The Rho GTP exchange factor Lfc promotes spindle assembly in early mitosis

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Communicated by Anthony J. Pawson, University of Toronto, Toronto, ON, Canada, May 22, 2005 (received for review January 31, 2005)

Rho GTPases regulate reorganization of actin and microtubule cytoskeletal structures during both interphase and mitosis. The timing and subcellular compartment in which Rho GTPases are activated is controlled by the large family of Rho GTP exchange factors (RhoGEFs). Here, we show that the microtubule-associated RhoGEF Lfc is required for the formation of the mitotic spindle during prophase/prometaphase. The inability of cells to assemble a functioning spindle after Lfc inhibition resulted in a delay in mitosis and an accumulation of prometaphase cells. Inhibition of Lfc's primary target Rho GTPase during prophase/prometaphase, or expression of a catalytically inactive mutant of Lfc, also prevented normal spindle assembly and resulted in delays in mitotic progression. Coinjection of constitutively active Rho GTPase rescued the spindle defects caused by Lfc inhibition, suggesting the requirement of RhoGTP in regulating spindle assembly. Lastly, we implicate mDia1 as an important effector of Lfc signaling. These findings demonstrate a role for Lfc, Rho, and mDia1 during mitosis.

Rho family GTPases, such as Rho, Rac, and Cdc42, are small GTPases that act as molecular switches to stimulate reorganization of the cytoskeleton. Although classically studied as regulators of actin, there is growing evidence that Rho GTPases can control the organization of other cytoskeletal proteins such as microtubules (1). The ability of Rho family GTPases to regulate cytoskeletal structures is not limited to interphase cells, because recent studies demonstrate that these proteins have an active and important role during mitosis (2–4).

The Dbl family of Rho guanine nucleotide exchange factors (RhoGEFs) are the primary activators of Rho GTPases in cells (5), and Lfc is a Dbl RhoGEF that was initially identified on the basis of the protein's ability to transform NIH 3T3 cells when overexpressed (6). Subsequent studies demonstrated that Lfc, or its human homologue GEF-H1, can activate RhoA and Rac1 *in vitro* (7, 8), and functions as a RhoA GEF *in vivo* (9). Overexpressed Lfc is associated with interphase microtubules (9, 10), and endogenous Lfc colocalizes with the mitotic spindle (11). The unique localization of Lfc at the mitotic spindle suggested that Lfc might have a role in regulating spindle function. To dissect the potential role of Lfc during mitosis, we used a combination of approaches to inhibit the activity of Lfc, and downstream effectors of Lfc, at specific mitotic stages. Our studies reveal a role for Lfc, Rho, and mDia1 in promoting assembly of the mitotic spindle in early mitosis.

Materials and Methods

Cell Culture, Constructs, and Transfection. Rat-2, Ptk-1, Xlk-1, and HeLa cells were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (HyClone). Transfection of Rat-2 fibroblasts was performed in six-well dishes by using Effectene (Qiagen, Valencia, CA).

Preparation of pBINNS1 LFC and Syk Short Hairpin RNA (shRNA) Vectors. A target shRNA sequence, corresponding to bases 2220–2242 of *Homo sapiens* LFP40 gene [National Center for Biotechnology Information (NCBI) accession no. U72206], was introduced

into the *Asp718* and *XbaI* sites of the human H1 RNA pol III promoter-based shRNA vector pBINNS1 (12) by using the sense and anti-sense-strand oligonucleotides 5'-GTACCAACCTTCAA-TGGCTCCATTGAACCAAGAGAGTTCAATGGAGCCATTGAAGGTTTTTTTGGAAAT-3' and 5'-CTAGATTTCCAA-AAAAACCTTCAATGGCTCCATTGAAGGTTG-3', respectively. The LFC shRNA target sequence is also found in the murine LFC gene (NCBI accession no. AF1177032) from bases 2235–2257. The Syk tyrosine kinase shRNA vector was prepared by using a target sequence directed toward bases 781–802 of the *H. sapiens* Syk gene (NCBI accession no. XM.053535). The sense strand oligonucleotide was 5'-GTACCAAGCAGATGGTTTGTAAAGAGTTCAAGAGAACTCTTAACAAACCATCTGCTTTTTTTGGAAAT-3' and the anti-sense strand was 5'-CTAGATTTCCAAAAAAGCAGATGGAAAGTTAAGAGTTTCTCTTGAAGTCTTAACAAACCATCTGCTTG-3'. The authenticity of the resulting vectors was confirmed by DNA sequencing.

