum-starved for 3 h, stimulated with 25 nM thrombin for 10 min, fixed, and stained with anti-Myc antibody and Alexa Fluor 594 phalloidin. The amount of F-actin was measured using Zeiss Enhanced Colocalization Tool software. Shown are representative images from three independent experiments. The changes in the relative amount of F-actin were expressed as the ratio of the F-actin area to the area of the whole cell. Data were collected from 100 cells for each experiment. *, p < 0.05.

FIG. 8. A proposed model of LIMK1 regulation of the actin cytoskeleton and microtubules. We propose that in resting endothelial cells LIMK1 is associated with microtubules. Ligand-induced activation of the Rho-ROCK pathway activates LIMK1, which in turn causes MTs destabilization and release of LIMK1. Consequently, activated LIMK1 associates with actin, thereby inducing its polymerization via cofilin phosphorylation.
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