LIM Kinase-mediated Cofilin Phosphorylation during Mitosis
Is Required for Precise Spindle Positioning

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The interaction of astral microtubules with cortical actin networks is essential for the correct orientation of the mitotic spindle; however, little is known about how the cortical actin organization is regulated during mitosis. LIM kinase-1 (LIMK1) regulates actin dynamics by phosphorylating and inactivating cofilin, an actin-depolymerizing protein. LIMK1 activity increases during mitosis. Here we show that mitotic LIMK1 activation is critical for accurate spindle orientation in mammalian cells. Knockdown of LIMK1 suppressed a mitosis-specific increase in cofilin phosphorylation and caused unusual cofilin localization in the cell cortex in metaphase, instability of cortical actin organization and astral microtubules, irregular rotation and misorientation of the spindle, and a delay in anaphase onset. Similar results were obtained by treating the cells with a LIMK1 inhibitor peptide or latrunculin A or by overexpressing a non-phosphorylatable cofilin(S3A) mutant. Furthermore, localization of LGN (a protein containing the repetitive Leu-Gly-Asn tripeptide motifs), an important regulator of spindle orientation, in the crescent-shaped cortical regions was perturbed in LIMK1 knockdown cells. Our results suggest that LIMK1-mediated cofilin phosphorylation is required for accurate spindle orientation by stabilizing cortical actin networks during mitosis.

The accurate establishment of the positioning and orientation of the mitotic spindle is essential for cells to determine the axis and plane of cell division and, consequently, to specify the sizes, positions, and fates of daughter cells after mitosis. Proper spindle positioning and orientation are fundamental for both asymmetric and symmetric cell division. Asymmetric cell division, the spindle orientation is controlled by a set of conserved “spindle-positioning proteins,” such as Inscuteable, Partner of Inscuteable (Pins), a G protein α subunit, and the Par3-Par6-aPKC complex, which asymmetrically localize in the cell cortex (1–3). Recent studies have shown that during symmetric cell division of cultured mammalian cells, integrin-mediated cell adhesion guides the proper spindle orientation parallel to the substrate plane through the regulation of the cortical actin cytoskeleton (8), and disruption of the actin organization causes spindle misorientation (8, 9). These findings suggest that the proper regulation of actin cytoskeletal dynamics in the cell cortex plays an important role in the spindle positioning. However, little is known about how cortical actin dynamics are regulated during mitosis and how they contribute to the spindle orientation and specific localization of spindle-positioning cues in symmetric or asymmetric cell divisions.

Cofilin is a key regulator of actin filament dynamics and reorganization by stimulating depolymerization and severance of actin filaments (10–12). The activity of cofilin is reversibly regulated by phosphorylation and dephosphorylation at Ser-3, with the phosphorylated form being inactive. LIM kinase (LIMK) phosphorylates cofilin specifically at Ser-3 and thereby inhibits the actin filament-disrupting activity of cofilin (13, 14). Because cofilin contributes to maintain the actin monomer pool in the cell, LIMK activation increases the F-actin ratio and results in the stabilization or accumulation of cellular actin filaments (13–16). LIMK-mediated cofilin phosphorylation is critically involved in a variety of cellular responses and physiological and pathological processes, including chemotactic migration, axon guidance, angiogenesis, and tumor invasion (17–22). The inactive Ser-3-phosphorylated cofilin (P-cofilin) is dephosphorylated and reactivated by Slingshot (SSH) family protein phosphatases (23).

We have previously shown that the level of P-cofilin periodically changes during the cell cycle in HeLa cells; it increases in

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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the early stage of mitosis, reaches a maximum level in metaphase, and then gradually decreases to the basal level as cells enter into anaphase, telophase, and cytokinesis (24). LIMK1 and SSH1 activities also change during the cell cycle in a pattern that correlates with the periodical change in P-cofilin; LIMK1 activity increases, and SSH1 phosphatase activity decreases in metaphase, and then LIMK1 activity decreases, and SSH1 activity increases gradually in later stages of mitosis until cytokinesis (24, 25). Inhibition of cofilin dephosphorylation at later stages of mitosis by the overexpression of LIMK1 or a phosphatase-dead SSH1 mutant results in the formation of multinucleated cells; this indicates that cofilin dephosphorylation and reactivation in the later stages of the cell cycle are important for completing cytokinesis (24, 25). In contrast, the role of cofilin phosphorylation in metaphase remains to be determined.