Antibodies. Polyclonal sheep antibodies (Exalpha Biologicals, Maynard, MA) were raised against bacterially produced, recombinant GST-Lfc41–487 (amino acids 41–487 of mouse Lfc) fusion protein. Anti-Lfc antibodies were then purified against bacterially produced GST-Lfc41–487. For antibody microinjection experiments, anti-Lfc, anti-mDia1, or control IgG were dialyzed against PBS overnight before microinjection. The final concentration of antibody used in the microinjection experiments was between 1 and 1.2 $\mu\text{g}/\mu\text{l}$.

Mouse monoclonal antibodies against bovine α -tubulin, Alexa Fluor 594 donkey anti-sheep, Oregon green 488 goat anti-mouse, Alexa Fluor 594 goat anti-mouse, and Alexa Fluor 594 goat anti-rabbit, were from Molecular Probes. Mouse anti-phosphohistone H3 was from Cell Signaling Technology (Beverly, MA). Mouse anti-His was from Santa Cruz Biotechnology. Rabbit anti-pericentrin was from Covance (Richmond, CA). CREST serum was obtained from Cortex Pharmaceuticals, Irvine, CA. Mouse monoclonal anti-mDia1 was obtained from Becton Dickinson.

Recombinant Proteins. cDNA corresponding to amino acids 41–487 of mouse Lfc were amplified from full-length Lfc mouse cDNA and was inserted into pGEX-4T vector (Amersham Pharmacia Biotech). Protein induction and purification of GST fusion proteins were carried out as described (13). His-tagged recombinant RhoAL63 was obtained from Cytoskeleton (Denver). The Rho effector loop mutant library was a kind gift of R. Treisman.

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Abbreviations: RhoGEF, Rho GTP exchange factor; shRNA, short hairpin RNA; MDCK, Madin-Darby canine kidney; ROCK, Rho-associated kinase.

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(London Research Institute, London). The F1F2, D1, and KA3 proteins were generous gifts from J. Copeland (University of Ottawa, Ottawa).

Microinjections. Injections were performed essentially as described (14). For mitotic injections, cells were seeded at low confluency onto coverslip and incubated for 48 h in 10% calf serum. Prophase, metaphase, or telophase cells were identified by nuclear and membrane organization among the asynchronously growing cells and injected over a period of 5 min and fixed at the times described. To inject cells during G₂, hydroxyurea was added to the media $\approx$ 22 h before injection, and washed from the media $\approx$ 4–6 h before injection. To identify injected cells in each experiment, dextran (molecular mass = 30 kDa) labeled with either Oregon Green, or Alexa Fluor 594 (Molecular Probes) was coinjected into cells with either PBS, antibody, C3 transferase, or recombinant protein.

Microscopy. Microscopy was carried out by using the Leica (Deerfield, IL) DMRX microscope and the inverted Leica DMIRB microscope, both equipped with fluorescence and transmitted light optics. Images were obtained by using the Olympus (Melville, NY) 1X-70 inverted microscope equipped with fluorescence optics and DeltaVision deconvolution microscopy software (Applied Precision, Bratislava, Slovakia).

Rho Activation Assays. A mutated serum-response element (SRE), SRE.L (kindly provided by S. Gutkind, NIH, Bethesda), which lacks the c-Fos ternary complex-binding site and responds to RhoA more specifically than the wild-type SRE promoter, was used to monitor activation of RhoA as described by Wells *et al.* (15). Rho activation using the Raichu probes was performed as described in ref. 16. Cdc42 and Rac activation assays were performed by using kits from Cytoskeleton and Upstate Biotechnology (Lake Placid, NY).

Results

To confirm previous observations that Lfc colocalizes with the mitotic spindle during mitosis, we used an affinity-purified antibody that specifically recognizes Lfc (Fig. 4A, which is published as supporting information on the PNAS web site) in immunofluorescence staining of mitotic Rat-2 cells. Consistent with previous studies (11), we observed that endogenous Lfc colocalized with the mitotic spindle (Fig. 5A, which is published as supporting information on the PNAS web site). We tested the hypothesis that Lfc may function during mitosis by knocking down Lfc expression by using shRNA.

