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Dissecting the Role of Rho-mediated Signaling in Contractile Ring Formation[D] [V]

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In anaphase, microtubules provide a specification signal for positioning of the contractile ring. However, the nature of the signal remains unknown. The small GTPase Rho is a potent regulator of cytokinesis, but the involvement of Rho in contractile ring formation is disputed. Here, we show that Rho serves as a microtubule-dependent signal that specifies the position of the contractile ring. We found that Rho translocates to the equatorial region before furrow ingression. The Rho-specific inhibitor C3 exoenzyme and small interfering RNA to the Rho GDP/GTP exchange factor ECT2 prevent this translocation and disrupt contractile ring formation, indicating that active Rho is required for contractile ring formation. ECT2 forms a complex with the GTPase-activating protein MgcRacGAP and the kinesinlike protein MKLP1 at the central spindle, and the localization of ECT2 at the central spindle depends on MgcRacGAP and MKLP1. In addition, we show that the bundled microtubules direct Rho-mediated signaling molecules to the furrowing site and regulate furrow formation. Our study provides strong evidence for the requirement of Rho-mediated signaling in contractile ring formation.

INTRODUCTION

During cytokinesis, F-actin and bipolar myosin II assemble at the cell equator and form the contractile ring. The motor activity of myosin II is believed to generate the constriction force that cleaves a parent cell into two daughter cells (Robinson and Spudich, 2000; Glotzer, 2001). Proper positioning of the cleavage plane ensures chromosome separation and asymmetric cell division—critical steps for genome stability and development, respectively. However, the spatiotemporal mechanism that regulates positioning of the contractile ring is poorly understood.

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Abbreviations used: C3, C3 exoenzyme; GAP, GTPase-activating protein; GEF, GDP/GTP exchange factor; GFP, green fluorescent protein; MRLC, myosin regulatory light chain; PAV, Pavarotti; PBL, Pebble; RNAi, RNA interference; siRNA, small interfering RNA; TCA, trichloroacetic acid.

The central spindle seems to be the source of the signal for positioning of the contractile ring, yet the nature of the signal is unknown (Cao and Wang, 1996; Wheatley and Wang, 1996). After the onset of anaphase, the microtubules between separating chromosomes are bundled into the central spindle. Central spindle assembly requires a kinesinlike protein, MKLP1/Pavarotti (PAV)/ZEN-4, and a Rho GTPase-activating protein (GAP), MgcRacGAP/RacGAP50C/CYK-4 (Jantsch-Plunger *et al*., 2000; Mishima *et al*., 2002). These proteins form a complex, known as centralspindlin, that catalyzes microtubule bundling in vitro (Mishima *et al*., 2002). MKLP1/PAV/Zen4 is phosphorylated by Cdk1 and the microtubule-bundling activity of centralspindlin is suppressed until the onset of anaphase (Mishima *et al*., 2004).

Centralspindlin is localized at the midzone of the central spindle, where microtubules interdigitate in an antiparallel direction. In *Drosophila*, the RacGAP50C-PAV complex (centralspindlin) interacts with Pebble (PBL), which is required for contractile ring formation (Somers and Saint, 2003). The mammalian orthologue of PBL is ECT2. ECT2 is a Rho GDP/GTP exchange factor (GEF), which activates the small GTPase Rho, and is required for cytokinesis in mammalian cells (Tatsumoto *et al*., 1999).

Although involvement of Rho in cytokinesis has been reported, its exact role is still unclear. A contribution of Rho in contractile ring formation has been reported previously (Kishi *et al*., 1993; Mabuchi *et al*., 1993). However, in fluorescence resonance energy transfer (FRET)-based analysis, it has been concluded that Rho activation is not required for contractile ring formation (Yoshizaki *et al*., 2003). Others reported that Rho is required for cytokinesis in later stages, such as constriction of the contractile ring or severing of the midbody (Drechsel *et al*., 1997; O'Connell *et al*., 1999).

In this study, we show that Rho acts as a microtubule-dependent signal that specifies the position of the contractile ring. We show that active Rho is required for contractile ring formation and that ECT2 RhoGEF, MgcRacGAP, and MKLP1 kinesin regulate translocation of Rho to the equatorial region. We also show that the Rho-mediated signaling for furrow positioning is spatiotemporally regulated by the postmitotic spindle structure.

MATERIALS AND METHODS

Antibodies

Rabbit anti-human MgcRacGAP antibody was raised against the glutathione *S*-transferase (GST)-fusion protein. The ECT2 antibody was reported previously (Tatsumoto *et al*., 1999). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-RhoA (26C4); anti-RhoA, B, and C (SC-179); anti-MKLP1 (N-19); and anti-Citron-kinase (C-20). Other antibodies used are as follows: anti-α-tubulin (Sigma-Aldrich, St. Louis, MO); anti-Rac1, anti-Citron-kinase, and anti-ROCK-I (BD Biosciences, San Jose, CA); anti-Cdc42 and anti-ROCK-II (Upstate Biotechnology, Lake Placid, NY); anti-pericentrin (Covance, Princeton, NJ); and anti-mono-phosphorylated myosin regulatory light chain (MRLC) (Cell Signaling Technology, Beverly, MA).

Cell Culture and Drug Treatments

HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For blebbistatin and latrunculin B experiments, HeLa cells were incubated with 100 μM blebbistatin (Calbiochem, San Diego, CA) or 20 μM latrunculin B (Calbiochem) for 6–8 h. For roscovitine experiments, HeLa cells were synchronized with 40 ng/ml nocodazole (Sigma-Aldrich) for 2.5 h, and the cells were harvested by shake-off. The harvested cells were plated on poly-D-lysine-coated coverslips and incubated with 40 ng/ml nocodazole for additional 30 min. Then, nocodazole was washed out, and the cells were incubated with 100 μM roscovitine (A. G. Scientific, San Diego, CA). For pretreatment with roscovitine, 100 μM roscovitine was added 15 min before nocodazole washout.

Stable Expression of Histone H2B-GFP and MRLC-GFP in HeLa Cells

The coding sequences for human Histone H2B and myosin regulatory light chain (MRLC) were obtained from HeLa cell cDNA by PCR amplification and were cloned into an expression vector, pEGFP-N1 (BD Biosciences), containing neomycin resistance. HeLa cells were transfected with the expression vectors by FuGENE 6 (Roche Diagnostics, Indianapolis, IN) and were cultured in the presence of 1 mg/ml G418 (Invitrogen, Carlsbad, CA) for 14 d. Stable colonies that express the green fluorescent protein (GFP)-fusion proteins were selected.

RNA Interference (RNAi)

The custom-synthesized small interfering RNAs (siRNA) were purchased from Dharmacon (Chicago, IL). Pools of 20- to 21-bp siRNAs were produced in vitro (Myers *et al*., 2003) by using recombinant Dicer (Stratagene, La Jolla, CA)). The siRNA sequences used in this study are listed in Table S1. Luciferase siRNA was used as a control. Transient transfection of siRNA was carried out, as described previously (Harborth *et al*., 2001).

Scrape-Loading with C3 Exoenzyme into HeLa Cells

HeLa cells were loaded with C3 exoenzyme (Calbiochem) by the scrape-loading method (McNeil *et al*., 1984) with slight modification. Briefly, HeLa cells in a 35-mm dish (1×10^5 cells) were scraped in 100 μl of 0.1 mg/ml C3 dissolved in phosphate-buffered saline (PBS) with a rubber policeman and incubated at room temperature for 10 min. The cells were washed with DMEM and seeded on coverslips.

Flow Cytometry

HeLa cells were harvested by trypsinization. The cells were washed with PBS and fixed with 2% formaldehyde in PBS. Then, the cells were incubated with 40 μg/ml RNase A for 1 h at 37°C followed by addition of 0.05 mg/ml propidium iodide (Sigma-Aldrich). Flow cytometry analysis was carried out using the FACScan system (BD Biosciences). In each preparation, 2×10^4 cells were counted.

Immunoprecipitation

Immunoprecipitation was carried out as reported previously (Mishima *et al*., 2002), with slight modification. Briefly, HeLa cells were synchronized using double thymidine block and nocodazole arrest. The cells were lysed with buffer A (20 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml chymostatin) with 0.5% Triton X-100, 1–1.5 h after nocodazole washout. For immunoprecipitation, 0.5 μg of IgG was added to the lysates, and the mixture was incubated for 1 h, followed by incubation with protein A-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) at 4°C. The beads were washed with buffer A and subjected to SDS-PAGE followed by immunoblotting.

Yeast Two-Hybrid Analysis

A Two-hybrid analysis was carried out as described previously (James *et al*., 1996).

Immunostaining and Microscopy

HeLa cells on coverslips were fixed with 4% formaldehyde in PBS. For staining of Rho or Citron-kinase, cells were fixed with 10% trichroloacetic acid (TCA) in PBS. The cells were permeabilized with 0.1% Triton X-100 in PBS and were blocked with 2% bovine serum albumin for 1 h. Then, the cells were incubated with primary antibodies. The cells were washed and incubated with fluorescence dye-coupled secondary antibodies (Invitrogen). The coverslips were mounted on glass slides with 80% glycerol. For staining of MKLP1, MgcRacGAP, or ECT2, cells were fixed with 100% methanol at −20°C and processed as described above, except that the permeabilization and blocking steps were omitted.

The double staining with two different rabbit antibodies are as follows. First, the methanol-fixed cells were incubated with a primary antibody, followed by incubation with the secondary antibody conjugated with Alexa Fluor 568 (Invitrogen). Then, the cells were stained with a different primary antibody labeled by Zeon Alexa Fluor 488 rabbit IgG labeling kit (Invitrogen).