In this study we examined the role of LIMK1 activation and cofilin phosphorylation during mitosis in human cells. We show that suppression of LIMK1 expression by small interfering RNA (siRNA) or inhibition of LIMK1 activity causes an unusual localization of cofilin in the cell cortex, instability of cortical actin organization and astral microtubules, irregular rotation and mispositioning of the mitotic spindle, and a delay in the onset of anaphase. Furthermore, a knockdown of LIMK1 perturbs the cortical localization of LGN (named after the repetitive Leu-Gly-Asn tripeptide motifs in the protein), a mammalian orthologue of Drosophila Pins, which is known to be an essential regulator of spindle positioning during asymmetric division in both Drosophila and mammalian cells (26–30). Our results suggest that LIMK1-mediated cofilin phosphorylation during mitosis plays an important role in the precise spindle positioning in mammalian cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Latrunculin A (Lat-A) was purchased from Molecular Probe. Cytochalasin-B (Cyto-B) and nocodazole were purchased from Sigma. The S3 and RV peptides were designed and synthesized as described previously (17, 31). The rabbit polyclonal antibodies specific to P-cofilin, cofilin (COF-1), and LIMK1 (C-10) were prepared as described previously (24, 32). The anti-cofilin monoclonal antibody (MAB-22) was provided by Dr. T. Obinata (Chiba University) (33). The anti-α-tubulin and anti-β-actin monoclonal antibodies were purchased from Sigma.

Plasmid Construction—To generate the plasmid coding for yellow fluorescent protein (YFP)-tagged tubulin, the α-tubulin cDNA was amplified by PCR using primers 5′-GGGGATTCCCGTGAGTGCATCTCCATC-3′ and 5′-ATGTCAACTTAGTATTCTCTCCCTC-3′, digested with BamHI and SalI, and subcloned into the BglII and SacII site of the pEYFP-C1 vector (Clontech). The plasmid for YFP-LGN was constructed by subcloning the mouse LGN cDNA (purchased from Invitrogen) into pEYFP-C1 vector. Plasmids coding for C-terminally YFP- or Discosoma red fluorescent protein (DsRed)-tagged cofilin and its S3A and S3E mutants, cyan fluorescent protein (CFP)-tagged histone H2B (CFP-H2B), and YFP-actin were constructed as described previously (16, 24, 25). A LIMK1 siRNA plasmid (pSUPER-LIMK1) that targets the human LIMK1 mRNA sequence (nucleotide positions from the start ATG codon, 1416–1434; GAATGTGGTGGTGGCTGAC) was constructed as described (34). As a control, a mutated LIMK1 siRNA plasmid was constructed by substituting two bases in the target sequence (GAATGTGGTGGTGGACCC).

Cell Culture, Synchronization, and Transfection—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. To synchronize cells at mitosis, cells were synchronized at prometaphase with 100 ng/ml nocodazole. For cell staining or time-lapse experiments, HeLa cells were transfected with the Lipofectamine method (Invitrogen) following the manufacturer’s protocol. Cells were then cultured in the thymidine-containing medium for 36 h and released for 12–15 h in fresh Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum as described (24).

Immunoprecipitation and Immunoblotting—HeLa cell lysates were prepared as described previously (24). After centrifugation, the lysates were subjected to SDS-PAGE and immunoblotted with the antibodies against P-cofilin or cofilin as described previously (24). To analyze endogenous LIMK1, the cell lysates were immunoprecipitated and immunoblotted with the anti-LIMK1 antibody (24).

Cell Staining—HeLa cells were fixed in 4% formaldehyde in phosphate-buffered saline for 20 min and permeabilized with absolute methanol for 10 min at 20 °C. After blocking with 1% bovine serum albumin in phosphate-buffered saline for 30 min, cells were stained with the anti-P-cofilin antibody or anti-cofilin antibody (MAB-22) followed by a rhodamine-conjugated anti-rabbit IgG or fluorescein isothiocyanate-conjugated anti-mouse IgG (Chemicon). For microtubule staining, cells were stained with the anti-α-tubulin monoclonal antibody followed by the rhodamine-conjugated anti-mouse IgG (Chemicon). 4,6-Diamidino-2-phenylindole (DAPI, Molecular Probes) was used for staining DNA. After washing with phosphate-buffered saline, images were obtained using fluorescence microscopy (model DMLB, Leica).