Endogenous Lfc protein turns over quantitatively in 24 h (Fig. 4B), and thus the expression of endogenous Lfc protein was analyzed 24 h posttransfection of shRNA vector pBINNS1-Lfc plasmid (12). Lfc protein levels decreased in a dose-dependent manner to transfected shRNA plasmid (Fig. 4C). Endogenous Lfc was significantly reduced in the majority of cells 24 h after transfection of the pBINNS1-Lfc plasmid as detected by Western blot and by immunofluorescence (Fig. 4D).

The mitotic index of cells with reduced levels of Lfc was increased compared with control cells as determined by anti-phosphohistone H3 staining ($12.0 \pm 1.0\%$ vs. $6.4 \pm 0.6\%$, respectively) (Fig. 5B). The number of prometaphase cells was 5-fold greater in Lfc RNA interference (RNAi)-treated cells compared with control cells ($43.4 \pm 6.8\%$ vs. $7.9 \pm 1.8\%$, respectively) (Fig. 5C). Thus, a reduction in endogenous Lfc protein levels in asynchronous cells delayed mitotic progression at the prometaphase–metaphase transition. After knockdown of endogenous Lfc, the microtubules of both prometaphase (Fig. 5D) and metaphase cells (Fig. 5E) were shorter, fewer in number, and directionally disordered compared with control cells (Fig. 5D and E). The interpolar distance was reduced, and the spindle structures were often “collapsed” onto the chromatin in Lfc-deficient cells compared with control cells (Fig.

5E, arrowhead). The localization of spindle pole-associated proteins such as pericentrin (Fig. 5D) or γ -tubulin (data not shown) was unaffected in Lfc knockdown cells. Twenty percent of cells that completed mitosis demonstrated satellite or micronuclei compared with $<1\%$ of control cells (Fig. 5F).

To further examine the role of Lfc during mitosis, we investigated the effects of overexpressing full-length Lfc or a catalytically inactive version of Lfc (Lfc T247A). Lfc T247A encodes an alanine at amino acid 247 instead of a threonine. This residue in Lfc is predicted by sequence alignment to be analogous in function to residues in the Dbl homology domains in other proteins shown to be critical for catalysis of nucleotide exchange (17–20). By two independent assays, we confirmed that the T247A mutation abolishes the exchange activity of Lfc (Fig. 6, which is published as supporting information on the PNAS web site). Overexpression of full-length Lfc in mitotic cells resulted in the formation of monopolar spindles with aberrantly long, extensively bundled microtubules in nearly all cells (Fig. 5G). Expression of Lfc T247A resulted in the nearly total depolymerization of microtubules during mitosis in $\approx 25\%$ of cells (Fig. 5H and I). Approximately half of the cells overexpressing Lfc T247A had shrunken monopolar spindles composed of attenuated and disorganized microtubules (Fig. 5H and I). Thus, overexpression of either full-length Lfc or the catalytically inactive Lfc T247A perturbs microtubule organization during mitosis and demonstrates that Lfc catalytic activity is important in mediating its role in regulating spindle assembly.

To examine the role of Lfc during mitosis, prophase cells were microinjected with anti-Lfc antibodies and fixed 30 min later. Anti-Lfc antibody-microinjected cells showed mitotic spindle defects in prometaphase with failure to polymerize, organize, and recruit microtubules to the spindle poles (Fig. 1B). Cortical microtubules failed to be incorporated into a central spindle structure and accumulated at the periphery of the cell (Fig. 1B, arrowheads). Late prometaphase cells contained shortened and disorganized microtubules emanating from two poles (Fig. 1B arrowheads). The chromatin of anti-Lfc antibody-injected cells that progressed to metaphase was not well organized into a metaphase plate structure, and the spindles were attenuated and collapsed onto the chromatin (Fig. 1A and B, metaphase cells). The microtubules of antibody-injected cells observed in anaphase, telophase, or at cytokinesis were similar to those seen in control cells. Reminiscent of the effects of Lfc RNA interference, chromosomal segregation defects such as anaphase bridges linking daughter nuclei were consistently observed in cells microinjected with anti-Lfc antibodies (Fig. 1B Insets). The number of abnormal anti-Lfc antibody-injected cells harboring abnormal spindle structures was quantified in Fig. 3E.