The specimens were observed, using an Axiovert S100 fluorescence inverted microscope (Carl Zeiss, Thornwood, NY) equipped with an OrcaER (Hamamatsu Photonics, Bridgewater, NJ) cooled digital camera. Time-lapse microscopy was carried out on the Axiovert S100 equipped with an environmental chamber (Carl Zeiss) maintained with 5% CO_2.

RESULTS

Essential Genes for Cytokinesis in Rho-mediated Signaling

To clarify which genes are essential for cytokinesis, we treated HeLa cells with siRNAs to the genes involved in Rho-mediated signaling. These included RhoGEF (ECT2), GAP (MgcRacGAP), kinesinlike proteins (MKLP1 and CHO1), Rho GTPases (RhoA, Rac1, and Cdc42), and Rho-effector kinases (ROCK-I, ROCK-II, and Citron-kinase). We used siRNA to firefly luciferase as a control. Treatment with each siRNA yielded 90–99% depletion of the target protein 72 h after transfection (Figure 1). Because MKLP1 and CHO1 are the longer and the shorter isoforms, respectively, encoded by the same kinesin gene, siRNA to MKLP1 depleted both MKLP1 and CHO1 (Figure 1A).

To evaluate cytokinesis failure, these siRNA-treated cells were examined by two different procedures: flow cytometry and morphological analysis. The flow cytometry profiles showed that ECT2, MgcRacGAP, MKLP1, and Citron-kinase RNAi increased the ratio of tetraploid (4N) and polyploid (8N and 16N) cells (Figure 2A). Morphological analysis confirmed increase in the percentage of bi/multinucleate cells in ECT2, MgcRacGAP, MKLP1, and Citron-kinase RNAi cells (Figure 2, B and C). To further verify essential genes for cytokinesis, we continued siRNA treatment for 6 d. ECT2, MgcRacGAP, MKLP1, and Citron-kinase RNAi led to giant cells with multinuclei (Figure S1A) or multipolar spindles (Figure S1B), indicating multiple failures of cytokinesis. Thus, our combined flow cytometry and morphological analyses show that in HeLa cells, ECT2, MgcRacGAP, MKLP1, and Citron-kinase are required for cytokinesis, whereas CHO1, RhoA, Rac1, Cdc42, ROCK-I, and ROCK-II are not essential for cytokinesis.

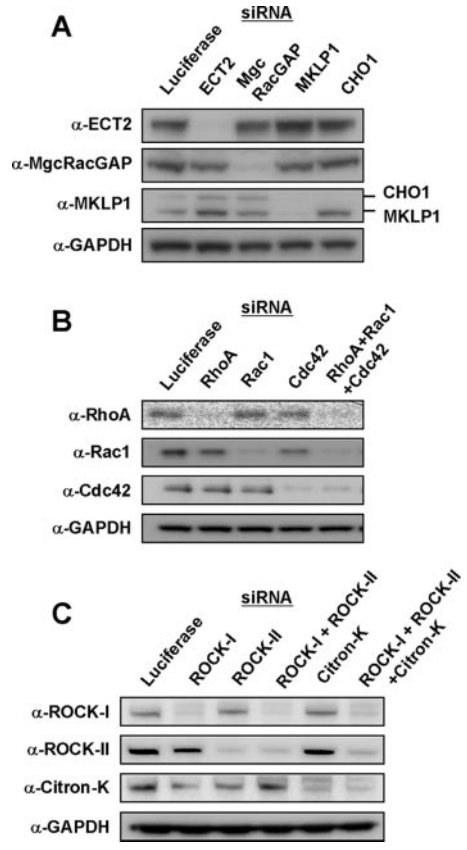

Figure 1. Immunoblots showing siRNA-mediated depletion of the proteins involved in Rho-mediated signaling. HeLa cells were treated with the indicated siRNAs for 72 h. GAPDH, glyceraldehyde-3-phosphate dehydrogenase, for a loading control.

Translocation of Active Rho Subfamily Proteins to the Equatorial Region Is Required for Contractile Ring Formation

Unexpectedly, RhoA RNAi did not inhibit cytokinesis. In the RhoA-depleted cells, the cleavage furrow was observed (Figure 3A), suggesting that RhoA is dispensable for cytokinesis. In mammalian cells, the Rho subfamily includes three close homologues, RhoA, RhoB, and RhoC, which might have redundant functions in cytokinesis. To confirm this, we did immunostaining and immunoblotting of RhoA-depleted cells, using RhoA-specific and pan-RhoA, B, and C antibodies (Yonemura *et al*., 2004). Both antibodies stained the cleavage furrow in control luciferase RNAi cells (Figure 3A, a and c). In RhoA RNAi cells, RhoA-specific antibody failed to stain the cleavage furrow (Figure 3A, b), but pan-RhoA, B, and C antibody staining remained at the cleavage furrow (Figure 3A, d). Thus, besides RhoA, other Rho subfamily proteins, such as RhoB or RhoC, are localized at the cleavage furrow and may compensate for the loss of RhoA function.

C3 exoenzyme (C3) is an ADP-ribosyltransferase specific for Rho (but not Rac1 or Cdc42) and inactivates its functions (Fiorentini *et al*., 1998). It is reported that C3-microinjected HeLa cells frequently detach from the dish or fail to initiate anaphase (O'Connell *et al*., 1999). To avoid these problems, we used the scrape-loading method to deliver C3 to HeLa cells (McNeil *et al*., 1984). The advantage of the scrape-loading method enabled us to analyze a large number of C3-loaded cells without decreasing cell viability. Treatment with C3 increased binucleate cells to ~80%, 24 h after loading (Figure S2, A and B). In the flow cytometry profile, C3 increased the population of tetraploid and polyploid cells (Figure S2C). Giant cells with multinuclei or multipolar spindles occurred 5 d after loading with C3 (Figure S2D). These findings support the idea that Rho plays an essential role in cytokinesis.

The localization of Rho during cell division is inconclusive. It is reported that the endogenous or tagged Rho protein is localized at the cell cortex but is not concentrated at the contractile ring (Drechsel *et al*., 1997; Yoshizaki *et al*., 2003). Recently, Rho has been detected at the cleavage furrow, using the TCA fixation method (Yonemura *et al*., 2004). We used this TCA fixation method and examined the localization of Rho in HeLa cells at various mitotic stages. Before the onset of anaphase, Rho was localized in the cytoplasm (Figure 3C, a). We discovered that after chromosome separation, Rho translocated to the equatorial region before furrow ingression (Figure 3C, b). As reported previously (Yonemura *et al*., 2004), Rho accumulated at the ingressed cleavage furrow and concentrated at the midbody (Figure 3C, c and d). Next, we examined the localization of Rho in C3-loaded cells. We found that C3 delocalized Rho from the equatorial region (Figure 3D). Thus, Rho (presumably active) accumulates at the equatorial region before furrow ingression.

Rho Activity Is Required for Accumulation of Myosin II at the Equatorial Region

To clarify the relationship between Rho and contractile ring formation, we scrape-loaded cells with the Rho-specific inhibitor C3 and examined the localization of myosin II and actin. C3 inhibited accumulation of both myosin heavy chain and actin at the equatorial region (Figure 3E). Thus, Rho is required for acto-myosin contractile ring formation.

To directly visualize the assembly of the contractile ring in living cells, we established HeLa cells that stably express MRLC-GFP. First, we determined the distribution of MRLC-GFP in PBS-loaded control cells during cell division. MRLC-GFP accumulated at the equatorial region after chromosome separation (Figure 4A, +4, +6 min; and Movie S1). Then, the cleavage furrow ingressed as MRLC-GFP accumulated (Figure 4A, +10 to +18 min). The furrow constricted to form the midbody (Figure 4A, +28 min). Finally, the midbody was severed and two daughter cells were separated (Figure 4A, +268 min). Next, we examined the localization of MRLC in C3-loaded cells. MRLC-GFP did not accumulate at the equatorial region even after chromosome separation (Figure 4B, +6, +10 min; and Movie S2). The cells spread without cleavage furrow ingression (Figure 4B, +20 to +90 min) and binucleate cells were generated (Figure 4B, +90 min). We monitored the scrape-loaded cells for 24 h by time-lapse microscopy. Although mock cells completed normal cell division (>99%; n = 188), C3-loaded cells failed to form the cleavage furrow (63%; n = 90). These observations reveal that during cytokinesis, Rho regulates myosin dynamics and contractile ring formation.