Time-lapse Fluorescence Analysis—For time-lapse imaging, HeLa cells were plated on a 35-mm glass-bottom dish and transfected with plasmids encoding the YFP- and CFP-fused proteins and/or the siRNA plasmid. Fluorescent images were collected every 3 min for 60 min using a confocal imaging system (LSM 510; Carl Zeiss MicroImaging) equipped with a plan Apo NA 1.4 63× oil immersion objective lens (Carl Zeiss MicroImaging).

Spindle Positioning Assay—The position of the mitotic spindle was evaluated by measuring the distance between the spindle center and the cell center and the angle between the spindle axis and the horizontal axis. To do this, HeLa cells expressing YFP-tubulin and CFP-H2B were fixed, and metaphase cells were photographed every 0.7 μm of z-section from the bottom to the top of the cell. The images were constructed into a three-dimensional image using a laser scanning confocal imaging system (Carl Zeiss MicroImaging). The distance and angle were analyzed by IP Lab image analysis software using the obtained x-y image and z image, respectively (Scanalytics, Fairfax, VA).

RESULTS

Cofilin Localization Changes during the Cell Cycle—We have previously shown that the level of P-cofilin increases during
mitosis (24). Because cofilin loses its actin binding ability by phosphorylation, an increase in the P-cofilin level during mitosis appears to affect its subcellular localization. To examine the role of cofilin phosphorylation during mitosis, we analyzed the localization of total cofilin and P-cofilin in HeLa cells during the cell cycle by immunostaining with an anti-cofilin antibody (which recognizes both phospho- and non-phospho-cofilin) and an anti-P-cofilin antibody (which specifically recognizes P-cofilin), respectively (Fig. 1A). Cell cycle progression was monitored by staining with DAPI. In interphase cells, total cofilin signals accumulated intensely in the cell periphery, whereas weak P-cofilin signals were detected diffusely throughout the cytoplasm; this indicates that non-phospho-cofilin is the major component of cofilin signals accumulated in the cell periphery. In contrast, in metaphase, P-cofilin signals markedly increased in the cytoplasm, and total cofilin signals were also diffusely distributed throughout the cytoplasm; this indicates that a large portion of cofilin molecules is phosphorylated and translocated from the cell cortex to the cytoplasm in metaphase. In early and late telophase, P-cofilin signals in the cytoplasm gradually decreased, and total cofilin signals accumulated in the contractile ring. When the cells were stained with the anti-cofilin antibody pre-adsorbed with antigenic cofilin or the second antibody alone for anti-P-cofilin antibody, no intense signal for cofilin or P-cofilin was detected (supplemental Fig. S1), which indicates that the signals observed in Fig. 1A represent the localization of endogenous cofilin and P-cofilin. These results demonstrate that cofilin dynamically changes its localization during the cell cycle, and the localization largely depends on the level of its phosphorylation. Similar to the observations in HeLa cells, the mitosis-specific cofilin phosphorylation and translo-
Role of LIM Kinase in Spindle Positioning