We quantified the frequency of chromosomal segregation defects in daughter cells that had transited mitosis 24 h after the microinjection of anti-Lfc antibodies into interphase cells. Approximately one third of daughter cells demonstrated numerous segregation defects (Fig. 1C). The chromatin segregation defects observed after injection of Lfc antibodies into prophase cells could have resulted from either perturbation of normal spindle assembly early in mitosis or to abnormalities during cytokinesis. To investigate which mitotic stage was most sensitive to Lfc inhibition, anti-Lfc antibodies were microinjected into cells at prometaphase, metaphase, and telophase. The cells were then fixed rapidly after injection. Prometaphase-injected cells manifested defects identical to those observed after injection of prophase cells (Fig. 1D, 12/15 injected cells). Strikingly however, the spindles of cells injected with anti-Lfc antibody in metaphase ($n = 50$) and anaphase ($n = 60$) were normal, and the spindle poles were well separated from the chromatin (Fig. 1D arrowheads). No defects in cleavage furrow formation or chromatin segregation were observed in anaphase-injected cells that had progressed to telophase (Fig. 1D), or in telophase-injected cells (data not shown). These data are consistent with those observed in the Lfc knock-down cells and suggest that the temporal interval where Lfc function is required for spindle

Fig. 1. Injection of anti-Lfc antibodies into prophase cells. (A) Cells injected with control IgG at prophase, fixed 30 min postinjection, and stained with anti-tubulin antibody (green) and DAPI (blue). (B) Cells injected at prophase with anti-Lfc antibody ($1.2 \mu\text{g}/\mu\text{l}$), fixed 30 min after injection, and stained with DAPI (blue, *Top*) and anti-tubulin antibody (green, *Middle*). (*Bottom*) The merged image is shown. (C) Rat-2 fibroblasts were injected with anti-Lfc antibody and fixed 24 h postinjection. Only one interphase cell was injected per field of view. Cells were then scored on the basis of chromosome organization defects such as satellite or micronuclei, or binucleated and linked. Two hundred cells of each injection were counted. Tubulin is shown in green. (D) Prometaphase, metaphase, or anaphase Rat-2 cells were injected with anti-Lfc antibody, fixed 10 min postinjection, and stained with DAPI (blue, *Top*), anti-tubulin antibody (green, *Middle*). (*Bottom*) The merged image is shown. Anaphase-injected cells typically had progressed to telophase by the time of fixation. (E) Control IgG-injected or anti-Lfc-injected mitotic Rat-2 cells stained by using anti-tubulin antibody (red), CREST serum (green), and DNA (blue). (*Right*) High magnification images of microtubules and kinetochores in the vicinity of the chromatin. (F) Prophase Rat-2 cells were microinjected with either control IgG or anti-Lfc antibody. We acquired 3- to 4- μm sections, and the images were rotated on the axis corresponding to the long axis of the spindle (shown by red lines). The angle of rotation was then determined by measuring the degree of rotation of each spindle pole from the plane of the attachment surface. In control IgG-injected cells, spindles do not rotate significantly from the plane of attachment. However, in anti-Lfc-injected cells, the spindle poles were often significantly rotated. (All scale bars: 10 μm .)

assembly is largely restricted to prometaphase rather than later stages of mitosis. Therefore, the chromosomal defects observed in Lfc knockdown or antibody-microinjected cells likely reflect the failure of the mitotic spindle assembled in early mitosis to properly execute its function during the later stages of mitosis.

Defects in chromatin segregation after Lfc inhibition may result from a defect in the microtubule capture or stabilization at kinetochores (21), and it has been previously observed that Cdc42 can regulate microtubule attachment to kinetochores after their initial capture (2). To visualize microtubule attachment to kinetochores, we stained metaphase cells with anti-tubulin antibodies and CREST serum, which recognizes CENP-A localized at the kinetochore. In control IgG-injected cells, kinetochores were frequently observed in intimate proximity to spindle microtubules and were organized into parallel bundles (Fig. 1E arrows). Kinetochores not associated with microtubules were rarely observed in control cells. However, in prophase cells injected with anti-Lfc antibody, many kinetochores seemed unattached to microtubules, and the microtubules in the vicinity of the chromatin seemed disorganized (Fig. 1E).