Phosphorylation of MRLC leads to bipolar myosin filaments, resulting in the formation of acto-myosin fibers (Applegate and Pardee, 1992). It has been reported that MRLC is phosphorylated at the cleavage furrow (Matsumura *et al*., 1998; Murata-Hori *et al*., 1998; Ueda *et al*., 2002). We found that phosphorylated MRLC colocalized with Rho at the equatorial region before furrow ingression (Figure 3F, Mock). C3 suppressed accumulation of phosphorylated MRLC at the equatorial region (Figure 3F, C3). Because MRLC is a substrate of Citron-kinase (Yamashiro *et al*., 2003) and Citron-kinase is localized at the cleavage fur-

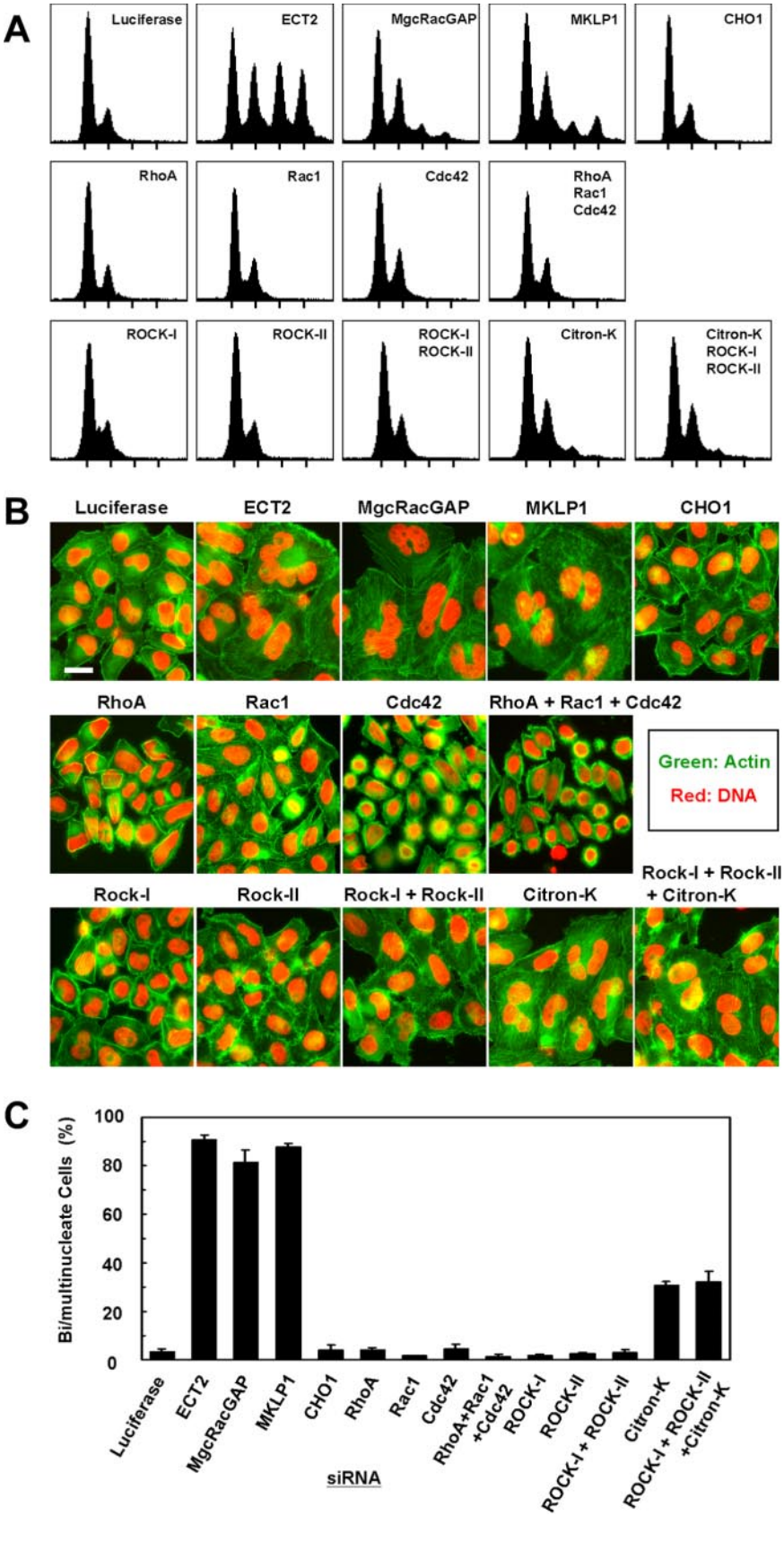

Figure 2. Effects of RNAi on cytokinesis. HeLa cells were treated with the indicated siRNAs for 72 h. (A) Flow cytometry profiles; horizontal axis, DNA content; vertical axis, cell counts. Positions corresponding to 2N, 4N, 8N, and 16N are indicated. (B) Morphology of siRNA-treated cells stained with 4,6-diamidino-2-phenylindole for DNA (red) and phalloidin for F-actin (green). Bar, 25 μm. (C) Frequencies of bi/multinucleate cells. In each of three independent experiments, 250 cells were examined. Values are shown as mean (%) ± SD.

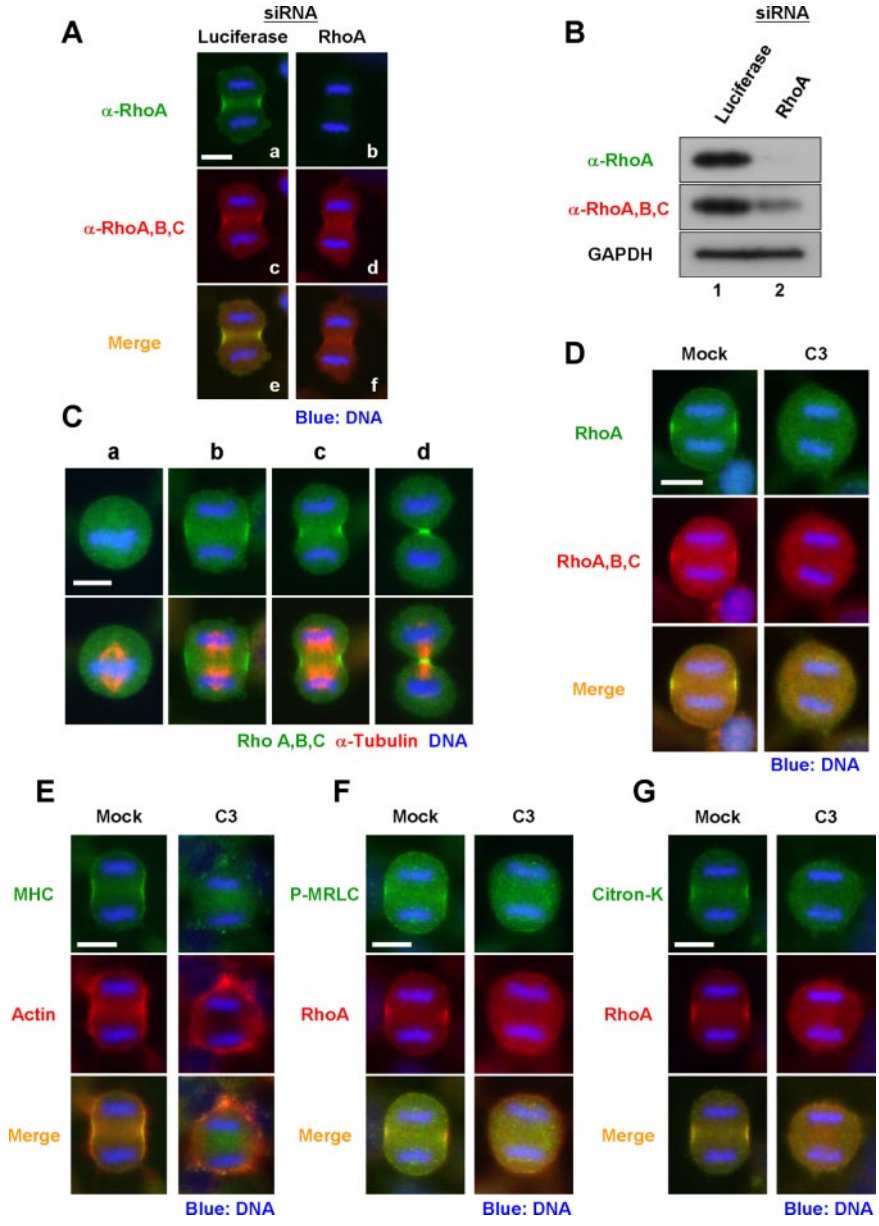

Figure 3. Translocation of active Rho to the equatorial region is required for contractile ring formation. (A and B) RhoA RNAi. Cells were treated with siRNA to Luciferase or RhoA for 48 h. (A) Cells stained with RhoA-specific and pan-RhoA, B, C antibodies. Bar, 10 μm. (B) Immunoblots, using RhoA-specific and pan-RhoA, B, and C antibodies. (C) Localization of Rho during mitosis. HeLa cells were fixed with 10% TCA and stained with pan-RhoA, B, and C antibody. a, metaphase; b, anaphase A; c, anaphase B; and d, telophase. Bar, 10 μm. Note that Rho translocated to the equatorial region before furrow ingression (b). (D) C3 delocalizes Rho from the equatorial region. Cells were stained with anti-RhoA and anti-RhoA, B, and C antibodies. Bar, 10 μm. (E–G) Active Rho is required for contractile ring formation. Bars, 10 μm. HeLa cells were loaded with PBS (Mock) or C3. Cells were stained with anti-myosin heavy chain (MHC) antibody and phalloidin (Actin) (E); anti-phosphorylated myosin regulatory light chain (P-MRLC) and anti-RhoA antibodies (F); anti-Citron-kinase (Citron-K) and anti-RhoA antibodies (G). In mock cells, myosin heavy chain and F-actin accumulated at the equatorial region (E; Mock); phosphorylated MRLC and Citron-kinase colocalized with RhoA at the equatorial region (F and G; Mock). In C3-loaded cells, acto-myosin contractile ring formation was disrupted (E; C3); accumulation of phosphorylated MRLC and Citron-kinase at the equatorial region (F and G; C3) were inhibited.

row (Madaule *et al*., 1998), we examined the localization of Citron-kinase. Like phosphorylated MRLC, Citron-kinase accumulated at the equatorial region before furrow ingression, and C3 inhibited this accumulation (Figure 3G). Thus, Rho is required for accumulation of phosphorylated MRLC and Citron-kinase at the equatorial region.