FIGURE 3. LIMK1 knockdown causes a delay in mitotic progression and the instability of spindle positioning. A, time-lapse images of mitotic HeLa cells transfected with control or LIMK1 siRNA. HeLa cells transfected with CFP-H2B (blue), YFP-tubulin (green), and control or LIMK1 siRNA were synchronized by thymidine block and released for 12–15 h. Cells in metaphase were analyzed every 3 min for 30–60 min by time-lapse fluorescence microscopy. The phase-contrast and fluorescence images were merged. Numbers indicate the time in minutes after the first frame. Scale bar, 10 μm. B, quantitative analysis of the effects of LIMK1 siRNA on mitotic progression. The time from metaphase (beginning of observation) to anaphase was analyzed by time-lapse microscopy, as in A. Each point represents the cumulative percentage of cells that entered into anaphase at the time indicated on the abscissa. C, measurements of spindle orientation. HeLa cells transfected with CFP-H2B (cyan), YFP-tubulin (green), and control or LIMK1 siRNA or empty vector were synchronized and fixed. Orientation of the mitotic spindles was analyzed using the x-y image (top panel) and z image (bottom panel). The yellow line indicates the spindle pole-to-pole axis, and the red spot indicates the geometric center of the cell. The cell borders are outlined by white dotted circles. Orientation of the mitotic spindle was evaluated by measuring the distance between the cell center and the midpoint of the spindle axis (x-y image) and the angle between the spindle axis and the basal surface (z image). D, scatter diagrams of the distance from the cell center (left) and the angle of the spindle (right). Red spots indicate the average values. The error bars represent S.E., p < 0.01 (*) and p < 0.002 (**) compared with vector-transfected cells.

 Knockdown of LIMK1 Suppresses Cofilin Phosphorylation and Causes Cortical Accumulation of Cofilin in Metaphase—We have previously shown that the kinase activity of LIMK1 increases during mitosis (24). To examine the role of LIMK1 activation in metaphase, we suppressed LIMK1 expression in HeLa cells using siRNA. Transfection of an siRNA plasmid targeting LIMK1 reduced the expression of endogenous LIMK1 to nearly half that seen in control cells transfected with a vector or a control siRNA plasmid in which two bases in the target sequence were mutated (Fig. 2A). Based on the transfection efficiency of the HeLa cells (~50%) under our experimental conditions, LIMK1 expression appeared to be almost completely suppressed in cells that were transfected with LIMK1 siRNA. When HeLa cells were cotransfected with LIMK1 siRNA and CFP (as a marker for transfection), synchronized at prometaphase, and stained with an anti-P-cofilin antibody, the P-cofilin signals were markedly reduced in LIMK1 siRNA cells compared with neighboring non-transfected cells (Fig. 2B, bottom panels). Transfection of control siRNA had no apparent effect on the P-cofilin staining in prometaphase (Fig. 2B, top panels). To further examine whether LIMK1 is involved in mitotic cofilin phosphorylation, HeLa cells were cotransfected with cofilin-YFP and LIMK1 siRNA, and the levels of cofilin-
YFP phosphorylation in lysates from cells in interphase and mitosis were analyzed by immunoblotting with an anti-P-cofilin antibody (Fig. 2C). Cells that were transfected with the vector or the control siRNA exhibited higher cofilin-YFP phosphorylation in mitotic cells than those in interphase. In contrast, the mitosis-specific increase in the P-cofilin level was not evident in LIMK1 siRNA cells. These results indicate that LIMK1 is primarily involved in the elevation of P-cofilin in mitotic cells.

We also examined the effect of LIMK1 knockdown on cofilin localization in metaphase. HeLa cells cotransfected with CFP-H2B, cofilin-YFP, and LIMK1 or control siRNA in the molar ratio of 1:4:15 were synchronized, and the localization of cofilin-YFP in metaphase was analyzed. The cells that expressed both CFP and YFP fluorescence were regarded to be the siRNA-transfected cells. In control siRNA cells, cofilin-YFP was diffusely distributed in the cytoplasm (Fig. 2D, top panels). In contrast, in LIMK1 siRNA cells, cofilin-YFP was partially mislocalized in the cell cortex, and the cell morphology was irregular (arrowheads in Fig. 2D, bottom panels), in a pattern similar to the one observed when cofilin(S3A)-YFP accumulated in the cortex of metaphase cells (see Fig. 1B, middle panels). Similar results were obtained for cofilin-YFP localization in LIMK1-knockdown HEK293T cells (supplemental Fig. S4). Taken together these findings suggest that LIMK1 plays a crucial role in cofilin phosphorylation and its diffuse distribution in the cytoplasm during metaphase.