The appearance of collapsed spindles after inhibition of Lfc by RNA interference or antibody injection suggested that Lfc was required for the proper tethering of astral microtubules to the

cortex (22). We hypothesized that failure of astral microtubules to be captured and/or attached at the cortex might also result in rotation of the mitotic spindle. We therefore measured the degree of rotation (along the z axis) between the long axis of the mitotic spindle and the plane of attachment of the cells (the x - y plane) when Lfc function was inhibited. The long axis of the mitotic spindle was parallel to the plane of attachment in either uninjected or control IgG-injected Rat-2 cells, demonstrating that the mitotic spindle normally lies parallel to the cellular substratum (Fig. 1F). However, the spindles in anti-Lfc antibody-injected cells were frequently rotated along the z axis and therefore had lost their normal planar orientation within the cell (Fig. 1F).

Rho Activity Is Required for Normal Spindle Assembly. We next investigated which downstream components of the Lfc signaling pathway are required for proper spindle assembly. The major target of Lfc *in vivo* is the Rho subfamily of GTPases (7, 9, 11). However, Rho activity has not been previously shown to be required for mitotic spindle assembly. Our results led us to test the possibility that Rho may be involved in regulating the formation of the bipolar spindle at early stages of mitosis. Prophase cells were injected with varying concentrations of C3 transferase from *Clostridium botulinum* (23, 24) and fixed 30 min postinjection (Fig. 2B). Roughly half

Fig. 2. Rho GTPase activity is important in normal spindle assembly in Rat-2, Xlk-1, MDCK, and Ptk-1 cells. (A) Uninjected or PBS prophase-injected Rat-2 cells fixed 30 min postinjection and stained with DAPI (blue, Left) and anti-tubulin antibody (red, Center). (Right) The merge image is shown. (B) Rat-2 cells injected with 20 μ g/ml (Left) or 40 μ g/ml (Right) C3 transferase, fixed 30 min postinjection and stained with DAPI (blue, Left) and anti-tubulin antibody (red, Center). (Right) The merged image is shown. (C) Rat-2 cells injected with C3 transferase (20 μ g/ml) during G₂, prophase, prometaphase, and metaphase. Cells injected during G₂ were fixed 4–6 h postinjection. Cells injected at prophase, prometaphase, and metaphase were fixed 10 min postinjection. The cells were stained with DAPI (blue, Left), and anti-tubulin antibodies (green, Center). (Right) The merged image is shown. (D) Xlk-1 cells were injected at prophase with either PBS (Left) or 20 μ g/ml C3 toxin (Right), fixed 45 min postinjection, and stained with anti-tubulin antibody (green), and DAPI (blue). (E) MDCK cells were injected at prophase with either PBS (Left) or C3 toxin (Right), fixed 30 min postinjection, and stained as in D. (F) Ptk-1 cells were injected at prophase with PBS (Left) or C3 toxin, fixed 45 min postinjection, and stained as in D. (G) Prophase HeLa cells were injected with 20 μ g/ml C3 toxin, fixed 30 min postinjection, and stained as in D. (All scale bars: 10 μ m.)

(45%) of the C3-injected cells in mitosis had severe defects in spindle assembly, with short spindle microtubules that were poorly organized (Fig. 2B). In some C3-injected cells, we observed ectopic areas of microtubule polymerization in addition to those at the spindles (Fig. 2B arrowheads). None of the dextran injected control cells were observed to have spindle defects (Fig. 2A). The results are summarized in Fig. 3E. We conclude that Rho activity is important for proper assembly of the mitotic spindle.

To investigate when Rho activity is most important in spindle organization during mitosis, C3 transferase was microinjected into Rat-2 cells released from a G₁/S block (G₂ cells), or cells at prophase, prometaphase, metaphase, or telophase. Microinjection of C3 transferase into G₂ (12/14 injected cells), prophase (18/20 injected cells), or prometaphase cells (10/15 injected cells) resulted in spindles with severely attenuated microtubules (Fig. 2C). However, after injection of C3 toxin into metaphase cells ($n = 30$ cells), we observed no effect on spindle organization or microtubule polymerization (Fig. 2C). Taken together, these data suggest that RhoA is required for early mitotic spindle assembly in prophase/prometaphase. Notably, we did not observe that injection of C3 toxin inhibited cytokinesis in Rat-2 cells, an observation that is remarkably consistent with a recent report from Yoshizaki *et al.* (25), which demonstrated that C3 toxin did not prevent cytokinesis in Rat-1a cells.