Rho Localization Is Independent of Myosin and Actin

Inhibition of myosin II ATPase activity does not prevent contractile ring formation (Straight *et al*., 2003). To determine whether myosin is required for the localization of Rho, we treated cells with blebbistatin, a myosin II ATPase inhibitor. Blebbistatin blocked furrow ingression but not contractile ring formation (Figure 5, K and N). Rho and the downstream signals of Rho, Citron-kinase and phosphorylated MRLC, accumulated at the equatorial region (Figure 5, B, E, and H). Thus, the motor activity of myosin II is not required for translocation of Rho to the equatorial region.

It has been reported that in yeast, accumulation of myosin at the cleavage furrow does not depend on F-actin (Bi *et al*., 1998; Motegi *et al*., 2000). To test whether actin is required for Rho localization, we disrupted F-actin structure, using latrunculin B (Figure 5O). Rho accumulated at the equatorial region (Figure 5C), indicating that F-actin is not required for Rho localization. Also, Citron-kinase and phosphorylated MRLC accumulated at the equatorial region (Figure 5, F and I). Myosin heavy chain accumulated at the equatorial region but the distribution of myosin heavy chain was abnormal (Figure 5L, granular structures), suggesting the requirement of actin for proper distribution of myosin II.

ECT2, MgcRacGAP, and MKLP1 Regulate Translocation of Rho to the Equatorial Region

Overexpression of oncogenic Dbl GEF has been shown to translocate Rho to the plasma membrane in interphase cells (Michaelson *et al*., 2001). We examined whether ECT2 Rho-

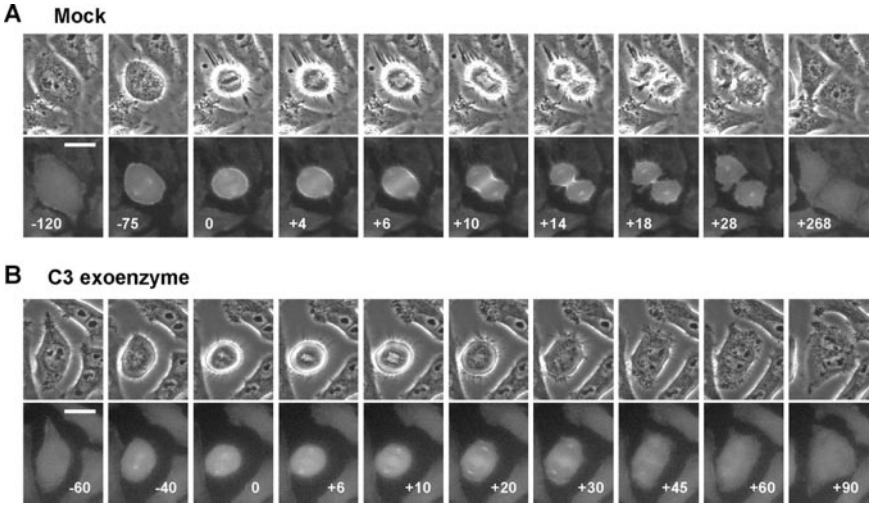

Figure 4. C3 exoenzyme inhibits contractile ring formation. (A and B) HeLa cells that stably express MRLC-GFP were loaded with PBS (Mock) or C3 by the scrape-loading method. Selected images of time-lapse recording of mock cells (A; Movie S1) and C3-loaded cells (B; Movie S2). Time in minutes from the onset of anaphase (time point 0) is indicated. Bars, 10 μm. C3 inhibited accumulation of MRLC to the equatorial region (B).

GEF is responsible for translocation of Rho to the equatorial region in anaphase. Depletion of ECT2 by RNAi diminished both RhoA and RhoA, B, and C staining at the equatorial region (Figure 6A, b). In ECT2-depleted anaphase cells, RhoA was delocalized from the equatorial region or exhibited aberrant localization (79 and 16%, respectively; Figure 6, B and C). Thus, ECT2 regulates translocation of Rho to the equatorial region.

MgcRacGAP and MKLP1 form a complex at the central spindle in anaphase and are required for completion of cytokinesis (Mishima *et al*., 2002). To test whether MgcRacGAP and MKLP1 regulate contractile ring formation, we examined the localization of Rho in MgcRacGAP- or MKLP1-depleted cells. As in ECT2 RNAi cells, Rho was delocalized or exhibited abnormal localization in MgcRacGAP or MKLP1 RNAi cells (Figure 6, A–C). Thus, MgcRacGAP and MKLP1 are also responsible for translocation of Rho to the equatorial region.

Because Citron-kinase and phosphorylated MRLC are the downstream signals of Rho, we examined the localization of these proteins in the siRNA-treated cells. Like C3 treatment, ECT2, MgcRacGAP, or MKLP1 RNAi inhibited accumulation of phosphorylated MRLC and Citron-kinase at the equatorial region (Figure 6D). Thus, ECT2, MgcRacGAP, and MKLP1 regulate the downstream signals of Rho for contractile ring formation.

ECT2, MgcRacGAP, and MKLP1 Regulate Contractile Ring Formation

The requirement of ECT2, MgcRacGAP, and MKLP1 for completion of cytokinesis (severing of the intercellular bridge or abscission) has been reported (Tatsumoto *et al*., 1999; Jantsch-Plunger *et al*., 2000; Kuriyama *et al*., 2002; Matuliene and Kuriyama, 2002; Minoshima *et al*., 2003). To confirm which step of cytokinesis is regulated by these genes, we treated HeLa cells with siRNAs and recorded time-lapse images. ECT2, MgcRacGAP, or MKLP1 RNAi caused a variety of defective cytokinesis phenotypes, including no cleavage furrow formation, incomplete furrowing, and regression (Figure S3, A and B). These observations suggest that ECT2, MgcRacGAP, or MKLP1 regulates multiple steps of cytokinesis, including contractile ring formation, furrow ingression, and completion of cytokinesis. Alternatively, they may only regulate the initial step of cytokinesis (contractile ring formation), which affects later cytokinetic events.

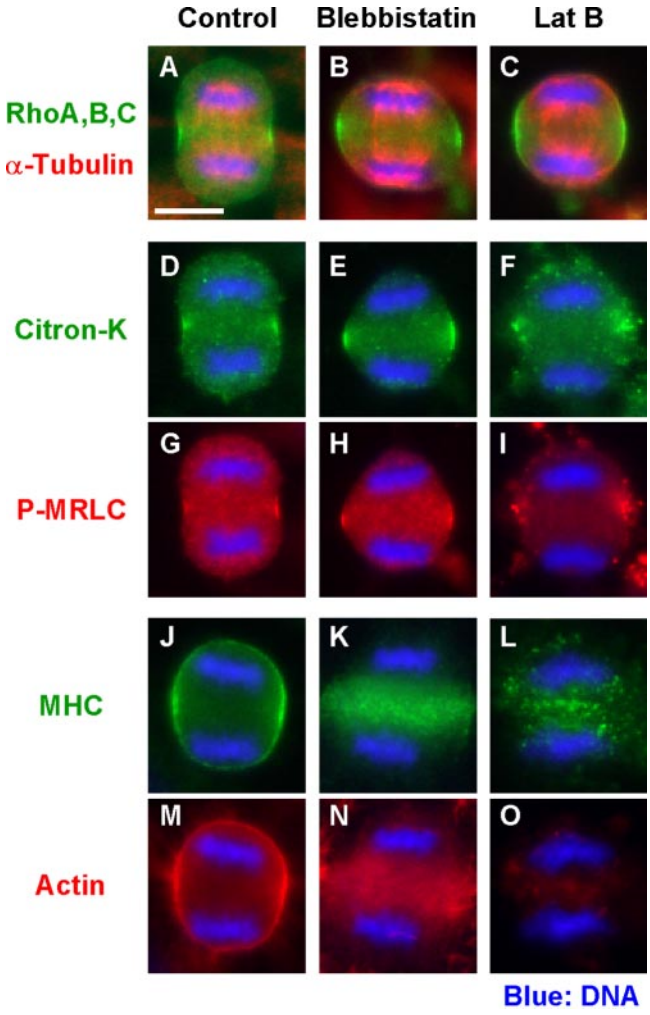

Figure 5. Translocation of Rho to the equatorial region is independent of actin and myosin. HeLa cells were treated with DMSO (Control), blebbistatin, or latrunculin B (Lat B). Cells were fixed and stained with anti-Rho A, B, and C, anti-α-tubulin, anti-Citron-kinase (Citron-K), anti-phosphorylated MRLC (P-MRLC), anti-myosin heavy chain (MHC) antibodies, and phalloidin (Actin).