Knockdown of LIM Kinase in Spindle Positioning

We next examined whether LIMK1 knockdown would affect the mitotic progression and spindle positioning in HeLa cells using time-lapse fluorescence microscopy. The mitotic spindle was visualized by the expression of CFP-H2B and YFP-tubulin. HeLa cells that had been cotransfected with CFP-H2B, YFP-tubulin, and siRNA plasmids in the ratio of 1:4:15 were cultured in thymidine-containing medium for 36 h and released for 12–15 h, before cells in metaphase were subjected to time-lapse observations. In cells that were transfected with control siRNA, the mitotic spindle was stably positioned near the geometric center...
Role of LIM Kinase in Spindle Positioning

FIGURE 5. Overexpression of cofilin(S3A) causes a delay in mitotic progression and the instability of spindle positioning. A, time-lapse images of mitotic HeLa cells expressing CFP-H2B (blue) and cofilin(WT)-YFP or cofilin(S3A)-YFP (green). Numbers indicate the time in minutes after the first frame. Arrowheads indicate the accumulation of cofilin(S3A) in the cell cortex. Bar, 10 μm. B, quantitative analysis of the effects of overexpression of control YFP, cofilin(WT)-YFP, cofilin(S3A)-YFP, or cofilin(S3E)-YFP on mitotic progression. The time for entry into anaphase was measured, as in Fig. 3B. C, quantitative analysis of the effects of overexpression of control DsRed, cofilin(WT)-DsRed, cofilin(S3A)-DsRed, or cofilin(S3E)-DsRed on spindle positioning. Scatter diagrams of the distance from the cell center and the angle of the spindle are shown as in Fig. 3D. p < 0.005 (**).}

The basal surface was measured as shown in Fig. 3C. In LIMK1 siRNA cells the distance between the spindle center and the cell center was longer, and the angle of spindle axis was larger than those in the cells transfected with empty vector or control siRNA (Fig. 3D). Thus, LIMK1 seems to play a crucial role in ensuring the accurate positioning and orientation of the spindle.

Inhibition of LIMK1 Activity Suppresses Mitotic Cofilin Phosphorylation and Causes a Delay in Mitotic Progression and Irregular Spindle Orientation—To examine the role of the kinase activity of LIMK1 during mitosis, we used a cell-permeable LIMK1 inhibitor (S3 peptide), which contains the phosphorylation site of cofilin and the cell-permeable sequence motif of penetratin. As a control, the RV peptide containing the reverse sequence of cofilin and the penetratin sequence was also tested. We have previously demonstrated that the S3 peptide, but not the RV peptide, inhibited the kinase activity of LIMK1 (17, 31). When interphase or mitotic HeLa cells were treated with the S3 or RV peptide and the levels of cofilin phosphorylation were analyzed by immunoblotting with an anti-P-cofilin antibody, the mitotic increase in cofilin phosphorylation was suppressed by the addition of S3 peptide but not RV peptide (Fig. 4A). Thus, the S3 peptide has the potential to inhibit the mitotic elevation of cofilin phosphorylation. We next analyzed the effects of the S3 peptide on mitotic progression and spindle positioning. HeLa cells expressing YFP-tubulin and CFP-H2B were treated for 30 min with the S3 or RV peptide, and then mitotic progression was analyzed by time-lapse fluorescence microscopy. In the cells treated with the S3 peptide, the mitotic spindle was moved and rotated irregularly (Fig. 4B, bottom panels). In contrast, the spindle was stably positioned parallel to the substrate plane in cells that were treated with the RV peptide (Fig. 4B, top panels). Quantitative analysis showed that treatment with the S3 peptide, but not the RV peptide, delayed the onset of anaphase compared with control untreated cells (Fig. 4C). We also analyzed the effects of the S3 peptide on the positioning and orientation of the spindle by measuring the distance between the spindle center and the cell center and the angle of the spindle axis (Fig. 4D). The cells that had been treated with the S3 peptide exhibited a longer distance and a larger angle, similar to that seen in LIMK1 siRNA cells (see Fig. 3D), than cells treated with the RV peptide or untreated cells. These findings indicate that LIMK1 activity is important for...
mitotic progression and normal spindle positioning in HeLa cells.