To test the general requirement for Rho activity for spindle assembly in other cell types, C3 transferase was injected into prophase Xlk-1 *Xenopus* fibroblasts, canine kidney epithelial Madin–Darby canine kidney (MDCK) cells, rat kangaroo kidney epithelium Ptk-1 cells, and human HeLa cells. C3 transferase injected into either prophase Xlk-1 (16/30 injected cells) or MDCK cells (21/27 injected cells) resulted in nearly complete depolymerization of the microtubule array during prophase/early prometaphase and disruption in the formation of the mitotic spindle during prometaphase and metaphase (Fig. 2D and E). In particular, spindles were disorganized, misshapen, and collapsed. A similar phenotype was observed after prophase injection of C3 toxin into Ptk-1 cells,

because the overall density and shape of spindle microtubules seemed affected by C3 (16/40 injected cells) (Fig. 2F). Notably, in Ptk-1 cells where astral microtubules are easily imaged, astral microtubule polymerization was completely inhibited by C3 toxin (Fig. 2F arrowheads). Previous reports have demonstrated that, whereas C3 transferase can prevent normal cytokinesis in HeLa cells, the toxin does not affect normal spindle assembly (26, 27). Consistent with these reports, we observed that C3 microinjection had no effect on spindle assembly in HeLa cells ($n = 60$ cells) (Fig. 2G). These observations suggest that the requirements for RhoA during both spindle assembly and cytokinesis are cell type-specific. Although Rho signaling has a critical role in spindle assembly in Rat-2, Xlk-1, MDCK, and Ptk-1 cells, its function in the highly transformed HeLa tumor cell line seems less important at this stage of mitosis.

To establish the importance of Rho activation by Lfc in spindle assembly, we sought to determine whether constitutively active forms of Rho could complement inhibition of Lfc. Prophase Rat-2 cells were microinjected with anti-Lfc antibodies in combination with recombinant RhoAL63, which is defective in its GTPase function and is thus constitutively active. Coinjection of RhoAL63 with anti-Lfc antibody resulted in near complete rescue of the spindle defects that resulted from microinjection of anti-Lfc antibodies alone (Fig. 3A and E). Furthermore, there was no delay in the prometaphase/metaphase transition as was seen in cells that were injected with anti-Lfc antibody alone (Fig. 3A and E). Normal spindle assembly could thus be fully restored in cells injected with active Rho, suggesting that Lfc acts as a RhoGEF during prophase/prometaphase to promote formation of the mitotic spindle.

To identify downstream signaling proteins activated by Rho GTPase required for spindle assembly, we exploited an allelic series of RhoA mutants that are uncoupled from specific RhoA effectors (28). In particular, we made use of constitutively active RhoA G14V mutants that lack the ability to bind either the mDia family of formins and/or Rho-associated kinase (ROCK) kinases, because both classes of proteins have recently been implicated in regulating