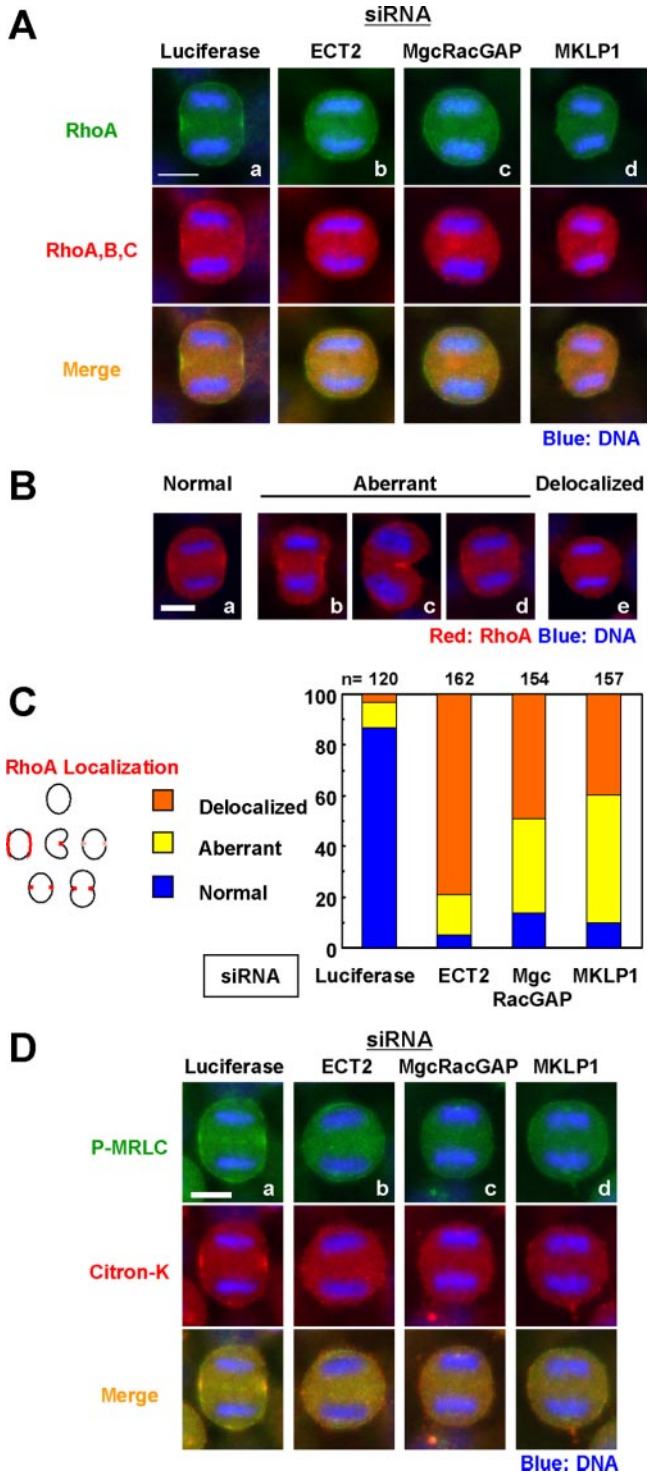

Figure 6. ECT2, MgcRacGAP, and MKLP1-dependent translocation of Rho to the equatorial region. HeLa cells were treated with siRNA to luciferase, ECT2, MgcRacGAP, or MKLP1 for 36 h. The cells were fixed with 10% TCA. Bars, 10 μm. (A) Cells stained with RhoA-specific and pan-RhoA, B, and C antibodies. ECT2, MgcRacGAP, or MKLP1 RNAi diminished the level of staining for both RhoA and RhoA, B, and C. (B and C) Abnormal localization of Rho in ECT2, MgcRacGAP, or MKLP1 RNAi cells. HeLa cells were stained with anti-RhoA antibody and the localization of RhoA was examined. (B) Examples of normal and abnormal localization of RhoA. Shown are MKLP1 RNAi cells. Localization of RhoA was classified as follows: a, Normal; b–d, Aberrant; and e, Delocalized. (C) Scoring of RhoA localization in anaphase cells. (D) Cells stained with phosphorylated MRLC (P-MRLC) and Citron-kinase (Citron-K). ECT2, MgcRacGAP, or MKLP1 RNAi inhibited accumulation of Citron-kinase and phosphorylated MRLC at the equatorial region.

Oceguera-Yanez *et al*. (2005)reported that ECT2 and MgcRacGAP regulate bipolar spindle assembly in prometa- to metaphase. However, by our time-lapse microscopy, we were not able to detect major detects in mitosis, such as prolonged prometaphase or chromosome mis-separation, in ECT2 or MgcRacGAP RNAi cells.

To analyze the relationship between the depletion level of the ECT2, MgcRacGAP, or MKLP1, and defects in contractile ring formation, we treated MRLC-GFP-expressing cells with siRNA to luciferase, ECT2, MgcRacGAP, or MKLP1. When ECT2, MgcRacGAP, or MKLP1 were almost completely depleted, MRLC-GFP did not accumulate at the equatorial region and furrow ingression was prevented (Figure S4F). In contrast, residual amounts of ECT2, MgcRacGAP, and MKLP1 led to accumulation of MRLC-GFP at the equatorial region and ingression of the furrow (Figure S4, C–E). Therefore, it seems that the severity of abnormal cytokinesis phenotypes correlate with the depletion level of each protein.

To show failure of contractile ring formation directly, we treated MRLC-GFP-expressing cells with siRNAs and recorded time-lapse images. Luciferase RNAi cells exhibited normal furrow ingression with accumulation of MRLC at the equatorial region (Figure 7A and Movie S3). In contrast, ECT2 RNAi inhibited accumulation of MRLC at the equatorial region, indicating that contractile ring formation was disrupted (Figure 7B, 0 to +20 min; and Movie S4). These cells spread without dividing, resulting in binucleate cells (Figure 7B, +25 to +72). MgcRacGAP or MKLP1 RNAi caused similar defects in accumulation of MRLC without forming the cleavage furrow (Figure 7, C and D, and Movies S5 and S6, respectively). Thus, ECT2, MgcRacGAP, and MKLP1 all regulate myosin dynamics and contractile ring formation during cytokinesis.

ECT2 Forms a Complex with MgcRacGAP and MKLP1 at the Central Spindle

In *Drosophila*, PBL RhoGEF forms a complex with RacGAP50C and PAV kinesin. RacGAP50C and PAV are colocalized at the central spindle but PBL is localized at the equatorial cell cortex (Somers and Saint, 2003). We examined the localization of mammalian orthologues ECT2, MgcRacGAP, and MKLP1 during cell division. In metaphase, they were localized in the cytoplasm. (Figure 8A, Metaphase). After the onset of anaphase, MgcRacGAP and MKLP1 accumulated at the central spindle (Figure 8A, a, Anaphase). Although, in *Drosophila*, PBL RhoGEF does not colocalize with RacGAP50C and PAV at the central spindle (Somers and Saint, 2003), we found that in mammalian cells, ECT2 colocalized with MgcRacGAP and MKLP1 at the interdigitating portion of the central spindle (Figure 8A, b and c, Anaphase).

To examine whether ECT2 forms a complex with MgcRacGAP and MKLP1, we coimmunoprecipitated these proteins, using an extract from HeLa cells synchronized in anaphase to cytokinesis. MgcRacGAP was coimmunoprecipitated with MKLP1 (Figure 8B, b, lane 5) and MKLP1 was coimmunoprecipitated with MgcRacGAP (Figure 8B, c, lane 4), confirming formation of an equimolar complex known as centralspindlin (Mishima *et al*., 2002). We found that ECT2 was coimmunoprecipitated with MgcRacGAP and MKLP1

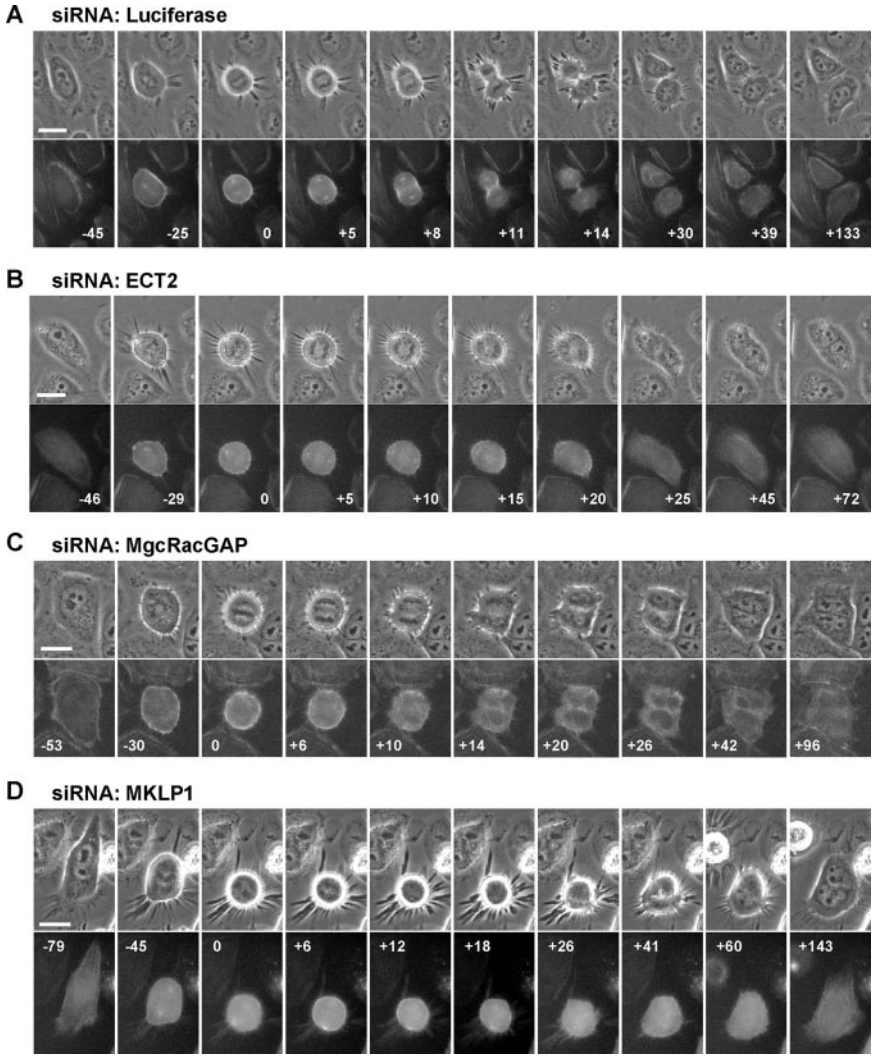

Figure 7. Formation of the contractile ring depends on ECT2, MgcRacGAP, and MKLP1. (A–D) Distribution of MRLC during cell division. Selected images from time-lapse recording. HeLa cells that stably express MRLC-GFP were treated with siRNA to Luciferase (A; Movie S3), ECT2 (B; Movie S4), MgcRacGAP (C; Movie S5), and MKLP1 (D; Movie S6). Bars, 10 μm. Time in minutes from the onset of anaphase (time point 0) is indicated. ECT2, MgcRacGAP, or MKLP1 RNAi inhibited the accumulation of MRLC at the equatorial region (B–D).