Overexpression of Cofilin(S3A) Causes a Delay in Mitotic Progression and Irregular Spindle Orientation—To determine whether the delay in mitotic progression and abnormal spindle positioning in response to the LIMK1 knockdown or S3 peptide treatment was due to insufficient cofilin inactivation, we analyzed the effects of cofilin(S3A) overexpression on the mitotic processes in HeLa cells. Time-lapse fluorescence analysis of HeLa cells that had been cotransfected with CFP-H2B and YFP-tagged cofilin(WT) or cofilin(S3A) revealed that overexpression of cofilin(S3A), but not cofilin(WT), led to abnormal movement of the chromosome position and a delay in anaphase entry (Fig. 5A). Local accumulation of cofilin(S3A)-YFP in the cortex was also observed (arrowheads in Fig. 5A). Quantitative analysis of the time of anaphase entry showed that overexpression of cofilin(S3A) delayed the onset of anaphase compared with control cells expressing YFP, whereas the expression of cofilin(WT) or cofilin(S3E) had no apparent effect on mitotic progression (Fig. 5B). We also analyzed the effects of overexpression of cofilin(WT) or its mutants on the orientation of the spindle. The overexpression of cofilin(S3A), but not cofilin(WT) or cofilin(S3E), induced the mispositioning of the mitotic spindle compared with control cells (Fig. 5C). These results further support the idea that cofilin phosphorylation and inactivation in metaphase is necessary for ensuring the onset of anaphase and normal spindle positioning in HeLa cells.

Knockdown of LIMK1 Destabilizes Cortical Actin Organization and Astral Microtubules—LIMK1-mediated cofilin phosphorylation inhibits the actin-disrupting activity of cofilin; therefore, knockdown of LIMK1 may induce the unusual activation of cofilin and destabilization of cortical actin structures during mitosis. We examined the effect of LIMK1 knockdown on the organization of the actin cytoskeleton. HeLa cells were cotransfected with CFP-H2B, YFP-actin, and LIMK1 or control siRNA and synchronized at metaphase. In control siRNA-transfected cells, YFP-actin was localized uniformly on the cell cortex during metaphase and then accumulated to the cleavage furrow and contractile ring in anaphase and telophase (Fig. 6A, top panels). In contrast, in LIMK1 siRNA-transfected cells YFP-actin was distributed unevenly on the cell cortex during metaphase (Fig. 6A, bottom panels); this suggests that LIMK1 knockdown destabilizes the cortical actin filaments during metaphase.

We also examined the effect of LIMK1 knockdown on the organization of astral microtubules. HeLa cells that had been transfected with CFP-H2B and LIMK1 or control siRNA were fixed and stained with an anti-α-tubulin antibody. In control siRNA-transfected cells, the spindle was positioned near the cell center, and the astral microtubules extended evenly from the two spindle poles toward the cell cortex (Fig. 6B, top panels). In contrast, in LIMK1 siRNA-transfected cells, the spindle was often located away from the cell center, and the astral microtubules emanating from the spindle poles stained only weakly (Fig. 6B, bottom panels). These observations suggest that LIMK1 knockdown causes the destabilization of both cortical actin filaments and astral microtubules.

**FIGURE 6. LIMK1 knockdown affects cortical actin organization and astral microtubules in mitotic cells.** A, time-lapse images of actin organization in cells expressing LIMK1 or control siRNA. HeLa cells transfected with YFP-actin (green) and CFP-H2B (blue) were synchronized, and cells in metaphase were analyzed every 3 min by time-lapse fluorescence microscopy. Numbers indicate the time in minutes after the first frame. Scale bar, 10 μm. B, effects of LIMK1 knockdown on astral microtubules. HeLa cells transfected with CFP-H2B (blue) and LIMK1 or control siRNA were synchronized, fixed, and stained with anti-tubulin antibody (red).