Fig. 3. Lfc regulates spindle assembly via Rho and mDia1. (A) Rat-2 cells injected with anti-Lfc antibody and recombinant His-tagged RhoA163 at prophase, fixed 30 min after injection, and stained with anti-tubulin antibody (green) and DAPI (blue). The final concentration of anti-Lfc antibody was adjusted to be the same as injection of anti-Lfc antibody alone in Fig. 2 (1.2 $\mu\text{g}/\mu\text{l}$), and the final concentration of RhoA163 was 0.5 $\mu\text{g}/\mu\text{l}$. (B–D) Rat-2 cells injected with anti-Lfc antibody and recombinant GST-tagged RhoA G14V/T37Y/C20R (B), G14V/E40L (C), G14V/F39A (D) at prophase, fixed 30 min after injection, and stained as in A. (C) Rat-2 cells injected with anti-Lfc antibody and recombinant GST-tagged RhoA G14V/E40L (0.5 $\mu\text{g}/\mu\text{l}$) at prophase, fixed 30 min after injection, and stained as in A. (D) Rat-2 cells injected with anti-Lfc antibody and recombinant GST-tagged RhoA G14V/F39A (0.5 $\mu\text{g}/\mu\text{l}$) at prophase, fixed 30 min after injection, and stained as in A. (E) Cells were fixed 30 min after injection of the following: control IgG antibody, anti-Lfc antibody, 20 $\mu\text{g}/\text{ml}$ C3 transferase, anti-Lfc antibody and RhoA163, anti-Lfc antibody and RhoAG14V/E40L, anti-Lfc antibody and RhoAG14V/T37Y/C20R, or anti-Lfc antibody and RhoAG14V/F39A. Values represent the percentage of mitotic cells with defects in spindle and/or chromatin organization. A minimum of 50 injected cells were counted in each experiment. (F) Prophase Rat-2 cells microinjected with anti-mDia1 antibody (1 $\mu\text{g}/\mu\text{l}$) and fixed 30 min after injection. Tubulin is shown in green; DNA is shown in blue. (G–I) Prophase Rat-2 cells microinjected with F1F2 (G), D1 (H), or KA3 (I) recombinant protein (all at ≈ 1.0 $\mu\text{g}/\mu\text{l}$), fixed 30 min following injection, and stained as in F. Tubulin is shown in green; DNA is shown in blue. (Insets) Chromatin bridges linking two daughter nuclei. (J) Quantification of the degree of spindle rotation in anti-mDia1 antibody and D1 protein-injected cells. Each data point represents the angle of rotation of one spindle pole from the attachment plane. (K) Anti-mDia1-injected mitotic Rat-2 cell stained by using anti-tubulin antibody (red), CREST serum (green), and DNA (blue). (Right) High magnification images of microtubules and kinetochores in the vicinity of the chromatin. and/or chromatin organization. A minimum

spindle assembly (2, 29). Coinjection of the RhoA mutant T37Y/C20R, which lacks the ability to bind all known Rho effectors, was incapable of rescuing spindle defects resulting from Lfc inhibition. (Fig. 3 *B* and *E*). RhoA E40L, which is uncoupled from ROCK kinases, was fully capable of rescuing the defects resulting from anti-Lfc injection, suggesting that ROCK is not a critical factor in Lfc's ability to regulate spindle microtubules (Fig. 3 *C* and *E*). In support of this observation, Y-27632 had no effect on the assembly of the spindle in either prometaphase or metaphase Rat-2 cells, but cells treated with Y-27632 failed to complete cytokinesis, resulting in binucleated cells (data not shown). Our data demonstrate that Rho-mediated activation of ROCK does not seem to regulate spindle assembly in Rat-2 cells. The function of ROCK during spindle assembly seems to be highly dependent on cell type because the ROCK inhibitor Y-27632 was not reported to affect mitotic

events in HeLa or Rat-1a cells (25, 26, 30). Furthermore, the activity of ROCK's primary target in spindle assembly, myosin II, does not seem to be required in all cell types and organisms for spindle formation (31–35). Coinjection of the RhoA mutant G14V/F39A, which couples only to mDia-related formins but not to other known Rho effectors, was capable of rescuing spindle defects resulting from anti-Lfc antibody injection (Fig. 3C arrowheads and 3E).

Our results suggested that the mDia family of formins are a potential downstream target of Lfc activity during mitosis. mDia1 is localized to both the mitotic spindle and cortical regions during mitosis (27) and can induce microtubule alignment and stabilization at the cell cortex (36). Both mDia1 and mDia3 share the ability to bind the kinetochore protein CENP-A and thereby may link microtubules to kinetochores (2). To test the role of mDia1 in

regulating mitotic spindle assembly, prophase Rat-2 cells were microinjected with a specific mDia1 monoclonal antibody (Fig. 7, which is published as supporting information on the PNAS web site) directed against its Rho-binding domain and fixed 30 min postinjection. Anti-mDia1 antibody injection resulted in the accumulation of prometaphase cells with disorganized mitotic spindles. Distinct spindle poles were rarely observed in anti-mDia1-injected cells (Fig. 3F). The attenuated spindle structures were also collapsed onto the chromatin in metaphase cells. Injection of anti-mDia1 antibody resulted in rotation of the spindle (Fig. 3J), and spindle microtubules seemed unattached to kinetochores (Fig. 3L).