(Figure 8B, a, lane 4 and lane 5, respectively) and vice versa (Figure 8B, b and c, lane 3). Thus, ECT2 RhoGEF forms a complex with MgcRacGAP and MKLP1.

To identify which component of centralspindlin (MgcRacGAP or MKLP1) directly interacts with ECT2, we did a yeast two-hybrid analysis (Figure 8C). As expected, an interaction between MgcRacGAP and MKLP1 was detected. We found that ECT2 interacted with MgcRacGAP but not MKLP1.

We tested whether this complex formation determines the localization of ECT2, MgcRacGAP, and MKLP1 at the central spindle. First, we examined the localization of ECT2 in MgcRacGAP or MKLP1-depleted cells. Luciferase RNAi did not affect the localization of ECT2 at the central spindle (Figure 9A). In contrast, MgcRacGAP or MKLP1 RNAi delocalized ECT2 from the central spindle (Figure 9, C and D). Thus, the localization of ECT2 at the central spindle depends on both MgcRacGAP and MKLP1. Next, we examined the localization of MgcRacGAP and MKLP1. ECT2 RNAi did not affect the localization of these proteins (Figure 9, F and J). However, MgcRacGAP RNAi delocalized MKLP1 (Figure 9H) and MKLP1 RNAi delocalized MgcRacGAP (Figure 9K) from the central spindle. Thus, MgcRacGAP localization depends on MKLP1 and MKLP1 localization depends on MgcRacGAP.

Bundled Microtubules Direct the Furrowing Site via Rho-mediated Signaling

Because anaphase microtubules specify the position of the contractile ring, we examined the relationship between microtubules and Rho. The microtubule inhibitor nocodazole inhibits polymerization of microtubules and arrests cells in prometaphase. Therefore, nocodazole prevented the onset of anaphase and translocation of Rho to the equatorial region (Figure 10B, Nocodazole). Inactivation of Cdk1/cyclin B regulates the timing of mitotic exit and cytokinesis as well as the mitotic spindle array (Wheatley *et al*., 1997; Echard and O'Farrell, 2003; Mishima *et al*., 2004). Recently, it has been reported that the Cdk1 inhibitors BM-1026 and purvanalol A promote premature mitotic exit and furrow ingression (Niiya *et al*., 2005). To test whether the Cdk1 inhibitor roscovitine affects initiation of cytokinesis, we treated synchronized prometaphase cells with DMSO (control) or roscovitine (Figure 10A, a and b). Control cells entered anaphase 1–3 h after nocodazole release, and 88% (n = 65) of the cells completed normal mitosis and cytokinesis within 4 h (Figure 10, C and D, a; and Movie S7). Like other Cdk1 inhibitors (Niiya *et al*., 2005), roscovitine promoted premature exit from mitosis (Movie S8). The cells formed ectopic furrows (Figure 10C, b, arrows) and exhibited cortical contraction (81%; n = 115).

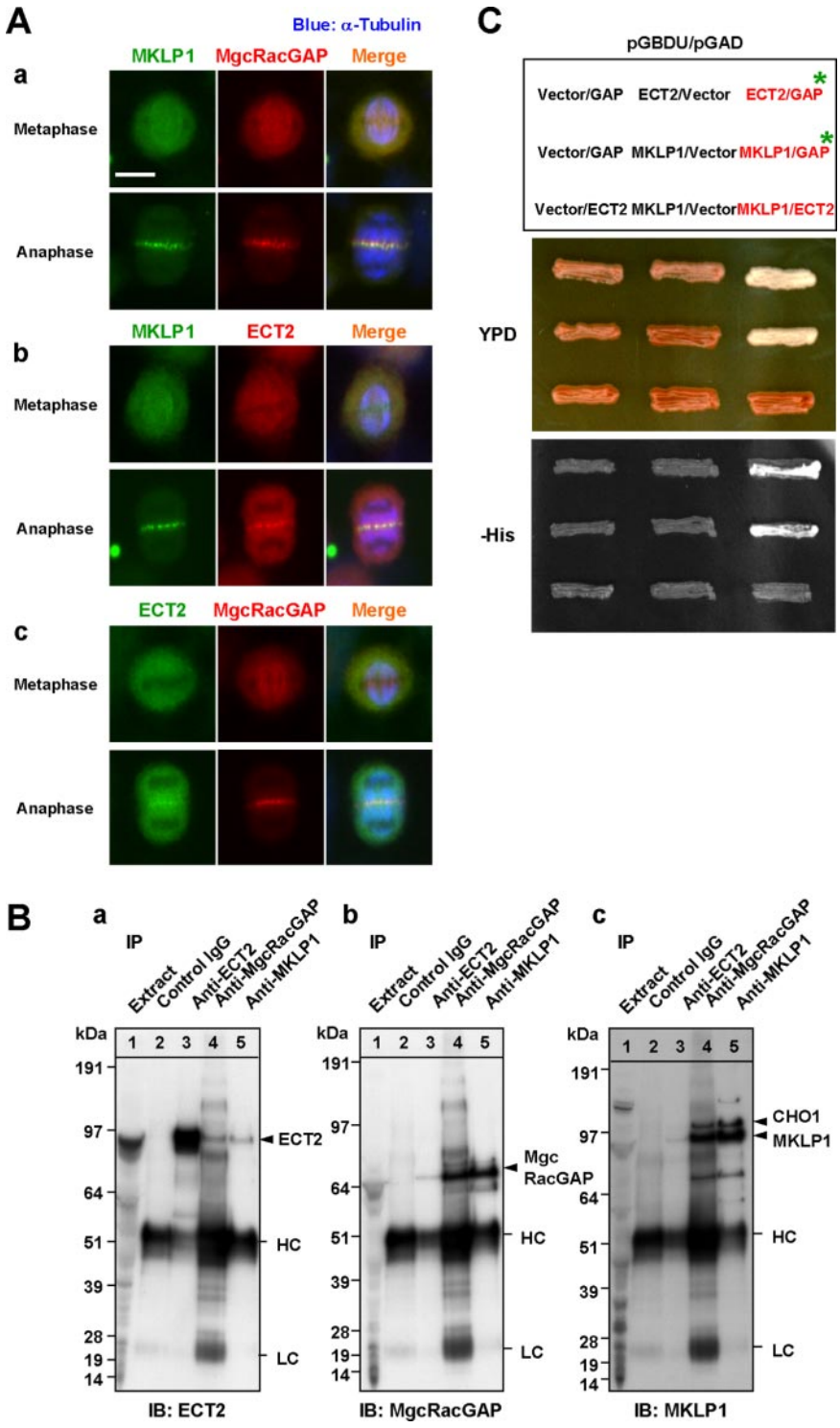

Figure 8. ECT2 forms complex with MgcRacGAP and MKLP1 at the central spindle. (A) Subcellular localization of ECT2, MgcRacGAP, and MKLP1 in metaphase and anaphase. HeLa cells were stained with indicated antibodies and anti-α-tubulin. Bar, 10 μm. In metaphase, MKLP1, MgcRacGAP, and ECT2 were found throughout in the cytoplasm. MKLP1 and MgcRacGAP were also found associated with the spindle. In anaphase, ECT2, MgcRacGAP, and MKLP1 were colocalized at the interdigitating portion of the central spindle. (B) Coimmunoprecipitation of ECT2 with MgcRacGAP and MKLP1. An extract from HeLa cells synchronized in anaphase to cytokinesis was immunoprecipitated with control IgG or antibodies to ECT2, MgcRacGAP, and MKLP1. Immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene diflouride membrane, and probed with indicated antibodies. (C) ECT2 interacts with MgcRacGAP but not MKLP1. Interactions were tested by a yeast two-hybrid system with full-length ECT2, MgcRacGAP, and MKLP1. Plasmids (pGBDU/pGAD) were transformed into PJ69-4A cells. After the transformants were selected, the cells were grown on nonselective medium (YPD) or selective medium (−His) at 25°C for 3 d. Asterisks indicate significant interactions. GAP:MgcRacGAP.

The cortical contraction started ~20 min after nocodazole release and lasted ~1 h. Finally, the cells spread out again without dividing.

To investigate whether ectopic furrow formation, like normal cleavage furrow formation, depends on Rho-mediated signaling, we examined the localization of Rho in roscovitine-treated cells. Rho accumulated at the ectopic furrows 30 min after nocodazole release (Figure 10C, b, arrows). Adjacent to the ectopic furrows, bundled microtubules were observed. Citron-kinase and phosphorylated MRLC, the downstream signals of Rho, also accumulated at the ectopic furrows (Figure 10E, b, arrows). Most cells completed cortical contraction 90 min after nocodazole release, when microtubule bundles and Rho accumulation disappeared (Figure 10D, b). These results suggest that ectopic furrow formation depends on Rho-mediated signaling.