**Effects of Actin Filament-disrupting Drugs on the Spindle Orientation and Stability of Astral Microtubules**—To further investigate the role of the actin cytoskeleton in spindle orientation, we examined the effects of the actin filament-disrupting drugs, Lat-A and Cyto-B, on the mitotic spindle orientation and the stability of astral microtubules. Time-lapse fluorescence analysis of mitotic HeLa cells expressing YFP-tubulin and CFP-H2B showed that treatment with Lat-A caused irregular movement and rotation of the mitotic spindle (Fig. 7A), as described previously (8). These cells did not elongate upon anaphase onset (Fig. 7A, 30 min) and often failed to undergo cytokinesis. Similar results were obtained when the cells were treated with Cyto-B (data not shown). The distance between the spindle center and the cell center and the angle of the spindle axis were quantitatively analyzed in cells that had been treated with the actin filament-disrupting drugs. Treatment with Lat-A or Cyto-B caused the mispositioning and misorientation of the mitotic spindle (Fig. 7B). When HeLa cells that had been treated with Lat-A were fixed and stained with the anti-tubulin antibody, the astral microtubules were only weakly stained compared with those in control cells, and the spindle was frequently positioned away from the cell center (Fig. 7C). These results suggest that the proper assembly of cortical actin filaments is required for normal spindle orientation and stable interaction of astral microtubules with the cell cortex. They also support the idea that LIMK1 is involved in spindle positioning by stabilizing cortical actin filaments during mitosis.
Cortical Localization of LGN Is Perturbed by LIMK1 Knockdown or Lat-A Treatment—LIMK1-mediated actin stabilization may be involved in the localization of cortical components that are required for the correct spindle orientation. To address this issue, we analyzed the localization of LGN, a mammalian orthologue of this issue, in HeLa cells (24). LGN localizes to the apical cell cortex during asymmetric cell division (26, 27). Similarly, LGN localizes to the apical cell cortex in mitotic basal epidermal cells in mammalian skin and appears to be involved in apical-basal spindle orientation and asymmetric cell division (30). In non-polarized mammalian cells in culture, such as HeLa and WISH cells, LGN localizes both in the spindle poles and in the regions of the cell cortex facing the spindle poles during mitosis (29, 35). When HeLa cells were cotransfected with CFP-H2B, YFP-LGN, and control siRNA, YFP-LGN was consistently concentrated within the control siRNA, YFP-LGN was consistently concentrated within the stable actin networks during mitosis.

Knockdown of LIMK1 by siRNA or the inhibition of its kinase activity by S3 peptide resulted in the mispositioning and irregular rotation of the mitotic spindle. Similar effects were observed in response to expression of cofilin (S3A) or treatment with actin-disrupting drugs. Based on these observations, we propose a model in which mitotic LIMK1 activation plays a crucial role in the correct positioning of the spindle by phosphorylating and inactivating cofilin and thereby stabilizing cortical actin organization during mitosis (Fig. 8C).

The interaction of astral microtubules with the cell cortex is essential for accurate spindle positioning, since disruption of astral microtubules with low doses of nocodazole impairs the spindle positioning (9, 36). Several microtubule-binding proteins, such as EB1, CLIP-170, APC, dynein-dynactin complex, and myosin X, are suggested to be involved in spindle positioning by controlling the tethering of astral microtubules to the cell cortex and by producing the pulling force on the microtubules (4, 36–40). We showed here that astral microtubules were weakened in LIMK1 knockdown cells or Lat-A-treated cells, which suggests that the stable anchoring of astral microtubules to the cell cortex requires the stable organization of cortical actin filaments. Thus, LIMK1-mediated cofilin phosphorylation probably contributes to spindle positioning by stabilizing the connection between the astral microtubules and the cell cortex through stabilization of the cortical actin cytoskeleton (Fig. 8C).

Spindle positioning is also regulated by various cell polarity proteins. In the asymmetric divisions of Drosophila neuroblasts (Fig. 8B). These results suggest that proper actin filament assembly is required for the localization of LGN in specific regions of the cell cortex.