To further test the requirement of mDia1 during mitosis, we next microinjected three different recombinant proteins during prophase: Δ N-F1F2 (F1F2), F1F2 (D1), and F1F2.KA3 (KA3) (Fig. 3G–J). F1F2 is a gain-of-function mutant of mDia1 that is comprised of mDia1's FH1 and FH2 domains. Overexpression of F1F2 has previously been observed to affect chromosomal segregation in HeLa cells (2). D1 is a loss-of-function mutant of mDia1 that has a deletion of the RBD and FH3 domains and an internal deletion of residues 750–770 between FH1 and FH2 (37). KA3 protein is a variant of F1F2 with a truncated FH1 domain and three lysine to alanine point mutations in the FH2 domain (K989/994/999A). The lysine to alanine mutations prevent the binding of mDia1 or mDia3 to CENP-A at the kinetochore (2) and disrupt mDia1-induced microtubule organization (36). KA3, like D1, inhibits activity of wild-type mDia1 *in vivo* and is defective for actin polymerization *in vitro* (37–39). Prophase injection of recombinant F1F2 resulted in defects in spindle organization during prometaphase, and strikingly, severe defects in chromosomal segregation (Fig. 3G), as has been described (2). Prophase injection of recombinant D1 (Fig. 3H) or KA3 (Fig. 3I) also resulted in the accumulation of prometaphase cells with disorganized mitotic spindles collapsed around the chromatin, and injection of recombinant D1 resulted in spindle rotation (Fig. 3J). The daughter chromatids of F1F2, D1, but not KA3-injected cells in anaphase were frequently observed to be linked by means of chromatin bridges which persisted through cytokinesis (Fig. 3G–I Insets). Thus, these data demonstrate that both constitutive activation and inhibition of mDia1 signaling during mitosis result in spindle defects. Taken together with our previous data that only activated forms of Rho that bind mDia formins can rescue spindle defects after Lfc

inhibition, these data suggest that Rho and mDia1 act directly downstream of Lfc during mitosis.

Discussion

We have used a combination of genetic and biochemical approaches to demonstrate that the RhoGEF Lfc, by means of the activation of RhoA and mDia1, regulates the assembly of the mitotic spindle during prometaphase. These data are in agreement with previous reports that have shown that both Rho GTPase and mDia1 promote microtubule stability and cortical microtubule attachment in yeast cells and in mammalian interphase cells (40–44). Similarly, mDia1 has been shown to mediate the stabilization and capture of microtubules in interphase cells (45, 46). Our experiments, however, cannot formally exclude a role for mDia3 in spindle assembly in Rat-2 cells as has recently been shown in HeLa cells (2). Both mDia3 and mDia1 bind equally well to CENP-A, whereas only mDia3 functions to attach mitotic microtubules to the kinetochore in HeLa cells (2). Cdc42 seems to be the operative Rho GTPase in HeLa cells that activates mDia3 during mitosis and is regulated by the RhoGEF Ect-2 and MgcRacGAP (2, 4). Therefore, Lfc/Rho/mDia1 operating in Rat-2 cells may control kinetochore attachment and serve a similar function to the Ect-2/Cdc42/mDia3 pathway in HeLa cells in addition to regulating the attachment of spindle microtubules to cortical sites. Notably, mDia3 binds equally well to Rho, Rac, and Cdc42, whereas RhoA binds to exclusively to mDia1 (2). Thus, Lfc may also be capable of activating both mDia1 and mDia3 in Rat-2 cells. One possibility is that, in Rat-2 cells, both mDia1 and mDia3 act to mediate the attachment of spindle microtubule to kinetochores, and that mDia1 functions exclusively to mediate the attachment of microtubules to cortical sites. Alternatively, the defects in the attachment of spindle microtubules to kinetochores after inhibition of Lfc/Rho/mDia1 may be secondary or indirect consequences of a failure in cortical attachment and spindle orientation.

We thank Exalpa Biologicals for assistance with antibody production. We thank Dr. Richard Treisman for providing the Rho effector mutants, Dr. John Copeland for providing mDia1 constructs, and Dr. Shuh Narumiya for helpful experimental advice. This work was supported by grants from the Canada Foundation for Innovation, the Canadian Institutes of Health Research (CIHR), and the National Cancer Institute of Canada (NCIC). A.W. is a Canada Research Chair in Molecular Medicine and Cell Biology.

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