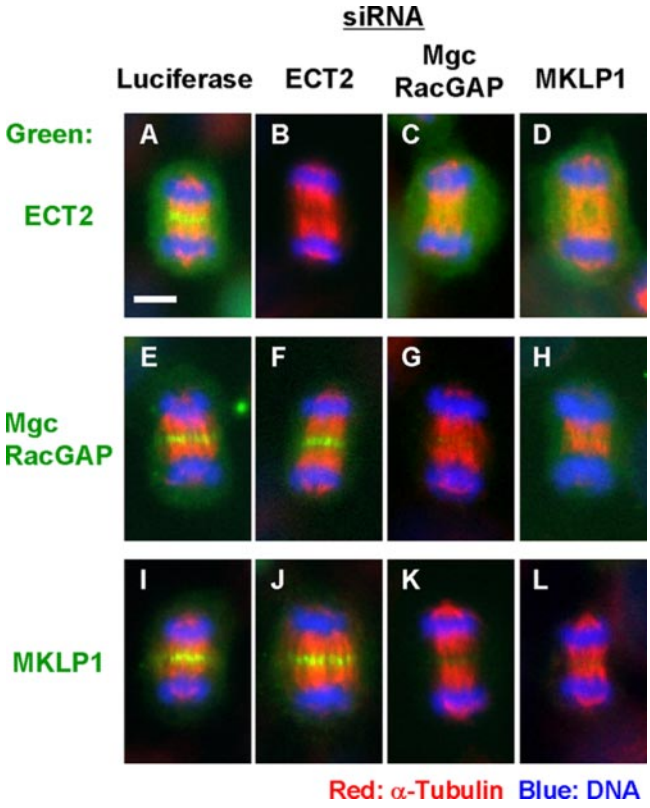

Figure 9. Localization of ECT2 at the central spindle depends on MgcRacGAP and MKLP1. Localization of ECT2, MgcRacGAP, and MKLP1 in cells depleted of these protein by RNAi. Bar, 10 μm. In control cells, ECT2 (A), MgcRacGAP (E), and MKLP1 (I) were localized at the interdigitating portion of the central spindle. MgcRacGAP or MKLP1 RNAi delocalized ECT2 from the central spindle (C and D) but ECT2 RNAi did not affect the localization of MgcRacGAP (F) and MKLP1 (J). The localization of MKLP1 and MgcRacGAP at the central spindle requires MgcRacGAP and MKLP1, respectively (H and K).

To test whether the timing of Cdk1 inhibition affects microtubule organization and furrow formation, we pretreated cells with roscovitine 15 min before nocodazole washout (Figure 10A, c). Roscovitine pretreatment suppressed cortical contraction to 11% (n = 82; Figure 10C, c), and cells spread without dividing (Figure 10D, c; and Movie S9). Microtubule bundling was not observed either in the presence of nocodazole (Figure 10B, Nocodazole + Roscovitine) or after washout of nocodazole (Figure 10, C and D, c). Ectopic furrow formation was suppressed, and Rho (Figure 10C, c), Citron-kinase or phosphorylated MRLC (Figure 10E, c) did not accumulate. These results suggest that proper temporal regulation of Cdk1 inhibition is required for microtubule bundling, and bundled microtubules spatially direct the localization of the Rho-mediated signaling molecules.

To confirm whether ECT2 RhoGEF regulates translocation of Rho and formation of the furrows, luciferase or ECT2 RNAi cells were treated with roscovitine. Luciferase RNAi cells exhibited cortical contraction (92%; n = 109; Movie S10). In contrast, as in the case of BMI-1026 treatment (Niiya *et al*., 2005), roscovitine-induced cortical contraction was suppressed by ECT2 RNAi (3%; n = 93; Movie S11). In luciferase RNAi cells, ECT2 accumulated at the bundled microtubules (Figure 10G, a) and Rho accumulated at the ectopic furrows (Figure 10H, a). Although microtubules were bundled by roscovitine treatment, ECT2 RNAi suppressed ectopic furrow formation (Figure 10G, b) and accumulation of Rho (Figure 10H, b). These results show that the ECT2-Rho pathway is the link between microtubules and furrow formation.

DISCUSSION

Although microtubules specify the position of the contractile ring, the nature of the signal is poorly understood. In this study, we have demonstrated that Rho serves as a microtubule-dependent signal that specifies the position of the contractile ring. Rho translocates to the equatorial region before furrow ingression, and active Rho is required for contractile ring formation. ECT2 RhoGEF is localized at the central spindle and is responsible for translocation of Rho to the equatorial region. ECT2 forms a complex with MgcRacGAP and MKLP1 kinesin. This complex formation is required for localization of ECT2 to the central spindle. Using the Cdk1 inhibitor roscovitine, we have shown that the postmitotic spindle structure spatiotemporally regulates the localization of the Rho-mediated signaling molecules and the position of the furrow. These findings indicate that the Rho-mediated signaling is the link between the central spindle and contractile ring formation (Figure 11).

Positioning of the Contractile Ring by Rho-mediated Signaling

Although the requirement of Rho in cytokinesis is reported, the role of Rho in contractile ring formation has been controversial. The TCA fixation method (Yonemura *et al*., 2004) enabled us to analyze the localization of endogenous Rho during cell division. We have found that Rho translocates to the equatorial region before furrow ingression. C3-loading or ECT2 RNAi inhibits this translocation and subsequent contractile ring formation. These observations support the following model: ECT2 RhoGEF activates Rho at the central spindle, and activated Rho translocates to the equatorial region and determines the location of the contractile ring. Consistently, using the Cdk1 inhibitor roscovitine, we have demonstrated that Rho accumulates at the ectopic furrows and ECT2 RNAi suppresses both accumulation of Rho and formation of ectopic furrows.

If ECT2 RhoGEF is localized at the central spindle, how does Rho accumulate at the equatorial region (equatorial cell cortex)? In interphase cells, Rho translocates to plasma membranes upon activation (Kranenburg *et al*., 1997; Michaelson *et al*., 2001; Yonemura *et al*., 2004). The equatorial cell cortex is the nearest plasma membrane to the central spindle. Therefore, it is possible that Rho, which is activated by ECT2 at the central spindle, accumulates at the equatorial cell cortex.

MgcRacGAP and MKLP1 form a complex at the interdigitating portion of the central spindle (Mishima *et al*., 2002). We have found that ECT2 RhoGEF colocalized with MgcRacGAP and MKLP1 at the central spindle before furrow ingression. We showed that ECT2 interacts with the MgcRacGAP-MKLP1 complex, using coimmunoprecipitation and yeast two-hybrid assays. We also demonstrated that MgcRacGAP or MKLP1 RNAi delocalizes ECT2 from the central spindle. Thus, the MgcRacGAP-MKLP1 complex determines the localization of ECT2 at the central spindle. Like ECT2 RNAi, MgcRacGAP or MKLP1 RNAi causes delocalization of Rho from the equatorial region, suggesting that MgcRacGAP and MKLP1 are upstream regulators of the ECT2-Rho pathway.