DISCUSSION

We have previously shown that LIMK1 is highly activated, and more than half of the total cofilin is phosphorylated during metaphase in HeLa cells (24); however, the functional role of the mitosis-specific LIMK1 activation and cofilin phosphorylation remained to be determined. In this study we showed that knockdown of LIMK1 suppresses mitotic increase in cofilin phosphorylation and induces the unusual localization of non-phosphorylated (active) cofilin in the cell cortex and an unstable organization of cortical actin cytoskeleton with the distorted cell morphology. Our results indicate that mitotic LIMK1 activation is critically involved in the phosphorylation and inactivation of cofilin, the dissociation of cofilin from the cell cortex, and consequently the stabilization of cortical actin networks during mitosis.
and sensory organ precursor cells, the spindle orientation is controlled by spindle-positioning proteins, such as Insuteable, Pins, a G subunit of a heterotrimeric G protein, and the Par protein complex (Par3, Par6, and aPKC), which localize asymmetrically on the apical cortex of mitotic cells (1–3, 41). LGN, a mammalian orthologue of Pins, also localizes on the apical cortex of mitotic epidermal cells in mouse embryo skin and is implicated in the orientation of the spindle toward the apical-basal axis (30). In non-polarized cultured mammalian cells, LGN localizes in the spindle poles and the cell cortex regions that are close to the spindle poles (29, 35, 42). LGN associates with membrane-anchored Go protein and NuMA, which binds to microtubules and dynein (29, 35). LGN also associates with Lgl2, which forms a complex with aPKC and Par-6 (42). Thus, these interactions seem to play important roles in the specific localization of LGN in the cell cortex and the connection of astral microtubules with the cell cortex in mammalian cultured cells. We observed that LGN was consistently localized in the bipartite crescent-shaped regions in the cortex facing the two spindle poles in mitotic HeLa cells. Its localization was perturbed by LIMK1 knockdown or Lat-A treatment, which suggests that LIMK1-mediated stabilization of cortical actin networks contributes to the local accumulation of LGN in the specific regions in the cell cortex and, thereby, the correct spindle orientation (Fig. 8C).

Recent studies have shown that integrin-mediated cell adhesion to the extracellular matrix is important for both the spindle orientation parallel to the substrate plane and the planar spindle orientation in non-polarized mammalian cells in culture (8, 9). In both cases integrin-mediated spindle orientation was perturbed by actin filament disruption (8, 9); the planar spindle orientation was accompanied by a polarized distribution of the actin-binding proteins cortactin and ezrin (9). It is possible that LIMK1-mediated actin stabilization is involved in the pathway from integrin-mediated cell adhesion to the polarized localization of actin-binding proteins; this may contribute to the local accumulation of LGN in the cell cortex. In this respect it is noted that inhibition of the integrin signal causes LGN mislocalization and spindle misorientation in mammalian skin cells (30). These results suggest that LGN localization is important for spindle orientation in both asymmetric and symmetric cell divisions. Further studies are required to answer the questions of how the crescent-shaped localization of LGN is established in nonpolarized cells, how actin dynamics and actin-binding proteins contribute to LGN localization in the cortex, whether the mechanism of LGN localization in symmetric cell division is similar to those in asymmetric cell division in Drosophila and mammalian cells, and how LGN regulates the spindle positioning.

We have also shown that LIMK1 knockdown or cofilin(S3A) overexpression causes the delay of the anaphase onset in HeLa cells. Previous studies also showed that in prostate cancer cell suppression of LIMK1 expression by antisense RNA induces the retardation of cell growth and cell cycle arrest at the G2/M phase, and overexpression of LIMK1 causes the appearance of abnormal multiple centromeres and multipolar spindle, transient cell cycle arrest at the G1/S phase, and delayed progression through the G2/M phase (43, 44). Thus, the level of LIMK1 expression seems to play an important role in proper cell cycle progression and chromosomal stability. Further studies are required to understand the mechanisms linking the LIMK1-mediated actin cytoskeletal changes to the regulation of cell cycle progression and centrosome duplication.

In summary, our data indicate that mitotic LIMK1 activation plays a crucial role in regulating the proper spindle orientation by controlling the stability of cortical actin networks and the
Role of LIM Kinase in Spindle Positioning

cortical localization of LGN in metaphase. Although HeLa cells undergo a symmetric cell division that gives rise to two identical daughter cells after mitosis, LIMK1 may also function in asymmetric cell division by regulating the cortical actin dynamics and asymmetric cortical localization of LGN and other spindle-positioning proteins. The intrinsic and environmental cues, such as cell-cell and cell-substrate interactions, may spatially regulate the cortical actin dynamics and, thus, the astral microtubule-anchoring regions in the cell cortex through the regulation of cofilin activity. Further studies will shed light on the mechanisms governing the spatial and temporal regulation of cortical actin dynamics during the cell cycle and how they regulate the localization of spindle positioning cues and the anchoring of astral microtubules to the cell cortex to determine spindle positioning and orientation during symmetric and asymmetric cell division.

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