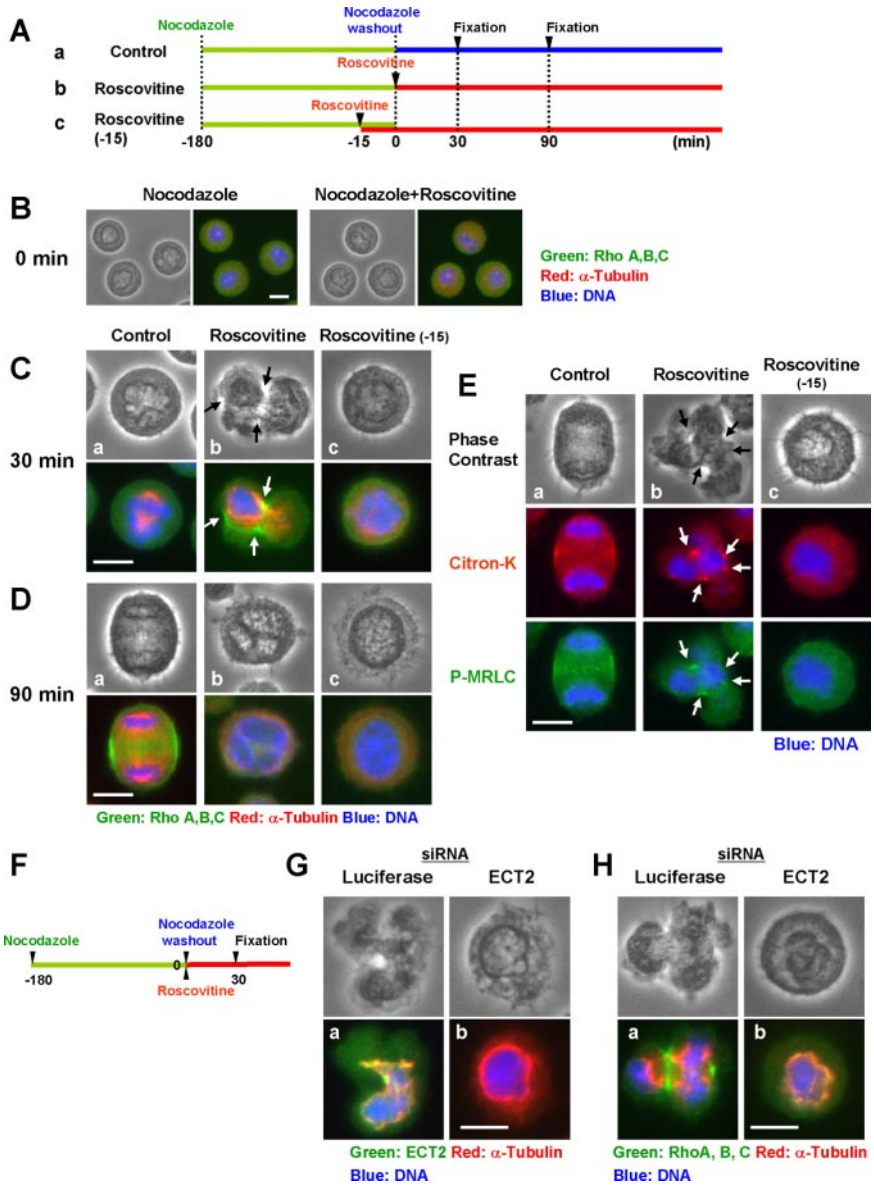

Figure 10. Bundled microtubules direct the Rho-signaling molecules to the furrowing site. Note that a, b, and c in C–E correspond to the experimental diagram shown in A. (A) Schematic diagram showing roscovitine treatment. Cells were treated with nocodazole for 3 h, and then nocodazole was washed out. Cells cultured without a or with roscovitine b. (c) Cells pretreated with roscovitine 15 min before nocodazole washout and then cultured with roscovitine [roscovitine (−15)]. (B) Localization of Rho (green) and microtubules (red) in cells treated without (Nocodazole) or with (Nocodazole + Roscovitine) roscovitine in the presence of nocodazole (corresponding to time point 0 min in A, a and c, respectively). In the presence of nocodazole, accumulation of Rho was inhibited. (C) Localization of Rho (green) and microtubules (red) in roscovitine-treated cells 30 min after washout of nocodazole. a, control cell at prometaphase. b, roscovitine-treated cell. Rho accumulated at ectopic furrows (arrows). Microtubules were bundled adjacent to the ectopic furrows. c, pretreatment with roscovitine suppresses furrow formation. Microtubules were not bundled and Rho did not accumulate. D, localization of Rho (green) and microtubules (red) in cells 90 min after washout of nocodazole. a, control cell in anaphase. Rho accumulated at the equatorial region. b, roscovitine-treated cells terminated contraction. Bundling of microtubules and accumulation of Rho disappeared. c, roscovitine-pretreated cells spread out again. (E) Localization of Citron-kinase (Citron-K; red) and phosphorylated MRLC (P-MRLC; green). a, 90 min; b and c, 30 min after nocodazole washout, respectively. In roscovitine-treated cells, Citron-kinase and phosphorylated MRLC accumulated at the ectopic furrows (b; arrows). Roscovitine pretreatment prevented formation of ectopic furrows and accumulation of Citron-kinase and phosphorylated MRLC (c). (F–H) RhoGEF ECT2 regulates translocation of Rho to the furrows. (F) Schematic diagram showing roscovitine treatment in luciferase or ECT2 RNAi cells. Cells were treated with siRNA to luciferase or ECT2 for 24 h and synchronized with nocodazole for 3 h. Then, nocodazole was washed out and cultured in the presence of roscovitine. (G) ECT2 staining in roscovitine-treated luciferase (a) or ECT2 (b) RNAi cells. (H) Localization of Rho in roscovitine-treated luciferase (a) or ECT2 (b) RNAi cells. In ECT2 RNAi cells, microtubules were bundled but Rho did not accumulate.

In *Drosophila*, PBL RhoGEF forms a complex with RacGAP50C and PAV kinesin during cytokinesis (Somers and Saint, 2003). RacGAP50C and PAV are localized at the interdigitating portion of the microtubules. However, unlike ECT2, PBL is localized at the equatorial cell cortex instead of the central spindle (Prokopenko *et al*., 1999). PBL at the contractile ring interacts with the RacGAP50C–PAV complex at the astral microtubules. Therefore, the RacGAP50C–PAV complex at the central spindle does not interact with PBL (Somers and Saint, 2003). In contrast, we have found that in human cells, ECT2 is colocalized with MgcRacGAP and MKLP1 at the central spindle. This may be because of the fact that the spindle that specifies the cleavage plane varies among different species; it is either the astral spindle or the central spindle or both (Robinson and Spudich, 2000; Glotzer, 2001). The astral spindle in *Drosophila* and the central spindle in mammalian cells probably play a major role in positioning of the contractile ring.

Bundled Microtubules Direct Furrow Positioning

Using the Cdk1 inhibitor roscovitine, we have shown that microtubule bundling requires inactivation of Cdk1 in proper timing. It has been reported that furrows are formed near the site of high numbers of stabilized microtubules (Canman *et al*., 2003). We demonstrate that the bundled microtubules lead to accumulation of Rho-mediated signaling molecules and cause ectopic furrowing. This indicates that spatiotemporal regulation of microtubule bundling is important for directing the Rho-mediated signaling molecules and positioning of the cleavage furrow.

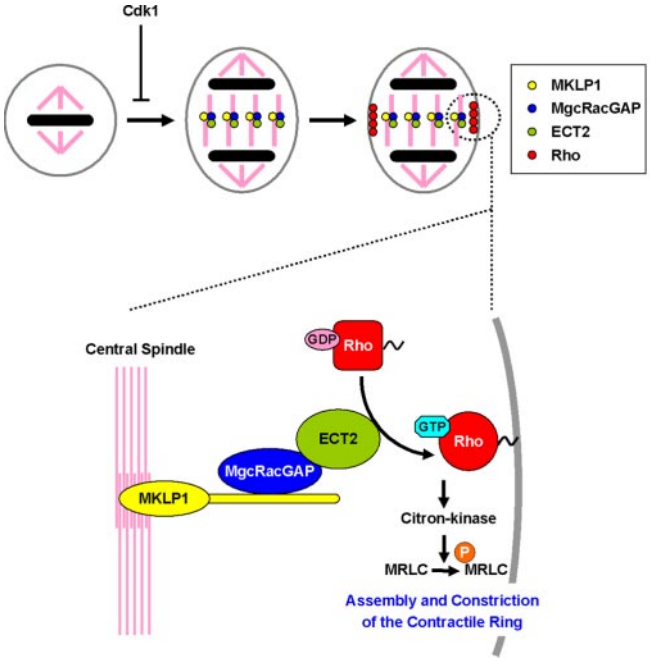

Figure 11. Rho-mediated signaling serves as a microtubule-dependent signal for furrow positioning.

Assembly of the Contractile Ring

The mechanism that leads to accumulation of actin and myosin filaments at the contractile ring is not well understood. In yeast, myosin II localization at the equatorial region is independent of actin (Bi *et al*., 1998; Motegi *et al*., 2000). We have shown that the actin inhibitor latrunculin B does not prevent recruitment of myosin II to the equatorial region. Thus, assembly of myosin filaments probably precedes formation of the acto-myosin contractile ring.

What signal regulates myosin assembly? It has been reported that MRLC is phosphorylated at the cleavage furrow (Matsumura *et al*., 1998; Murata-Hori *et al*., 1998) and that this phosphorylation occurs before furrow ingression (Matsumura *et al*., 1998). In this study, we have shown that the Rho inhibitor C3 and siRNA to ECT2, MgcRacGAP, or MKLP1 inhibit accumulation of phosphorylated MRLC at the equatorial region. Phosphorylation of MRLC promotes self-assembly of myosin II to the bipolar filaments and facilitates formation of the acto-myosin fibers in vitro (Applegate and Pardee, 1992). Therefore, phosphorylation of MRLC might regulate assembly of the myosin filaments and subsequent acto-myosin contractile ring formation.

MRLC is a substrate of Citron-kinase (Yamashiro *et al*., 2003) and mutations in Citron-kinase lead to defects in furrow formation and ingression (Madaule *et al*., 1998; D'Avino *et al*., 2004). We have shown that Citron-kinase is essential for cytokinesis and that the localization of Citron-kinase depends on Rho, ECT2, MgcRacGAP, and MKLP1. Also, we have shown that Citron-kinase colocalizes with phosphorylated MRLC at the equatorial region before furrow ingression. These results suggest that Citron-kinase might be responsible for phosphorylation of MRLC at the equatorial region during cytokinesis.

In conclusion, we propose the microtubule-dependent Rho-mediated signaling mechanism for positioning and assembly of the contractile ring as follows (Figure 11). In anaphase, as Cdk1 is inactivated, microtubules are bundled to the central spindle between separated chromosomes. ECT2 RhoGEF forms a complex with MgcRacGAP and MKLP1 at the central spindle. Then, ECT2 activates Rho, which translocates to the equatorial region. Rho recruits Citron-kinase, which phosphorylates MRLC at the equatorial region. The phosphorylated myosin bundles the acto-myosin contractile ring and generates constriction force. Therefore, the relationship between the positioning and the assembly of the contractile ring seems to be inseparable. Further testing of this model should provide general insights into the molecular mechanism of cytokinesis.

During the course of our manuscript revision, three reports on the requirement of Rho-mediated signaling for contractile ring formation have been published (Bement *et al*., 2005; Yuce *et al*., 2005; Zhao and Fang, 2005). Bement *et al*., 2005 reveal the active zone of RhoA at the equatorial region before furrow formation, using the Rho GTPase binding domain of Rhotekin-GFP-fusion protein. Yuce *et al*., 2005 and Zhao and Fang, 2005 show that ECT2 RhoGEF regulates translocation of Rho to the equatorial region and contractile ring formation. They also show the central spindle localization of ECT2 is controlled by MgcRacGAP and MKLP1.

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